

# Chapter 2

## Fibril Formation by Short Synthetic Peptides

Andrew Smith

**Abstract** Nature produces an array of self-assembled fibres from proteins and peptides with a wide range of functionalities. This has inspired scientists to design peptides that exploit specific protein folds to form simple yet multi-functional self-assembled fibres. Of the various protein folds the most commonly used has been the  $\beta$ -sheet fold as it is easily accessible and produces nanoscale fibres which have a wide range of stabilities. Research has also been driven by the relationship to the various amyloid diseases, which produce  $\beta$ -sheet rich fibres. Here we will discuss the use of natural protein sequences as the basis of peptides that self-assemble to  $\beta$ -sheet rich fibres followed by peptide sequences that have been designed de novo purely based on the rules for the formation of a  $\beta$ -sheet. How changes in the amino acid sequence of these various peptides affects the properties of the fibres and also the macroscopic materials formed by these peptides will be discussed in each case. We will then look into how these structures have been utilized for applications as scaffolds for cell culture and tissue regeneration, followed by their use in the nanotechnology field.

**Keywords** Self-assembly · De novo peptide · Beta-sheet · Peptide design

### 2.1 Overview

In this chapter we will discuss the design and application of peptides that self-assemble into  $\beta$ -sheet structures reminiscent of the amyloid structure. Firstly I will introduce the field of peptide self-assembly and the various areas of research before concentrating on those peptides based on the  $\beta$ -sheet fold. These  $\beta$ -sheet peptides can be broken down into two groups, those based on naturally occurring sequences and those that are designed purely on the rules for forming  $\beta$ -sheets. For the natural sequences there are only a few examples of amyloid based sequences due to the obvious potential ramifications of using a sequence that can cause disease for applications. However, non-disease related sequences have been used to create beta-sheet based fibers. These natural sequences have been studied extensively and we will

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discuss the sequence-property relationship of those peptides specifically, the sequence-property relationships related to disease will be discussed elsewhere. The designed peptides purely utilize the rules for the  $\beta$ -sheet fold and self-assembly to generate a sequence whose properties can be varied by specific alterations to the amino acid sequence. Lastly we will discuss the varied applications of self-assembling  $\beta$ -sheet peptides in the fields of 3D cell culture, tissue regeneration and engineering and also nanotechnology.

## 2.2 Introduction to Peptide Self-assembly

Throughout history Man has utilized biological materials to create functional as well as decorative objects from the construction of houses and furniture made from the wood of trees down to the production of fine garments and wound dressings using silk (Gerritsen 2002). In the modern era our understanding of biological materials at the molecular level offers the potential to allow us to use the fundamental components of biological systems, proteins for the development of new materials (Bhushan 2009; Tamerler and Sarikaya 2009). At the molecular level cells construct large complex networks of fibers that are formed by the self-assembly of individual proteins, a process sometimes referred to as bottom-up assembly. This is of major interest to material scientists as the current method of producing micro- and nanoscale structures is by a process known as top-down, where the fine structure is cut out or molded out of a larger structure similar to how the majority of materials have been modified by Man (Whitesides et al. 1991). Bottom-up processes involve the organization of small components to create a larger structure and as a consequence can be more efficient in the use of available materials. Biology is an expert at this process with fascinating structures such as virus capsids and diatoms being produced (Yao et al. 2011), which have a level of structure and reproducibility which is currently beyond that of human capabilities. The two mentioned systems have at their core a protein structure which creates the structure and in the case of diatoms acts as a scaffold for biomineralization (Jeffryes et al. 2011). Such protein structures have obvious applications in cell culture and tissue engineering as simplified replacements for the extracellular matrix, which is protein based. Additionally, biomineralized protein structures and even the naked protein structures have potential in nanotechnological applications. Of course, to exploit such structures successfully it is desirable to understand the process of self-assembly in biological systems. As such the early areas of interest have been one dimensional self-assembled structures, basically fibers, as these are one of the simplest structures to make. Fibrous systems offer the potential to generate materials that could be utilized as nanoscale fibers for applications such as nanoelectronics or as bulk materials where the density of nanoscale fibers becomes sufficiently high that a hydrogel is produced which has a range of applications including cell culture and tissue regeneration.

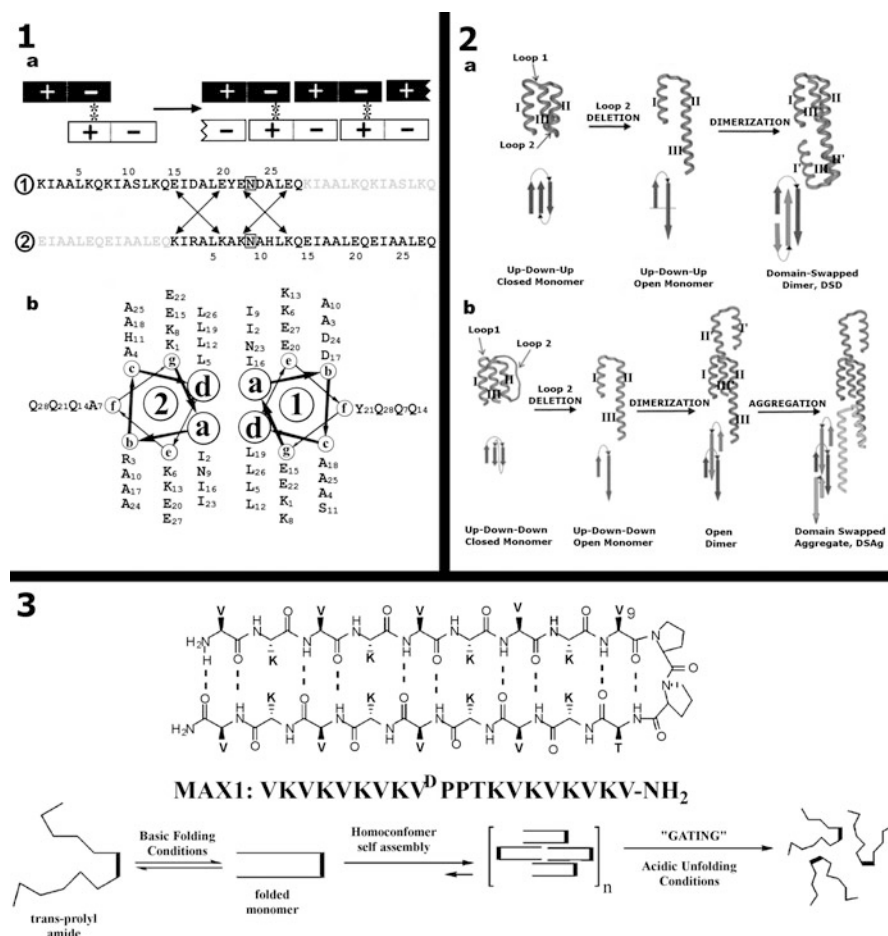
In general peptides that are designed to self-assemble either interact with themselves or a related peptide under specific conditions to assemble longitudinally into a

fiber with nanoscale width (1–100 nm) and lengths in the order of microns or higher. These fibers can be either rigid rod-like structures with 2D crystal properties, or worm-like fibers with a low persistence length, or even branched fibers. As a consequence at high concentration it is possible for a hydrogel to form due to either the entanglement of long fibers or the formation of a physically cross-linked network of fibers, this is especially true for the latter two types of fibrous structures. Some fibers are also specifically designed to assemble onto a surface which results in the underlying surface properties affecting the fiber network and structure.

A variety of protein folds have been utilized for the formation of long nanoscale fibrous structures. These have included designs based both on coiled-coils and beta-sheets (Fig. 2.1). Here we are mostly interested in designs for fibrous structures that utilize the beta-sheet fold for structure formation, so only a brief description of systems based on coiled coils will be given.

### 2.3 Coiled-coil and Helical Bundle Fibers

There are several examples of coiled coils or helical bundles being used for the formation of fibrous structures, most utilize sequences that have properties/arrangements not normally seen in coiled coils. This is due to the fact that all natural examples of coiled coils are “blunt-ended”, basically all the chains of the coiled-coil structure end at the same point with no overlap of one helix of a coiled coil into the structure of another protein (Lupas 1996). This precludes self-assembly in the longitudinal direction of the coiled-coil, however, due to the level of understanding of the coiled-coil folding motif it has been possible to manipulate this. A coiled-coil structure is defined by a heptad repeat of polar and hydrophobic residues and the relationship of the hydrophobic residues to the oligomerization state of the coiled-coil, be it dimer or trimer, parallel or anti-parallel is known (Walshaw and Woolfson 2003). As a result it has been possible to design sequences that have an overlap or “sticky-end”. This “sticky end” is one helix of the coiled coil extending beyond the main coiled-coil region which can then interact with another peptide to form a complete coiled-coil thus allowing the formation of longitudinal assemblies. This has been demonstrated several times using dimeric coiled coils (Pandya et al. 2000; Bromley et al. 2010) and also with trimeric coiled coils (Ogihara et al. 2001; Zimenkov et al. 2006) as well as helical bundles (Zou et al. 2011). One example of a dimeric parallel coiled-coil fiber utilized two peptides that we designed with four heptads, however the last two heptads interact with the first two of the second peptide, and vice versa (Pandya et al. 2000). As a result the formation of a coiled-coil between two peptides results in both peptides having either their first two or last two heptads exposed to interact with further peptides. This allows the assembly of these peptides into a fiber, through modification of the sequence it has been shown that it is possible to alter the stability, and properties of the fiber for various applications (Fig. 2.1, Panel 1) (Papapostolou et al. 2007; Smith et al. 2005; Smith et al. 2006; Ryadnov and Woolfson 2005). In the case of a trimeric coiled coil a peptide was designed to utilize a domain swap



**Fig. 2.1** Schematics of a four different peptide based self-assembly systems. *Panel 1:* Two peptides designed to fold to a parallel coiled-coil with “sticky ends” to allow longitudinal self-assembly. Reprinted with permission from (Pandya et al. 2000). Copyright 2000 American Chemical Society. *Panel 2:* A domain swapped dimer can be formed by partial unfolding of a trimeric helical bundle (a), by modifying the loop a extended domain swapped aggregate can be formed (b) (Ogihara et al. 2001) Copyright (2001) National Academy of Sciences, USA. *Panel 3:* A schematic of a designed beta hairpin, MAX1 utilizing a forced turn sequence containing a L- and D-proline. Reprinted with permission from (Schneider et al. 2002) Copyright 2002 American Chemical Society

that converts a protein dimer into a protein oligomer. By altering the loop that allows one of the helices to interact in the normal dimer, it forces the structure to always have one exposed helix which can then interact with a further peptide to allow self-assembly into an oligomeric fiber rather than a dimer (Fig. 2.1, Panel 2) (Ogihara et al. 2001).

## 2.4 Beta-sheet and Turn Based Fibers

A large number of research groups have investigated the development and application of a variety of beta-sheet based self-assembling fibers. The earliest examples are based on sequences from natural proteins that have the potential to form large beta-sheet fibers. This has then led to an exploration of peptides with related sequences and the development of sequences designed using the basic parameters for a beta-sheet fold, alternating hydrophobic/hydrophilic residues. Recently designs based on sequences seen in amyloids have been explored as a fibrous system. In this chapter the discussion of amyloid sequences will be purely limited to those that have found a use as biomaterials. Many of these self-assembling peptides have been further redesigned to allow a wider range of applications from a basic design. Many of these applications are in the field of cell culture and tissue regeneration due to their potential as replacement extracellular matrices (ECM), as they are protein based, like the natural ECM, and have desirable properties such as biodegradability and responsiveness. It has also been proposed and demonstrated that these self-assembled systems could potentially be used in nanotechnological applications as nano-wires and quantum dots.

As such we will initially discuss the development of beta-sheet fibers that are based on natural sequences and their derivatives before discussing those peptides that have been purely designed based on the rules for the beta-sheet fold. After this we will discuss the applications of these materials together as many have similar potential applications.

### 2.4.1 *Peptides Based on Natural Sequences*

The first example of a beta-sheet based self-assembled fiber developed from a natural protein came from the yeast protein zootin (Zhang et al. 1993). The peptides developed from this protein have become the basis for an entire group of peptides often referred to as the ionic complementary peptides. These peptides consist of alternating charged and hydrophobic residues and form a beta-sheet based structure. Zootin was identified in the early 1990's, this protein contained a repeating sequence of alternating hydrophobic and charged residues, (Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)<sub>2</sub>. This sequence became known as EAK16, based on the fact that it only contains the amino acids glutamic acid, alanine and lysine, and is 16 residues long. Since the discovery of this first peptide further studies have been undertaken by a range of research groups using peptides derived from this sequence. These peptides maintain the basic alternating pattern of charged and hydrophobic amino acids and use oppositely charged amino acids in the same peptide so only one peptide sequence is needed for self-assembly to occur. As a consequence a library of peptides have been studied to investigate which properties of the amino acid side-chains influence the self-assembly of the peptides into  $\beta$ -sheet fibrils. The peptides tend to be named after

the 3 amino acids used in the peptide, two oppositely charged and one hydrophobic amino acid, the peptide length and the spacing of charges, indicated by roman numerals I-IV, where the value determines the charge spacing between the opposite charges. For example EAK16-I, would be AEAKAEAKAEAKAEAK, where the sign of the charge changes at every charge residue, while the peptide EAK16-II would have the sequence AEAEAKAKAEAEAKAK, the original natural sequence, where the sign of the charge changes at every second charged residue.

One method by which these peptides have been characterized and compared is the change in critical concentration for self-assembly upon sequence alteration. There are several methods by which the critical concentration for self-assembly of these and other peptides have been determined. One of the main techniques used to study these peptides is rheometry as the self-assembly process tends to generate a hydrogel at 1 wt % concentrations. An alternative method for monitoring the effect of sequence changes is the surface tension of a peptide solution, which again indicates the critical concentration for self-assembly (Hong et al. 2004; Hong et al. 2003; Fung et al. 2003).

