Extracellular matrix components modulate different stages in β₂-microglobulin amyloid formation

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ABSTRACT

Amyloid deposition of wild-type human β₂-microglobulin (WT-hβ₂m) in the joints of long-term hemodialysis patients is the hallmark of dialysis-related amyloidosis (DRA). In vitro, WT-hβ₂m does not form amyloid fibrils at physiological pH and temperature unless co-solvents or other reagents are added. Therefore, understanding how fibril formation is initiated and maintained in the joint space is important for elucidating WT-hβ₂m aggregation and DRA onset. Here, we investigated the roles of collagen I and the commonly administered anticoagulant, low-molecular-weight (LMW) heparin, in the initiation and subsequent aggregation phases of WT-hβ₂m in physiologically relevant conditions. Using thioflavin T (ThT) fluorescence to study the kinetics of amyloid formation, we analyzed how these two agents affect specific stages of WT-hβ₂m assembly. Our results revealed that LMW-heparin strongly promotes WT-hβ₂m fibrillogenesis during all stages of aggregation. However, collagen I affected WT-hβ₂m amyloid formation in contrasting ways: decreasing the lag time of fibril formation in the presence of LMW-heparin and slowing the rate at higher concentrations. We found that in self-seeded reactions, interaction of collagen I with WT-hβ₂m amyloid fibrils attenuates surface-mediated growth of WT-hβ₂m fibrils, demonstrating a key role of secondary nucleation in WT-hβ₂m amyloid formation. Interestingly, collagen I fibrils did not suppress surface-mediated assembly of WT-hβ₂m monomers when cross-seeded with fibrils formed from the N-terminally truncated variant ΔN6-hβ₂m. Together, these results provide detailed insights into how collagen I and LMW-heparin impact different stages in the aggregation of WT-hβ₂m into amyloid which lead to dramatic effects on the time course of assembly.

Dialysis-related amyloidosis (DRA) is a severe condition that leads to progressive bone and joint atrophy in the majority of long-term haemodialysis patients(1-5). This disorder results from the deposition of amyloid plaques formed predominantly of wild-type human β₂-microglobulin (WT-hβ₂m) in joints and cartilage
tissue(3,6-8). In its non-pathogenic role, WT-hβ2-m constitutes the light chain of the major histocompatibility complex class I (MHC I), which functions in presenting antigens to T-cells(9). After dissociation from the MHC I complex, WT-hβ2-m is normally degraded and excreted by the kidneys(10). In renal failure, the concentration of β2-m in the plasma is increased up to >60-times in comparison with that of healthy individuals(3,6-8). Aggregation of WT-hβ2-m then leads to the formation of amyloid plaques that are deposited almost exclusively in skeletal tissues(11), which are rich in extracellular matrix (ECM) components, including collagens and the glycosaminoglycans (GAGs) heparan sulfate and hyaluronic acid(12-15). The mechanism of recruitment of WT-hβ2-m specifically to skeletal tissues is not fully understood, but ECM components, such as low molecular weight (LMW)-heparin (a GAG mimic, relevant here since this is given to all patients undergoing renal replacement therapy), apolipoprotein E and collagen, have been found to enhance WT-hβ2-m aggregation in vitro(13,16-18). Amyloid fibrils have also been found associated with collagen fibrils in ex vivo deposits from DRA patients(13), and monomers of both WT-hβ2-m and its natural proteolytic product, ΔN6-hβ2-m, which lacks the N-terminal six amino acids, have been shown to have weak (Kp: 4.1×10^-4 M and 4.9×10^-6 M, respectively(15)) affinities for collagen I at the pathophysiologic pH of 6.4(12). Despite this evidence of the importance of LMW-heparin and collagen in amyloid formation, the mechanism(s) by which interactions with the reagents affect aggregation of WT-hβ2-m and ΔN6-hβ2-m remain unclear.

