Current Topics in Peptide & Protein Research
Vol. 13, 2012

Review

The effect of heparin on amyloid aggregation and toxicity

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ABSTRACT

Charged polyelectrolytes such as glycosaminoglycans are frequently found associated with the proteinaceous deposits in the tissues of patients with amyloid diseases. Experimental evidence indicates that they can play an active role in favoring amyloid fibril formation and stabilization. Binding of glycosaminoglycans to amyloid fibrils occurs prevalently through electrostatic interaction involving the negative polyelectrolyte charges with positively charged side chains residues. Similar to catalyzed reactions, glycosaminoglycans favor aggregation, nucleation and amyloid fibril formation functioning as a structural template for the self-assembly of highly cytotoxic oligomeric precursors rich in β-sheet into harmless mature amyloid fibrils. Moreover, the amyloid promoting activity of glycosaminoglycans can be facilitated through specific interactions via binding sites between amyloid polypeptide and glycosaminoglycan molecules. Numerous studies have identified common structural features in the heparin/heparin sulphate binding sites of proteins. Clusters of basic amino acid residues and consensus sequences consisting of alternating basic and non basic residues are capable to bind the negatively charged heparin as well as other glycosaminoglycan molecules in a variety of proteins that are induced to form β-structure upon interaction with the polyelectrolyte. Interestingly, heparin induces amyloid aggregation of globular proteins that do not exhibit any tendency to aggregate under physiological conditions. The well documented acceleration of fibril formation may open new strategies to limit the cytotoxicity of the early pre-fibrillar aggregates in patients suffering from amyloid diseases.

KEYWORDS: amyloid aggregation, glycosaminoglycans, heparin

INTRODUCTION

Charged polyelectrolytes have frequently been found associated with the proteinaceous deposits in the tissues of patients with amyloid diseases [1, 2]. A careful examination of diseased tissues has revealed the presence of a significant quantity of polysaccharide belonging to the glycosaminoglycan family (GAGs). Among these species, heparan sulfate is the most common, being found in a variety of amyloid disorders including Alzheimer's disease, type II diabetes, light chain amyloidosis, and the prion related diseases [3-4]. Despite the ubiquitous presence of polyanions in amyloid deposits, the degree of specificity and the nature of the interactions involved are controversial. In this review, we analyze some of the most recent results showing that proteins containing exposed cluster of basic residues, undergo amyloid-like aggregation in the presence of GAGs. This occurs also for proteins not involved in amyloid diseases.

Amyloid aggregation

Amyloid diseases are related to anomalies in the folding process of certain proteins that may form insoluble fibril deposits. The aberrant assembly of one of more than 40 human proteins into insoluble

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fibrillar deposits is the hallmark of human amyloid diseases, among which neurodegenerative disorders such as Alzheimer's disease, and non neuropathic conditions such as type-II diabetes [5].

Amyloid diseases differ from each other by the specific protein deposited in the extracellular space and the specific tissues subjected to deposition and degeneration [6]. Although the exact molecular structure(s) of the proteotoxic species and the mechanism(s) of proteotoxicity are still unclear, cell and animal model studies of several amyloidogenic proteins suggest that the oligomers, which may or may not be on pathway to fibril formation, are more deleterious than mature fibrils [7-11]. Amyloid fibrils share common structural features despite the considerable diversity in the primary sequence of the constituent proteins. Amyloids extracted from tissues are typically composed of nonbranching 7 to 10 nm fibrils assembled from two to three 3 nm filaments (protofilaments) twisted around each other. They are rich in β-sheet structures and the ordered regions adopt a classic cross-β structure in which individual strands in the β-sheets run perpendicular to the long axis of the fibril with the inter β-sheet hydrogen bonds oriented parallel to the fibril axis [12-14]. Each disease-specific amyloid contains a unique polypeptide that by a complex and poorly understood *in vivo* mechanism becomes misfolded, forming prefibrillar aggregates that then assemble into highly ordered tissue deposits.

