Aggregation characteristics of non-aromatic polar amino acids and its association to amyloids

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Abstract: Aggregation of amino acids to amyloid like structures is known to have implications in the pathophysiology of single amino acids based inborn-errors of metabolism (IEMs). Studying the aggregation properties of amino acids is of crucial interest also to understand the association of these IEMs to amyloid associated diseases. Hence, herein we have studied the self-assembly of different non-aromatic charged/uncharged polar amino acids namely L-Glutamine (Gln), L-Aspartic acid (Asp), L-Glutamic acid (Glu) L-Histidine (His), L-Arginine (Arg), L-Serine (Ser) and L-Threonine (Thr) whose amyloid characteristics have still not been explored by ageing them for varying time intervals from 0-15 days in aqueous solution. The structure formation by the self-assembly of these amino acids were then studies by microscopy. Notably, of all amino acids glutamine revealed amyloid like febrile morphologies as observed in case of aromatic amino acids. The MTT assay also revealed a relatively more cytotoxic nature of glutamine assemblies as compared to other amino acid aggregates and suggests it may have amyloid like characteristics. Along with Gln, Asp and Glu also revealed formation of some unique self-assembled structures. The thioflavin T assay suggests these aggregates may have amyloid nature. Hence, the aggregation studies of these amino acids may have important
implication in the pathogenesis of disease caused by the accumulation of glutamine, aspargine and aspartic acid.

1. Introduction:
Aggregation of proteins or peptides lead to formation of amyloid structures which lead to different types of diseases such as Alzheimer’s, Parkinson’s and type-II diabetes to name a few.\textsuperscript{1} Parkinson's disease is caused by self-assembly of \(\alpha\)-synuclein,\textsuperscript{2} while Alzheimer’s disease is caused by self-assembly of A\(\beta\) peptides\textsuperscript{3} and Insulin amyloid polypeptide (IAPP) aggregates in diabetes mellitus (type 2 diabetes).\textsuperscript{4} etc. Gazit and coworkers for the very first time reported self-assembly of aromatic single amino acid Phenylalanine (Phe) and its implications in the etiology of phenylketonuria. Subsequently, they reported apoptosis inducing fibers formed by self-assembly of Tryptophan (Trp).\textsuperscript{5} Further, they illustrated formation of antibodies against tyrosine fibres. Hence, the studies by Gazit and coworkers suggest etiology of IEMs like Phenylketonuria,\textsuperscript{6} Tyrosinemia,\textsuperscript{7} Hypertryptophanemia may be associated to amyloid diseases and hence they proposed a generic amyloid hypothesis, which suggest a common pathophysiology for the wide range of diseases via amyloidogenic pathway.\textsuperscript{8} Subsequently, studies by other research groups re-affirmed the hypothesis. In this context, Bowers and co-workers investigated self-assembled structures of phenylalanine through ion-mobility mass spectrometry and found the presence of sideways \(\pi\)-stacking interactions, between the tubes (T-shaped \(\pi\)-stacking), together with \(\pi\)-stacking along the fibril axis within the tubes. This structure explained the cytotoxic nature of Phe fibers as their hydrophobic outer surface will facilitate cell penetration while hydrophilic inner core will disrupt ion channel leading to cell death. Kar \textit{et al.} illustrated intrinsic property of phenylalanine to trigger protein aggregation and hemolysis and showed its direct relevance to phenylketonuria.\textsuperscript{9} Kar \textit{et al.} recently also reported the ability of
tyrosine to seed aggregation of other cellular protein and create a "lethal trap" which ultimately resulted in cytotoxicity and cell death.\(^9\) Our group has also reported amyloid like toxic self-assembled structures formed by the aliphatic amino acids Cysteine and Methionine, and suggested its implications in etiology of cystinuria, Hypermethioninemia.\(^{10}\) Sarkar et al. reported amyloids formed by methionine and its cross seed with Phe disrupts DMPC lipid membrane, and deforms red blood cells. Recently we also reported unusual aggregates formed by proline, hydroxyproline, and lysine which also revealed cytotoxicity to ShS5Y neural cell lines as confirmed by MTT assays.\(^{11}\) Hence, motivated by the previous research studies and our own investigation on single amino acid self-assembly and its implications in diseases, we were motivated to study the aggregation properties of other non-aromatic amino acids (Gln, Ser, Thr, Ala, Asn, Arg, Asp, Glu) and one aromatic acid (His) extensively under varying concentration and ageing time. The excess of these amino acids in body causes disease conditions like hyperserinemia,\(^{12}\) histidinemia\(^{13}\) and threoninemia.\(^{14}\) Thus, in the present study, we studied aggregation properties of Gln, His, Ser, Thr, Ala, Arg, Asn, Asp and Glu in neutral conditions extensively via microscopic studies.
Figure 1. Chemical structure of (a) L-Alanine (b) L-Asparagine (c) L-Arginine (d) L-Serine e) L-Threonine f) L- Glutamine g) L-Histidine h) L-Aspartic acid and i) L-Glutamic acid.

