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Self–Assembled Peptide Nanostructures for Biomedical Applications: Advantages and Challenges

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1. Introduction

Over the last 20 years, self-assembled nanostructures based on peptides have been investigated and presented as biomaterials with an impressive potential to be used in different bionanotechnological applications such as sensors, drug delivery systems, bioelectronics, tissue reparation, among others. Several advantages (mild synthesis conditions, relatively simple functionalization, low-cost and fast synthesis) confirm the promise of these biological nanostructures as excellent candidates for such uses.

Through self-assembly, peptides can give rise to a range of well-defined nanostructures such as nanotubes, nanofibers, nanoparticles, nanotapes, gels and nanorods. However, there are several challenges that have yet to be extensively approached and solved. Issues like controlling the size during synthesis, the stability in liquid environments and manipulation have to be confronted when trying to integrate these nanostructures in the development of sensing devices or drug-delivery systems. The fact that these issues present difficulties is reflected in the low number of devices or systems using this material in real applications.

The present chapter discusses these challenges and presents possible solutions. A review of the state-of-the-art work concerning the use of peptide self-assembled structures in biomedical applications is given. Additionally, our findings regarding the on-chip synthesis of peptide self-assembled nanotubes and nanoparticles, their controlled manipulation, as well as electrical and structural characterizations are introduced. Our latest results showing the interaction of peptide self-assembled structures with cells for the development of a combined sensing/cell culture platform and the use of these material in clean-room processes together with the stability of the biological structures in liquid are also presented.

2. Peptide nanostructures formed by self-assembly

The field of biological self-assembly is very diverse and the structures formed can vary tremendously in both shape and size. For this reason, a full description of all possible self-assembled structures and the monomers forming them is beyond the scope of this chapter. Rather, the focus will be on the applications and challenges that one needs to be aware of when working with such structures. For this, it is important to have a certain understanding

of the process behind the formation and this section therefore provides a brief introduction to the concepts behind self-assembly along with a short description of the most important structures that can be formed though self-assembly. Reviews have been written about each of the different structures and we therefore by no means claim to provide an exhaustive account of these configurations.

The structures formed by hydrophobic dipeptides and those formed by diphenylalanine will be given special attention since they are able to give rise to nanotubes, nanofibers and nanoparticles depending on the formation conditions. Furthermore, these structures will serve as model materials throughout the chapter to illustrate the challenges faced when working with the self-assembly of structures. Table 1 gives an overview of some self-assembly structures. We recommend that the interested reader consult the references in the table for further information.

Structure	Example of Monomer	Relevant references
Nanotubes	Hydrophobic dipeptides, cyclic peptide, linear peptides	(Bong et al., 2001, Gorbitz, 2001, Nelson et al., 1997)
Nanofibers	Hydrophobic peptides, beta sheets	(Kim et al., 2010, Scanlon & Aggeli, 2008, Wiltzius et al., 2009)
Nanoparticles	Boc – Diphenylalanine	(Adler-Abramovich & Gazit, 2008, Bohr et al., 1997, Krebs et al., 2007, Nigen et al., 2010, Slotta et al., 2008)
Nanotapes	P_{11}-II	(Fairman & Akerfeldt, 2005, Fishwick et al., 2003)
Gels	K24	(Aggeli et al., 1997, Fairman & Akerfeldt, 2005)

Table 1. Overview of several structures that can be formed through self-assembly. For each category, examples of monomers capable of forming the particular structure are given. Further information regarding the different structures can be found in the articles mentioned in the last column.

Even though the specific configuration of the different monomers, as well as the structures formed from them, vary a lot both in shape and size, the overall driving force behind the formation procedure is the same: entropy. Self-assembly of the monomers occurs when it is thermodynamically favorable for them to be incorporated in the particular self-assembled structures rather than be exposed to the solvent from all sides. The structures listed in Table 1 are described in more detail below.

2.1 Nanofibers

Self-assembling nanofibers have long been a focal point for researches all around the world and for good reason. These structures have received much attention as potential building blocks for the next generation of biosensors. They have been considered both as a fabrication material and as important components in the final device (as electrode modification or as the central part of a biological field effect transistor (BioFET)).

Many different structures have been shown to self-assembly into nanofiber-like conformations. The most well-known of these are undoubtedly the amyloid fibrils. These

beta sheets of aminoacids stack together in aggregates to form long insoluble fibrils. The insolubility of the structures can be harmful in the body and, for instance, Alzheimer's disease is caused by such an aggregation of the amyloid beta 42 protein fragments. However, in biosensor applications the insolubility of the self-assembled nanofibers is highly desirable since it will insure the long-term stability of the sensor.

2.2 Nanotubes

Overall, the shape of nanotubes is very similar to that of the nanofibers discussed above; the difference being that the nanotubes are hollow. Nanotubes can therefore be used in much of the same applications as nanofibers and can furthermore be employed in implementations where the cavity inside the structures is loaded with drugs or for reducing metal ions to form nanowires of metal covered with a peptide shell for easy functionalization purposes (Reches & Gazit, 2003).

Nanotubes can be obtained from a large variety of monomers such as cyclic peptides, as demonstrated in (Ghadiri et al., 1993) and (Tarek et al., 2003), linear peptide fragments such as phenylene ethynylene oligomers (Kim et al., 2010, Slotta et al., 2008) and disc-shaped motifs. Another structure that shares the tubular configuration is that of the rosette nanotubes formed from a heteroaromatic bicyclic base (Fenniri et al., 2002). Figure 1 offers illustrations of these different formation processes.
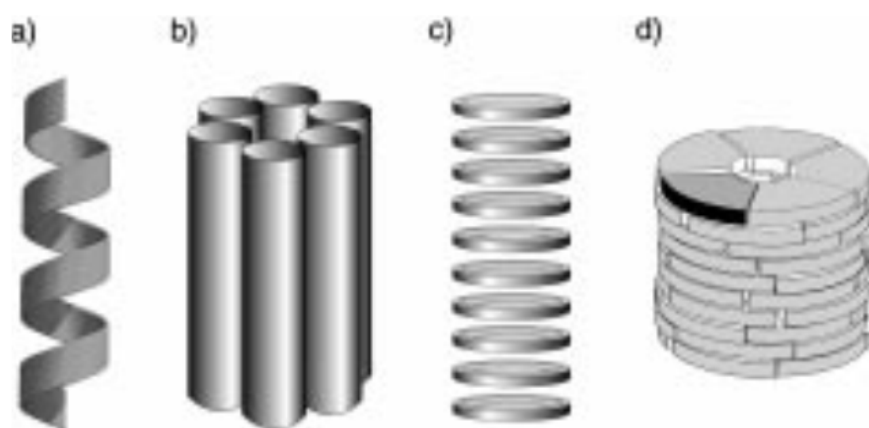

Fig. 1. Examples of self-assembly processes that can give nanotube structures: coiled peptide fragment similar to the alpha helices in proteins, supramolecular assembly of tubular structures to form larger tubes, stacking of cyclic peptide motifs, and an arrangement of disc-shaped motifs in larger nanotubes. Reprinted from (Bong et al., 2001).

