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## Short Peptide Based Self-Assembled Nanostructures: Implication In Drug Delivery And Tissue Engineering

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**Abstract:** Self-assembly of biomolecules facilitates the creation of a diverse range of hierarchical nanostructures from a wide range of polymeric and non-polymeric materials. Peptides and specifically short peptides are very attractive in this respect due to their unmatched biocompatibility, ease of synthesis, functionability as well as tunable bioactivity along with the availability of rich chemistry for fine-tuning the structure and function of peptides according to environmental conditions. Self-assembled peptide based nanostructures such as tubes, filaments, fibrils, hydrogels, vesicles, monolayers have been studied by many research groups and found application as three-dimensional cell growing scaffolds, dental implants, neural tissue engineering scaffolds and as carriers for drugs, proteins and genes, nucleotides. Nanostructures are also being developed from designed or modified amino acids to have enhanced cellular as well as *in vivo* stability. These modified nanostructures showed enhanced drug delivery properties both under *in vivo* and *in vitro* conditions.

**Key Words:** Nanostructures, peptides, drug delivery, tissue engineering, self-assembly.

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**Introduction:** Self-assembly is defined as the autonomous organization of components into ordered patterns or structures.<sup>1</sup> Living cells consist of many self-assembling systems that work in synchronization to achieve a defined goal. Self-assembly, as a process contributes significantly in biology such as maintaining the cell integrity, performing important cellular functions as well as inducing abnormalities that cause disease.<sup>1</sup> Thus, understanding life necessitates a better understanding of self-assembly. Concepts of self-assembly have also been used in many disciplines for constructing useful materials. Molecular self-assembly is in fact a very practical way of making ensembles of nanostructures. The ubiquitous existence of self-assembly processes in living systems along with the prevalence of various non-covalent interactions (van der Waals, electrostatic, and hydrophobic metal—ligand,  $\pi$ - $\pi$  stacking interactions, hydrogen and coordination bonds) in biology, have resulted in rapid development of self-assembling biomaterials as a promising research area.<sup>2-4</sup> Self-assembly provides the flexibility of developing novel materials with tailored morphologies and desired functions through single-molecule design and engineering. This results in controlling the bulk properties of the resultant material by modulating individual monomeric building blocks. Thus, by modulating structural changes in constituent molecules it becomes possible to dictate the behavior of the end product.

In recent past, many self-assembling nanomaterials are being generated from various organic polymers, carbohydrates, nucleic acids, proteins etc. either to gain a better understanding of the phenomenon or to use them for applications ranging from molecular devices to delivery systems or scaffolds.<sup>1,2</sup>

For example, different polysaccharides due to their many merits have been investigated as templates for the synthesis of nanomaterials. Polysaccharides have natural origin, they are biodegradable, non-toxic and safe. They have abundant natural resources and have low cost of purification. Polysaccharides such as alginate, pectin, dextran, chitosan, hyaluronic acid etc. have been studied for constructing nanostructures. Polysaccharide nanostructures are prepared by methods such as covalent crosslinking, ionic crosslinking, polyelectrolyte complex formation, and by the self-assembly of polysaccharides modified with hydrophobic groups. However, the cross-linking agent such as glutaraldehyde could be a limiting factor in the potential biomedical application of these nanostructures prepared by cross-linking method. Polysaccharides like chitosan, dextran either in native or modified form or in association with other polymers form nanoparticles that can encapsulate various drugs, genes, proteins, peptides.<sup>5</sup> Chitosan has been investigated for making variety of nanocarriers,<sup>6,7</sup> and chitosan nanocarriers have been shown to encapsulate drugs like retinol,<sup>8</sup> utilized for DNA delivery.<sup>9</sup> Carboxymethyl chitosan nanoparticles have been investigated for doxorubicin

delivery. Also, Calcium-alginate nanoparticles have been shown to encapsulate anti-TB drugs like isoniazid, pyrazinamide and rifampicin with high efficiency and loading resulting in increased bioavailability of these drugs.<sup>10</sup>

Furthermore, biomolecules like DNA, RNA and proteins are endowed with many desirable properties which make them suitable templates for constructing self-assembled nanostructures. DNA nanostructures with either two or three dimensions and with varied shapes like, icosahedral, tubular, tetrahedral, Y-shaped geometries are being constructed and have been shown to deliver cargos like, drugs (anticancer, various antibiotics), oligo-nucleotides, dyes, inorganic nanoparticles, proteins etc. to different cells or to display groups that induce immunostimulation.<sup>11</sup> The superiority of DNA nanoarchitectures as compared to other self-assembling systems lies in the flexibility of finetuning the geometry and size of DNA nanoarchitectures very accurately because of the well-known self-recognition properties of DNA and the knowledge of the exact structure of the double helix on the atomic level. This allows the ability of controlling spatial distribution of cargoes and ligands on DNA nanostructures.

Multifunctional DNA nanostructures simultaneously decorated with targeting agents, drug payloads as well as imaging agents are also being investigated for theranostic applications.<sup>12</sup> Hybrid nanoparticles like DNA-gold nanoparticles have additional features like magnetic properties, plasmonic effects or the ability of fluorescence quenching that could find application in imaging, detection and as transfection agents and gene regulation materials.<sup>13-15</sup> DNA-block copolymer micelles (DBC) have also been shown to have applications in biomaterial purification,<sup>16,17</sup> templated synthesis<sup>18</sup> and in nanoelectronics.<sup>19</sup> For *in vitro* and *in vivo* experiments usually larger quantities of biomaterials are needed. However due to cost issues, it is important that while designing DNA nanostructures the number of strands per DNA nanostructure should be kept at the minimum and this is one of the major limitations of DNA nanostructures.

Like DNA, RNA molecules by virtue of their inter and intra molecular interactions can self-assemble to form nanostructures by both template or non-template mediated assembly. RNA self-assembly will have significant role in nanofabrication by virtue of their ability to form 3D nanostructures, to produce reversible self-assembly and self-repair. RNA nanoparticles have shown application in pathogen detection, drug/gene delivery, and various other therapeutic applications.<sup>20</sup> For instance, motor pRNA of the bacteriophage phi29 formed nanostructures of various shapes like twins, tetramers, rods, triangles, and 3D arrays of micron size via interaction of programmed helical regions and loops. These structures were resistant to a wide range of temperatures, salt concentrations, and pH and have shown potential to deliver oligonucleotides like ribozyme or siRNA to kill cancer cells both *in vitro* and *in vivo* or Hepatitis B virus.<sup>21,22</sup>

Like DNA, RNA and carbohydrates, proteins due to the existence of varied structures, shapes and chemical properties and availability of a large data base can act as nice templates for molecular self-assembly and many protein based nanostructures are being investigated.<sup>23-25</sup>

Though all these systems have their own demerits and limitations, their relative instability within living systems and other deleterious effects can hinder the potential applications of these systems for safe human use.

The idea of generating peptide based biomaterials essentially came from the observation that many soluble cellular proteins could self-assemble to form well-ordered tubular deposits in neurodegenerative diseases such as Alzheimer's, Parkinson's and prions.<sup>26</sup> Though, disastrous to normal physiology,<sup>26</sup> these protein assemblies formed the basis of developing novel nanobiomaterials.<sup>27</sup> These observations have led to many efforts focusing on studying self-assembling peptides and several peptide based nanostructures have been described/developed.<sup>28-30</sup>

There are many advantages associated with using peptides as building blocks to make ensembles of nanostructures. These include the availability of detailed structural and functional information for many peptides and proteins, easy and rapid chemical synthesis, tunability to various environmental conditions (like pH, temperature, ionic strength), adoption of well defined helical or  $\beta$ -hairpin/sheet secondary structures that can energetically favor self-association and assembly.<sup>31</sup> Peptides however generally are labile to enzymatic degradation, but if being developed as delivery agents, there are different strategies to circumvent this problem. For example, by introducing non-coded residues in the peptide design, the peptide based nanostructures can be made more resistant to enzymatic degradation.<sup>32, 33</sup>

Nanostructures generated by peptide self-assembly: Peptides, based on their design, can assemble into different kind of supramolecular architectures (Fig.1) such as ribbons,<sup>34</sup> nanotubes<sup>35,36</sup>, monolayers with nanoscale order and vesicular structure.<sup>28-30</sup> Nanostructures have been developed from different types of peptides including cyclic peptides, amphiphilic peptides, peptides containing  $\beta$ -sheeted motifs, helical-structures or  $\beta$ -turns.

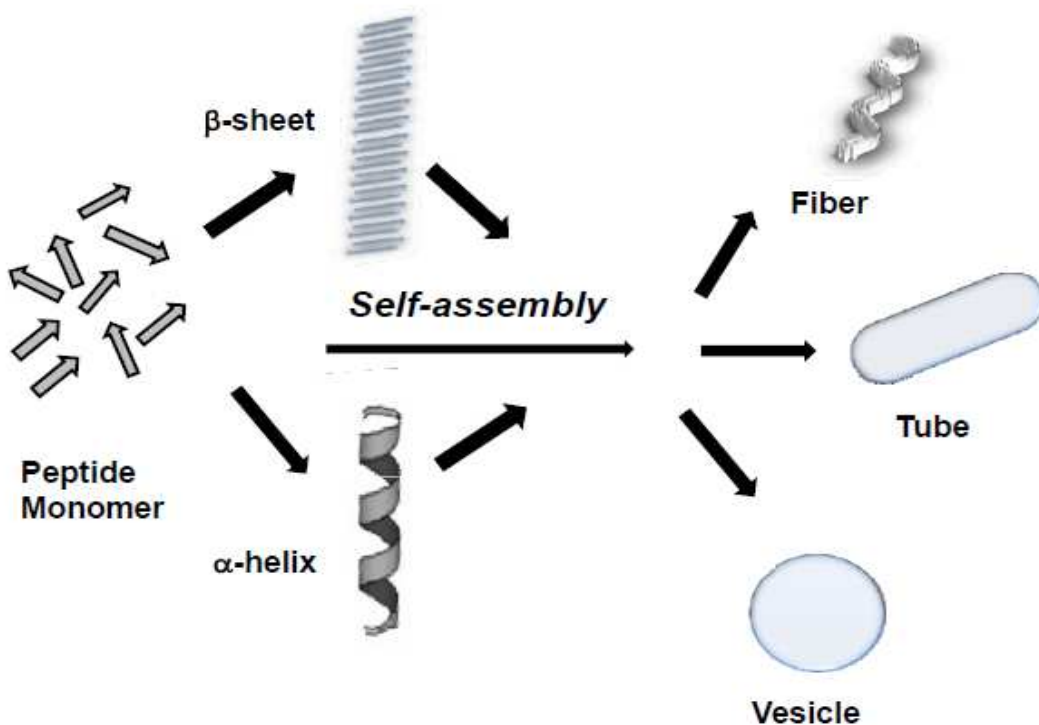


Fig. 1: Self-assembly of peptides into different types of nanostructures.