Compared with the intransigence of WT-hβ2-m to form amyloid fibrils at pH 6-7(19,20), ΔN6-hβ2-m readily forms amyloid at these pH values in vitro(21,22). ΔN6-hβ2-m comprises ca. 30% of the β2-m present in DRA deposits(21,23) and contains a non-native anti-X-P32, a pre-requisite for amyloid formation(22,24) that is retained in the amyloid fibril structure itself(25). Weak interactions between the apical loops of ΔN6-hβ2-m and WT-hβ2-m have been shown to promote amyloid formation of the normally innocuous WT-hβ2-m (26,27), suggesting a potential role of ΔN6-hβ2-m in initiating fibril assembly of the WT protein. How ΔN6-hβ2-m, collagen and LMW-heparin together influence amyloid formation of WT-hβ2-m, however, has remained unclear.

Here we used detailed analysis of the kinetics of amyloid formation to determine the role of collagen I, LMW-heparin and ΔN6-hβ2-m, and their mixtures, on amyloid fibril formation of WT-hβ2-m. The results reveal that LMW-heparin and collagen I influence multiple phases of WT-hβ2-m amyloid formation, including initiation, elongation and secondary nucleation processes. Additionally, we found that the effects of collagen I on amyloid formation depend on whether fibril growth of WT-hβ2-m is self-seeded or cross-seeded by ΔN6-hβ2-m fibrils. Overall, the results shed new light on the mechanisms by which biologically-relevant factors influence WT-hβ2-m amyloid assembly. More generally, they reveal how the local environment can have a dramatic effect in defining the rate and mechanisms of protein assembly into amyloid.

RESULTS

LMW-heparin and collagen I have a synergistic effect in the initiation of amyloid formation of WT-hβ2-m

The hallmark of DRA is formation of proteinaceous deposits comprised of WT-hβ2-m and ΔN6-hβ2-m in the ECM-rich joint spaces(3). As native WT-hβ2-m does not form amyloid at neutral pH, or at the slightly acidified pH (pH 6.2) in affected joints(18), unless co-solvents or copper ions are added(13,16-19,28-32), (Figure 1A), we investigated how collagen I that is found in the ECM, and GAGs (represented by LMW-heparin), affect the kinetics of aggregation of WT-hβ2-m. Previous studies have demonstrated a role of these components in hβ2-m amyloid assembly(13,15,17,18,33), but the precise mechanism(s) by which they affect aggregation, and the possible synergy between these different components, remained unclear. Using the enhancement of thioflavin T (ThT) fluorescence as a probe of amyloid formation and measurement of the resulting fibril growth kinetics showed that LMW-heparin (0.1 mg/ml) induces fibril formation of WT-hβ2-m (0.47 mg/ml) within ~30 h, resulting in the formation of long, straight fibrils typical of amyloid (Figure 1A,B). By contrast, collagen I did not induce amyloid formation in the absence of LMW-heparin over the timescale measured here (Figure 1C,D) (note, however, that collagen can
promote amyloid fibril formation over much longer timescales, as previously reported(13). Addition of both components revealed that collagen I modulates the kinetics of LMW-heparin-driven WT-h\(\beta\)\(2\)m fibril formation in a complex manner (Figure 1E,F). At low concentrations (0.03-0.12 mg/ml), collagen I accelerates LMW-heparin-induced aggregation of WT-h\(\beta\)\(2\)m, decreasing the lag time relative to the effect of LMW-heparin alone (Figure 1E, light green colours, compared with Figure 1A, blue). However, addition of higher concentrations of collagen I (≥0.47 mg/ml) in the presence of LMW-heparin, retards fibril formation by increasing the lag time (Figure 1E, dark green colours, see also Supplemental Figure S1A). Enhancement of WT-h\(\beta\)\(2\)m amyloid formation by collagen I is consistent with previous results which have shown that collagen I alone can induce aggregation of WT-h\(\beta\)\(2\)m(13). WT-h\(\beta\)\(2\)m has also been shown to bind to both collagen I and LMW-heparin(12,14,15,18). At high concentrations of collagen I, we assume that competition between collagen I and LMW-heparin binding to each other (\(K_D\): 7.9×10^{-8} M)(34) and binding to WT-h\(\beta\)\(2\)m may reduce the availability of these components to interact with WT-h\(\beta\)\(2\)m slowing its aggregation. Sequestration of WT-h\(\beta\)\(2\)m nuclei/aggregates on the surface of the collagen I fibrils, which would be favoured at high concentrations of collagen I, could also disfavor amyloid formation (see below) and contribute to the complex dose-dependent behavior observed.