2. Materials and Methods:

Amino acids used in the experiment are of analytical grade obtained from Sigma aldrich and used without further purification. The sample preparation was done in deionized water. Each amino acids were prepared 10 mM as stock solution. Further dilute it to 1-10 millimolar (mM) concentration in water. From stock solution (fresh to 15 days samples) of each concentration 20μl aliquot were taken and putted on glass slide and allowed it to dry for 12 hours. After proper drying images were taken by using Optical microscopy (Leica DM2500) in 40 X and 63 X
magnifications. In addition to that, Phase contrast images were taken (40X) by using the same microscopy. For Tetrahydrofuran (THF)- Water study, the THF is prepared 5-80% in water and followed the above procedure for taking optical microscopic images. Methanol-Water study were performed with 5-80% of methanol in water. In addition to that, we analysed the structural change in acidic (pH 2) as well as basic (pH 10). For Thioflavin T (ThT) binding assay Co-incubate the sample with 20 μM concentration of ThT for half hour and put it under red and blue filter of fluorescence microscopy.

3. Result and Discussion:

To understand the aggregation characteristics of non-aromatic amino acids having functional groups in their side chaing we studied self-assembly of different aliphatic charged/uncharged amino acids by incubating them in aqueous solution for varying time intervals. Hence, the amino acids were incubated for varying time intervals from 0 to 15 days using 1mM, 3mM and 5mM concentration. The structure formation in amino acids were assessed by studying their morphologies through optical microscopy (OM) or Scanning Electron Microscopy (SEM). The FESEM images of alanine (Ala), histidine (His), serine (Ser), threonine (Thr), arginine (Arg) and aspargine (Asp) are shown in Figure 2a-f.

The FE- SEM image suggest amyloid like fibrillar structure are not formed in either of these amino acids under all concentrations and conditions. Ala is conventionally, used as negative control in amyloid studies of amino acids hence the results were expected and some crystalline aggregates could be observed in fresh condition which do not revealed any structural change during the course of ageins (Figure 2a) FESEM image of Histidine shows twisted nanosheet like morphologies (Figure 2b) in fresh conditions. After ageing, no significant morphological transitions could be observed and the structures could be discerned in fresh conditions only. The
FESEM image of L-Serine show thick tape like aggregates after 15 days of ageing (Figure 2c) while L-Threonine also revealed branched crystalline tape like aggregates after 15 days (Figure 2d). L-Arginine-HCl come twisted bundle could be seen after 10 days while in L-Asparagine no specific structure could be discerned neither in fresh or aged conditions (Figure 2e, f). However, for Glutamine we could observe amyloid like fibrillar structures after 10 days of ageing (Figure 2g) and certain interesting unusual uniform self-assembly after 10 days of incubation could also be noted in Aspartic acids and Glutamic acid (Figure 2h-i).

**Figure 2.** SEM image of amino acids after incubating it in water varying time interval at 37.4°C. a) alanine fresh; b) histidine fresh; c) serine after 15 days of incubation; d) threonine after 15 days of incubation; e) arginine after 10 days of incubation and f) asparagine after 10 days of incubation.
Notably, if the aggregation in amino acids follows an amyloidogenic pathway it is expected that the structures formed will induce significant cytotoxicity. Hence, to understand the cytotoxic nature of the structures formed by amino acids we analysed the MTT assay on mouse fibroblast L929 Cells and mouse neural cell lines by incubating the cells with varying concentration of amino acids. The MTT assay were performed by adding the amino acid solution aged for 24 h and 10 days. Notably for all amino acids more cytotoxicity was observed after ageing. Figure 3 illustrates MTT assay on neural cell lines when 10 days aged sample of amino acids were coincubated with the cells. Notably, of all amino acids glutamine revealed significant cytotoxicity. Similar observations were noted when MTT assay was performed on musue fibroblast cells (Figure 4).

![Bar charts of MTT assay](image)

**Figure 3.** MTT assay on N2a mouse neural cell lines with varying concentrations of aged amino acids.
Figure 4. MTT assay of the amino acids after ageing on L929 mouse fibroblast cell lines with varying concentrations of aged amino acids.

