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

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Abstract: Aggregation of amino acids to amyloid like structures is known to have implications in the pathophysiology of single amino acid based inborn-errors of metabolism (IEMs). Studying the aggregation properties of amino acids is of crucial interest also to understand the etiology of these IEMs from an amyloid perspective. 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 in aqueous solution for varying time intervals from 0-15 days. Notably, of all amino acids glutamine revealed amyloid like fibrillar morphologies as observed in case of aromatic amino acids reported previously after ageing. Further, aspartic acid and glutamic acids also revealed uniform self-assembled morphologies after 10 days of ageing. The MTT assay also corroborated with microscopic observations and a relatively more cytotoxic nature of glutamine assemblies as compared to other amino acids could also be envisaged. The Thioflavin T binding assays suggest the structures formed by Gln, Asp and Glu may have amyloid nature. Hence, the results presented in this manuscript may have crucial implications in understanding the patho-
physiology of IEMs caused by the excess of Gln, Asp and Glu and suggest a possible extension of generic amyloid hypothesis to these diseases.

1. Introduction:
Aggregation of proteins or peptides lead to formation of amyloid structures which are hallmark in the pathogenesis of diseases such as Alzheimer’s, Parkinson’s and type-II diabetes to name a few.\textsuperscript{1-3} Parkinson's disease is caused by the self-assembly of α-synuclein,\textsuperscript{4,5} while Alzheimer’s disease is caused by the self-assembly of Aβ peptides\textsuperscript{6,7} and Insulin amyloid polypeptide (IAPP) aggregates in diabetes mellitus (type 2 diabetes).\textsuperscript{8,9} 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.\textsuperscript{10} Subsequently, they reported apoptosis inducing fibers formed by self-assembly of Tryptophan (Trp).\textsuperscript{11} Further, they illustrated formation of antibodies against tyrosine fibers.\textsuperscript{12} The studies by Gazit and coworkers suggest etiology of IEMs like Phenylketonuria, Hypertryptophanemia, Tyrosinemia may be associated to amyloid diseases\textsuperscript{10-12} and hence, they proposed a generic amyloid hypothesis, which suggest a common pathophysiology for the wide range of diseases via amyloidogenic pathway.\textsuperscript{13} Subsequently, studies by other research groups re-affirmed the hypothesis. In this context,

Kar and coworkers illustrated intrinsic property of phenylalanine to trigger protein aggregation, hemolysis and showed its direct relevance to phenylketonuria.\textsuperscript{14} Further, the research team, 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.\textsuperscript{15} Subsequently, Kar group also reported amyloid mimicking assemblies of artificial sweetener aspartame\textsuperscript{16} and dopamine.\textsuperscript{17} Our group has also reported amyloid-like toxic self-assembled structures formed by the aliphatic amino acids Cysteine, Methionine and suggested its implications in etiology of
cystinuria, Hypermethioninemia.\textsuperscript{18} Sarkar et al. reported amyloids formed by methionine and its cross seed with Phe disrupts DMPC lipid membrane, and deforms red blood cells.\textsuperscript{19} A similar observation was noted by Gazit and coworkers wherein through computation studies they reported cell penetrating nature of phenylalanine and tryptophan assemblies.\textsuperscript{20}

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.\textsuperscript{21} 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 non-aromatic polar amino acids (Gln, Ser, Thr, Ala, Asn, Arg, Asp, Glu and His) extensively under varying concentration and ageing time. Chemical structure of all amino acids used in this study were shown in Figure 1.
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. Our studies suggest, of all amino acids Gln, Asp and Glu have a tendency to aggregate after prolonged incubation. ThT binding assay suggest amyloid like nature of these aggregates. The aged samples of Glutamine also produce significant cellular toxicity, thereby hinting at its amyloidogenic nature. These results are of significant interest as they have implication in pathogenesis associated with defects of glutamate and Aspartate metabolism.22-26

2. Materials and Methods:
Amino acids (AAs) used in the experiment were of analytical grade obtained from Sigma aldrich and used without further purification. The sample preparation was done in deionized water. Each amino acid was 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 analyzed 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 an hour and observed it under red and blue filter of fluorescence microscopy.
3. Results and Discussion:

To understand the aggregation characteristics of non-aromatic amino acids having functional groups in their side chains we studied self-assembly of different aliphatic charged/uncharged amino acids by incubating them in aqueous solution for varying time intervals. Thus, 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), asparagine (Asp), glutamine (Gln), aspartic acid (Asp) and glutamic acid (Glu) were shown in Figure 2a-i.