**Cyclic peptide based nanostructures:** Ghadiri *et al.* for the first time developed peptide nanotubes based on cyclic polypeptides with an even number of alternating D- and L- amino acids. These peptides assembled by virtue of inter-molecular hydrogen bonding and formed self-assembled nanotubes with internal diameter ranging from 7-8 Å (Fig. 2).<sup>37, 38</sup> Because of the alternating D and L amino acid sequence, the peptide side chains in these assemblies lie on the outward direction creating a hollow tubular structure. The diameters of these nanotubes could be varied depending on the cyclic peptide ring size. Lanreotide growth hormone inhibitor, a natural cyclic peptide has also been shown to self-assemble to form ordered nanotubes by virtue of aromatic and hydrophobic interactions.<sup>39</sup> These nanotubes showed potential for a wide range of applications such as antimicrobial agents, biosensors, catalyst etc.<sup>40, 41</sup>

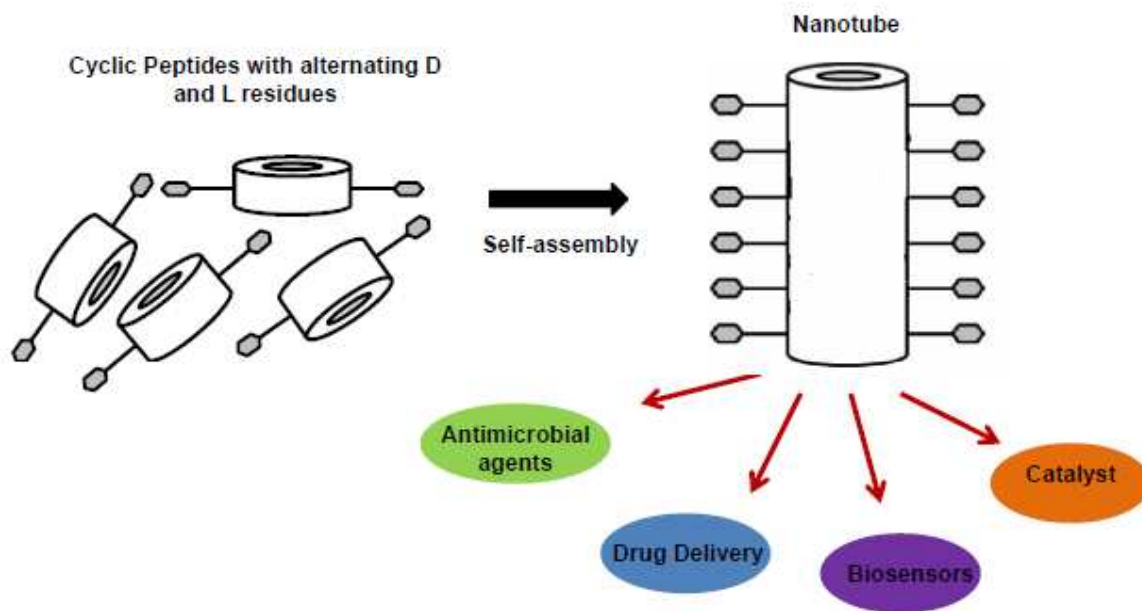


Fig. 2: Self-assembly of cyclic peptides with alternating D and L residues and their potential applications.

**Nanostructures generated from beta-sheeted or alpha-helical peptides:** A large number of peptides have been shown to self-assemble by forming beta-sheeted secondary structures.<sup>42</sup> Beta-sheeted peptides have been used to synthesize a variety of supramolecular architectures such as ribbons,<sup>43, 44</sup> nanotubes,<sup>36, 45, 46</sup> monolayers with nanoscale order,<sup>47</sup> macrocycles etc.<sup>48, 49</sup> An example of a pH responsive self-assembling beta sheeted peptide is P11-4 (QQRFEWFEQQ). This peptide is pH sensitive due to the ionizable glutamate and arginine side chains. At concentrations below <math>10\text{ mg/ml}</math> and at neutral pH, the peptide is soluble but adopts a hydrogel state at low pH (pI: 4.2). This occurs due the formation of anti-parallel  $\beta$ -sheet tapes, which then stack together to form fibrils. At its critical gelling concentration of  $10\text{ mg/ml}$ , the peptide also form hydrogel like structures in culture medium or in presence of  $140\text{ mM}$  salt.<sup>43</sup> Similarly, MAX1, a 20 residue long peptide, with two  $\beta$ -strands with alternating valine and lysine residues connected through a type II'  $\beta$ -turn, when kept under basic conditions, first folds into a  $\beta$ -hairpin like structure which then self-assembles into a hydrogel network.<sup>50, 51</sup>

Powers *et al*, also investigated the self-assembly of designed  $\beta$ -hairpin peptides and found that these peptides can self-assemble at air/water interface to form various nanostructures.<sup>52</sup> Other designed peptides like MAX-8, the modified version of MAX-1 with the lysine at 15<sup>th</sup> position replaced by glutamic acid and MAX-3 with 7<sup>th</sup>, 12<sup>th</sup> and 16<sup>th</sup> valine residue of MAX-1 replaced by threonine



residues, have been shown to undergo triggered folding in response to pH and/or temperature changes, to adopt a  $\beta$ -hairpin conformation that undergo self-assembly to form various nanostructures.<sup>49, 50, 53</sup> Large polypeptides with alternating segments of polar and non-polar residues like the 16 residue peptides RADA-16 I and II have been shown to form  $\beta$ -sheeted structures that self-assembled into pH responsive hydrogels.<sup>54</sup> RADA16 (RADARADARADARADA) is also marketed under the name of PuraMatrix™ (3DM, Inc., Cambridge, MA, USA). This 16 amino acid peptide readily self-assembles into a nanofiber network. Gel formation can also be triggered by an increase in ionic strength or by a change in pH. In another approach, linear peptides were assembled into nanotubes by utilization of highly directional metal-ligand interactions.<sup>55</sup> The ability of these peptides to form polymeric  $\beta$ -sheets can be modulated by pH and salts in a manner that is dictated by the number and positioning of charged amino acid residues.

Though  $\beta$ -sheeted peptide motifs are being widely scrutinized as templates for forming self-assembled nanoscale materials,<sup>50,56</sup> the design of  $\alpha$ -helical nanoscale materials has seen only limited advancement. As models of fibrillar structures based on  $\alpha$ -helical coiled coil motifs are prevalent in body such as in cytoskeleton and extracellular matrix<sup>57</sup>,  $\alpha$ -helical synthetic peptides have been attractive targets for de novo design of fibrillar nanostructures. For instance, filamentous nanostructures were generated from coiled-coil peptide motifs 25-50 residues long. These peptides were based on heptad repeats (abcdefg)<sub>n</sub>, with positions a and d being occupied by hydrophobic residues and polar residues anywhere else in the sequence. Hydrophobic residues formed inter-helical hydrophobic core, providing a stabilizing interface between the helices. Charged residues at positions “e” and “g” participate in electrostatic interactions contributing to coiled-coil stability. These peptides form 2-5 helices that wrap around each other in a superhelical fashion to form nanoscale fibers.<sup>58-60</sup> Woolfson *et al.* designed peptides (29-36 residue long) based on helical coiled-coils,<sup>61, 62</sup> that self-assembled into nanofibers. Their peptides were also based on a heptad sequence repeat, abcdefg, with hydrophobic residues isoleucine and leucine at the ‘a’ and ‘d’ sites, respectively. The hydrophobic residues promoted helix oligomerisation through hydrophobic collapse. To promote staggered assembly of peptides and fibril formation, lysines were incorporated at the ends of the peptides with central glutamates to allow ionic interactions resulting in fibrous assembly.<sup>63,64</sup> Hartgerink *et al.* have also used heptad repeats producing helical coiled-coils that form nanofibres in a concentration-dependent manner. They used pH and ionic strength as triggers for self-assembly with incorporation of isoleucine and leucine residues at positions ‘a’ and ‘d’ of the heptad, and glutamates at positions ‘e’ and ‘g’ providing an acidic region. Hence, at low pH ionic repulsion was eliminated and carboxylic acid side chains hydrogen bonded with each other to form assembly.<sup>64</sup>

Hydrogels are three dimensional (3 D) networks with capacity to imbibe and retain water. They have been shown to possess a wide range of biomedical applications.<sup>65, 66</sup> Hydrogels were also being developed from self-assembly of helical polypeptides based on ‘aba’ triblock motifs that are composed of a central random coil block flanked by two coiled-coil forming sequences. The self-assembly occurred as a balance between the oligomerization of the helical ends and swelling of the central water-soluble random coil segment.<sup>67-69</sup> Interestingly, it was observed that hydrogel formation and their physical properties could be fine-tuned by changing the structure and length of the coiled-coil building blocks. Consequently, stimuli responsiveness (to temperature and/or pH, salt) could be introduced in the self-assembled hydrogels by modulating amino acid sequence of the coiled-coil domains. Denaturation of coiled-coil domains by guanidine hydrochloride (GdnHCl) resulted in disassembly of the hydrogels and removal of GdnHCl by dialysis caused coiled-coil refolding and hydrogel reassembly.<sup>68</sup>

Various research groups have designed leucine zippers (a condition where the interacting surface between the helices contained leucine) for constructing nanostructures with different properties.<sup>70,71</sup> O’Shea *et al.* were the first to propose a peptide design in which one strand with acidic residues and the other with basic residues yielded a parallel heterodimer to form a model called ‘Peptide Velcro’.<sup>70</sup> In this design, a single asparagine residue in the sequence forms a hydrogen bond with corresponding asparagine residue in the other subunit contributing to the directional specificity of the helix orientation and oligomerization state. Thus, it appears that simple sequences can be designed to have a very high preference to pair with each other to form useful structures. These ‘Peptide Velcro’s can be utilized to bring two molecules together, which may be useful in sensing applications.

***Self-assembly of amphiphilic peptides:*** Zhang and co-workers demonstrated that 7-8 residue long surfactant-like peptides, characterized by well-defined hydrophilic and hydrophobic residues that could self-assemble in water to form well-ordered nanotubes and nanovesicles.<sup>72</sup> Hydrophilic head group of these peptides were composed of aspartic acids and their tails were composed of hydrophobic amino acids such as alanine, valine, or leucine. Like other surfactants, self-association by hydrophobic interactions was the major governing force for the self-assembly of these peptides.<sup>72</sup> Amphiphilic peptides generally consist of a hydrophilic peptide headgroup and a hydrophobic alkyl tail where the tail participates in aligning the head group to form various secondary, super-secondary and tertiary conformations.<sup>73-75</sup> These peptides self-assembled to form a variety of morphological structures with nanodimensions such as micelles, vesicles or tubules.<sup>36, 44, 54, 72</sup> Tsonchev *et al.* demonstrated that the self-assembly of amphiphilic peptides was driven by both hydrophobic as well as electrostatic interactions.<sup>76</sup>

Bolaamphiphiles are also a class of amphiphilic molecules consisting of two hydrophilic groups flanked by a hydrocarbon chain.<sup>77</sup> Their peptide segment undergo  $\beta$ -sheet hydrogen bonding to form various supramolecular nanostructures such as fibers, rods, tubes, ribbons, and spheres etc.<sup>78-81</sup> Bolaamphiphilic nanotubes have been shown to have several interesting applications such as use in viral assays,<sup>82,83</sup> protein sensing<sup>84</sup> etc.

All of the above described strategies of making assembled structures have utilized large polypeptide sequences with the use of either purely L-amino acids, D-amino acids or both. However, the associated expense and complexity of synthesis of large linear peptides, cyclic and dendritic structures have strongly limited the applicability of such peptides for practical purposes. Moreover, their proteolytic instability has also been a major concern for their application in biological conditions or in *in vivo* situations.

