DESIGN, FABRICATION AND CHARACTERIZATION OF A SELF ASSEMBLING PEPTIDE HYDROGEL FOR POTENTIAL TISSUE ENGINEERING APPLICATIONS

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DESIGN, FABRICATION AND CHARACTERIZATION OF A SELF ASSEMBLING PEPTIDE HYDROGEL FOR POTENTIAL APPLICATION IN TISSUE ENGINEERING

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MS THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: **Shama Nazir, NUST201260331MASAB91012F**, Titled: **"Design, fabrication and characterization of self assembling peptide hydrogel for potential tissue engineering applications"** be accepted in partial fulfillment of the requirements for the award of **Master's of Science (Industrial Biotechnology)** degree with A grade.

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DEDICATION

Dedicated to the most inspirational and enthusiastic person of my life

My Husband

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LIST OF ACRONYMS

%	Percentage
°C	Degree Celsius
α	Alpha
β	Beta
3D	Three Dimensional
Å	Angstrom
CGC	Critical Gelation Concentration
cm	Centimeter
ECM	Extracellular Matrix
FDA	Food and Drug Authority
Fmoc	Fluorenylmethyloxycarbonyl
FTIR	Fourier Transform Infrared
HPLC	High Performance Liquid Chromatography
IKVAV	Oleucine-lysine-valine-alanine-valine
M	Molar
mg	Milligrams
mg/ml	Milligram per millilitre
ml	Milliliter
NaCl	Sodium chloride

NaOH	Sodium hydroxide
PCL	Poly-caprolactone
PEG	Poly ethylene glycol
pH	Power of H+
PLGA	Poly lactic-co-glycolic acid
PVA	Poly vinyl alcohol
RGD	Arginine-Glycine-Aspartic acid
TFA	Trifluoroacetic acid
μm	Micro meter

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ABSTRACT

The design and development of an efficient biomaterial which is capable of supporting growth of cells in 3D, is an important milestone for biomedical applications. Self assembling peptide hydrogels are promising biomaterial owing to their biocompatibility and biodegradability. Their physical and mechanical properties can also be easily tailored. In the present study we designed two ionic complementary hexapeptides that differ in hydrophobicity i.e., FK6 (FEFGFK) and VK6 (VEVGVK) where F is phenylalanine, G is glycine, K is lysine, E is glutamic acid and V is valine. The aim of this study was to investigate the effect of hydrophobicity, concentration, pH and temperature on the gelation of these peptides. Morphological characterization of the peptide hydrogels was performed by Fourier Transformed Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) which revealed that the peptides self assemble into beta sheet structures when in solution form. These beta sheet structures align to form nanofibrils. Hydrogel formation for FK6 was observed at a specific concentration with stability at optimum pH and temperature required for cell culture, whereas, no gelation was observed in case of VK6 indicating a direct role of hydrophobicity in the gel formation. The hemocompatibility of the designed peptide hydrogels was also observed, where in FK6 showed barely any hemolysis of red blood cells (RBCs). Therefore, the FK6 peptide hydrogel is proposed to be a promising scaffold for various tissue engineering applications.

Keywords: Tissue engineering, Peptide scaffold, Hydrogel, Self assembly, Biomaterial

Chapter 1

1. INTRODUCTION

Peptides are an ideal building block for fabrication of supramolecular structures. Peptides have the ability to adopt precise secondary structures which can easily be tailored by variation in their amino acid sequence, this directly affects the physical properties and nano features of the 3D macromolecular structures they make. Self-assembling peptide systems presently are the point of interest of scientists, as peptides are natural material they are biocompatible and biodegradable (Fichman and Gazit, 2014). Due to ease of their synthesis and as their properties can be manipulated they are good candidate for tissue culture (Hauser and Zhang, 2010) and other biomedical applications including drug delivery (Altunbas *et al.*, 2011), wound healing (Schneider, Garlick and Egles, 2008) and biosensors (Scanlon and Aggeli, 2008). Till date a number of macromolecular assemblies, e.g. fibrillar networks, tubules, membranes and amphiphilic micelles etc have been synthesized by the self assembly of various peptide sequences (Zhang, 2003; Matson, Zha and Stupp, 2011). Through the study of various naturally occurring protein motifs, scientists have been able to reveal the molecular interactions i.e. electrostatic attraction, hydrogen bonding, ionic interaction, hydrophobic bonding, π- π stacking interactions, and van der waals interactions that are responsible for peptide self-assembly and is highly specific in nature (Adhikari and Banerjee, 2012).

Self assembling peptides are a centre of attention as they have ability to form stable hydrogels. Hydrogels are hydrophilic polymeric networks that have the tendency to retain a significant amount of water (up to 90 % of its dry weight) while maintaining a distinct three-dimensional structure (Kopeček and Yang, 2009). They have shown great potential to be used as scaffolds for tissue engineering applications (Fichman and Gazit, 2014).

The gelation properties and the self-assembly process of peptide hydrogels can be utilized to tailor them for a wide range of bio-medical applications. Peptide hydrogels also have potential to promote cell recruitment by creating a microenvironment rich in nanofibres when they are directly injected in situ (Miller and Saiani, 2010). Peptide hydrogels can be easily tailored by controlling the process of self assembly and gelation. To be used in regenerative medicine a hydrogel scaffold should be easy to handle at 37 °C and at physiological pH; the gelation should be rapid and reproducible under mild conditions. The mechanical properties of hydrogel should resemble those of the natural tissue. The structure of the gel at nano, micro and macroscopic level should be uniform and it should not hinder the long term culture of the cell types (Jayawarna *et al.*, 2009).

Regenerative medicine and tissue engineering require biomaterials in the form of scaffolds that mimic the ECM - extracellular matrix, which can maintain and monitor the growth of cells, their migration and could help in their differentiation and function (Kopecek *et al.,* 2009). The material should be highly porous so that the diffusion of oxygen, nutrients and metabolites across it becomes possible (Ancar *et al.*, 2007). The mechanical properties of the scaffold should resemble the tissue that is required to be generated. For wound dressings usually such scaffolds are required that decompose after successful tissue regeneration (Chen, Ushida and Tateishi 2000; Chen, Ushida and Tateishi, 2002). Therefore a lot of research is underway to construct an ideal scaffold which could meet all the requirements of generating an artificial tissue graft.

Self assembly in proteins was first discovered by Zhang in 1990s in a DNA binding protein, Zoutin. The peptide AEAK16-II (AEAEAKAKAEAEAKAK), motif contains repeats of alternating hydrophobic and hydrophilic amino acids that fold into beta sheet structures in water with distinct hydrophilic and hydrophobic surfaces (Zhang et al, 1993). New sequences were then designed, RADA16-I (RADARADARADARADA) and RADA16-II (RARADADARARADADA) by

replacing the lysine and glutamate residues in AEAK16 by arginine and aspartate. In these peptide motifs nanofiber formation in the presence of salts was observed. Further new sequences were designed based on this concept in which alanine was replaced by more hydrophobic motifs were replaced (i.e. IKIE16, VKVE16 and FKFE16). Also the new sequences containing less amino acids i.e. octa peptides were developed which upon self assembly form hydrogels having less critical gelation concentration as that of the 16-mer sequence due to a competition between entropy and enthalpy (Caplan *et al.,* 2002). A number of peptide based fibrous scaffolds are presently developed that permit growth of cells in 3D environment analogous to the ECM (Jayawarna *et al.,* 2006).

These peptides were further modified by attaching various bioactive motifs to the original peptide sequence. A heparin-binding peptide was integrated into the peptide amphiphile structure to observe its effect on the cell behavior. Heparin- a highly sulfated glycosaminoglycan binds angiogenic growth factors that promote formation of new blood vessels. It is observed that the heparin molecules bind to the PA fibres through electrostatic interactions. This system has been shown to encourage the blood vessels formation (Rajangam *et al.,* 2006).

In another study self assembling peptide RAD16‑I was modified with sequences taken from laminin, fibronectin, collagen (for cell adhesion, differentiation), and bone marrow homing peptides. No difference in the gelation of functionalized peptide was found from that of the nonfunctionalized peptide. Mouse neural stem cells were then cultured into it. The cells showed good adhesion and differentiation, as indicated by the gene expression studies (Gelain *et al.,* 2006). RAD16‑I has also been evaluated for production of artificial skin. Aqueous solution of RAD16-I was impregnated with fibroblasts and cultured for 3 weeks to form a synthetic dermis. To which keratinocytes were added on the surface to form an epidermal layer. After one week the expression of collagen I was observed by fibroblasts which aggregated in the clusters. Three to five layers of keratinocytes were observed in the epidermis. The

number of fibroblasts in the synthetic dermis increased up to 2 weeks of culture, then remained constant for 5 weeks (Kao, Kadomatsu and Hosaka, 2009). A functional motif 'IKVAV' derived from laminin was linked to the $RADA_{16}$ and the functional scaffold was used for the reconstruction of the injured brain. The peptide solution with encapsulated neural stem cells was directly injected into a brain surgery model of rat. The hydrogel perfectly filled the injured cavity and adhesion, proliferation and differentiation of neural stem cells was observed. The histological analyses showed that the functionalized peptide hydrogel (RADA16-IKVAV) enhanced the persistence of encapsulated NSCs and also reduced glial astrocytes formation (Cheng *et al.,* 2013).

With the understanding of the self-assembly process and the gelation properties of peptide hydrogels, two ionic-complementary hexapeptides were designed FEFGFK and VEVGVK (where F is phenylalanine, G is glycine, K is lysine, E is glutamic acid and V is Valine) having hydrophilic and hydrophobic residues that self assembles into beta sheet structures. The effect of hydrophobicity was also investigated by comparing the gelation properties of two peptide sequences as VK6 is less hydrophobic then FK6. The effect of concentration and pH on the gel is also assessed. To study the stability of hydrogel at different temperatures phase experiments are conducted. Finally the hemocompatibility of the peptide is evaluated through RBC hemolysis assay.

Chapter 2

2. LITERATURE REVIEW

Tissue engineering is an evolving interdisciplinary field in biomedical sciences which has shown remarkable promise in generating biological substitutes for harvested tissues, prostheses, and implants as a potential alternative to restoration or improvement of impaired tissue function (Yang *et al.,* 2002; Andrée, Haverich and Hilfiker, 2013). The first generation technology mainly focused on restoring damaged tissues with non-biological biomaterials such as metals, synthetic polymers and ceramics (Langer and Vacanti, 1993). A number of strategies are currently being used to engineer tissues. A combination of scaffold materials, cells, engineering methods, and physiological and biochemical factors is employed to create the desired tissue substitute (Andree, Haverich and Hilfiker, 2013). Another strategy, autologous to tissue regeneration, in this patient's own cells are introduced into a scaffold (ex vivo), which is followed by cell attachment and proliferation resulting in the formation of a new tissue (Lu *et al.,* 2011).

The scaffold serves as a synthetic ECM to integrate cells into a 3D architecture which then directs the growth and development of a desired tissue substitute (Lu *et al.,* 2011). The most critical thing in the technique is the choice of scaffold material. Therefore, a number of natural as well as synthetic materials are being considered as potential tissue engineering scaffolds (Kretsinger *et al.,* 2005; Lee and Mooney, 2001). As an alternative, a variety of hydrogels are also being employed as a scaffold material. They can provide a micro environment for cell growth and proliferation that mimics the natural tissue, and allow the diffusion of nutrients as well as cellular waste through the porous network. Due to their high water content they take precedence over other types of polymeric scaffolds (Kretsinger *et al.,* 2005).

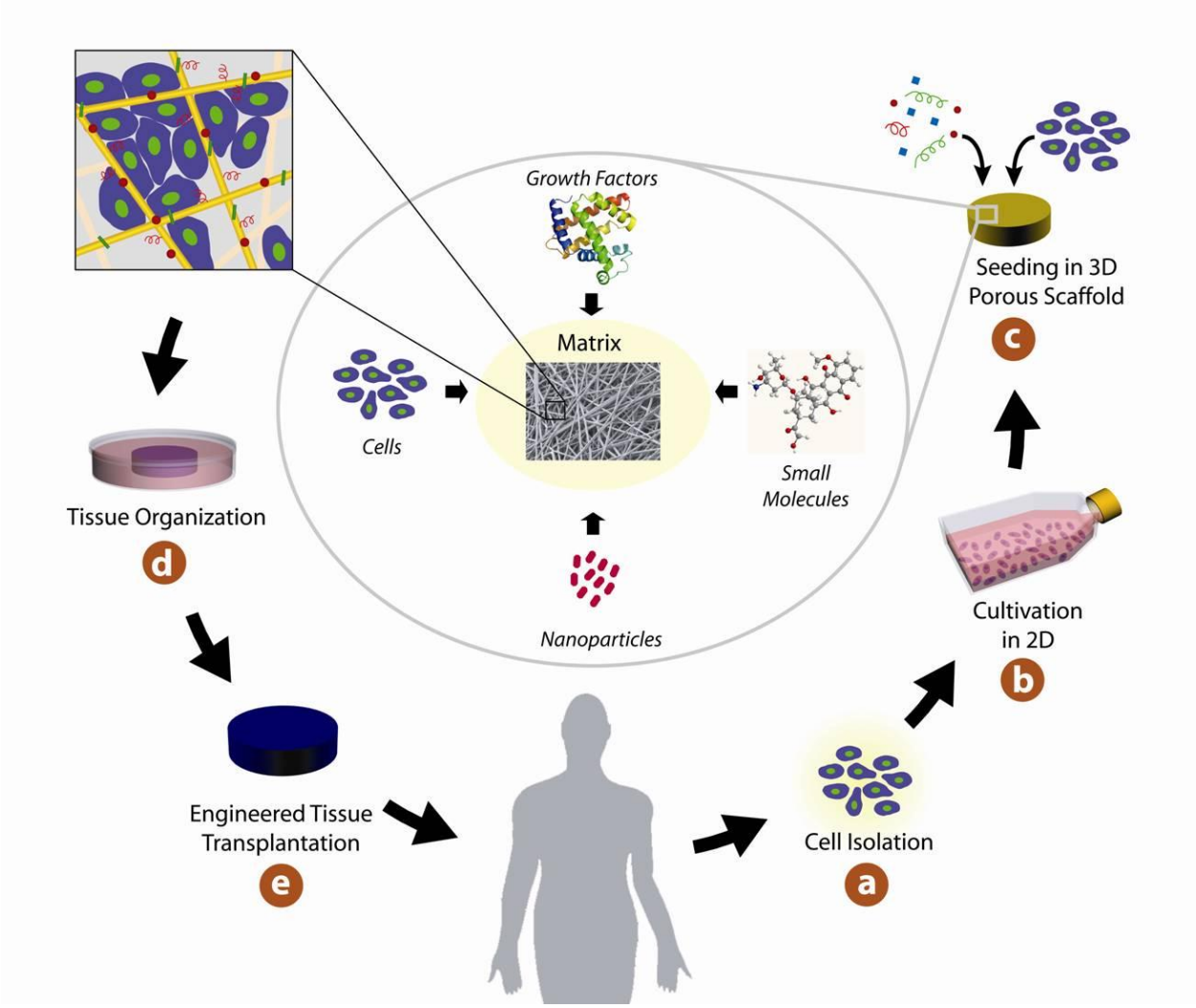


Fig 1: Basic principles of Tissue Engineering (adapted from Dvir *et al.*, 2011)

2.1 SCAFFOLD DESIGN

Scaffold serves the purpose of a substrate for the embedded cells acting as a physical support to monitor the formation of the new cells as well as it is capable of supporting 3D tissue formation (Griffith, 2002). An Ideal multi-purpose tissue engineering scaffold is difficult to design since each tissue requires precise matrix with well-defined characteristics. The scaffold defines a region that shapes the

developing tissue, may also perform temporary function at the defect site while new tissue develops as well as allows the attachment of seeded cells and transport of growth factors required for the tissue regeneration (Carletti, Motta and Migliaresi, 2011).