Hence, from the cytotoxicity analysis, it may be inferred that of all amino acids glutamine assemblies appear to be toxic both on neural as well as L929 cell lines. Further, Gln in particular formed amyloid like fibrillar structures during the course of ageing. Hence, we were motivated to study the self assembly of glutamine further by varying microscopy analysis. The aggregation properties of L-Glutamine (Gln) were studied under varying concentration and ageing time. Figure 1 illustrates the different FESEM images of Gln in fresh condition as well as aged sample. The SEM images, of Gln showed random aggregates in fresh condition which after 10 days changed to dense thick fibers and after 15 days converted into long thick bundle like fibrillar morphologies (Figure 5a-f). The optical microscopy studies of 10 days aged sample of Gln also revealed fibrillar structure which could bind ThT (Figure 5g-i).
Figure 5. (a,c) SEM images of L-glutamine at lower magnification in (a) fresh condition; (b) 10 days aged sample; (c) 15 days aged sample (1mM); (a,c) SEM images of L-glutamine at lower magnification in (a) fresh condition; (b) 10 days aged sample. (c) 15 days aged sample (1mM); Optical microscopy image of 10 days aged sample of glutamine g) bright field; h) Thioflavin T stained structures under green filter i) Thioflavin T stained structures under red filter.

AFM images of L-glutamine in fresh, 10 days and 15 days aged sample were shown in Figure 6 a-c. AFM analysis of glutamine shows sphere like morphologies in fresh condition which after ten days converted into thin ring like fibers and after 15 days thick febrile bundles could be observed (Figure 6 a-f).
Figure 6. AFM images of L-glutamine a) fresh; b) 10 days aged; c) 15 days aged.

The thin ring like febrile assemblies might be too soft in nature due to which they might melt with high electron beam. Further, we also studied the effect of solvents on self-assembly to decipher the role of hydrophobic and hydrophilic balance in aggregation. Hence, we studied self-assembly of Gln under varying % of THF:water and methanol:water. Firstly, 25 mM stock solution of L-Amino acids were prepared followed by its dilution with THF:water or methanol:water mixture as shown in (Table S-1, ESI†). From the studies it was evident that as the amount of THF increases the fibers were broken as it is aprotic relatively non-polar solvent due to which it may solubilize the aggregates (Figure S-1, ESI†). We further also pursued methanol:water study to understand the change in the structure formation with increasing percentage of methanol (Figure S-2, ESI†). From the result it is revealed that, the Gln fibers are not broken that much in the presence of methanol:water system as compared to THF:water system. PH dependent studies showed that, in acidic pH as well as basic pH there is a noticeable
variation in the structure formation of Gln (Figure S-3, ESI†). The fibers were broken in both acidic as well as basic pH. Thioflavin T binding assay were performed for Gln under optical microscopy which shows that the formed fibers of glutamine are amyloid in nature (Figure S-4, ESI†). Further the effect of urea and tannic acid

The self-assembling properties of Asp and Glu were also studied under different time intervals since they also revealed formation of some interesting self-assemblies during ageing studies (Figure 2). The FESEM images of L-Aspartic acid in fresh condition, 10 days and 15 days aged sample were shown in Figure 7 a-c. The FESEM images revealed that L-Aspartic acid self-assembled into tiny flakes like morphology in fresh condition. Bundles of fiber were observed in 10 days aged sample followed by conversion of these fibrous morphology into dense fibers after 15 days (Figure 7 a-c). The FESEM images of L-Glutamic acid in different ageing times (fresh, 10 days and 15 days) at same concentration (1mM) were shown in Figure 7 d-f. The FESEM images of L-Glutamic acid shows flakes like structure in fresh condition. L-Glutamic acid self-assembled into globular fibrous structure in 10 days. These globular structure converted into globular fiber of larger size after 15 days of ageing (Figure 7 d-f).
Figure 7. SEM images of L-aspartic acid a) fresh condition b) 10 days aged sample c) 15 days aged sample and L-glutamic acid d) fresh condition e) 10 days aged sample f) 15 days aged sample.

Finally, ThT assays were performed on aged sample of Gln, Asp and Glu to understand their amyloid nature. It is known that amyloids have propensity to bind ThT and its fluorescence is enhanced on binding amyloidogenic structures. Figure 8a represent ThT assay with aged sample of different amino acids. Notably of all amino acids studied Gln, Asp and Glu again revealed enhanced fluorescence as compared to other amino acids. Notably, the amyloid nature of Asn is already reported. The enhanced fluorescence of Gln, Asp and Glu as compared to Asn again confirms the self-assembled aggregates formed by these three amino acids may have more amyloid like character. Hence the pathogenesis in IEMs caused by the excess of these three amino acids may be associated to amyloid like aggregation.
Conclusion:

We have tried to study the self-assembly of non aromatic charged/uncharged polar amino amino acids under varying ageing time to assess its aggregation properties. The main aim of this study was to decipher the aggregation characteristics of these amino acids from an amyloid perspective. The MTT and ThT assay along with microscopy data suggest three amino acids namely Gln, Asp and Glu may aggregate to amyloid like structures and may have potential implications in the pathogenesis of IEMs associated with the excess of these amino acids.
References:


