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ORIGINAL PAPER

Comparison of aggregation enhancement and inhibition as strategies for reducing the cytotoxicity of the aortic amyloid polypeptide medin

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Abstract Aortic medial amyloid (AMA) occurs as localised non-atheromatous plaques in virtually all individuals over the age of 50. The major protein component of AMA is the 50-residue polypeptide medin. Here we propose two methods of manipulating medin aggregation to reduce the cytotoxic species of medin: either by promoting formation of larger benign species or retaining small non-cytotoxic species. Medin co-localises with a variety of factors including glycosaminoglycans (GAGs). The first approach shows that the GAG heparin enhances the rate of medin aggregation and alters the morphology of the amyloid fibrils. Cellular viability measurements suggest that heparin eliminates small cytotoxic species of medin, promoting formation of benign fibrils. The second approach applies a previously successful approach of designing small peptide moieties that are complementary to the key amyloidogenic sequence but which contain modified amino acids known to disrupt hydrogen bonding and therefore prevent aggregation of the target protein. This approach also reduces cellular toxicity of medin at all stages of the aggregation process examined exhibiting a different mode of action to heparin. These results raise the question of whether enhancement of medin aggregation by GAGs is beneficial, by eliminating toxic oligomers, or has deleterious effects by reducing arterial plasticity associated with increased fibril load and whether small peptide inhibitors can be applied as drug candidates for amyloid diseases.

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Abbreviations

$AMed_{42–49}$	Peptide corresponding to residues 42–49 of medin
AMA	Aortic medial amyloid
BA	β-Alanine
DLS	Dynamic light scattering
GABA	γ-Aminobutyric acid
GAG	Glycosaminoglycan
HSPG	Heparin sulphate proteoglycan
MTT	(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
TEM	Transmission electron microscopy
ThT	Thioflavin T
SRE	Self-recognition element

Introduction

Protein misfolding disorders and amyloid plaques have become pathological watchwords owing to their association with dementias and prion diseases. Some 30 polypeptides aggregate into amyloid fibrils under physiological conditions, and many of these are associated with extracellular deposits or intracellular inclusions that are the pathological hallmark of misfolding disorders known collectively as amyloidoses (Chiti and Dobson 2006). The prevalence of amyloid diseases in the aging population has become a major health concern for Western countries and consequently much resource is being invested into developing therapies (Merlini and Westermark 2004). The design and discovery of agents that modulate the onset or propagation of protein aggregation

could furnish future drugs for a range of protein misfolding diseases.

The exact nature of the pathogenic amyloid species is a matter of intense debate, but there is increasing evidence that oligomers or intermediates rather than fibrils are responsible for cytotoxicity and associated cell death in amyloid diseases (Bucciantini et al. 2002; Kayed et al. 2003; Lashuel et al. 2002). One therapeutic option is to design small molecules to block aggregation or stabilise benign oligomers formed on- or off-pathway to the fibrils (Ehrnhoefer et al. 2008; Masuda et al. 2006). However, there is evidence that promoting the formation of insoluble aggregates could lower the concentration of the toxic intermediates associated with disease and protect against pathological damage (Bodner et al. 2006a, b; Kvam et al. 2009).

Aortic medial amyloid (AMA) is found in 97% of individuals over the age of 50 and is the most common form of localised amyloid (Peng et al. 2001). The impact of AMA on cardiovascular health is unknown, but there is speculation that the amyloid deposits contribute to age-related diminished elasticity of aortic vessels. The main constituent of AMA is a 50 amino acid polypeptide medin derived from the proteolysis of lactadherin, a mammary epithelial cell–expressed glycoprotein that is secreted as part of the milk fat globule membrane (Haggqvist et al. 1999). Medin assembles into filamentous aggregates bearing all the hallmarks of amyloid, with typical cross-β structure and Congo red birefringence (Haggqvist et al. 1999). The amyloid-promoting region appears to be confined to the 18–19 C-terminal residues of medin, within which the segment N^{42}FGSVQFV ($AMed_{42–49}$) forms fibrils independently of the full-length polypeptide (Haggqvist et al. 1999; Larsson et al. 2007). Recent experimental evidence suggests that prefibrillar intermediates of medin are cytotoxic and may underlie the pathogenesis of sporadic thoracic aortic aneurysm (Peng et al. 2007), a life-threatening condition associated with degenerative changes in the media caused by weakening of the aortic wall.