A wide range of proteins and peptides that do not form amyloid *in vivo* can be induced to do *in vitro* and this has led to the hypothesis that the ability to form amyloid is a general property of polypeptide chains [13]. Amyloid fibril formation in bulk solution occurs through a nucleation-dependent polymerization process consisting of two phases, i.e., nucleation and extension. The time course of the conversion of a peptide or protein into its fibrillar form typically includes a lag phase that is followed by a rapid exponential growth phase. The lag phase is assumed to be the time required for "nuclei" to form. The initial step of nucleus formation consists in the association of monomers (Figure 1). This process is thermodynamically unfavorable and is the rate limiting step of the fibrillation process. Once a nucleus has formed, the further addition of monomers to the nucleus becomes thermodynamically favorable and results in rapid extension of amyloid fibrils *in vitro* [15]. As with many other processes dependent on a nucleation step, addition of preformed fibrillar species to a sample of a protein under aggregation conditions ("seeding") causes the lag phase to be shortened and ultimately abolished when the rate of the aggregation process is no longer limited by the need for nucleation [5].

The path of fibril formation usually begins with prefibrillar kinetic precursors, collectively indicated as protofibrils or soluble oligomeric intermediates, which appear as globules 2.5-5.0 nm in diameter or larger, with an intrinsic tendency to further organize into pore-like annular and tubular structures [16-19]. The interest for such prefibrillar intermediates has recently grown, since in most of cases, they have been shown to display the highest cytotoxicity, whereas mature fibrils appear less toxic or even harmless [20-24]. The specific mechanism by which these species appear to mediate their toxic effects is not completely understood; probably, toxicity is mediated by common structural features shared by prefibrillar precursors [25, 26]. These results have led to the proposal that the molecular basis of cell and tissue impairment may be related to the transient appearance of prefibrillar assemblies, under conditions where their intracellular levels rise due to any dysfunction of the cellular clearing machineries [27].

Until very recently, it was thought that only polypeptide chains associated with clinical disorders were able to form amyloid fibrils. A number of recent studies have, however, shown that proteins unrelated to diseases, under suitable conditions, can form aggregates *in vitro* with structural and cytotoxic properties that closely resemble those of the amyloid fibrils that are formed in diseased tissues [28-34]. These observations have led to the hypothesis that the ability to form amyloid structures is a generic property of proteins resulting from stable interactions primarily involving the main chain that is common to all polypeptides [35-37].

Heparin-induced amyloid aggregation

Glycosaminoglycans (GAGs) are the most abundant heteropolysaccharides in the body. They are long

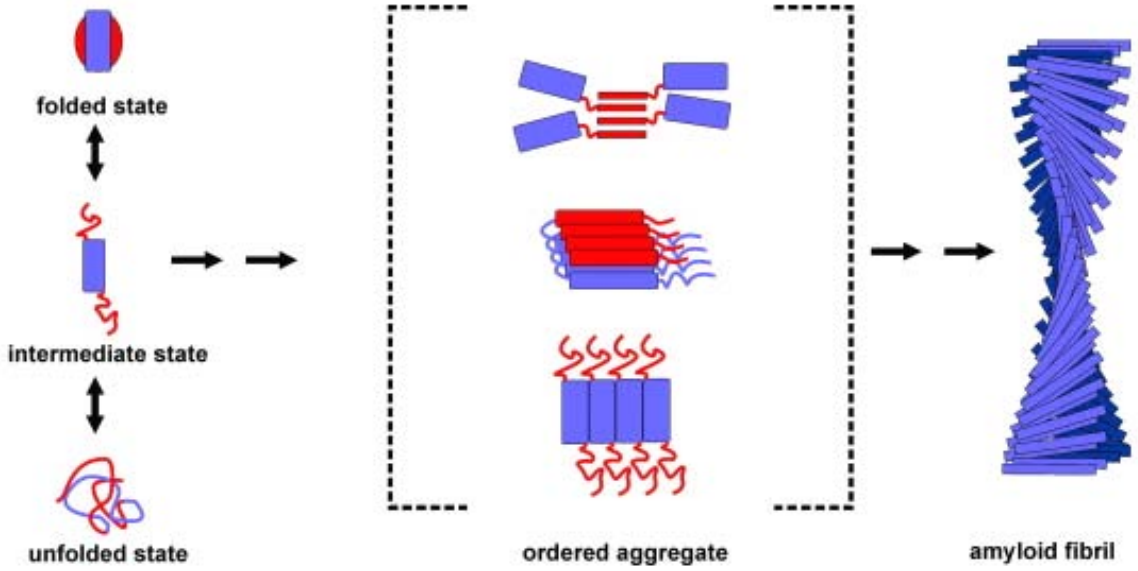

Figure 1. Partly folded polypeptide chains, released from ribosomes (the protein-synthesizing machines), normally collapse into correctly folded, functional proteins. Partly folded polypeptides sometimes associate with similar chains to form aggregates. Aggregates vary in size from soluble dimers and trimers up to insoluble fibrillar structures. Both soluble and insoluble aggregates can be toxic to cells through interactions with cell membranes.