***Self-assembly of short peptides into different nanostructures:*** Lately, it was demonstrated that very small peptides could also self-assemble into various nanomorphologies, thereby minimising the difficulty and cost of their synthesis and simultaneously enhancing their stability (Fig. 3). These short peptide fragments were mostly discovered in a quest to determine the minimal required sequence for amyloid formation. An example for this is, NFGAIL (hIAPP22–27), a hexapeptide fragment of the islet amyloid polypeptide (IAPP) that forms well-ordered amyloid fibrils similar to that formed by the full length polypeptide.<sup>85</sup> FGAIL (hIAPP23–27) a pentapeptide fragment of IAPP polypeptide, also formed fibrillar structure.<sup>85</sup> Similarly, Westermark *et al.*, discovered NFLVH and NFGSVQ to be the minimal active amyloidogenic peptides fragment of the Aortic Medial Amyloid.<sup>86, 87</sup>

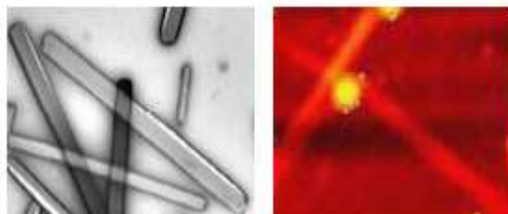
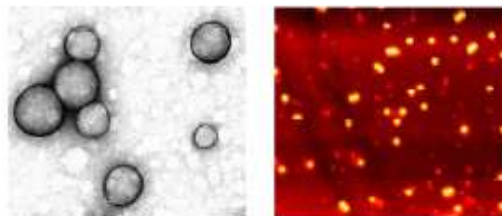
## Self-assembly of short peptides ( $\leq 5$ amino acid) into nanostructures

### Vesicle forming sequences

CFF, E $\Delta$ F, K $\Delta$ F

R $\Delta$ F, M $\Delta$ F

Phg-Phg, Acp-TE, di-para-nitro-F



### Tube forming sequences

FF, (D)-F-(D)-F, NH<sub>2</sub>-FF-NH<sub>2</sub>, Boc-FF

F $\Delta$ F, LL, LF, di-pentafluoro-F, NFGSV

FL, IL, WG,  $\beta$ AA,  $\delta$ AvaF, Ac-FF

### Fibril forming sequences

NFGAI, FGAIL

NFLVH

DFNK, GAIL, GFIL, KLVFF, DFNKF

NFGSV, Boc-AUV-Ome, Boc-AUI-OMe

F $\Delta$ F, Cbz-FF, IF, Fmoc-F, Fmoc-FF

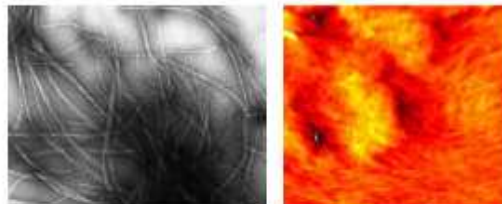


Fig. 3: Sequences of short peptides ( $\leq 5$  amino acids long) that can self-assemble into nanostructures.

KLVFF, a pentapeptide fragment of the amyloid  $\beta$ -peptide A $\beta$ -42, also self-assembled in phosphate buffered saline (PBS) to form a hydrogel.<sup>88</sup> It was hypothesized that the electrostatic charges of the peptide by salt ions favored  $\beta$ -sheet formation that self-associated to form a nanofibrillar gel network. Later, DFNKF, a pentapeptide fragment of human calcitonin, was also found to form well-ordered amyloid fibrils similar to those formed by the full length polypeptide.<sup>89</sup> In a quest to find out the minimal requisite peptide sequences of the protein TAR–DNA binding protein (TDP 43) that are involved in aggregation and plaque formation in amyotrophic lateral sclerosis and frontotemporal lobar degeneration, Akash *et al.* discovered 10-13 residue peptides (like EDLIKGISV, MNFGAFSINPAMM) that formed well ordered fibrillar structures similar to that formed by a full length protein.<sup>90</sup> All these studies revealed that almost all of the amyloid forming peptides have minimal active shorter peptide fragments which carries the capacity to form amyloid fibrils similar to those formed by large native polypeptides. These studies also suggested that aromatic residues in general play critical role in amyloid fibril formation.<sup>91</sup>

Hauser and co-workers created a unique class of natural tri- to heptapeptides made of simple, non-aromatic amino acids that self-assembled in water to form hydrogels.<sup>92</sup> The amphiphilic peptide motif consisted of a tail of aliphatic nonpolar amino acids (N terminus) with decreasing hydrophobicity and a hydrophilic head group of acidic, neutral, or basic nonaromatic polar amino acids (C terminus). This assembly involved a conformational transition of the structurally unorganized monomers into metastable  $\alpha$ -helical intermediates that terminated in cross- $\beta$  structures. The peptides had a characteristic sequence motif consisting of an aliphatic amino acid tail of decreasing hydrophobicity capped by a polar head, which endowed them amphiphilicity. Decrease in hydrophobicity from the N-terminus (acetylated to suppress charge effects) to C-terminus strongly improved ease of self-assembly, stability and strength of nanostructures. It was observed that the length of the hydrophobic tail and the polarity of the head group were integral elements that supported facile hydrogel formation. Hexamers typically formed gels more readily than pentamers, tetramers, and trimers. Stronger gels were derived from head groups with acidic residues (D and E), followed by neutral (S and T) and basic (K) polar, nonaromatic amino acids.

Hauser *et al.* also carried out morphological evaluation of diverse nanostructures formed by varying amino acid sequence and concentration of a class of small self-assembling peptides. They modified these peptides by replacing the aliphatic amino acid at the C-terminus with different aromatic amino acids. The best gelling hexamer LIVAGD and the smallest trimer peptide IVD, were modified by replacing the aspartic acid residue at the C-terminus with an aromatic amino acid (F, W and Y) residue and tracked for the effect of the introduced aromatic residues on self-assembly and morphology of resulting nanostructures. Where, aliphatic peptides formed long, helical fibers that entangled into hydrogel meshes, the modified peptides contrastingly formed short, straight fibers with a flat morphology. No helical fibers were observed for the modified peptides. Such study dealing with the assembly of small peptides derived from simple aliphatic amino acids is significant and may be relevant and helpful in understanding chemical evolution leading to the origin of life on Earth. These aliphatic peptide based self-assembled nanostructures were proposed to have a variety of potential applications in bioengineering and nanotechnology.<sup>93</sup>

Johansson *et al.* showed that even tetrapeptides are capable of forming amyloid fibrils. They hypothesized that hydrophobic interactions alone are not sufficient enough for the self-assembly of these peptides<sup>94</sup> and there are other factors which favour peptide self-assembly into fibers.

Working with smaller peptides, Banerjee *et al.* demonstrated that tripeptides like Boc-AUV-OMe, Boc-AUI-OMe and Boc-AGV-OMe, where U is  $\alpha$ -amino isobutyric acid, self-associated to form

super-molecular  $\beta$ -sheet structures which further assembled into amyloid-like fibrils.<sup>95</sup> In another piece of work Banerjee *et al.* demonstrated pH-responsive nanostructural transition of a tripeptide, TUA, from nanotubes to nanovesicles.<sup>96</sup>

***Self-assembly of dipeptides and their modified versions into distinct nanostructures:*** Moving ahead in this direction researchers tried to explore the self-assembly of peptides as small as dipeptides (Fig. 4). The first report for dipeptide self-assembly came from Gazit's group who investigated the nature of self-assembly of the dipeptide, FF, a core motif of amyloid  $\beta$  (A $\beta$ ) polypeptide segment. This dipeptide self-assembled into highly ordered nanotubes/microtubes,<sup>46,97-99</sup> nanowires<sup>100</sup> and nanoforests.<sup>101</sup> Interestingly, FF nanotubes were found to be thermally stable, a unique property desired to be present in any biologically inspired material.<sup>102</sup> Gazit *et al.* further used FF to construct arrays of nanotubes that can act as high surface-area electrodes for storing energy and making microfluidic chips.<sup>97</sup> A newer type of assembly method based on vapor deposition was used for high scale production of FF nanotubes. This method was not only novel but also provided the handle to fine-tune length and density of nanotubes by controlling monomer supply from gas phase. This further demonstrated that mode and nature of assembly of a given structure can be fine-tuned based on the method and conditions provided.<sup>97</sup> FF based self-assembled nanotubes and nanowires have been investigated further for other mechanical applications such as construction of nanodevices, nanobiosensors and low resistance conducting nanowires.<sup>103</sup> Ihee and his group demonstrated a fascinating morphological transformation between diphenylalanine based nanowires and nanotubes. Nanotubes were obtained by dissolving the peptide in water by sonication followed by heating, whereas, nanowires were obtained in water at high ionic strength. These two morphologies are interconvertible.<sup>104</sup>

Görbitz *et al.* showed that hydrophobic dipeptides like LL, LF, FL, and IL self-assembled into nanotubes by forming head to-tail (NH<sub>3</sub><sup>+</sup> · · -OOC) hydrogen bonds.<sup>105-109</sup> Water-filled nanotubes of the dipeptide WG also demonstrated the ability of negative thermal expansion.<sup>110, 111</sup> These workers also showed that dipeptides VA and FF can form nanoporous structures.<sup>105, 108, 109, 112, 113</sup> Ripmeester's work further demonstrated that dipeptide-based nanoporous materials could adsorb inert gases, such as Xenon.<sup>114, 115, 116</sup>

Gazit *et al.* further demonstrated that addition of a thiol group in FF changed its assembly from tubular to spherical structures.<sup>117</sup> Gazit *et al.* further demonstrated the assembly of other aromatic homodipeptides into nano-spheres, nano-plates, nano-fibrils and hydrogel.<sup>118</sup> These peptide nanostructures can be used as casting mold for the fabrication of metallic nano-wires and coaxial



nano-cables,<sup>46,119</sup> and can have biomedical applications like biosensing, tissue engineering, and molecular imaging etc.<sup>120-126</sup>

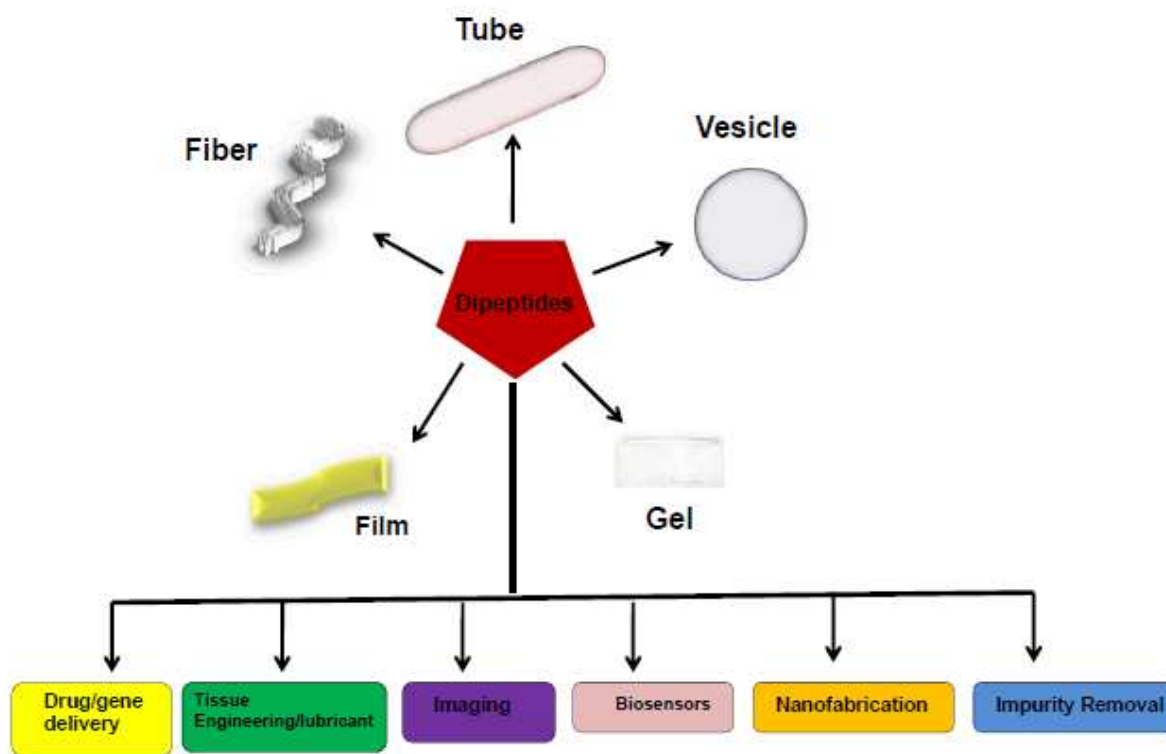


Fig. 4: Formation and potential biomedical applications of dipeptide based nanostructures.