Amyloid deposits in vivo comprise not only protein aggregates but also nucleic acids, heparan sulphate proteoglycans (HSPGs) and glycosaminoglycans (GAGs; van Horssen et al. 2003), and various other polyelectrolytes (Calamai et al. 2006). The presence of polyanionic species such as GAGs during amyloid aggregation has been shown to profoundly influence the growth rate and morphology of fibrils and intermediates from various polypeptides (Castillo et al. 1998; Konno et al. 2007; van Horssen et al. 2003). Virtually nothing is known about the effect of GAGs on the aggregation rate of medin or on fibril morphology, but a recent study of medin interactions with anionic phospholipid membranes suggest that the monomeric peptide associates electrostatically with biological interfaces displaying a negative potential (Olofsson et al. 2007). Here we report that heparin, a highly sulphated naturally occurring GAG that is used widely as an anticoagulant, has a profound effect on the rate of aggregation, fibril morphology and cytotoxicity of medin.

In this work we compare the effects of two classes of agents previously shown to modulate the aggregation of amyloidogenic polypeptides, either by accelerating fibril formation or by blocking steps in the aggregation pathway. The enhancement of amyloid aggregation by the GAG heparin is first examined. We next compare the effects of heparin with the anti-aggregation properties of β-alanine (BA) substituted peptides. We recently demonstrated that amyloid aggregation can be blocked by BA- and γ-aminobutyric acid (GABA)-substituted peptides that have sequences complementary to a self-recognition element (SRE) of the target amyloid polypeptide (Madine et al. 2009b). We evaluate the effects of both classes of compounds on the rate of aggregation, morphology and cellular toxicity of the 50 amino acid polypeptide medin associated with AMA.

Medin is a poorly characterised form of amyloid, and our investigations aim to enhance understanding of medin aggregation, whilst simultaneously investigating approaches that can be applied as therapeutic applications for amyloid diseases. To our knowledge this is the first time potentiation and inhibition have been directly compared as methods of reducing cytotoxicity associated with amyloid aggregation.

Experimental section

Preparation of samples

Synthetic medin, $AMed_{42–49}$ and BA peptides were purchased with free amine and carboxyl groups from Peptide Protein Research (UK). Fibrils were prepared as follows. The peptide was subjected to three dissolution-evaporation cycles with hexafluoroisopropanol to break up any initial aggregates. The peptides were then dissolved in the minimum volume of DMSO to ensure solubility and added to ddH_20 to a final DMSO concentration of 10% v/v and a peptide concentration of 0.25 mM (1 mM for $AMed_{42–49}$). Solutions were incubated with agitation at 25°C for up to 8 days, and fibrils were harvested by spinning down in a benchtop centrifuge for 30 min. Samples were taken at time points throughout the incubation, with time 0 corresponding to the freshly prepared sample, and flash frozen or analysed straight away. Comparisons were carried out between frozen and freshly prepared samples to ensure that flash freezing samples did not affect the oligomeric states. In experiments to monitor the effects of heparin, fibrils were grown in the presence of equimolar heparin with a mean molecular mass of 16 kDa (Sigma-Aldrich UK). In experiments to monitor

the effect of modified peptides on aggregation, peptides were present at equimolar concentrations.