2.3 Nanoparticles

The field of nanoparticles is somewhat diverse and covers the small well-defined structures formed by different monomers. Such structures range from nanospheres with a hollow core to various solid structures. The hollow particles have received much attention as possible drug delivery candidates and their non-hollow counterparts as biological variants of nanobeads.

2.4 Nanotapes

Peptide nanotapes are formed from the stacking of peptide beta sheets as described in (Fishwick et al., 2003). The formed tape structure often interacts to form double layers with

similar nanotape structures. When the concentration of these nanotapes exceeds a certain threshold, they tend to form hydrogels as described below (Aggeli et al., 1997).

2.5 Hydrogels

Hydrogels based on peptide structures constitute interesting "smart" materials in which the properties of the gel change depending on various parameters such as pH, ionic strength, temperature and salinity (Aggeli et al., 2003). These hydrogels can be formed by a number of different self-assembled structures such as for instance the nanotapes mentioned above (Fairman & Akerfeldt, 2005).

2.6 Hydrophobic dipeptides

Several structures formed through a self-assembly process have been described very briefly above. To provide the reader with an idea of the structure of such self-assembled materials, a more thorough description of the conformations formed by the hydrophobic dipeptide diphenylalanine is presented below.

Hydrophobic dipeptides, and in particular diphenylalanine, are rather unique monomers since – depending on the formation condition – they can self-assemble into nanotubes or nanofibers. Moreover, with addition of a protective group at the amino group of the peptide, they can also form spherical nanoparticles. The structures formed from this material will be used to illustrate the various topics covered in the present chapter and are therefore described in more detail.

The diphenylalanine monomer has named a full class of self-assembling molecules known as the FF class in which the monomers have in common that they are hydrophobic dipeptides with somewhat bulky side-chains. Some of the monomers in this structure class are illustrated in Figure 2. This section offers only a brief description of the assembled structure, whereas a more detailed one of the self-organizing process from hydrophobic dipeptides can be found in (Aggeli et al., 2003, Gorbitz, 2007, 2001, 2006).
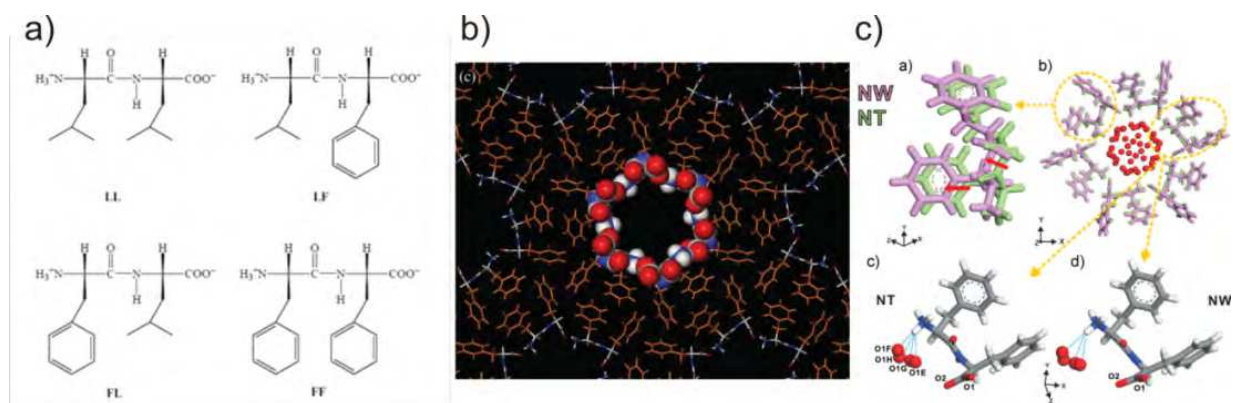

Fig. 2. Structures of different hydrophobic dipeptides that form nanotubes according to the diphenylalanine peptide shown in the lower right corner. Reprinted with permission from (Aggeli et al., 2003, Gorbitz, 2007, 2001).

Figure 2a) illustrates different examples of hydrophobic dipeptides, ranging from the bulky diphenylalenine to the somewhat smaller dileucine. All of the structures shown in the figure have in common that the side-chains of both the involved amino acids are somewhat hydrophobic - hence the name and the fact that they as any peptides have a hydrophilic

backbone along with free amino- and carboxylic groups in either end of the molecule. The most favorable configuration of the peptide is with two side-chains sticking out on opposite sides. However, when both side-chains possess hydrophobic properties, it is thermodynamically favorable to have them both on the same side of the peptide backbone, thereby facilitating the shielding of these hydrophobic regions. Figure 2b provides the structure of the nanotube formed by this monomer and Figure 2c compares the position of the monomers in the nanotubes and nanofibers.

The nanotubes are formed in solution by the addition of the peptide monomers to water, whereas the nanofibers are grown from an amorphous peptide film on a substrate in an aniline vapor. To form nanoparticles with this particular monomer, a tertbutoxycarbonyl group is attached to the amino group of the dipeptide (Adler-Abramovich & Gazit, 2008). When these modified dipeptides are emerged into a mixture of ethanol and water the nanoparticles are formed within minutes.
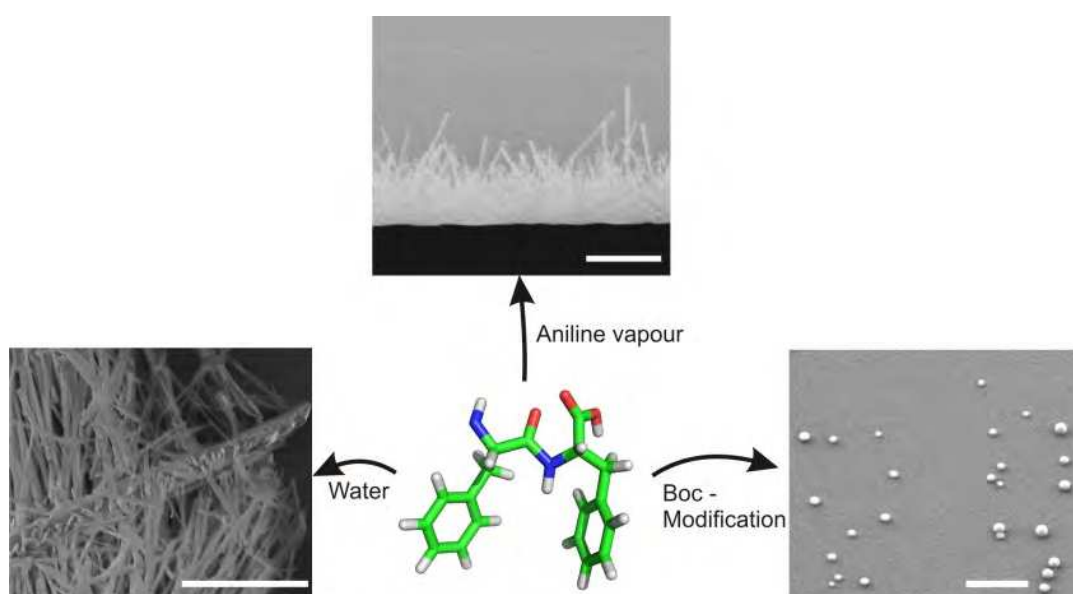

Fig. 3. SEM images of the three different structures that can be obtained from dipeptide diphenylalanine. Right) The nanotubes are formed in aqueous solution. Top) The peptide nanofibers that are grown from an amorphous film in an aniline vapor. Left) Nanoparticles formed from a modified version of the peptide monomer where the amino group is protected by a Boc group. The scale bar in the images corresponds to 1 μm.

Figure 3 illustrates the structure of the nanotubes formed by the diphenylalanine dipeptides. On the left one can see an image of how these smaller nanotubes assemble into larger microcrystals with radii up to a couple of micrometers. Both scale bars correspond to 1 μm and hence the single nanotube formed from diphenylalanine has a diameter around 200 nm. The top image presents a SEM image of the peptide fibers grown from a substrate. Here, it can be seen that the nanofibers are somewhat similar in width and length (both depend on the condition during formation). Finally, the right image shows the nanoparticles formed from the Boc modified diphenylalanine. The size of these spherical nanoparticles ranges from 50 nm to hundreds of nanometers.

All of the organization processes mentioned above are driven by entropy through hydrophobic - hydrophilic interactions and are further stabilized by hydrogen bonding

between the individual peptide fragments of which the structures consists. This fundamental knowledge will prove very important when the stability of the structures is assessed later in this chapter. The next section is devoted to different approaches for characterizing such structures. In order to understand the results, a general idea behind the formation process is necessary.

3. Characterization of self-assembled peptide nanostructures

Before one starts working with new materials it is important to characterize the materials in question to determine in which application the structures can be used. If for instance the structure turns out to have isolating properties, it may prove challenging to employ it in biological field effect transistors and one should either modify the materials or find another application. This section presents different means of characterizing the self-organizing structures with respect to their electrical, physical and chemical properties. Of course, these characteristics can be determined in a multitude of ways and the optimal approach would be to verify the results using several techniques investigating the same properties. A few examples on how such characterizations can be conducted are described below. The structures formed by diphenylalanine monomers presented in the previous section will be used as an illustrative case.

3.1 Atomic Force Microscopy (AFM)

An atomic force microscope (AFM) can be used in many different modes for the characterization of a number of parameters ranging from the configuration of the formed structures over the conductivity of these to the determination of the Young modulus. This section describes a few of these characterization techniques. The simplest task involves investigating the geometry of the formed structures directly through any of the standard AFM imagining techniques. When the outer geometry of the formed structures has been determined this information can be used later in the investigation. One can for instance employ AFM as an electrical force microscope (EFM) where a potential difference is applied between the tip and the sample. This would determine whether or not the structure is hollow. In such an approach the structure itself is first imaged using standard AFM techniques, after which the tip is raised some nanometers above the surface and kept at this distance using the data from the AFM measurement. The cantilever is then vibrated and the phase shift of the cantilever, which is strongly dependent on the material between the AFM tip and the substrate, is monitored. As a result, the appearance of the signal will differ depending on whether the sample is hollow or solid. A more detailed description of this technique can be found in (Clausen et al., 2011, Clausen et al., 2008).

AFM can also be utilized to determine the conductance of the formed structures using the AFM tip and gold electrodes placed on the substrate in a standard two-point electrical measurement. For this type of investigation, the structures need to be placed in a way where one end of them is on top of a metal electrode on the substrate and the other end extends over the isolating material. The AFM tip can then be positioned on top of the part of the structure that extends outside the metal surface. This way, the distance over which the I-V curve is measured can be varied and, by following the evolution of the signal, the contact resistance can be isolated from the resistance of the structure itself. If the contact resistance dominates the measurement, the resistance of the structure should not change with the distance. However, if the contact resistance can be neglected, the resistance should drop with the distance.

When the geometrical shape of the structures is known, AFM can be used in combination with a modeling approach to determine the Young modulus of the structure in question. The AFM tip is positioned on top of the structure and pressed into it. From the force distance curves, the Young modulus can be derived directly by a theoretical model as described in (Niu et al., 2007) or by a comparison to a simulation as presented in (Kol et al., 2005). One of the main criteria for a successful characterization is knowledge of the overall structure of the material. Since the models that the data are compared to are strongly dependent on the employed structure, the results would vary greatly for different assumptions. If for instance a structure is assumed hollow in the model when it is in fact solid, the interpretation of the force distance curves would lead to a much larger Young modulus than the real value.

The last example involves the direct observation of the thermal stability of the structures under dry conditions as described in (Sedman et al., 2006). In this approach, the AFM tip is placed on top of the structure to be investigated and the position of the tip is monitored as its temperature is increased. The structure thereby degenerates, which causes the tip to move downward as an indication that the temperature limit for this particular structure has been reached.

3.2 Electron microscopy and related techniques

Another widely used characterization tool is the electron microscope, including techniques ranging from scanning electron microscopy (SEM) to transmission electron microscopy (TEM) and related approaches such as Focused Ion Beam (FIB) milling. This section gives a few examples on how such methods can be used to characterize self-organizing structures. As for the AFM analysis, the simplest starting point for any investigation is to determine the geometry of the structure directly. Again, the surface shape of the formed structures can be readily visualized. However, more information can be gained from a thorough SEM analysis when combined with other methods.

By using TEM, it can be easily verified whether the formed structures contain cavities or not. In TEM, high-energy electrons are sent through the material so that the intensity of each point in the image corresponds to the number of electrons passing through that specific point in the sample. In such images – if the structure is indeed hollow – its central part will appear lighter than its walls (Clausen et al., 2011).

In combination with a simple hotplate, the thermal stability of the structures can be investigated as described in (Adler-Abramovich et al., 2006). In this approach, the formed structures are heated to a specific temperature and then investigated with SEM to determine whether they seem unharmed or not. This kind of investigation takes advantage of the good resolution of SEM combined with the fast imaging possibilities.

Finally, the strength of the structure can be investigated using SEM with a built-in FIB source. The structure to be analyzed is simply placed inside a FIB SEM and one measures the amount of time it takes to mill through the sample. By comparing the time to values known for other materials, it is possible to get an idea of the stability of the structure. If a FIB SEM is not available, similar experiments can be conducted in a reactive ion etching (RIE) machine as described in (Larsen et al., 2011).

3.3 Other characterization techniques

Not every parameter of the structures can be determined using these two classes of instruments and many other techniques are available for the characterization of the

parameters mentioned above with others. This section presents a few of these tools and focuses on the characterization of parameters that have not been brought up above.

Even though many structures are very stable under dry conditions, the results may be quite different when the same structures are submerged in a liquid. To test the stability of the formed configurations when wet, one needs to find a way of monitoring them when submerged. When this is not possible or when clearer results are demanded, the solution in which the structures are submerged can be monitored for the concentration of the monomers. For this purpose, a sample containing the structures is placed in a fresh solution with no structures or monomers present. Samples are then taken from the solution at different times so that the concentration of the monomers in the formerly fresh solution can be monitored as a function of time. (The concentration of the monomers can be measured using for instance HPLC). If the concentration of the monomers increases continuously over time until equilibrium is reached, this is an indication of the structures being dissolved in that solution. However, if the concentration of the monomers remains constant throughout the experiment then either the structures do not dissolve or dissolve very quickly. The process is described in more detail in (Andersen et al., 2011).

This section has focused on how standard lab tools and traditional imaging equipment can be used in the characterization of various parameters for self-assembled structures. Other standard characterization techniques such as Fourier Transformed Infrared spectroscopy (FTIR), x-ray diffraction (XRD), Differential scanning calorimetry (DSC), Thermogravimetric analysis (TGA) etc. can be employed on self-assembled structures as well as on traditional materials (Ryu & Park, 2008).

4. Advantages of self-assembled peptide nanostructures for biomedical purposes

There are many applications in the biomedical field where self-assembled peptide nanostructures could play an important role as part of biosensing platforms, as efficient drug-delivery systems, as contrast image agents or as a hydrogels for tissue reparation. This section presents the advantages that make this biomaterial such a promising candidate for such applications.

4.1 Synthesis

One of the more interesting properties of self-assembled peptide nanostructures is the fact that their synthesis takes place under non-harsh conditions. These types of biological supramolecular structures are normally fabricated at room temperature, aqueous environments and without using specialized equipment. These parameters mark a huge difference between these biological nanomaterials and nanomaterials traditionally used in nanotechnology such as carbon nanotubes or silicon nanowires of which fabrication implies high temperatures, specialized equipment and in some cases clean-room facilities increasing their production cost.

Additionally, the synthesis of these self-assembled peptide nanostructures varies from a few seconds to several days of incubation. Depending of the building block used, different shapes can be obtained. A special case is the short aromatic dipeptide, diphenylalanine. By varying the synthesis conditions, nanotubes, nanofibers or nanoparticles can be generated (Reches & Gazit, 2006). All these mild fabrication conditions are reflected in the low cost of the fabrication process.

Recently, our group reported on the on-chip fabrication of self-assembled peptide nanotubes and nanoparticles (Castillo-León et al., 2011). These on-chip fabricated structures displayed a more uniform size and shape as compared to counterparts prepared according to the traditional method. Figure 4 shows the microfluidic chip used for the synthesis of self-assembled peptide nanotubes and nanoparticles. In less than a minute, hundreds of these on-chip fabricated self-assembled structures were obtained.
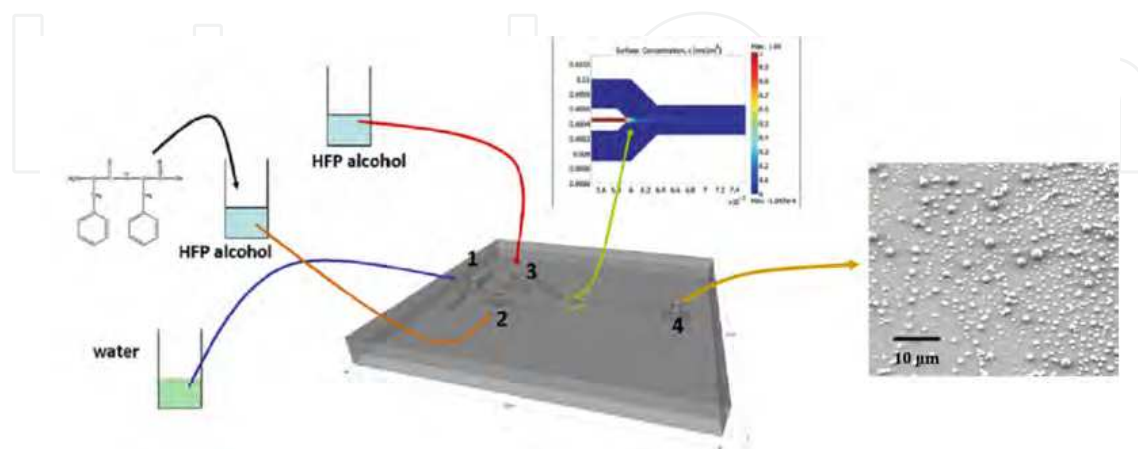

Fig. 4. An illustration of the designed microfluidic chip. The inset shows a simulation of the mixing of different solutions inside the merging channel, where the nanostructures are expected to be formed. SEM image of the on-chip formed self-assembled nanoparticles. Reprinted from (Castillo-León et al., 2011) Copyright (2011), with permission from Elsevier.

As mentioned before, the fabrication of self-assembled peptide nanostructures can, in almost all the cases, be done at room temperature under aqueous conditions. However, two interesting approaches that involve the use of temperatures between 100 and 220 °C were presented by Ryu and Park for the fabrication of vertically well-aligned peptide nanofibers and Adler-Abramovich et al. for the formation of peptide nanotube arrays by chemical vapor deposition (Adler-Abramovich et al., 2009, Ryu & Park, 2008). In both cases the synthesis was performed under non-aqueous conditions, giving nicely organized nanostructure arrays.

As presented in Table 1, there is a long list of peptide monomers that can be used to synthesize self-assembled nanostructures. The final shape and size of the obtained nanostructure will depend on the choice of peptide monomers as well as on synthesis parameters such as pH, solvent polarity, temperature, etc.