Crystalline dipeptides like AV, VA, IV and VI have been shown by Sozzani and his co-workers to self-assemble into nanoporous materials with capacity to adsorb, separate, and store various gases such as methane, carbon dioxide, and hydrogen.<sup>127</sup> Ventura and his co-workers reported that a dipeptide, IF, at 1.5 wt% and at pH 5.8 self-associated into a transparent, thermo-reversible gel composed of a network of nanofibrous structure in water.<sup>128</sup>

Modified dipeptides have also been explored as templates for making biologically functional self-assembled nano or micro structures owing to their enhanced proteolytic stability. Two modified dipeptides [ $\beta$ -AA;  $\delta$ -Ava-F] containing an N-terminally positioned  $\omega$ -amino acid residue [ $\beta$ -alanine ( $\beta$ -A/ $\delta$ -amino valeric acid ( $\delta$ Ava))] self-associated to form nanotubes in the solid state as well as in an aqueous solution. TEM images of these two dipeptides revealed the formation of uniform and well ordered hollow nanotubular structures with varying dimensions.<sup>129</sup> Interestingly, the nanotubular structures formed by these peptides in the solid state and in solution differed significantly demonstrating their differential assembly behavior and packing arrangements in these two states. These nanotubes were found to be stable over a wide range of pH values and temperature.<sup>129</sup> The

above studies, indicated that water molecules by virtue of their intermolecular hydrogen bonding capacity, always play a pivotal role in the formation and stabilization of the nanotubular assemblies.<sup>129, 130</sup> In another study three water-soluble short peptides each having a common motif, a hybrid of  $\beta,\alpha$ -amino acid residues ( $\beta$ -A-Xaa, Xaa = V/I/F) were found to self-assemble to form hollow nanotubes. These nanotubes could tolerate heat up to 80° C, a wide range of pH (2–10), and were resistant to proteolytic degradation. These dipeptide-based robust crystalline nanotubes have been used as suitable templates for fabricating dipeptide-stabilized gold nanoparticles on their outer surfaces.<sup>131</sup>

Dipeptide-based nanoporous materials obtained from two water-soluble synthetic dipeptides namely  $\beta$ -A-Phg (Phg: phenylglycine) and Phg- $\beta$ -A have shown the capacity to adsorb N<sub>2</sub> gas. Interestingly, these nanoporous materials obtained from dipeptides were degradable by soil bacterial consortium suggesting their ecofriendly nature.<sup>132</sup> They were not only very different from the existing type of nanoporous materials generated from zeolites, metal organic framework (MOF) and others, but also had the advantage of biodegradability and eco-friendliness generally lacking in other organic and polymeric systems.<sup>132</sup>

Fmoc-FF, formed hydrogels with nanofibrillar morphology in an aqueous solution with physical properties superior to those of hydrogels formed by longer polypeptides,<sup>126</sup> whereas, an uncharged peptide analogue, Ac-FF-NH<sub>2</sub>, self-assembled into tubular structures.<sup>133</sup> Gazit *et al.* further explored other amine and carboxyl modified diphenylalanine peptide analogues and revealed that these dipeptides formed ordered tubular structures at the nanometric scale.<sup>133</sup> Ulijn and co-workers demonstrated that Fmoc dipeptides, derived from a combination of four different amino acids, namely glycine, alanine, leucine and phenylalanine, formed hydrogels whose structural and physical properties varied depending on the nature of the amino acids present in the peptide building blocks.<sup>134</sup> Peptides containing various aromatic moieties like Fmoc,<sup>126, 135-137</sup> naphthalene,<sup>138, 139</sup> and pyrene<sup>140, 141</sup> have been proven to be good templates for making nanofibrillar hydrogel networks by the virtue of  $\pi$ - $\pi$  stacking and hydrophobic interactions. The dipeptide amphiphile Fmoc-LG has been shown to self-assemble into thin surface supported hydrogel gel films and gap-spanning hydrogel membranes whose thickness can be closely controlled from tens of nanometers to millimeters. The films and membranes were stable once formed and could be reversibly dried and collapsed, then reswollen to regain the gel structure.<sup>142</sup>

Dipeptides formed by the substitution of phenyl groups with naphthyl groups such as di-D-1-Nal and di-D-2-Nal also formed ordered fibrillar nanostructures. di-D-1-Nal (Nal: naphthalene) formed fibers with 10 nm diameter. Whereas, di-D-2-Nal (Nal: naphthalene) peptide assembled into wider tubular



structures with a diameter of about 50 nm and were more bundled than the fibrils formed by di-D-1-Nal peptide. Interestingly, these naphthylalanine-based peptide structures showed single or low-number of walls as compared to multi-walled structures of the diphenylalanine tubes.<sup>118</sup> Self-assembly of diphenylalanine peptides with nitro and phenyl groups has also been investigated. Di-para-nitro-Phe at a concentration of 5 mg/ml formed spherical nanostructures with different diameters embedded in fibrillar nanostructures. Di-4-phenyl-Phe homo-dipeptide self-assembled into square plates with various dimensions. These square plates appeared to be very thin and symmetrical with clear borders.<sup>118</sup> It was also observed that the aromatic dipeptide, diphenylglycine, self-assembled into well ordered closed-caged nanospheres.<sup>143</sup>

Similar to FF, the dipeptide, (D)-F-(D)-F made up of two D-amino acids, self-assembled in water to form nanotubular structures with diameters ranging from 2 nm to 100 nm. Interestingly, both vesicles and nanotubes were found upon dilution of the solution with an appropriate volume of water suggesting that the concentrations of peptide can play a key role in determining the formation of nanotubes alone or formation of a mixture of nanotubes and nanovesicles.<sup>144</sup> Junbai Li and his co-workers also showed nanotube to nanovesicle conversion of dipeptides. They demonstrated that a cationic dipeptide [FF-(NH<sub>2</sub>) • HCl] could form self-assembled nanotubes at physiological pH, those spontaneously converted into spherical vesicle-like structures after dilution.<sup>145, 146</sup>

Yet in another interesting example, the peptide Acp-YE (Acp,  $\epsilon$ -amino caproic acid) demonstrated a concentration dependent nanovesicle to nanotube transformation. At a concentration of 6.9 mg ml<sup>-1</sup>, Acp-YE formed vesicles and nanotubular structures were obtained at a peptide concentration of 2.3 mg ml<sup>-1</sup>, whereas an intermediate concentration of 3.4 mg ml<sup>-1</sup> of the peptide led to the formation of an array of fused vesicular structures that fused to form nanotubular structures upon dilution. Thus peptide concentration played a significant role modulating peptide assembly from nanovesicles to nanotubes or fused vesicular structures.<sup>147</sup>

Our own work has been focussed on the development and biomedical applications of dipeptides containing an unnatural amino acid  $\alpha,\beta$ -dehydrophenylalanine ( $\Delta$ Phe;  $\Delta$ F) in the peptide backbone. We found that the dipeptide F $\Delta$ F, similar to FF assembled into distinct tubular structures with mean diameter of 27-30 nm in water (Fig. 5), which were stable over a broad range of pH conditions and also in presence of proteases.<sup>32</sup> FTIR and CD studies demonstrated that the dipeptides adopted beta-turn like structure in tubular state.<sup>32</sup> High stability of the self-assembled tubes over a broad range of pH conditions and to a highly nonspecific proteolytic enzyme, Proteinase K, makes these tubes interesting candidates for future applications in drug delivery.

Two amphiphilic  $\Delta F$  containing dipeptides, E $\Delta F$  and K $\Delta F$ , self-assembled into anionic and cationic vesicular structures respectively.<sup>148</sup> E $\Delta F$  assembled into pleomorphic spherical structures ranging from 50-200 nm with a mean diameter of  $\sim 110$  nm, whereas, vesicles formed by K $\Delta F$  appeared mostly spherical with a mean diameter of  $\sim 370$  nm and a relatively narrow size range (250-450 nm). They were also stable to proteinase K. Light scattering studies of these particles demonstrated that E $\Delta F$  formed particles with a mean size of approximately  $370 \pm 30$  nm and K $\Delta F$  formed vesicles with a mean hydrodynamic diameter of  $400 \pm 160$  nm.

We further extended these studies with remaining dipeptides with C-terminal  $\Delta F$  but with varying N-terminal residue, where the n-terminal residue was any one of the 20 naturally occurring amino acid. Out of these, the ones with aromatic N-terminal amino acid formed nanotubes, whereas those with charged N-terminal residues formed vesicles. The dipeptides with hydrophobic N-terminal residues also formed vesicles (unpublished work) (Fig. 5). It was further observed that  $\Delta F$  dipeptides with hydrophobic groups at their N-terminus formed visible assemblies whereas those with hydrophilic N-termini formed assembled structures invisible to the naked eye. Such an observation is interesting and points towards flexibility in peptide design which can be exploited to modulate overall nanostructure morphology. We also found some dipeptides like M $\Delta F$ , I $\Delta F$  and L $\Delta F$  that self-assembled in a mixture of methanol and water (50:50, v/v) to form various nanostructures can also load hydrophobic drugs. In a 50:50 mixture of methanol and water, L $\Delta F$  formed large visible aggregates with hydrodynamic diameter in micrometer range, whereas I $\Delta F$  formed nanostructures with mean diameter of approximately 600 nm. The mean hydrodynamic diameter of M $\Delta F$  nanoparticles was found to be approximately 160 nm. TEM showed that in a 50:50 mixture of methanol and water, all three dipeptides exhibited different assembly behaviour and formed nanostructures with varied morphologies. I $\Delta F$  formed micelle-like structures with an average diameter of 20 nm. L $\Delta F$ , with equal molecular mass but differing in the position of a side chain methyl group of leucine, assembled into fibrillar structures, with diameters in the nanometer range. M $\Delta F$  formed very regular vesicular structures with a mean diameter of  $\sim 40$  nm.<sup>149</sup>

Another interesting study showed that the dipeptide F $\Delta F$ , at a higher concentration and under appropriate assembling conditions could form stable hydrogels having dimensions in nanometer range. The dipeptide gel was colorless and translucent in appearance (Fig. 6 a). The gel was elastic in nature with higher storage modulus ( $G' \sim 209$  kPa) than loss modulus ( $G'' \sim 19.7$  kPa) and also had higher mechanical strength (evident from stable  $G'$  and  $G''$  values at changing frequency). Electron micrographs further showed that the gel matrix was composed of a highly dense network of fibers, which provided it with high mechanical strength and solvent retention properties. The gel also showed

responsiveness towards various environmental conditions such as salt concentration, temperature and pH, a desirable feature of hydrogels used in drug delivery.<sup>33</sup>

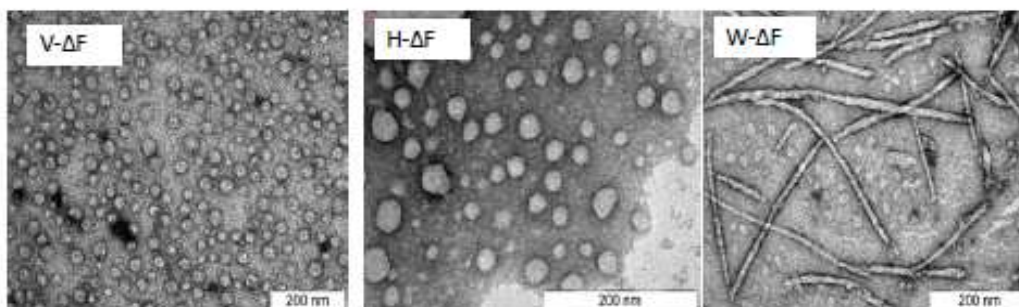


Fig. 5: TEM image of  $\Delta F$  dipeptide nanostructures (nanotubes and nanovesicles).