The ideal scaffolds provide a framework and initial support for the cells to attach, differentiate and proliferate, and form an extracellular matrix (ECM) (Agrawal and Ray, 2001). Besides being biocompatible, materials used in tissue engineering applications must follow a certain biochemical and physical design criteria. Few of the desirable properties of an ideal scaffold are listed below

- The topography and chemistry of the scaffold surface must facilitate cell adhesion, proliferation and differentiation for which the wettability, softness, stiffness and roughness of the scaffold material must be appropriate (Chang and Wang, 2011).

- The scaffolds should be biocompatible, non-immunogenic and non-inflammatory, neither the polymer nor the by-products of its degradation should cause inflammation in vivo.

- The scaffold should be biodegradable and eliminated eventually; the rate of degradation must be directly proportional to the rate of tissue formation as much as possible.

- The microstructure of scaffold should provide ample space for the attachment of cells, regeneration of extracellular matrix, and during culture diffusional constraints should be minimal. The porosity, pore size, pore structure, pore interconnectivity, surface/volume ratio of the scaffold must be appropriate to allow cell migration, nutrient transport, gaseous exchange and elimination of waste products (O'Brien *et al.,* 2005; Chang and Wang, 2011).

On the other hand, pore radii should be restricted to the micron scale in order to limit the diffusion rate of biomolecules (Zhang, Gelain and Zhao, 2005). Pore size also depends on the type of tissue to regenerate. The pore size required for mammalian tissue growth and differentiation ranges from 20-300um whereas larger pore size of the order of 450um is required for growth and regeneration of bone cells (Chang and Wang, 2011).

- The material should be mechanically strong and have required elasticity, rigidity, stress to strain ratio and tensile strength (Engler *et al.,* 2006) to resist environmental stresses and maintain sufficient structural integrity. In addition to the support provided for cell growth, scaffolds also influence differentiation of the stem cells depending on their mechanical properties. For instance, human mesenchymal stem cells were found to differentiate into distinct cell types by modulating the elasticity* of the material used. Neuron-like lineages were obtained with relatively soft (E' ~ 1 kPa) matrices, muscle lineages with moderate (E' ~ 10 kPa) matrices and bone lineages with rigid (E' ~ 100 kPa) matrices (Engler *et al.*, 2006).

- The industrial constraints of the scaffold consist of cost effectiveness, reproducibility and ease of handling.

A number of 3D scaffolds have been fabricated from natural as well as synthetic materials and are being used for culturing cells. Numerous techniques have been employed to fabricate tissue engineering scaffolds that include solvent casting, phase separation, particulate leaching (Liu and Ma, 2004; Zhao *et al.,* 2002), gas foaming, freeze drying (Shen *et al.,* 2000; Whang *et al.,* 1995) and fibre-meshes (Freed *et al.*, 2006).

2.2 TYPES OF SCAFFOLDS

Scaffolds for tissue engineering can be formed by a number of materials either obtained naturally or prepared synthetically. Biomaterials from both origins have their own pros and cons. Mechanical strength of synthetic polymers is reasonably good in addition to their thermal stability and their rate of degradation can be easily altered. Few of the disadvantages include their hydrophobic surfaces and lack of signals for cell-recognition (Zhu and Marchant, 2011; Sionkowska, 2011). Naturally derived biomaterials have the potential advantage of biological recognition that may help in cell adhesion and function, have good biocompatibility and biodegradability, but they have poor mechanical properties.

A blend of natural and synthetic materials is also used for creating biomaterials with required properties. These blends offer better mechanical and physical properties then the single component alone.

Table 2: Characterization of different Scaffolds and their Applications (Croisier, Florence and Christine, 2013)

Scaffold	Specifications	Advantages	Disadvantages	Biomedical applications
Hydrogels (3D)	Physically associated (reversible)	Soft, non-toxic, flexible	Unstable (dissolution may occur), Low mechanical resistance, Variation in pore size	Tissue replacements , growth factor delivery, engineering drug
	Chemically cross-linked (irreversible)	Soft, controlled pore size, stable, flexible	Crosslinking may affect intrinsic properties, May be toxic	

Sponges (3D)		Highly porous	May shrink	filling material in tissue engineering
	Free-standing	Soft	Low porosity	Skin substitutes, Wound dressings
Films (2D)	Thin (LB)	Coating	Construction of multilayers is difficult	Skin substitutes, Wound dressings, Scaffold coatings
	Thin (LBL)	Multilayer construction, Coating	Many steps	
Porous Membranes (2D)	Nanofibers	Highly porous		Skin substitutes, Wound dressings, Scaffold coatings
		Skin ECM mimic	Isolation difficult	

2.3 SYNTHETIC POLYMERS

Synthetic polymers are attractive for tissue engineering applications as their chemical and physical properties can be easily tailored e.g. their production with specific mechanical strength, block structures, molecular weights, degradable linkages, cross-linking modes and gelation kinetics can be easily attained. This influences their cross-linking density, gel formation dynamics, degradation kinetics and mechanical properties (Armentano *et al.,* 2010). Among the most extensively used biopolymers for tissue engineering are polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), polymethyl methacrylate (PMMA) and their copolymers (Shalumon *et al.,* al., 2012; Kreja *et al.,* 2012; Hajiali *et al.,* 2011; Pereira *et al.,* 2012).

Table 1: Synthetic Based Biomaterials and their applications in tissue Engineering

Polymer	Applications	References
PLA	biodegradable surgical suture	Shalumon *et al.*, 2012
	Culturing anterior cruciate ligament-derived fibroblasts	Kreja *et al.*, 2012
PGA/gelatin	vascular tissue engineering	Hajiali *et al.*, 2011
PCL	bone tissue engineering	Pereira *et al.*, 2012
PLGA	cardiac tissue engineering	Yu *et al.*, 2014

PEG, owing to its unique properties, is the most extensively studied polymer to make hydrogels. It is non immunogenic, nontoxic, soluble in water and other organic solvent (Buxton *et al.*, 2007; Yang *et al.*, 2005). In addition to this various functional groups (such as thiol, acrylate and carboxyl) and bioactive agents can be attached to the end hydroxyl groups of PEG molecules., PEG chains can be cross-liked by photo-polymerization allowing the formation of polymeric scaffolds (Zhu, 2010).

Ferdinand *et al.* has reported PEG-based hydrogels to be a suitable 3-D scaffold for soft tissue engineering applications. The crosslinking of gel is not lethal to the cells as it can occur in the absence of free-radical initiators. The mechanical properties of this polymer are similar to that of adipose tissue. These hydrogels have indicated to provide appropriate microenvironment for differentiation of stem cells to adipocytes (Brandi *et al.*, 2010). One of the main advantages of this polymer is its non-immunogenicity since it is known to be inert to most bio-molecules, including proteins (Peppas *et al.*, 2002).

Polyvinyl alcohol (PVA) has been used in a number of biomedical applications including drug delivery, contact lenses, tendon repair and ophthalmic materials (Baker

et al., 2012; Gibas and Janik, 2010). These hydrogels have been formed by physical crosslinking using freeze-thaw processes and chemical cross-linking with aldehydes, or radiation (Zhang *et al.,* 2013). They can be tailored to produce materials with mechanical properties appropriate for a desired application (Jiang, Liu and Feng, 2011). Further cell attachment, migration, proliferation and differentiation can be achieved by attachment of different bioactive molecules to the pendent hydroxyl groups (Stephens-Altus *et al.,* 2011; Ranjha and Khan, 2013).

Schmedlen *et al.,* reported polyvinyl alcohol hydrogels (PVA) for use as tissue engineering scaffolds (Schmedlen *et al.,* 2002). With increasing polymer concentration, the ultimate tensile strength and The Young's modulus of PVA hydrogels increases also hydrogels with increased elasticity can be formed by modifying the number of cross-linkable groups which are attached to chain (Gibas and Janik, 2010; Baker *et al.,* 2012) . Fibroblasts when seeded within PVA hydrogels of 3mm thickness remained viable during two weeks in culture and no differences in viability was observed across the thickness of the hydrogel. Extracellular matrix proteins were also found to be produced by the cells seeded within the PVA hydrogels which was indicated by the production of hydroxyl-proline in the culture. These PVA hydrogels were functionalized by attaching RGDS motif and were observed to support the attachment and spreading of fibroblasts intrinsically. These results suggest that photo-polymerizable PVA hydrogels are promising for tissue engineering applications (Schmedlen *et al.,* 2002).

Poly glycolic acid (PGA) is one of the most extensively used polymers for scaffolds. It is highly crystalline due to the regularity of its chain structure. It rapidly degrades in vivo or in aqueous solutions and its mechanical integrity diminishes within 2 to 4 weeks. Its degradation however depends on its molecular weight, physical structure as well as on the degradation conditions. PLA is another polymer also used in tissue engineering applications which is more hydrophobic than PGA, because of the extra methyl group in its repeating units. PLA scaffolds maintain their

mechanical integrity for months to years in vitro and in vivo. To obtain an intermediate degradation rate between PLA and PGA, their copolymer PLGAs with varying ratios of each polymer is synthesized (Gao and Ma, 2014).

Polycaprolactone (PCL) is a semi-crystalline polymer and has a very low glass-transition temperature of around -60 ºC (Chang *et al.*, 2013). Therefore it always occurs in a rubbery state and is highly permeable under physiological conditions. Its degradation rate is very low as compared to PLA, PGA and PLGA (McNeil, Griffiths and Perrie, 2011). Due to inadequate mechanical strength, hydrophobicity and low bioactivity, it is less attractive for general tissue engineering applications, mostly used in hard tissue engineering, for long-term implants and drug-delivery systems (Nitya *et al.*, 2012). However it can be degraded enzymatically under physiological conditions by intracellular mechanisms. In a study electrospun nanofibres of PCL have been used for culturing chondrocytes In vitro (Silva *et al.*, 2013). But the properties of synthetic polymers can be tailored by blending them with other polymers e.g. PCL can be blended with another polymer PLGA which is more hydrophilic, has less mechanical strength and is more biodegradable as compared to PCL. A blend of PLGA/PCL has been successfully used for culturing human mesenchymal stem cells and osteoblast cells (Curran, Tang and Hunt, 2008; Tang and Hunt, 2006). The addition of PLGA as a doping agent in the PLGA/PCL increased the value of this biomaterial for tissue engineering applications by enhancing the biocompatibility, cell attachment and cell proliferation (Hiep and Lee, 2010).

Despite the versatility of copolymers and cross-linkers available to tune their characteristics to the required properties, some synthetic polymers remain potentially harmful for tissue engineering applications. Components involved in the production of these materials can indeed be toxic in some cases, making hydrogels prepared from not entirely pure polymers inadequate for such applications (Sionkowska, 2011).

2.4 NATURAL POLYMERS

Natural polymers have been used in regenerative medicine owing to their biocompatibility and biodegradability. They include proteins, such as gelatin, collagen, silk, fibrin and genetically engineered proteins as well; and polysaccharides, such as alginate, chitosan, hyaluronic acid and agarose. However, there are concerns in use of natural hydrogels due to their poor mechanical properties and potential immunogenic reactions (Zhu and Marchant, 2011). They can be used in the form of hydrogels, films and foams/sponges.

Gelatin is among extensively used polymers for tissue engineering owing to its biocompatibility and biodegradability (Mao *et al.,* 2003). Gelatin-based cryogels have been used as potential bone tissue-engineering scaffolds especially for craniofacial bone tissues (Inci *et al.,* 2013). Genipin cross-linked gelatin scaffolds, produced by electrospinning, were tested as a platform to include decellularized rat brain extracellular matrix as an active agent to provide fundamental biochemical cues to the seeded cells. Demonstrated the effectiveness of the proposed decellularization protocol and the cytocompatibility of the resulting brain matrix. Then, the *in vitro* biological assays of the conditioned electrospun scaffolds, using rat allogeneic mesenchymal stromal cells, confirmed their biocompatibility and showed a differentiative potential in presence of just 1% w/w decellularized rat brain extracellular matrix (Baiguera *et al.,* 2014).

Chitosan is a known natural polymer obtained by alkaline deacetylation of chitin (Martino, Sittinger and Risbud, 2005). Chitosan and chitin are commercially acquired from shellfish sources e.g. shrimps and crabs. Chitosan is one of the widely used biomaterials for tissue engineering due to its biocompatibility, good mechanical properties, porosity, healing capacity and anti-microbial activity (Khor and Lim, 2003; Dutta, Rinki and Dutta, 2011). It has surface properties that promote cell attachment, proliferation and differentiation (Croisier and Jerome, 2013). It can be shaped into a

variety of forms, such as film, gel, powder, sphere, and fiber. Many researchers have chosen chitosan or a blend of chitosan with other polymers to culture different cell types (Khor and Lim, 2003; Charernsriwilaiwat *et al.*, 2012).

A chitosan/gelatin blend was formulated into membranesand cultured with human MG-63 osteoblast-like cells. The cells exhibited good adhesion to the scaffold, suggesting that these chitosan/gelatin membranes are beneficial for biomedical applications (Nagahama *et al.,* 2009). Sandra *et al.,* modified the chitosan surface with argon plasma and nitrogen and cultured L929 fibroblasts on it. Surface modified chitosan membranes were found to promote the proliferation of fibroblasts as compared to untreated chitosan membrane. They observed improved cell attachment and adherence (Luna *et al.*, 2011).

Nanofibres formulated by electrospinning of Chitosan/PEO (Polyethylene oxide; a synthetic polymer) exhibit cellular biocompatibility. The scaffold has been found to promote cell attachment and viability. The diameter of nanofibres was found to increase with the addition of UHMWPEO (Ultra high molecular weight polyethylene) and decrease with the addition of PVP to the electrospinning solution. So by blending natural polymers with synthetic polymers the scaffold with desired properties can be designed (Croisier and Jerome, 2013). Chitosan/PVA (polyvinyl alcohol) blends are also used for a variety of biomedical applications (Huang, GE and XU, 2007). Three dimensional CAD (Computer Aided Design) scaffolds for tissue engineering have also been developed using methacrylamide-modified gelatin (GelMOD). These scaffolds showed stability in tissue culture medium and supported the adhesion and proliferation of porcine mesenchymal stem cell. The seeded cells differentiated into the anticipated lineage upon applying osteogenic stimulation (Ovsianikov *et al.,* 2011).

Hyaluronic acid (HA), a glycosaminoglycan, found in ECM and is the main inorganic component of the bone. It is extensively used in biomedical applications as

it is highly biocompatible. HA stimulates osteoconduction and after implantation it is slowly replaced by the host bone (Collins and Birkinshaw, 2013). HA based scaffolds have been used for cell encapsulation for example cartilage regeneration using auricular chondrocytes (Kim *et al.,* 2012, Erickson *et al.,* 2012). It is sometimes blended with other polymers e.g. HA/Collagen based scaffolds (Zhang *et al.,* 2010), HA/Chitosan (Tan *et al.,* 2009) and have also been used for cartilage regeneration. The engineering of heart valves has been difficult on polymeric surfaces even when they were modified with the peptides.

In a recent study Espander and his colleagues cultured human adipose-derived stem cells in vivo, successfully on a HA-derived scaffold and also observed the human cornea-specific proteins expression (Espandar *et al.,* 2012). To enhance the regeneration of bone tissue HA hydrogels have also been used as a coating on scaffolds produced from PLA and PLGA (Antunes *et al.,* 2010; Kang *et al.,* 2011).

Synthetic polymers have more reproducible chemical and physical properties in comparison to the natural polymers and are critical factors for the production of tissue engineering scaffolds. Presently, synthetic polymers have become an important choice for the fabrication of hydrogel scaffolds as there mechanical strength, biodegradability, molecular weights and block structures can be easily tailored. Biodegradable, non-biodegradable and bioactive polymers are the three main types of the synthetic polymers.

2.5 SELF-ASSEMBLING PEPTIDES SYSTEMS

Polypeptides constitute a class of materials that is between natural and synthetic polymers. This class of biomaterials can be created synthetically and mimics the behavior of natural scaffold in the form of hydrogel. These peptide hydrogels have been used in a number of biomedical applications due to their favorable structural features, high water content and biocompatibility, including wound healing (Loo *et al.,*

2014), tissue engineering (Van Vlierberghe, Dubruel and Schacht, 2011) and drug delivery (Altunbas and Pochan, 2012).

2.5.1 Molecular Self Assembly

Self-assembly is a bottom-up approach in which small building blocks arrange autonomously in nature to create ordered and functional molecular architectures. The structures produced are usually in an equilibrium state. This attainment of spontaneous equilibrium state of molecular structures is based on the balance between different types of attractive and repulsive non-covalent or weak covalent forces, which are created between neighboring molecules (intermolecular interactions) and within molecules (intramolecular interactions) (Whitesides and Grzybowski, 2002). A number of self-assembling peptide systems has been developed. These peptide systems can be tailored for specific applications and have been classified on the basis of nanostructures of the peptides like nanofibers, nanotapes, nanotubes and nanovesicles (Luo *et al.,* 2013; Kopeček and Yang, 2009). Self assembling peptides have been widely used in regenerative medicines (Luo *et al.,* 2013).

2.5.2 Forces Controlling Self Assembly

A number of noncovalent interactions are involved in controlling the process of self assembly e.g. van der waals forces, electrostatic interactions, hydrogen bonds, hydrophobic and aromatic interactions.

Hydrogen bonds are very dominant among these forces. Hydrogen bond forms between hydrogen atom which is covalently bonded to a strong electronegative atom such as oxygen or nitrogen and an electronegative atom which exist in its vicinity. In peptides hydrogen bonds commonly involve nitrogen and oxygen atoms. Hydrogen bonding is a directional force in which the two electronegative atoms are usually ~ 3 Å apart (Nelson and Cox, 2000).

Attractive electrostatic interactions develop when positively polarized species associate with negative ones. The presence of salts in solution is known to weaken electrostatic interactions as salt molecules act as counter-ions screening the charges of the compounds (Alberts *et al.,* 1994). The electrostatic interaction between lysines and arginines (positively charged groups) and aspartic and glutamic acids (negatively charged groups) on the nanoscale results in the assembly of supramolecular structures (Gazit, 2008).

Van der Waals interactions occur when the electron cloud of one atom is attracted by nucleus of non-bonded neighboring atom and vice versa. The nucleus is positively charged, while the electrons around it are negatively charged. The effect is greatest when the groups are most polarizable, usually between the methyl groups e.g. in case of valine. Atoms that are close together and only 5 Å or less apart can participate in these interactions (Nelson and Cox, 2000).

Hydrophobic interactions arise when the peptides assemble in such a way that water excludes from their hydrophobic core and the polar parts that are hydrophilic reside on the aqueous surface. A well ordered structure of the molecules is obtained as a result (Gazit, 2008).

Aromatic interactions (π-π interactions) rise from the attraction of π-electron clouds existing on the surfaces of aromatic moieties (Steed and Atwood, 2009). Butterfield and Waters discovered that aromatic interactions can stabilize an α-helix in water by providing bond energy up to –3.3 kJ mol^{-1} between two phenylalanines (Butterfield, Patel and Waters, 2002).

Any of the above-mentioned forces, or combinations thereof, can drive the self-assembly process. As compared to the covalent bonds these forces are feeble, however when acting cooperatively they can create stable supramolecular assemblies.

2.5.3 Molecular Structures

Self assembling peptides are mainly characterized by an interchanging arrangement of negative and positive charged residues. The distribution of the positive and negative charges should follow an alternating pattern in order to have ionic complementarity. The most simple and widely studied types are type I (− +), type II (− − + +), type III (– – – + + +) and type IV (− − − − + + + +). The presence of complementary groups within each peptide molecule governs the molecular self-assembly. Self assembly can be triggered in the system by a number of factors which result in the spontaneously assembly of the oligo peptides to a variety of macroscopic structures (Zhang, Gelain and Zhao, 2005).

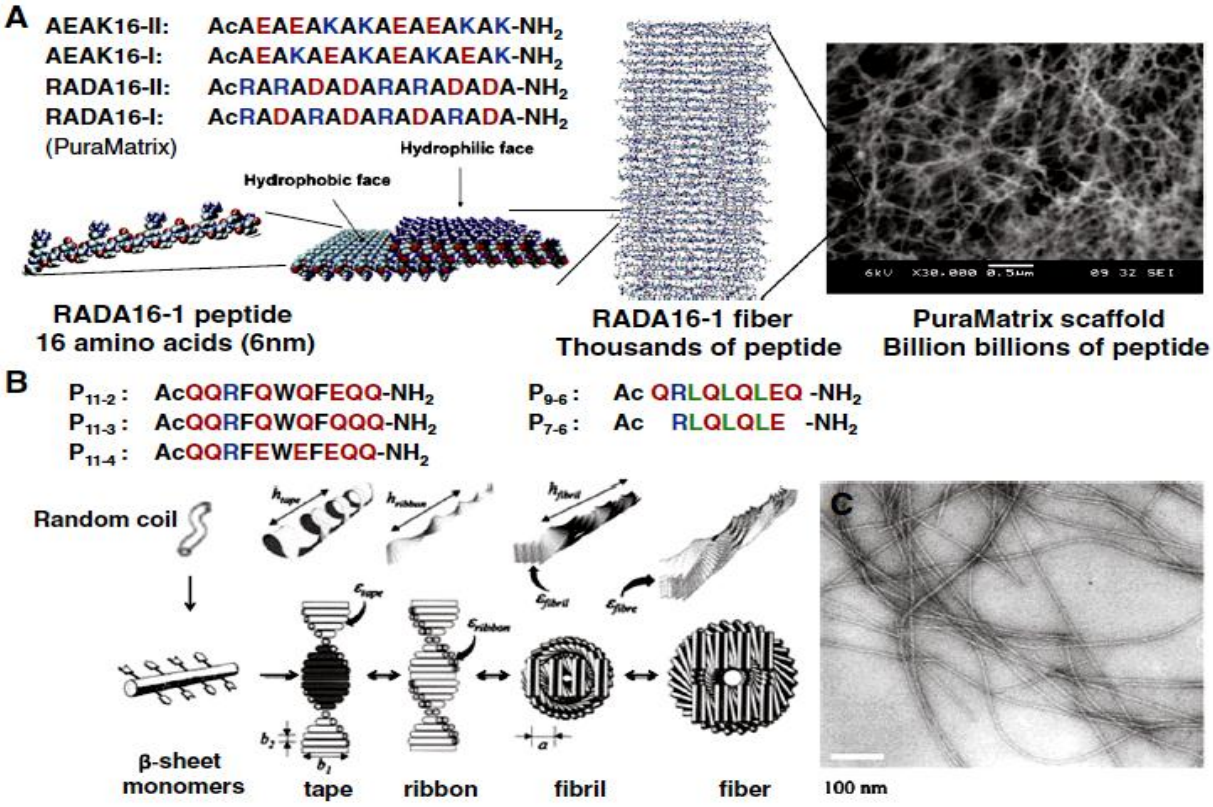


Fig.2: (A) β-Sheet forming short peptides RADA16-I (PuraMatrix™); **(B)** Short amphiphilic peptide nanotapes (Aggeli *et al.*, 2001); **(C)** TEM of a P11-4 gel in water (Aggeli *et al.*, 2003) (adapted from Loo, Zhang and Hauser, 2012)

2.6 AMINO ACIDS, PEPTIDES AND PROTEINS

2.6.1 Amino acids

Amino acids are the unit structures of proteins. There are twenty different types of amino acids.

Each amino acid consists of :

- central carbon atom (C)
- carboxyl group (COO)
- amino group (NH_3)
- hydrogen atom (H)
- side chain (R) group

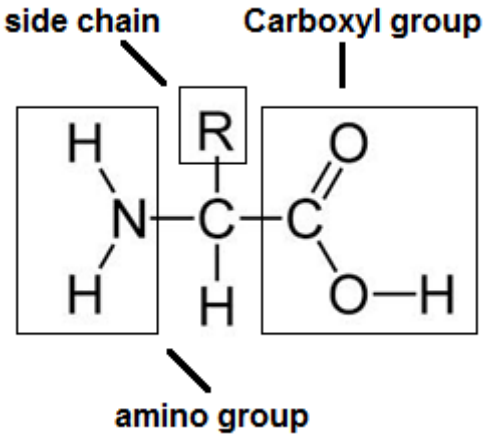
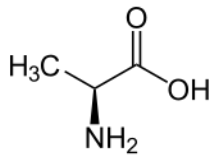
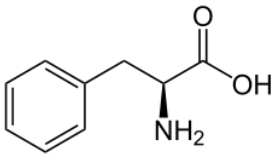
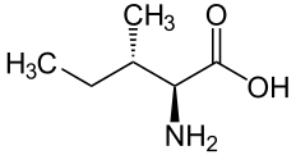
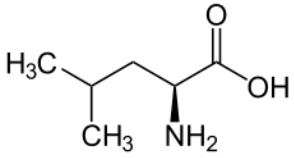
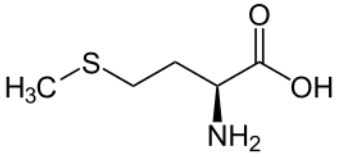
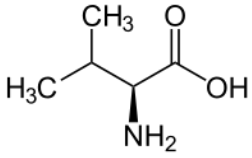
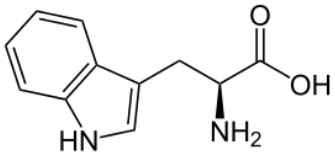
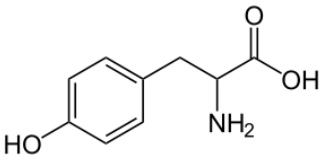
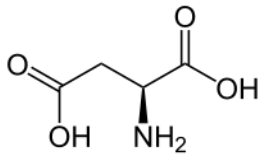
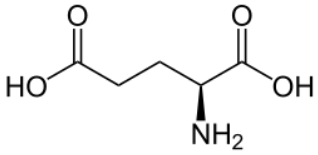
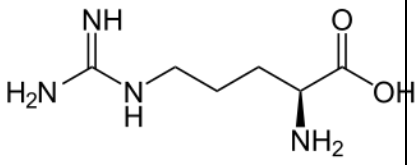
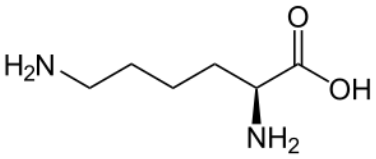
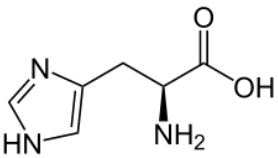
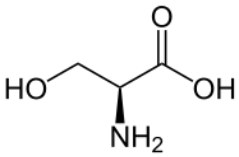
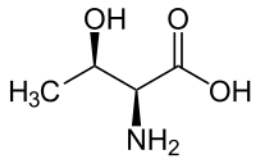
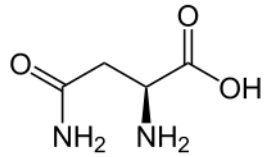
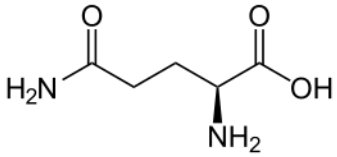
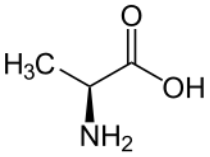
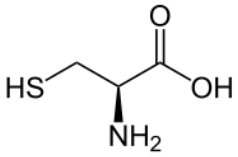
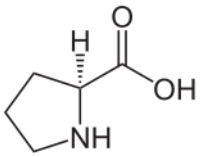


Fig 3: Basic structure of an Amino acid

Table 3: Amino Acids Characterization and their Structures

Non-Polar / Hydrophobic amino Acids		
Name	**Abbreviations**	**Structures**
Alanine	Ala, A	
Phenylalne	Phe, F	
Isoleucine	Ile, I	
Leucine	Leu, L	
Methionine	Met, M	
Valine	Val, V	
Tryptophan	Trp, W	

Tyrosine	Tyr, Y	
Acidic amino acids		
Aspartic Acid	Asp, D	
Glutamic Acid	Glu, E	
Basic amino acids		
Arginine	Arg, R	
Lysine	Lys, K	
Histidine	His, H	
Polar / Hydrophilic amino acids		
Serine	Ser, S	

Threonine	Thr, T	
Asparagine	Asn, N	
Glutamine	Gln, Q	
Special amino acids		
Glycine	Gly, G	
Cysteine	Cys, C	
Proline	Pro, P	

2.7 PEPTIDE BOND

A peptide bond is formed as a result of a condensation reaction between two amino acids, which links all the adjacent amino acid residues in a protein chain. In a protein chain the amino N, α carbon, carbonyl carbon sequence is repeated throughout and the side-chain groups (R) extend from the backbone of a protein chain.

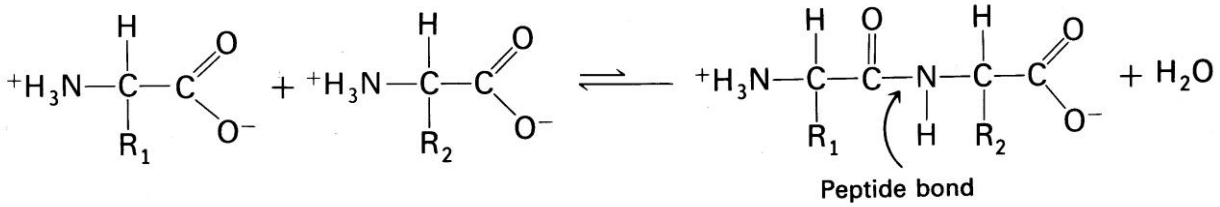


Fig 4: Formation of a peptide bond

2.8 PROTEINS

Proteins are polymers that consist of chains of amino acids also known as polypeptides. Each type of protein has a distinct sequence of amino acids. They are further folded into one or more specific spatial conformations, driven by a number of non-covalent interactions such as Van der Waals forces, hydrogen bonding, ionic and hydrophobic interactions.

