Self-assembly of Homogentisic acid may have implications in Alkaptonuria

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Abstract: Alkaptonuria is a rare inborn-error of metabolism caused by the accumulation of Homogentisic acid (HA). Herein, we have studied the self-assembling and aggregation properties of HA with the aim to assess the effect of accumulation of HA inside the body. To our surprise, we noted aggregation of HA follows an amyloidogenic pathway and the fibrillar assemblies made from small globules are formed after ageing HA solution. These assemblies were characterized by conventional microscopy tools and their amyloidogenic nature was assessed by Thioflavin T binding assays. The cytotoxicity analysis by MTT assay suggest cellular viability was decreased in both mouse neuronal and normal fibroblast cells when they were co-incubated with the aged solution of HA. Hence, the results presented in the manuscript are of significant interest in understanding the etiology of alkaptonuria form an amyloid perspective and may have possible implication in its therapeutic cure in future

Introduction

Alkaptonuria is a rare inborn-error of metabolism caused by the deficiency of homogentisate dioxygenase (HGD) enzyme which is responsible for the metabolism of tyrosine and phenylalanine.\textsuperscript{1-4} As a result, accumulation of homogentisic acid (HA) occurs which causes build up of a dark ochronotic pigment called Alkapton.\textsuperscript{2} The therapeutic cure for the rare disease is still unknown despite several efforts.\textsuperscript{4} Hence, it is imperative to understand the etiology or molecular mechanism behind the pathogenesis of Alkaptonuria which can greatly aid to unravel its therapeutic cure. Recently, the concept of metabolite amyloids and its implications in the pathogenesis of rare in-born errors of metabolism has garnered keen
interest. Hence, the molecular mechanism behind the pathogenesis of Alkaptonuria should also be investigated from an amyloid perspective and the aggregation characteristic of the associated metabolite which is present in excess in this disease should be studied.

Conventionally, the word amyloid was referred to aggregated formed by misfolding of proteins and peptides which cause diseases such as Alzheimer’s, Parkinson’s and a plethora of Prion diseases. However, the ground breaking research by Gazit and co-workers which revealed amyloid like toxic structure formation by single amino acid Phenylalanine and its implications in the etiology of Phenylketonuria paved the way for the extension of generic amyloid hypothesis to the metabolites and illustrated the crucial role of amyloidogenic pathway in the pathogeneses of wide range of IEMs. Hence, the further studies illustrated amyloid like structures formed by tyrosine which induces cross-seeding in nearby protein and produce a lethal trap. Similarly, fibrillar structures formed by tryptophan which induces apoptosis in cells and haemolysis caused by phenylalanine fibres was reported. Further, it was also reported that metabolite assemblies formed by aromatic amino interact with cell membrane and can penetrate inside it thus causing membrane disruption. Our group for the very first time illustrated amyloid-like structures formed by non-aromatic aminoacid cysteine and methionine. Subsequently we also reported unusual aggregates formed by proline, hydroxyproline and lysine. We are currently also studying the aggregation characteristics of non polar aromatic amino acids and its association to amyloid. Wangoo and co-workers have also studied self-assembly of amino acids extensively and reported amyloid like structures formed by the self-assembly of Aspargin. Apart from amino acids other non proteinaceous metabolites like uracil, cystine, orotic acid were also reported to form amyloid like structures. The fibrillar assemblies formed by quinolinic acid induced apoptosis. Accumulation of adenine and its fibrillation has been studied in yeast model. Similarly oxalic acid fibres are reported to induce retinopathy. Recently, glucocerebrosides have also
been illustrated to form amyloid like assemblies and it was surmised that such aggregation may play a crucial role in the pathogenesis of Gaucher’s disease. Further, Kar and co-workers recently reported amyloid mimicking assemblies formed by aspartame and dopamine.

Hence, inspired by these literature studies and our own interest in self-assembly and amyloid studies, we investigated the self-assembling properties of homogentisisic acid with the aim to understand its implications in the etiology of Alkaptonuria. Hence, we investigated the self-assembled structure formation by HA under varying time period and to our surprise noted HA assembled to fibrils when it is incubated in solution for longer time. The Thioflavin T assay also suggest formation of thin amyloid like fibrils after ageing. The atomic force microscopy suggest these febrile clusters are made of small globules clustered over one another. Small globuler structure can also been seen besides clustered fiber like deposit in SEM. Finally the MTT assay reveled increased cytotoxicity of HA upon ageing and it was noted that HA induced toxicity both in mouse normal fibroblast and neural cell lines. Notably the cell viability was deacreased upto 80% at 10 mM concentration of HA when coincubated with mouse neural N2a cells.

**Result and Discussion**

The chemical structure of HA has both phenolic as well as acidic group (Figure 1a). Hence, both pi-pi stacking as well as hydrogen bonding interactions can be surmised to induce self-assembly. Hence HA was dissolved in varying concentration 100µM, 1mM and 10mM in deionized (DI) water to assess its self-assembly. The self-assembled structures were initially studied by optical microscopy which suggests formation thick fibre like bundles at random places for 1mM HA. The observation suggested aggregation propensity of HA Hence the solution was diluted to 100 µM with the aim to achieve amyloid likeness fibrillar assemblies. However, at 100 µM very small aggregates were seen, hence the solution was aged for 10
days, after which thin fibrillar structures formed by aggregation of small globules could be observed. The SEM micrograph also revealed small random aggregates at 100µM HA (Figure 1b). However, after 10 days of ageing fibre like bundles adhered to surface could be observed. Interestingly, at high resolution small globules could be observed, which suggests the fibrillar morphologies are formed by aggregation of small globules. Further, as observed in case of OM, thick fiber like bundle could be observed in fresh HA at 1mM which after ageing formed clustered bundle like aggregates which also reveal globular structures at random places.

Fibres, to confirm observations of OM and SEM, we also resorted to atomic force microscopy which also complemented the observations of SEM as before ageing HA revealed random aggregates at isolated places but after ageing clustered globular structures were observed. The observation confirmed that small clustered globules appear like fibrils.
bundles adhered to surface in SEM and OM. The 2D and 3D micrograph of AFM before and after ageing the samples of 100µM HA are shown in Figure 2.

![Atomic Force Microscopy analysis of HA aggregates before and after ageing.](image)

**Figure 2.** Atomic Force Microscopy analysis of HA aggregates before and after ageing.

Further, to assess the underlying forces driving the self-assembly of HA we coincubated HA with urea and tannic acid (TA). Urea is a known hydrogen bond breaker. Hence, if urea is co-incubated with the assemblies the role of hydrogen bonding interaction could be surmised. Interestingly when urea was added the febrile morphology observed change to globular sphere like structures. The observation suggest crucial role of hydrogen bonding interactions and suggest in absence of hydrogen bonding the pi-pi stacking interactions which will occur in HA due to the presence of aromatic ring will get strablized and drive formation of globules. However, when hydrogen bonding is there the globules tend to fuse with each to form thick bundle like febrile morphology. Similarly TA is a well-known amyloid inhibitor which works
by disrupting pi-pi stacking interaction. In presence of TA also the morphologies transformed to small globules however the aggregation propensity was much less as compared to that observed after urea addition. The observation again confirmed that if hydrogen bonding alone is the driving for aggregation could take place, however, the pi-pi stacking is the most crucial interaction. The interplay of hydrogen bonding as well as pi-pi stacking will caused clustered fibrillar bundle in HA. However, when these forces are present alone small globular aggregates will be seen. The study also present Pi-PI stacking interaction as stronger dring force for aggregation in HA as compared to hydrogen bonding.

![Figure 3](image_url)

**Figure 3.** Cross-seeding experiment of HA with urea, Phe and TA.

Next, we also cross-seeded phenylalanine fibers with HA assemblies. Phe is also the smallest reduction model for amyloid studies. The co-incubation studies suggest fibrillar structure of Phe changes to small globules as HA might interfere with the pi-pi stacking interactions present in Phe alone. These studies also confirm cross talk of HA assemblies with Phe and suggest the aggregation propensity of Phe is enhanced in presence of HA and the HA assemblies induces pi-pi stacking interactions which are acting unidirectionally in case of Phe to make it birediction resulting in transition from fibrillar to globular shapes (Figure 3).
Further, to study the amyloid character of HA assemblies both in solution and solid state we performed Thioflavin T (ThT) binding assays. The fluorescence of ThT is enhanced in solution when co-incubated with amyloid fibrils. Phe is the most studied metabolite whose amyloid nature is well known. Hence, in our studies we took Phe as a control. ThT binding assay in solution suggest enhanced fluorescence of ThT when aged HA samples assemblies were co-incubated with it. Further the enhancement in fluorescence was higher in case of HA as compared to Phe. Further, when the two assemblies were co-incubated the fluorescence was enhanced more suggesting the amyloid character is enhanced. The ThT binding was also studied microscopically and it was observed that aged HA samples form fibrillar assemblies which could bind ThT. On the other hand in fresh sample the ThT could be seen binding only at borders where the HA gets accumulated due to drying.

![Figure 4. Thioflaving T binding assays of HA. (a)](image)

Finally, if HA assemblies have amyloid character they should also induce toxicity in cells. Hence the Fresh and aged sample of HA was co-incubated with mouse neural N2a Cells and mouse normal fibroblast L929 cells. The MTT assay suggest aged samples of HA caused drastic decrease in cellular viability in both the cell lines (Figure 5). Even the fresh sample of HA reduced cell viability. However after ageing, the cellular viability was decreased by more that 80% in both neural as well as normal fibroblast. The study confirms cytotoxic nature of aged assemblies.
Conclusions

The self-assembling properties of HA have been studied in detail by different microscopy methods. The self-assembly studies suggest aggregation of HA is concentration as well as time dependent. At low concentration bundle-like fibres adhered to the surface appear after ageing for 10 days. The SEM and AFM studies suggest small globular structures fuse with each and appear like fibres. The role of pi-pi stacking and hydrogen bonding in aggregation of HA were investigated by co-incubation studies with urea, TA and Phe. The MTT assay suggests cytotoxicity is significantly enhanced in cells after addition of aged samples of HA implicating the ageing process induce formation of amyloid-like self-assemblies. Hence the
results presented may have implications in understanding the patho-physiology of Alkaptonuria, a disease associated with the accumulation of HA from an amyloid perspective.

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**References**


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