Amyloid like aggregates formed by the self-assembly of proline and Hydroxyproline

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Abstract: Single amino acid based self-assembled structures have gained a lot of interest recently owing to their pathological significance in metabolite disorders. There is plethora of significant research work which illustrate amyloid like characteristics of assemblies formed by aggregation of single amino acids like Phenylalanine, Tyrosine, Tryptophan, Cysteine and Methionine and its implications in pathophysiology of single amino acid metabolic disorders like phenylketonuria, tyrosinemia, hypertryptophanemia, cystinuria and hypermethioninemia respectively. Hence, studying aggregation behaviour of single amino acids is very crucial to assess the underlying molecular mechanism behind metabolic disorders. In this manuscript we report for the very first time the aggregation properties of non-aromatic single amino acids Hydroxy-proline and Proline. The morphologies of these were studied extensively by Optical microscopy (OM), ThT binding fluorescence microscopy, Scanning Electron Microscopy (SEM) and Atomic force microscopy (AFM). It can be assessed that these amino acids form globular structures at lower concentrations and gradually changes to tape like structures on increasing the

concentration as assessed by AFM. ThT and CR binding assay reveal the aggregates do have amyloid like characteristics. Further MTT assays on SHSY5Y neural cell lines reveal cytotoxicity and the aggregates caused significant cell death in dose dependent manner. These results have important implications in understanding the pathophysiology of single amino acid disorders like Hyperprolinemia and Hydroxyprolinemia in association with amyloid diseases. The symptoms of these diseases are also accompanied by extensive neurological problems like intellectual disability, seizures and psychiatric problems which further evince amyloid like etiology for these rare in-born errors of metabolism.

Introduction

Single amino acid metabolic disorders are in-born error of metabolisms (IEM's) caused by their accumulation due to the errors caused in the metabolic pathways. The pioneering work of Gazit and co-workers for the very first time reported the self-assembly of the phenylalanine to amyloid like toxic fibres.[1] They further proposed 'generic amyloid hypothesis' relating the etiology of phenylketonuria to amyloid associated diseases. As an extension of their hypothesis, Gazit research group, further reported the formation of apoptosis-inducing amyloid fibrils by the self-assembly of tryptophan[2] and the formation of antibodies against cytotoxic tyrosine assemblies.[3] Following the work of Gazit, several other research groups have observed similar results for single amino acid assemblies and their findings implicate the involvement of amyloid like aggregation in the patho-physiology of metabolic disorders.[4,5]

In this context, our research group, recently, reported the self-assembly of non-aromatic single amino acids Cysteine (Cys) and Methionine (Met) to amyloid-like structures[6].

In the present manuscript, we report for the very first time self-assembled aggregates formed by the single amino acid Proline (Pro) and Hydroxyproline (Hyp). Pro and Hyp assemblies were characterized in detail by the various microscopic and spectroscopic techniques.. We have also tried to understand implications of these aggregates in metabolite disorders and amyloid studies and henceforth studied these structures by various techniques like amyloid binding assay, solid state FTIR, NMR and cytotoxicity analysis by MTT assay. The self-assembling behaviour of Pro and Hyp was similar and the concentration dependent studies reveal formation of tape like aggregates at higher concentrations (5 mM, 10 mM) while the formation of globular morphologies at 1 mM and 3 mM concentrations as assessed by AFM. Notably, SEM images of Pro and Hyp could only be recorded without gold coating and at lower magnifications since the assemblies were extremely soft in nature and starts melting with higher energy electron beam. Phase contrast microscopy and XRD also supported this observation amorphous nature of assemblies confirmed amorphous non crystalline nature of aggregates confirming they are formed by the process of self-assembly and not crystallization (ESI). The nature of interactions which lead to structure formation of Hyp and Pro were purported by co-incubating the assemblies in urea and FTIR and NMR analysis. The cytotoxicity assays on neural SHSY5Y cell lines suggest both Pro and Hyp aggregates reveal cytotoxic property and caused reduction in cell viability in a dose dependent manner.[6,7] The cytotoxicity analysis by MTT assay suggest important implications of these aggregates in rare genetic in-born errors of metabolisms like Hyperprolinemeia and Hydroxyprolinemia and relate it to amyloid associated diseases. Notably the symptoms of these diseases which are caused by accumulation of proline and hydroxyproline include seizures, intellectual disability, schizophrenia and major neurological and psychiatric