**Self-assembly behavior of single amino acids or modified single amino acids:** Single amino acids with various chemical modifications have also been investigated for their potential to make self-assembled structures. Ryan *et al.* studied self-assembly of modified single amino acid and showed that Fmoc-F formed self-assembled hydrogels. They demonstrated that side chain functionalization of Fmoc-F can have significant effect on its self-assembly and hydrogelation behaviour.<sup>150</sup> Fluorinated derivatives of Fmoc-F, such as penta-fluorophenylalanine (5-FI-Phe), tri-fluorophenylalanine (3-FI-Phe), when dissolved in water demonstrated spontaneous assembly into fibrils which later on formed a hydrogel network. Ryan *et al.* further investigated the effect of end group functionalization on the self-assembly and hydrogelation pattern of Fmoc-F derivatives by converting the C-terminal carboxylic acid moieties of Fmoc-5-FI-F-OH and Fmoc-3-FI-F-OH into amide and methyl ester groups. Their results depicted that though, C-terminal amide derivatives showed faster assembly than the parent carboxylic acids, but the resultant hydrogels were weak and unstable to shear stress because of the lower water solubility of the amide functionality. On the other hand C-terminal esters owing to their high hydrophobicity, self-assembled into only fibrous structure. From these results it became clear that the monomer/ solvent interactions are in general very complex and they influence the self-assembly and hydrogelation pattern to a great extent. Overall, variation of either the fluorinated aromatic side chain or N-terminal functionalization influenced hydrogelation pattern of these molecules. This also signified that fluorous and  $\pi$ - $\pi$  interactions as the primary determinants for molecular recognition and self-assembly. A better understanding of these interactions would facilitate the development of optimal amino acid based low molecular weight (lmw) hydrogelators.<sup>150</sup> Fmoc protected tyrosine (Fmoc-Y) also self-assembled into hydrogels by controlled enzyme-triggered dephosphorylation of Fmoc-phosphotyrosine.<sup>151</sup> Such enzymatic hydrogelation of small molecules

has also been investigated for various applications.<sup>152-153</sup> In a very recent and interesting work, Gazit *et al.* demonstrated self-assembly of just a single amino acid phenylalanine into ordered fibrils at pathological concentration found in the mental disorder phenylketonuria. The fibrils had amyloid-like morphology and exhibited well-ordered electron diffraction pattern. These assemblies exhibited cytotoxicity towards PC12 cells that was neutralized by the antibodies generated against the fibrils. These fibrils were shown to be present in the hippocampus mice model and in parietal cortex brain tissue from individuals with phenylketonuria.<sup>154</sup>

Apart from experimental investigations a lot of work has also been done on the theoretical modelling and computer simulations of self-assembling systems. Schatz, Ratner, and group used bead and packing models to study self-assembly of peptide amphiphiles (PAs).<sup>155-157</sup> in their study of cone-shaped amphiphiles, they found that electrostatic interactions between charged residues of peptides, induced a void volume in the hydrophobic tail region which guided the cluster to a cylindrical rather than a spherical shape. McCullagh and co-workers using molecular modelling demonstrated that fiber formation was dependent on choice of peptide residues.<sup>156</sup> Lee *et al.* studied the relaxation of self-assembled structures of 144 PA molecules, consisting of a hydrophobic alkyl chain attached to the N-terminus of the sequence SLSLAAAEIKVAV, into cylindrical nanofibers using atomistic molecular dynamics simulations in explicit water with physiological ion concentration. Self-assembly of the molecules is initiated in a cylindrical configuration based on prior experimental and theoretical investigations, and the resulting cylindrical configuration was found to be stable during 40 nano second simulations. It was observed that water and sodium ions can penetrate into the peptidic part of the fiber but not between the alkyl chains. The electrostatic interactions between the PAs and the sodium counterions and the van der Waals interactions between the PAs were found to be the most important interactions stabilizing the nanofiber architecture.<sup>158</sup> Lee *et al.*, also used a CG MD simulation based on the MARTINI force-field to study self-assembly process of 140 IKVAV epitope bearing PAs for 16  $\mu$ s and found that PAs first formed spherical micelles (during first 0–0.05  $\mu$ s), which then formed a three-dimensional network with neighboring micelles via van der Waals interactions, then the tails of different micelles merge and formed fibers.<sup>159</sup> Velichko *et al* developed a simplified coarse-grained model to study the influence of hydrogen-bond formation on the self-assembly of PAs and found that fibrous assemblies were formed by both hydrophobic interactions and the network of hydrogen bonds.<sup>160</sup> It has been suggested that the formation of  $\beta$ -sheets parallel to the axis of the fiber is the driving force for the formation of cylindrical fibers rather than spherical micelles.<sup>161-163</sup>

In order to understand A $\beta$  fibrilization mechanism as well as structural properties of FF nanostructures, simulation studies were carried out using both all atom<sup>164,165</sup> and coarse-grained (CG) peptide models.<sup>166,167</sup> Tamamis *et al.* studied association of FF peptides using an implicit water model, and found transient formation of ring-like structures which are reminiscent of nanotubular structure.<sup>165</sup> However, these studies used only few peptide molecules (96 FF chains) or short simulation time and only resulted in disordered aggregates. Energy minimization<sup>168</sup> simulations and molecular dynamics<sup>164</sup> simulation studies of FF showed the formation of cylindrical reminiscent of tubular structures.<sup>169</sup> Simulations of the crystal structure of FF suggested that strongest interactions occurred between side chains and the charged termini formed salt bridges.<sup>76, 156</sup> Guo *et al.*<sup>170</sup> used coarse-grained peptide model to study FF nanostructures and their concentration dependence. They suggested that FF peptide behaved like a surfactant that first formed vesicles and then these vesicles fused to form nanotubes. Their MD trajectories showed the formation of ordered spherical, vesicular or tubular nanostructures and a formation of either vesicles or tubes was concentration dependent. At low concentrations fusion of vesicles or the fusion of vesicles with a bilayer occurred, whereas at high concentrations, first a bilayer is formed, that bend and close to form tubes.<sup>170</sup>

Recently, Jeon *et al.* explored the initial stages of FF assembly by carrying out molecular dynamic simulations on zwitter ionic and capped FF nanotubes. They showed that electrostatic interactions between peptides led to the formation of ordered dimers and trimers, whereas hydrophobic interactions between side chains were involved in deciding the structures of larger oligomers.<sup>171</sup> Also FF peptides with charged termini because of electrostatic steering first formed dimer or trimer ladders that further facilitated hydrophobic association of side chains and formed more ordered and compact structures as compared to that of uncharged FF peptides that associated by hydrophobic interactions.<sup>171</sup> All these studies along with experimental investigations shed light on the potential phenomenon behind peptide assembly and significantly improved understanding of these systems.

***Potential applications of peptide based self-assembled nanostructures in drug-delivery:*** Different types of biocompatible, inorganic nanomaterials have been developed for drug delivery purpose.<sup>153, 172</sup> However, many of them contain potentially toxic elements<sup>173-176</sup> and have been proven to be less promising for human use. For instance, positively charged lipid-based nanoparticles are known to trigger strong immune responses. Liposomes present technological limitations such as poor reproducibility and stability, and low drug entrapment efficiency and poor control of drug leaching. Polymer nanosystems may be potentially useful alternatives, but their surface functionalization for improving drug-targeting is usually complicated and rather ineffective. Besides, most nanostructures

based on naturally occurring polymers have problems of eliciting unwanted immune response and also present lot to lot variability which makes it difficult to predict their behavior in living systems.

Designed peptide based nanoparticles, due to their biocompatibility, ease of synthesis and functionality, in principle can be excellent candidates for drug and gene delivery. Encapsulation by self-assembled peptide nanostructures holds particular promise in the delivery of biological molecules, including DNA, water insoluble drugs and tagged molecules for imaging.

**Drug delivery using nanostructures generated from long peptides:** Tanaka *et al.* developed TV-XIIa, a 11-residual peptaibol derived carrier peptide for the delivery of antisense oligonucleotides. TV-XIIa was derivatised with a 10-mer of lysine at the C-terminus to make the designed carrier peptide, Ac-U-N-I-I-U-P-L-L-U-P-I-K-K-K-K-K-K-K-K-K-K-OH (U:  $\alpha$ -aminoisobutyric acid), which electrostatically interacted with oligodeoxynucleotides (ODNs) and formed a complex with ODNs, capable of crossing membranes of NIH3T3 cells to accumulate in the cytoplasm and the nucleus.<sup>177</sup>

A novel class of core-shell like self-assembled nanoparticles developed from an amphiphilic peptide, Cholesterol-G3R6YGRKKRRQRRR, abbreviated as CG3R6TAT, where TAT is the transcriptional activator protein of the human immunodeficiency virus type-1, by Yang *et al.* demonstrated powerful antimicrobial activities against a variety of microbes (bacteria, yeasts and fungi).<sup>178</sup> The nanoparticles possessed a broad spectrum of antimicrobial activities and were active against variety of gram-positive as well as drug-resistant gram-positive bacteria, fungi and yeast with low minimal inhibitory concentration (MIC) values and very low haemolysis. More interestingly, the peptide nanoparticles could cross the blood brain barrier (BBB) in a *Staphylococcus aureus*-induced meningitis rabbit model and minimised bacterial growth in brain without causing any significant toxicity to the major organs. These properties indicate that nanoparticles can be developed as efficient antimicrobial agents in treating brain infections. Yang *et al.*, further demonstrated that the efficacy of these peptide nanoparticles could be extended to other infectious diseases such as Methicillin-resistant *Staphylococcus aureus* associated infections, *Candida albicans*-caused brain infections, *Stachybotrys chartarum* infections. Taken together, these nanoparticles were found to be promising antimicrobial agents that can be used to treat brain infections and other infectious diseases.<sup>178</sup> The cyclic peptide nanotubes developed by Ghadiri *et al.*, also served as nanocontainers for effective drug delivery<sup>179</sup> and also as anti-microbial agents.<sup>180, 181</sup>

Peptide-Based-Nanoparticle Devices (PBND), which are described as short amphipathic peptides with a capacity to form stable nanoparticles with proteins and/or nucleic acids have also been explored for



their potential as a drug and gene delivery vehicles. Divita *et al.* developed a PBNB named MPG, a 27-residue-long primary amphipathic peptide (acetyl-GALFLGFLGAAGSTMGAWSQPKKKRKVCysteamide), containing three distinct domains: a N-terminal hydrophobic motif (GALFLGFLGAAGSTMGA) derived from the fusion sequence of the HIV-1 gp 41 (glycoprotein 41) for interaction with the lipid moiety of the cell membrane, a hydrophilic domain (KKKRKV) derived from the nuclear localization sequence of simian virus 40 large T-antigen for promoting interactions with nucleic acids and intracellular trafficking of the cargo, and a linker domain (WSQP), which improves the flexibility and integrity of the hydrophobic and the hydrophilic domains. This PBNB showed siRNA condensing properties by virtue of a basic peptide domain and membrane destabilizing properties due to the presence of hydrophobic peptide sequence, formed stable nanoparticles with siRNA and entered cell independently of the endosomal pathway to efficiently deliver siRNA into a variety of cell lines as well as *in vivo*.<sup>182, 183</sup> Other PBNB-family peptides named CADY and PEP also acted as gene carriers that entered a variety of challenging cells independently of the endosomal pathway for efficient delivery.<sup>184</sup>

Nanocarriers generated from chondroitin sulfate A (CSA) coated and PEGylated poly-L-lysine-based dendrimers have also been developed for controlled and sustained delivery of an antimalarial drug chloroquine phosphate (CQ). Entrapment in the peptidic carrier led to a significant reduction in the cytotoxicity as well as hemolytic activity of the drug. Also a significant reduction in levels of ring and trophozoite stages of *Plasmodium falciparum* were found after being treated with drug loaded CSA coated dendrimers as compared to free drug.<sup>185</sup>

Yang *et al.* developed self-assembled micelles generated from synthetic oligopeptide amphiphiles (i.e. A<sub>12</sub>H<sub>5</sub>K<sub>10</sub> and A<sub>12</sub>H<sub>5</sub>K<sub>15</sub>) that acted as efficient gene delivery vehicles.<sup>186</sup> In another study they developed nanostructures from an oligopeptide amphiphile, Ac-(AF)<sub>6</sub>-H<sub>5</sub>-K<sub>15</sub> (FA32), and evaluated them as carriers for co-delivery of the anticancer drug doxorubicin (Dox) with luciferase reporter and p53 genes. Co-delivery of drug and gene using FA32 micelles demonstrated synergistic cytotoxic effect between p53 gene and Dox with an increase in p53 mRNA expression level as well as end point cytotoxicity towards HepG2 cells.<sup>187</sup>

**Drug delivery using nanostructures generated from short peptides:** Peptide-based vectors owing to their easy functionability and better design strategies can be developed as efficient gene delivery vehicles. Zhang *et al.* developed a series of surfactant peptides like LLLLLLKK, which are composed of a hydrophobic tail attached to a polar head group at the C- or N-terminus. This peptide self-assembled into nanovesicles and nanotubes that acted as DNA delivery vehicles.<sup>188, 189</sup> In DNA

solution, the positively charged peptides self-assembled into a tube that encapsulated the negatively charged DNA. This 'minivan' was then able to deliver DNA to growing cells, at least in some cases. These systems can further developed as smarter materials by tagging the minivan surface with a cell specific marker.<sup>190</sup> Using  $\beta$ -sheeted peptide-based nanoribbons as scaffolds, filament-shaped artificial viruses for gene and drug delivery have been developed.<sup>191</sup>

Stimuli-responsive peptide nanostructures are particularly attractive as drug delivery vehicles since these can have stimuli triggered drug release at target sites. For example, stimuli responsive peptide nanovesicles can be loaded with drugs and other important biomolecules to release them in response to environmental systems like pH, temperature and others.<sup>147</sup> Tripeptide derivatives conjugated with olsalazine, an anti-inflammatory prodrug, self-assembled in water to form prodrug-containing supramolecular hydrogels.<sup>192</sup> It was also shown that the controlled release of an anti-inflammatory agent 5-aminosalicylic acid could be achieved from the gel by the disruption of the supramolecular hydrogel caused by the reduction of the azo group. This method can be generalised for developing new nanobiomaterials for site specific drug delivery.<sup>192</sup> Banerjee *et al.*'s group developed nanovesicles from dipeptides containing glutamic acid residues at the C terminus. Though these vesicles were stable over a wide range of pH, they showed responsiveness towards the presence of calcium ions. These vesicles encapsulated the anticancer drug Dox, fluorescent dyes, various biologically active molecules and were capable of releasing them in response calcium ions. They have also been shown to act as delivery agents for biologically active molecules, such as cyclic adenosine monophosphate within the cells, while preserving their biological activity.<sup>193</sup> Another fascinating example of peptide nanostructure for drug delivery was the one generated from the oligopeptide Acp-YE (Acp,  $\epsilon$ -amino caproic acid), which showed nanotube to vesicle transition and was responsive to calcium ions in the solution as well as to pH change.<sup>147</sup> These nanovesicles could entrap Dox and release it in triggered manner in presence of calcium ions.

***Peptide based self-assembled hydrogels in drug delivery:*** Hydrogels are important class of biological materials with wide range of applications in drug delivery and tissue engineering. Peptide hydrogels/nanogels are particularly important biomaterials as they do not use harmful chemicals (*e.g.*, toxic cross-linkers etc.) to initiate gelation, they are non-toxic, non-immunogenic, biodegradable and degrade into natural amino acids. It has been shown that coiled-coil polypeptide based on, aba, tri-block polymers self-associated to form hydrogels with potential application in sustained protein delivery.<sup>152</sup>

Hydrogels prepared from MAX1 and MAX8 encapsulated and released model biomacromolecules in a controllable fashion. Fluorescence recovery after photobleaching (FRAP) measurements and bulk



release studies of a series of FITC-labeled dextrans within gel networks of differing peptide weight percents, demonstrated that probe diffusion inside the hydrogel network depended on probe size, peptide sequence, and the mesh size of the gel. Further, the electrostatic interactions between a given macromolecule and the gel network also influenced their release pattern. This study suggested that the release of macromolecules with varied characteristics such as size and charges from these self-assembling  $\beta$ -hairpin peptide hydrogels, could be controlled and fine-tuned by simply modifying the amino acid sequences and the peptide weight percent of the gel.<sup>194</sup>

Hydrogels generated from oligopeptides, GAIL and GFIL, demonstrated thermo and pH responsive behaviour. At their minimum gelling concentration, GAIL and GFIL gels were shown to entrap  $8.62 \times 10^{-3}$  (M) and  $3.79 \times 10^{-3}$  (M) of Dox respectively. They also showed controlled drug release behaviour with almost 85% and 90% of the drug molecules getting released from the gel matrix after 45 h respectively.<sup>195</sup>

Self-assembled hydrogels generated from Fmoc-diphenylalanine encapsulated various enzyme bioreceptors (e.g., glucose oxidase or horseradish peroxidase) and fluorescent reporters [e.g., CdTe and CdSe quantum dots (QDs)]. Enzyme loaded hydrogels were smartly used for biosensing purpose and for the detection of analytes such as glucose and toxic phenolic compounds by using a photoluminescence quenching of the hybridized QDs. These results suggest that the peptide hydrogel can act as intelligent optical biosensing platforms by virtue of their simple fabrication method (by self-assembly), efficient analyte diffusion, and high encapsulation efficiencies for fluorescent reporters and bioreceptors.<sup>196</sup>

Dipeptide hydrogels derived from beta-amino acids by Banerjee *et al.* have been shown to encapsulate vitamins like B<sub>2</sub> and B<sub>12</sub> and sustain their release for 3 days at physiological pH (7.46) and temperature (37 °C). This depicts the potential of these gel-based biomaterials for sustained release of drugs and other important biomolecules.<sup>197</sup>

**Drug delivery using  $\Delta F$  dipeptide Nanostructures:** Since last decade, our group has been working on the design and synthesis of  $\Delta F$  dipeptide based nanostructures for drug delivery applications. Dipeptide nanovesicles formed by amphipathic dipeptides K $\Delta F$  and E $\Delta F$  could encapsulate bioactive molecules such as vitamin B<sub>12</sub>, amodiaquin (antimalarial), ampicillin (antibiotic), mitoxantrone (anticancer), polypeptide insulin, synthetic anti-microbial peptides etc. The ability of these vesicles to entrap proteins of various sizes such as recombinant malaria vaccine candidates namely, merozoite surface protein-119 (Pf MSP-119; 11.2 kDa), merozoite surface protein-3N (Pf MSP-3N; 25 kDa), and *Plasmodium falciparum* Histidine Rich Protein-2 (Pf HRP-II; 32.9 kDa), in addition to chicken

egg lysozyme (16.2 kDa), bovine serum albumin (BSA; 66.4 kDa), and anti-mouse goat IgG (150 kDa) was also investigated. Results showed that all proteins interacted with the nanovesicles to varying degrees. TEM imaging carried out to determine the position of proteins on the dipeptide vesicles showed that protein Pf HRP-II was encapsulated in both the vesicles, whereas Pf MSP-119 localized preferentially on the surface of the vesicles. However, TEM studies also suggested that some other proteins like BSA, lysozyme, Pf MSP-3N, and IgG destabilized the nanovesicles. The dipeptide vesicles were taken up by mammalian cells and were not cytotoxic.<sup>148</sup>

Other  $\Delta$ F dipeptides like R $\Delta$ F, L $\Delta$ F formed vesicular and micellar nanostructures. R $\Delta$ F also formed self-assembled nanostructures with a mean hydrodynamic diameter of approximately  $250 \pm 50$  nm. TEM showed that the dipeptide formed vesicular nanostructures with mean size ranging between 30-50 nm. L $\Delta$ F with a hydrophobic N-terminus, assembled into micellar structures with hydrodynamic diameter of  $\sim 250$  nm.<sup>198,199</sup> Nanostructures formed by the F $\Delta$ F, R $\Delta$ F, L $\Delta$ F and E $\Delta$ F entrapped drugs like riboflavin, niacin, amodiaquin, mitoxantrone, ampicillin with varying entrapment efficiency. They also showed non-cytotoxicity towards variety of mammalian cells. R $\Delta$ F nanostructures could be labeled with the radio isotope technetium-99 (Tc99) with stannous tartarate as the reducing agent. Biodistribution studies carried out using Tc-labeled R $\Delta$ F nanostructures, showed accumulation in kidney and bladder along with some radioactivity in heart and blood vessels 1 hour post-injection. Interestingly, the biodistribution profile revealed that R $\Delta$ F nanostructures can evade uptake by reticulo endothelial system (RES) organs such as liver (no significant accumulation) to remain in blood circulation for long time.<sup>198</sup>

Delivery of hydrophobic drugs has always been a challenge. Thus in order to improve the loading of hydrophobic drugs, we tried to develop novel self-assembled dipeptide nanostructures in an aqueous/organic mixture. We chose curcumin as the model drug and tried to load it in  $\Delta$ F dipeptide nanoparticles. Among all the dehydridipeptides tested, M $\Delta$ F demonstrated maximum curcumin loading efficiency and could release the drug in a sustained manner. Loading of curcumin in M $\Delta$ F nanoparticles, increased its solubility and improved cellular availability. Curcumin-M $\Delta$ F nanoparticles showed an enhanced cytotoxic effect in different cancerous cell lines such as human cervical cancer cell line (HeLa), human breast cancer cell line (MCF-7) and human hepatocarcinoma cell line (HuH-7), as compared to native curcumin. These nanostructures also enhanced curcumin's *in vivo* efficacy towards inhibiting tumor growth in Balb/c mice bearing a B<sub>16</sub>F<sub>10</sub> melanoma tumor.<sup>149</sup> Such dipeptide-nanoparticles are also expected to improve the delivery of other potent hydrophobic drug molecules with poor cellular uptake, bioavailability, and efficacy.

Dipeptides E $\Delta$ F, K $\Delta$ F, R $\Delta$ F and D $\Delta$ F, with charged amino acids at N-termini, were synthesized and investigated for their assembly behavior. Out of the four, R $\Delta$ F formed vesicular nanoparticles that could be easily derivatized with folic acid. Folic acid derivatized nanoparticles showed enhanced cellular-uptake in various cancer cells like human breast adenocarcinoma (MDA-MB-231), HeLa that over-expressed folic acid receptors. Folic acid derivatized R $\Delta$ F nanoparticles also exhibited high Dox encapsulation efficiency and Dox loaded nanoparticles demonstrated enhanced cytotoxicity towards cancer cells over-expressing folic acid receptors (Fig. 6). Biodistribution and tumor distribution studies carried out using Tc99 labeled R $\Delta$ F and folic acid-R $\Delta$ F nanoparticles in Earlich ascetic tumor bearing Balbc mice, exhibited enhanced tumor targeting and accumulation as compared to underivatized nanoparticles. In comparison with underivatized nanoparticles or native drug, Dox loaded folic acid-R $\Delta$ F nanoparticles showed enhanced tumor regression in breast tumor bearing nude mice as well as ascetic tumor bearing Balbc mice.<sup>199</sup>