2.8.1 Protein Structures

The linear arrangement of amino acids in the form of a chain constitutes the primary structure of a protein. The amino acids in the primary structure are joined by peptide bonds. These peptide chains can adopt several spatial arrangements. The resulting organization is called secondary structure which includes random coil structure, which when stabilized forms a spiral rod like structure called an α helix; and a planar structure formed by the arrangement of two or more β strand, called β sheet structures. The secondary structure is stabilized by the formation of hydrogen bonds between amino acid residues. Next higher level of structural organization in three dimensions is called tertiary structure organization. In addition to hydrogen bonds the hydrophobic interactions and disulfide bonds are also involved in the stabilization of a tertiary structure. The random coils, α helices, β strands and β turns are organized in a compact arrangement in a tertiary protein structure. For single polypeptide proteins the

highest level of organization is tertiary structure. Multimeric proteins that consist of more than one polypeptide chains attain higher level of organization called quaternary structure. The quaternary structure is a three dimensional structure of a protein having multiple subunits with specific number and position of the subunits. The quaternary structure is stabilized in the same fashion as that of a tertiary structure.

2.9 STRUCTURAL PROPERTIES OF IONIC COMPLEMENTARY PEPTIDE SYSTEMS

The following section reviews examples of peptide based self-assembling systems designed to create nano-structured architectures with potential applications in tissue engineering.

2.9.1 α-Helices

Ionic complementary peptides have three-dimensional nanofiber structures. Siani and coworkers studied the self assembly of two ionic complementary alanine based octapeptides, AEAKAEAK and AEAEAKAK (A=alanine; K= lysine; and E= glutamic acid). The charge distribution for the peptides is - + - + and - - + + respectively. Self assembly for AEAKAEAK was not observed whereas AEAEAKAK self assembled into thick rigid fibres having a diameter of 6 nm which exhibited ‘‘pearl-necklace’’ morphologies consisting of two laterally connected fibrils (Saiani *et al.,* 2009). The secondary structure of the peptide depends upon its amino acid sequence for example, Ala, Lys, Glu, and Gln are found more often in a-helices. AEAEAKAK which is an alpha based peptide formed alpha helical confirmation which was revealed from its FTIR spectra. Repeating pattern of polar and non polar amino acids helps the peptide to adopt a-helical confirmation in which hydrophobic residues are buried in and hydrophobic interactions between alanine residues and electrostatic interactions between charged residues i.e. lysine and glutamic acid further

stabilize the structure. Gelation was not observed for the peptide AEAEAKAK even at 100mg/ml concentration which means these peptides cannot be formulated into 3D structures. As well no evidence has been reported in literature for their use in tissue engineering (Mohammed, Miller and Saiani *et al.*, 2007).

Many systems based on the self assembly of linear peptides that adopt a-helical confirmations were documented earlier. Kojima and fellows were the first one to describe one in 1997. They described a single peptide system, α3-peptide that assembles into several micron long and 5-10nm thick fibres. Their peptide consisted of three repeats of a heptad sequence LETLAKA. It assembled into a-helical fibrils 5-10nm wide at pH 6.0 and low salt concentration. With increase in salt concentration larger fibres were observed.

In 2000 Woolfson and coworkers introduced a self assembling fiber system which consisted of two leucine-zipper peptides having sticky ends which assembled into coiled coil fibrils when joined end to end due to leucine and isoleucine residues. These fibrils further joined to form thick fibers 50nm wide and 10mm in length. Later on they introduced a number of self assembling fiber system supplemented with functional moieties, cross links and branches into the fibrous system (Ryadnov and Woolfson, 2003; Woolfson and Ryadnov, 2006). These SAFs were then modified chemically to develop specific interactions which directed the crystallization of the peptides within the fibers. As a result the fibers with increased thickness and more ordered structure formed which easily settled out of the solution and synthetic peptides with molecular weight in the mDa range formed. With a change of certain amino acids in the SAF peptide its gelation properties also changed. In 2009 Banwell and coworkers proposed hSAF peptides that formed temperature-responsive hydrogels. They also tested hSAFs in comparison with the commonly used Matrigel, and reported that hSAFs support cell growth and differentiation of adrenal pheochromocytoma cells of rat for sustained periods in culture. So these gels could be utilized as engineerable scaffolds for tissue engineering and cell culture (Banwell *et al.*, 2009).

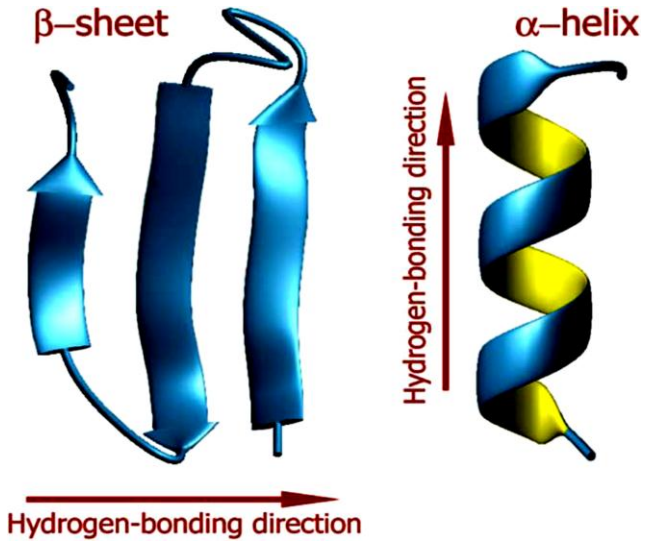


Fig 5: Diagrammatic representation of the two main types of hydrogen-bonded structure in proteins, the a-helix and the b-sheet (adapted from MacPhee and Woolfson, 2004)

Another single peptide system named as magic wand peptide was reported which self assembles to form thickened and extended fibers with structures similar to the SAFs, but with much enhanced thermal stability. Moreover the fibers formed by magic wand are straight and do no form branches (Gribbon *et al.*, 2008).

2.9.2 β Sheet Structures

In the early 1990s, Zhang and his coworkers serendipitously discovered a natural protein motif AEAK16-II in yeast DNA which self-assembled in water into β-sheet peptide motifs. Its sequence was AEAEAKAKAEAEAKAK and was named as Zuotin (a Z-DNA binding protein) (Zhang *et al.*, 1993). The sequence comprises of cyclic repeats of ionic hydrophobic and hydrophilic amino acids which causes the peptides to assemble into β-sheet with distinct hydrophilic and hydrophobic surfaces (Zhang *et al.*, 1993). Self assembly takes place in the aqueous environment, where the hydrophobic alanines form corresponding hydrophobic interactions, and the peptides

with positive and negative charges pack together in a zig zag pattern on the hydrophilic side through intermolecular ionic interactions. These β-sheets then stack to form nano-fibers of approximately 10 nm in diameter. These nano-fibers then aggregate into a 3D scaffold that contains more than 99% of water.

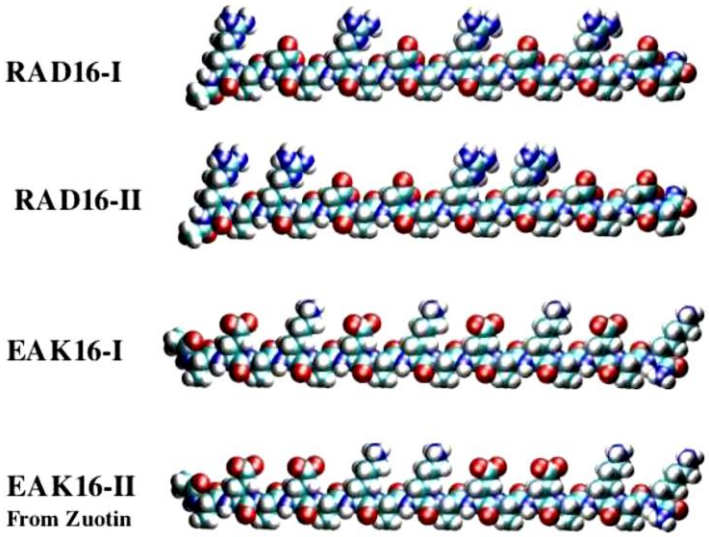


Fig 6: Molecular models of several self-assembling peptides, RAD16-I, RAD16-II, EAK16-I and EAK16-II (adapted from Zhang and Zhao, 2007)**.**

Each molecule is around 5nm long with alanines arranged on one side and the alternating negative and positive charged amino acids on the other side. By substituting glutamate and lysine in AEAK16 with aspartate and arginine residues, further ionic complementary peptides were formed which were RADARADARADARADA (RADA16-I) and RARADADARARADADA (RADA16-II). These newly formed peptides followed same process of self-assembly in which they burry their hydrophobic residues to exclude water and expose the polar surfaces to water. (Zhang et al, 1993).These peptide hydrogels were reported to support the attachment of the cells. Some of these gels have also been reported to support cell proliferation, differentiation (Loo, Zhang and Hauser, 2012), migration and production of their own ECM (Zhang 2003). Among them, the most studied peptide is

RADA16-I, which is commercially available and the trade label is PuraMatrix (Kyle *et al.,* 2009; Zhang *et al.,* 1993).

Later on Zonghli Lou and coworkers used L-form amino acids instead of D-form amino acids and proposed a new self-assembling peptide scaffold d-EAK 16. It behaved similar to L-EAK 16 in 3-D cell culture environment. It supported cell growth with high cell viability and a low-level cell apoptosis. There was no significant difference to the L-form self-assembling peptide rather D-form peptides have an advantage over L-form i-e they are resistant to degradation by proteases (Luo *et al.,* 2013).

Moreover ionic-complementary peptides can be easily formulated by using different amino acids with the same distribution of charged residues. By substituting alanine with iso-leucine (IKIE), phenylalanine (FKFE) or valine (VKVE), more self assembling peptides are formed with difference in hydrophobic residues. Also by reducing the number of peptides in a unit molecule they require lower critical concentrations for the β-sheet formation (Ulijn and Smith, 2008).

Zhang et al also reported the self-assembly of peptides comprising 8 amino acids. Such as KFE8 (Zhang *et al.,* 2002). KFE8 (FKFEFKFE) consists of two repeats of lysine (K), phenylalanine (F) and glutamic acid (E) with a charge distribution of +-+-. The self assembly of the peptide is triggered on dissolution into water. Hydrophobic and electrostatic interactions in the presence of alternating hydrophobic (F) and hydrophilic (K is positively charged and E negatively charged) ultimately lead to the formation of β-sheets (Zhang *et al.,* 1993).

Another group of researchers Saiani *et al.,* designed ocapeptides FEFEFKFK and FEFKFEFK and studied their gelation properties and self assembly. Both of the peptides formed gel at a concentration greater then 8mg-ml (Saiani *et al.,* 2009; Miller and Saiani, 2010). The peptide solutions were analyzed for their structural

morphology by AFM, TEM and SANS. AFM and TEM results confirmed the presence of a dense network of fibres with a diameter of 4-5 nm. FTIR was conducted to check the fibre morphology. Adsorption band was obtained at 1625 cm^{-1} and 1695 cm^{-1}. These bands specify the occurrence of antiparallel beta sheets in the structure (Bartha and Zschrp, 2002). Moreover the fibre density was checked by Small Angle Neutron Scattering (SANS) experiments which showed that as the concentration of peptide was increased; fibre density increases while the porosity and pore size decreases. For the two peptides it was 30± 3 nm for the concentration 10mg/ml which decreased to 15± 3 nm at concentration 40 mg/ml of sample. So the concentration of peptide can control the porosity and mesh size of the gel (Saiani *et al.*, 2009).

For the phenylalanine based peptides the beta sheets had a hydrophobic face and a hydrophilic face as the polar groups were arranged on one side and the phenyl rings on the opposite side. So, this way two beta sheets come closer and bury their hydrophobic face as shown in fig 7 (Saiani *et al.*, 2009).

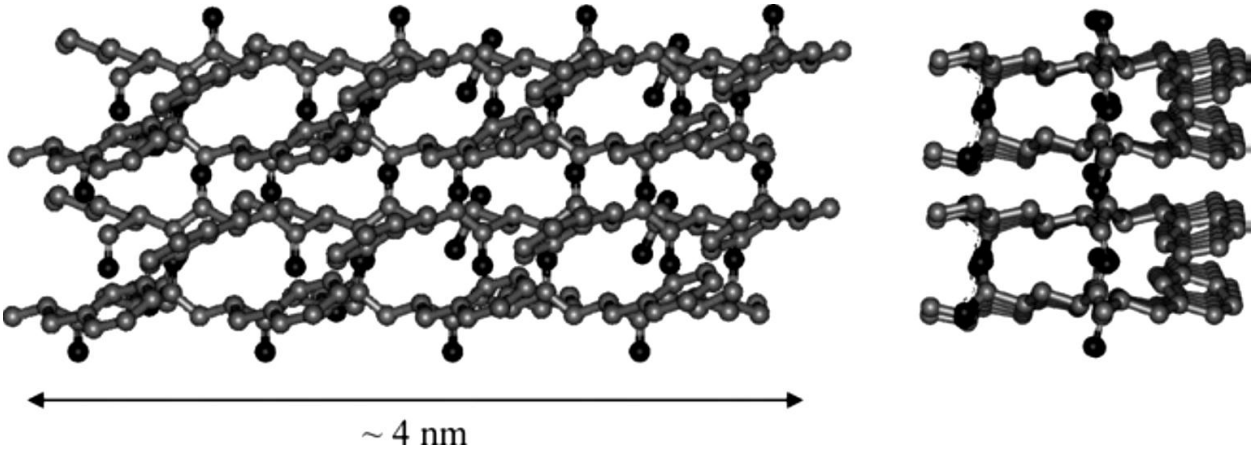


Fig 7: Structural representation (left shows the top view and right shows the side view) of four FEFEFKFK peptides in an anti-parallel β-sheet (adapted from Saiani *et al.*, 2009).

2.9.3 β Hairpin Structures

A beta hair pin forming self-assembling peptide system has been proposed by Schneider *et al.* consisting of 20 amino acids, MAX1. The sequence (H2N-VKVKVKVKVDPPTKVKVKVKV-CONH2) includes alternating Valine and Lysine residues which fold into a beta hairpin structure at high pH or in the presence of salt. The folding and self-assembly is brought about by the abolition of charge repulsion between neighboring lysine residues (Rughani and Schneider, 2008). The peptide folds into beta hairpin structures that align themselves and self-assemble into fibrils.

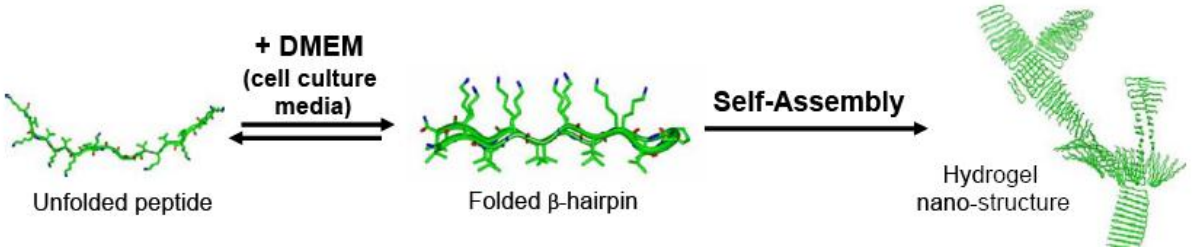


Fig 8: Proposed mechanism for the folding and self-assembly of MAX hydrogels (adapted from Branco *et al.*, 2009).