4.2 Functionalization

In order to use these biological nanostructures as contrast imaging agents or as part of a biosensing platform they need to be decorated with appropriate functional molecules providing them with specific properties. Functional compounds such as antibodies, magnetic or metallic particles, enzymes, quantum dots or fluorescent compounds have been incorporated into the structure of self-assembled peptide nanostructures (Reches & Gazit, 2007). Ryu and co-workers developed photoluminescent peptide nanotubes by the in-situ incorporation of luminescent complexes composed of photosensitizers such as salicylic acid, cf. Figure 5 (Ryu et al., 2009). Based on this idea, the same group later developed an optical biosensor for the detection of neurotoxins and compounds such as glucose and hydrogen peroxide (Kim et al., 2011, Kim et al., 2011).

Fig. 5. Optical image of fluorescent self-assembled peptide nanostructures. The nanotubes were functionalized with salicylic acid in order to enhance its fluorescence following the method developed by (Ryu et al., 2009).

An interesting approach for the functionalization of biological self-assembled nanostructures was developed by Kasotakis et al. It involved using a self-organized peptide building block as a scaffold for the systematic introduction of metal-binding residues at specific locations within the structure. By employing an octapeptide from the fiber protein of adenovirus, three new cysteine-containing octapeptides were designed. These synthesized fibrils were able to efficiently bind silver, gold, and platinum nanoparticles (Kasotakis et al., 2009). The metallic-decorated fibers are considered being used in photothermal therapy and in the development of surface-enhanced Raman scattering (SERS) biosensors for detecting DNA by taking advantage of the Raman signal enhancement due to the presence of silver and gold particles. Figure 6 displays transmission electron microscopy (TEM) images of metallic decorated nanofibrils.
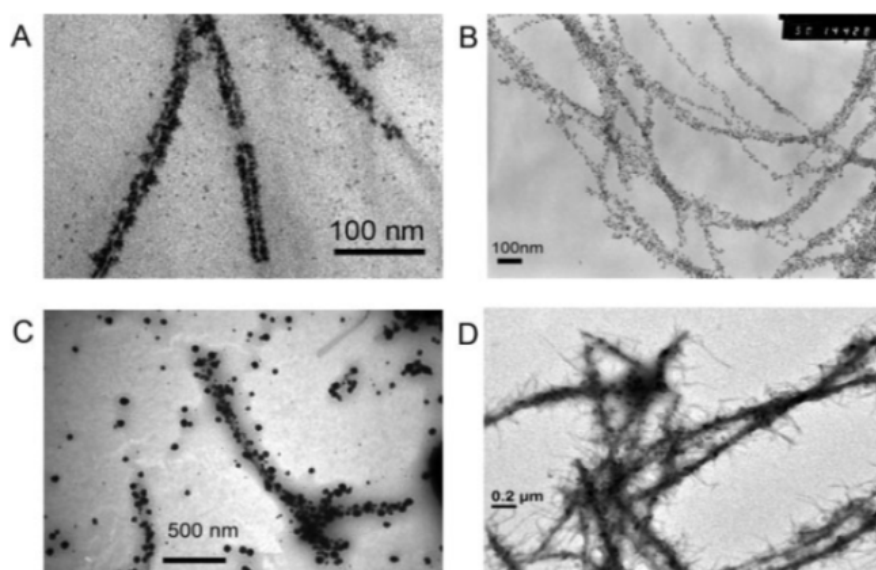

Fig. 6. TEM images of peptide fibrils after incubating with a platinum solution. (A) Fibrils formed from NSGAITIG peptide, scale bar = 100 nm. (B) the NCGAITIG peptide, scale bar = 100 nm. (C) the CNGAITIG peptide, scale bar = 500 nm. (D) CSGAITIG peptide, scale bar = 0.2 μm. Reprinted with permission from (Kasotakis et al., 2009) Wiley 2009.

Another example of the advantages of functionalization of self-assembled peptide nanostructures was presented by Jayawarna and colleagues; they introduced chemical functionality into Fmoc-peptide hydrogels by adding Fmoc-protected amino acids with varying side groups. In this way, an improved 3D in-vitro cell culture system was developed. The functionalized gel showed an enhanced compatibility with the cell culture. Additionally, the properties of the gel could be tuned depending of the type of cell used in order to obtain even better results in terms of compatibility (Jayawarna et al., 2009).

4.3 Biocompatibility

Despite the high levels of attention given to this type of biomaterial, an advanced study to evaluate the biocompatibility and immunogenicity of these nanostructures is still lacking. Such an investigation will bring important information that will define the possibility to use this biomaterial in applications such as drug-delivery systems or tissue reparation in humans. The available studies are limited to growth cells and tissues in the presence of self-assembled peptide nanofibers or hydrogels and assessed whether the exposed cell or tissue growth was affected by the presence of the biomaterial. Next, a couple of examples will be presented and more studies involving the interaction between self-assembled nanostructures and cells or tissues will be listed in Table 2.

Mahler et al. fabricated a self-assembled hydrogel using the Fmoc-diphenylalanine peptide. This hydrogel was used as a cellular support to grow Chinese hamster ovary (CHO) cells. The cellular viability was analyzed using a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. The obtained results demonstrated that cells grown on the hydrogel scaffold showed a viability of more than 90%(Mahler et al., 2006).

In another study, Jayawarna and co-workers investigated the use of Fmoc-dipeptides with different aminoacids as three-dimensional cell culture platforms. Their work confirmed the use of this peptide building block as a good option for cell culture. The investigation involved growing chondrocyte, human dermal fibroblasts and 3T3 fibroblast cells in two and three dimensions (Jayawarna et al., 2006, Jayawarna et al., 2008, Jayawarna et al., 2009, Jayawarna et al., 2007).

Liebmann and collaborators synthesized self-assembling peptide hydrogels to generate patterned 3D cell cultures within patterned silicon microstructures (Liebmann et al., 2007). In their study, astrocyte, MDCK and COS7 cells were used. The results proved how the peptide-derivative hydrogel simplifies both handling and loading of the gel to the microstructures and how the composition and matrix density of the gel could be fully controlled by chemical tailoring processes.

Our group utilized a nanoforrest of self-assembled peptide nanofibers previously used for electrochemical detection of dopamine as a cellular support for the growth of HeLa and PC12 cells (Sasso et al., 2011). As shown in Figure 7, the cells grew and divided without difficulty, proving that the presence of peptide nanofibers did not hinder cell growth. The next step in this study will be to combine the sensing properties of the functionalized nanofibers with the cell culture characteristics in order to develop a combined biosensing/cell culture platform where the cells releasing dopamine will be in intimate contact with the biorecognition element.

A more detailed and complete biocompatibility and immunogenicity assessment will clarify the risks of biomedical use of these materials in humans. More examples of employing self-assembled peptide hydrogels in applications involving interaction with cells or tissues are presented at the end of the chapter in Table 2.
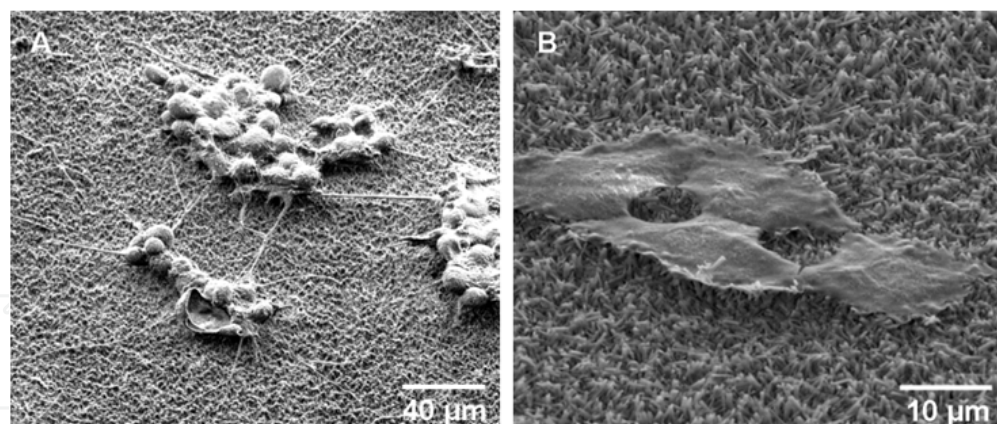
Fig. 7. SEM image of the growth of A) PC12 and B) HeLA cells on a self-assembled peptide nanofiber forest.

5. Challenges when using self-assembled peptide nanostructures for biomedical purposes

Despite the many advantages making self-assembled peptide nanostructures a great candidate for various biomedical applications, challenges regarding size control during synthesis, manipulation and immobilization as well as stability in liquid environments need to be overcome. In certain applications such as the development of drug delivering systems, the size of the nanostructures as well as their stability in different solutions (buffers, acid or basic solutions) is of vital relevance in order to obtain a correct behaviour. If the nanostructure selected for drug-cargo is bigger than the target cell, the delivery process will be difficult and will most probably fail. The same will occur if the biological nanostructure is unstable in liquid solutions causing its structure to disappear after a few seconds in contact with the solution (MaHam et al., 2009). This example highlights the importance of controlling some of these parameters during the synthesis and application of self-assembled structures for biomedical purposes.

This section discusses challenges regarding size control during synthesis, stability in liquid environments, manipulation and conductivity. Moreover, some alternatives to overcome them are presented.

5.1 Size control

As previously mentioned, the control of the size of self-assembled peptide nanostructures is very important in applications such as biosensors or drug-delivery systems. The presence of nanostructures of equivalent dimensions will be reflected in the fabrication of biosensing platforms with similar and reproducible characteristics.

Due to the nature of the self-assembled process, the control of their size during the synthesis is extremely difficult to obtain. However, changes in the fabrication parameters or the use of templates could help to obtain self-assembled nanostructures of similar dimensions.

With polycarbonate membranes as templates, Porrata and co-workers were able to synthesize peptide nanotubes with controlled diameters. The diameters of the peptide nanotubes were as small as 50 nm Porrata (Porrata et al., 2002). A different approach was later applied by Han et al. in order to fabricate self-assembled peptide nanostructures with defined structures and sizes by changing the polarity of the solvents used during the fabrication process (Han et al., 2008). Their results demonstrated that by altering the polarity

of the solvent used during the synthesis, diverse morphologies ranging from nanotubes to nanoribbons and nanofibers could be achieved. Despite the importance of their work with regard to understanding natural self-assembly into complicated architectures, their approach allows only a structural control and not one of the sizes of the obtained structures. An elegant study to obtain size-tuneable assemblies of peptide nanofibers was presented by Park et al. One-dimensional self-assembled peptide nanofibers with tuneable sizes were obtained by changing the initial concentration of the monomer used to fabricate them and by controlling the pH environment. Peptide nanofibers with diameters from 110 to 600 nm and lengths from 1.95 to 18 nm were obtained following this method (Park et al., 2009).

5.2 Stability in liquid environments

The use of self-assembled peptide nanostructures in biomedical applications were supramolecular structures will be in direct contact with liquid solutions, e.g., their use as drug delivery systems, imaging contrast agents or as biosensors, requires a good stability in the solution in question. In order to illustrate the importance of this point, we will take as an example the self-assembled nanostructures based on the aromatic dipeptide diphenylalanine. They have been presented as versatile materials with potential uses in different fields like biosensing, microelectronics, drug-delivery, and tissue reparation among others (Scanlon & Aggeli, 2008). However, it has been noted that the nanotubes formed by the diphenylalanine peptide were not very stable once they were in contact with liquid solutions such as water or phosphate buffers, i.e., liquids that are commonly use in biomedical studies (Andersen et al., 2011, Ryu & Park, 2008).

A detailed study was done to evaluate the behavior of these structures under liquid conditions using solvents of common use for biomedical purposes. The investigation made it clear that the structures were completely unstable when dissolved in water, phosphate buffer or methanol, cf. Figure 8. These results will certainly limit their use in many applications. Surprisingly, the stability of nanofibers fabricated using the same dipeptide showed completely different results; they were stable in the presence of these liquids (Andersen et al., 2011).
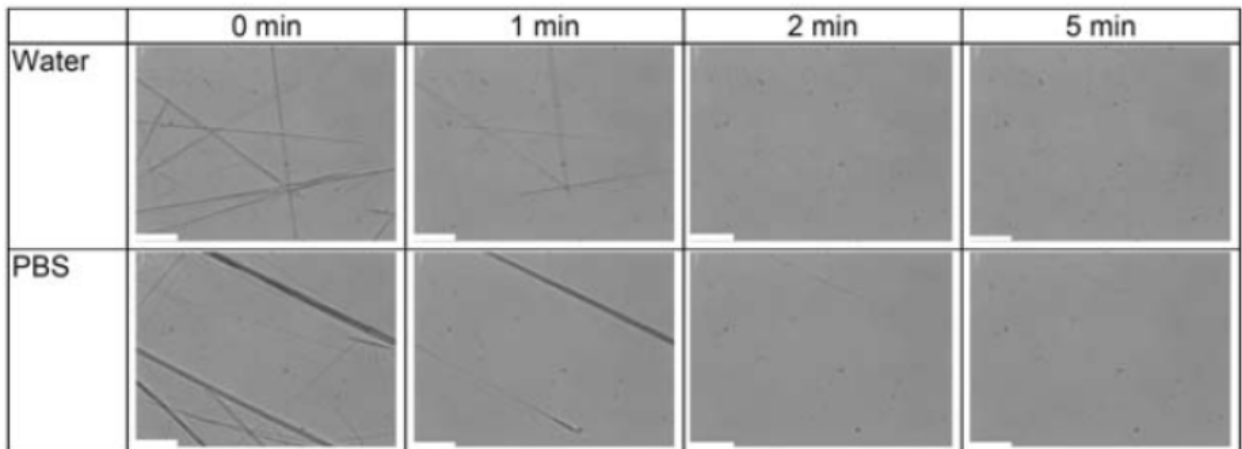