unbranched molecules consisting of disaccharide repeating subunits, having molecular weights of roughly 10-100 kDa (Figure 2). The disaccharide units contain either of two modified sugars, *N*-acetylgalactosamine (GalNAc) or *N*-acetyl-glucosamine (GlcNAc), and a uronic acid such as glucuronate or iduronate. GAGs are highly negatively charged molecules, with extended conformation that imparts high viscosity to the solution. There are two main types of GAGs. Non-sulphated GAGs include hyaluronic acid, whereas sulphated GAGs include chondroitin sulphate, dermatan sulphate, keratan sulphate, heparin and heparan sulphate.

GAGs are located primarily on the surface of cells or in the extracellular matrix (ECM) of multicellular organisms, where they can be found either covalently linked to the protein core of proteoglycans or as free macromolecules. Moreover, GAGs have been found to be closely associated with all amyloid fibrils isolated from humans [1], and there is evidence that they play an active role in favoring amyloid fibril formation and stabilization [38, 39]. Snow and Kisilevsky [40] reported an increase in GAG levels at the time of serum amyloid A deposition. More recently, it was demonstrated that inhibition of heparin

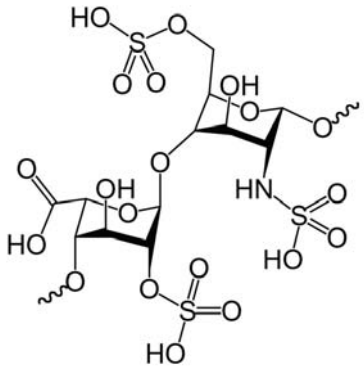

Figure 2. GAGs are highly negatively charged molecules, with extended conformation consisting of disaccharide repeating subunits. The disaccharide units contain either of two modified sugars, *D*-glucosamine or *N*-acetylglucosamine (GlcNAc), and a uronic acid such as glucuronate or iduronate.

sulfate biosynthesis is directly correlated with loss of amyloid deposition in amyloid A animal models [41-43].

Evidence for the relation between GAGs and amyloid comes also from *in vitro* studies. GAGs, particularly heparan sulfate (HS) and its highly sulfated derivative heparin, stimulate, *in vitro*, the formation of amyloid fibrils from the Alzheimer

Aβ protein [44-46]; heparin and, to a lesser extent, heparan sulfate have been reported to increase significantly the rate of fibrillation of tau protein [47], α-synuclein [48], gelsolin [49], β2-microglobulin [50, 51], acyl-phosphatase [52], islet amyloid polypeptide (IAPP) [53], immunoglobulin light-chain protein [54] and the aortic amyloid polypeptide medin [55]. Heparan sulfate has also been found to convert the prion protein from the PrP^{C} to the PrP^{SC} form [56].

Generally, among GAGs, heparin is particularly effective in accelerating fibril formation probably because of its high content of sulfate groups [44]. Several studies have demonstrated that electrostatic interactions are important in the binding of heparin to amyloid fibrils. In particular, removal of all sulfate groups from heparin or the addition of magnesium or calcium ions suppresses these interactions, thereby indicating their electrostatic nature [44, 51]. Moreover, it has been postulated that the amyloid promoting activity of heparin is facilitated through specific amyloid polypeptide-heparin interactions via binding sites [57-64].

Notwithstanding the large body of data associating heparin and other GAGs with amyloidogenesis, little is known about the mechanism by which heparin promotes amyloid formation or about its effect on the overall aggregation pathway. It has been supposed that, similar to catalyzed reactions, GAGs favour aggregation, nucleation and amyloid fibril formation by a mechanism substantially different from that occurring in bulk solution [65]. The data available suggest that heparin can influence and promote the misfolding of polypeptides into pro-amyloidogenic intermediates rich in β-sheet and may also function as a structural template for self-assembly. Recent studies on acetyl phosphatase have shown that heparin sulfate induces changes in the aggregation process by splitting it in a parallel manifold faster pathway [66].