**Role of the collagen sequence and conformation in LMW-heparin-induced aggregation of WT-h\(\beta\)\(2\)m**

Collagen I can adopt a hierarchy of structures within the ECM (Figure 2A). The canonical collagenous sequence consists of Gly-Xaa-Yaa triplets, where Xaa and Yaa can be any amino acid, but are most often Pro and hydroxyproline (Hyp/O), respectively. Single collagen polypeptide chains fold into polyproline type II (PPII) helices. Three such chains then twist together to form a triple helix that is stabilized by a network of inter-chain hydrogen bonds between Gly and Xaa of a neighboring chain(35). The triple helices further self-assemble into higher order fibrils. To determine the effects of collagen sequence and conformation on the aggregation kinetics of WT-h\(\beta\)\(2\)m, the kinetics of LMW-heparin-induced aggregation were monitored in the presence of two different collagen mimetic peptides (CMPs), as well as denatured full-length collagen I. The CMP, Ac-(Pro-Hyp-Gly)\(_{10}\)-GY-NH\(_2\) (named POG\(_{10}\)), forms a stable triple helix conformation without additional native sequence fragments(36). However, this peptide does not form intermolecular interactions required to proceed to collagen fibril formation. Removal of only one Gly from the middle POG repeat (named Gly-), disrupts the triple helix conformation(37,38). By contrast with the complex effects of collagen I fibrils on the lag time of LMW-heparin induced aggregation of WT-h\(\beta\)\(2\)m (Figure 1E), incubation of WT-h\(\beta\)\(2\)m monomers (0.47 mg/ml) with up to 1 mg/ml POG\(_{10}\) or Gly-peptide, in the presence of 0.1 mg/ml LMW-heparin did not significantly affect the lag time of fibril formation relative to the lag time in the absence of peptide (Figure 2B,D,E, and Figure S1A,B,D). Full-length collagen I denatured into single chains also had no significant effect on the lag time of aggregation (Figure 2C, E and Figure S1C). Hence, adoption of a triple helical collagen structure with a native sequence is required for collagen I to modulate the rate of LMW-heparin-induced WT-h\(\beta\)\(2\)m aggregation, highlighting the specificity between the different surfaces involved in amyloid assembly.

**Surface-mediated aggregation of WT-h\(\beta\)\(2\)m is protected by collagen I**

To determine whether a stable complex is formed between WT-h\(\beta\)\(2\)m monomer and/or fibrils and collagen I, samples were taken at different times during aggregation in the presence or absence of 0.1 mg/ml LMW-heparin, added to collagen I (1.0 mg/ml) and pelleted 10 mins later by centrifugation at 5000 xg (Experimental Procedures). At this low centrifugation speed only collagen I fibrils sediment, while WT-h\(\beta\)\(2\)m (monomers, oligomers and fibrils) remain in the supernatant. Whether WT-h\(\beta\)\(2\)m monomers/small oligomers and/or fibrils bind collagen I was then determined by monitoring the band intensity of WT-h\(\beta\)\(2\)m that pelleted with collagen I on an SDS-PAGE gel. These experiments showed that WT-h\(\beta\)\(2\)m co-precipitates with collagen I fibrils only after an incubation time of 35 h (Figure 3A-C), (ca. the \(t_{50}\) of aggregation in the presence of 0.1 mg/ml LMH-heparin (Figure 1A)). A higher extent of co-precipitation was observed after 85 h, by which time WT-h\(\beta\)\(2\)m amyloid formation has reached.
completion in the presence of LMW-heparin (Figures 1A and 3A,C). These results show that the fibrillar form of WT-h\(\beta\)m interacts with collagen I fibrils most tightly, while species formed in the lag time appear not to bind collagen I tightly, at least at the detection limit of these experiments. Consistent with these results, interactions between WT-h\(\beta\)m monomers and LMW-heparin or collagen I were found to be weak as assessed by \(^{1}H-^{15}N\) HSQC NMR spectra. This was assessed by 1 h co-incubation of 80 \(\mu\)M \(^{15}\)N-WT-h\(\beta\)m with 0.2 mg/ml LMW-heparin and/or 2 mg/ml collagen I and measurement of the chemical shifts of backbone resonances. No significant chemical shift perturbations (or linewidth) were observed in these experiments (Figure S2), suggesting that these interactions are too weak to detect by these methods under the conditions employed.