The FESEM image suggest amyloid like fibrillar structure are not formed Ala, His, Ser, Thr, Arg under all concentrations studied and ageing time. Ala is conventionally, used as a 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 ageing (Figure 2a). FESEM image of histidine shows twisted nano sheet 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 Ser show branched thin aggregates after 10 days of ageing (Figure 2c), while Thr also revealed branched thin tape-like aggregates after 10 days (Figure 2d). Arg revealed random twisted bundles after 10 days, while in Asp thick twisted bundle like fibrous morphology as observed previously was also noted (ref) (Figure 2e, f). However, for Gln 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 Asp and Glu (Figure 2h-i).

![SEM images of amino acids](image)

**Figure 2.** SEM image of 10 days aged sample of (1mM) amino acids incubated at 37.4°C. a) Ala; b) His; c) Ser; d) Thr; e) Arg; f) Asp g) Gln; h) Asp; i) Glu.

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 analyzed the MTT assay on mouse fibroblast L929 Cells and mouse neural cell lines by co-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, 10 days aged sample of AAs revealed favorable structure formation hence it was considered optimal period of ageing. MTT assay suggest coincubation with aged AAs induced more cytotoxicity as compared to fresh AAs (Figure 3, ESI). Figure 3 illustrates MTT assay on neural cell lines when 10 days aged sample of amino acids were coincubated with the cells. From Figure 3 it is evident that Gln revealed more cytotoxicity as compared to other AAs. Similar observations were noted when MTT assay was performed on mouse fibroblast L929 cells (Figure 4).

Figure 3. MTT assay on N2a mouse neural cell lines after addition of varying concentrations of aged amino acids.
Hence, from the cytotoxicity analysis, it may be inferred that among all amino acids glutamine assemblies appear to be toxic both in neural as well as L929 cell lines. Further, since 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 5 illustrates the different FESEM images of Gln in fresh condition as well as aged sample. The SEM images, of 5 mM 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 also corroborated with SEM studies (Figure 5g-i). The phase contrast image suggest soft nature of Thioflavin T binding assay were performed for Gln under optical microscopy which shows that the formed fibers of glutamine are amyloid in nature (Figure 5i).

**Figure 5.** (a-f) SEM images of 5mM Gln at low and high magnification respectively. (a, d) fresh condition; b, e) 10 days aged sample; (c, f) 15 days aged sample (1mM); (g-i) Optical microscopy images of 10 days aged sample of glutamine (g) bright field, (h) Phase contrast and (i) Thioflavin T stained images.
AFM images of Gln in fresh, 10 days and 15 days aged sample are shown in Figure 6 a-f. AFM analysis of glutamine shows sphere like morphologies in fresh condition which after ten days converted into thin ring like fibers at random places along with thick fibrillar bundle and after 15 days dense fibers were found as observed in SEM images (Figure 6 a-f).

![AFM images of Gln](image)

**Figure 6.** AFM images of L-glutamine: a) Fresh condition, b) 10 days aged sample and c) 15 days aged sample d) 3D image in fresh condition, e) 3D image of 10 day aged sample, f) 3D image of 15 day sample.

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 (Table S-1, ESI†). From these studies it was evident that as the amount of THF is increased the fibers are broken. Since THF is relatively...
non-polar aprotic solvent hence it may solubilize the aggregates (Figure S-1, ESI†). We further also pursued methanol:water study to understand the changes 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 since methanol is polar solvent. 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 and remains stable only at neutral pH.