These observations suggest that GAGs could play an active role in amyloidogenesis *in vivo*, perhaps even a protective role, by quaternary structural conversion of proteotoxic soluble oligomers into less toxic amyloid fibrils and related cross-β-sheet aggregates. While a variety of hypotheses have been put forward to explain the mechanisms by which GAGs facilitate amyloid fibril formation, scant mechanistic data are available, and the precise mechanism by which GAGs accelerate amyloidogenesis is subject to debate.

Motamedi-Shad *et al*. [52] showed that heparan sulfate accelerates the conversion of acylphosphatase from the native state into the amyloidogenic, yet monomeric, partially folded state. They also indicate that heparan sulfate does not simply accelerate the conversion of the resulting partially folded state into amyloid species, but splits the process into two distinct pathways occurring in parallel: a very fast phase in which heparan sulfate interacts with a fraction of protein molecules causing their rapid aggregation into β-sheet containing oligomers and a slow phase resulting from the normal aggregation of partially folded molecules that cannot interact with heparan sulfate. Overall, the results indicate that heparan sulfate can both destabilize the initial folded state, accelerating its transition to the aggregation prone state, causing a manifold acceleration on the subsequent self-assembly of partially unfolded monomers into amyloid aggregates, with the latter effect appearing to be larger than previously thought.

More recently, Bourgault *et al*. [67] proposed that sulfated GAGs accelerate TTR amyloidogenesis without influencing the initial steps of the TTR amyloidogenesis cascade, which includes tetramer dissociation, partial misfolding of the released monomer to form the amyloidogenic monomer, and formation of TTR oligomers. The sulfated polymeric surface of GAGs interacts with TTR oligomers, primarily through electrostatic interactions, concentrating TTR oligomers and possibly orienting them so as to accelerate the formation of larger aggregates by quaternary structural conversion (Figure 3). The high density of sulfate groups and the polymeric nature of GAGs seem to be essential for binding to multiple TTR oligomers simultaneously and converting them into higher molecular weight aggregates, possibly by preferentially aligning them. The binding of heparin to amyloid proteins has been reported to increase the degree of order of the protein within the aggregates, thus favoring the fibrillation process [51].

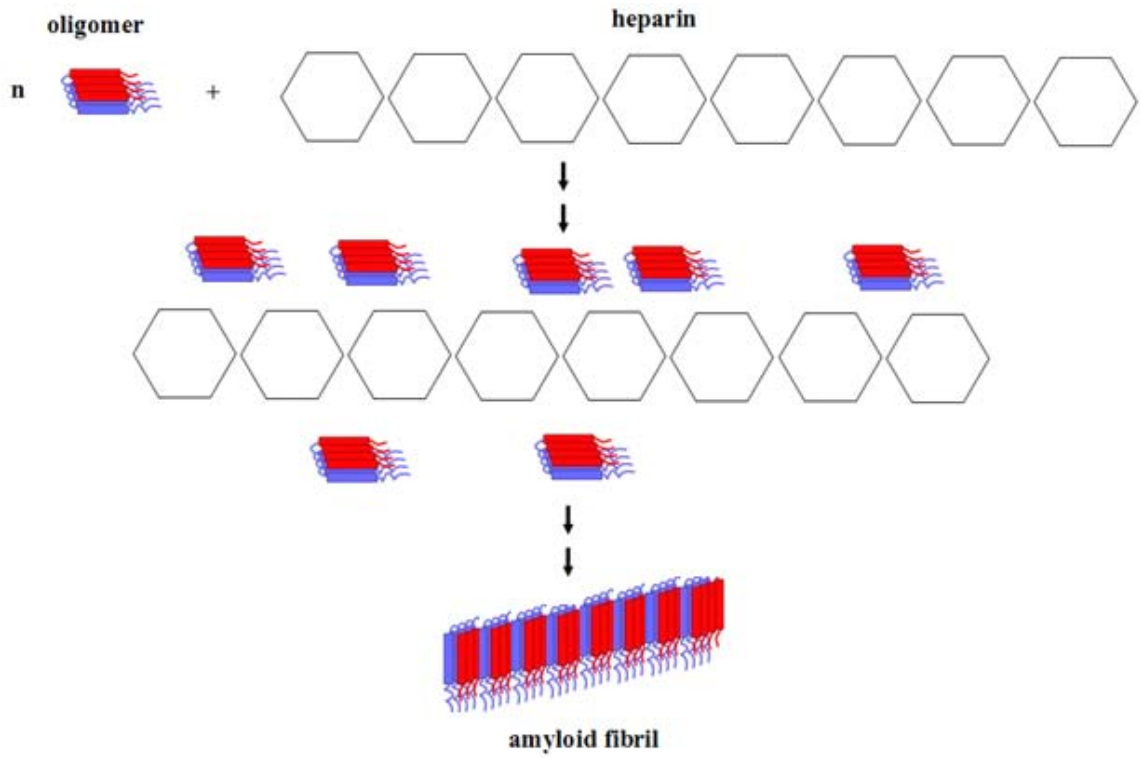

Figure 3. Alignment of TTR oligomers on heparin molecule accelerates the process of fibril formation.