Since WT-h\(\beta\)m fibrils and collagen I were shown to interact by the co-pelleting assay, we next analysed the effect of collagen I on seeded growth of WT-h\(\beta\)m fibrils (using WT-h\(\beta\)m fibril seeds produced in the presence of LMW-heparin (Experimental Procedures)). As expected, the addition of WT-h\(\beta\)m seeds (5-30 % \(v/v\)) enhances the rate of formation of WT-h\(\beta\)m fibrils, dependent on the seed concentration (Figure 4A). Note that under these quiescent conditions and without seeds no fibrils form(22,24,26). Interestingly, a biphasic curve is generated in the presence of fibril seeds, with the first (relatively small) increase in ThT fluorescence intensity occurring in the first 2 h (Figure 4A and inset). This phase presumably monitors the elongation of fibril ends by WT-h\(\beta\)m monomers. Addition of 1 mg/ml collagen I does not affect this phase (Figure 4B). By contrast, the second phase, with larger ThT amplitude (Figure 4A), is significantly retarded by the addition of 1 mg/ml collagen I (Figure 4B, Figure S3). In the presence of low concentrations (5 % \(v/v\)) of seeds and under quiescent conditions, these fibril-mediated interactions are the dominant processes of fibril formation. Hence, in these conditions, the interaction of collagen I with WT-h\(\beta\)m fibrils, observed by co-precipitation (Figure 3A), protects against fibril surface-mediated growth of WT-h\(\beta\)m amyloid by masking the fibril surface.

The specificity of collagen I for WT-h\(\beta\)m fibrils was next probed by monitoring the effect of collagen I on reactions in which pre-formed seeds of ΔN6-h\(\beta\)m were used to cross-seed amyloid formation of WT-h\(\beta\)m monomers (Figure 4C,D). WT-h\(\beta\)m and ΔN6-h\(\beta\)m are known to co-aggregate(13,18,23), forming fibrils of a different morphology in vitro than those formed by each protein alone(27). The results showed that the effect of collagen I on the seeded aggregation of WT-h\(\beta\)m is highly dependent on the identity of the seeds added. Addition of ΔN6-h\(\beta\)m seeds to WT-h\(\beta\)m monomers also results in biphasic fibril growth curves (Figure 4C). However, the rate of the initial phase is slower under all conditions for the cross-seeded reactions compared with the self-seeded reactions (Figure S3A). Most notably, the secondary process, that occurs after rapid fibril elongation, is much less affected by collagen I in the cross-seeded reactions than when self-seeded (compare Figures 4 B and D, see also Figure S3B). Thus, the interference of collagen I with surface-mediated growth of WT-h\(\beta\)m fibrils depends on the morphology of the h\(\beta\)m fibril seeds which differ when self-seeded and cross-seeded by ΔN6-h\(\beta\)m(27).

LMW-heparin promotes assembly of WT-h\(\beta\)m fibrils in both self-seeded and cross-seeded reactions

Finally, the effect of LMW-heparin on fibril formation of WT-h\(\beta\)m was monitored in seeded reactions, to determine whether the addition of this GAG can out-compete the effect of collagen I on aggregation. When mixed, LMW-heparin is able to rescue the inhibitory effect of collagen I on secondary processes whether the reaction is self- or cross-seeded (Figures 5A-D and Figure S3). Thus, collagen I and LMW-heparin have different effects on WT-h\(\beta\)m aggregation at multiple phases of fibrillation. Collagen I acts primarily on the secondary surface-mediated growth of self-seeded fibrils and is dependent on whether the reaction is self-seeded or cross-seeded (compare Figures 4A,B with 5A,B)). By contrast, LMW-heparin can enhance growth at all stages of aggregation and is insensitive to the distinct amyloid conformations produced by self-seeding or cross-seeding with ΔN6-h\(\beta\)m.