Analysis of fibril growth and morphology

Aggregation was monitored by DLS for 0.25 mM medin (1 mM for $AMed_{42–49}$) in 10% v/v DMSO/ddH_2O. A series of 20 measurements of 10 s each were taken at 30°C on a Zetasizer Nano DLS machine from Malvern Instruments, and averaged profiles were produced. Amyloid formation was monitored with the fluorescent dye Thioflavin T (ThT). Peptide sample (50 µl in 10% v/v DMSO/ddH_2O) was added to 950 µl of 10 µM ThT in 10 mM Tris, pH 7.5, vortexed and transferred to a 1 cm path length cuvette. Spectra were recorded on a Cary Eclipse Varian fluorescence spectrophotometer at 25°C with excitation at 450 nm and emission at 482 nm with band pass 5 nm. A new excitation band at 450 nm and enhanced emission at 482 nm are observed when amyloid is present. Morphologies of the insoluble aggregates were analysed by transmission electron microscopy (TEM) using negative staining methods (2% uranyl acetate). Peptide suspensions (10 µl in 10% v/v DMSO/ddH_2O) were loaded onto carbon-coated copper grids and visualised on a Tecnai 10 electron microscope at 100 kV.

Cell viability

Aliquots of peptide solutions were taken at specified time points throughout the incubation process and frozen until required. Bovine aortic endothethial cells (ECACC) were plated on 96-well plates at 30,000 cells/well and grown for 24 h. Peptide samples (preincubated at 0.25 mM in 10% v/v DMSO/ddH_2O) were added in aggregation buffer and incubated for 48 h at 37°C prior to addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, and further incubation for 2 h. Absorbance was read at 570 nm. Percentage cell viability was calculated based on the absorbance measured relative to that of cells exposed to buffer or heparin alone as appropriate, with dead cell controls representing 0% cell viability.

Results

Rates of aggregation

Aggregation of medin was followed using DLS to monitor the particle sizes formed by the peptide freshly dissolved in aqueous buffer and after incubation for up to 8 days, alone or with heparin. Using standard bond angles and lengths for medin present in a fully extended conformation, a theoretical maximum hydrodynamic radius for the monomeric protein would be ~18 nm. In the absence of heparin, medin initially forms small species less than 50 nm in diameter, consistent with the size of trimers, dimers or monomers. Further aggregation occurs after 1 day into larger species some 1,000–1,500 nm in diameter (Fig. 1a) and these remain the predominant species after 3 days of incubation. After 8 days, medin is present as predominant species of around 2,500 nm in diameter. The solution becomes cloudy by this stage and insoluble aggregates can be isolated by centrifugation. In the presence of heparin, medin initially forms a species of around 1,000 nm in diameter, with no evidence of smaller species (Fig. 1b). After 1 day the solution becomes cloudy and the predominant species is ~2,500 nm in diameter. Little further change is observed in the sample after 3 days or at the 8 day end-point.

The nature of the species formed initially and at day 3 and day 8 was examined by monitoring fluorescence changes in the dye ThT, which binds to the cross-β structure of amyloid (Levine 1993). A pronounced enhancement in ThT fluorescence accompanies the time course of aggregation, consistent with the formation of cross-β elements (Fig. 1c). At each time point, fluorescence was highest for medin in the presence of heparin; heparin alone had no effect on ThT fluorescence (data not presented). The DLS and ThT data together indicate that heparin accelerates the aggregation of medin into amyloid species.

Three peptides were derived from the amyloidogenic sequence $N^{42}FGSV$ (Haggqvist et al. 1999; Madine et al. 2009a) at the C-terminus of medin by replacing glycine with BA, and these were screened against medin aggregation. Figure 2 shows the aggregation properties of medin incubated in the presence of the modified peptides NF(BA)SV, (BA)SV, and F(BA)S. DLS profiles for medin coincubated with NF(BA)SV and (BA)SV show aggregation into ~2,500 nm particles after 3 days (Fig. 2a, b). However, no aggregates larger than ~100 nm are formed when medin is coincubated with F(BA)S for up to 8 days (Fig. 2c). A broad peak representing a very small percentage of the sample volume can be observed at ~2,100 nm at all time points (Fig. 2c); it is unclear what this minor species is. ThT binding to the same samples is consistent with these results (Fig. 2d), suggesting that F(BA)S impedes amyloid formation of medin over a time period up to 8 days. This peptide was thus selected as the medin inhibitor and NF(BA)SV and (BA)SV were not examined further.

