Amyloid like Property of Large Protein Fibers & its application as a Biomaterial

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Amyloid like Property of Large Protein Fibers & its application as a Biomaterial

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Supervisors' Certificate

This is to certify that the work presented in the dissertation entitled: ***Amyloid Like Property of Large Protein Fibers & its application as a Biomaterial*** submitted by *Kaustuv Das,* Roll Number 216BM2014, is a record of original research carried out by him under our supervision and guidance in partial fulfilment of the requirements of the degree of *M.Tech Biotechnology* in *Department of Biotechnology and Medical Engineering*. Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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Dedication

To My Parents and God

For whom I see this Day.

Department of Biotechnology and Medical Engineering
National Institute of Technology Rourkela

May 28,2018

Declaration of Originality

I, *Kaustuv Das*, Roll Number *216BM2014* hereby declare that this dissertation entitled *Amyloid like Property of large protein fibers & its application as Biomaterial* presents my original work carried out as a Masters student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the sections "Reference" or "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

Kaustuv Das

May 28, 2018

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Abstract

Amyloids are protein fibrillar aggregates which possess a definite cross β structure providing them rigidity and resistant against cleavage by proteases. The orientation of the β strands corresponding to the axis of the β sheets is what gives them there characteristic cross β architecture. The occurrences of amyloids are implicated in diseases like Alzheimer's and Parkinson's where the tissue functionality is turned inactive due to protein aggregation. However, natural conditions like In Skin Melanosomes and In Pituitary the peptide hormones are seen to have amyloid conformations. In non-mammalian sources like Curli proteins in the biofilm of E.coli, APLP2 proteins in xenopus and spider silk also possess amyloid like conformation. The specific modulus of amyloid is comparable to steel and the ease of amyloid preparation which is also cost effective makes them a prospective biomaterial like Silk. Amyloids are prepared from different protein sources in-vitro subjected to various physiological conditions.

Keeping in view of these aspects we prepared amyloid from Pepsin which is reported. The reason for selecting pepsin is their ability to form denatured aggregated fibrils at the physiological pH of the body. In this study preparation of large amyloid fibers from pepsin is reported. Proteins are known for emulsifying capacity in food industry. Amyloids are structures possessing exposed β sheet arrangement keeping in view of such morphology we evaluated its role as an emulsifier. The emulsion stability was assessed using inverted tube assay and centrifugation. Such a property of amyloids if optimized can be used efficiently as lipophilic drug delivery agent.

Spider silk is reported to be a structural sub-class of amyloid cross β conformation. In domain of such finding we assessed the structural conformation of three kinds of wasp nest fibers (Common wasp, Yellow Jacket wasp and Mud dauber wasp). The overall structural morphology and the protein secondary structure distribution were observed and was compared to that of amyloid. The findings suggested that the nest fibers may possess some amyloid like conformation due to their Thioflavin T binding property but also the presence high number of native β sheets proposes some unique secondary structure arrangement like silk. Biocompatibility of the materials were performed which included MTT Assay and

Hemolysis Assay. To evaluate their possible role as biomaterials in bone tissue engineering Alkaline Phosphatase Activity, Glycosaminoglycan secretion and Cell adhesion study were also carried out. The findings proposed that like Natural Silk the Natural Wasp Fibers can also be used as a future biomaterial for tissue engineering.

Keywords - Amyloids; cross β architecture; Emulsion; Natural fibers.

List of Abbreviations

1.	Scanning Electron Microscopy …….	**SEM**
2.	Environmental Scanning Electron Microscopy …….	**ESEM**
3.	Confocal Laser Scanning Microscopy ……..	**CLSM**
4.	X-Ray Diffraction ……..	**XRD**
5.	Fourier Transform Infrared Spectroscopy ……..	**FT-IR**
6.	Wasp Nest Fibers ……..	**WNF**
7.	Mud dauber Nest Fibers ……	**MNF**
8.	Yellow Jacket Nest Fibers …….	**YNF**

List of Tables

List of Figures

CONTENTS

Chapter 1

INTRODUCTION

1.1 Background study and Significance of Work

Amyloids are insoluble β stack aggregated protein fibers which are found to deposit in various organs in disease conditions. Alzheimer's and Parkinson's are some of the widely known diseases where amyloid formation is implicated. Literatures reported the formation of synthetic amyloid in laboratories under certain physiological conditions. Ionic strength, pH & temperature are some of the conditions whose variability seems to cause amyloidgenesis in proteins [1]. It is reported that synthetic amyloid of variable diameters and shapes is dependent on the distribution of hydrophobic amino acid residues in the primary structure [2]. However, cited mostly in diseases existence of amyloid in nature as a functional entity proves it is not a randomly misfolded structural entity. In Humans existence of peptide hormones in pituitary gland [3], in melanosomes [4] are reported to have amyloid like conformation. Non-mammalian protein fibers like Spider Silk [5] ,Curli in E.coli [6] are reported to have similar β sheet conformations like amyloid.

Proteins are amphipathic agents known to acts as emulsifiers. On that aspect we evaluated role of large amyloid fibers as emulsifiers. In addition to that, Wasp Nest fibers (WNF) and Yellow Jacket Nest fibers (YNF) and Mud dauber Nest Fibers (MNF) are the three natural fibers specimens which were evaluated to understand their secondary structure distribution and to investigate their resemblance with amyloid like structure. As it is known that amyloid possess fibrillar structures and natural fiber like spider silk resembles partially to amyloids. X-Ray Diffraction, Fourier Transform Infrared Spectroscopy and Thioflavin T binding assay were done to analyze the structural diversity. Confocal Microscopy was used to visualize amyloid fibers using amyloid specifc dye Thioflavin T. The study also assessed the role of the nest fibers as a scaffold material for tissue engineering. Biocompatibility of YNF and WNF was checked through MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and blood compatibility was evaluated by hemolysis assay. Cell adhesion study on the

materials was investigated by CSLM using Human osteosarcoma cell line MG-63. Alkaline Phosphatase assay and release of glycosaminoglycan was also assessed for WNF and YNF. The study revealed the relative structural morphology of synthetic amyloid and natural occurring nest fibers. The distribution of similar β stack structure in natural fibers showed the necessity of such structural distribution pertaining to rigidity and integrity of the overall structure of the nest. Similar to spider silk as reported [5] the structural composition of silk fibers constituting YNF, WNF and MNF can be defined as a structural sub-class of amyloid β stack conformation.

1.2 Pepsin - An Acid Protease

Pepsin is an endopeptidase present in stomach which helps to digest proteins. It is active at an optimum pH of 2 and possesses two aspartate residues in the active site. Pepsin is produced from pepsinogen after cleavage of basic amino acid residues in the acidic pH of stomach. The amino acid composition of pepsin includes majorly Aspartic acid, Serine, Threonine, Glycine and Isoleucine. Some industrial applications of pepsin are :-

1. Production of soy protein and gelatin along with substitution of rennet for production of cheese.
2. Pepsin is used for bating of hides to remove the hair , fats for leather industry.
3. In medical applications it is used to produce F'(ab) fragment from antibodies as the Fc fragment tend to create immune reactions.

1.3 Synthetically Prepared Amyloid

Amyloids though are implicated in disease conditions but due to its structural integrity and make-up it has come up to be an effective biomaterial. The cross β architecture of the amyloids is the chief reason for its robustness and hence the scientific community is aspiring to bring it for commercial use like silk. Silk extracted from cocoons are already in market as a biomaterial. Apart from Aβ-42 which is naturally an amyloidgenic protein [7] formed after cleavages of APP, a number of different proteins are also transformed into amyloid conformation under certain physiological conditions. We will see the proteins which are used

to form amyloids under certain conditions and along with that few applications of amyloid as a biomaterial.

1. **Crystallin** is an eye lens protein which forms amyloid fibers when subjected to acidic pH of 2.0 in presence of denaturing agent TFE at room temperature. Further incubation at 60°c showed enhanced fibril formation [8].
2. **Lysozyme** is an antimicrobial enzyme found in tears, saliva and mucus. Fibers formation in lysozyme is noted to be caused by both alkaline and acidic pH in room temperature and at higher temperature respectively [9].
3. **α-Synucelin** a protein found in the tip of nerve cells in brain and is implicated in Parkinson's disease due to the formation of insoluble fibirllar aggregates. Invitro alpha-synucelin reportedly form amylid fibers at pH7.4 in presence of sodium or potassium chloride at room temperature [10].
4. **Pepsin** is an aspartate protease found in mammalian stomach. Pepsin is reprted to from amyloid fibers at room temperature at physiological pH of 7.1. In our study as well we used pepsin as the source of protein form amyloid preparation [11].

The reason for using Pepsin as the protein source is that it is native in acidic pH but form amyloid in neutral pH. Hence pepsin if used as the amyloid source to be used as biomaterial will remain in such state inside the body without reverting back to native state and causing tissue damage.

1.4 Large Amyloid Fibers

Amyloid fibers that are reported to be prepared in-vitro are mostly in nanometer scale which is in coherence with the amyloid fibers seen in the diseased tissues of Alzheimer's or Parkinson's patients. However, micrometer fibers collectively are also reported which indicated the prospect that macroscopic features of amyloids can be controlled and designed at molecular level [12]. The shape of fibers can be rectangular or cylindrical possessing tapering ends which is dependent on the distribution of the hydrophobic amino acids. The modulus of such structures can also be varied accordingly. Such an aspect of amyloids to be

made into larger structures is necessary for it possess modulus like silk and specific modulus parallel to steel [13].

1.5 Application of Amyloid as a Biomaterial

1. Construction of amyloid hydrogels which is used for the differentiation of the mesenchymal stem cells to neurons [14].
2. Preparation of hybrid nanocomposite by deposition of Hydroxyapatite on amyloid fibers which possess bone biomimetic features [15].
3. An immobilsing platform using grapheme oxide coated on amyloid fibers for glucose sensing activity [16].
4. Construction of gold coated amyloid aerogels with bulk auto-fluorescence for bio-catalysis [17].

1.6 Non-Mammalian Amyloid like Structures

Apart from animal proteins some other fibrous structures have shown similar understanding with amyloid like conformations. Curli proteins of E.Coli , Silk and spider silk to name a few which are widely researched for their structural similarity with amyloid conformations.

1. Curli proteins are an essential component for biofilm formation in enterobactericeae family. The csg family of operons regulates curli production which is one of the functional amyloid like conformation found in nature [6].
2. APLP2 is an APP subclass amyloid precursor protein which is found in Xenopus sp. a south African claw toed frog. This protein is also a functional form of amyloidgenic protein which is found in the melanotrope of pituitary glands [18].
3. Similar to the above structures is the spider silk which as reported by Slotta et. al is a structural subclass of amyloid fibers [5]. With the presence of 3_1-helical and random coiled structure spider silk shows a unique structural composition which is regarded as a sturdy material.

All the above structures were evaluated with similar characterizations as of in-vitro prepared amyloid.

1.7 Wasp Nest

Fibers like silk are also found in nest of various other insects like mud dauber, Yellow Jacket, common wasp, etc. The intention is to understand & find the similarity between such structures and amyloids. As we know from existing literatures that wasp synthesize their nest from oral secretion along with processed plant parts. The processing is carried in the mouth itself. The oral secretions are proteins observed to have three conserved amino acids among certain wasp species which are serine, glycine and alanine [19]. The conserved amino acids among the wasp's oral secretion can help to categorize the different wasp species based on their habitat as reported. The mucoproteins in the saliva provide both adhesive as well as hydrophobic properties which are the reason for it water resistant capability [20]. In case of mud dauber wasp it was found that the saliva secretion tends to bind water along with clay particles which make up the nest [21]. The saliva acts as a cement material and the clay soil is used specifically for making up the hive. The presence of cellulose like material are also found as the wasps tend to use plant parts which are processed and along with oral saliva is made to bring up the whole nest as discussed above.

1.8 Emulsions

Emulsions are basically the presence of one immiscible liquid in another. In normal case of oil and water the bi-phasic system when shaken results in oil droplets which over time coalesce to form two separate phases. The possible reason of coalescence can be attributed to four factors Creaming, Brownian Flocculation, Sedimentation flocculation and disproportionation [22]. The factors act simultaneously or in any succession. The presence of a component which has both a hydrophilic end and a hydrophobic end act to stabilize the oil droplets inside the aqueous solvent thereby preventing their coalescence is known as an Emulsifier. Emulsifiers have wide scale implications in food industry. Oil in water and Water in Oil are the generally two types of food emulsion. Proteins in general have an amphipathic nature and thus are widely used as emulsifiers in food industry. It was reported that the emulsification behavior of the globular protein are influenced largely by solubility, surface hydrophobicity and molecular flexibility [23]. In context with emulsification property of proteins we encounter two terms Emulsifying capacity and Emulsion stability. Emulsifying

capacity of a protein is dependent on charge, shape, neutrality of the dipole, hydrophobicity and Emulsion stability is dependent on the magnitude of these factors [24]. Casein and whey in milk are natural emulsifiers which help to stabilize the fat globules inside the aqueous phase. In our study we wanted to evaluate the role amyloids which are denatured protein fibrillar aggregates for their emulsifying capacity. The reason was the exposure of the hydrophobic groups which formed the β sheets. The application of amyloid as an emulsifier if implemented can act as a major carrier for lipophilic drugs.

Objectives

1. Preparation of Large amyloid fibers from Pepsin and its Characterization.
2. To evaluate the ability of Amyloid to act as an Emulsifier.
3. To understand the structural distribution of naturally occurring fibers and their application as a biomaterial.

Chapter 2

Literature Review

Amyloids are fibrillar protein aggregates implicated mainly in disease conditions like Alzheimer's and Parkinson's. The reason for formation of amyloid in our body which renders the tissue inactive is not yet known but is linked to some amyloid precursor proteins like Aβ-42. Amyloid precursor proteins are protein entities which are prone to amyloid formation in our living system. The reason for amyloid being so dreadful is its cross β architecture which is not only irreversible but also resistant to degradation. The cross β patterns consists of β sheets perpendicular to the axis of the arrangement. The intra strand spacing in a β sheet and inter β sheet spacing is constant over a certain range. Intra strand spacing is around 4-4.7Å and inter sheet spacing is between 10-11Å. This structural architecture of amyloid provides rigidity and robustness which is the reason for its increasing popularity to be used as a biomaterial. In the last decade however researchers found that occurrence of amyloids in natural conditions was exhibited in peptide hormones in pituitary, in melanosomes and in non-mammalian sources like Curli proteins in E.coli and APLP2 proteins in Xenopus sp. The findings suggested that occurrence of amyloids are not restricted to diseases alone rather nature used such a structural morphology of amyloids for some specific functions.

In-vitro synthesis of amyloids using a wide variety of proteins have been carried out for understanding the fundamental aspect of amyloid formation and also to use it as a biomaterial. To identify amyloid formation Thioflavin T and Congo Red dye binding Assay, X-Ray Diffraction, Circular Dichorism, Fourier Transform Infrared Spectroscopy and Electron Microscopy Imaging are the characterization which proves its synthesis. Thioflavin T is one of the amyloid specifc dyes which binds in between the side chains of the cross β structure and is one of the basic characterizations done for amyloid identification. The dye on binding with the amyloid fibers fluoresces when excited at 450nm with an emission optimum at 480nm. Congo Red is also used for diagnosis of amyloid in tissues which when binds to amyloids gives red/green birefringence under polarized microscope. However studies showed that Congo Red is significantly unspecific for amyloids compared to ThioflavinT. X-Ray

diffraction is one of the characterization which specifically evaluates a sample for the presence of the cross β structure. The equatorial and meridonial bands at 10A and 4.7A specifically denotes cross β structure. Ciruclar Dichorism is used to determine the secondary structure distribution in a protein structure. In case of amyloids presence of extensive β sheets and a minima between 210nm and 220nm designates formation of amyloid fibers. Fourier transform infrared spectroscopy similar to CD spectra analysis shows secondar structure distribution. Deconvolution of amide I region(1600-1700cm^{-1}) and a peak at 1605-1625cm^{-1} shows amyloid type β sheet formation. To confirm the presence of amyloid fibers electron microscopy with SEM or TEM is necessary for visual affirmation. Fibers are generally of nanometer size diameter however Ridgley et. al presented fibers of 10-20μm diameter as well which is dependent on the amino acid distribution and hydrophobic amino acid residues in adder proteins.

Amyloids are extensively being used as Biomaterials below are some of the application of amyloid as a Biomaterial.

1. Amyloid fibers were made from lysozyme and β-lactoglobulin .The aim of the experiment was to create a new form of hybrid nano-composite which shows bone-mimetic features. The composite was made from HAP(Hydroxyapatite) and brushite which were later combined with amyloid fibers. Amyloid fibers made suspension of HAP and brushite colloidially stable in water by adsorption of the platelets on the surface. By varying the fibril fraction it was observed that the moduli and the density of the composite was comparable with that of normal cancellous bone. Finally it was noted that pre-osteoblastic cells were able to adhere and grow on the hybrid nano-composites.
2. Aerogels are basically air filled gels where the liquid inside it is replaced by air leaving solid gel network intact. In this work, amyloid fibers and gold nanoparticles are combined to form functionalized aerogels. β-lactoglobulin was the source for amyloid fibril formation. The work showcases the formation of a new form of ultraporous biomaterial with high surface area , low density, soft and non-brittle. Another important property of the new aerogel was that it exhibited bulk auto-fluorescence. Its usage lies in the area of bio-catalysis, bio-detection, etc.