DISCUSSION

A detailed study of the effects of the local environment on the course of protein aggregation is
Examining the effects of two macromolecules that are present in conditions relevant to DRA, collagen aggregation in an *in vitro* setting may act synergistically to alter the course of amyloid formation, including how the different biologically-relevant and other compounds on I and the GAG LMW-heparin. Since WT-β2m can provide important information on the role of secondary nucleation (39-41,43,48). Such studies can provide important information on the role of biologically-relevant and other compounds on amyloid formation, including how the different factors may act synergistically to alter the course of aggregation in an *in vitro* setting(17,30).

Here we have adopted this approach by examining the effects of two macromolecules that are present in conditions relevant to DRA, collagen I and the GAG LMW-heparin. Since WT-β2m is not prone to amyloid formation in the absence of co-factors at pathophysiological pH and temperature, understanding how the molecular components of the local environment affect WT-β2m amyloid formation and impact the kinetics of fibril formation is important for developing an understanding of hβ2m amyloidogenesis in DRA. In addition, how the biological environment affects the ability of ΔN6-β2m, which makes up ~30% of the hβ2m component in DRA plaques(21,23), to stimulate aggregation of WT-β2m may also shed light on how the aggregation of WT-β2m may be initiated *in vivo*.

Understanding amyloid formation of WT-β2m in mechanistic detail in the context of the ECM in joints and cartilage is extremely challenging, given the multi-component composition it presents. Here, we have started to investigate aggregation in this environment by determining how different components relevant to DRA (collagen I and LMW-heparin) impact the different kinetic stages of WT-β2m aggregation. The studies presented show that LMW-heparin, collagen I and ΔN6-β2m have different effects on the course of WT-β2m aggregation, which compete for the different stages of aggregate formation. Firstly, under all conditions, LMW-heparin is able to promote the self-assembly of WT-β2m, decreasing the lag time and increasing the rate of fibril formation by affecting secondary stages, whether self-seeded or cross-seeded by ΔN6-β2m. Previous studies have shown that LMW-heparin binds and stabilizes WT-β2m amyloid fibrils, while the non-sulfated GAG hyaluronic acid has no effect on fibril stability or the rate of fibril formation, suggesting that ionic interactions between the GAG and WT-β2m must be involved(17). Determining the origins of molecular recognition between different species (monomers, oligomers and fibrils) of WT-β2m and ΔN6-β2m and GAGs will require further exploration, for example by varying the patterns of sulfation, the identity of the carbohydrate moieties that differ between GAGs, and the length of the GAG, which have been shown previously to affect amyloid-GAG recognition(49,50).

Analysis of the effects of heparan sulfate, the most abundant GAG in the joint ECM, would be particularly important for hβ2m, although previous studies have shown that heparan sulfate and LMW-heparin have similar effects on seeded elongation of fibril growth using WT-β2m(17).