Fig. 8. Microscopy images of the peptide nanotubes dissolved in distilled water and PBS (pH 7,4). From these images, the nanotubes were found to lose their structure in some of the solutions in which it has been claimed that they were stable. All scale bars in the images correspond to 20 μm. Reprinted from (Andersen et al., 2011)- Reproduced by permission of The Royal Society of Chemistry.

As in the case of the size control, the focus of this study was to change the parameters during the synthesis in order to obtain more stable nanotube structures. When changing the pH value of the solution used for the self-assembly of the peptide nanotubes, more stable structures were obtained. The nanotubes fabricated using a buffer of pH 3 instead one of pH 7 displayed a better stability in water and phosphate buffer. This is just a first step towards the fabrication of stable diphenylalanine nanotubes. More studies are required in order to evaluate the implications of the pH change on the properties of this biological nanostructure.

It is also worth mentioning that the discovery of the instability of the diphenylalanine nanotubes in water gave rise to the idea to use these structures as an etching mask material for the rapid fabrication of silicon wires (Larsen et al., 2011). This application is beyond the scope of the present chapter but we mention it to encourage the reader to see the positive side of the results that could initially appear as negative.

5.3 Manipulation

The development of biosensing platforms using self-assembled peptide nanostructures requires the connection between these structures and the appropriate transducers (electrodes). Due to their dimensions, the manipulation of micro and nano-scale biomaterials is a critical issue. Linking of our macroscopic world to the nanoscopic one of nanotubes, nanofibers and nanoparticles is a technological challenge. Fortunately, thanks to advances in micro- and nano-fabrication, several techniques and instruments have emerged to help scientists overcome the obstacles of size when interacting with tiny biological entities (Castillo et al., 2009, Castillo et al., 2011).

Apart from being able to find, grasp, push or move these biological objects to a desired location it is important to avoid any damage to the nanostructure as a result of the interaction between the manipulation instrument and the object to be moved. Altering or changing the structure of the self-assembled nanostructure during the manipulation process could affect the behavior of these structures used in a specific biomedical application.

The manipulation of self-assembled peptide nanostructures could involve the direct contact of the instrument as well as the biological structure, like when using atomic force microscopy (AFM) tips. Another option is to perform the manipulation through non-contact methods as in the case of dielectrophoresis (DEP) or optical tweezers. Next, examples of available manipulation methods are presented.

The thermomechanical manipulation of aromatic peptide nanotubes was demonstrated by Sedman et al. at the University of Notthingham (UK). Using an atomic force microscope as a thermomechanical lithographic tool, Sedman and co-workers were able to manipulate self-assembled nanotubes formed by two aromatic peptides; diphenylalanine and dinapthylalanine. Indents and trenches were thermally etched into the nanostructures, suggesting their possible use as nano-barcodes (Sedman et al., 2009).

Magnetic alignment is a versatile contact-free manipulation method that is effective over the whole sample. With this technique, the manipulation is achieved by magnetic beads that are attached or engulfed by the biological entity to be manipulated. Reches and Gazit aligned nanotubes assembled from diphenylalanine monomers using a external magnetic field as shown in figure 9 (Reches & Gazit, 2006).

However, a principal drawback of magnetic manipulation is the fact that the aligning forces are very small. Consequently, the alignment will occur only when the molecules present in

the sample are very large and contain moieties with high susceptibility as in the case of aromatic units.
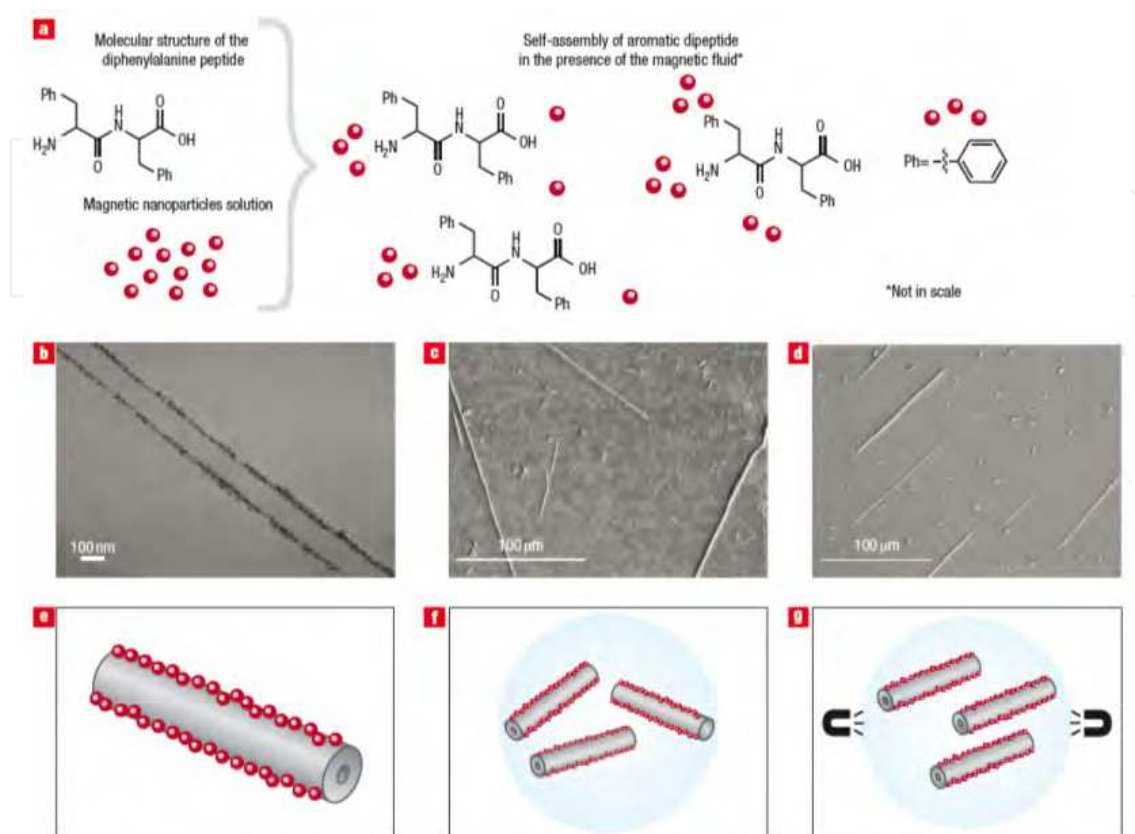