problems. Thus, the symptoms are similar to those associated with amyloid disorder like Alzheimer's and Parkinson's and evince an amyloid like etiology for these diseases.

Experimental Section

L-Proline and L-Hydroxyproline were purchased from sigma and used without further purification. The purity of all the amino acids procured was minimum 99%. Milli Q water was used for preparing all the solutions (Millipore). No additional organic solvent was added. The optical microscopy (OM) images were taken under Leica DFC450c microscope with 40X, 63X and 100 X magnifications. OM samples were prepared on glass slides and 20 μL of 5 mM solution of peptides were dispensed and dried at room temperature. Scanning electron microscopy (SEM) images were taken using a Nova Nano FEG-SEM 450 microscope (the accelerating voltage ranged from 5 to 15 kV). SEM samples were prepared on silicon wafers and 10 μL of 5 mM solution of peptides were dispensed and dried at room temperature. The samples were analyzed without gold coating under low vacuum. The concentration dependent AFM studies were done using 1 mM, 3 mM, 5 mM and 10 mM amino acids. The samples were placed on freshly cleaved muscovite mica surfaces followed by imaging with an atomic force microscope (INNOVA, ICON analytical equipment, Bruker, operating under the acoustic AC mode (AAC or tapping mode), with the aid of a cantilever (NSC 12(c) from MikroMasch, Silicon Nitride Tip) by NanoDrive™ version 8 software. The force constant was 2.0 N/m, while the resonant frequency was ~276 kHz. The images were taken in air at room temperature, with the scan speed of 1.5 lines/sec. The data analysis was done by using of nanoscope analysis software. The sample-loaded substrates were dried at dust free space under 40W lamp for 30 minutes followed by high vacuum drying and subsequently examined under AFM.

The MTT assays were done by following previously reported methodology for assessing the cytotoxicity of single amino acid structures.[6,7] The Pro and Hyp structures were co-incubated with SHSY5Y cell lines. For these studies SHSY5Y cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM Thermo-scientific) supplemented with L-glutamine (5 mM, Gibco), antibiotics (penicillin/streptomycin 10000 U, Gibco) and 10% heat-inactivated foetal bovine serum (Gibco) at 37 °C and 5% CO2 in a humidified atmosphere. Cell lines were seeded (10^4 /100µL) in 96 well plates and incubated overnight. After attachment, the medium was replaced with 2% FBS. For cytotoxicity studies, treatment with amino acid samples with varying concentrations of 1, 3, 5 and 10 mM was given for 12 hours. Phenylalanine cytotoxicity is well reported so it was taken as positive control.[6] The cell viability was determined by MTT assay. Tetrazolium solution (5 mg/mL) was added to each well and incubated overnight. The medium was replaced with DMSO to dissolve the purple precipitate and the absorbance intensity was measured at 540 nm, using an ELISA plate reader. Cell viability was calculated as a percent of the control group (untreated 100%).

Results and Discussions

Pro and Hyp solutions were prepared using deionized water at varying concentration from 1 mM to 10 mM. The self-assembling properties of 1 mM, 3 mM, 5 mM and 10 mM solution were assessed by optical microscopy (OM) measurement. The aggregation properties at 1 mM and 3 mM were not visible in OM. However, aggregation of Pro and Hyp solution could be observed at 5 mM and 10 mM concentrations. Notably, Pro and Hyp self-assembly is still not reported in literature. Hence, we were motivated to characterize the structures formed by Pro and Hyp in more detail through more sophisticated techniques like SEM and AFM. Figure 1a and 1d displays OM image of the structures formed by the 5 mM solution of Pro and Hyp while Figure

1b and 1d reveal its SEM image. The self-assemblies formed by both Pro and Hyp shows very long tape-like aggregates. The SEM for the Pro and Hyp samples could only be recorded without gold coating since the structure melted as they were very soft in nature by gold coating. Another interesting observation was that both Pro and Hyp assemblies tend to melt down as voltage or exposure time of e-beam was increased in SEM. This observation implicated that both Pro and Hyp structures are indeed soft in nature formed exclusively via self-assembly process and not by crystallization. The Pro and Hyp structures were also analyzed by phase contrast microscopy. Phase contract microscopy can be very useful to get preliminary information about the surface characteristics of the structure and may indicate if it has crystalline properties. The images of Pro and Hyp structures under 63X resolution in phase contrast microscopy indicated that assemblies are amorphous in nature and lack any crystalline property (ESI).

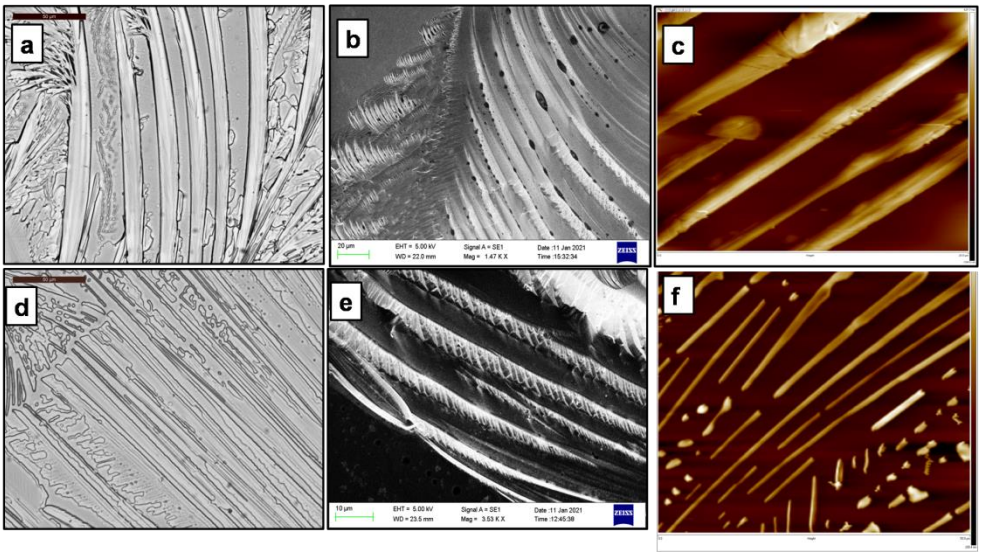

Figure 1: Self assembled structures formed by 5 mM solution of Pro and Hyp. (a) Optical microscopy image of of Pro; (b) SEM image of Pro; . (c) AFM image of Pro; . (d) Optical microscopy image of Hyp; (e) SEM image of Hyp; (f) AFM image of Hyp.

AFM analysis of Pro and Hyp at 5 mM further confirmed these observations and long tape like network could be assess with globular structures present at isolated places. Figure 1c and 1e shows representative AFM images of Pro and Hyp at 5 mM concentrations. The presence of globular morphologies in AFM indicated the network and tape like structures are formed by its coagulation. Hence, concentration dependent morphological studies by AFM were studied at 1 mM, 3 mM, 5 mM and 10 mM solutions of Pro and Hyp.

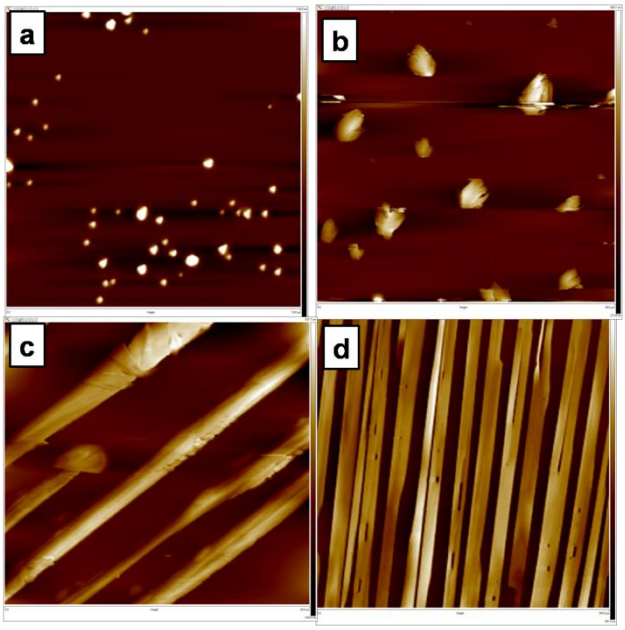

Figure 2: AFM images of Pro at (a) 1 mM concentration; (b) 3 mM concentration; (c) 5 mM concentration; (d) 10 mM concentration.

AFM indeed revealed formation of very small globules at 1 mM which increases in size by merging at 3 mM. At 5 mM closed network of these globules are formed and tape like structures can also be observed while at higher 10 mM concentration morphologies of Pro and Hyp changed to regular tape like structures which are arranged in like a lattice.

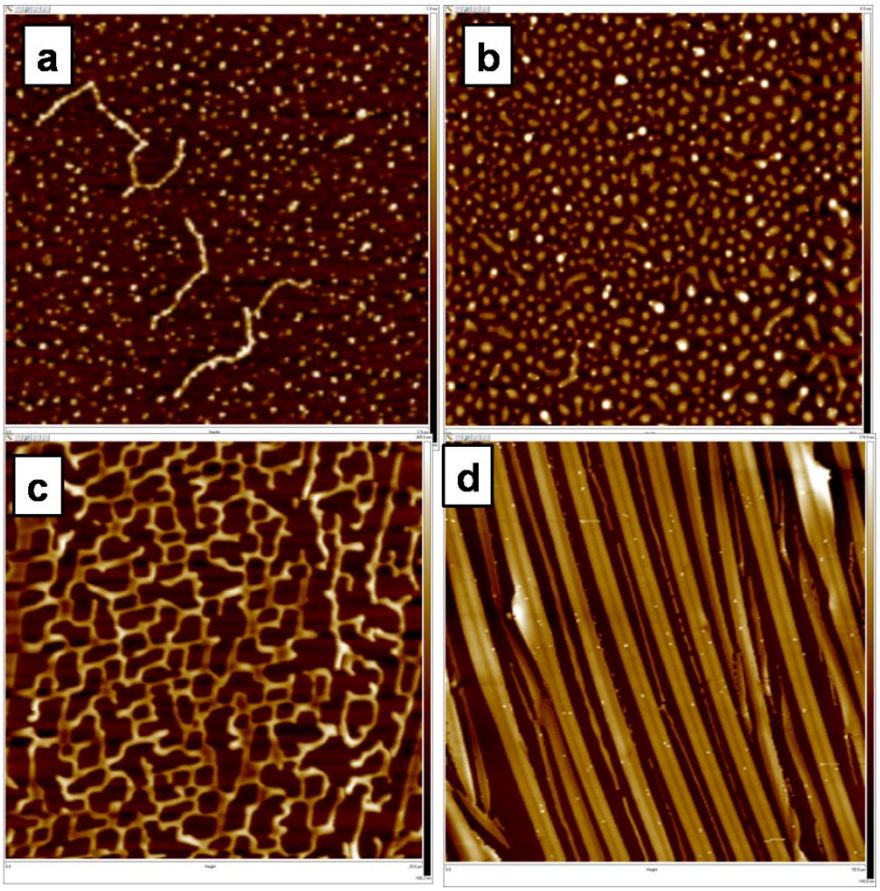

Figure 3: AFM images of Hyp at (a) 1 mM concentration; (b) 3 mM concentration; (c) 5 mM concentration; (d) 10 mM concentration.

These observations were indeed very interesting and suggest morphologies and be controllably assembled based on its concentration. (Figure 2, Figure 3). The Pro and Hyp assemblies were also analyzed under varying pH (ESI) which suggest optimal condition for self-assembly under neutral condition and the structures gets disrupted in slightly acidic or alkaline environment.

To probe the role of hydrogen bonding in structure formation, Pro and Hyp assemblies were co-incubated with urea. Figure 4b and 4d illustrates representative AFM image of Pro and Hyp

assemblies after co-incubation with urea. Both Pro and Hyp assemblies are disrupted completely on co-incubation with urea henceforth suggesting crucial role of hydrogen bonding in both Pro and Hyp aggregation (ESI). Further to understand the role of hydrogen bonding with neighboring water molecule we did control studies using aprotic solvent mixture 5% DMSO:DCM. Due to the aprotic nature the solvent with not be able to form hydrogen bond with Pro and Hyp Interstitially, when we assessed self-assembling behavior of Pro and Hyp in this solvent system we could not assess any structure formation for both Pro and Hyp. (ESI) Hence these studies further confirmed that tape like self-assemblies of Pro and Hyp are formed via hydrogen bonding interaction with adjacent water molecules as well as intramolecular hydrogen bonding.

Further, to get insight into the prevalent solution structure of Pro and Hyp assemblies and to envision its amyloid nature, Thioflavin T and Congo red binding assays were performed. ThT shows remarkable enhancement in its fluorescence on binding with amyloid.[20] The fluorescence spectra of ThT with Pro and Hyp indeed revealed enhancement in fluorescence and confirms amyloid like structure formation by these single amino acids (Figure 4). Since thioflavin T binds to hydrophobic pockets of beeta sheets present in amyloid fibres, it further confirmed that the Pro and Hyp tape like structure do exist in solution. Further the structures also got stained with Thioflavin T as assessed by fluorescence microscopy. The amyloid nature of the assemblies was further confirmed by congo red binding assay in solution. Although the nature of binding of CR with amyloidogenic peptides is still under debate, it is universally accepted that regardless of the manner of binding, CR displays an increase in absorbance intensity and a red-shift on binding with amyloid.[21] Spectra obtained of Pro and Hyp with CR (Figure 4d) revealed a slight red shift in spectra and increase in absorbance. As control we co-incubated other single amino acids Phe, Tyr, Cys, Met and Trp with CR since their self-assembling behavior to amyloid like structure is

already known. All reported single amino revealed increase in absorbance and similar red shift with CR. CR binding assays thus suggest that CR binds to structures formed by Pro and Hyp and hence the assemblies may have amyloid like characteristics. (Figure 4)

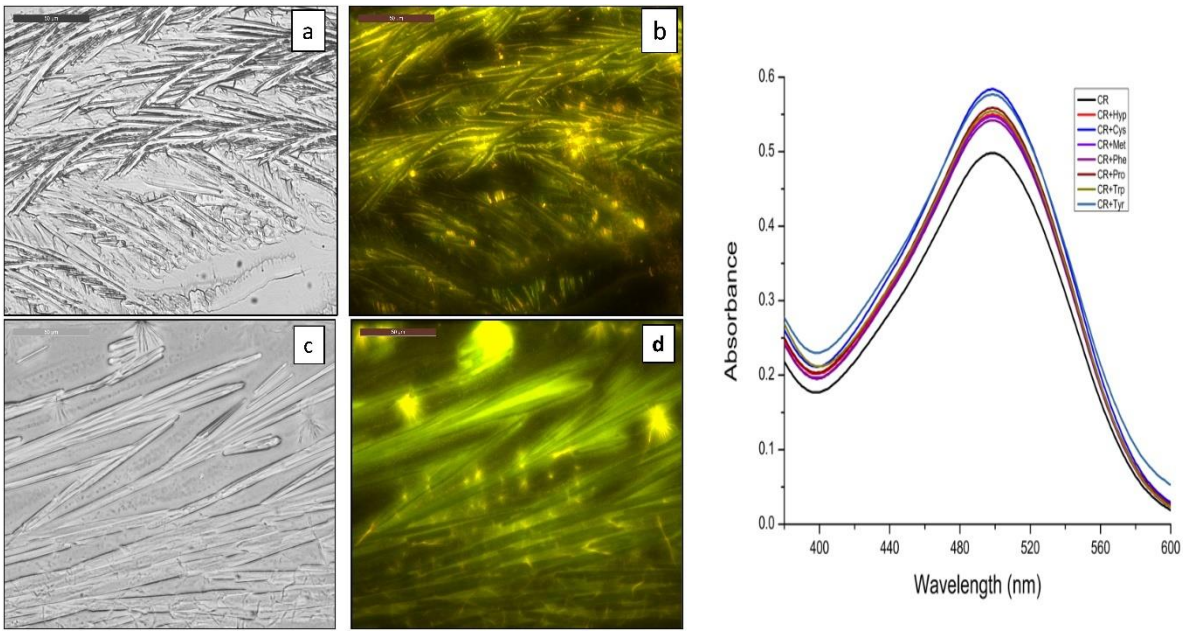

Figure 4

Figure 4 (a) Optical microscopic image of Hyp at 40X (b) Hyp fibers bind with thioflavin T and shows a green fluorescence (c) Optical microscopic image of Pro at 40X (d) Pro fibers bind with thioflavin T and shows a green fluorescence (e) CR binding assay with different single amino acid assemblies showed an increase in intensity, suggesting amyloid characteristics for both Hyp and Pro.

It is very well reported that amyloid assemblies are neurotoxic in nature and accumulation of Aβ peptide induces cell death. MTT assay is a reliable in vitro assay which has been used for assessing the effects of neurotoxic compounds and has been used in cultures of different neuroblastoma cells, for the quantitative measurement of Aβ toxicity.[22] The MTT assay analysis reveals that structures formed by Pro and Hyp were cytotoxic in a dose dependent manner (Figure 5). The cytotoxicity anaysis was done at four different concentrations of amino acids: 1 mM, 3 mM, 5 mM and 10 mM to assess the dose dependent cytotoxicity. To assure formation of fibrils amino acids were heated with cell medium (excluding FBS) at 90 ^{o}C for 30 minutes and gradualy cooled and incubated overnight before treating it with SH-SY5Y cell lines. Phenylalanine was taken as positive control since its toxicity is well reported. [4]