By contrast with LMW-heparin, collagen I has a more complex role on WT-β2m assembly into amyloid, affecting the lag time of fibril formation and secondary growth phases in different ways, dependent on the concentration added, the presence of LMW-heparin, the structural organization of the collagen added and whether fibril growth WT-β2m is self-seeded or cross-seeded by ΔN6-β2m fibrils. A decrease in the lag time of WT-β2m assembly occurs upon addition of low concentrations of collagen I and LMW-heparin relative to the addition of LMW-heparin alone, suggestive of one route for the initiation of WT-β2m assembly at pathophysiological pH and temperature. However, at high concentrations of collagen I the lag time is extended. Collagen I fibrils also interact strongly with WT-β2m amyloid fibrils suppressing surface mediated growth (Figure 6, top) by competing for interactions with the WT-β2m fibril surface. Notably, a different fibril morphology formed by cross-seeding WT-β2m with ΔN6-β2m fibrils, does not show this marked suppression of fibril formation by collagen I (Figure 6, bottom). Whether self-seeded or cross-seeded with ΔN6 fibril seeds, LMW-heparin is able to outcompete...
the binding of collagen I to WT-hβ2m fibrils, releasing the potential of the amyloid fibril surface to enhance fibril formation via secondary nucleation processes. These results are consistent with previous studies that also showed enhanced aggregation of WT-hβ2m in the presence of LMW-heparin(18,24). In addition to its role in suppressing amyloid formation, interactions between collagen I and WT-hβ2m fibrils may prevent the clearance of amyloid from the joint space, providing an explanation for the localization of DRA plaques to joint and cartilage tissues. Other factors not investigated here, such as the presence of chaperones and/or other proteins, oxidation, glycation, or other post-translational modifications of the hβ2m sequence, the presence of Cu²⁺ ions, and shear flow within the joint space may also contribute to amyloid formation(17,19,31,40,51,52). Overall, therefore, the results portray a marked complexity in amyloid formation in the ECM, in which a finely tuned balance of different components (in this case collagen I, LMW-heparin and ΔN6-hβ2m) affect the progression of hβ2m aggregation and its sequestration in the joints to give a pattern of amyloid deposition that is the hallmark of DRA.

EXPERIMENTAL PROCEDURES

Protein preparation
WT-hβ2m and ΔN6-hβ2m were expressed and purified as described previously(22). For NMR experiments, ¹⁵N- and ¹³C-labelled WT-hβ2m and ΔN6 hβ2m were prepared as described in(53).

Collagen preparation
Collagen type I (354249) from rat tail was purchased from BD Biosciences. Collagen was diluted to 3 mg/ml in 0.1 M acetic acid. Before use, it was dialysed into 50 mM MES, 120 mM NaCl at pH 6.2. For preparation of collagen I fibrils, the dialyzed collagen was incubated at 37 °C for 1 h. For preparation of collagen single chains, the dialyzed collagen was incubated for 30 min at 70 °C. CMPs POG₁₀ and Gly- were purchased from LifeTein and Tufts University Core facility, respectively, and were directly diluted into 50 mM MES, 120mM NaCl at pH 6.2 to the concentrations specified.

Formation of fibril seeds
Fibrils of WT-hβ2m were assembled in 50 mM MES buffer, 120 mM NaCl at pH 6.2 in the presence of 0.1 mg/ml LMW-heparin (Iduron) in a BMG Fluostar Optima plate reader at 37 °C at 600 rpm. The fibrils were sonicated for 1 min, distributed in aliquots and frozen in liquid nitrogen. The size of the seeds was determined using negative stain transmission electron microscopy (TEM).

Kinetic measurement of aggregation
WT-hβ2m and ΔN6-hβ2m fibrils were assembled in 50 mM MES buffer, 120 mM NaCl at pH 6.2. Fibril growth was performed in a BMG Fluostar Optima plate reader at 37 °C at 600 rpm (or quiescently in the case of seeded reactions). A final concentration of 10 μM ThT (Sigma) and 40 μM β2m was used. When required, seeds (5-30 % v/v) were added. The fibril yield was measured by centrifuging 50 µl of the end points at 14000 g, where amyloid fibrils are found in the pellet and soluble material remains in the supernatant and can be quantified spectrophotometrically.

Determination of the lag time of fibril growth
The lag times of fibril growth under different conditions were determined by fitting a tangent to the curve at the midpoint of the elongation phase and extrapolating this time to the baseline signal in the lag phase. The intersection point of these two lines was considered the lag time.

Determination of the elongation rate and the half time of kinetics in the presence of seeds
ThT fluorescence curves were normalized to the final time point where ~100% of the protein was converted to fibrils. In cases where this was not the case, and therefore there was no plateau in the fluorescence curves (such as in Figures 4B and 4D), the ThT signal was normalized to the corresponding value in the presence of LMW-heparin (Figures 5B, 5D) where the fibril yield was 100%. The observed elongation rate was calculated by fitting straight lines to the ThT fluorescence curves in the first hour of the normalized aggregation kinetics divided by the concentration of seeds. The t₅₀ is the time taken to reach 50% of the maximal ThT fluorescence.