Fibril morphology

The ultrastructure of medin aggregates formed after 8 days was visualised by TEM (Fig. 3). Medin alone forms a dense network of filaments several hundred nanometers long and ~10–20 nm in diameter (Fig. 3a). Interestingly, in the presence of heparin, the filaments become longer and more

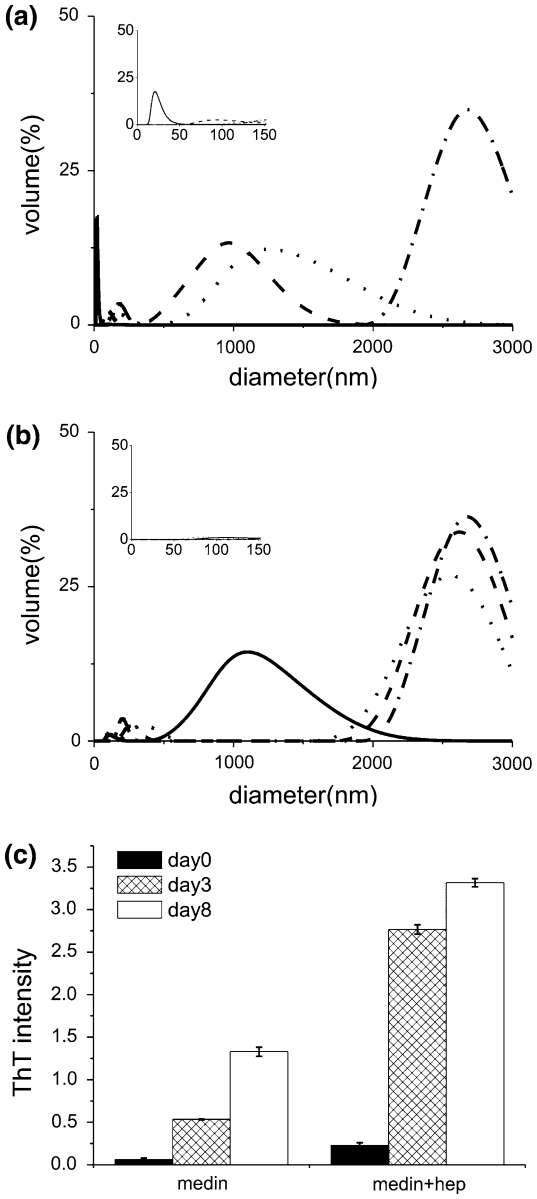

Fig. 1 Experiments to monitor the effect of heparin on the rate of medin aggregation. **a** DLS profiles of particle size in an aqueous solution of medin alone, and **b** in the presence of heparin, immediately after preparation (day 0; *solid line*) and after incubation at 25°C for 1 day (*dotted line*), 3 days (*dashed line*) and 8 days (*dot-dash line*). *Insets* show the region 0–150 nm in more detail. **c** ThT fluorescence intensity of medin alone and coincubated with heparin