Molecular recognition of heparin by proteins

Numerous studies have identified common structural features in the heparin/heparin sulphate binding sites of proteins. Different structural (NMR spectroscopy and X-ray crystallography) and molecular modeling approaches have been used to elucidate the GAG-protein interactions [68]. Side-chains of Asn, Asp, Glu, Gln, Arg, His and Trp are more likely to form the binding sites for non-sulphated carbohydrates than other amino acids [69-71]. The aromatic indole ring of Trp residue can pack against the hydrophobic face of a sugar molecule and has a significantly higher mean solvent accessibility in carbohydrate binding locations. The aliphatic residues of Ala, Gly, Ile and Leu, which are usually buried inside proteins, do not appear to participate in sugar binding. Clusters of positively charged basic amino acids can form ion pairs with spatially defined negatively charged sulphate or carboxylate groups on heparin chains. Glycosaminoglycans interact with residues that are prominently exposed on the surface of proteins. The main contribution to binding affinity comes from ionic interactions between the highly acidic sulphate groups and the basic side chains of arginine, lysine and, to a lesser extent, histidine [72]. The relative strength of heparin binding by basic amino acid residues has been compared and arginine has been shown to bind 2.5 times more tightly than lysine. The guanidino group in arginine forms more stable hydrogen bonds as well as stronger electrostatic interactions with sulphate groups. The ratio of these two residues determines, in part, the affinity of a binding site in a protein for GAGs [73].

Although the interactions of GAGs with proteins also involve a variety of different types of interactions, including van der Waals forces, hydrogen bonds and hydrophobic interactions with the carbohydrate backbone, the formation of ion pairs between positively charged side-chains and negatively charged GAG's group is certainly the most prominent cause of GAG-protein interaction. It has also been observed that heparin-binding domains contain amino acids such as asparagine and glutamine which are capable of hydrogen binding. The affinity of heparin-binding proteins for heparin/heparan sulphate is also

enhanced because of the presence of polar residues with smaller side chains like serine and glycine.

To understand the molecular recognition of heparin by proteins, Cardin *et al*. [74] determined the structure of the heparin-binding regions of apolipoprotein (apo) B-100, the major protein constituent of human plasma low density lipoproteins (LDL). They showed that LDL contains five to seven heparin-binding sites of high positive charge density, the amino acid sequences of which were determined [75]. The same regions were also identified by Weisgraber and Rail [76]. The amino acid sequence of the heparin binding regions of apo B-100 was found very similar to that of apo E1819 and human vitronectin (Vn)20 with respect to the organization of basic and hydropathic residues [77, 78]. Starting from these considerations, Cardin and Weintraub [79] suggested that heparin binding domains usually contain the consensus sequences XBBBXXBX or XBBXBX, where B is a lysine or arginine (with a very rare occurrence of His) and X is a non basic amino acid. The residues Asn, Ser, Ala, Gly, Ile, Leu and Tyr are more common at positions 'X'. Residues such as Cys, Glu, Asp, Met, Phe and Trp exhibit a very low occurrence at positions 'X' in either the α-helical or β-sheet domains of heparin binding proteins. Depending on the secondary structure of the protein, very few residues in these consensus sequences may actually participate in GAG binding sites. In particular, if the consensus sequence XBBBXXBX belong to an α-helix, basic side-chains are usually displayed on one side forming an amphiphatic helical arrangement. Therefore, in order to interact with a linear GAG chain, it would be predicted that the positively charged amino acid residues in α-helical proteins would have to line up along the same side of the protein segment.

In β-strands, the positively charged residues in a GAG-binding protein are located in a different way compared with α-helical structures. The basic amino acids in the sequence XBBXBX line up on one face of a β-strand, whereas the hydropathic residues points back into the protein core. However, many proteins that bind heparin do not possess these sequences [78, 80]. One model has suggested that a spacing of 20 Å between two basic amino acids is a critical determinant of heparin binding ability [81]. Such spacing can be achieved by a peptide in α-helical conformation by basic amino acids spaced 13 residues apart or, in β-strand conformation, 7 residues apart. Clusters of basic amino acid residues capable of binding to the negatively charged heparin molecules have been also described in a variety of proteins that are induced to form β-structure upon heparin interaction.