Collagen co-precipitation
40 μM WT-hβ2m (in the presence/absence of 0.1 mg/ml LMW-heparin) was incubated at 37 °C
at 600 rpm in a BMG Fluostar Optima plate reader. At different time points, 40 µl aliquots were taken and mixed with 1 mg/ml of collagen I fibrils and incubated for 10 mins. The sample was centrifuged at 5000 x g for 10 min. Samples in the absence of LMW-heparin were used as a control. The pellet was washed once with the incubation buffer and the centrifugation step was repeated. The pellet and the supernatant were then separately analyzed using 15% (w/v) polyacrylamide Tris-Tricine gels. Gels were stained with Coomassie Instant Blue (Expedeon).

**NMR spectroscopy**

Samples of 15N-labeled protein (40-80 µM) in 50 mM MES buffer containing 120 mM NaCl, pH 6.2 90% (v/v) H2O/10% (v/v) D2O were used for NMR experiments. 1H-15N HSQC spectra were collected in a Varian INOVA NMR spectrometer performing at 600 MHz and were processed in NMRPipe and analyzed using programmes available in CCPNMR analysis(54,55).

**Electron microscopy**

At the end of fibril assembly, 10 µl of sample were applied to carbon-coated EM grids. The grids were then carefully dried with filter paper before samples were negatively stained by the addition of 10 µl of 2% (w/v) uranyl acetate. Micrographs were recorded on a JEOL JEM-1400 electron microscope.
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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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References

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FOOTNOTES

1 The abbreviations used are: h$\beta_2$m, human $\beta_2$-microglobulin; DRA, Dialysis related amyloidosis; ECM, extracellular matrix; ThT, thioflavin T; MHC I, major histocompatibility complex class I; GAG, glycosaminoglycan; LMW, low molecular weight; CMP, collagen mimetic peptide; TEM, transmission electron microscopy.
Figures and Figure Legends

Figure 1

**Figure 1.** (A) Amyloid fibril formation of WT-hβ2m (40μM) in the absence (purple) or presence (blue) of 0.1 mg/ml LMW-heparin. (B) Negative stain transmission electron micrograph (TEM) at the end of the aggregation reaction (80 h) for WT-hβ2m in the presence of 0.1 mg/ml LMW-heparin. (C) Effect of collagen I on WT-hβ2m aggregation in the absence of LMW-heparin. Collagen I concentrations are indicated in the panel in mg/ml. (D) Negative stain TEM of the sample at 1 mg/ml collagen after 80 h of incubation. (E) Effect of collagen I on WT-hβ2m aggregation in the presence of 0.1 mg/ml LMW-heparin. For each condition, a single ThT fluorescence trace
representative of the mean aggregation kinetics taken over at least three replicate experiments, with three samples in each is shown (see also Figure S1). (F) Representative negative stain TEM of the end point of the aggregation process in the presence of 1 mg/ml collagen I and 0.1 mg/ml LMW-heparin. Scale bar in all TEM panels = 100 nm. Note the schematic drawings used to annotate the different reagents present in different experiments (WT-hβ2m (purple), LMW-heparin (grey) and collagen I fibrils (yellow/red bundle)). These symbols are used throughout the manuscript to denote the additives included in each experiment.
**Figure 2**