The self-assembly process of the ionic complimentary peptides is affected by several factors centering on the competition between the drive to sequester the hydrophobic residues of the peptide from water and the effect of charge repulsion from the charged amino acids. Changes in the value of the critical coagulation concentration give a good indication of the effect of the mutation on the competition between hydrophobicity and charge repulsion. At neutral pH self-assembly occurs due to the overall neutral charge of these peptides, while at low pH the peptides remain in solution due to the overall positive charge of the peptides. This has been shown to relate to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory which allows a rational method of designing these peptides so that under specific conditions self-assembly will occur (Caplan et al. 2002). Using a peptide KFE12 (FKFEFKFEFKFE) it was possible to demonstrate that at low pH the addition of salt masks the charges on the peptides from one another allowing self-assembly to occur (Caplan et al. 2000). The addition of salt at low pH is relevant to many of these peptides as most are synthetically produced, which means that the peptides contain residual trifluoroacetic acid from the purification process. As a consequence when the peptides are dissolved in pure water they tend to have a pH of around 3, which means that the peptide has an overall positive charge due to the protonation of the glutamic acid side chains in the case of KFE12.

The salt masking effect is dependent on the valence of the relative counterion to the charge on the peptide at a specific pH. Higher valence counterions require lower concentrations to mask the charge on the peptides and trigger self-assembly (Caplan et al. 2000). At neutral pH the balancing of the charges on the peptides results in the self-assembly of the peptides in the absence of salt. This has been further demonstrated by altering the sequence to replace the glutamic acid residues with glutamine, the non-charged counterpart to this residue, in the peptide KFE12, which is now KFQ12 which then requires the presence of salt at pH values up to pH 10 (Caplan et al. 2002). Additionally, an AFM study into EAK16-II showed that at low peptide concentrations <0.1 mg/mL the dimensions of the fibers formed by the peptide is dependent on the NaCl concentration up to a salt concentration of 20 mM

highlighting that charge repulsion is affecting the self-assembly process (Hong et al. 2005).

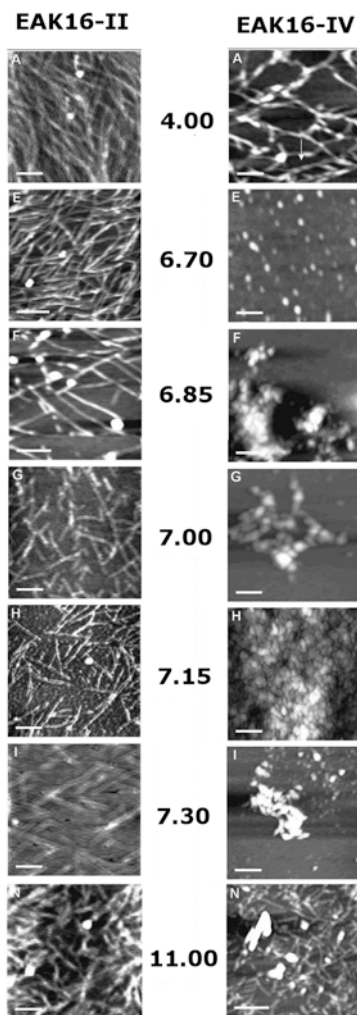
The other key factor that influences the critical coagulation concentration relates to the hydrophobicity of the side chains in the peptide. Through experiments using different hydrophobic residues in the peptides it is clear that there is a competition effect between the charge repulsion and the hydrophobicity of the peptide. Using a series of peptides KFE12, KVE12, and KIE12, it was found that switching from valine to isoleucine results in a decrease in the concentration of counterions needed to trigger self-assembly (Caplan et al. 2002). With a change to phenylalanine there is less correlation between the concentration of counterions and its calculated hydrophobicity. This however, is more related to the varying methods of defining the hydrophobicity of phenylalanine, some methods indicate it is more hydrophobic than isoleucine and others indicate that it is less. Taken as a whole though there is a clear correlation between an increase in the hydrophobicity of the amino acids used and a decrease in the required amount of salt for self-assembly to occur (Caplan et al. 2002).

Another factor is the length of the peptide, however this has a more complicated effect. Shortening or lengthening the peptide from 12 amino acids to 8 or 16 in both cases results in an increase in the critical concentration. This has been proposed to be related to a competition between increasing entropy as the peptide length increases and a decrease in the effect of charge due to the Debye length of charge effects being limited (Caplan et al. 2002).

Changing the spacing between the charges in the peptide has been looked at experimentally and computationally. Experiments indicate that at pH 7 and low concentration both EAK16-I and EAK-16-II form fibrillar structures while EAK16-IV forms small aggregates (Hong et al. 2003; Jun et al. 2004). At other pH values EAK16-IV forms fibrillar structures similar to the other peptides, however, the fibrils are twice as thick (Hong et al. 2003). Simulations of these peptides indicate that in the case of EAK16-I and IV the unassembled peptides favor an extended worm-like chain structure and a folded beta hairpin like structure, respectively, while EAK16-II can adopt either structure and that this starting fold has an impact on the final self-assembled structure (Jun et al. 2004). At low peptide concentrations where the peptides have sufficient time to fold prior to self-assembly it is possible to detect by AFM a change in the morphology of EAK16-IV. At low concentration it forms small aggregates due to the formation of internal electrostatic interactions and the exposure of its hydrophobic face to the solvent which results in aggregation (Fig. 2.2). At higher concentrations intermolecular interactions dominate due to the closer proximity of individual peptides to one another resulting in the formation of fibrillar structures similar to that seen for EAK16-I and II (Fung et al. 2003).

One of the key features that make these peptides and other self-assembly peptides interesting is their self-healing ability. It has been shown that an example peptide RADA16-I can have its macroscopic fibrous structure disrupted by sonication of a hydrogel of this peptide until it is a liquid. The molecular structure however, remains as a beta-sheet as determined by circular dichroism. If the sample is then allowed to rest after the sonication process the reassembly of fibers can be visualized after

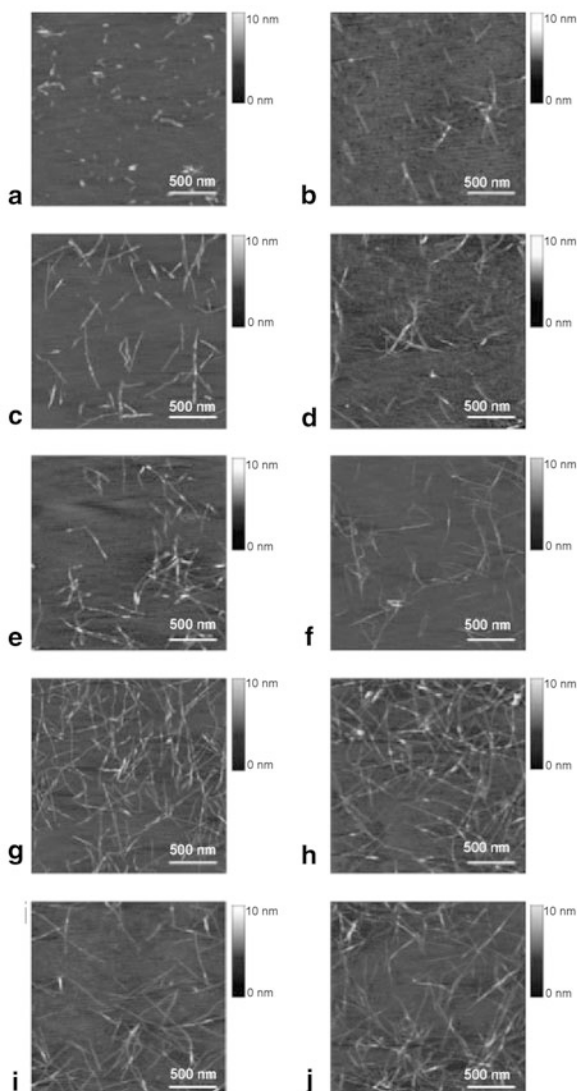
**Fig. 2.2** The self-assembled structures of EAK16-II and—IV differ due to the potential of EAK16-II to adopt either an extended beta-sheet or a folded beta-turn at different pH values. Adapted with permission from (Hong et al. 2003). Copyright 2003 American Chemical Society



a couple of minutes, these fibers then continue to grow over time. It can be seen in Fig. 2.3a and further images that the fibers extend from the remaining fiber fragments to form long fibers again. The storage modulus,  $G'$ , of the sample increases over time during this process indicating that the solution is reverting to a hydrogel. Disruption of the hydrogel can be repeated several times, and within 4 h the hydrogel is nearly completely returned to its original state, after 24 h it has identical properties. It is proposed that sonication causes the long fibrils of the hydrogel to break into very short sections that can then reassemble with one another, especially as the hydrophobic interaction between the beta-sheets is non-specific allowing sheets to slide over one another (Yokoi et al. 2005). This effect is often seen with self-assembled peptides, where the fibrillar structure can be disrupted by sonication or shear forces and will

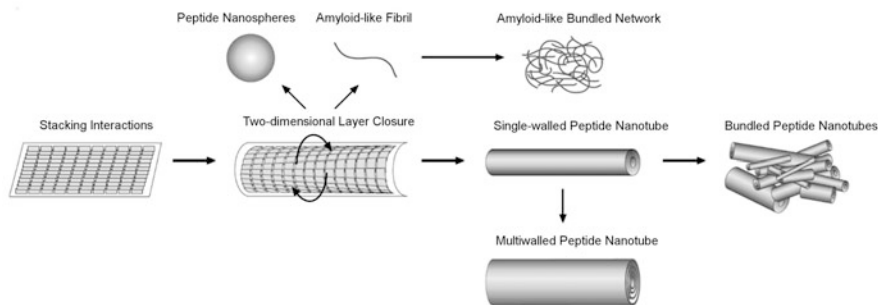


**Fig. 2.3** AFM images of RADA16-I nanofiber at various time points after sonication. 1 min (a), 2 min (b), 4 min (c), 8 min (d), 16 min (e), 32 min (f), 64 min (g), 2 h (h), 4 h (i), and 24 h (j). Fibers can be seen to elongate over the time course of the experiment indicating the re-assembly of the initial fibers. (Yokoi et al. 2005)  
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then re-assemble over a period of time of rest, this effect has been used in a number of systems for applications (Altunbas et al. 2011; Haines-Butterick et al. 2007).

Through the study of amyloid structures and the core sequence of Abeta it was proposed that diphenylalanine would self-assemble into a fibrous structure. This is indeed the case and it has been shown to self-assemble into fibrous structures from aqueous solvents and additionally it will form a range of structures in various organic solvents (Rechtes and Gazit 2003, 2004, 2006a). The molecular structure is



**Fig. 2.4** Schematic model for the formation of the various structures by the aromatic homodipeptides. Varying structures could be formed by alternative organization of the ordered layers. The specific organization of the layers could lead to tubular (single-walled or multiwalled), spherical, or fibrillar structures. Due to the geometrically restricted interactions of the aromatic moieties and their complex hydrophobic and electrostatic nature, various changes in the electronic environment of the aromatic system in the context of very small peptide, can significantly affect the organization of the assembled formed (Reches and Gazit 2006b)

proposed to consist of the pi-stacking of the aromatic side chains with one another, while the backbone hydrogen bonds form a beta-sheet structure (Amdursky et al. 2009; Reches and Gazit 2004). In organic solvents this can then form tubes as well as particles (Reches and Gazit 2003, 2004). If the aromatic side chains are replaced with aliphatic side chains of similar hydrophobicity the peptide does not self-assemble in aqueous solvents, however, they can still be made to assemble in some organic solvents (Mishra and Chauhan 2011). Spectroscopic studies of these structures indicate that they have a beta-sheet structure, however the shortness of the sequence means that they may not have all the traditional features of a beta-sheet structure (Reches and Gazit 2006b). This is due to the presence of only one peptide bond which means that there is the potential to adopt more conformations than the normal observed conformations in peptides. Crystal structure studies of a range of dipeptides indicates that diphenylalanine forms a structure where both side chains are on one side of the peptide backbone to allow for packing of the side chains, a high energy configuration, but this is energetically off-set by the packing of the side chains (Gorbits 2007). Models of this peptide propose that the self-assembly process proceeds to sheets which can then either remain as sheets in the traditional amyloid like structures of they can roll up to form tubular structures (Fig. 2.4) (Reches and Gazit 2006b).

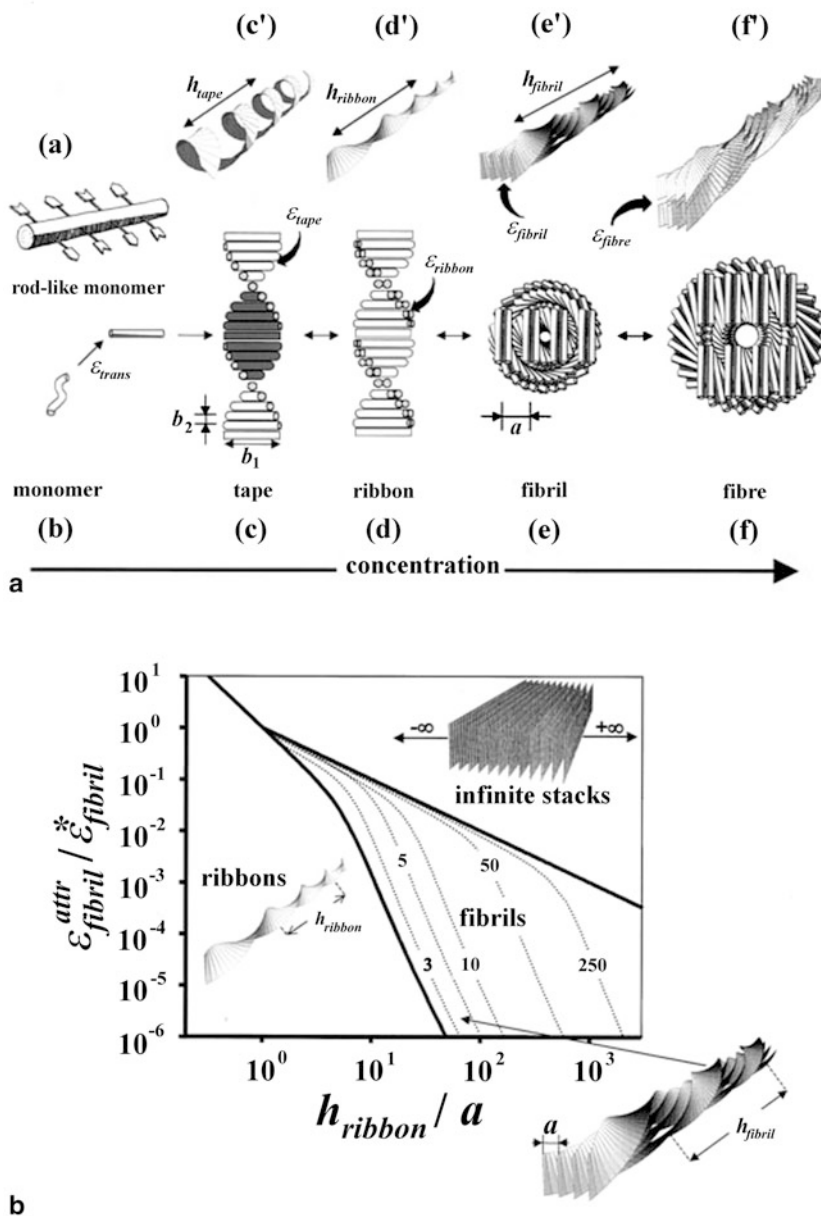
With organic solvents it has been shown that letting the solvent evaporate allows the formation of 3D structures that have been termed nano-forests due to the appearance of many vertical tubes all packed next to one another. These structures have been demonstrated to be formed from a number of solvents. Characterisation of the structures formed by X-ray and electron diffraction indicate that the tubular structures have a crystalline structure with a hexagonal symmetry (Reches and Gazit 2006a).

Derivatives of these very short peptides have also been investigated that include an additional hydrophobic group on either the N- or the C-terminus. These groups tend to be either 9-fluorenylmethoxycarbonyl (Fmoc) (Jayawarna et al. 2006; Smith et al. 2008; Zhang et al. 2003), or tert-butoxycarbonyl (Boc) (Mahler et al. 2006), in the case of the N and C-terminus respectively, which are convenient to produce as the last deprotection step in the synthesis of these peptides is missed out during synthesis to give these products. Additionally, groups such as naphthalene have been used (Jayawarna et al. 2007). These peptides readily form hydrogels made up of a fibrous network that has at its core a beta-sheet structure. In the case of Fmoc dipeptides it is necessary to use a pH of 7 or below, in the case of diphenylalanine, and for other dipeptide sequences pH 3–5, for a hydrogel to occur. This pH requirement is related to the protonation of the unprotected C-terminus as it is the only charged group in the peptide due to the N-terminus being blocked by the Fmoc group. Interestingly, the self-assembly process which the peptides undergo results in the apparent pK<sub>a</sub> of the C-terminus shifting by over 6 pH units for the assembly of individual fibers (Tang et al. 2009). This highlights the fact that strong hydrophobic interactions seen in the self-assembly of peptides can sometimes result in the apparent pK<sub>a</sub> values of amino acid side chains and the peptide termini shifting significantly away from their expected values.

There are a range of peptides that have been developed that are based on the use of short peptides with additional moieties appended to aid self-assembly. These include small drug molecules (Yang et al. 2004; Yang et al. 2005) to the large range of peptide amphiphiles that utilize alkyl chains attached to short peptides. However, these do not inherently rely on the beta-sheet structure for self-assembly and will not be further discussed here but these reviews cover these structures (Hamley 2011; Matson and Stupp 2012).

### 2.4.2 *Peptides Based on Designed Sequences*

Due to the simplicity of the sequence requirements of a beta-sheet there have been many attempts to design beta-sheets that will self-assemble. The vast majority of beta-sheet based peptides used for self-assembly utilize an alternating hydrophobic/hydrophilic pattern similar to the previously described systems, however many incorporate turns or differing arrangements of charges to create more complex and responsive self-assembled systems. An early example of a designed peptide was DN1 which was designed by the Aggeli group with the sequence CH<sub>3</sub>CO-Gln-Gln-Arg-Phe-Gln-Trp-Gln-Phe-Glu-Gln-Gln-NH<sub>2</sub> (Aggeli et al. 1997). DN1 was designed to self-assemble in water due to the hydrophobic interaction of the CH<sub>2</sub> groups of the glutamine side chains as well the hydrophobic interaction of the phenylalanine and tryptophan side chains. The charged residues, glutamic acid and arginine provide a level of specific recognition to the self-assembly process. DN1 has been shown to self-assemble at 15 mg/mL in water to a thermally stable hydrogel with an



**Fig. 2.5** Model of hierarchical self-assembly of chiral rod-like units. **a** Local arrangements (c–f) and the corresponding global equilibrium conformations (c'–f') for the hierarchical self-assembling structures formed in solutions of chiral molecules (a), which have complementary donor and acceptor groups, shown by arrows, via which they interact and align to form tapes (c). (e and f) The front views of the edges of fibrils and fibers, respectively. **b** Phase diagram of a solution of twisted ribbons that form fibrils. The areas divided by the thick lines reveal the conditions where ribbons, fibrils, and infinite stacks of completely untwisted ribbons are stable. (Aggeli et al. 2001) Copyright (2001) National Academy of Sciences, USA

anti-parallel beta-sheet structure, as determined by FTIR spectroscopy, which fits to the intended design of the structure (Aggeli et al. 1997).

Further designs based on the peptide DN1 utilized either only positively or negatively charged amino acids as the charges in each peptide. As the peptides have an overall charge they only self-assemble under conditions where the charge on the peptide is neutralized to some degree. In this way it was possible to relate the fluid/hydrogel transition to the charge state of the peptides and also determine the shifts in the pKa values of the side chains. It was found that the incorporation of one oppositely charged residue into the peptide, to give it a single charge once the main charged residues in the peptide are neutralized, helped prevent the formation of a flocculated material and maintained a stable hydrogel over a wider pH range (Aggeli et al. 2003b).

By designing peptides with opposite charges it was possible to create a two component system where even though the individual peptides are both in solution at pH 7, when mixed they immediately self-assemble with one another to form a hydrogel in a one to one ratio (Aggeli et al. 2003a). Using an excess of one of the peptides resulted in the solution containing the unfolded excess peptide, while the other peptide and the equivalent amount of excess peptide was found in the hydrogel. At either higher or lower pH values for the positively and negatively charged peptides, respectively, the peptide is capable of self-assembling with itself due to neutralization of the charged residue at these pH values the other peptide remaining in solution.

The self-assembly process for peptides based on DN1 can be described by a mathematical model relating the peptide concentration to a number of parameters which makes it possible to determine the degree of self-assembly at various concentrations (Fig. 2.5). This model defines the self-assembly process as a series of stages each with a critical concentration of the previous structure required for the next stage to be initiated (Fig. 2.5a). The model describes the process from the transition of an unfolded peptide to a folded beta-sheet structure and then its assembly into tapes and through several association events up to a fiber that consists of multiple tapes. The model also relates the helical pitch of the fiber to the assembly process of the fibers due to the energy requirements to fit two helical tapes together (Fig. 2.5b) (Aggeli et al. 2001). This model effectively describes how a number of other similar peptide designs, and even natural peptides associate to form fibrillar structures.

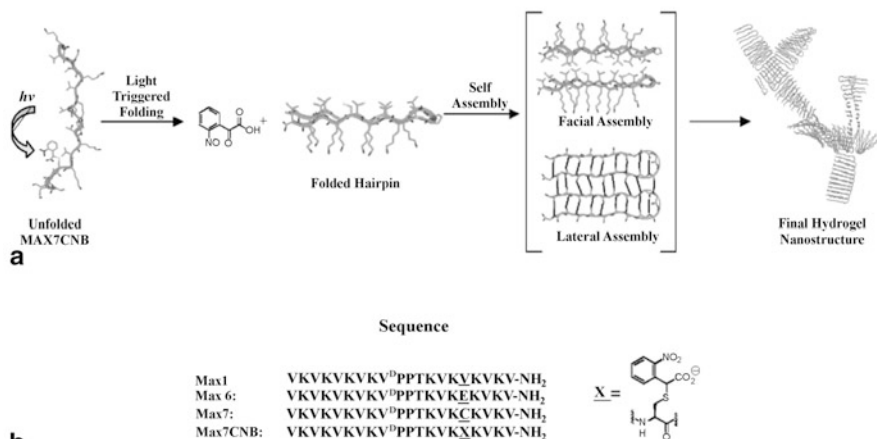
Further designs related to DN1 were made which change the charge residues in the peptide and replace the terminal glutamine residues with serine residues, which alters the overall peptide charge and hydrophobicity, respectively. These changes resulted in a change in the hydrogel phase diagram, for example, altering the overall charge of the peptides from negative to positive, results in a switch between gelation at high and low pH respectively. This demonstrated that similar charge effects related to the DLVO theory are observed for these peptides as for the ionic complimentary peptides (Carrick et al. 2007) which shows that these effects are generic to peptide self-assembly.

The previous designed peptides self-assemble through the association of individual beta-strands. Other designed beta-sheet based self-assembled fibers utilize peptides that form one or more turns that triggers the self-assembly process into a

fibrous structure. Some of these designs have utilized the fact that synthetic peptides can incorporate non-natural amino acids which allows specific conformations to be favored by the peptides. In the case of the peptide MAX1, a beta-hairpin has been designed that incorporates a D-proline as well as a naturally occurring L-proline, which forces the adoption of a type II' turn (Fig. 2.1, Panel 3). In this way it is possible to favor the formation of a hairpin conformation by the peptide. The presence of positively charged residues in the sequence ensures that self-assembly only occurs at high pH or in the presence of high salt and that the peptide undergoes self-assembly at temperatures higher than 25 °C (Schneider et al. 2002). The process of self-assembly of these peptides takes hours for unstirred solutions indicating that in the case of these peptides the rate limiting step is the self-assembly process rather than the folding of the peptide as when the concentration is raised from the micromolar concentrations used in spectroscopic measurements to the millimolar concentrations used in rheology measurement the self-assembly process is complete within minutes (Schneider et al. 2002). By careful redesign altering the charged lysine residues in the peptide to glutamate residues in a systematic way it has been possible to find a peptide sequence that can undergo self-assembly at physiological conditions of pH 7.4, 150 mM NaCl, and 37 °C (Rajagopal et al. 2009). These beta-hairpin structures also demonstrate the same shear sensitivity and repair ability of the purely beta-sheet based structures described earlier, which seems to be an inherent ability of many peptide based self-assembled fibers and hydrogels (Schneider et al. 2002).

Further responsiveness has been built into these peptides through the incorporation of various switches which makes the peptides sensitive to external stimuli, only self-assembling after stimulation. In the first case a light sensitive switch was incorporated into the peptide.  $\alpha$ -carboxy-2-nitrobenzyl a photocage was incorporated into the hydrophobic face of the peptide design. The photocage is negatively charged and it was demonstrated with a test peptide that the presence of a negative charge in the hydrophobic face of these peptides prevents self-assembly (Fig. 2.6b). With the photocage present the peptide remains in solution, however, on exposure to UV light the photocage is removed leaving just the cysteine residue to which it was attached and then folding of the peptide and self-assembly can occur (Fig. 2.6a). Analysis shows that at least 60 % of the peptides are degraded and that disulphide bonds are not forming between the peptides during this process (Haines et al. 2005). An alternative to using light to trigger self-assembly is the use of a metal binding site, 3-amidoethoxyaminodiacetoxy-2-aminopropionic acid, again negatively charged in the absence of  $Zn^{2+}$ , but neutrally charged once it has bound the metal ion. This non-natural amino acid was incorporated at the C-terminal position of the designed peptide where it would be in the hydrophobic face of the folded structure. As a consequence the negative charge again prevents folding of the peptide to a beta-hairpin and thus assembly, while the presence of zinc ions permits folding and hence self-assembly can occur (Micklitsch et al. 2011).

The basic design discussed above utilized a single beta-hairpin, a recent design has attempted to utilize multi-stranded peptides that incorporate more than one hairpin. This has been achieved with a design TSS1 that has two hairpins, again consisting of a



**Fig. 2.6** **a** Light-induced material formation. UV illumination of MAX7CNB results in side-chain decaging and  $\beta$ -hairpin intramolecular folding. Subsequent facial and lateral self-assembly ultimately produces a hydrogel material. **b** Sequences of carboxyamided peptides.  $\alpha$ -carboxy-2-nitrobenzyl protection of the cysteine in MAX7 gives MAX7CNB. Reprinted with permission from (Haines et al. 2005). Copyright 2005 American Chemical Society

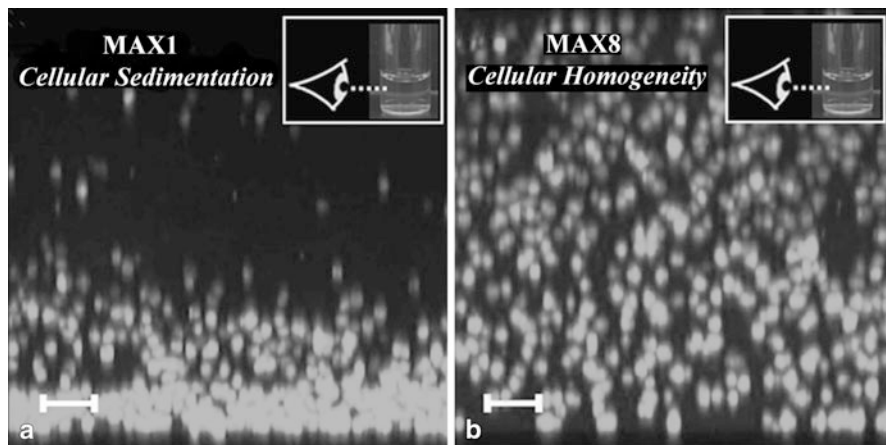
D- and L- proline to create the type II' turn and strands consisting of alternating valine and lysine residues. This design has been shown to self-assemble when the temperature is raised to 37 °C probably related to the greater impact of the hydrophobic effect at higher temperatures (Rughani et al. 2009).