The effect of heparin on apomyoglobin

Apomyoglobin (a protein that does not show any tendency to aggregate and to form fibrils under physiological conditions) contains three consensus sequences corresponding to the consensus sequences identified by Cardin and Weintraub that are localized in the turn regions between helices C–D, E-F, and F-G. Moreover, clusters of basic amino acids that do not conform to consensus sequences are present in the primary structure at the end of the B helix, i.e., RLFKSH, the beginning of the E helix, i.e., LKKHG, and at the end of the G-helix, i.e., HVLHSRH (Figure 4).

The addition of heparin to wild type apomyoglobin causes amyloid aggregation and fibril formation that is much more evident on lowering the pH from 7.0 to 5.5 (Figure 5). Vilasi *et al*. [82] monitored the structural evolution of the forming aggregates using FTIR spectroscopy, ThT fluorescence and electron microscopy. In particular, they found that the FTIR spectra of the wild-type apomyoglobin at pH 5.5 in the presence of heparin recorded 2 days after the onset of aggregation shows an amide I' maximum close to 1625 cm^{-1}, which clearly indicates the presence of the cross-β structure. The ThT fluorescence confirmed this result. As expected, in the absence of heparin, ThT fluorescence did not increase at either pH 7.0 or pH 5.5. Conversely, ThT reactive aggregates were readily formed in the presence of heparin (Figure 5A). Finally, electron microscopy showed the presence of fibrillar structures (Figure 5B). The effect of the heparin-induced wild-type apomyoglobin aggregates on cell viability was also examined by the same authors. Aggregates were added to the cultured cells at various times after aggregation onset (Figure 5C). The protein aggregates formed at the start of aggregation at

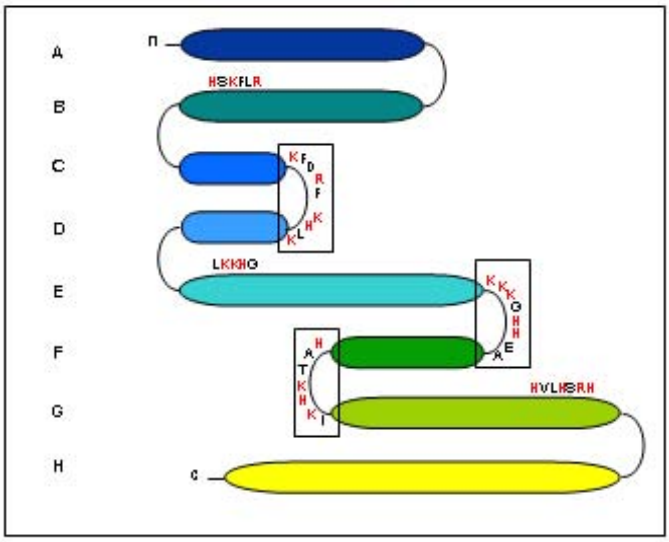

Figure 4. Consensus sequences for heparin binding along the helices in apomyoglobin structure. Each of the 8 α-helices is marked with a letter and is represented by an ellipsoid of size proportional to the length of the sequence. Residues corresponding to Cardin and Weintraub motifs are boxed. Basic residues are shown in red.