**Figure 2**: The effect of CMPs and denatured collagen chains on LMW-heparin-induced WT-h\(\beta_2\)m fibril formation. (A) Schematic of collagen hierarchical conformations, from the primary amino acid sequence, single chain PPII helix, triple helix of three single chains, and higher order fibrils. LMW-heparin-induced aggregation kinetics of WT-h\(\beta_2\)m (40\(\mu\)M) in the presence of (B) POG10 peptide (which forms a stable collagen triple helix), (C) collagen I in the single chain form, and (D) Gly-peptide (that does not form a stable triple helix). The concentration of the peptides added is indicated by color in the inset. All traces were obtained with 0.1 mg/ml LMW-heparin. Negative stain TEM micrographs of the end points are shown as an inset for each condition (scale bar = 100 nm). (E) Box plot of the lag time for the WT-h\(\beta_2\)m aggregation in the presence or absence of 1 mg/ml collagen I fibrils (data taken from Figure 1E), denatured collagen, or CMPs. WT (WT-h\(\beta_2\)m alone (no collagen or CMPs added)), Fibril (WT-h\(\beta_2\)m plus collagen I fibrils); POG (WT-h\(\beta_2\)m plus POG10 peptide), SC (WT-h\(\beta_2\)m plus single chain collagen I), Gly- (WT-h\(\beta_2\)m plus Gly-peptide). The data are representative of three replicate experiments, with three samples in each. Asterisk denotes p<0.002.
Figure 3

(A) Co-pelleting of WT-hβ2m aggregated for different times with collagen I fibrils with/without 0.1 mg/ml LMW-heparin. SDS PAGE analysis of the whole sample (w) and the pellet after sedimentation of collagen I by low speed centrifugation (p). Molecular weight markers with masses in kDa are shown alongside. (B) Negative stain TEM micrograph of WT-hβ2m incubated in the presence of 1.0 mg/ml collagen I and 0.1 mg/ml LMW-heparin for 15 h, showing the presence of collagen I fibrils but no WT-hβ2m amyloid. (C), as (B) but image taken after incubation for 65 h. WT-hβ2m amyloid can be clearly seen in this image alongside collagen I fibrils. Scale bar in each micrograph is 500 nm.
Figure 4

Figure 4: The effect of collagen I fibrils on self-seeded and cross-seeded growth with WT-hβ2m monomers. (A) Aggregation kinetics of WT-hβ2m in the presence of preformed WT-hβ2m seeds (5-30% (v/v)). The inset shows an expanded plot over the first ∼ 2 hours. (B) As in (A), but in the presence of 1 mg/ml collagen I. (C) Aggregation kinetics of WT-hβ2m in the presence of preformed ΔN6-hβ2m seeds (5-30% (v/v)). The inset shows an expanded plot over the first ∼ 2 hours. (D) As in (C), but upon addition of 1 mg/ml of collagen I. Three replicate experiments, with three samples in each were measured. Here, a single trace representative of the mean aggregation kinetics is shown. See also Figure S3 for statistics.
Extracellular matrix modulates $\beta_2$m amyloid formation

Figure 5

Figure 5: The effect of LMW-heparin on self-seeded and cross-seeded growth with WT-h$\beta_2$m monomers. (A) Aggregation kinetics of WT-h$\beta_2$m in the presence of preformed WT-h$\beta_2$m seeds (5-30% (v/v)) and 0.1 mg/ml LMW-heparin. The inset shows an expansion of the first ~ 2h. (B) As in (A), but in the presence of 0.1 mg/ml LMW-heparin and 1 mg/ml collagen I. (C) Aggregation kinetics of WT-h$\beta_2$m in the presence of preformed $\Delta N6$-h$\beta_2$m seeds (5-30% (v/v)) in the presence of 0.1 mg/ml LMW-heparin. The inset shows an expansion of the first ~ 2h. (D) As in (C), but in the presence of 0.1 mg/ml LMW-heparin and 1 mg/ml collagen I. Three replicate experiments, with three samples in each were acquired. See also Figure S3.
Figure 6: Schematic of the effect of ECM components on seeded WT-h\(\beta\)\(_2\)m aggregation. (Top) WT-h\(\beta\)\(_2\)m forms amyloid through self-seeded fibril growth. A secondary phase, fibril surface mediated growth, is enhanced by LMW-heparin and a co-mixture of LMW-heparin and collagen I, but is suppressed by collagen alone. (Bottom) Cross-seeding WT-h\(\beta\)\(_2\)m monomer with \(\Delta N6\)-h\(\beta\)\(_2\)m seeds forms fibrils with a different conformation(27). The fibril surface mediated growth of these fibrils is also enhanced by LMW-heparin and the LMW-heparin and collagen I co-mixture, but is not affected by collagen I alone.