3. Various scientific groups have shown that amyloid fibers have the unique property of allowing mammalian cells to spread, adhere & grow. In this study, amyloid made from α-Synuclein protein was used to make amyloid hydrogels. Human mesecnchymal stem cell (hMSCs) were seeded on the hydrogels and was transplanted in mice. It was observed that the hydrogels contained the cells, improved their survival time compared to as reported earlier and also helped in differentiation of hMSCs into neuronal cell lineage without evoking any immune response. The hydrogels thus created has the potential to be used as a biomaterial for stem cell therapeutics in tissue engineering.
4. Bombyx mori silk solutions were prepared by firstly when sericin proteins were extracted when silks were boiled in 0.02 M Na2CO3 solution for 20 min. Then the aqueous solutions were prepared through dissolving the extracted silks in 9.3 M LiBr solution at 60oC for 4 h and dialyzing against distilled water for 72 h. The solution was further centrifuged at 9,000 rpm for 20 min at 40°C to remove silk aggregates and to generate optically clear SF solutions with final concentration of about 6 wt%. Further,fabrication of SF-coated HA nanoparticles are done by aqueous precipitation reaction by keeping SF as template and surface stabilizer. Firstly an emulsion were obtained and from further centrifugation SF coated HA nanoparticles were obtained. Finally nanoparticles further modified to fabricate into SF coated HA nanofibers. Finally BMP-2 loaded SF/HA composites were prepared.

Pepsin

Pepsin is an acidic protease possessing aspartate residues in its active which is optimally active at a pH of 2. The secondary structure distribution of pepsin includes 14% α helices and 44% of β sheets along with the presence of random coils. Pepsin helps in partial digestion of protein producing fragmented peptides which are acted upon by pancreatic enzymes to get absorbed and assimilated in the body. Pepsin is produced from its precursor molecule Pepsinogen which contains 44 more amino acids compared to native pepsin. At the acidic pH of the stomach the additional amino acid residues are hydrolyzed. Two aspartate residues are present in the active site of the molecules and cleaves peptide bonds between aromatic or hydrophobic amino acids. Pepsin extracted from porcine stomach is mainly used of research purposes.

Industrial Applications of Pepsin include:-

(i) Production of gelatin from collagen and soy proteins. (ii) Helps in removal of hairs and fats from processed hides in leather industry. (iii) For the synthesis of F'(ab) fragment from antibodies to reduce immune reaction from Fc. (iv) As a substitute of rennet for making cheese.

Emulsions

Emulsions are basically the presence of one immiscible liquid within another. Oil droplets in water when shaken form globules inside it resulting in emulsion but after a while coalesce back to form separate phases leading to destabilsation of the emulsion. This destabilsation of the emulsion is attributed to mainly 4 factors Brownian flocculation, Creaming, Sedimentation and disproportionation. All 4 factors can act in order or simultaneously leading to separation of organic solvent from the aqueous one. But in our daily used products and also naturally numerous examples of emulsions which are stable for a significant period of time are present. The component which prevents the coalescence of the organic droplets is known as an Emulsifier. Emulsifiers are amphipathic compounds possessing both hydrophobic and hydrophilic groups. Hydrophobic residues bind oil whereas hydrophilic ones attach with the aqueous solvent thereby stabilizing it. Milk is a natural oil in water emulsion where the Casein protein acts as an Emulsifier. Egg albumin is also a natural product used for emulsion stabilization. Proteins are naturally amphipathic compounds having a hydrophobic core and hydrophilic surrounding. To evaluate a material for its emulsifying property, the stability of emulsion is checked with the help of centrifugation, filtration, shaking or stirring and heating. Whey Proteins and egg proteins like albumin and lecithin is actively used for emulsification purpose. To distinguish between an emulsifier and emulsion stabilizer we can say that the former acts to basically bind the oil components inside an aqueous solvent while the later helps to maintain stability of the homogenous distribution of the organic solvent in the aqueous phase. Emulsion falls under the category of detergents due to the presence of both hydrophobic and hydrophilic ends.

Pickering Emulsions are a form of oil in water emulsion where solid colloidal particles actually help to stabilize the oil inside the aqueous solvent. The adsorption of solid colloids

on the oil surface reduces oil and water interface which prevents coalescence of droplets. A wide range of compounds are available like Bacterial Cellulose Nanocrystals(BCN), silica Titanium dioxide and Magnesium Aluminum silicate which are known to stabilize oil in water emulsions. TiO2 is known for use in both O/W and W/O emulsion. Applications of pickering Emulsions include for health and cosmetics.

Wasp Nest

Wasps are neither bee nor ant they fall under the order Hymenoptera and subclass Apocrita. Wasps in Indian subcontinent which are found in Yellow Jacket, Mud dauber and common wasp To understand how these insects build up their hives which resistant against daily natural hostilities including climate and predators we need to look up their morphology. Earlier the nest material which was believed to be made of chitin is disapproved and researchers have found that those are basically proteins. In a study based on polistine wasp *Polybia paulista* it was found that the oral secretion along with plant parts together combined made up the nest. The oral secretion are proteins with three conserved amino acids among certain wasp species which are serine, glycine and alanine. The conserved amino acids among the wasp's oral secretion can help to categorize the different wasp species based on their habitat as reported. The mucoproteins in the saliva provide both adhesive as well as hydrophobic properties which are the reason for it water resistant capability. In case of mud dauber wasp it was found that the saliva secretion tends to bind water along with clay particles which make up the nest. The saliva acts as a cement material and the clay soil is used specifically for making up the hive. The presence of cellulose like material are also found as the wasps tend to use plant parts which are processed and along with oral saliva is made to bring up the whole nest as discussed above.

Chapter 3

Materials & Methods

3.1 Synthesis of In-vitro Amyloid - Pepsin was the source of protein which was used for in-vitro amyloid formation. As reported [11] the pepsin solution at 9 different concentrations 70mg/ml to 120mg/ml at an interval of 10 was incubated for 3 weeks at 40°c in phosphate buffer pH 7.1 for evaluating emulsifying ability of amyloids. For in-vitro amyloid preparation similar physiological conditions were maintained at 2mg/ml concentration. The longer duration of incubation was observed as a key factor for optimally stabilizing an emulsion.

3.2 Preparation and Characterization of Emulsion - The emulsion was prepared by mixing Hexane as the organic solvent with that of amyloid solution without any additional dilution. The oil in water emulsion included the organic solvent to the amyloid solution in 3:7 ratio. The organic solvent was of 0.3ml while the amyloid solution was 0.7ml which was vortexed followed by ultrasonication in a bath sonicator for 1 min. The biphasic system was kept stable in undisturbed condition for 5 mins to allow stable emulsion.

3.3 Emulsion Stability - The stability of the formed emulsion was evaluated by using centrifugation and also by inverted tube assay. The emulsions were inverted to check both the stability and the time taken for the solution to stabilize the oil in water. Centrifugation was performed at 2000g for 2mins and the depth of the creamy later was noted using a graded scale both before and after the centrifugation.

Droplet characterization was also evaluated using light microscopy. The droplet size of the emulsion was noted for 4 different concentrations with two concentrations stabilizing the emulsions maximally and other two least stabilizing emulsion.

3.4 Sample Preparation of Natural Fibers

Yellow Jacket Nest Fibers (YNF) - Aerial nest of Yellow Jacket was collected. The sample was initially washed twice with distilled water followed by a wash with 70% isopropanol for

disinfection. YNF was then subsequently exposed to UV radiation for 10 minutes before characterizing it. The sample was made sure to be free from any form of dust or insect parts.

Wasp Nest Fibers (WNF) - Wasp nest was collected from house corner. WNF similar to YNF was washed twice with distilled water and then with 70% isopropanol for disinfection. WNF was made sure to be free from any form of insect larvae. It was the exposed to UV radiation for 10 minutes before any characterization was performed.

Mud dauber Nest Fibers (MNF) - Mud dauber nest was collected from house corner. WNF similar to YNF was washed twice with distilled water and then with 70% isopropanol for disinfection. WNF was made sure to be free from any form of insect larvae. It was the exposed to UV radiation for 10 minutes before any characterization was performed.

3.5 Scanning Electron Microscopy - Electron microscopy images of pepsin amyloid, YNF, WNF and MNF was done under Scanning Electron Microscope (Nova Nanosem 450) Pepsin amyloid was centrifuged at 500g for 10 mins after which the pellet was collected air dried and then presented for imaging. YNF, MNF and WNF were washed as described previously placed on glass slides and given for imaging. Overall structural assessment of the nest Fibers were done through SEM.

3.6 Environmental Scanning Electron Microscope - ESEM (FEI,USA) was done for visualizing the amyloids around the hexane droplets as it provides the provision for allowing semi-wet samples for imaging. The emulsion after preparation was taken in a glass slide which was dried and then presented for imaging without any coating. The drying of the sample and also due to the focusing of the electron beam inside the system led to inflated bubble due to the volatile hexane solvent escape. The imaging was performed in ESEM mode (with water) and at partial vacuum.

3.7 Spectrofluorimetry - Pepsin amyloid prepared in laboratory was diluted in phosphate buffer at concentration of 2mg/ml. Thioflavin T dye binding assay was evaluated by using spectrofluorimetry to confirm amyloid formation. Thioflavin T stock solution was prepared by dissolving 8mg of Thioflavin T in 10ml of phosphate buffer followed by syringe filtration

(0.22μm). The working solution of the dye was prepared by diluting the stock solution in phosphate buffer at 1:50 ratio. The sample bound dye was excited at 450nm at the emission window was kept between 460 to 500nm.

3.8 X-Ray Diffraction (XRD) - The X-ray diffraction pattern of the samples was obtained using Ultima IV Multipurpose X-ray Diffractometer (Rigaku Co., Tokyo, Japan). The monochromatic x-ray source with a wavelength of 0.154nm corresponding to Cu-Kα. X-Ray diffraction study was carried out for pepsin amyloid, WNF, YNF and MNF respectively. All the samples were dried and cleaned before putting it for the study. The 2theta scan range for all the samples were kept between 5° to 50° with step size of 0.02 and scan rate of 5°/min.

The d-spacing was calculated to corresponding 2theta value from Bragg's equation :

$$n\lambda=2d\sin\theta$$

3.9 Fourier Transform Infrared Spectroscopy (FT-IR) - The Infrared spectra of YNF, WNF, MNF and pepsin amyloid were recorded using ATR-FTIR, AlphaE, Bruker,USA in the spectroscopic range of 500-4000 cm^{-1} at a scanning range of 12 . All the measurements were carried out in room temperature on KBr disks. In order to determine the distribution of secondary structures deconvolution of the amide I region (1600-1700cm^{-1}) was performed. The deconvolution was done by second order derivitisation and Gaussian peak fitting methods. All the samples peak fitting was done till 5 iterations.

3.10 Confocal Laser Scanning Microscopy (CLSM) – Binding of Thioflavin T dye to the amyloid Fibers prepared from pepsin was checked through CLSM. The sample along with the dye was excited by Argon laser gun of 454nm and the PMT channel was kept between 470-500nm. To observe the presence of amyloid surrounding the emulsion droplets CLSM was used. Thioflavin T and Congo red both amyloid specific dyes were used for visualization and affirmation of the amyloids stabilizing the emulsion droplets. For Congo Red argon laser of 561nm was used with width PMT channel kept between 575 to 610nm.

CLSM was also used to study the **Cell adhesion property** of YNF,MNF and WNF. Both the samples were disinfected with 70% isopropanol twice before cell adhesion study was done.

Cells were seeded on the sample at a density of 10^3 cells per well. Cell seeded samples were incubated for 72 hours at 37°c and 5% CO_2. After 72 hours the samples was washed twice with PBS to remove unadhered cells and then cell staining dye (Calcein AM) was added. The dye was incubated with the samples for 40 mins with before imaging. The esterase activity of live cells cleaves the dye when it enters the cell due to which fluorescence is observed when excited at the wavelength of 405nm.

3.11 Biocompatibility

The biocompatible nature of the naturally found fiber samples were assessed by performing a cell viability assay using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT). YNF , WNF and MNF were washed and sterilized with water and isopropanol (70%) respectively followed by UV exposure for 10 minutes. Human osteosarcoma cell line (MG-63) was used for the study. The cell density for the study was 10^3 cell per well incubated with the sample for 96 hours in 37°c and 5% CO_2. After 96 hours MTT(5mg/ml) was added to the cells followed by incubation for 3.5 hours. Following that MTT was removed and the produced formazan crystals were dissolved in Dimethyl Sulfoxide (DMSO). Results were obtained by reading the absorbance of the purple formazan product at 570nm. The study was done in triplet to evaluate the standard deviation.

3.12 Hemolysis Assay

Blood was collected from a healthy goat placed in 3.8% Sodium citrate and diluted in 0.9% (w/w) saline in 5:4 ratio. The diluted blood was taken as negative control and blood along with 0.01(N) Hydrochloric acid as positive control. The sample was washed and sterilized followed by incubation with diluted blood for 60 minutes at 37°c. After 60 mins the sample was centrifuged at 3000 rpm for 10 mins. The OD of the supernatant having the lysed RBCs was calculated using spectrophotometer at 545nm.

$$\text{Hemolysis \%} = (O.D_{sample} - O.D_{\text{Negative Control}}) / (O.D_{\text{Positive control}} - O.D_{\text{Negative control}}) \times 100.$$

3.13 Alkaline Phosphatase Activity

Alkaline Phosphatase actvity was evaluated using MG-63 cells. Cells were intially seeded on WNF, YNF and MNF and incubated for 7 days at 37°c and 5% CO_2. After 7 days the cells were first trypsinized & then lysed with 0.1% of Triton-X followed by addition of *p*NPP substrate. The substrate along with lysed cells were incubated for 1 hour at 37°c. The reaction was stopped using 0.5N NaOH. The result was measured by reading absorbance at 405nm.

3.14 Release of Sulfated Glycosaminoglycan

The gross release of glycosaaminoglycans(GAG) by the MG-63 cells when seeded on WNF & YNF was evalauted by Dimethylmethylene Blue Assay(DMMB). The experiment duration was for 7 days. Samples were intially seeded with cells and incubated for 7 days at 37°c and 5% CO_2. After 7 days 20µl of the media having GAG was taken and added with 200µl of prepared DMMB dye.The abosrbance reading was taken at 525nm.

Chapter 4

Results & Discussion

4.1.1 Formation of pepsin amyloid

Pepsin is an acidic protease possessing aspartate residues in its active which is optimally active at a pH of 2. The secondary structure distribution of pepsin includes 14% α helices and 44% of β sheets [25]. The reason for considering pepsin as the protein source of amyloid synthesis is for its property to turn inactive at body's physiological pH. This aspect of pepsin compared to other proteins generally used for in-vitro amyloid synthesis gives us an edge to use it as a biomaterial. As reported [11] Pepsin amyloid was prepared by incubating at phosphate buffer at pH 7 for a definite period of time. In this study we followed similar physiological conditions with a protein concentration of 2mg/ml at 40°C. The solution was incubated for 7 days after which characterizations including X-Ray Diffraction, Fourier Transform Infrared Spectroscopy, Circular Dichorism, Spectrofluorimetry and Confocal Microscopy using Thioflavin t was carried out to evaluate the pepsin turned amyloids. Pepsin Assay was done to assess the activity of the pepsin amyloid compared to native pepsin. Keeping in view of the amphipathic nature of the amyloid fibers due to the exposure of the β sheets we evaluated its role as an Emulsifier. A significant finding was that at concentrations below 10mg/ml the amyloid solutions were unable to stabilize the oil in water emulsion. Hexane was taken as the organic solvent to evaluate the role of amyloid as an Emulsifier.

4.1.2 Morphology of Synthetically Prepared amyloid

Visual affirmation of the pepsin prepared amyloid was performed by scanning electron microscopy. Fig (4.1) shows the formation of aggregated fibrillar structures indicating amyloidgeneis of pepsin at neutral pH. Compared to as reported [11] the fibers formed in this study were of larger aggregated fibers of micrometer size. As reported by Ridgley et. al the formation such large amyloid fibers can be attributed to the hydrophobic amino acid distribution. In this case however the large amyloid fibers can be due to higher concentration

of the protein and longer incubation time. The results were further validated with X-Ray Diffraction, Spectrofluorimetry and Confocal Scanning Laser Microscopy.

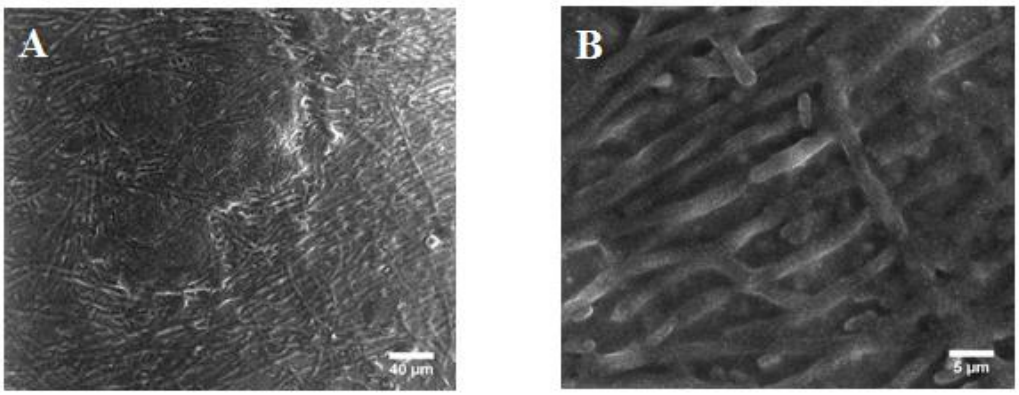


Fig. 4.1 - (A) Scanning Electron microscopy Images of porcine pepsin turned amyloid fibers. (B) Large amyloid fibers observed under Scanning Electron Microscope after incubating for 7 days at 40°C.

4.1.3 X-Ray Diffraction

The cross β conformation of amyloid fibers is a distinct feature which can be harnessed by the help of X-Ray Diffraction. As reported the 2θ peaks at signifies the intra-strand spacing and inter-sheet spacing respectively.