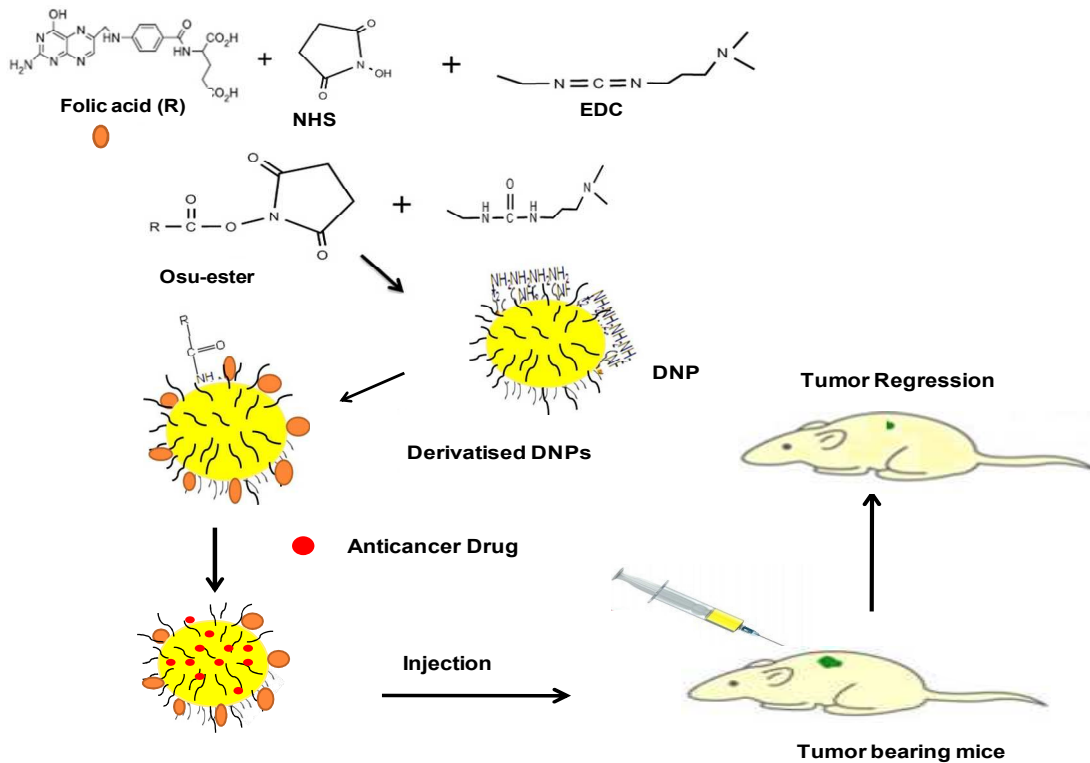


Fig. 6: Tumour targeted delivery by folic acid derivatized  $\Delta$ F dipeptide nanoparticles (DNPS).

Recently, we have also shown the potential of F $\Delta$ F nanotubes for intravitreal delivery of pazopanib, a multi-targeted tyrosine kinase inhibitor with efficacy in treating various cancers as well as ocular

disease like choroidal neovascularisation in wet age related macular degeneration. The drug could be loaded in the nanotubes by both pre and post-loading methods. Pre-loading method was found to be more efficient in loading the drug in nanotubes (25% w/w pazopanib loading and ~55% loading efficiency) compared to the post-loading (8 % w/w pazopanib loading and ~17% loading efficiency) . Plain and peptide loaded nanotubes were non-cytotoxic to retinal pigment epithelial cells. The tubes sustained in vitro release of pazopanib for 35 days. They also demonstrated sustained back of the eye delivery for a period of 15 days following an intravitreal injection using a 33 gauge needle.<sup>200</sup>

We also investigated gene delivery potential of nanoparticles synthesized from cationic  $\Delta F$  dipeptides R $\Delta F$  and K $\Delta F$  and found that the cationic dipeptides condensed plasmid DNA into discrete vesicular nanostructures with R $\Delta F$  forming more regular and ordered vesicular nanostructures.<sup>201</sup> Dipeptide nanoparticles were non-cytotoxic and showed enhanced cellular uptake. They protected DNAs condensed inside them from enzymatic degradation and could successfully deliver these DNAs to different types of cancer cells such as HeLa and HuH 7 (Fig. 7). GFP encoding plasmid DNA loaded dipeptide Nps showed positive gene transfection as well as gene expression in HuH 7 cells. By virtue of their simple dipeptide origin, ease of synthesis, high enzymatic stability as well biocompatibility, these nanostructures can be foreseen to be developed as vehicles for effective gene therapy.

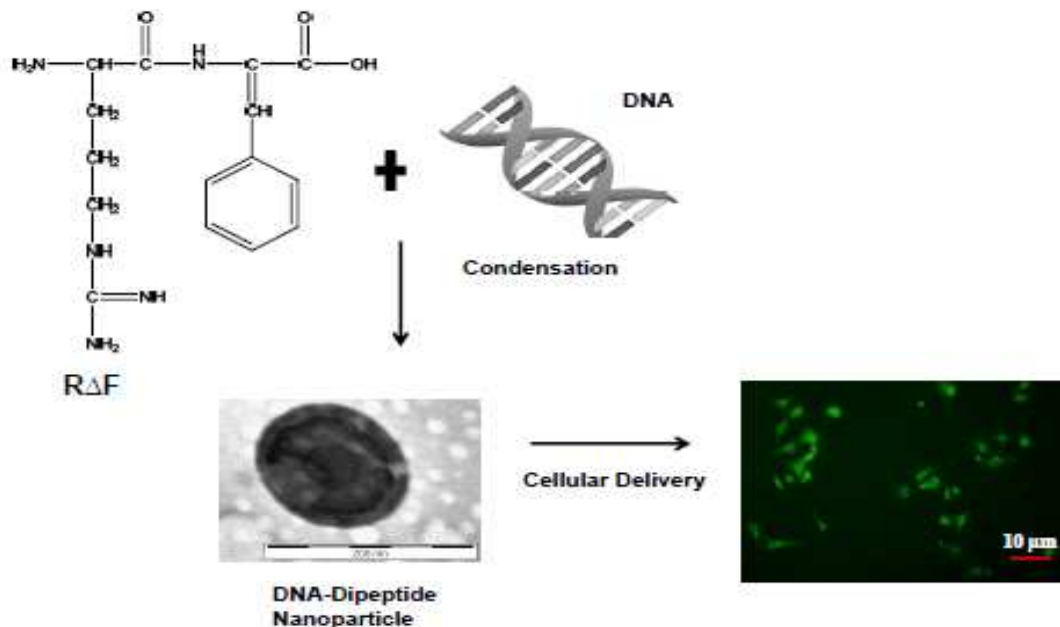


Fig. 7: Cationic dipeptide-DNA nanoparticles for cellular delivery.

Thus, all our work showed that the  $\Delta F$  dipeptide nanostructures can act as potential candidates for drug delivery applications. The most important point here is that these dipeptide nanostructures are

biocompatible with no adverse side effects both *in vitro* and *in vivo*. Due to the presence of  $\Delta F$  residue in the peptide design, these dipeptide nanostructures are expected to show enhanced stability to enzymatic degradation<sup>202, 203</sup> and thus would have high *in vivo* half-life.

Similarly, F $\Delta$ F hydrogel also showed drug loading and release behavior (Fig. 8). The peptide gel could effectively entrap vitamins like ascorbic acid, riboflavin, and vitamin B12, antibiotics like ampicillin and chloramphenicol, antimalarial drugs such as amodiaquin, anticancer drugs like fludarabine and mitoxantrone and anti-tuberculosis drugs like L-cycloserine and isoniazid. The polypeptide insulin was also entrapped in the dipeptide gel. All the entrapped molecules in the hydrogel, exhibited sustained release behavior from the gel matrix with their diffusion coefficient (D) values ranging between  $D = 2.9 \times 10^{-11}$  to  $5.6 \times 10^{-10}$ . The release rate of drugs from the gel matrix (D values) were also found to be dependent on the nature of drugs like their molecular weight, ClogP values and net charge. The gel further demonstrated stimuli responsive behavior and released entrapped molecules in response to change in environmental pH or salt-concentration.<sup>33</sup> The ability of the dipeptide hydrogel to encapsulate a diverse kind of bioactive molecules within its matrix and their “depot-like” sustained release behavior, as well as increased proteolytic stability, makes the described dipeptide gel a promising candidate for potential drug delivery applications.

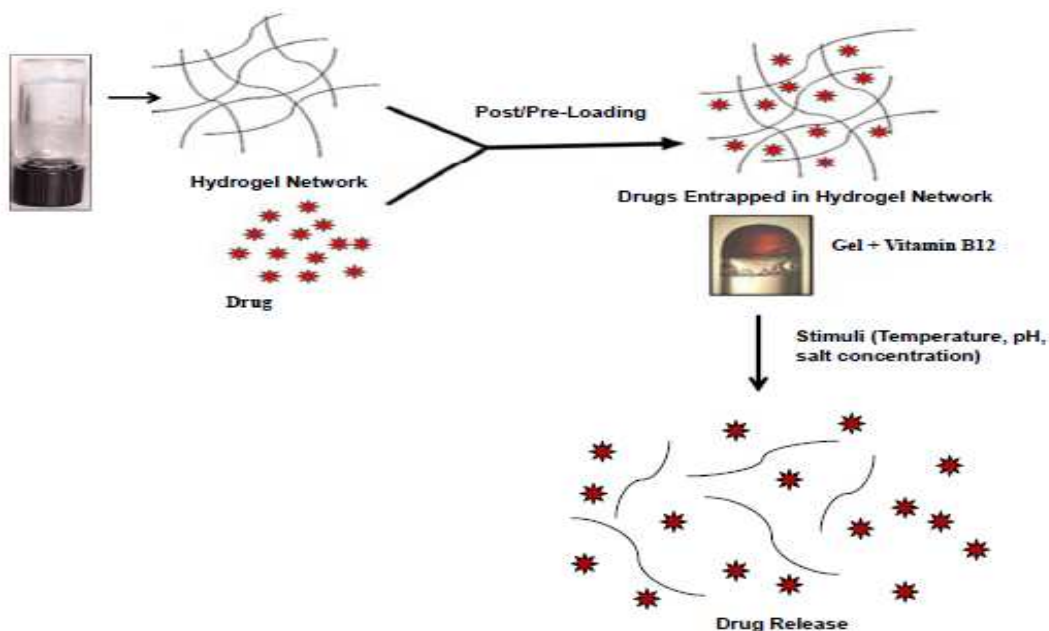


Fig. 8: Schematic diagram showing drug entrapment and release in a peptide (F $\Delta$ F) hydrogel.

**Potential application of self-assembling peptides in tissue engineering:** Successful tissue regeneration requires that cells should be provided with an environment appropriate for regeneration,

induction, and development of neo-tissue. The behavior pattern of cells changes when they are constrained to two dimensional (2 D) culture in petri dishes, after being isolated from their complex native tissue environment. Hence, it is necessary that cells should be grown in a dimensional (3D) matrix to maintain their natural phenotypic shape and behavior patterns. Scaffolds required for the 3D growth of cells to generate specific organs should ideally be biodegradable with nontoxic degradation products easily eliminated by the host. One important aim in the design of synthetic tissue scaffolds is to mimic the structure and function of the extracellular matrix (ECM) for providing the cells a microenvironment similar to those encountered *in vivo*. The ECM organizes cells into tissue, affects overall cellular architecture, provides pathways for migratory cells, participates in signal transduction pathways and strengthens tissues. The architecture of natural ECM components has been an inspiration for researchers to synthesize materials with similar structure for biomedical applications such as hydrogels, for cell-based therapeutics<sup>204,205</sup> and soft tissue engineering scaffolds.<sup>206</sup>

The present day cell-based therapeutic hydrogels, either derived from natural biopolymers (such as collagen, hyaluronate, fibrin, alginate, agarose, and chitosan) or from synthetic polymers (such as poly(acrylic acid), poly(ethylene oxide), poly(vinyl alcohol), and polyphosphazene).<sup>206</sup> The natural polymers suffer from limitations such as presence of variable properties based on their source of origin or presence of viral contaminations, whereas, synthetic polymers usually lack functional sites for cellular interactions and may show adverse side-effects.