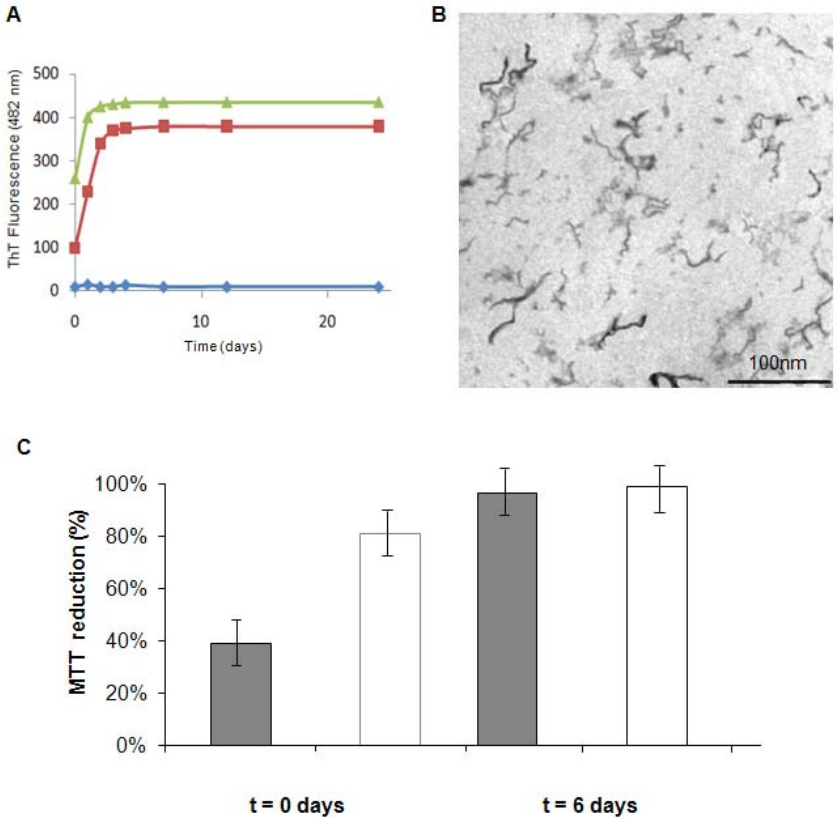

Figure 5. Effect of heparin on wild-type apomyoglobin amyloid aggregation and cytotoxic activity. Panel A: ThT fluorescence in the absence (♦) and in the presence of 0.1 mg/mL heparin at pH 5.5 (▲) and at pH 7.0 (■). Panel B: Electron microscopy image of the protein in the presence of 0.1 mg/mL heparin at the beginning of the aggregation process. Panel C: Cell viability of NIH-3T3 cells exposed to wild-type apomyoglobin aggregates formed in the presence of 0.1 mg/mL heparin at pH 7.0 (grey) and at pH 5.5 (white) detected by MTT assay. Aliquots of protein were taken at 0, and 6 days from the onset of the aggregation process and incubated for 24 h with cells. Data are expressed as average percentage of MTT reduction +/- SD relative to cells treated with medium plus heparin, from triplicate wells from 5 separate experiments.

pH 7.0 killed about 60% of cells, whereas the aggregates formed at pH 5.5 were harmless. Six days after aggregation onset, the aggregates formed at both pH values were not cytotoxic. The different cytotoxicity of the aggregates formed at the start of aggregation at pH 7.0 and pH 5.5 could be related to their different compactness. At pH 7.0, the low number of electrostatic interaction between heparin and protonated hystidyl residues makes the aggregates less compact thereby determining an increase of their exposed hydrophobic area [83]. This different toxicity could also be due to the acceleration of the fibrillization process that occurs at pH 5.5 (Figure 5A). In fact, it could well be that under these conditions, a reduced steady-state level of early toxic aggregates is reached consequent to the increased rate of oligomer growth into harmless higher order assemblies, as recently reported for TTR aggregation in the presence of heparin [67].

The observation that the addition of salt at pH 5.5 does not influence the heparin induced aggregation profile indicates that the increased aggregation is not only related to histidine protonation but also to a greater propensity of the protein to undergo structural modifications. It has been shown that lowering pH from 7.0 to 5.5 reduces the conformational stability of apomyoglobin by about 2-3 kcal/mole thereby making the protein more susceptible to perturbing agents [84, 85]. In this context, the proton gradient formed in proximity of the heparin surface is likely to modify the structural properties of the protein and possibly favor its misfolding.

Taken together, the results obtained by Vilasi *et al*. [82] with wild-type apomyoglobin indicate that heparin is able to induce an amyloid aggregation process that readily terminates with the formation of a fibrillar species rich in cross-β structure.

CONCLUSIONS AND PERSPECTIVES

A number of challenges lie ahead in the investigation of the interactions of GAGs with proteins from a structural and functional point of view. For example, heparin is known to interact with a number of proteins but the precise mechanism of interaction and the induced effect are not perfectly known. In the case of amyloidogenic proteins, the findings so far reported collectively may suggest the use of these compounds as potential therapeutic agents for amyloid associated diseases for their ability to reduce the concentration of highly cytotoxic species [86, 87]. However, radical new insights into structure-function relationships concerning GAGs-protein interaction will help to resolve many of the outstanding problems in this field.

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