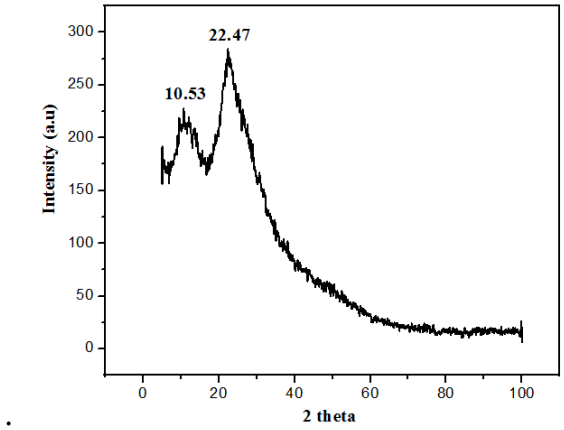


Fig 4.2 shows the two peaks obtained from the synthetically prepared pepsin amyloid. The peaks acquired at 22.47° and 10.53° which corresponds to d-spacing of 4Å and 8.5Å respectively which justifies the cross β conformation indicative of amyloid formation.

The cross β conformation is the reason for rigidity and robustness of amyloid along with its property to resist degradation by any enzymes. The orientation of the β strands perpendicular to the axis of the sheets is the reason for the cross β architecture which is also the reason for Thioflavin T binding.

4.1.4 Dye Binding Assay

The binding of amyloid specific dye , Thioflavin T to amyloid fibers is one of the confirmatory analysis for synthetically prepared amyloid. Thioflavin is a cationic benzothialonic dye which binds with the cross β structure of the amyloid fibers with a simultaneous increase in the fluorescence intensity. The excitation of Th t bound fibers at 450nm with contemporaneous emission at 480nm is indicative for amyloid fibrillar formation. Fig (4.3) shows a peak at 488nm when excited at 450nm using spectrofluriometry. The peak suggests the formation of amyloid from pepsin as reported. However it is necessary to note that the binding of Thioflavin T to nucleic acid is also reported [26] which is due to ionic interaction. The reason for Thioflavin T binding to amyloid proteins hence can also be attributed to the charge distribution of the protein in that particular pH. However existing studies have couples Thioflavin T binding to the cross β architecture of amyloid fibers to the other characterizations like X-Ray Diffraction, FT-Infrared Spectroscopy, Circular dichorism along with Electron Microscopy.

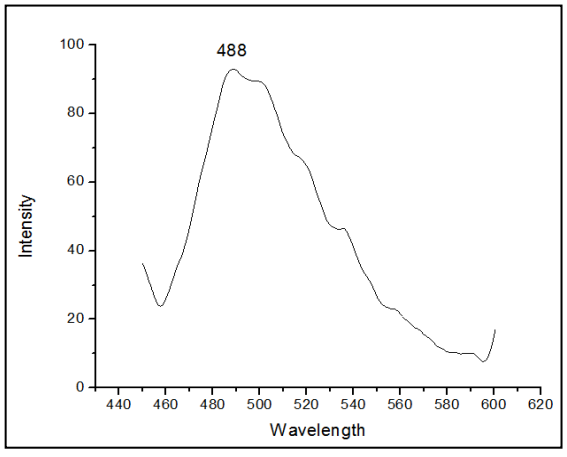


Fig 4.3 - Fluorescence spectra of Thioflavin T in pepsin after incubating at 40°C in pH 7.1 for 7 days.

In our study we have presented Th T study along with CD spectra analysis, SEM and X-Ray diffraction to validate the formation of amyloid fibers from pepsin.

4.1.5 Confocal Laser Scanning Microcopy (CLSM)

Amyloid specific dye Thioflavin T is reported to diagnose the presence of amyloid in tissues using fluorescent microscopy. Similarly in-vitro amyloid synthesis is checked with fluroscent spectroscopy to determine the cross β architecture. Keeping in view of Th T use for identification & diagnosis of amyloid in tissues [27] we used the similar concept by the help of CSLM. The florescence of Thioflavin T on binding the amyloid fibers as observed in spectrofluorimtery is also evaluated using CSLM. The dye bound amyloid solution was incubated for 15 mins before exciting at 454nm (Argon Laser). The PMT channel was kept open between 470-520nm to maximally recover the emission spectrum. CLSM study proves that apart from electron microscopy confocal imaging can also be used for evaluating the presence of amyloid fibers in presence of amyloid specific dye like Thioflavin T. Fig (4.4) shows the Th T bound amyloid fibers which authenticate the presence of large amyloid fibers as was observed in SEM imaging.

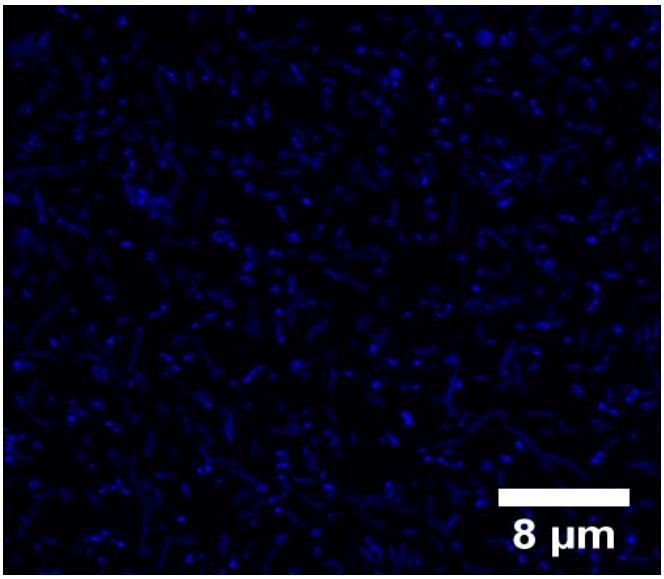


Fig 4.4 - Fluorescent amyloid fibers in presence of Thioflavin T as viewed under Confocal Laser Scanning microscopy when excited by argon laser of 454nm.

4.1.6 Pepsin Assay

Activity of pepsin after forming amyloid was also evaluated to validate the extent of denaturation of pepsin. The extent of degradability of hemoglobin under acidic condition by pepsin and pepsin turned amyloid was assessed. Fig (4.5) shows the activity of native pepsin with respect to after forming amyloid. It can be seen that pepsin amyloid lost its activity significantly which validates the denaturation of pepsin and its subsequent transformation to amyloids.

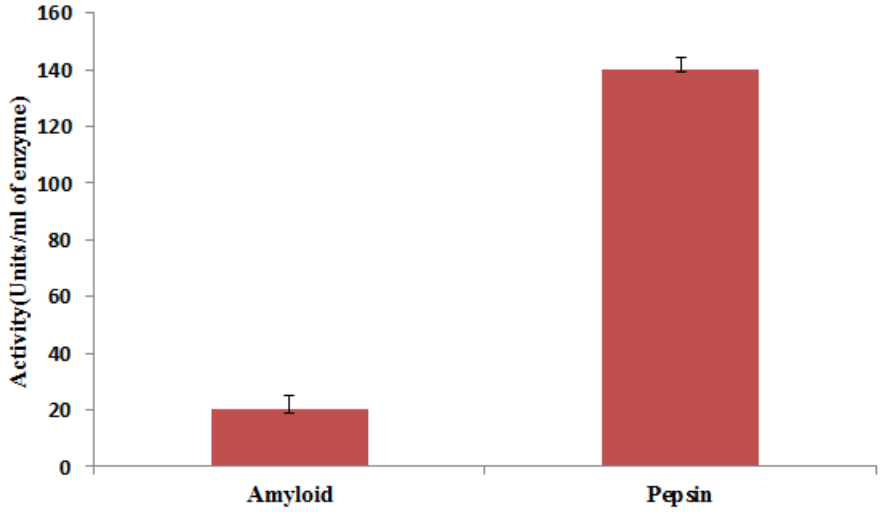


Fig 4.5 - Pepsin Activity after amyloid formation.

4.1.7 Role of Pepsin Amyloid as an Emulsion Stabilizer

Emulsions are basically the presence of one immiscible liquid in another. In normal case of oil and water the bi-phasic system when shaken results in oil droplets which over time coalesce to form two separate phases. The possible reason of coalescence can be attributed to four factors Creaming, Brownian Flocculation, Sedimentation flocculation and disproportionation [24]. The factors act simultaneously or in any succession. The presence of a component which has both a hydrophilic end and a hydrophobic end act to stabilize the oil droplets inside the aqueous solvent prevents their coalescence and is known as an Emulsifier.

In this study we investigated the ability of amyloid to stabilize emulsions. Proteins like whey are reported to act as an emulsifier [28]. The natural structure of a protein which contains a hydrophobic core and hydrophilic periphery is imperative to act as a good emulsifier [29].

Porcine pepsin at acidified pH where it is native structure was taken as control. Fig (4.7) shows that with time pepsin lost its capability to hold the organic solvent and the phases separated out. This finding suggests that native protein do have the property to stabilize emulsion but for a brief period of time. As they slowly starts to revert back to their native states they lose the ability to hold the organic solvent in the aqueous surrounding leading to separation of the phases. However the denaturing of a protein to amyloid configuration is seen Fig (4.6) to optimally hold the oil in water for a longer duration compared to the control. The exposure of the β sheet rich hydrophobic structure is stabilizing organic solvent and at the same time bonding with the aqueous solvent by the hydrophilic residues.. We evaluated the stability of the emulsions using centrifugation and observed the optimum concentrations that were maximally stabilizing the emulsions. Droplet characterization was also carried which showed that with increasing concentration the droplet size decreased.

4.1.7.1 Emulsion Preparation

The amyloid was prepared by incubating 9 different concentration of porcine pepsin at 37°c for a month. Phosphate buffer at pH 7.1 was the dissolving medium. The necessity to use higher concentration of protein can be attributed to the formation of large amyloid fibers which are much more efficient to hold the organic solvent in water. Protein concentration below 2mg/ml which is reported to form nanometer amyloid fibers was inefficient to hold the oil in water. Hexane was used as an organic solvent and was dispersed in amyloid solution at 3:7 ratio [24]. The biphasic system was ultrasonicated and kept in undisturbed condition to form stable emulsions. Fig (4.6) shows the 9 different concentration of pepsin amyloid stabilizing the oil in water emulsion. Fig (4.9) shows the time dependent study of amyloid stabilizing the emulsion. It was observed that 3 days is the optimum time taken by amyloid to stabilize emulsion.

Confocal Microscopy and ESEM Imaging were performed to validate the presence of amyloid surrounding the emulsion droplets. Thioflavin T and Congo Red the two amyloid specific dyes were used to visualize the amyloids. Fig (4.8) shows the emulsion droplets surrounded by fluorescently labeled amyloid fibers. The presence of amyloid fibers surrounding the inflated hexane droplets can be viewed in Fig (4.10). It should be noted that along with

amyloid fibers some aggregated amyloid like denatured protein structures were also formed which played synergic role in stabilizing the emulsions. Stability of an emulsion was evaluated by centrifugation process. Centrifugation leads to coalescence of the organic solvent droplets which ultimately leads to destabilization of the oil in water emulsion. In this study centrifugation was done to understand the particular concentration which was optimally holding the oil droplets inside the aqueous solvent.

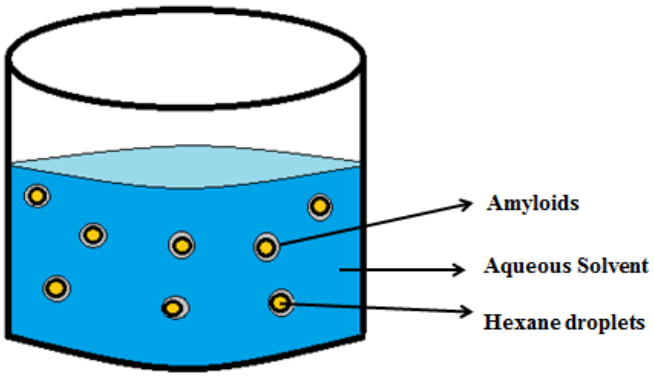


Fig 4.6 - Schematic representation of the organic solvent Hexane stabilized by amyloid fibers in the aqueous solvent. Oil in Water (O/W) Emulsion.

Fig 6 through a schematic representation depicts the possible phenomenon where the hydrophobic residues of the amyloid fibrils seem to stabilize the organic solvent inside the aqueous solvent of the amyloid solution.

Fig 4.7 - Hexane/ Amyloid solution oil in water biphasic system after ultrasonicating for 1 min.

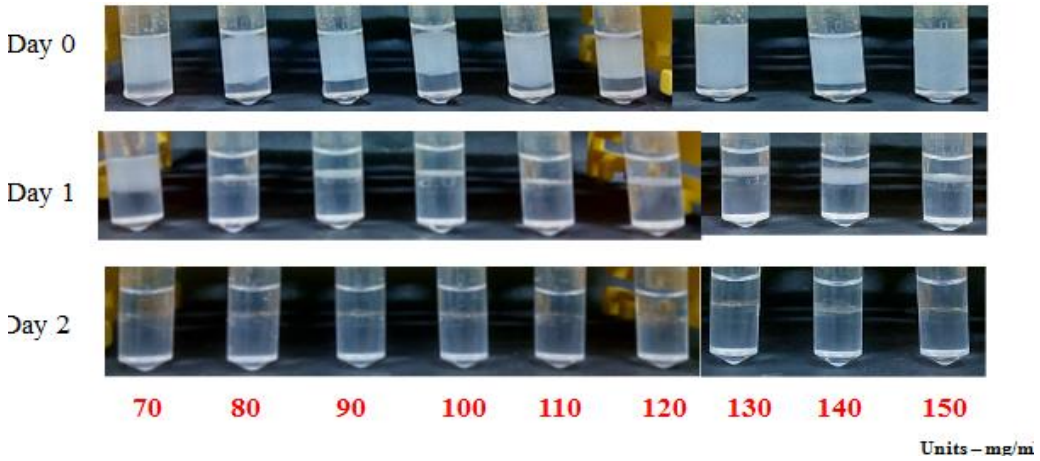


Fig 4.8 - Depicting the destabilization of emulsion leading to phase separation in presence of native pepsin under similar condition used for pepsin turned amyloid study.

Fig (4.8) shows that with time the ability of native pepsin to stabilize organic solvent decreased which ultimately led to destabilization of the emulsion leading to phase separation. The phenomenon as described previously can be attributed to the fact that during ultrasonication the pepsin structure got denatured which led to exposure of the hydrophobic residues thereby helped in stabilization of hexane. However with time the protein mat have reverted back to its native form leading to the hydrophobic amino acids in the core structure which ultimately led to the coalescence of the organic solvent leading to separation of the bi-phasic system.

4.1.7.2 Validation of the Role of Amyloids as an Emulsifier

Confocal Microscopy (CLSM) and Environmental Scanning Electron Microscopy was used to validate the presence of amyloid surrounding the hexane droplets as exhibited in Fig 4.9,4.10. The amyloid specific dyes proved amyloid presence by florescence activity while E-SEM helped to visually affirm the presence of small fibril in an round the inflated hexane droplets. The emulsions were dried before placing for E-SEM which may be the reason for the inflated bubble formation.

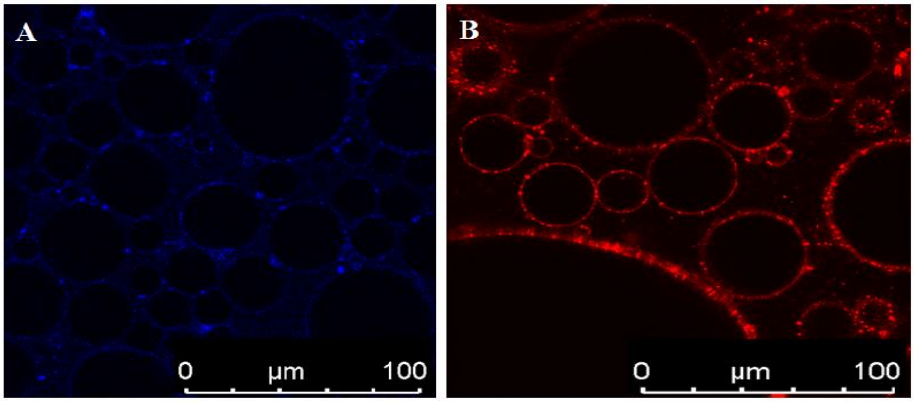


Fig 4.9 - Confocal Laser scanning micrograph of hexane droplets stabilized by amyloids. (A) Amyloid stained by Thioflavin T (ex/em-454/480nm) (B) Amyloid stained by Congo Red (ex/em-561/580nm).

Both Thioflavin T and Congo Red are dyes used for amyloid identification. However Thioflavin is much more specific for the cross β architecture of the amyloids compared to Congo Red whose specificity for amyloids are ambiguous. The Thioflavin T fluorescence in Fig (4.9) provides support for the presence of amyloids in an around the organic solvent.ESEM Imaging was performed for visual affirmation of the amyloids in their role for stabilizing the organic solvent. The presence of the amyloid fibers around a single inflated droplet on which the electron beam was focused proves its occurrence. However presence of denatured protein aggregates also may have accounted for the emulsifying property of the amyloid solution. This is because of the high protein concentration which is generally not reported to be used for in-vitro amyloid preparation.

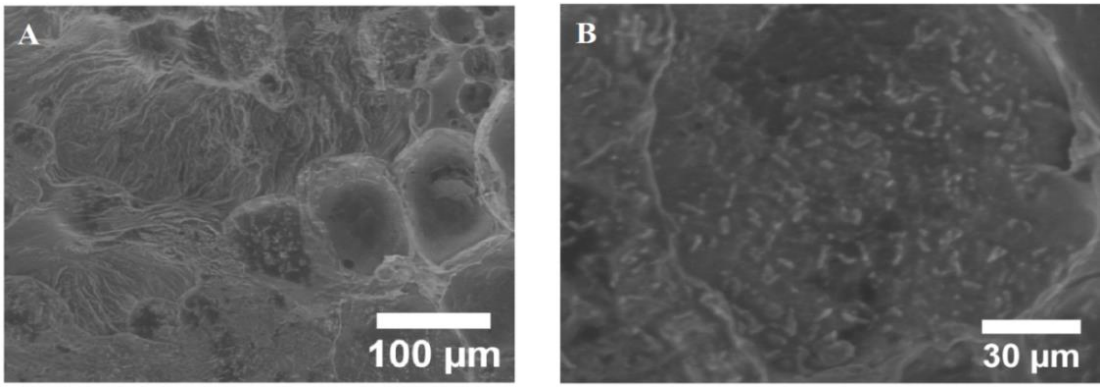


Fig 4.10 - (A) Inflated Hexane Droplets (B) Amyloid fibrils around the hexane droplet**.**

4.1.7.3 Emulsion Stability

An Emulsion is considered stable when subjected to various physical conditions like filtration, centrifugation, heating, etc. All the above methods cause destabilization of the emulsions which ultimate leads to coalescence of the droplets. Creaming is a natural process and an emulsion is said to be stable as long as it can prevent aggregation of the droplets(23). In our work we used inverted tube assay and centrifugation to observe and note the concentration which was optimally stabilizing the emulsion. The inverted tube assay Fig(8) shows the time required by amyloid to stabilize emulsion.