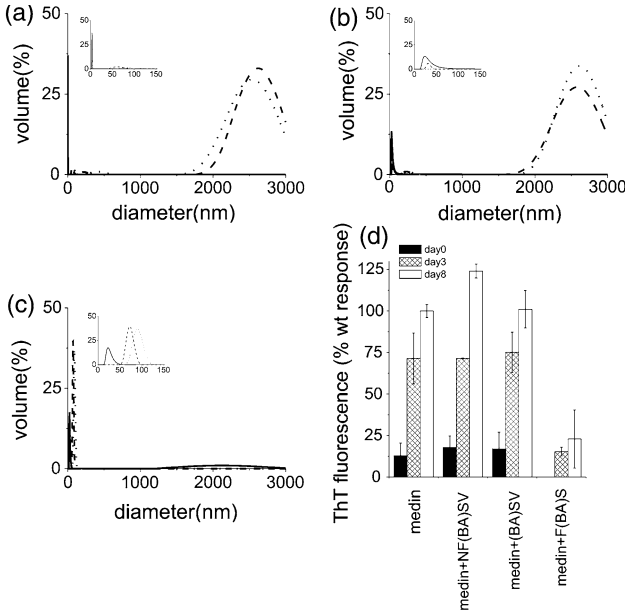

Fig. 2 Experiments to monitor the effect of modified peptides on the rate of medin aggregation. DLS profiles of particle size in an aqueous solution of medin in the presence of **a** NF(BA)SV, **b** (BA)SV and **c** F(BA)S, immediately after preparation (day 0; *solid line*) and after incubation at 25°C for 3 days (*dotted line*) and 8 days (*dashed line*). *Insets* show the region 0–150 nm in more detail. **d** ThT fluorescence intensity of medin alone and coincubated with modified peptides as shown

slender than medin alone (Fig. 3b), despite the enhancement of ThT fluorescence evident in Fig. 1. Similar observations have been made for fibrils of α-synuclein and the Alzheimer's polypeptide $A\beta_{1-40}$ grown in the presence of heparin (Cohlberg et al. 2002; Snow et al. 1994). In the presence of F(BA)S, very few fibrils of medin are observed, with average size less than 500 nm (Fig. 3c), alongside smaller, sparsely occurring granular structures.

Medin cytotoxicity

Medin toxicity against bovine aortic endothelial cell lines was examined using the MTT assay. The fresh (day 0) peptide solution and solutions preincubated for 1, 3 or 8 days were added to the cells to measure the effect on cell viability at the same stages of the aggregation process monitored by DLS. At a final concentration of 50 μM, upon addition to the cells the peptide alone reduces the viability of cells by ~35–40% at day 0, and this effect is maintained after allowing the peptide to aggregate for up to 3 days (Fig. 4). The toxic effect is lost after preincubation of the peptide at 0.25 mM for 8 days, with 95% of the cells remaining viable after adding the peptide (Fig. 4). These results support the earlier conclusion (Peng et al. 2007) that medin aggregation intermediates present at early time points are cytotoxic, progressing to non-cytotoxic species upon aggregation to fibrils. The size distribution of particles at the corresponding time points (Fig. 1a) suggests that freshly prepared medin and samples taken following incubation for up to 3 days contain prefibrillar dimers, trimers and higher oligomers, which are known to be toxic for other amyloid polypeptides (Peng et al. 2007). However, the ~2,500 nm species observed by DLS after 8 days may correspond to insoluble fibrils that are less toxic to the cells.