The resulting hydrogel was also found to be nontoxic, non immunogenic and promote cell proliferation NIH 3T3 murine fibroblasts (Kretsinger., *et al.*, 2005). It also has been shown to support the growth of cementoblasts and direct the processes of biomineralization in the development of bone tissue (Gungormus *et al.*, 2010). The hydrogel developed by Schneider *et al.* is mechanically robust, shear recoverable optically transparent, and cytocompatible and can be ideally used as a tissue engineering scaffold (Haines-Butterick *et al.*, 2007).

MAX1 gels have also been reported to have antibacterial activity which is exhibited by their surfaces although they are nonhemolytic to human erythrocytes. The broad spectrum antibacterial activity against S. pyogenes, S. epidermidis, K. pneumoniae, S. aureus, and E. coli, has been observed. These gels therefore are

proposed to be an attractive candidate for use in tissue regeneration, even in nonsterile atmosphere (Salick *et al.,* 2007).

Their group has also proposed another b-hairpin peptide (MAX8), which has a design analogous to that of MAX1 but has a lysine replaced by glutamic acid at position 15. MAX8 peptide has been observed to self assemble into hydrogel faster than MAX1. The substitution of one amino acid has been hypothesized to reduce the electrostatic repulsion between the chains resulting in increased rate of self assembly and mechanical strength of the gels (Hule *et al.,* 2008). MAX8 also has been checked for cell culture applications, by seeding NIH 3T3 murine fibroblasts and mesenchymal C3H10t1/2 stem cells on these gels (Kretsinger *et al.,* 2005; Haines-Butterick *et al.,* 2007). The gel was observed to be cyto-compatible and mechanically stable under cell culture conditions.

2.9.4 Peptide Amphiphiles

Amphiphiles have surfactant like structures; they generally contain both hydrophilic and hydrophobic regions that self-assemble to form nanofibres, vesicles and micelles. Vauthey *et al.,* 2002 designed surfactant peptides with one or two aspartic acid residues at the C termini and six successive hydrophobic amino acids with acetylation on N terminus (V6D, V6D2, A6D, A6D2 and L6D2). By modifying the aliphatic side groups of the amino acids, the overall hydrophobicity of these peptides can be changed. These peptides self assembled to form nanovesicles and nanotubes of diameter 30–50nm with a helical twist. The hypothesized pathway of the self assembly is that monomeric peptides with a diameter of 2 nm join together to form a bilayer ring, the water is excluded as the hydrophobic tails pack together and on inner and outer side of the tube the hydrophilic heads are exposed to water as shown below in fig 9. The tubular arrangements may consequently stack to form longer nanotubes through noncovalent interactions which can then be engineered to form vascular implants (Vauthey *et al.*, 2002).

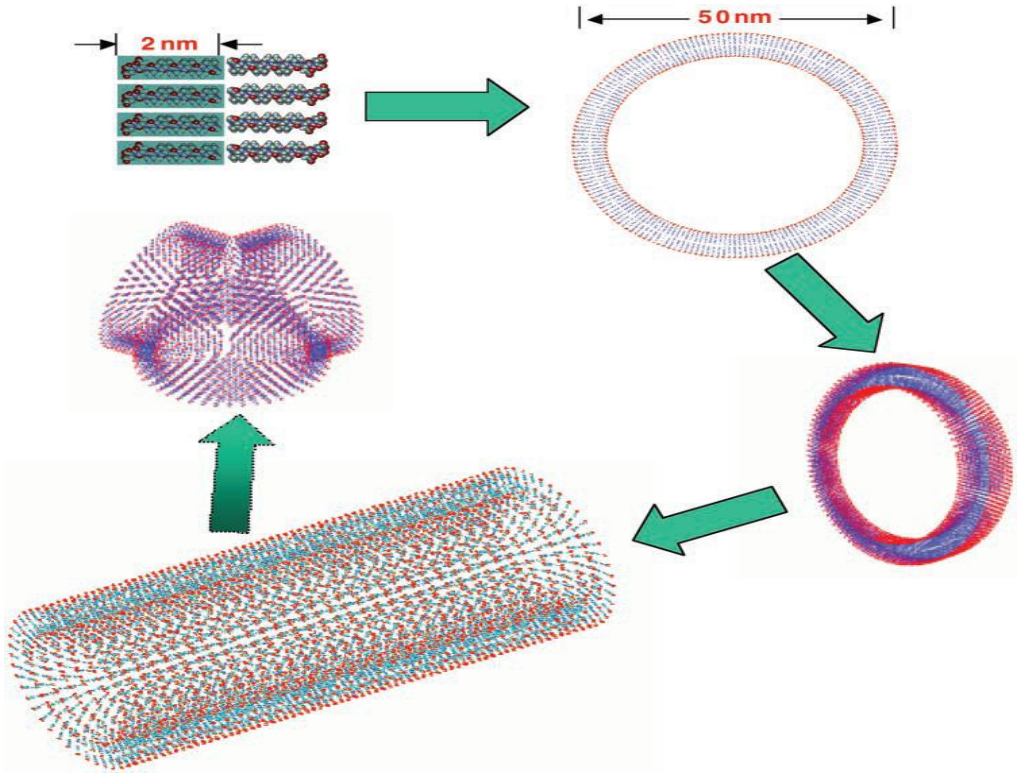


Fig 9: Nanotube formation by packing of V6D peptide (adapted from Vauthey *et al.*, 2002).

Later Stupp and his coworkers developed a series of peptide amphiphile (PA) molecules to be used for tissue engineering. Their peptide amphiphiles were composed of four structural domains; region 1 is a long alkyl tail which is hydrophobic and the length of which can be changed to impart different properties; region 2 is a short peptide sequence which is responsible for the formation of beta sheets by the formation of hydrogen bonding, this region can be modified to control the mechanical properties of the resulting nanofibers; region 3 is hydrophilic and contains charged amino acids which give it the property of pH and salt responsive self assembly; region 4 consisted of bioactive moiety for example RGDS or IKVAV, that is displayed on the surface and helps in the interaction with cells growth factors and proteins (Cui, Webber and Stupp, 2010). Self assembly of their peptide amphiphiles has been reported by adjustment of pH, use of metal ions, temperature and ionic strength.

Silva *et al.* (2004) used peptide amphiphiles with IKVAV motif and were able to support the growth of NPCs, and their differentiation into neurons. Mata *et al.,* 2010 has reported that the peptide amphiphile nanofiber networks can be utilized for recreation of nanostructure of the bone when mineralized with hydroxyapatite.

2.9.5 Aromatic peptides

Like amphiphilic peptide systems short aromatic peptides also spontaneously self assemble based on hydrophobic interactions mainly aromatic i-e π-π bonds (Gazit, 2007). They are short peptides e.g. diphenylglycine peptide also called amyloid forming peptides as they form a similar structure as that of the β-amyloid polypeptide, found in the Alzheimer's disease due to the interaction between aromatic moieties (Mahler *et al.,* 2006). These peptides at the nanoscale self-assemble into hollow tubular structures. These discrete nanotubes are tremendously rigid and form spontaneously when mixed into aqueous solution or by changing the temperature of an aqueous solution of the peptides (Kol *et al.,* 2005). Other analogs such as diphenylglycine and the Fmoc-diphenylalanine form nano spherical assemblies and nanofibrils respectively. The peptide nanotubes are reported to be robust and stable under extreme conditions including boiling, autoclave temperature, and exposure to various organic solvents (Orbach *et al.,* 2009; Adler-Abramovich *et al.,* 2006). Hydrogels made from Fmoc di-phenylalanine and Fmoc di-glycine peptides in 1:1 ratio as well as from Fmoc di-phenylalanine and Fmoc lysine peptides in 1:1 have been shown to support the growth of bovine chondrocytes for 7 days in cell culture conditions and the cells are also reported to maintain their metabolic activity Jayawarna *et al.,* 2006). In another study Fmoc-FF/RGD peptide hydrogel has also been reported to support the proliferation of human dermal fibroblasts in 3D cell cultures invitro (Zhou *et al.*, 2009; Zhao *et al.*, 2010).

2.10 APPLICATION OF PEPTIDE SYSTEMS IN TISSUE ENGINEERING

Self assembling peptide systems have improved performance as compared to the natural or synthetic acellular scaffold to be used as potential scaffold for tissue engineering applications. A number of invitro studies have used peptide hydrogels for culturing cells where they have proved good being biocompatible and by providing cell adhesion, their proliferation and differentiation. Scaffolds fabricated from peptide based hydrogels have been shown to support neurite outgrowth (Gazit, 2008).

Self-assembling peptide nano-fiber scaffold has also been shown to be a promising biomaterial for restoration of brain tissue. It has capability to be used as filling material in brain cavities formed due to trauma or neurosurgical procedures (Guo *et al.,* 2009).

It is unlikely that hydrogels developed from self-assembling peptide scaffolds, either with or without cell seeding, could fulfill the biomechanical requirements for use as scaffolds for the replacement of functional cardiovascular or musculoskeletal connective tissues, such as blood vessels, heart valves, ligaments or tendons. Here, there is a need for macro-, meso- and micro-scale biomechanical functionality in order to deliver the appropriate macro-, meso- and micro-strains to cells for appropriate mechanosensing and restoration of physical function (Nune *et al.,* 2013).

Chapter 3

3. MATERIAL AND METHODS

3.1 MATERIALS AND SOLUTIONS

Two hexapeptides FK6 (FEFGFK) and VK6 (VEVGVK) were designed and purchased from 'Synpeptide Co Ltd' and used without any further purification. Purity of peptides (94 %) was determined by HPLC and mass spectrometry The Molecular weight of the peptide FK6 and VK6 was 773.89 g/mol and 629.76 g/mol respectively . For pH adjustment 0.5 M NaOH solution was prepared by dissolving 4.0 g of NaOH (Sigma-Aldrich) in 200 ml of autoclaved distilled water (HPLC grade). pH of the solutions was checked using universal pH indicator purchased from Merck Germany. For hemolysis assay triton X-100 (1%) solution was made by dissolving 1 ml of Triton X-100 stock (Sigma-Aldrich) in 9 ml of distilled double autoclaved water. 1X Phosphate-buffered saline (PBS) was prepared by dissolving NaCl: 8 g, KCl: 0.2 g, Na_2HPO_4: 1.44 g and KH_2PO_4: 0.24 g in 1000 ml of distilled double autoclaved water.

3.2 FORMATION OF PEPTIDE HYDROGEL (FK6 AND VK6) AT VARYING CONCENTRATIONS

Both of the hexapeptides in the form of powder were weighed using an electronic balance in sterile 2 ml microtubes according to the required concentration (10 mg, 20 mg, 30 mg and 40 mg), water (1 ml) was then added to each microtube with the help of a micropipette (to make a solution of 10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml respectively). The tubes were then vortexed for a few seconds for homogeneous mixing and then placed in an oven at 90 ºC for two hours for complete dissolution.

Microtubes were then taken out of the oven and the solutions were allowed to cool down at room temperature after which their pH was checked. 0.5 M NaOH was then added slowly to adjust the pH and the solution was observed for gelation for a few minutes. The gelation was checked by the tilt-test.

3.2.1 Effect of pH on gelation

Peptide solutions were prepared by the same method as described in the above section. The pH was varied by adding 0.5M NaOH solution with the help of a micropipette to the peptide solution in the microtubes. The tubes were then vortexed for 10-15 sec after adding NaOH and the pH was then checked by using universal indicator (Merck Germany). Gelation at each pH was checked by the tilt-test.

3.2.2 Effect of Temperature on gelation

The effect of temperature on peptide gel at different concentrations (10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml) was checked at a range of temperatures (25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, 85 °C and 95 °C). The oven was set at a specific temperature prior to the incubation. The gel containing microtubes were placed at that temperature for 30 mins. The microtubes were then taken out; tilt-test was performed to check the stability of the gel at that temperature. The microtubes were then placed at room temperature for 30 mins to stabilize the gel before repeating the above steps for the other listed temperatures.

3.3 FORMATION OF PEPTIDE HYDROGEL (BLEND OF FK6 AND VK6)

Peptide solution with a mixture of two peptides FK6 and VK6 was formed at concentration 30 mg/ml with different ratios of two peptides (VK6 and FK6; 30:70,

50:50, 70:30). Weighed amount of both the peptides was added in 2 ml microtubes. 1 ml water as solvent was then added. The solution was vortexed and then kept at 90°C for two hours in the oven. After which the pH of the solution was checked. The solution was cooled to room temperature and kept for 10 minutes. Gelation was then checked by the tilt test.

3.3.1 Effect of pH on the FK6/VK6 blend

The peptide solution was prepared by the same procedure as described in the above section and the pH of the solution was varied by adding 0.5 M NaOH to the solution. The pH of the solution was subsequently checked by using universal indicator (Merck Germany). Effect of change in pH on the gel formation was checked by the tilt test.

3.4 CHARACTERIZATION OF PEPTIDE GEL

In this section an account of the theoretical and technical aspects of the methods employed to morphologically characterize the studied systems is given. For each technique a brief theoretical overview of its usage as well as the experimental procedure will be described.

3.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

3.4.1.1 Theory

Fourier transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of emission, absorption, Raman scattering / photoconductivity of a solid, liquid or gas. An FTIR spectrometer collects spectral data in a wide spectral range simultaneously. The technique provides information of the secondary structure of proteins.

The infrared spectrum consists of three groups i.e. near (λ = 1–2.5 μm), mid (λ = 2.5–25 μm) and far (λ > 25 μm). The Infrared spectroscopy is usually done for the mid frequency range of IR (f = 4000 to 400 cm^{-1}). A compound when subjected to IR, results in a transfer of energy due to the interaction between chemical bonds and the IR waves. Infrared absorption (IR) gives rise to vibrational spectra in which vibrational bands are produced by characteristic groups of atoms near the same frequency irrespective of the molecule in which they are found. Peptide-bond angles, hydrogen-bonding patterns and other inter and intramolecular effects give rise to particular wavenumbers of the bands within a specific range.

FTIR is adapted to study peptide structures. The amide bonds in the peptide molecule absorb infrared radiation at specific frequencies termed as amide bands. These frequency values are linked with different secondary structures of proteins as shown in table 4. A number of amide bands are observed in vibrational spectra of proteins but the amide I band is mainly used to identify the secondary structure of protein.

Table 4: Amide I frequency values (in cm^{-1}) Characteristic of Protein secondary structures in water (adapted from Pelton and Mclean, 2000)

Type of conformation	**Frequency range (cm^{-1})**
α-helix	1650–1657
Parallel β-sheet	1626–1640
Antiparallel β-sheet	1612–1640(strong); 1670–1690 (weak)
β-turn	1655–1675
Random coil	1640–1651

3.4.1.2 Experimental Procedure

Identification of secondary structure acquired by hexapeptides upon dissolution was carried out using Fourier transform infrared spectroscopy (FTIR). FTIR was performed using a Perkin Elmer FTIR spectrometer aided with Spectrum 100 software. The FT-IR spectrum of the peptide gel at 10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml concentrations was analyzed at pH 7, using the KBr disc technique and 5 scans per sample were recorded.