The emulsions were centrifuged at 4000g for 2 mins[24] and the decrease in the creamy emulsion layer was observed. Fig (4.9) shows the emulsion stability after centrifugation. The ratio between the emulsion volume before and after centrifugation was calculated which revealed the concentration which was optimally stabilizing the emulsion. The ratios are represented in a bar graph Fig (4.12) showcasing the two concentrations 70mg/ml and 90mg/ml which were optimally stabilizing the emulsion.

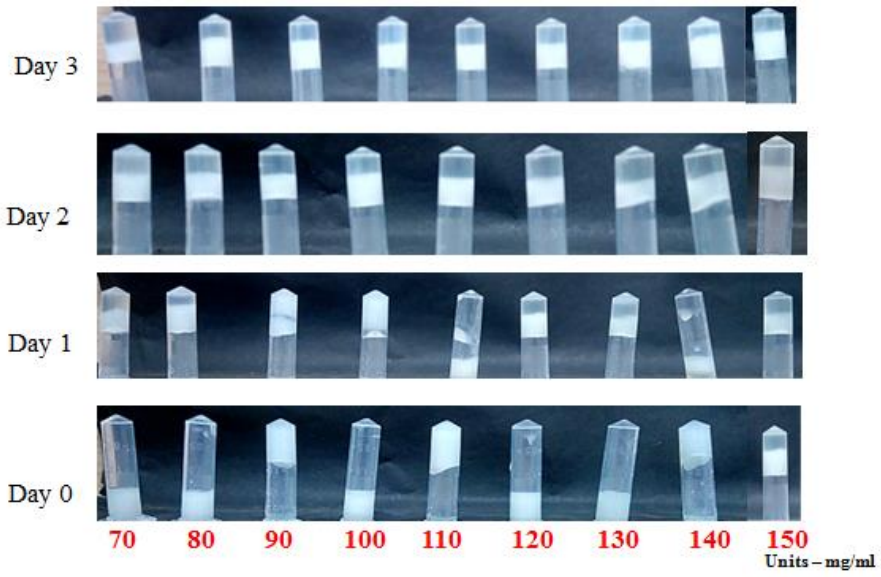


Fig 4.11 - Hexane/Amyloid solution oil in water emulsion inverted tube assay depicting emulsion stability and time required by amyloid to stabilize the organic solvent optimally.

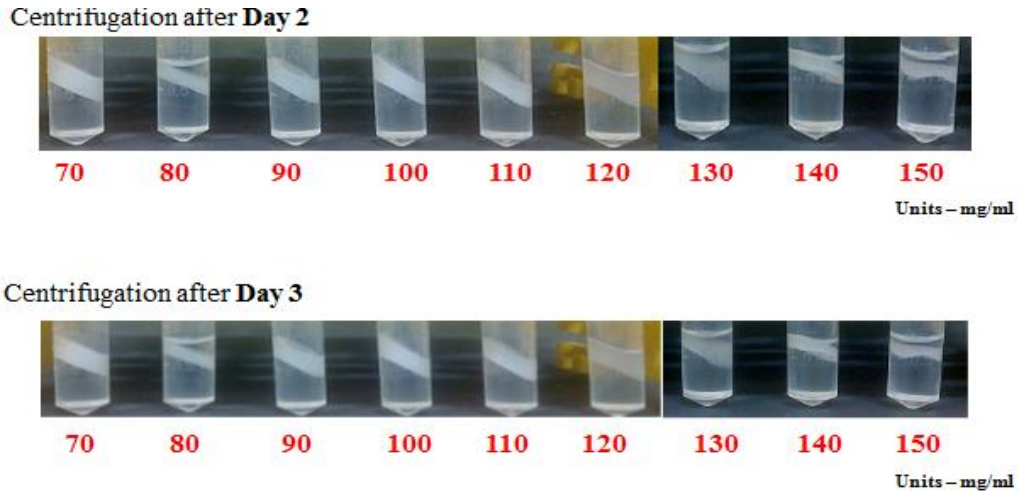


Fig 4.12 - Hexane/ aqueous Amyloid solution oil in water emulsion stability after centrifuging at 4000g for 2 mins for increasing protein concentrations from 70mg/ml to 120mg/ml.

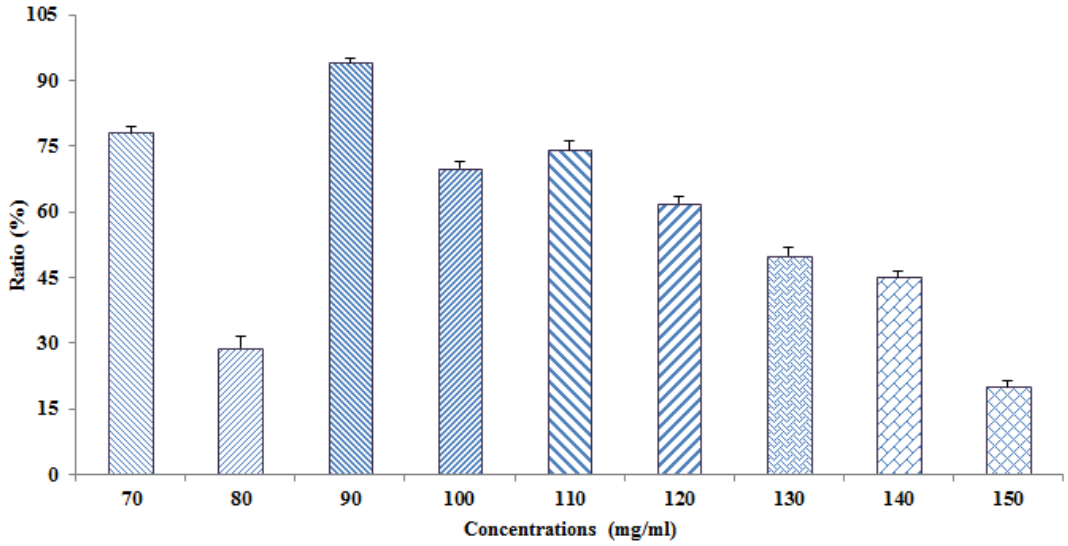


Fig 4.13 - Ratio of Hexane in the emulsion (v/v) after centrifugation at 4000g for 2 mins to the increasing amyloid concentration from 70mg/ml to 120mg/ml.

4.1.7.4 Droplet Characterization

Droplet mean diameter was measured of 4 different concentration of pepsin amyloid. Two were 70mg/ml and 90mg/ml which were optimally stabilizing the emulsions and other two were 110mg/ml and 120mg/ml which were least stabilizing emulsion. The graph shows a clear indication of decreasing droplet size with increase in amyloid concentration. The finding suggests that with higher concentration more uniform fibers were formed which were leading to smaller droplets. However the reason for decreased stability at highest concentrations cannot be comprehended in such preliminary studies.

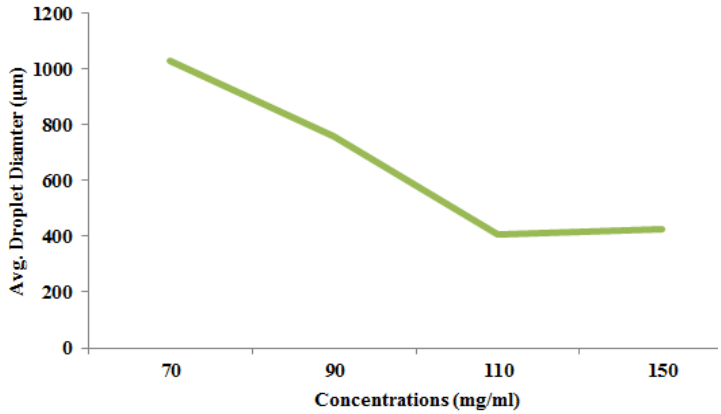


Fig 4.14 - Droplet diameter vs protein amyloid concentration in an emulsion having Hexane as the organic solvent at 7: 3 ratio.

It should be noted that aggregated protein structures were also viewed in confocal microscopy and ESEM as the concentrations of protein taken amyloid formation was higher than usually reported hence it is expected that denatured pepsin along with amyloids played an active role as emulsifiers. Both amyloid and the denatured components of proteins were synergistically acting as emulsion stabilizers and not amyloids independently as discussed above. Hexane was considered as a hydrophobic model for studying the role of amyloid as an emulsifier. Further studies using Hexadecane and long chain hydrocarbons are necessary to further assert amyloid's role as an emulsifier for higher. This aspect of amyloid in conjunction with higher lipids like olive oil [30] or linseed oil [31] which are used for lipophilic drug delivery can be carried out for efficient drug delivery applications.

4.2.1 Nest Samples and its corresponding Insects

Nest Hives **Insects**

Common Wasp (*Vespula vulgaris* **)**

Yellow Jacket Wasp (*Vespula germanica* **)**

Mud dauber Wasp (*Sceliphron caementarium)*

Fig 4.15 - Nest Structure and its respective Insects.

4.2.2 Surface Characterization of the Natural fibers

4.2.2.1 Wasp Nest

Scanning Electron Micrograph imaging was performed to assess the overall fiber morphology of WNF. Fig (4.16) depicts the fibrous network of WNF providing an idea about the fiber morphology and architecture. Fig (4.16B) reveals the dimeric nature of the fibers constituting the nest. The average diameter of the dimeric fibers were of 7.5µm with a range of 6µm(lowest) to 9µm(highest). It is expected that the dimeric nature of the fibers is the reason for the rigidity and integrity of the overall structure of the nest.

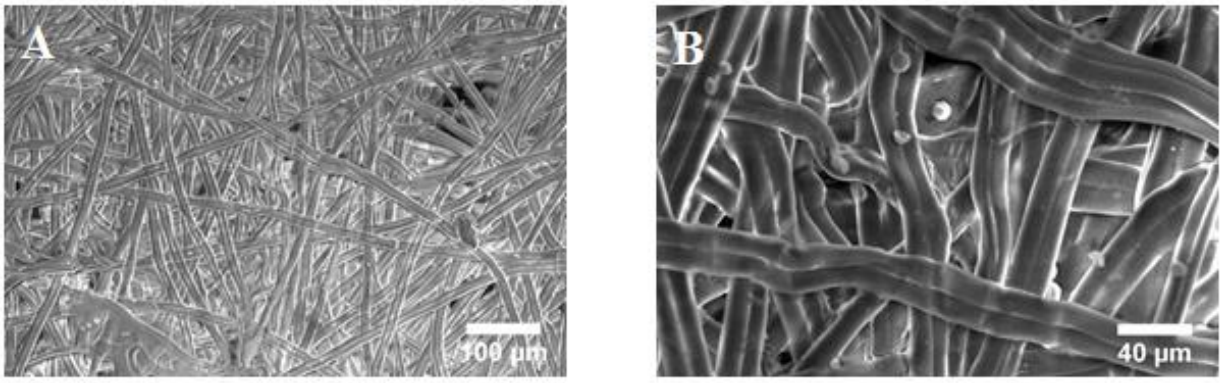


Fig 4.16 - (A) Scanning Electron Micrograph images of WNF to elucidate the structural makeup of the nest.(B) Scanning Electron Micrograph image depicting the dimeric nature of the WNF.

4.2.2.2 Wasp Nest after Boiling in in Sodium Carbonate

When the fibers were boiled with sodium carbonate 0.2M (Na_2CO_3) [32] for 1 hour it was observed that the dimeric structure was dissolved. The dimeric fibers became monomeric indicating that the interaction among them may be chiefly ionic in nature. As was observed previously the single fibers had an average diameter of 3.5µm. Fig (4.17) shows the monomeric fibers after dissolving in Na_2CO_3. The salt on dissolving creates an ionic environment which is the probable reason for the separation of the fibers in presence of heat

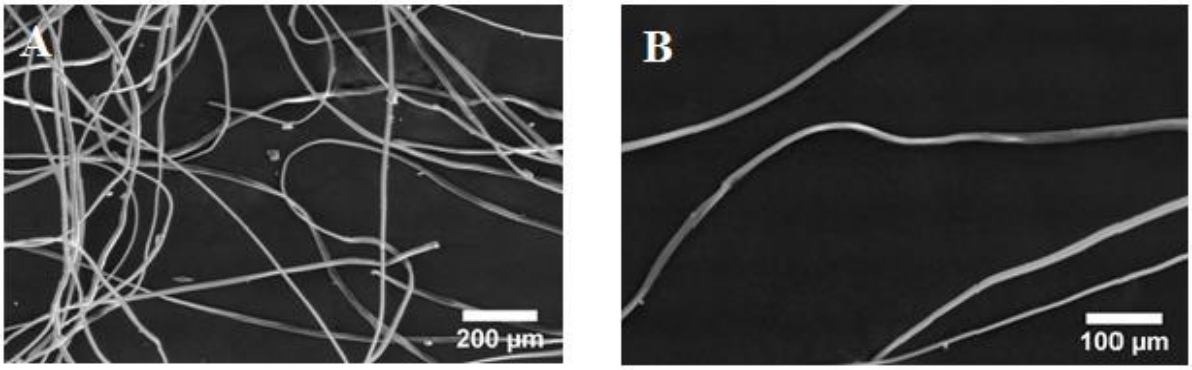
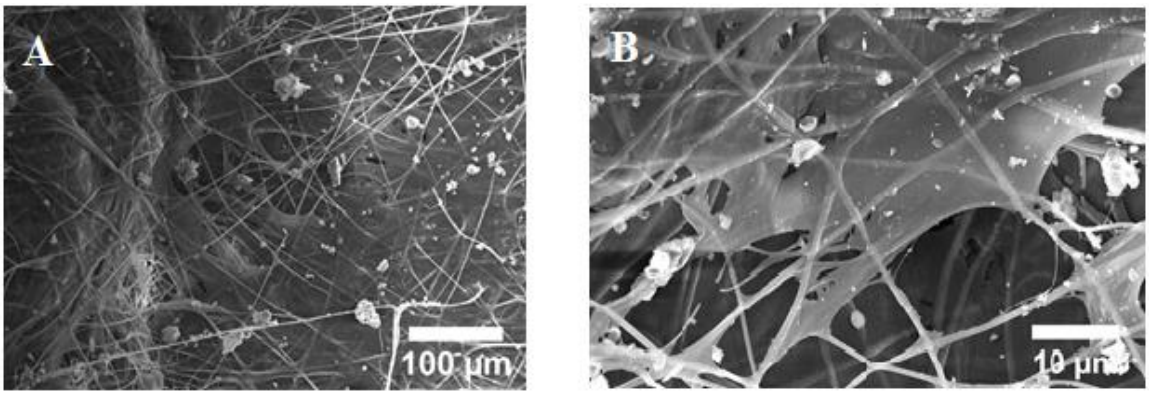


Fig 4.17 - (A)Scanning Electron Micrograph images of Wasp nest fibers after boiling with Na_2CO_3 for 1 hour. (B) Scanning Electron Micrograph Image of single separated fibers of WNF.

4.2.2.3 Mud dauber Nest

Mud dauber nest as observed under scanning electron microscope having fibrous structure is depicted in Fig (4.18A) which gives a probable idea of how it is able to bind the soil particles around it. The fibers have an average diameter of 1.8µm with a range of 1.5µm (lowest) to 2µm (highest) approx. Apart from the fibers, there are membranous sheet like structures which are connecting the fibers and thus consolidating the entire architecture for nest formation. The membranous sheet as shown in Fig (4.18B) can be finer fibers in nanometer range holding the framework. It is to be noted that the mud dauber nest possess these fibrous structures inside the nest unlike the other hives where the fibers are exposed. The nest fibers are present inside the clay soil which actually binds & holds the soli particles within it. Single consolidated fibers of MNF are depicted in Fig 4.18(C,D).



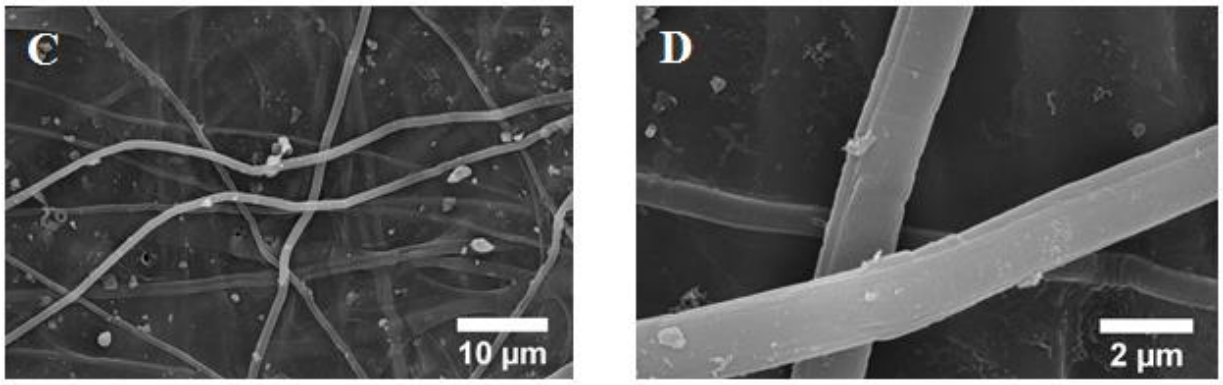


Fig 4.18 - (A) Scanning Electron Micrograph of Mud dauber Nest fibers for understanding the structural architecture of the nest. (B) Scanning Electron Micrograph Image to show the presence of membranous structure in between the fibers. (C,D) To understand the singular fiber structure of MNF.

4.2.2.4 Yellow Jacket Nest

Similar to WNF and MNF Scanning Electron Micrograph imaging showed the structural morphology of the yellow jacket nest. The fibrous network is visible but the diameters of fibers are found to vary unlike other two structures where uniformity was seen. Fig (4.19) shows the overall nest fibers arrangement in YNF. YNF showed variability in the distribution of varied fiber diameters from 4µm (lowest) to 15µm (highest) with an average of 8.2µm .

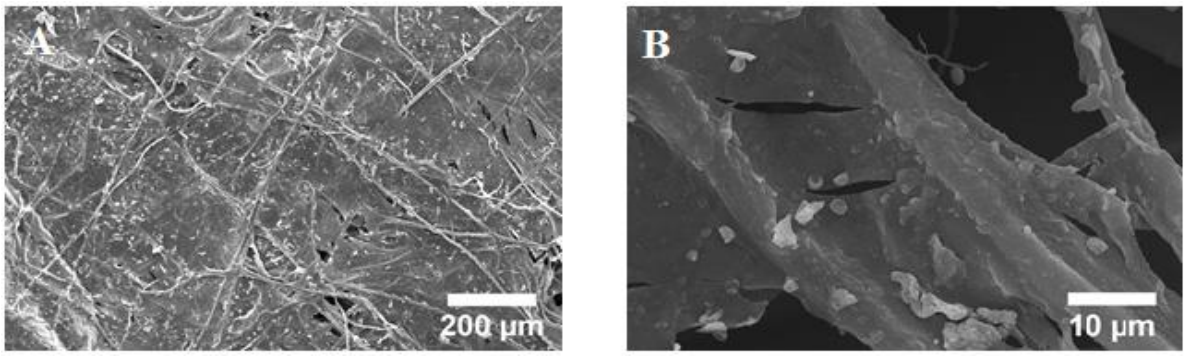


Fig 4.19 – (A)Scanning Electron Micrograph of Yellow Jacket nest fibers showing overall morphology of the nest. (B) Scanning Electron Micrograph image of YNF depicting the presence of membrane in between two fibers.

The presence of membranous sheet similar to MNF is also visible in Fig (4.19B) which can be the connecting structure of among the fibers imparting the nest's overall structural integrity. The membrane is similar to mud dauber nest where it is much more conspicuous and can be dense fibirllar aggregates forming an even network.

4.2.2.5 Comparison of the diameters of YNF, MNF and WNF

A comparison was carried out for YNF,WNF and MNF on the basis of fiber diameters constituting the nest to understand their significance in accordance to their place of occurrence. For the three samples 20 fibers were counted for their diameter before making the statistical comparison. As mentioned above YNF showed variance in different type fiber diameters. Lowest diameter fibers are of MNF as observed. Fig (20) depicts a bar graph indicating average fiber diameters w.r.t the three samples.

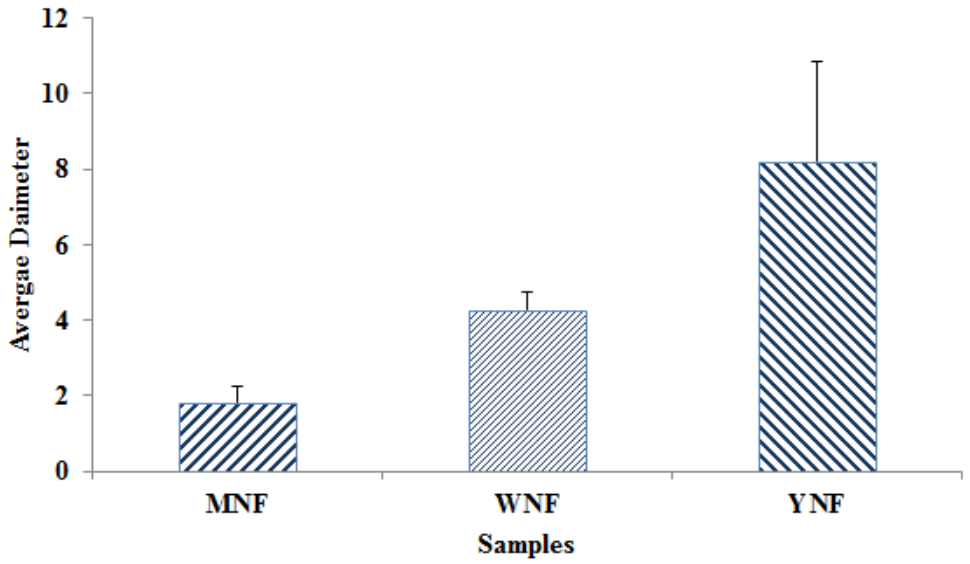


Fig 4.20 - Comparison of the average diameters of YNF, WNF and MNF. The standard deviation of YNF indicates the presence of varied fibers of different diameters in the nest structures. In the above graph all the fibers chosen were singular indicating that single fibers of WNF were considered while counting the average.

4.2.3 Structural Study of the Natural Fibers

4.2.3.1 Fourier transform infrared spectroscopy (FTIR)

FT-IR was performed to validate the presence of protein and simultaneously analyze the distribution of secondary structures in the naturally occurring nest fibers. The amide I region of the peptide bond from 1600-1700cm^{-1} is assigned to C=O stretching which corresponds with the protein secondary structures [33]. It is one of the prominent and sensitive vibrational bonds of the protein backbone. The Amide II and Amide III are assigned to N-H and C-N stretching which are weaker vibrations and hence Amide I is mostly considered. The study of the protein secondary structures based on FT-IR is reported to be evaluated by studying the respective Circular Dichorism spectra analysis and X-Ray Diffraction data [34].

4.2.3.1.1 Wasp Nest Fibers

FT-Infrared Spectroscopy performed on WNF as depicted in Fig (4.21) shows a single maxima at 1639cm-1 within the amide I region which shows the presence of β-sheets distribution .The major peak at 1639cm-1 reveals the of presence native β sheets rather than amyloid β sheet conformation. On deconvoluting the amide I spectra three maxima at 1623cm^{-1}, 1641cm^{-1} and 1662cm^{-1} were obtained which proved the distribution of native β-sheets (1620-1640cm^{-1}) along with distinct presence of β-turns or antiparallel β strands(1662cm^{-1}).

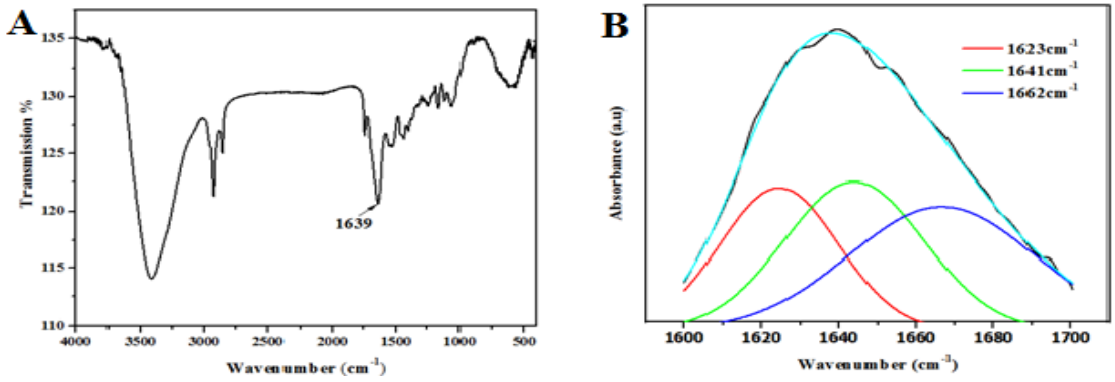


Fig 4.21 - (A) FT-IR spectra of the Wasp Nest fibers. (B) After Deconvolution of the amide I region from 1600-1700cm-1.

4.2.3.1.2 Mud dauber Nest Fibers (MNF)

FT-IR spectra of mud dauber nest at the amide I region of 1632cm^{-1} gave an indication of the proteinaceous characteristic of nest. The major peak at 1632cm^{-1} as shown in Fig (4.22) indicates the presence of β sheet conformations. On further deconvolution of the amide I spectra 3 peaks were obtained which were at 1629cm^{-1}, 1639cm^{-1} and 1667cm^{-1}. Both 1629 and 1639 which falls within the amide I region indicates the presence of native β sheets structures along with the presence of antiparallel β sheets or turns. As reported earlier for silk fibroin [35] spider silk[5] similar observations for the presence of β sheet distribution were observed with transmission peaks in the amide I region at 1630cm^{-1} and 1624cm^{-1} respectively. The extensive presence of such β sheet structure can be attributed for the nest fibers property of having rigidity and tensile strength. In both the cases of spider silk and silk fibroin distribution of alanine and glycine residues are reported as the reason for the presence of such β sheet structure.

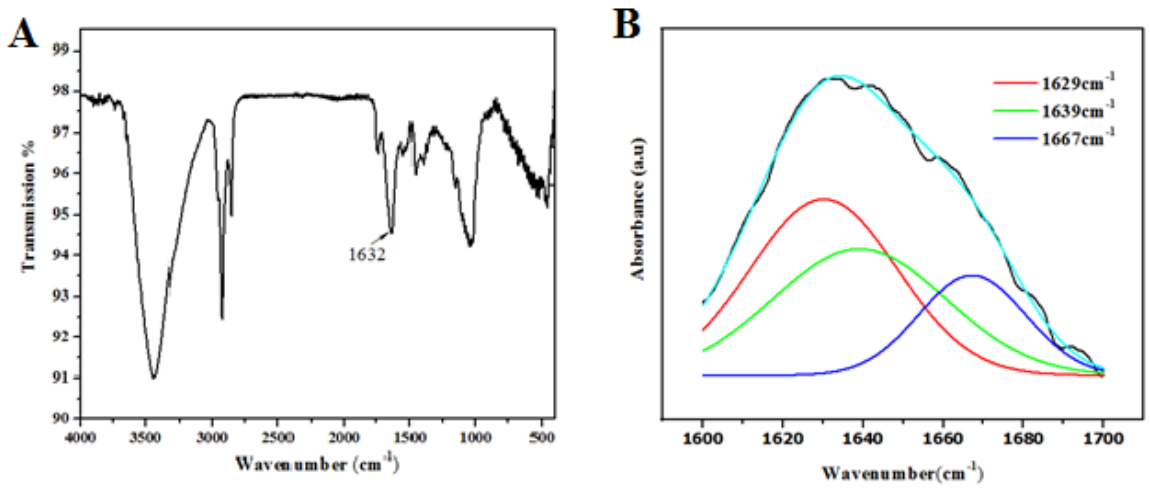


Fig 4.22 - (A) FT-IR spectra of Mud dauber nest fibers (B) After deconvolution the amide I region from 1600 cm-1 to 1700cm-1.

The study suggests the extensive presence of β sheets and turns indicating their significance in such naturally occurring structural makeup.

4.2.3.1.3 Yellow Jacket Nest Fibers (YNF)

Infrared spectroscopy revealed the distribution of secondary structures in YNF. Compared to the above two structures of Wasp and Mud dauber observation show similar maxima at 1639cm-1 region indicative of β sheet composition (Fig 4.23). On deconvoluting the amide I region however something significant was found. The three peaks that were obtained are at 1625cm-1, 1642cm-1 and 1658cm-1. In Fig (4.23B) the first peak at 1625cm-1 indicates the presence of amyloid type β sheet conformation [35], at 1642cm-1 like Mud dauber and Wasp nest structures shows the presence of native β sheet structures. However at 1658cm-1 the peak indicating the presence of alpha helix structure is an unique understanding. In the yellow jacket nest composition shows the distribution of amino acids pertaining to the nest formed from salivary fluid content of the insects. The secondary structure of Yellow Jacket nest as understood from FT-IR study proves the necessity of both alpha helix and β sheets for stability of the structures.

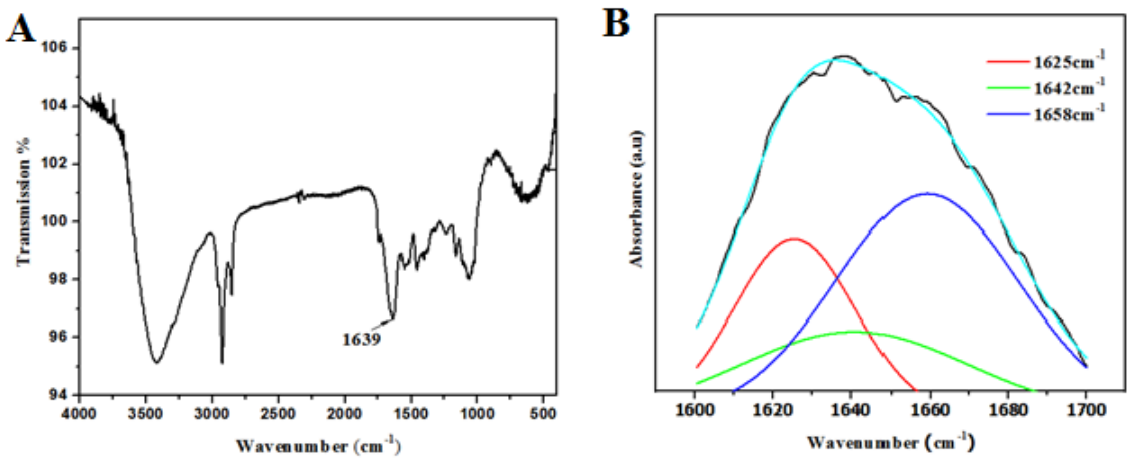


Fig 4.23 - (A) FT-IR spectra of the Yellow Jacket Nest fibers (B) After Deconvolution the amide I region from 1600cm^{-1} to 1700cm^{-1}.

For all the above samples the secondary structure distribution was analyzed by Gaussian fits after the deconvoluting the amide I spectra as described for each. The Gaussian curve fitting was done till 5 iterations for all the samples. The R^2 value for all the cases were above 99%.

4.2.3.2 X-Ray Diffraction

X-Ray Diffraction was performed to analyze the structural distribution of the naturally occurring nest fibers and to support the findings of FT-Infrared Spectroscopy.

4.2.3.2.1 Wasp Nest fibers

Figure (4.24) shows two distinct peaks at 13.5° and 23.29° 2theta values respectively. The corresponding d-spacing of the two peaks are at 6.55Å and 3.81Å indicating the presence of β sheet [36] like structure along with distribution of α helix patterns. The presence of 3.81Å peaks corresponds close to the silk fibroin alternative β sheet spacing of 3.5Å [37]. However the other silk fibroin β sheet spacing is around 5.5Å approximately [37]. The finding suggests that native β sheets are present but may possess their own distinct structural distribution like silk fibroin. Further studies to extensive understand the secondary structures are necessary.

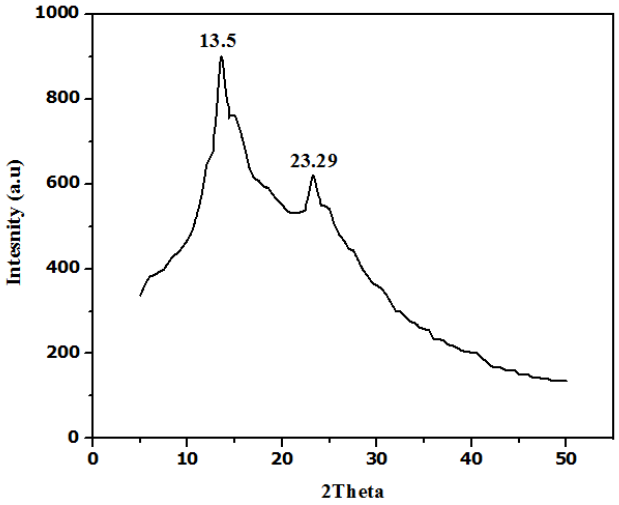


Fig 4.24 - X-Ray Diffraction curve of Wasp Nest fibers depicting 2θ peaks at 13.5° and 23.29° respectively.

4.2.3.2.2 Mud dauber Nest fibers

X-Ray diffraction of mud dauber nest structure showed two significant peaks at 13.7° and 27.01° respectively with corresponding d-spacing being 6.455Å and 3.29Å respectively in Fig 25. The findings are similar to that of wasp nest fibers indicating an uniform distribution of β sheet conformations stabilizing the overall nest structure. Similar to WNF peaks at d-spacing

of 3.29Å shows proximity towards silk fibroin like morphology. However like WNF. MNF may also possess unique distribution of β sheet conformation which cannot be conclusively presented from the preliminary findings through X-Ray Diffraction.

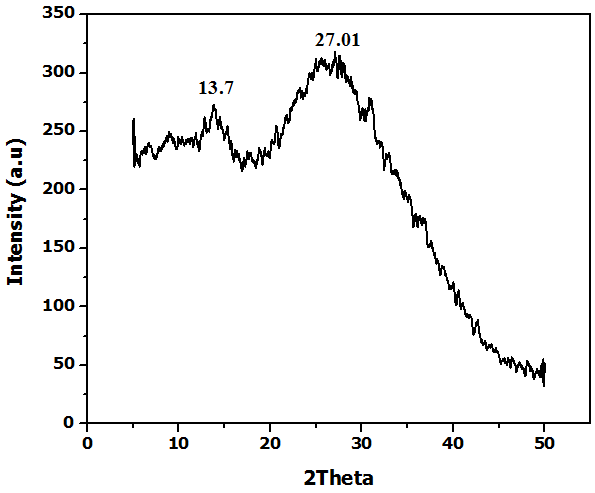


Fig 4.25 - X-Ray Diffraction curve of Mud dauber Nest fibers portraying 2θ peaks at 13.7° and 27.01° respectively.

4.2.3.2.3 Yellow Jacket Nest Fibers

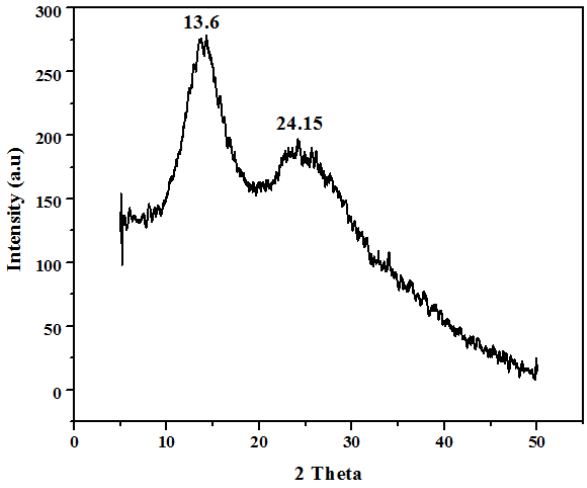


Fig 4.26 - X-Ray Diffraction curve of Wasp Nest fibers depicting 2θ peaks at 13.6° and 24.15° respectively

The X-Ray diffraction study of the Yellow Jacket Nest fibrous structure shows Fig (4.26) two peaks at 13.6° and 24.15° which corresponds to d-spacing of 6.5Å and 3.68Å respectively.

Yellow Jacket is also a form of wasp whose salivary secretion is reported to be needed for making up the nest fibrous structure. X-Ray diffraction graph of YNF is expected to portray the structural arrangement. Similar to WNF and MNF, YNF also possess a sub-class of silk fibroin conformation. All the 3 structures show similarity in the d-spacing which indicates the partiality towards β sheet conformations which provides rigidity to the structures.

4.2.4 Binding of Amyloid Specific Dye

For evaluating the presence of amyloid like conformation in the naturally occurring fibrous structures we performed Thioflavin T and Congo Red dye binding assay both of which are amyloid specific dye. However the specificity of Thioflavin T to bind with the cross β conformation is much more compared to Congo Red but as both are used in amyloid identification hence we applied it in this study as well.

Thioflavin T upon binding with the cross β structure of the amyloid fibers the dye's absorption spectra shifts from 385nm to 440nm. We performed the assay with the help of Confocal Laser Scanning Microscopy (CLSM). Confocal Microscopy was performed keeping in view that Th T fluoresces at 480nm when bounds to amyloid. As reported previously Thioflavin T was used to diagnose amyloid in tissues [23] under fluorescent microscopy keeping this aspect in mind confocal microscopy was used to visualize amyloid fibers.

Fig (4.27), Fig (4.28) and Fig (4.29) shows the WNF , YNF and MNF fibers in presence of Thioflavin T and Congo Red under CLSM.. Argon laser of 454 nm was used to excite thioflavin t bound samples. The fluorescence emission was collected by keeping the PMT channel between 470-495nm to specifically collect the emission radiation. For Congo Red Argon Laser of 561nm was used with the PMT channel between 575nm to 610nm. When bound to the fibrous structures of WNF, MNF and YNF Thioflavin T showed enhanced fluorescence at 480nm. Spider silk was also seen to have enhanced Thioflavin T binding whose FT-IR indicated a presence cross β architecture among other secondary structures. Thioflavin T is also reported to bind the nucleic acids by ionic interaction hence the dye binding assay using Thioflavin T is not a full proof assay exclusively for the identification of

amyloid fibers. FT-IR spectra and X-Ray Diffraction data retrieved from the naturally occurring fibers as discussed above supports the distribution of β sheet structures and the fluorescence of Thioflavin T upon binding the fibers by which we can say that the naturally occurring nest fibers possess some form of similar amyloid like conformation..

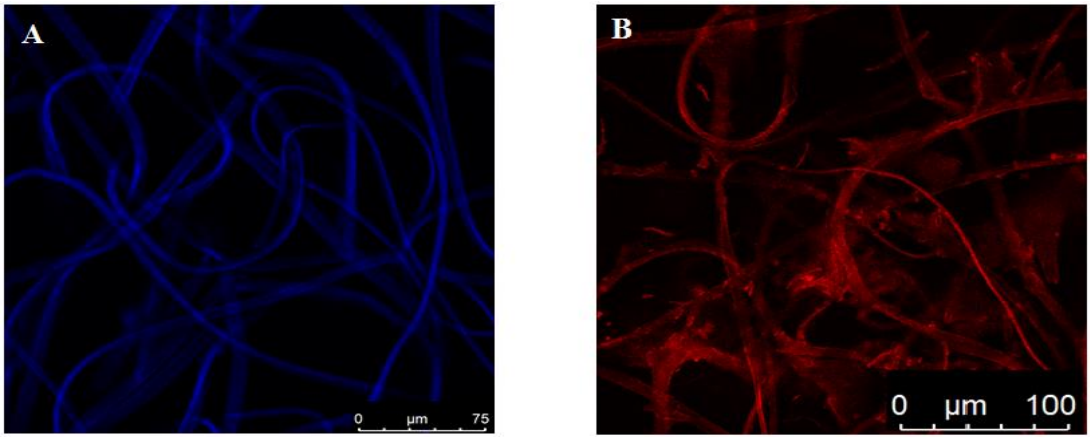


Fig 4.27 - (A) Thioflavin T bound fluorescent WNF excited by 454nm argon laser (B) Congo Red bound fluorescent WNF excited by 561nm Argon Laser.

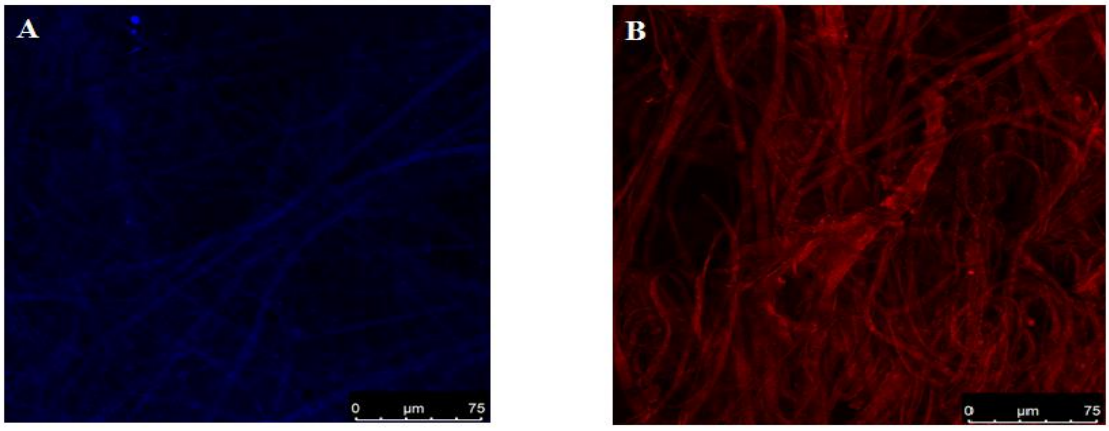


Fig 4.28 - (A) Thioflavin T bound MNF excited at 454nm with emission spectrum b/w 475nm to 490nm (B) Congo Red bound MNF excited at 540nm with emission between 560nm to 590nm.

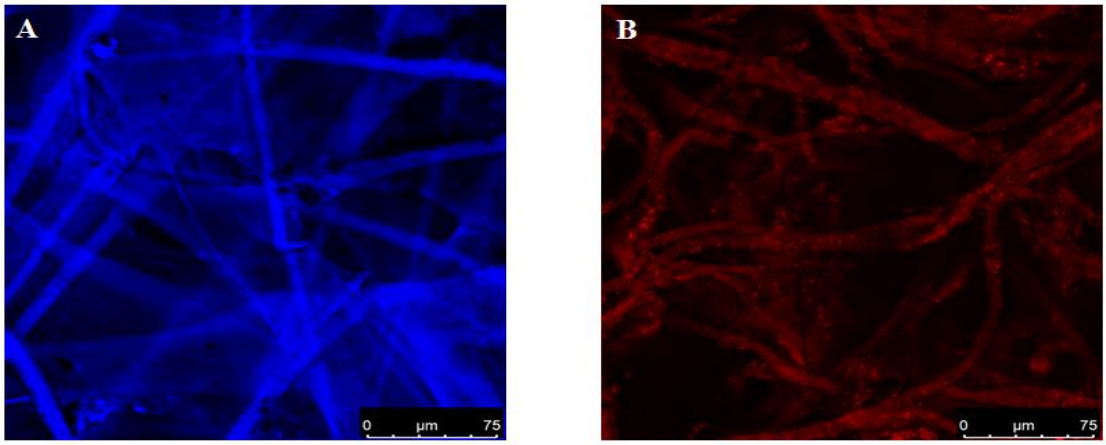


Fig 4.29 - (A) Thioflavin T bound YNF excited at 454nm with emission spectrum b/w 475nm to 490nm (B) Congo Red bound YNF excited at 561nmnm with emission between 575nm to 5610nm.

The membranous layer in between the fibers of Mud dauber nest and Yellow Jacket also took up fluorescence indicating the amyloid like nature of nanometer sized fibers present. Laser intensity for both the dyes with regard to all the samples were kept constant.

4.2.5 Cell Viability Assay

The cell viability assay was performed to understand the bio-compatibility of the naturally occurring fibers. The cells were incubated with the samples for 4 days after which MTT assay was performed. The results of the assay were evaluated using spectrophotometer. Optical density was measured at 570nm. Fig (4.30) shows the cell viability in presence of the samples which shows the cells grew more than the control. The finding suggests that the materials are not cytotoxic for the cells. The non-toxic nature of the samples can be helpful for allowing it to be used as a biomaterial like silk in future. To further evaluate their role in tissue engineering we performed Alkaline Phosphatase activity and Glycosaminoglycan secretion after evaluating their non-cytotoxic nature.

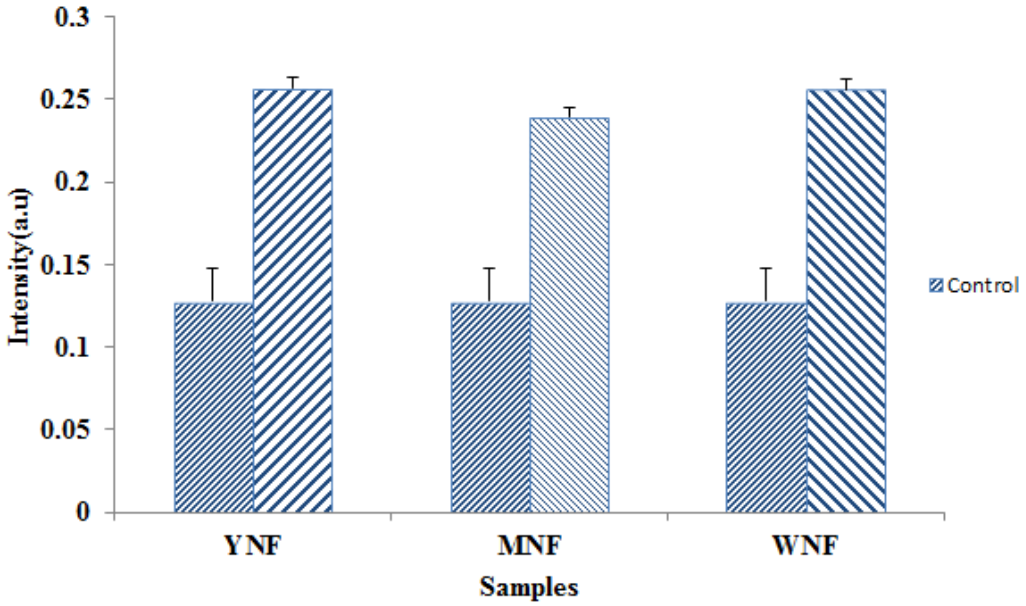


Fig 4.30 - Cell viability of MG-63 cells in presence naturally occurring fibers by the help of MTT assay.

4.2.6 Hemolysis Assay

Hemolysis assay was performed to observe the effect of WNF, YNF and MNF on the erythrocyte degradation. Blood compatibility is an important for any material to be used for biomaterial purpose. The negative control was diluted RBCs and the positive control had RBCs along with 0.01(N) HCl. By assuming Positive control is 100% hemolytic while negative 0% accordingly we assessed the hemolytic percentage. All the samples was seen to have less than 5% hemolysis percentage indicating its blood biocompatible nature [36]. Wasp Nest fibers seem to comparatively less blood compatible compared to YNF and MNF however more evaluations are necessary ascertain the role of WNF causing lysis of RBCs even in negligible amount. Fig (4.31) shows the hemolysis percentage of all the naturally occurring fibers.

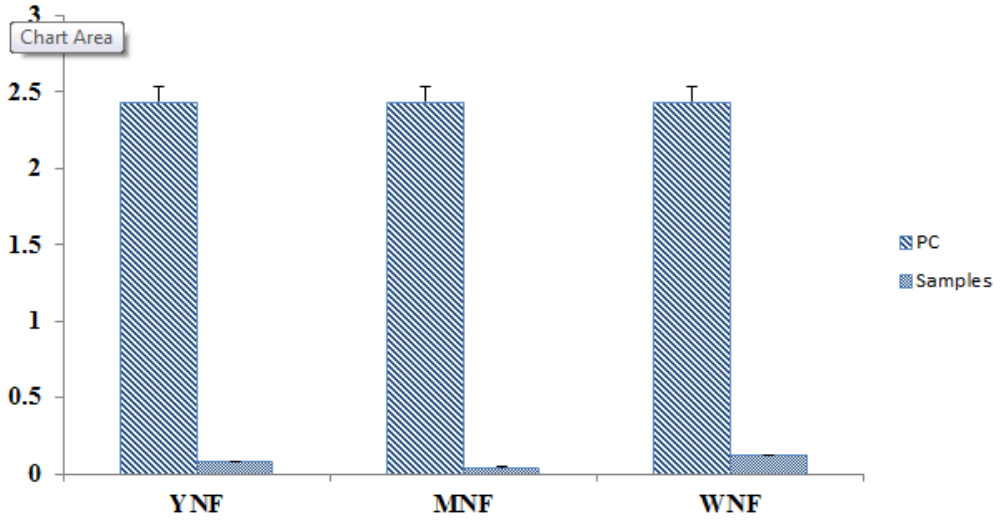


Fig 4.31 - Hemolysis graph of YNF, MNF and WNF with respect to Positive Control (PC).

Table 1.1 - Hemolytic percentage of the natural wasp fibers compared to Positive Control

Sl. No.	Samples Tested for Hemolytic activity	Hemolysis Percentage
1.	Wasp Nest Fibers	0.126% ± 0.0007
2.	Mud dauber Nest Fibers	0.042% ± 0.002
3.	Yellow Jacket Nest Fibers	0.083% ± 0.001
4.	Positive Control (Blood in 0.01N HCl)	100%

4.2.7 Cell Attachment Study

Confocal Microscopy was performed discretely to show the binding and agglomeration of cells on WNF, YNF and MNF. The experiment duration was 72 hours after the cells were seeded on to the samples. Calcein AM dye was used to stain the live cells. Calcein AM bound live cells were excited at 405nm Argon Laser and PMT channel was kept between 420nm to 450nm for retrieving the emission spectrum. Proliferation of the cell was noted as the findings suggested in viability assay. With the increase in incubation time increase in cell proliferation was observed indicating biocompatibility of the material. The images depicts the cells adhered to WNF, YNF & MNF respectively. The cells as viewed in each samples are rounded rather than extended. Such cellular morphology can be attributed to the decreased RGD motif like sites on YNF , WNF and MNF which allows cell integrin to bind optimally to the surface [38]. The cellular agglomeration suggests the cells are bound to ECM secreted from few cells on which the other cells are attached with.

From the Fig (4.32, 4.33 and 4.34) it can be portrayed that the materials do have cell binding ability but not firmly which is needed as the nest holds the progeny eggs but not too firmly so as to inhibit the growth of the young insects.

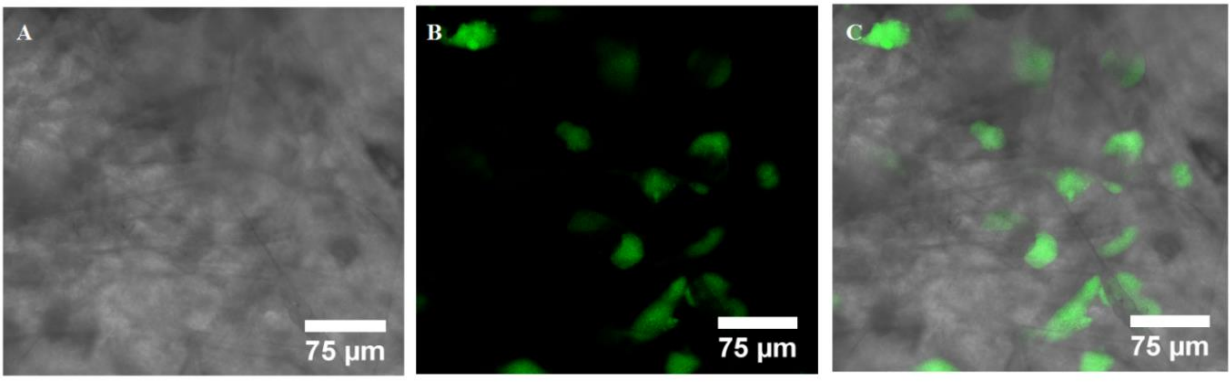


Fig 4.32 - (A) Phase contrast microscopy image of WNF as viewed under CLSM. (B) Live Cell imaging using Calcein AM dye upon WNF. (C) Overlapped Images viewing the adherence of the MG-63 live cells on WNF.

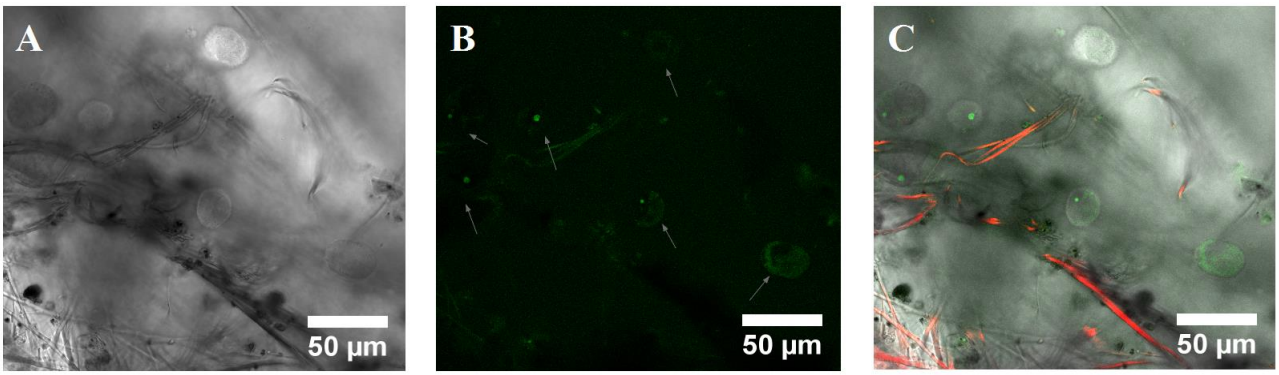


Fig 4.33 - (A) Phase contrast microscopy image of MNF as viewed under CLSM. (B) Live Cell imaging using Calcein AM dye upon MNF. (C) Overlapped Images viewing the adherence of the MG-63 live cells on MNF.

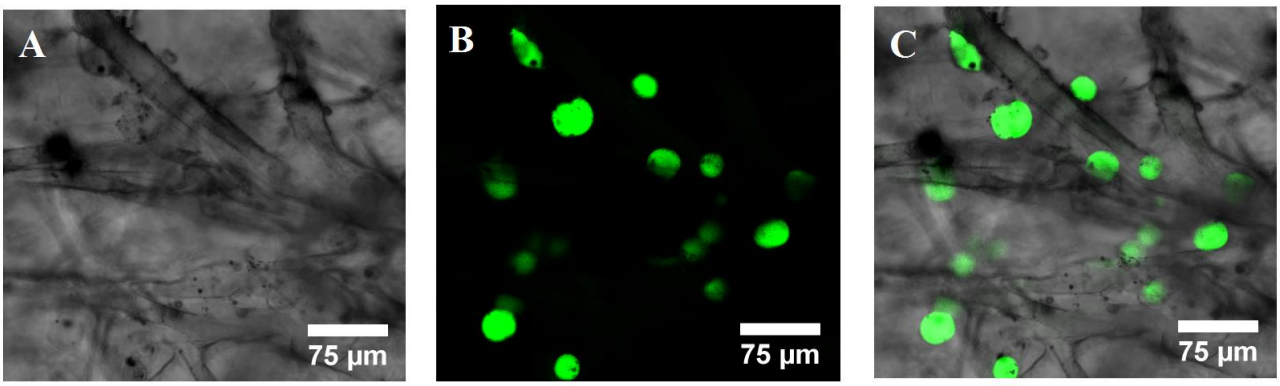


Fig 4.34 - (A) Phase contrast microscopy image of YNF as viewed under CLSM. (B) Live Cell imaging using Calcein AM dye upon YNF. (C) Overlapped Images viewing the adherence of the MG-63 live cells on YNF.

4.2.8 Release of Sulfated Glycosaminoglycan (GAG)

Glycosaminoglycans (GAG) are charged glycosidic resdiues linked with protein cores. GAGs are dimer of N-acetylglucosamine or N-acetylgalactoseamine linked with glucuronic or iduronic acids. The negative charge on GAGs due to presence of a sulfate group helps to retain water and thus is important for maintaining the bio-mechanical properties of the tissue [39]. The samples were evaluated for its role in GAG secretion by the human osteosarcoma cell line (MG-63). The cells were seeded on the samples and were incubated for 7 days at 37°c and 5% CO_2 before quantifying the extent of GAG secretion. Fig (4.35) shows that w.r.t control which contained only cells the materials showed increased GAG secretion.

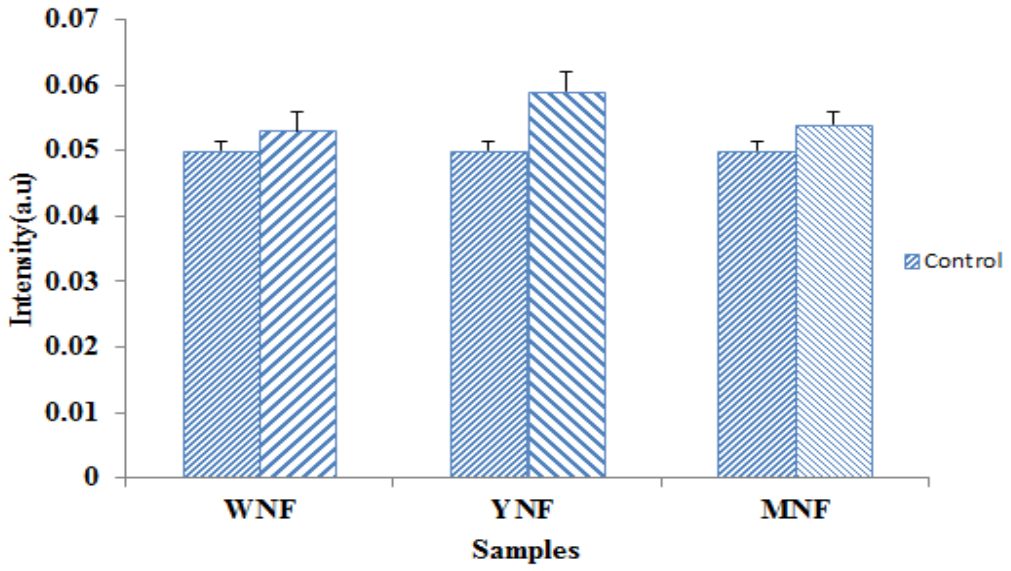


Fig 4.35 - Glycosaminoglycan secretion by MG-63 cell line when adhered to WNF, YNF and MNF. With respect to control possessing only cells the secretion of GAG in presence of YNF showed significant result.

4.3.10 Alkaline Phosphatase Activity

Alkaline phosphatase(ALP) activity is an early marker for bone formation. Increase in ALP indicates osteogenic differentiation [40]. The naturally occurring fibers were evaluated for their possible role in bone tissue engineering. After 7 days pNPP substrate was added to sample seeded cells. After cell lysis, extent of hydrolysis of pnPP substrate to p-nitrophenol indicates alkaline phosphatase activity. Results were obtained by measuring the optical density at 405nm. Fig(4.36) shows the ALP activity of the samples compared to control which consisted only cells. YNF showed maximum secretion followed by MNF and comparatively less was produced in presence of WNF. A comparative study shows that YNF is a more probable choice for tissue engineering if evaluated on the basis GAG secretion. WNF showed least ALP activity out of the 3 materials. However further studies are necessary to affirm the findings and to know the reason for such behavior of the osteosarcoma cell line on the natural fibers.

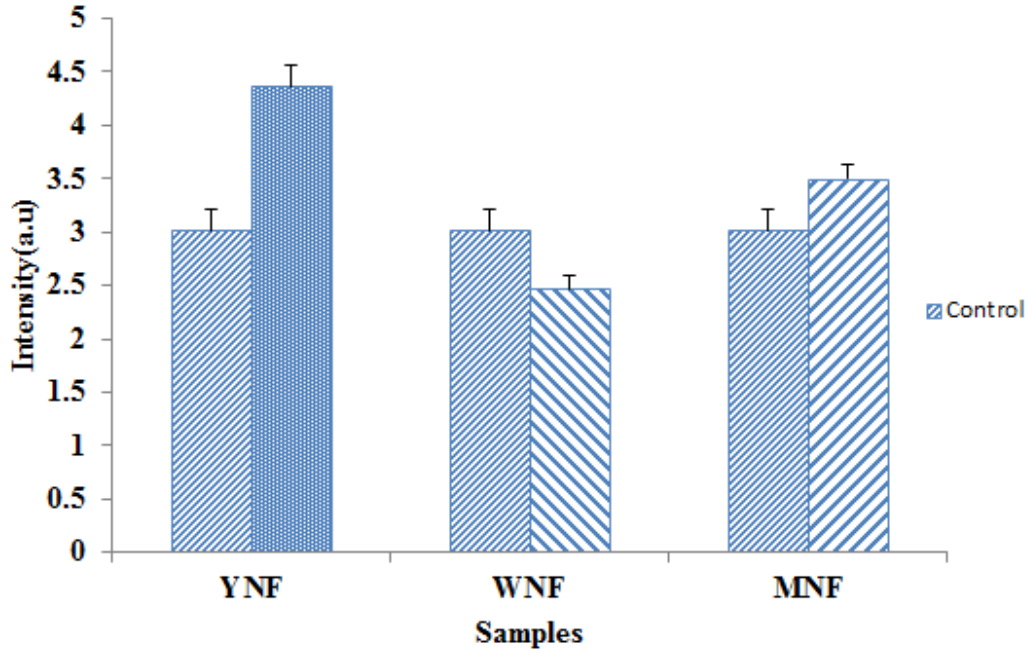


Fig 4.36 - Alkaline Phosphatase activity of MG-63 cells in presence of YNF, WNF and MNF. WNF showed lower ALP secretion compared to YNF and MNF and with respect to control which contained only cells.

4.3 Structural Comparison of Large Amyloid Fibers and Natural Wasp Nest Fibers

The structural similarity between Large amyloid Fibers of Pepsin and Natural fibers is evaluated by Scanning Electron Micrograph Images, X-Ray Diffraction and Fourier Transform Infrared Spectroscopy.

4.3.1 Scanning Electron Microscopy

Scanning Electron Micrograph Images of Large Amyloid fibers to the Natural fibers presents a distinct morphology. The Amyloid Fibers are seen to be made up of aggregated protein components which joined together to form extended Fibers as is known for amyloid fibril formation. In contrast to that natural fibers are smooth and elongated with much more consolidated structural make up. The membranous attachments in between the fibers of Yellow Jacket Nest & Mud dauber Nest are also finer Fibers of nanometer scale. Overall by Fig 36 we can construct an idea about the external structural build of the in-vitro made Fibers and the natural fiber.

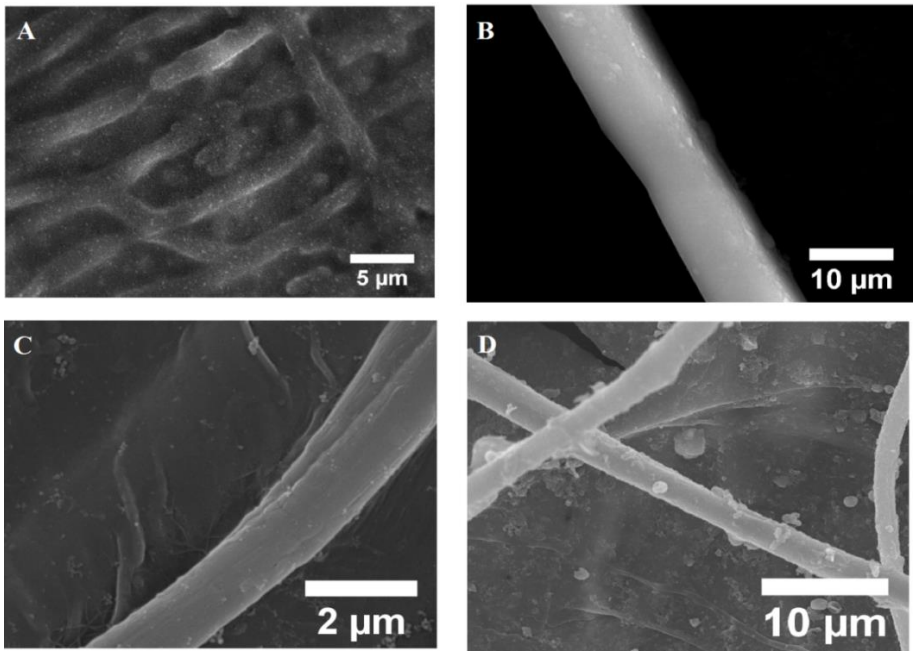


Fig 4.37 - (A) SEM Image of Large Amyloid Fibers (B) SEM Image of WNF (C) SEM Image of MNF (D) SEM Image of YNF.

4.3.2 X-Ray Diffraction

X-Ray fiber diffraction of the Large Amyloid Fibers showed an cross β architecture due to the two 2θ peaks at 10.53° and 22.47° which corresponds to the d-spacing of 4Å and 9Å respectively. The spacing of 4Å and 9Å shows the intra-strand and inter-sheet spacing respectively. In contrast to that the natural fibers had two peaks one around 13° and another at 24° except MNF which had the second peak at 27°. The corresponding d-spacing for the peaks are seen to lie at 3.5Å and 6.5Å respectively. The initial peak of 10.5° of amyloid Fibers is in close proximity with 13° of the natural fibers indicating the presence of β sheets as it corresponds to strand spacing. The second peak at 24° is in line with XRD peak of silk fibroin In lieu of the findings of Fourier Transform Spectroscopy we can see the prevalence of native β sheets which is unlike the cross β architecture of amyloids. From the initial findings it can concluded that the presence of β sheets are present which mimic more like silk fibers but their structural arrangement cannot be deduced for which further studies are necessary.

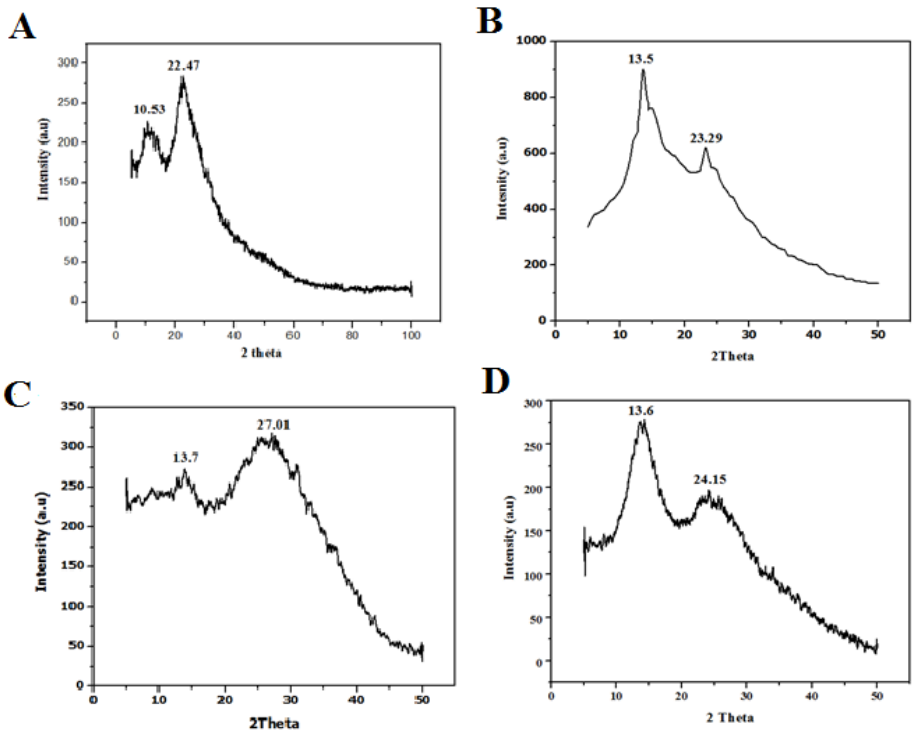


Fig 4.38 - X-Ray Diffraction curve (A) Large Amyloid Fibers (B) Wasp Nest Fibers (C) Mud dauber Nest Fibers (D) Yellow Jacket Nest Fibers.

4.3.3 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy of Amyloid Fibers shows a peak between 1605cm^{-1} to 1625cm^{-1} which indicate the presence of β sheets in amyloid like conformation. The FT-IR sepctra analysis of the natural fibers showed peaks at 1632cm^{-1} (MNF) and 1639cm^{-1} (YNF & WNF). The results show the presence of native β sheet conformation which is unlike the amyloid cross β arrangement. On further deconvolution of the amide I region (1600cm-1 to 1700cm-1) using gaussian peak fitting the results were confirmed but also proved the existence of antiparallel β sheets & turns at 1666cm^{-1} region along with some alpha helices at 1662cm^{-1} of YNF. The findings suggest the presence of native β sheets which mimics more like silk fibers rather than amyloid structural arrangement.