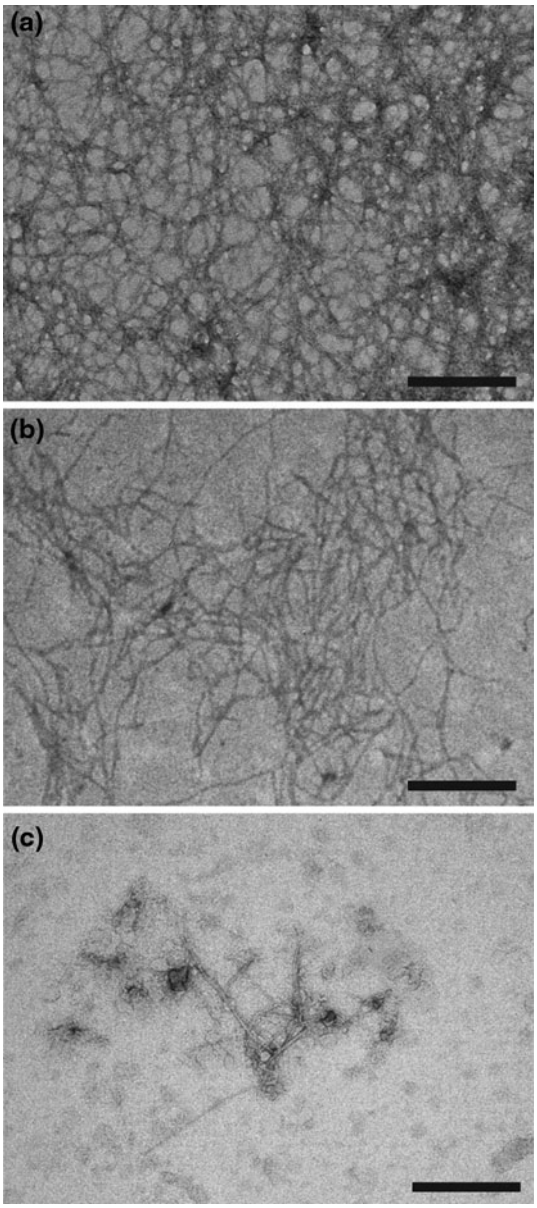

Fig. 3 Negative stain transmission electron micrographs of medin (harvested after incubation of 0.25 mM peptide in aqueous solution for 8 days). **a** Medin alone. **b** Medin with heparin. **c** Medin with F(BA)S. *Scale bar* is 500 nm

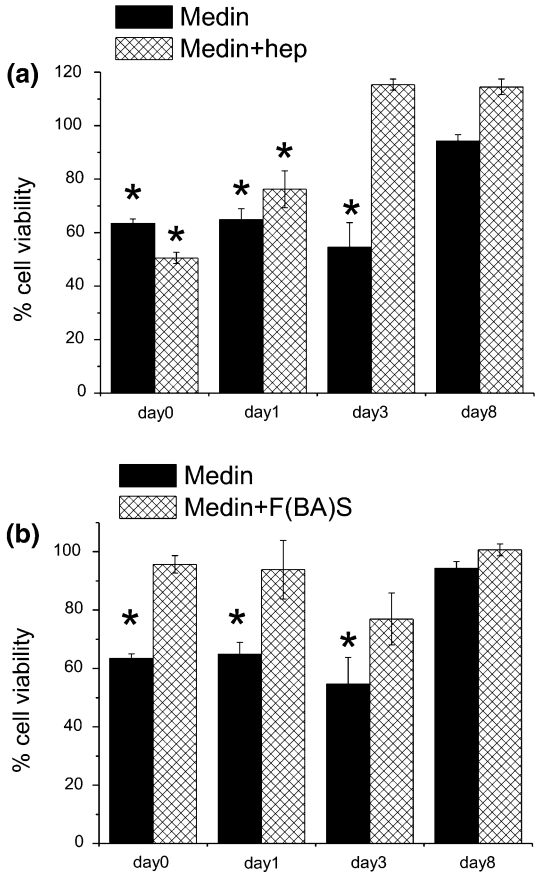

Fig. 4 Cell viability of bovine aortic endothethial cells upon addition of **a** medin alone preincubated at 0.25 mM and in the presence of equimolar heparin or **b** medin preincubated at 0.25 mM alone and in the presence of equimolar F(BA)S. Results were obtained from three independent experiments in which each experiment was carried out in triplicate (i.e., nine measurements in total). Statistically significant differences from control samples ($P < 0.05$, calculated from student's *t*-test) are indicated by an *asterisk*. Control samples contained buffer only, buffer with heparin or buffer with F(BA)S, as appropriate

These results were compared with the cytotoxicity of peptide samples coincubated with heparin and F(BA)S (Fig. 4a, b). At day 0 and day 1, the medin/heparin mixture displays similar cytotoxicity to the peptide alone (~50% reduction in cell viability), but after 3 days of incubation the toxic effect is lost completely (Fig. 4a). DLS indicates that heparin promotes the aggregation of medin into the ~2,500 nm species after just 1 day, and so it is reasonable to suggest that these are similar to the non-toxic species formed after 8 days in the absence of heparin. Taken together, DLS and cell viability studies indicate that addition of heparin promotes aggregation and concurrent loss of cytotoxicity of medin. However, DLS of the medin/heparin samples appears to show complete aggregation by day 1 in contrast to cell viability results, suggesting that there are still some toxic species present at day 1. One speculation for this discrepency is that a small proportion of low molecular weight species remain at day 1 and exert the cytotoxic effects observed, however, they represent such a small popoulation of the sample they are not observed by DLS. Addition of F(BA)S to fresh medin solutions appears to have a cytoprotective role showing no significant cell loss at any of the time points measured, confirming that F(BA)S impedes the formation of fibrils and is cytoprotective even in the early stages of medin aggregation.