## 2.5 Applications

### 2.5.1 Biological Applications of Beta-sheet Based Fibers

Many of the systems described above have been developed for specific applications or have later been modified towards applications. The obvious area of applications for a peptide based self-assembled fiber, especially those which can form hydrogels is as a scaffold in cell culture. One of the main challenges of cell culture is to provide an environment for cells to grow which is as natural as possible, this is especially true where tissue regeneration is desired as a three dimensional scaffold is required. These artificial extracellular matrices must be biocompatible and ideally be capable of interacting with the cells, as well as allowing the cells to replace the scaffold with a natural extracellular matrix once they have proliferated and grown sufficiently. As such a number of *in vitro* studies have been undertaken into a variety of the self-assembled systems described in the previous sections as well as using other peptide folds (Collier et al. 2010). Here we will discuss just a few of the studies that have looked into the viability of different cell lines on these materials.





**Fig. 2.7** Encapsulation of mesenchymal C3H10t1/2 stem cells in 0.5 wt % MAX1 and MAX8 hydrogels. Light microscopy images shows the incorporation of cells into a MAX1 gel leading to cell sedimentation (**a**) and into a MAX8 gel resulting in cellular homogeneity (**b**) (Scale bars: 100  $\mu\text{m}$ .) (Haines-Butterick et al. 2007) Copyright (2007) National Academy of Sciences, USA

The ionic complementary peptides based on the EAK16 peptides have been utilized in many cell culture studies. Early *in vitro* experiments tested the biocompatibility of these peptides and also investigated the incorporation of modified peptides that have cell interaction motifs either in the peptide or as an additive to the hydrogel. These hydrogels were shown to be capable of supporting cell attachment (Zhang et al. 1995). In one study several neuronal cell types were shown to extend neurites through RAD16-II hydrogels, with the neurites following the contours of the hydrogel scaffold (Holmes et al. 2000), this peptide is also commercially available under the name Affimatrix. It has also been shown that hydrogels of RAD16-I and II can support Human umbilical vein endothelial cells (HUVEC) and these cells will form interconnected capillary-like networks. However, hydrogels of KFE8 and KLD12 do not support cell attachment and growth which seems to indicate that the primary sequence of the peptides used in the fibers affects cell attachment (Sieminski et al. 2008).

As mentioned previously a lot of these self-assembling fibers which form hydrogels have shear thinning properties, which after removal of the shear forces re-assemble to a hydrogel. Some of these systems take hours to achieve this however, it is possible to redesign peptides to improve the recovery time. The designed peptide MAX1 was unsuitable for this purpose due to slow hydrogelation kinetics, resulting in the cells sedimenting before gelation was complete (Fig. 2.7a). The peptide was redesigned, MAX8, to incorporate a single negative charge into the positively charged sequence to increase the hydrogelation kinetics. A hydrogel made from MAX8 can be passed through a syringe needle due to the shear thinning properties of the hydrogel which then reforms rapidly to prevent cell sedimentation and give an even distribution of cells throughout the hydrogel (Fig. 2.7b) (Haines-Butterick et al. 2007). This peptide will self-assemble to a hydrogel in the presence of the cell



culture media DMEM. Thus the peptide can be mixed with a cell suspension in a syringe, and then be injected into tissue where it will reform its original hydrogel structure making this peptide suitable for 3D cell culture.

As many of the self-assembling peptides lack recognition motifs that aid cells in attachment and proliferation in the extracellular matrix, there have been attempts to incorporate these into the artificial systems. As such, mixed systems of the basic self-assembling peptides and a ratio of peptides that have been modified with one or more of these recognitions motifs have been developed. One example utilizes the transthyretin (105–115) peptide (TTR1) from the human transthyretin sequence as the basis of the self-assembled fibers, with the integrin binding sequence RGD or a control sequence RAD at the C-terminus of TTR1. The self-assembled structures of each peptide bound the normal amyloid dyes. The fibrils of the modified TTR1 were seen to be approximately twice as wide as the basic TTR1 sequence, possibly due to lateral association due to the appended sequences. Trypsin digestion of the self-assembled structures demonstrated the availability of the RGD and RAD motifs on the surface of the fibers. The addition of the dansyl fluorophore to the C-terminus of each peptide for immuno-gold labeling allowed the testing of mixed fibers consisting of the basic TTR1 peptide plus one of the modified peptides. TEM images showed that the spacing of the labels could be controlled by altering the ratio of unlabeled to labeled peptide with the distance between RGD motifs varying from 0.5 (100 % label) to 85 nm (1 % label), which covers the natural spacing of integrin receptors at 50 nm. Cell attachment studies with mouse fibroblast 3T3 cells showed that the RGD modified TTR1 had the capability of inhibiting cell attachment to fibronectin coated surfaces and also had comparable cell attachment properties to fibronectin coated surfaces (Gras et al. 2008). These results demonstrate that natural sequences can potentially be modified and utilized in cell culture.

Another example of mixed systems used the designed peptide Q11 (QKQ-FQFQFEQQ), a variant of the DN1 peptide (Aggeli et al. 1997), with an N-terminal RGDS sequence, an integrin binding sequence, or IKVAV, a sequence that modulates neuron cell attachment and neurite outgrowth. Incorporation of 10 % mol labeled peptides into the hydrogel does not disrupt the formation of the hydrogel or alter the hydrogels mechanical properties. The presence of the ligands had the expected effect of improving the attachment of HUVEC cells and modulating neurite outgrowth, for the incorporation of RGDS and IKVAV, respectively (Jung et al. 2009). The Q11 peptide has also been utilized to demonstrate an increased immune response to the OVA<sub>323–339</sub> (ISQAVHAAHAEINEAGR) sequence, a H-2<sup>b</sup>-restricted class II peptide containing multiple antigenic determinants. This peptide was attached to the N-terminus of the Q11 peptide through a short serine-glycine linker and shown to self-assemble into mixed fibers. The fibers were then shown to give higher IgG titers in mice compared to the peptide alone, and cross reactivity demonstrated that this was not due to the Q11 peptide. It is hypothesized that the effect is due to the multi-valent epitope surface of the self-assembled fibers, interestingly, it appears that the self-assembled structure of Q11 does not generate an immune response itself (Rudra et al. 2010).