Self-assembling peptides generally show favorable properties concerning biocompatibility, immunogenicity and biodegradability, producing non-toxic waste products. In recent past several self-assembled peptide based hydrogels have been explored as scaffolds for 3 D cell growth and tissue engineering purpose.<sup>207</sup> Self-assembling peptide nanofiber scaffolds with amino terminal with sequences derived from collagen IV or laminin demonstrated enhanced adhesion and spreading of human aortic endothelial cells.<sup>208</sup> In order to mimic ECM structure and porosity, peptide nanofibers were further combined with epithelial growth factors. The modified nanofibers showed improved rate of wound healing on human skin equivalent tissue model.<sup>209</sup> Such modified scaffolds have also showed enhanced growth properties in mouse neural stem cells<sup>210</sup> and pre-osteoblasts.<sup>211</sup> Zhang *et al.* developed a unique type of peptide based scaffolds which could be injected into myocardium, creating an intra-myocardial microenvironment for mouse endothelial cells that promoted vascular cell recruitment.<sup>212</sup>

Hartgerink *et al.* developed various peptide amphiphiles which could form different types of nanofibers.<sup>213</sup> These peptide amphiphile molecules were derivatised with biologically active peptides containing RGD for promoting cell adhesion and phosphorylated serine residue for promoting



hydroxyapatite mineralization. Linear and cyclic RGDS<sup>214</sup> as well as various other bioactive signals such as biotin<sup>215</sup> were also presented on N-terminal of peptide amphiphile nanofibers to promote better cell growth and differentiation. Peptide nanofibers were further assembled to form branched peptide amphiphiles, leading to greater accessibility of binding sites. These nanofibers were developed as bioactive scaffolds with improved epitope recognition for enhanced cell adhesion<sup>214, 216</sup> for tissue engineering applications,<sup>217, 218</sup> and magnetic resonance imaging.<sup>219</sup> Sargeant *et al.* fabricated hybrid bone implants by self-assembling peptide amphiphile nanofibers within porous titanium.<sup>220</sup> These implants demonstrated cell encapsulation, vascularization and mineralization of calcium phosphate with a calcium:phosphate ratio similar to that of hydroxyapatite.

Self-assembling nanofibers have also been shown to act as scaffolds for neural progenitor cells<sup>221</sup>, dental stem cells<sup>222</sup> as well as have been used for cell entrapment<sup>223</sup> and stimulation of angiogenesis.<sup>224</sup> RGD containing  $\beta$ -sheet forming peptide sequences with capacity to self-assemble into nanoribbons, have been shown to encapsulate hydrophobic molecules as well as cells through integrin receptors. Hydrogel scaffolds produced by the peptide Ac-(KLDL)<sub>3</sub>-CONH<sub>2</sub> has been shown to support the growth and differentiation of chondrocytes along with stimulating the synthesis and accumulation of extracellular matrix.<sup>225</sup> Self-assembling peptides were also being developed as scaffolds for cartilage repair and promotion of nerve cell growth.<sup>226</sup> A peptide amphiphile, C16-G3A4-IKVAV, shown to promote the re-growth of nerve cells in rats was made by including a neurite-promoting laminin epitope tag IKVAV.<sup>227</sup>

The  $\beta$ -hairpin hydrogel developed from the peptide MAX 1, promoted growth and proliferation of fibroblast cells.<sup>228</sup> Another study demonstrated hydrogel formation from a N-terminally protected peptide sequence Napthalene-FFGRGD.<sup>229</sup> This was used for surface coating on poly(3-caprolactone) (PCL) films, and to promote cell attachment and growth.

Similarly self-assembled fibers generated from RADA16 peptide were used in a wide range of biomaterial applications, including cartilage tissue repair,<sup>225</sup> osteoblast proliferation and differentiation,<sup>211</sup> bone regeneration<sup>230</sup> and axon regeneration.<sup>231</sup> Cardiomyocytes or non-differentiated stem cells loaded fibril-forming peptide gels when injected into damaged heart tissues led to the improvement of transplanted cell survival and wound healing after a myocardial infarction<sup>212, 232</sup>.

Scaffolds derived from Peptide-amphiphiles (PAs) have been shown to promote the attachment of primary human bladder cells, demonstrating the potential biological application of PAs for

augmenting the biocompatibility of polymeric materials conventionally used for tissue engineering.  
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Another wonderful application of PA-based nanofibers includes the adhesion and migration of neural cells *in vitro*.<sup>227</sup> Remarkably, these PA-based nanofibers exhibited extremely promising results in an animal model based on a spinal cord injury.<sup>233</sup> Another amphiphilic peptide construct, containing a heparin-binding site, exhibited very exciting results in promoting angiogenesis.<sup>234</sup> These types of peptides were further modified with biotin<sup>215</sup> and a Gd<sup>3+</sup> metal-chelating moiety suitable for detection by magnetic resonance imaging (MRI).<sup>219</sup>

Vescovi *et al.* reported about short assembling peptides containing the bone marrow homing peptide 1 (BMHP 1) functional motif (PFSSTKT) (BMHP1-SAPs). These peptides assembled to form nano or micro structures of varied shape like tubular fibers, twisted ribbons, tubes and sheets. Interestingly, despite of having heterogeneous nanostructure morphology and varied scaffold stiffness, all the BMHP1-SAPs exhibited  $\beta$ -sheets and  $\beta$ -turns like secondary structure. Few of these 10-mer peptides promoted adhesion, differentiation, and proliferation of human neural stem cells. These SAPs could be derivatized with the cell adhesion promoting motif like RGDGG, for promoting enhanced cell attachment. *in vivo* experiments carried out using these SAPs exhibited negligible side reactions towards host nervous tissue.<sup>210</sup> Hydrogels prepared from the P11-4 peptide also found to have tissue engineering applications such as enamel remineralization,<sup>235</sup> injectable scaffolds<sup>236</sup> and joint lubricants.<sup>237</sup>

Zhou *et al.* reported a peptide based bioactive hydrogel using molecular selfassembly of a mixture of two aromatic short peptide derivatives: Fmoc-FF (Fluorenylmethoxycarbonyl diphenylalanine) and Fmoc-RGD (arginine-glycine-aspartate). This biomimetic nanofibrous hydrogel acted as a 3D-scaffold for anchorage-dependent cells.<sup>134</sup>

An array of FF nanofibers grown on gold microelectrodes led to the development combined cell culture and biosensing platforms. Peptide nanowires were modified with conductive polymers for enabling detection of dopamine at physiological concentrations as well as to grow different cell lines such as PC12 and HeLa.<sup>238</sup>

Our group has also reported 3D growth of mammalian cells [HeLa and fibroblast (L929)] on chemically functionalized FAF hydrogel. The peptide hydrogel was functionalized with the cell adhesion motif “RGD” containing pentapeptide to facilitate cell growth and proliferation. This functionalized hydrogel provided a wonderful support for 3D cell growth for more than two weeks with maintaining cell viability and spreading. This study provides an excellent example of a simple peptide based-hydrogel to attain increased cell growth promoting properties, with high enzymatic



stability.<sup>239</sup> This gel-based soft material acts as a convenient template for 3D cell growth with a probable use in tissue engineering and cell biology.

All these results encourage the use of peptide-based biomaterials in regenerative medicine and pave the way for the development of novel self-assembling peptide sequences that may be useful for material science and regenerative medicine applications. In addition to the above mentioned applications peptide based self-assembled structures can have many more applications such as materials for surface engineering, biosensing devices and many more to be discovered in near future.<sup>29</sup>

**Conclusion:** Self-assembly is a marvelous strategy to make ensemble of nanostructures. Small peptide-based self-assembled nanostructures, due to their inherent biocompatibility, easy tunability, simple and cost-effective synthesis could offer a myriad of potential uses in biomedical applications. Peptides could spontaneously self-assemble into tubular or fibrillar or vesicular nanostructures. The nanostructures have been shown to entrap a wide range of bioactive molecules with a controlled release pattern. Peptide based hydrogels with nanofibrillar morphology has been shown to support 3D growth of various cell types along with promoting cell differentiation in many cases. Thus small peptides have tremendous potential to be developed as intelligent biomedical scaffolds with many biomedical applications. Thus peptide self-assembly is emerging as a new area of research and has spurred intense interest in the fabrication of nanoscale devices and demand for miniaturization in both academia and industries necessitate progress in this area.

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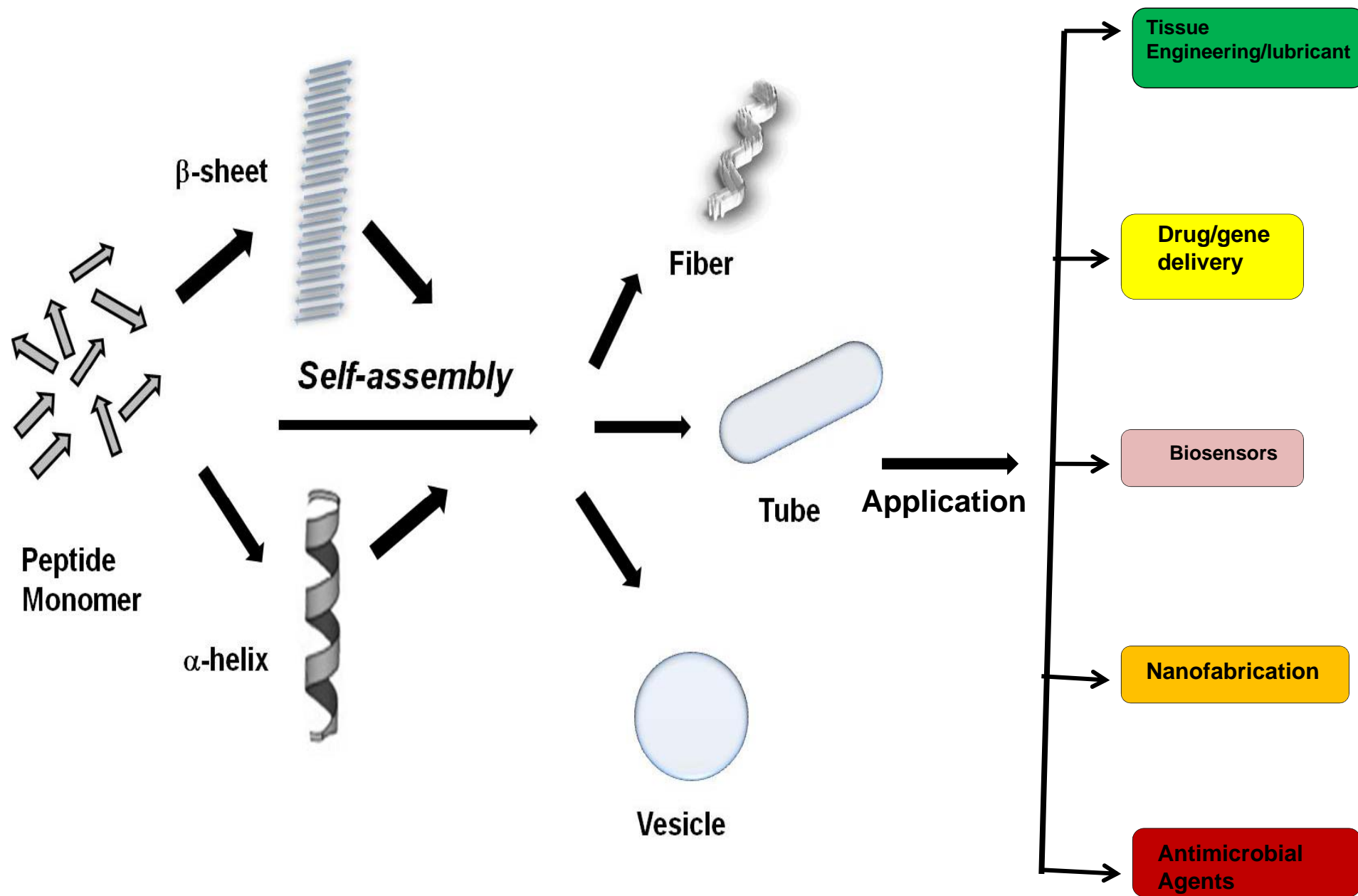
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Self-assembling peptides with many potential biomedical applications