The self-assembling properties of Asp and Glu was 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 structures converted into globular fiber of larger size after 15 days of ageing (Figure 7 d-f). ThT binding assay for aspartic acid and glutamic acid were performed under optical microscopy which revealed that the fibers formed by both amino acids were amyloid in nature (Figure 7g-l).
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; (g-l) Optical microscopy images of 10 days aged sample of aspartic and glutamic acid (g, j) bright field, (h, k) Phase contrast and (i, l) Thioflavin T stained images.
To understand the mechanism of structure formation namely in Gln, Asp, Glu we also studied the self-assembly of these three amino acids under varying solvent mixtures THF: water and methanol: water. Also the amino acids were co-incubated with urea and tannic acid (TA) to understand the role of hydrogen bonding and hydrophobic attractions in structure formation (Figure 9b-d and Figure 9f-h). Notably, urea is a well-known hydrogen bond breaker while TA is a generic amyloid inhibitor which breaks amyloid assemblies by disrupting $\pi-\pi$ stacking interactions in aromatic amino acids. However, in non-aromatic amino acids TA may work by destabilizing hydrophobic attractions. Addition of urea to aged sample of Gln, Asp and Glu result in disruption of the self-assembled structures and only small crystalline aggregates could
be observed at isolated places suggesting hydrogen bonding played an important role in self-assembled structure formation of these AAs. Coincubation of Gln, Asp and Glu with TA also revealed disruption of self-assemblies. Suggesting TA might interact with these structures via hydrophobic interactions causing its destabilization.

Further the self-assembled structures formed by AAs were also cross seeded with Phenylalanine (Phe) fibrils, a known reductionist model for amyloid studies to understand its effect on amyloid like aggregate formation. Further Phe fibrils also play a crucial role in etiology of phenylketonuria (Figure 8j-l). It was noted that Phe fibrils were disrupted when it was co-incubated with Gln. Further the fibrillar morphology of Gln alone could also not be observed. The microscopy study suggest both Phe and Gln fibrils are disrupted due to mutual interaction. However, the co-incubation of Glu self-assembled structures to Phe fibrils did not induce any structural changes albeit Phe fibrils appeared more dense which suggest Gln structure positively modulate Phe fibrillation. Co-incubation of Phe fibrils with Asp structures also lead to gel like structures which again suggest enhanced aggregation like Glu. Hence, from these cross seeding studies it may be surmised that the aggregation characteristics of Phe fibrils may be modulated by co-incubation with these AAs. Further, since Gln coincubation is causing disruption of Phe fibrils, it may possibly be used as therapeutic module for the treatment of phenylketonuria.
Finally, ThT assays were performed on aged sample of Glu, 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 9a represent ThT assay with aged sample of different AAs. Notably of all AAs studied Glu, Asp and Glu again revealed enhanced fluorescence as compared to other AAs. Notably, the amyloid nature of Asn is already reported. (ref) The enhanced fluorescence of Glu, Asp and Glu as compared to Asn again confirms the self-assembled aggregates formed by these three AAs may have more amyloid like character. Hence the pathogenesis in IEMs caused by the excess of these three AAs may be associated to amyloid like aggregation (Figure 9).
Conclusion:

We have studied the self-assembly of various non aromatic polar amino acids namely Gln, His, Ser, Thr, Asp, Glu and Arg under varying conditions to assess their aggregation properties. The main aim of this study was to decipher the aggregation characteristics of these AAs when they may be present in excess inside the body. Gln revealed amyloid like fibrillar morphologies while Asp and Glu also revealed interesting self-assembled morphologies after ageing. MTT assay suggest the structures formed by self-assembly of Gln are cytotoxic to both neural and normal fibroblast cell lines. Thioflavin T assays suggest structures formed by Gln, Asp and Glu may
have amyloid characteristics since the structures bind ThT which is evident by microscopy and fluorescence analysis. Hence, these results may have potential implications in studying the pathogenesis of diseases associated with the excess of Gln, Asp, and Glu from an amyloidogenic perspective.

**Funding Sources**

The work was supported by the SERB SPG/2021/000521 received by Dr. Nidhi Gour.

**Conflicts of interest**

There is no conflict of interest to declare.

**References:**