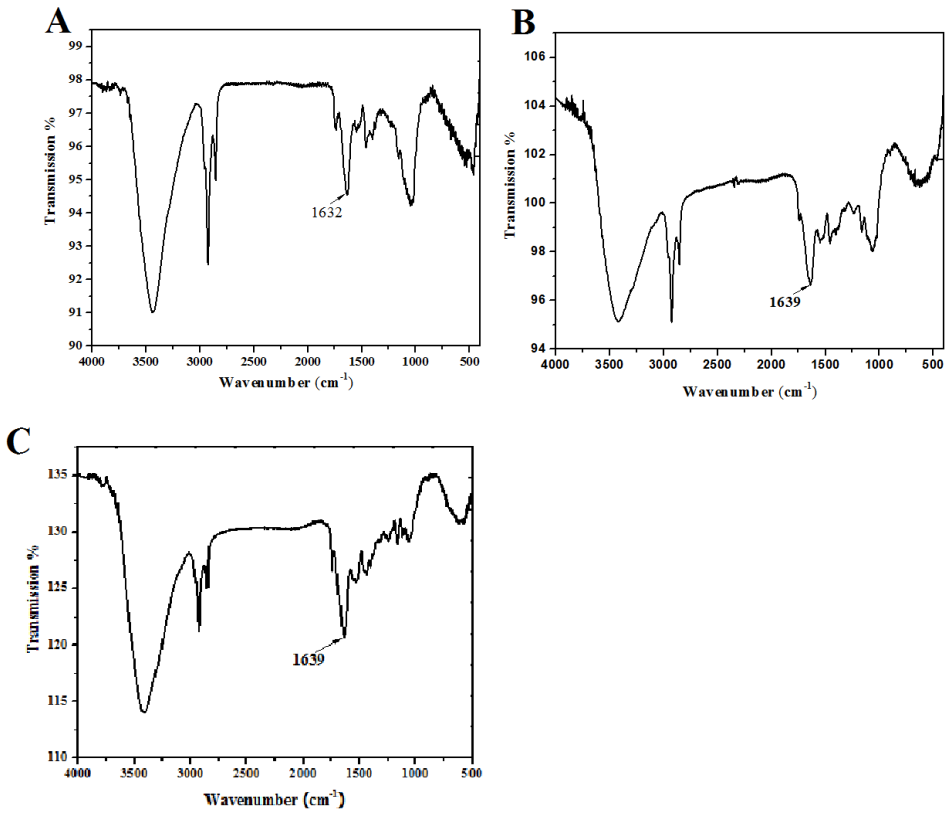


Fig 4.39 - Fourier Transform Infrared Spectroscopy of (A) Mud dauber Nest Fiber (B) Yellow Jacket Nest Fiber (C) Wasp Nest Fiber depicting the native β sheet conformation between 1625cm^{-1} and 1640cm^{-1}.

4.3.4 Confocal Laser Scanning Microscopy

Confocal Microscopy was used for Thioflavin T binding study of the amyloid fibers. As it is reported earlier that Thioflavin T was used for amyloid diagnosis using fluroscent microscopy we approached similar method but using CLSM. Amyloid Fibers from pepsin was confirmed by fluorescence spectroscopy, X-Ray Diffraction and SEM Imaging. Thioflavin T bound amyloids when excited at 454nm the amyloid Fibers were visible which proved that apart from Electron microscopy CLSM can also be a procedure of amyloid identification. Similar study when carried out for WNF, MNF and YNF fluroscent fibers were observed. Now XRD and FT-IR proved the presence of native β strands unlike cross β conformation of amyloids. The CLSM study of the natural fibers using Thioflavin T can be due to the presence of some amyloid like structures inside the fibers which is yet to be examined else it can be an ionic interaction. Thioflavin T is known to bind nucleic acids using ionic interaction.

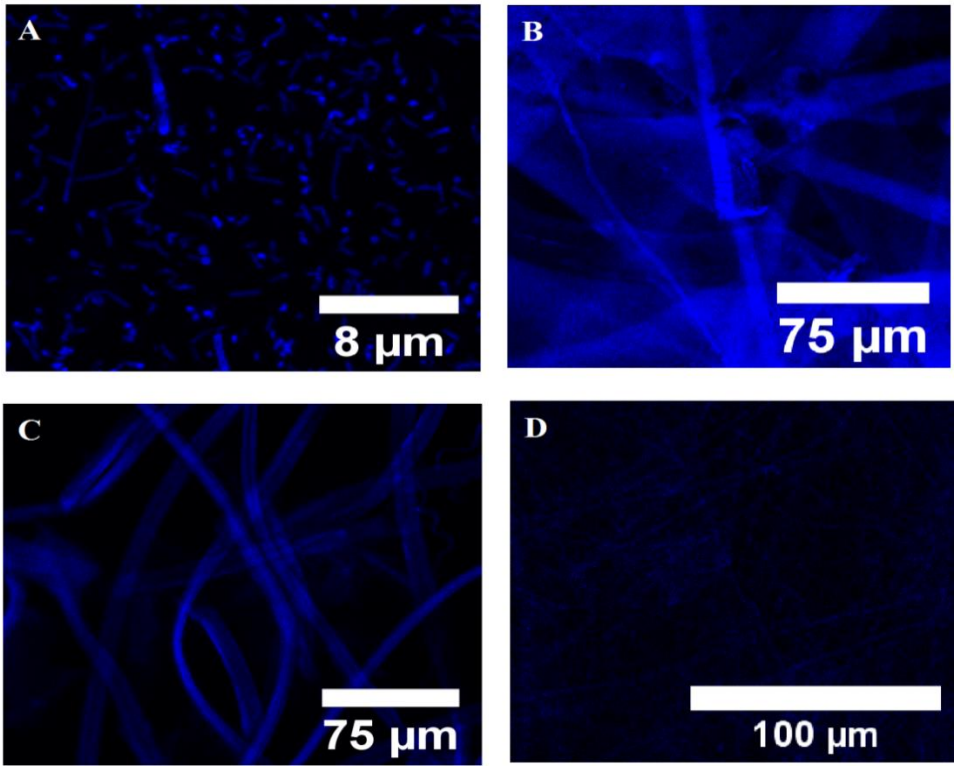


Fig 4.40 - Thioflavin T stained (A) Large Amyloid Fibers from pepsin (B) Yellow Jacket Nest Fibers (C) Wasp Nest Fibers (D) Mud dauber Nest fibers under CLSM.

Chapter 5

Conclusion

Large Amyloid fibers from pepsin was formed which was confirmed by SEM Imaging, Thioflavin T dye binding assay and X-Ray diffraction. The large amyloid fibers formed from pepsin can be attributed to the higher protein concentration of 2mg/ml which led to 2-3μm diameter fibers. Large amyloid fibers have higher rigidity and robustness compared to nanometer fibers and thus is more sought after as a biomaterial. The ability of such amyloid fibers to stabilize emulsions was also proved. Hexane was taken as the hydrophobic model and the findings showed athe amyloid property to stabilize emulsions. However the concentrations that were taken for amyloid formation were far higher than usually reported for amyloid fiber formation. In view of that we think that significant amount of denatured aggregated structures were also formed which also played an active role in oil in water emulsion. The presence of amyloids around the hexane droplets were verified by amyloid specific dye binding assay with the help of CLSM along with ESEM Imaging. The presence small fibers around the inflated Hexane droplets in ESEM verified the presence of amyloids. To summarize we synthesized large amyloid fibers and saw its emulsifying capacity. Future work remains to assess the ability of these formed amyloid fibers to stabilize large hydrophobic molecule like Olive oil or linseed oil for lipophilic drug delivery application.

X-Ray Diffraction and FT-Infrared Spectroscopy showed the extensive distribution of native β sheets along with β turns as the major structural backbone of WNF, MNF and YNF. FT-IR Peak at $1639cm^{-1}$, $1632cm^{-1}$ and $1639cm^{-1}$ proved that the β stacks of WNF, MNF and YNF respectively are of native β sheets exclusive of cross β conformation of amyloids. The XRD peak at d-spacing of 3.5Å is conserved of silk fibroin β sheets which indicates silk like property of the wasp fibers. However, fluorescence of Thioflavin T on interaction with the materials proved that the all the samples may possess some amyloid like β stack structure or can also be a charged interaction between the samples and dye micelles. Cell Viability and Hemolysis assay showed the bio-compatibilty and blood compatibility of WNF, MNF and YNF. Alkaline Phosphatase Activity and GAG release showed the applicability of MNF and

YNF to be used in tissue engineering apart from WNF which had significantly less ALP activity. Cell adhesion study of all the samples using osteosarcoma cell line was proved by combination of CLSM and Phase contrast microscopy. To summarize we presented 3 types of natural fibers which is made up of extensive native β sheet secondary structure which may be a structural subclass of amyloids like spider silk but have its own β sheet conformation like natural silk. In addition to that the preliminary investigation of the wasp fibers on their ability to allow osteosarcoma cells to secrete Alkaline Phosphatase and Glycosaminoglycans exhibits their prospect in tissue engineering as effective biomaterials. In depth study of the structural properties of the fibers and further evaluating the property of the fibers to act as effective biomaterials is what remains to be done.

References

1. Stine, W.B., Dahlgren, K.N., Krafft, G.A. and LaDu, M.J., 2003. In vitro characterization of conditions for amyloid-β peptide oligomerization and fibrillogenesis. *Journal of Biological Chemistry*, *278*(13), pp.11612-11622.
2. Ridgley, D.M., Rippner, C.M. and Barone, J.R., 2015. Design and Construction of Large Amyloid Fibers. *Fibers*, *3*(2), pp.90-102.
3. Maji, S.K., Perrin, M.H., Sawaya, M.R., Jessberger, S., Vadodaria, K., Rissman, R.A., Singru, P.S., Nilsson, K.P.R., Simon, R., Schubert, D. and Eisenberg, D., 2009. Functional amyloids as natural storage of peptide hormones in pituitary secretory granules. *Science*, *325*(5938), pp.328-332.
4. Bissig, C., Rochin, L. and van Niel, G., 2016. PMEL amyloid fibril formation: the bright steps of pigmentation. *International journal of molecular sciences*, *17*(9), p.1438.
5. Slotta, U., Hess, S., Spieß, K., Stromer, T., Serpell, L. and Scheibel, T., 2007. Spider silk and amyloid fibrils: a structural comparison. *Macromolecular bioscience*, *7*(2), pp.183-188.
6. Dueholm, M.S., Albertsen, M., Otzen, D. and Nielsen, P.H., 2012. Curli functional amyloid systems are phylogenetically widespread and display large diversity in operon and protein structure. *PloS one*, *7*(12), p.e51274.
7. Colvin, M.T., Silvers, R., Ni, Q.Z., Can, T.V., Sergeyev, I., Rosay, M., Donovan, K.J., Michael, B., Wall, J., Linse, S. and Griffin, R.G., 2016. Atomic resolution structure of monomorphic Aβ42 amyloid fibrils. *Journal of the American Chemical Society*, *138*(30), pp.9663-9674.
8. Kaur, M., Healy, J., Vasudevamurthy, M., Lassé, M., Puskar, L., Tobin, M.J., Valery, C., Gerrard, J.A. and Sasso, L., 2014. Stability and cytotoxicity of crystallin amyloid nanofibrils. *Nanoscale*, *6*(21), pp.13169-13178.
9. Swaminathan, R., Ravi, V.K., Kumar, S., Kumar, M.V.S. and Chandra, N., 2011. Lysozyme: a model protein for amyloid research. In *Advances in protein chemistry and structural biology* (Vol. 84, pp. 63-111). Academic Press.

10. Marsh, S.E. and Blurton-Jones, M., 2012. Examining the mechanisms that link β-amyloid and α-synuclein pathologies. *Alzheimer's research & therapy*, *4*(2), p.11.
11. Ghosh, S., Dolai, S. and Dey, J., 2013. Amyloid fibril formation by pepsin in neutral pH at room temperature. *Soft Matter*, *9*(48), pp.11457-11460.
12. Ridgley, D.M., Claunch, E.C. and Barone, J.R., 2013. Characterization of large amyloid fibers and tapes with Fourier Transform Infrared (FT-IR) and raman spectroscopy. *Applied spectroscopy*, *67*(12), pp.1417-1426.
13. Ridgley, D.M., Freedman, B.G., Lee, P.W. and Barone, J.R., 2014. Genetically encoded self-assembly of large amyloid fibers. *Biomaterials Science*, *2*(4), pp.560-566.
14. Das, S., Zhou, K., Ghosh, D., Jha, N.N., Singh, P.K., Jacob, R.S., Bernard, C.C., Finkelstein, D.I., Forsythe, J.S. and Maji, S.K., 2016. Implantable amyloid hydrogels for promoting stem cell differentiation to neurons. *NPG Asia Materials*, *8*(9), p.e304.
15. Li, C., Born, A.K., Schweizer, T., Zenobi-Wong, M., Cerruti, M. and Mezzenga, R., 2014. Amyloid-hydroxyapatite bone biomimetic composites. *Advanced Materials*, *26*(20), pp.3207-3212.
16. Wu, X., Li, M., Li, Z., Lv, L., Zhang, Y. and Li, C., 2017. Amyloid-graphene oxide as immobilization platform of Au nanocatalysts and enzymes for improved glucose-sensing activity. *Journal of colloid and interface science*, *490*, pp.336-342.
17. Nyström, G., Fernández-Ronco, M.P., Bolisetty, S., Mazzotti, M. and Mezzenga, R., 2016. Amyloid templated gold aerogels. *Advanced materials*, *28*(3), pp.472-478.
18. Orcholski, M.E., Zhang, Q. and Bredesen, D.E., 2011. Signaling via amyloid precursor-like proteins APLP1 and APLP2. *Journal of Alzheimer's Disease*, *23*(4), pp.689-699.
19. Kudô, K., Yamane, S., Mateus, S., Tsuchida, K., Itô, Y., Miyano, S., Yamamoto, H. and Zucchi, R., 2001. Nest materials and some chemical characteristics of nests of a New World swarm-founding polistine wasp, Polybia paulista (Hymenoptera Vespidae). *Ethology Ecology & Evolution*, *13*(4), pp.351-360.
20. Maschwitz, U., Dorow, W.H.O. and Botz, T., 1990. Chemical composition of the nest walls, and nesting behaviour, of Ropalidia (Icarielia) opifex van der Vecht, 1962 (Hymenoptera: Vespidae), a Southeast Asian social wasp with translucent nests. *Journal of Natural History*, *24*(5), pp.1311-1319

21. Kamalu, I.C., Nwakaudu, M.S., Nkuzinna, O.C., Uzondu, F.N., Effiong, E.E. and Obibuenyi, I.J., 2015. CHEMICAL COMPOSITION OF THE NEST AND MODELS OF THE METAMOPHIC POLYMORPHISM OF THE MUD DAUBER WASP. *International Journal of Applied Chemical Sciences Research*, *3*(2), pp.1-14.
22. Kalashnikova, I., Bizot, H., Cathala, B. and Capron, I., 2011. New Pickering emulsions stabilized by bacterial cellulose nanocrystals. *Langmuir*, *27*(12), pp.7471-7479.
23. Walstra, P.I.E.T.E.R., 1983. Formation of emulsions. In *Encyclopedia of emulsion Technology, vol. 1. Basic Theory*(pp. 57-128).
24. Pomeranz, Y. and Munck, L., 1981. Cereals, a renewable resource: theory and practice. In *International Symposium on Cereals, a Renewable Resource, Theory and Practice, Copenhagen (Denmark), 1981*. American Association of Cereal Chemists.
25. Berman, H.M., 2000. West brook J. *Feng Z., Gilliland G., Bhat T. N., Weissig H., Shindyalov IN, Bourne PE*, pp.235-242.
26. Hobbs, J.R. and Morgan, A.D., 1963. Fluorescence microscopy with thioflavine-T in the diagnosis of amyloid. *The Journal of Pathology*, *86*(2), pp.437-442.
27. KELIÉNYI, G., 1967. On the histochemistry of azo group-free thiazole dyes. *Journal of Histochemistry & Cytochemistry*, *15*(3), pp.172-180.
28. Zhu, D., Damodaran, S. and Lucey, J.A., 2010. Physicochemical and emulsifying properties of whey protein isolate (WPI)− dextran conjugates produced in aqueous solution. *Journal of agricultural and food chemistry*, *58*(5), pp.2988-2994.
29. Cabra, V., 2008. Emulsifying properties of proteins
30. Kalepu, S., Manthina, M. and Padavala, V., 2013. Oral lipid-based drug delivery systems–an overview. *Acta Pharmaceutica Sinica B*, *3*(6), pp.361-372.
31. Jaiswal, M., Dudhe, R. and Sharma, P.K., 2015. Nanoemulsion: an advanced mode of drug delivery system. *3 Biotech*, *5*(2), pp.123-127.
32. Rockwood, D.N., Preda, R.C., Yücel, T., Wang, X., Lovett, M.L. and Kaplan, D.L., 2011. Materials fabrication from Bombyx mori silk fibroin. *Nature protocols*, *6*(10), p.1612.
33. Hiramatsu, H. and Kitagawa, T., 2005. FT-IR approaches on amyloid fibril structure. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1753*(1), pp.100-107.

34. Ridgley, D.M., Claunch, E.C. and Barone, J.R., 2013. Characterization of large amyloid fibers and tapes with Fourier Transform Infrared (FT-IR) and raman spectroscopy. *Applied spectroscopy*, *67*(12), pp.1417-1426.
35. Zhang, H., Li, L.L., Dai, F.Y., Zhang, H.H., Ni, B., Zhou, W., Yang, X. and Wu, Y.Z., 2012. Preparation and characterization of silk fibroin as a biomaterial with potential for drug delivery. *Journal of translational medicine*, *10*(1), p.117.
36. Andiappan, M., Sundaramoorthy, S., Panda, N., Meiyazhaban, G., Winfred, S.B., Venkataraman, G. and Krishna, P., 2013. Electrospun eri silk fibroin scaffold coated with hydroxyapatite for bone tissue engineering applications. *Progress in biomaterials*, *2*(1), p.6.
37. Koh, L.D., Cheng, Y., Teng, C.P., Khin, Y.W., Loh, X.J., Tee, S.Y., Low, M., Ye, E., Yu, H.D., Zhang, Y.W. and Han, M.Y., 2015. Structures, mechanical properties and applications of silk fibroin materials. *Progress in Polymer Science*, *46*, pp.86-110.
38. D'Souza, S.E., Ginsberg, M.H. and Plow, E.F., 1991. Arginyl-glycyl-aspartic acid (RGD): a cell adhesion motif. *Trends in biochemical sciences*, *16*, pp.246-250.
39. Köwitsch, A., Zhou, G. and Groth, T., 2018. Medical application of glycosaminoglycans: a review. *Journal of tissue engineering and regenerative medicine*, *12*(1), pp.e23-e41.
40. Golub, E.E. and Boesze-Battaglia, K., 2007. The role of alkaline phosphatase in mineralization. *Current Opinion in Orthopaedics*, *18*(5), pp.444-448.