3.4.2 Scanning Electron Microscopy (SEM)

3.4.2.1 Theory

The SEM-Scanning Electron Microscopy is based on use of a focused beam of electrons with high-energy to generate a variety of signals on the surface of the sample under test. The signals that are generated reveal information regarding the samples' morphology, chemical composition, nano-structure and orientation of fragments making up the sample.

In SEM, a 2-dimensional image is generated from the data that is collected over a specific area of the samples surface. Approximately, Areas ranging from 1 cm to 5 μ in width can be viewed using conventional SEM techniques. A magnification of up to 20 X - 30,000 X can be achieved with a spatial resolution of 50 - 100 nm. Quantitative and qualitative analysis (EDS) of the sample can also be performed with SEM by observing the selected point locations.

Samples that are electrical insulators are coated with a thin layer of conducting material e.g.; gold, carbon or some alloy to avoid charge build-up. The selection of coating material depends on the data to be studied i.e. carbon is mostly used for

elemental analysis, whereas metal coating is used when high resolution images are required.

3.4.2.2 Experimental Procedure

The morphology of nanofibres within the peptide solutions was checked by scanning electron microscopy (SEM). Peptide solution at 5 mg ml^{-1} was sonicated, at room temperature for fibres to completely disperse in the solution thus easing the visualization. A drop of each solution was then separately placed on the metal grids and dried under light lamp. Metal grids with dried samples were then placed in JFC-1500, Ion sputtering device, 250 Å, to allow gold coating. Samples were then analyzed using JSM-6940A, Analytical SEM, JEOL-Japan.

3.4.3 Blood Hemolysis Assay

3.4.3.1 Theory

Hemolytic activity is a requirement to be tested for any blood contacting medical device. The test is based on erythrocyte lysis induced by contact, leachables, toxins, metal ions, surface charge or any other cause of erythrocyte lysis. Interactions among blood and biomaterials can cause erythrocyte lysis, hemolysis, particularly if the contact is for long duration or if the blood is in contact with large surfaces. Hemolysis may cause anemia and the release of iron and other bioactive substances. Hemolysis can be measured by incubating the biomaterial with erythrocyte suspension at 37 ºC for 24 hours and the release of hemoglobin can be measured by means of a spectrophotometer.

3.4.3.2 Experimental procedure

Healthy human blood was obtained in vials containing EDTA (ethylenediaminetetraacetic acid). 5 ml of blood sample was mixed with 10 ml of PBS in sterile centrifuge tubes. The mixture was centrifuged for 10 mins at 1200 rpm to separate the red blood cells from the serum in the form of a pellet. The pellet is then washed 6 times with PBS (40 ml). After which the pellet is dispersed in 30 ml of PBS. The peptide hydrogel at 20 mg/ml was added to the sterile eppendorfs. Further 2 fold dilutions were made from the original peptide solution (20 mg/ml) in seven new eppendorfs. 0.4 ml of suspended red blood cells and 0.4 ml of PBS solution were then added to all these eight eppendorfs. They were then incubated at 37 °C for two hours. After this the eppendorfs were centrifuged at 3000 rpm for 10 min to pellet down the RBCs. From these eppendorfs supernatant was obtained and its optical density was observed at 540 nm using an ultraviolet spectrophotometer. Triton X-100 and PBS were used as positive and negative controls respectively. All hemolysis experiments were carried out in triplicate. The hemolysis ratio (HR) of the red blood cells was calculated using the formula;

$$HR\ (\%) = \frac{OD\ (test\ \text{sample}) - OD\ (negative\ control)}{OD\ (positive\ control) - OD\ (negative\ control)} \times 100$$

In the above equation; OD test sample, OD positive control and OD negative control are denoted as the optical density of the sample, positive and negative controls, respectively.

Chapter 4

4. RESULTS

4.1 HEXAPEPTIDE FK6

4.1.1 Gel formation

The critical gelation concentration of the peptide was found to be 15 mg/ml i.e. peptide forms a viscous sol-gel at concentrations less than that. The formation of stable gel was checked by the tilt test in which the microtube containing the gel is inverted and tapped slightly to check if the contents of the microtube flow downwards. The stability of the gel is confirmed if the gel remains intact and do not drop on inversion of the microtube.

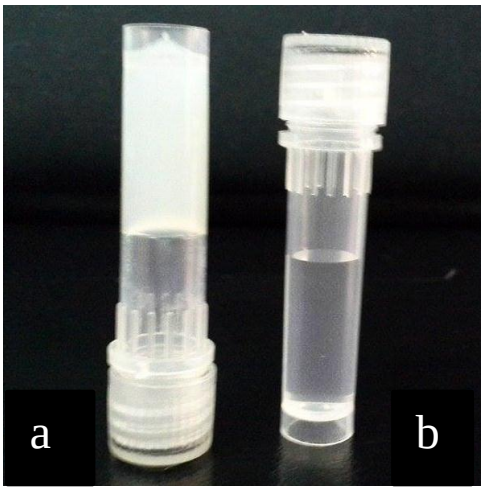


Fig 10: Hexapeptide FEFGFK (Peptide concentration 30 mg/ml; a) with NaOH b) without NaOH)

4.1.2 Effect of peptide concentration on gelation

The hexapeptide when completely dissolved in water at 90 °C for 2 hours showed no change in consistency when brought at room temperature. The pH of the peptide solution was 1 which means acidic due to the presence of TFA (trifluoroacetic acid) in the solution which is a reagent used in the synthesis of the peptide. At a concentration of 30 mg/ml and above the peptide solution forms gel when kept for 6-7 hours.

4.1.3 Effect of pH on gelation

The pH of the peptide solutions was changed by the addition of 0.5 M NaOH drop wise to the solution. The pH of the solutions was continuously checked. The peptide solution at 10 mg/ml turned slightly viscous while solution at 20, 30 and 40 mg/ml formed stable gels within few seconds on changing the pH. The gel was observed to form instantly at a range of pH from pH 3 to pH 10. The opacity of gel increased with the increasing pH as shown in fig 11.

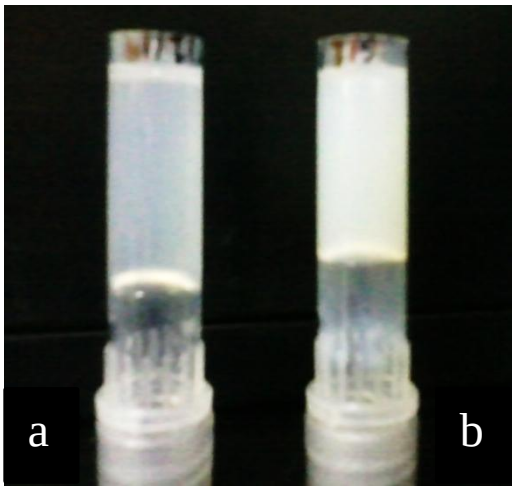


Fig 11: Hexapeptide FEFGFK (Peptide concentration 30 mg/ml; a) pH 3 b) pH 7)

4.1.4 Effect of Temperature on gelation

The phase diagram between peptide concentration and melting temperature were made to study the correlation between peptide concentration and gel transition temperature. The gel at different concentrations 10 mg/ml, 20 mg/ml, 30 mg/ml and 40 mg/ml was checked at different temperatures 25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, 85 °C and 95 °C. It was observed that the gel transition temperature increases with increase in peptide concentration. Peptide gel at 40 mg/ml concentration was stable up to 85 ºC and at 20 mg/ml concentration it was stable up to 55 ºC as shown in fig 12. Also gel at all the concentrations was found to be stable at 37 ºC which is the temperature required for cell culture.

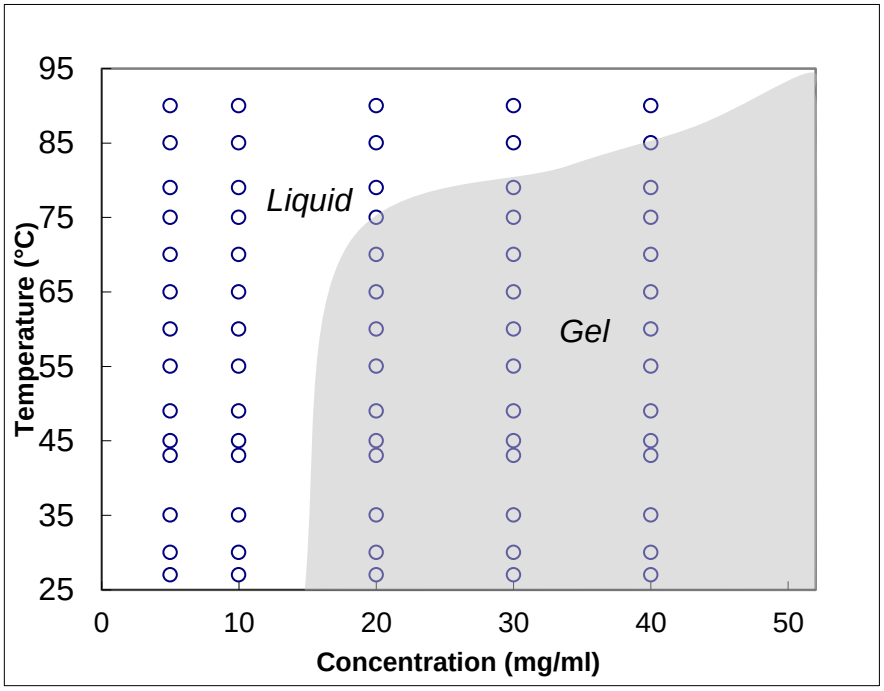


Fig 12: Phase diagram of peptide gel FEFGFK at pH 7, Concentration vs. Temperature

4.2 HEXAPEPTIDE VK6

4.2.1 Gel formation and the Effect and pH

The hexapeptide VK6 did not form gel at any concentration. On tilt test the contents of microtube had flown downwards. There was no effect of pH change and concentration (0-40 mg/ml) on the peptide solution. It remained in the solution form and no change in the color and texture of the solution was observed.

4.3 BLEND OF FK6 AND VK6

4.3.1 Gel formation and the Effect of pH

Gel formation in the peptide solution at concentration 30 mg/ml of the blend FK6 and VK6 at different ratios (30:70, 50:50 and 70:30) was observed. No change in color and texture of peptide solution at all these ratios was observed after complete dissolution. When NaOH solution was added to change the pH of the solution, gelation was observed for all the peptide solutions. The gel remained intact at a range of pH i.e. from pH 3 to 7.

4.4 MORPHOLOGICAL CHARACTERIZATION OF PEPTIDE SOLUTIONS

4.4.1 Peptide FK6

4.4.1.1 Fourier Transform Infra-red Spectroscopy

For the peptide solutions, ranging between 10-40 mg/ml and at pH 7, Fourier Transformed Infra Red (FTIR) Spectroscopy analysis was performed in order to

determine the characteristic secondary structure formed by the peptides in the solution. The results for FTIR analysis, as shown in fig 13 are displayed as a function of percentage transmittance (%) versus wave number (cm^{-1}). The spectra revealed that all the samples showed distinctive peaks lying in the range of 1638 to 1640 cm^{-1}, which fall in the amide I region. Peaks in this specific range are characteristic for structures comprising of β-sheets as reported by literature (Adochitei and Drochioiu, 2011).

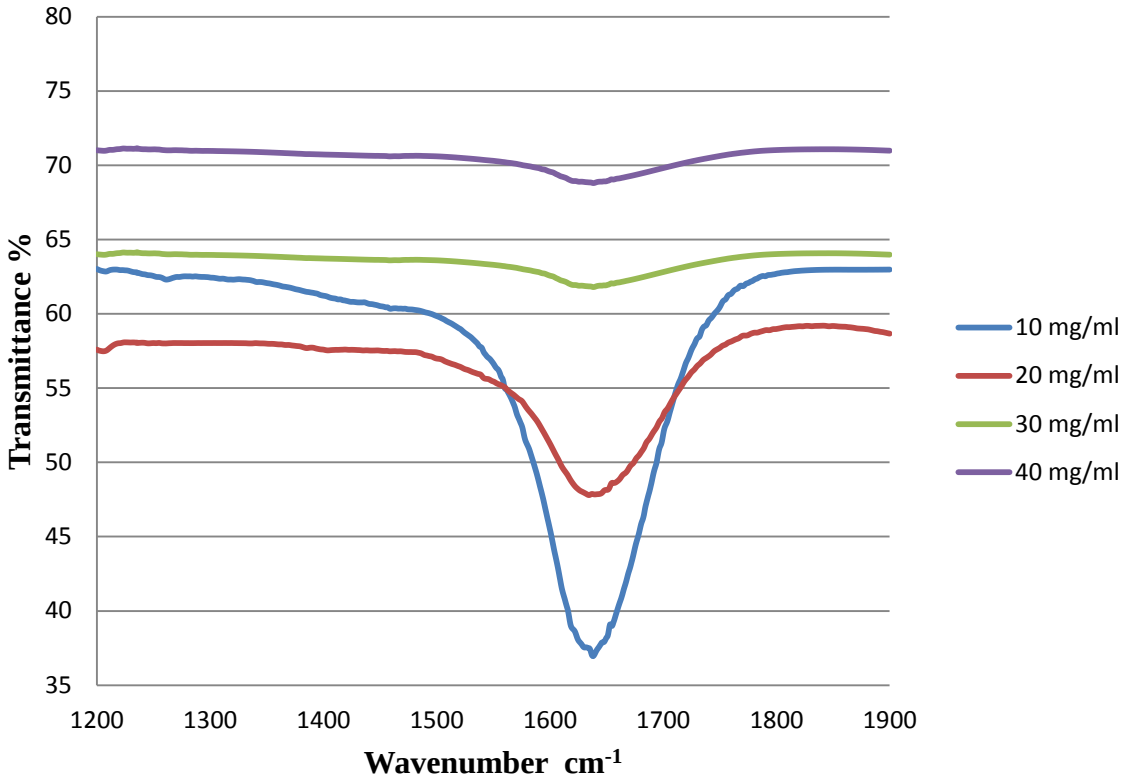


Fig 13: FTIR spectra of peptide (FK6) solutions; 10, 20, 30 & 40 mg/ml, at pH 7. Characteristic absorption peaks in the range of 1638 to 1640 cm^{-1} were observed at each concentration, which fall in Amide I region and are characteristic of β-sheet structures

4.4.1.2 Scanning Electron Microscopy

Scanning electron microscopy was done to analyze the structural morphology of peptides inside solution. An interconnected network of nanofibres was found in the images of peptide solution with NaOH. The network appeared like a mesh of fibres having small pores as can be seen in fig 14 (b and c). SEM images of peptide solution without NaOH lacked the interconnectivity between fibres and the fibre network was also less dense as in fig 14 (a). Fig d and e present a close view of the nanofibres seen in peptide solution.