Core region for medin aggregation

We investigated whether the modulation of medin aggregation by heparin and F(BA)S involves interactions with the

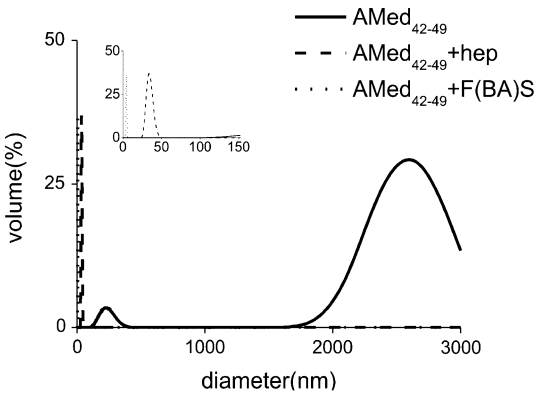

Fig. 5 DLS profiles of particle size in an aqueous solution of 1 mM $AMed_{42–49}$ alone (*solid line*) and in the presence of equimolar heparin (*dashed line*) or F(BA)S (*dotted line*) after incubation at 25°C for 4 days. *Inset* shows the region 0–150 nm in more detail

amyloidogenic C-terminal region (residues 42–49). The peptide Ac-NH-NFGSVQFV-COO^- ($AMed_{42–49}$) readily forms fibrils with morphology similar to the parent polypeptide (Haggqvist et al. 1999). Aggregation of $AMed_{42–49}$ in the presence and absence of heparin or F(BA)S was followed by DLS (Fig. 5). After 4 days incubation the peptide alone forms ~2,500 nm diameter species associated with insoluble fibrillar aggregates (Madine et al. 2009a). By contrast, in the presence of heparin, only small species (<50 nm diameter) are present after 4 days and the solution remains visibly clear (Fig. 5, dashed line). These small species could possibly represent small oligomeric (e.g. tetrameric, pentameric) mixtures of $AMed_{42–49}$ and heparin complex with a theoretical hydrodynamic radius of ~10 nm. In contrast to full-length medin, heparin thus appears to inhibit rather than promote $AMed_{42–49}$ aggregation. Over the same 4 day time period the presence of F(BA)S also prevents $AMed_{42–49}$ aggregating to form large species, with the major species observed at ~5 nm (Fig. 5, dotted line). F(BA)S appears to completely abolish $AMed_{42–49}$ aggregation retaining the peptide in a monomeric form.

Discussion

It is now widely accepted that extracellular amyloid deposits contain not only protein aggregates, but also nucleic acids, other associated proteins (Alexandrescu 2005), HSPGs and GAGs (van Horssen et al. 2003), and various other polyelectrolytes (Calamai et al. 2006). The presence of GAGs during aggregation are known to profoundly influence the growth rate and gross morphology of mature fibrils of the Alzheimer's Aβ and pancreatic amylin polypeptides as well as the size of their assembly intermediates (Castillo et al. 1998; Konno et al. 2007; van Horssen et al. 2003) and their pathogenic properties (Castillo et al. 1998; Cohlberg et al. 2002; Konno et al. 2007). Here heparin is shown to accelerate the aggregation of full-length medin and to impede aggregation by the fibrillogenic C-terminal octapeptide segment $AMed_{42–49}$. Heparin may inhibit $AMed_{42–49}$ aggregation by electrostatic interactions with the two polar residues N42 and Q46 or with the N-terminal amide, which here was not blocked by acetylation and so is positively charged at pH 7. This observation led us to hypothesise that heparin may enhance aggregation of full-length medin by interacting at sites outside the C-terminal region. The negatively charged sulphate moieties of GAGs are predicted to associate with proteins via positively charged residues (Cardin and Weintraub 1989). For example the N-terminus of $A\beta_{1–40}$ contains a consensus GAG binding motif of XBBXBX (where B is a basic residue) in the sequence V_{12}HHQKL (Watson et al. 1997). Medin does not contain an exact match to this consensus sequence but does have two basic residues within its N-terminus (R6 and K9) which could serve as an association site for heparin. This study of medin-heparin interactions provides a useful starting point for further experimental exploration of the role of GAGs in AMA.