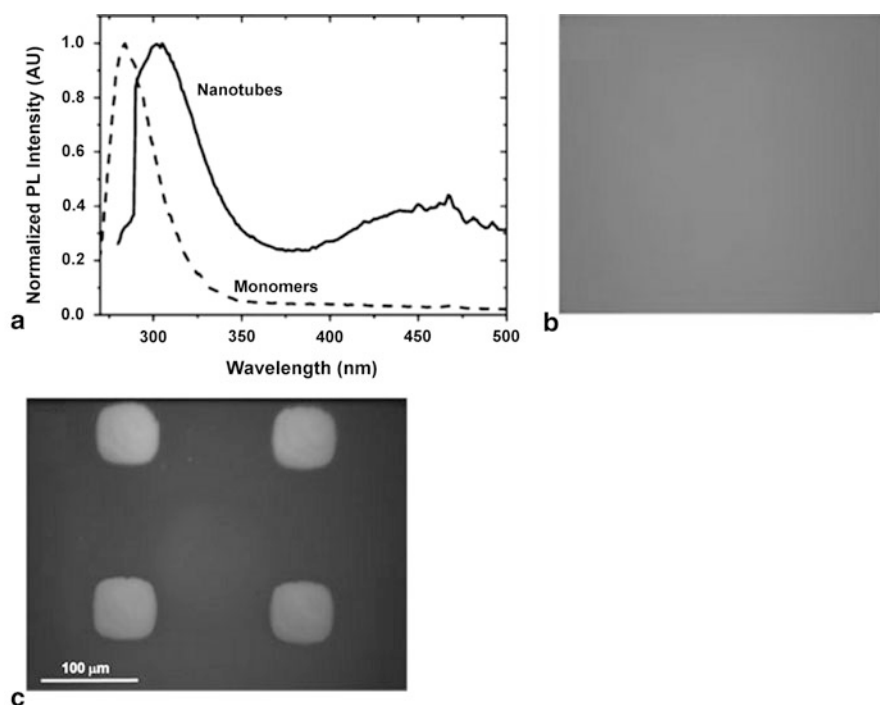
Other self-assembling fibers have been specifically developed for the purpose of acting as templates for the formation of a mineralized material. Hydroxyapatite is a major component of dental enamel. During tooth formation the hydroxyapatite deposition is controlled by enamel matrix proteins, which are anionic. However, these are not present in the mature tooth and hence replacement of hydroxyapatite in the tooth is not possible. Self-assembled fibers offer the potential to act as a replacement matrix which can then be used to deposit new hydroxyapatite. This has been demonstrated using a designed peptide similar to the DN1 peptide which is anionic from the Aggeli group. This peptide was shown to be capable of slowing the degradation of hydroxyapatite in lesions on a tooth during pH cycling and also the hydrogel of this peptide showed the potential to nucleate the formation of amorphous hydroxyapatite over a period of 7 days (Kirkham et al. 2007).

### ***2.5.2 Nanotechnological Applications of Beta-sheet Based Fibers***

While the previous examples have concentrated on the more common concept of utilizing a biological fibre as a replacement for natural biological materials others have looked at the use of these biological fibres in non-biological applications. In the case of di-phenylalanine the nanotubes formed from this material have been placed in a solution of silver ions and then through the use of counterions the silver ions have been precipitated to produce a peptide nanotube filled with silver, the peptide coating can then be removed in this case through the use of proteases, but it also possible to use chemical methods to remove the protein layer leaving the metal wire, which has defined nanometer widths (Reches and Gazit 2003).

This has been taken a step further for diphenylalanine as it turns out that as well as fibres it is possible to make small particles from this material (Fig. 2.4). By taking diphenylalanine and dissolving it in hexafluoroisopropanol and then diluting into either methanol or water, either particles or fibres are formed. The particles all have uniform dimensions and it is possible to switch between the two states by changing the solvent, indicating that the structures are related. Spectroscopic analysis of the particles and tubes indicate that they have the property of quantum confinement which means that the particles act as quantum dots and possess semi-conductor properties (Amdursky et al. 2010). These properties are related to the orientation and interaction of the peptide side chains to one another in the self-assembled structure and thus are limited to those peptides that have aromatic side chains.

By using vapour deposition of diphenylalanine it is possible to construct patterned arrays that possess interesting photonic and also piezo-electronic properties, highlighting the potential of biological based nanomaterials in nanoelectronics and other areas normally not associated with biological based materials. For example it is possible to pattern the diphenylalanine nanotubes onto a surface where they are fluorescent due to their structure acting as quantum wells (Fig. 2.8), which has potential in the development of biological lasers (Rosenman et al. 2011; Lee et al. 2011). There are an increasing number of research papers looking into this area of research and similar applications, these will not be further discussed here as the peptide is



**Fig. 2.8** **a** Photoluminescence (PL) of diphenylalanine peptide nanotubes (PNT) (solid line) and diphenylalanine monomers (dashed line); the excitation wavelength was 260 nm. **b** *blue* PL image of homogeneous PNT array deposited by vapor deposition technology. **c** PNT-light emitting devices (patterned array) (Rosenman et al. 2011)

being used mostly for its chemical and molecular properties and not for its peptide structure (Huang et al. 2011; Yan et al. 2010; Wang et al. 2011).

## 2.6 Summary

To summarise, peptides have been utilized to form self-assembling fibres. Of special interest here are the peptides based on the  $\beta$ -sheet fold, of which there are many such systems. The understanding of how  $\beta$ -sheets fold and the side chains interact with one another has allowed both designed and natural sequences to be successfully utilized for the formation of fibrous structures. While some sequences are based on those observed in amyloid forming proteins, most are derived from other natural sequences and designed sequences which rely on a combination of charge interactions and hydrophobic interactions as well as the backbone hydrogen bonding of the beta-sheet structure for self-assembly. These fibrous structures often form hydrogels due to the high density of fibers and in some cases the physical crosslinking of the fibers with one another. In general these hydrogels have been utilized for cell culture

based applications due to their biocompatibility and amenability to modification with biological markers and recognition motifs. This has allowed them to be tested with mammalian and human cells in both 2D layers on a hydrogel and also in 3D cell culture where the cells are incorporated into the hydrogel. While there is one example system used commercially for cell culture it has yet to be seen if these peptides can be taken further and become suitable for medical applications. Some systems have also been shown to be suitable for biomineralization such as the formation of hydroxypapatite crystals opening up the potential for their use in hard biological materials. Lastly the very short peptides have been shown to exhibit quantum confinement due to the arrangement of the side chains and structure formed by the peptides. This potentially means that these peptides have applications in technologies not normally associated with biological systems, such as in the formation of nanoscale electrical components. Thus the area of peptide self-assembly appears to continue to have a wide range of applications available to it and has much promise for the future.

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Systemic Amyloid Disease

Harris, J.R. (Ed.)

2012, XVIII, 650 p., Hardcover

ISBN: 978-94-007-5415-7