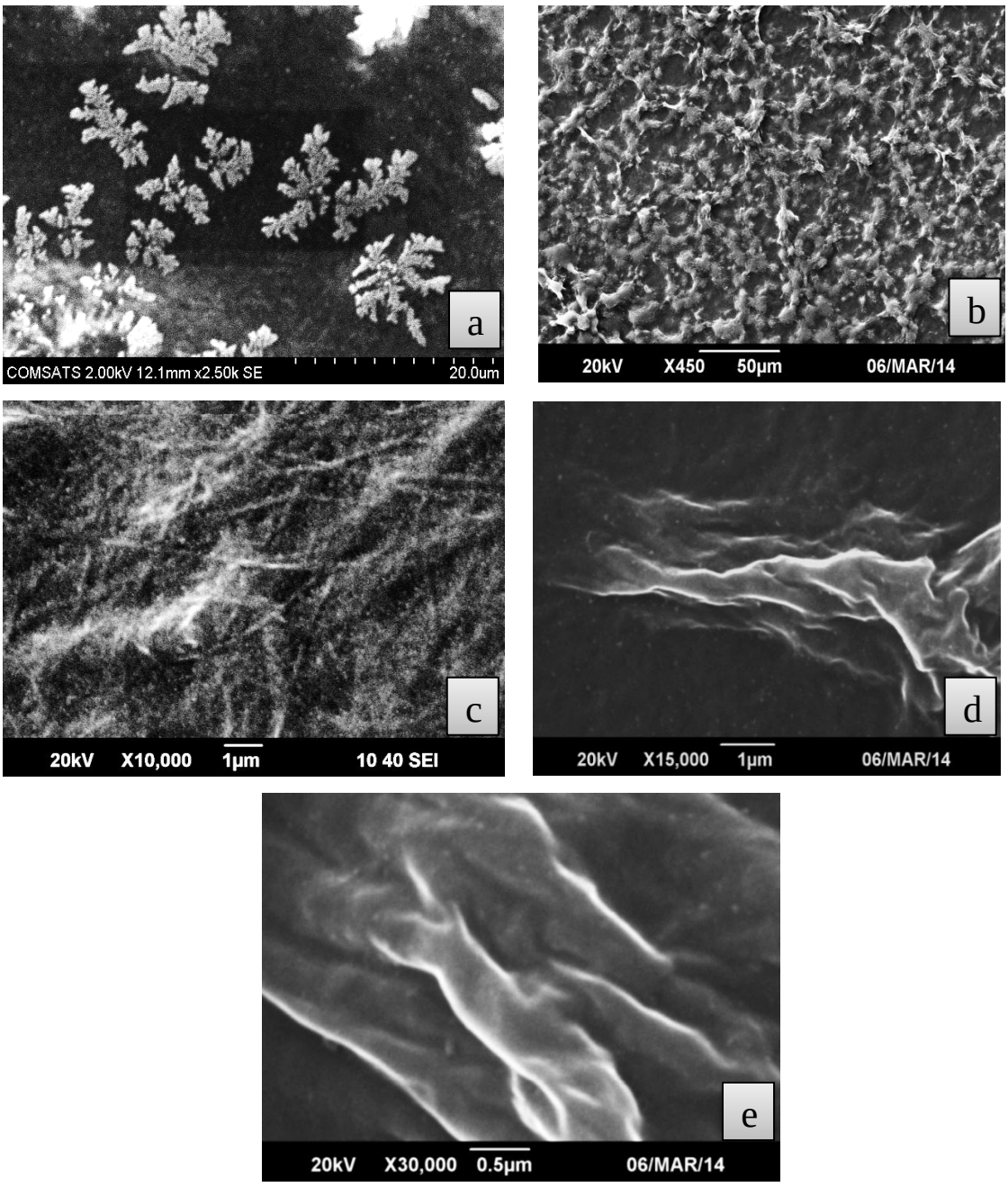


Fig 14: SEM micrographs of peptide (FK6) gel with a network of fibres present at peptide concentration 3 mg/ml (a) Without NaOH at pH 1 (magnification: X 2500) scale bar represents 20 µm; (b, c, d & e) With NaOH (pH 7) at magnification: X 450, X 10,000, X 15,000 and X 30,000 respectively scale bar represents 50 µm, 1 µm, 1 µm and 0.5 µm respectively

4.4.2 Hexapeptide VK6

4.4.2.1 Fourier Transform Infra Red Spectroscopy

FTIR spectra obtained for the peptide solution is presented in the graph below. It showed a sharp peak at 1638 cm^{-1}, which is a characteristic of presence of parallel beta sheets. These results indicate that hexapeptide VK6 self assembles into parallel beta sheet fibres although the gel formation is not observed.

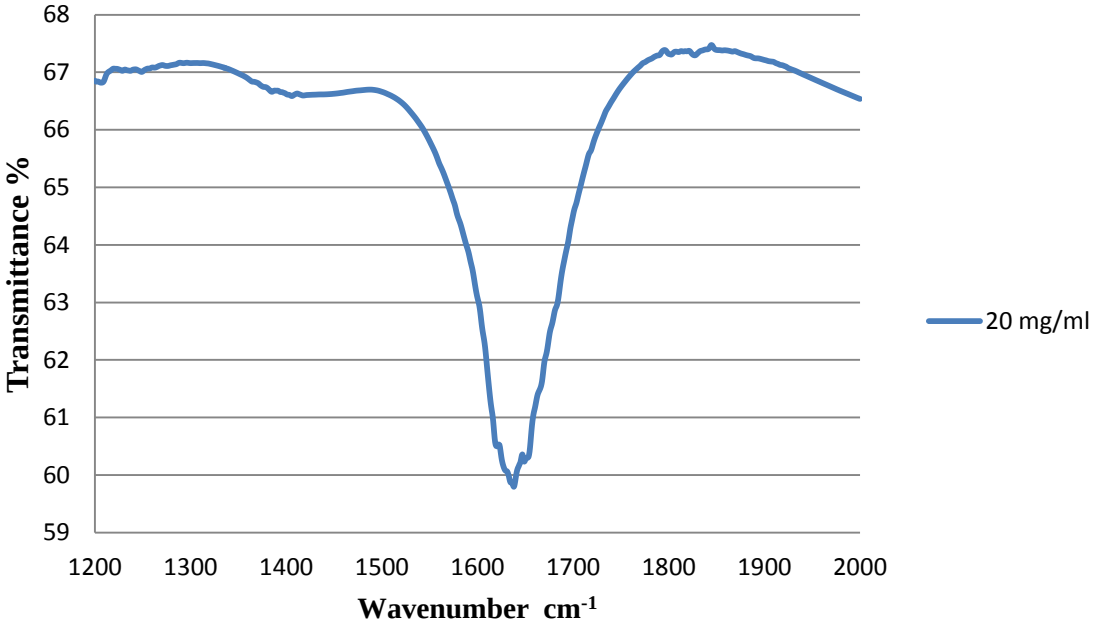


Fig 15: FTIR spectra of peptide (VK6) solutions; 20 mg/ml, at pH 7. Distinctive absorption peak in Amide I region, at 1638 cm^{-1} which represents a β-sheet secondary structure

4.4.2.2 Scanning Electron Microscopy

Nanofibres were also observed in SEM images of the peptide (VK6) solution indicating that self assembly also takes place in peptide VK6 solution as shown in fig 16.

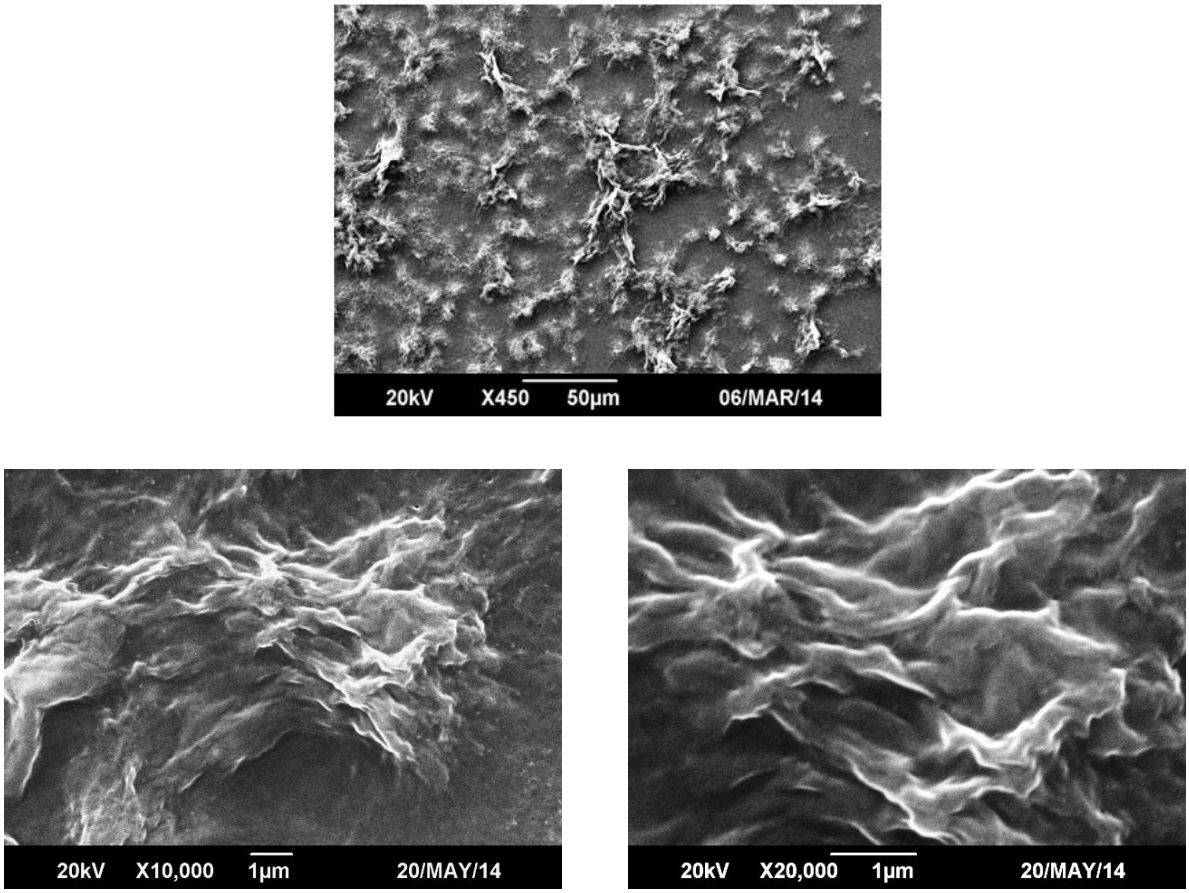


Fig 16: SEM micrographs of peptide (VK6) with nanofibres present at peptide concentration 3 mg/ml at pH 7; with magnification of X 450, X 10,000 and X 20,000 at a, b and c respectively. Scale bar in a represents 50 µm and in b and c represents 1 µm

4.4.3 Blend of FK6 and VK6

4.4.3.1 FTIR

Secondary structure formation was observed in the blend of two peptides. No change in the secondary structures formed by the blend was observed from those formed in the solution of individual peptide solutions. The FTIR spectra obtained for

the of peptide blend (FK6 and VK6) solutions, in ratio 30:70, 50:50 and 70:30 of VK6 and FK6 had strong absorption bands between 1632 to 1638 cm^{-1} which is a characteristic of parallel beta sheet structures. Bands in the same range that fall in amide-1 region were observed for the individual peptide solutions as well.

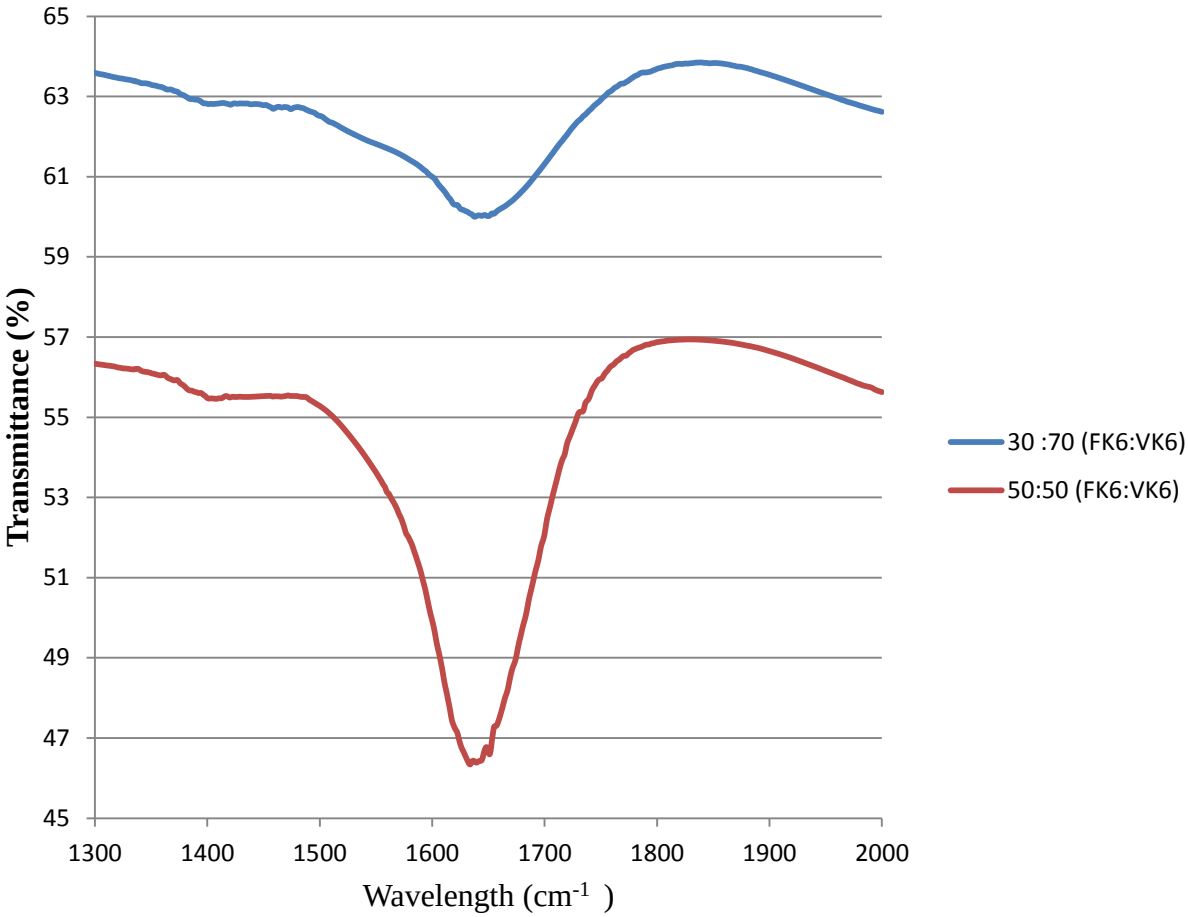


Fig 17: FTIR spectra of peptide blend (FK6 and VK6) solutions; 30 mg/ml, at ratio 30:70 and 50:50, at pH 7. Distinctive absorption peak in Amide I region, at 1638 cm^{-1} and 1639 cm^{-1} respectively, which represent a β-sheet secondary structure

4.5 HEMOLYSIS ASSAY

The hemocompatibility of the peptide gels was also evaluated by a hemolysis assay. Percentage hemolysis of peptide FK6 gel was almost negligible at all the concentrations indicating that the peptide is hemocompatible. In comparison to FK6, the percentage hemolysis of peptide VK6 gel was significant and around 14 % at a concentration of 20 mg/ml.