The modified peptide F(BA)S was designed to recognise and associate with the complementary region $F^{43}GS$ of medin through self-recognition. Our hypothesis is that this specific interaction interrupts the conventional β-sheet pattern of hydrogen bonding and deters further peptide target molecules from hydrogen bonding to the main chain of the aggregating species. If this hypothesis is correct, the peptide would also prevent the aggregation of $AMed_{42–49}$. Coincubation of F(BA)S with the target SRE peptide $AMed_{42–49}$ for 4 days retains the peptide in a monomeric form, consistent with the proposed hypothesis of action of the inhibitory peptide F(BA)S associating with the complementary recognition sequence and preventing peptide aggregation. Data presented here analysing medin aggregation in the presence of the inhibitory peptide F(BA)S show that fibril formation is prevented and cytotoxicity reduced. It is shown clearly that, under the conditions of analysis used here, the formation of toxic aggregated species of medin is prevented by F(BA)S. It is not possible to speculate at this stage as to the mode of action of F(BA)S. Further experiments will be required to ascertain whether F(BA)S prevents medin aggregation completely or drives aggregation via an alternative pathway or conformation.

In summary, we present two different methods for reducing amyloid toxicity in vitro using medin as the target protein. Results demonstrate that the relationship between the rate of medin aggregation, cytotoxicity and end-point fibril morphology and load is complex. There is nevertheless strong evidence that large (~2,500 nm) insoluble aggregates of medin lose the cytotoxicity associated with smaller

oligomeric species, and that heparin promotes the formation of these insoluble species. Increasing aggregation rate reduces the lag time for fibril formation, reducing the length of time toxic intermediates are present to cause cell death, shown here using heparin to accelerate aggregation. These results raise the questions of whether GAGs could be exploited as a therapeutic target against amyloid-associated diseases or are deleterious to health by increasing fibril load. This is a long-standing debate that is highly studied in amyloid research both in vitro and in vivo. There is contrasting evidence that promoting formation of intracellular amyloid inclusions in Parkinson's and Huntington's disease may protect against pathological damage (Arrasate et al. 2004; Bodner et al. 2006a, b) or may enhance oxidative stress promoting cell death (Kvam et al. 2009). The second approach presented here to reduce amyloid toxicity is inhibition of aggregation. We have shown a small modified peptide that successfully reduces the cytotoxicity of medin throughout the time scale of the aggregation process. Results presented here in Fig. 5 suggest that the mode of inhibitory aggregation action of the short modified peptide could result from association with medin through the amyloidogenic C-terminus of the protein. However, alternative modes of action cannot be ruled out based on this limited data, and further work is currently underway to elucidate and confirm the mechanism for this inhibitory effect.

Work presented here studies the effect of adding heparin and inhibitory peptides at equimolar concentrations as a starting point to identify compounds that are able to alter the aggregation pathway of medin in vitro. In order to progress towards therapeutic applications the next stage of this work is to test lower concentrations of these compounds to determine complete profiles of activity and effectiveness for potential drug incorporation.

The impact of AMA on cardiovascular health is not completely known, but there is speculation that the amyloid deposits contribute to age-related diminished elasticity of the vessels. Westermark and colleagues tested the hypothesis that widely spread medin amyloid deposits in the thoracic aorta are associated with aortic wall weakening, noting that other forms of amyloidosis involving arteries can lead to an increased risk for rupture (Peng et al. 2007). They hypothesised that prefibrillar aggregates of medin, rather than mature fibrils, may be involved in the pathogenesis of thoracic aortic aneurysm by being toxic to the surrounding smooth muscle cells. The different approaches modulating amyloid aggregation presented here represent two possible therapeutic methods that could be targeted in the search for preventative drugs for amyloid diseases.

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