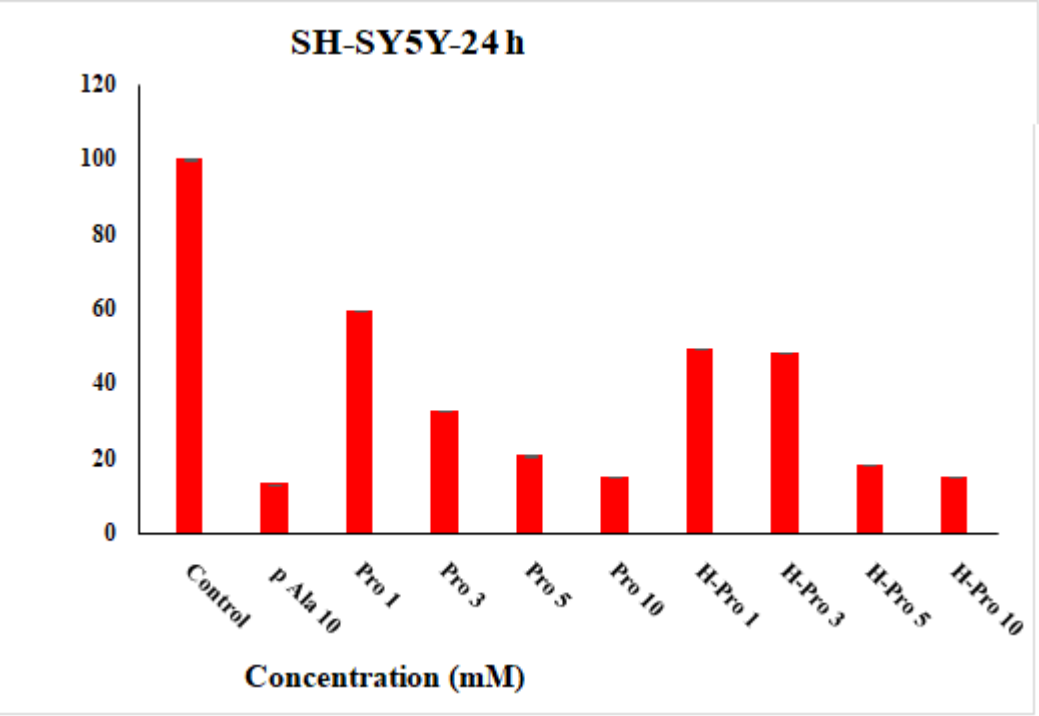

Figure 5. MTT assay with Proine and Hydroxyproline structures on neural cell lines.

Figure 5 reveals cytotoxicty analysis of Pro and Hyp to SHSY5Y cells. It may be noted that btoth Pro and Hyp aggregates were cytotoxic and even at 1mM concentration they cell viablity drastically as compared to control. The cell viablity was reduced to 40.3% in ase of Pro while it was reduced to 51.6% by Hp at 1mM concentration. As the concentration was increased to 10mM the cell viablity was only 15.6% for Pro while 14.8% for Hyp revealing its very cytotoxic nature at higher concentrations. Cells are incubated with these aggregates for short time as compared to physiological conditions, hence higher concetrations might be required to induce cytotoxicity. A comparative analysis of toxicity induced by Phe at 10mM is similar to that induced by Pro and Hyp (Figure 5c\).. This study implicates that Pro and Hyp structures may possess amyloid like characteristics similar to Phe. It further suggests that etiology of genetic inborn error like hyperprolinemia and hydroxyprolinemia may be related to amyloid associated diseases and need to be studied further.

CONCLUSIONS

In summary, we report for the very first time spontaneous structure formation by the self-assembly of single amino acid Proline and Hydroxyproline. The structures were characterized in detail by various microscopic and spectroscopic techniques. The amyloid binding assays and cell viability analysis reveals that Pro and Hyp aggregates are toxic and have amyloid like nature. These results are highly significant since to the best of our knowledge aggregation properties of Pro and Hyp are not reported so far. The formation of toxic aggregates by Pro and Hyp also suggest that the accumulation of these amino acid might lead to such cytotoxic structure formation due to which symptoms of neurological problems like intellectual disability and seizures appear in Hyperprolinemia and Hydroxyprolinemia the diseases which are causedby excess of these amino acids. Our future endeavours will be focussed to assess cytotoxicity data in

more detail by other cell based assay and histochemical analysis to confirm the role of Hyp and Pro aggregation in etiology of these in-born errors of metabolism.

Associated Content

The supporting information of this manuscript is available and contains additional figures and methods.

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Abbreviations

Pro - ProlineHyp - Hydroxyproline

Phe - Phenylalanine

Tyr - Tyrosine

Trp - Tryptophan

FF - Diphenylalanine

OM – Optical Microscopy

SEM – Scanning Electron Microscopy

TEM – Transmission Electron Microscopy

ThT- Thioflavin T

CR- Congo Red

ATR-FTIR - Attenuated Total Reflectance- Fourier Transform Infrared spectroscopy

NMR – Nuclear Magnetic Resonance

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