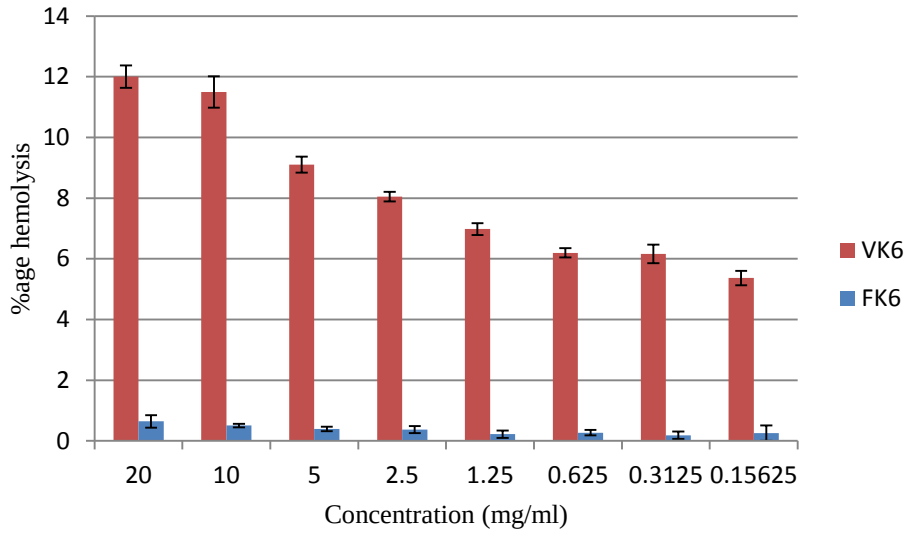


Fig 18: Percentage hemolysis of Hexapeptide FK6 (less than 2% at all the concentrations) and Hexapeptide VK6 (12% hemolysis at 20mg/ml)

Chapter 5

5. DISCUSSION

5.1 GELATION BEHAVIOR OF PEPTIDE GEL AND NANOGEL

Peptides are short chains of amino acids and are segments of a protein. Considering the bottom up approach, small peptide units can be designed which can self-associate in the solution into well-defined structural arrangements i.e. nanofibrils, nanotapes, nanotubes and hydrogels due to the noncovalent interactions (Kopecek and Yang, 2009). A large number of self assembling peptide systems have been designed and developed till date. One of the most widely studied systems is the 'ionic complementary peptides' first introduced by Zhang (Zhang *et al.*, 1993). These peptides has affinity to form gels, fiber networks or membranes, governed by their intrinsic and physicochemical properties such as sequence, concentration, pH and the presence of salts e.g. EAK16, RADA16, KFE8 and KLD12 are the peptides that belong to ionic complementary peptides (Zhang *et al.*, 1993, 2005; Kisiday *et al.*, 2002; Caplan *et al.*, 2002; Jun *et al.*, 2004).

Based on this understanding two hexapeptides FK6 (FEFGFK) and VK6 (VEVGVK) were designed which contain six amino acids each (F: phenylalanine; G: Glycine; E: glutamic acid; K: lysine and V: valine). The gelation behavior and the effect of change in pH, concentration and temperature on both the peptides separately and in the form of a blend were checked. Furthermore, the structural properties of the self assembled peptides were studied by Scanning Electron Microscopy, Transmission Electron Microscopy and Fourier Transform Infrared Spectroscopy.

The hexapeptide FK6 when completely dissolved in water at 90 ºC for two hours showed no change in consistency at room temperature. The pH of the peptide solution was highly acidic i.e. 1 mainly due to the presence of TFA (trifluoroacetic acid), a reagent used in the synthesis of the peptide. When the peptide solutions were kept for 6-7 hours the gel formation was observed in solutions having concentration 30 mg/ml and above. The gels formed at pH 1 are very weak and tend to collapse or flow when gently tapped.

The pH of the peptide solutions when altered by the drop wise addition of 0.5 M NaOH instantly caused the gelation of the peptide solution. The peptide solution at concentration of 10 mg/ml turned viscous when the pH was raised. However peptide solution at 15 mg/ml and above instantly formed stable gels upon changing the pH from 1 to 3 up till pH 10. The opacity and rigidity of gels at 30 and 40 mg/ml concentration increased with the increase in pH and formed stable self supporting gels within few seconds at range of pH 3-7. It can be concluded that the critical gelation concentration CGC of the peptide is greater i.e. 30 mg/ml at pH1 whereas lowers to 15 mg/ml at pH 3 and above. Saiani et al reported exhibition of similar behavior by their ionic complementary octapeptide (Boothroyd *et al.*, 2013). As peptides consist of different types of amino acids, the change in pH of the solution can have effect on the ionic state of the amino acids (Chen, 2005). The ability to form stable gel at a wide range of pH (pH 3 and above) specifically at physiological pH 7 implicated its role in cell culture (Yan *et al.*, 2008; Luo *et al.*, 2013).

The self-assembly in the peptide solution is responsible for the formation of gel, it is a natural process which takes place ubiquitously on its own (Zhang, 2003; Fichman and Gazit, 2014). Peptide molecules when in close vicinity acquire ordered arrangement due to the physical entanglements and non covalent interactions including hydrogen bonding, dipole dipole interactions, hydrophobic interactions and other vander waal forces (Maude, Ingham and Aggeli, 2013; Modepalli *et al.*, 2014). One of the reasons of this behavior is the arrangement of charge on the peptide

molecule. These peptide molecules, when present in a solution undergo ordered self assembly to form a network of fibres (Xiong *et al.,* 1995; Kabiri *et al.,* 2013). The periodic repeats of ionic hydrophilic and hydrophobic amino acids cause the peptides to fold into beta sheet conformation having characteristic hydrophilic and hydrophobic surfaces (Zhang *et al.,* 1993). In aqueous conditions hydrophobic interactions are formed by overlapping hydrophobic amino acids (phenylalanines). The ionic hydrophilic amino acids on the other hand form intermolecular ionic interactions. The hydrophilic and hydrophobic interactions consequently lead to the formation of beta sheet structures, which stack to form nanofibers. These nanofibers aggregate enclosing a lot of water resulting in the formation of hydrogel (Loo, Zhang and Hauser, 2012).

Each peptide molecule (FEFGFK) predominantly has a negatively charged hydrophilic amino acid i-e; E (glutamic acid) at one end and a positively charged hydrophilic amino acid i-e; K (Lysine) at the other end. However, the overall net charge on the peptide molecule is zero. The self assembling peptide which was discovered from Z-DNA binding protein also contained peptide amphiphiles joined by electrostatic interactions (Zhang *et al.*, 1992; Caplan and Lauffenburger, 2002). Niece *et al.* has reported that nanofibres can be formed by via electrostatic attraction between two oppositely charged peptide amphiphiles. So electrostatic attractions play a vital role in the self assembly of peptide in solution (Niece *et al.*, 2003).

Phenyl alanine (F) and Glycine (G) are the nonpolar hydrophobic residues present in the hexapeptide chain. Phenyl alanine being hydrophobic in nature greatly influences the self assembly of peptides. With the increase in hydrophobicity of the peptide molecules, lesser time is required for gelation to occur and the resulting gel will have enhanced mechanical properties. (Chow *et al.,* 2008; Kabiri *et al.,* 2013). Although Phenyl alanine and Glycine are nonpolar in nature, but at increase in the pH can bring change in their ionic state. May be that change triggers the system towards hydrophobic collapse and ultimately to the gel formation.

The peptide VK6 (VEVGVK) having similar sequence of charges and with same charged amino acids was designed in which phenyl alanine in the sequence was replaced with valine. Valine is less hydrophobic as compared to phenyl alanine. phenyl alanine contains an aromatic ring in its structure while valine lacks it. The aromatic ring present in phenyl alanine increases its tendency to participate in pi-pi interactions. The peptide VK6 is found to self assemble into beta sheet structures and also observed to form fibres but no gelation is observed in its case up to a concentration of 40 mg/ml. So the nanofibres are insufficient to form a hydrogel in this case. Similar behavior was observed by Saiani *et al.,* who studied the self assembly of four ionic complementary octapeptides (AEAEAKAK, AEAKAEAK, FEFEFKFK and FEFKFEFK) Gelation was observed in case of peptide containing phenyl alanine and no gelation was observed in case of alanine containing peptides in a concentration range of 0-100mg/ml, when the amino acid phenyl alanine was replaced by alanine in the same peptide sequence (Saiani et al, 2009). The decrease in hydrophobicity in the system can be the reason of no gelation. The mechanical properties of a macrostructure have been shown to increase with increase with the hydrophobicity of a peptide (Chen, 2005). The critical concentration for secondary structure formation was observed to decrease when more hydrophobic residues were substituted in place of Alanine i.e. IKIE16, VKVE16 and FKFE16 (Ulijn and Smith, 2008).

Further the gelation behavior of the blend of two peptides i.e. VK6 and FK6 was also observed in which peptide solutions containing FK6 and VK6 in a ratio of 30:70, 50:50 and 70:30 were formed and overall concentration of peptide was kept 30mg/ml. Peptide solutions were found to form hydrogel at all these concentrations. Formation of gel having peptide FK6 and VK6 in a ratio of 30:70 was very significant as it shows that the blend of FK6 and VK6 is forming a gel at a critical gelation concentration less than that of FK6 alone. So VK6 has some role in the gelation although it alone cannot form a gel. In a study Boothroyd *et al.* studied the self assembly of mixed ionic complementary peptides FEFEFKFK and

HHHHHHFEFEFKFK, in a ratio of 9:1 in comparison to non functionalized peptide FEFEFKFK. They found that critical gelation concentration in case of mixed peptide increased due the formation of thick nanofibes in case of mixed ionic complementary peptides (Boothroyd *et al.,* 2008).

In another study Miller *et al.* studied the gelation of a set of six octapeptides FEFEFKFK, VEVEVKVK, LELELKLK, FEFKFEFK, VEVKVEVK and FEFEFRFR. All the peptides except FEFEFRFR are reported to self-assemble into anti-parallel b-sheet conformations in solution. These beta sheet structures further associate to form nanofibres of diameter 8 nm, which when above CGC entangle to form a network of fibres and ultimately a gel. By replacing arginine in place of lysine in FEFEFKFK results in a change of fibre diameter from 8 nm to 20 nm). Peptide FEFEFKFK is observed to form a uniform 3D network of branching fibres. When arginine which is bulkier amino acid is introduced into the peptide, lateral recognition of the fibre is increased and it becomes capable of creating multiple ionic bridges. This leads to the creation of an entangled network instead of a branched network (Miller and Saiani, 2010).

To study the correlation between peptide concentration and sol-gel transition temperature, phase diagram studies were conducted. It was observed that due to change in temperature the system undergoes a transition from gel to liquid. For a specific concentration the temperature at the transition point can be termed critical gelation temperature (T_{gel}). It is observed that the peptide concentration significantly influences the T_{gel} value (fig 4a). Peptide gel formed at 40 mg/ml had higher T_{gel} i.e. 85 ºC than at 20 mg/ml concentration i.e. 65 ºC (Nanda *et al.,* 2012). For various biomedical applications specifically in vivo it is pertinent for gel to form at 37 ºC. The minimum concentration required forming stable gel at 37 ºC was determined to be 15 mg/ml and even with increase in temperature no gel was formed below critical gelation concentration (CGC) of 15 mg/ml. After being exposed to their respective melting temperature, gels were recovered upon cooling implying to be

thermoreversible in nature. As shown in Figure, the T_{gel} increased with increasing peptide concentrations.

5.2 STRUCTURAL CHARACTERIZATION OF THE PEPTIDE GEL

FTIR spectroscopy was carried out to analyze the secondary structures in peptide hydrogels. FTIR spectra obtained for all the peptide gels at concentration 10-40 mg/ml produced amide 1 band under IR at a frequency of 1638 cm^{-1}, reflecting amide carbonyl (>C=O) stretching, which is a characteristic of the presence of parallel beta sheets (fig 5) (Bartha and Zscherp, 2002; Pelton and McLean, 2000; Zandomeneghi *et al*, 2004). These results indicate that hexapeptide forms parallel beta sheet rich secondary structures. Each amino acid constituent has its own tendency to form particular secondary structures. The phenyl alanine based peptides have already been shown in the literature to form beta sheet structures (Saiani *et al.,* 2009; Zhang *et al.*, 1993). Peptide solution at 10 mg/ml does not form a gel but the existence of beta sheet conformations indicate that the self assembly occurs even at this concentration. Also the periodicity of hydrophilic and hydrophobic amino acid residues and the alternating hydrophilic and hydrophobic side groups can affect the secondary structure formed by a peptide (Chen, 2005).

Preliminary testing revealed that NaOH has significant role to play in gelation. To further elucidate the effect of NaOH on gel morphology, SEM was performed on samples with and without NaOH. In the absence of NaOH, a few nanofibres could be distinguished but they lacked interconnectivity. Whereas a network of highly dense, interconnected nanofibres was visible in sample with NaOH. Addition of NaOH resulted in increased pH which brought hydrophobic collapse and the nanofibres formed as a result of beta sheet assembly were driven to assemble laterally, subsequently giving rise to hydrogel (fig 6). So change in pH strongly affects the self

assembly by changing the ionic state of the amino acids. EAK 16-IV self assembles into globular nanostructures at neutral pH, due to bending of the peptide caused by strong electrostatic attractions. At low pH electrostatic attractions become weak as most of the negatively charged amino acids (glutamic acid) are neutralized, therefore no globular structures are formed instead fibrillar assemblies are observed. Similarly at high pH the electrostatic interactions are reduced due to neutralization of lysine residues again resulting in fibrillar assemblies (Hong *et al.,* 2003).

Chapter 6

6. CONCLUSIONS AND FUTURE PERSPECTIVES

By understanding the basic properties of amino acid motifs, peptides can be designed that self assemble into nanomaterials having versatile properties that can be exploited for various biomedical applications. We have investigated that the novel and cogently designed hexapeptide sequence FEFGFK (FK6) upon dissolution forms a hydrogel at a concentration of 15 mg/ml and above, that contains an interconnected network of nanofibres formed by self assembly as seen in SEM images. FTIR spectroscopy revealed that the nanofibres adopt a beta sheet confirmation. The hydrogel is stable at physiological pH (7) and temperature (37 °C). Two factors peptide concentration and pH are observed to trigger the formation of gel. At 30 mg/ml hydrogel forms at pH 1 and is stable at up to pH 10 whereas at 15 mg/ml the peptide solution remains clear and the gel formation is observed as the pH is increased to pH 3.

Another hexapeptide VEVGVK (VK6) less hydrophobic then FEFGFK (FK6) was simultaneously investigated to observe the effect of hydrophobicity on hydrogel formation. VK6 in solution also self assembles into beta sheet conformation. SEM images showed the formation of nanofibres in the solution but no gel formation at a concentration range of 0-40 mg/ml was observed for this peptide. Change in pH also had no effect on the peptide solution.

The red blood cell hemolytic assay was also conducted which showed that the peptide FK6 is impeccably hemocompatible but peptide VK6 showed a little hemolysis.

Further mechanical characterization by oscillatory rheology and characterization of fibre morphology and fibre diameter by TEM is required. Finally the cytocompatibility of hydrogel scaffold is required to be tested through cell culture.

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