Fig. 9. The self-assembly of the diphenylalanine-based peptide nanotubes in the presence of a ferrofluid and their exposure to an external magnetic field resulting in the control over their horizontal alignment. (a) Schematic representation of the dipeptide monomers self-assembled in the presence of a ferrofluid solution containing magnetite nanoparticles approx. 5 nm in diameter. (b) TEM image of a self-assembled peptide tube coated with magnetic particles. (c) Low-magnification SEM micrograph of the self-assembled magnetic tubes. (d) Horizontal arrangement of the self-assembled magnetic tubes after their exposure to a magnetic field, observed by low-magnification SEM. (e) Schematic representation of the self-assembled magnetic tubes. (f,g) Schematic representations of the magnetic tubes randomly oriented before exposure to the magnetic field (f) and horizontally aligned upon exposure to the magnetic field (g). Reprinted by permission from Macmillan Publishers Ltd: Nature Nanotechnology, (Reches & Gazit, 2006), copyright (2006).

Another contact-free method used for the manipulation of self-assembled nanostructures is dielectrophoresis. Dielectrophoresis occurs when a polarizable object is exposed to an inhomogeneous electric field, so that the Coulomb forces induced on the charges on each half of the dipole differ, causing a net force on the object. The manipulation of self-assembled peptide nanotubes was reported by our group in 2008. Using micro-patterned gold electrodes, peptide nanotubes were manipulated by adjusting the amplitude and frequency of the applied voltage, cf. Figure 10. The electrical characterization of the immobilized peptide nanotubes was studied, both for single peptide nanotubes and bundles of them (Castillo et al., 2008).
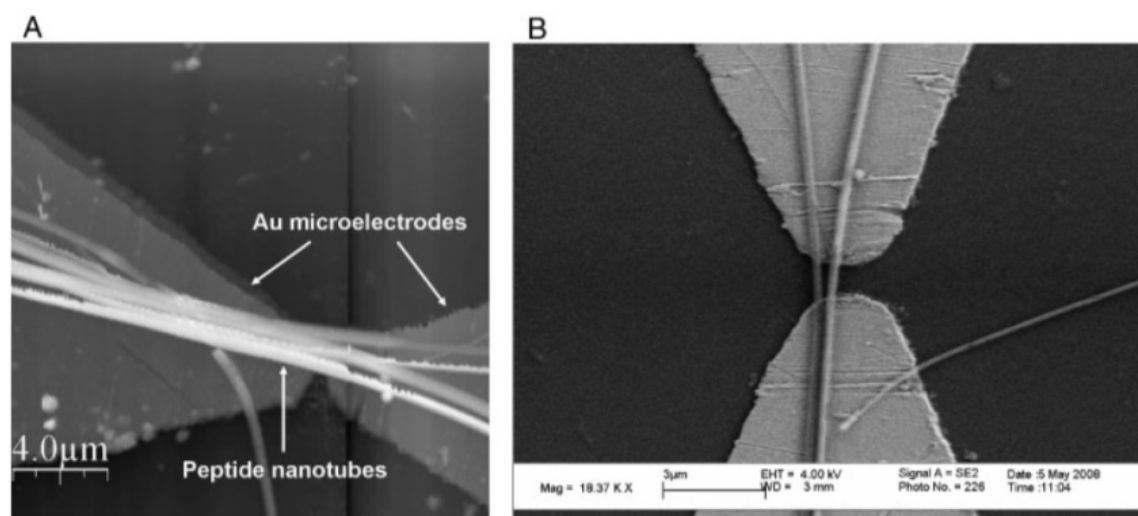
Fig. 10. (A) AFM image of the SAPNT bundles immobilized on Au electrodes using DEP. (B) SEM image of two amyloid peptide nanotubes immobilized on top of gold microelectrodes. Reprinted with permission from (Castillo et al., 2008) Wiley 2008.

At the same time, de la Rica and co-workers used DEP for the incorporation of antibody functionalized peptide nanotubes on top of gold electrodes for the development of a label-free pathogen detection chip. The peptide nanotubes were modified with antibodies against the herpes simplex virus type 2 (HSV-2), and thus the binding of the virus to its antibody was monitored by a capacitance change between the electrodes (de la Rica et al., 2008).

Zhao and Matsui developed a method for the accurate immobilization of antibody-functionalized peptide nanotubes on protein-patterned arrays by optimizing their ligand-receptor interactions (Zhao & Matsui, 2007). Peptide nanotubes self-assembled from bolaamphiphile peptide monomers and bis(N-α-amido-glycylglycine)-1,7-heptane dicarboxylate were coated with antibodies anchored on the amide groups of the nanostructure surface via hydrogen bonding. The antibody-modified nanotubes where then attached to complementary antigen-patterned surfaces as a function of antigen concentration.

Finally, Kumara et al. demonstrated the use of laser tweezers for the trapping and manipulation of self-assembled flagella protein nanotubes. Nanotubes with diameters below 50 nm were optically trapped using a biologically infrared wavelength (1064 nm) laser tweezer without affecting the nanostructure (Kumara et al., 2006).

In summary, manipulation of self-assembled peptide nanostructure was possible without altering the properties or the structure of these biological entities. Contact and contact-free techniques are available for the precise positioning of these samples on desired locations for their integration into more complicated structures such as biosensing platforms.

5.4 Conductivity

The low conductivity of self-assembled peptide nanostructures limits their use in the development of sensing or diagnosis platforms without involving a functionalization step that introduces compounds to help increase their conductivity. Self-assembled peptide nanotubes and nanofibers synthesized from various sources displayed low conductivity values and high resistance ($R > 10^{14} \, \Omega$) (Castillo et al., 2008, Scheibel et al., 2003). Conductive polymers, enzymes, or metallic particles are some of the materials used to functionalize the

self-assembled nanostructures thereby giving rise to an increase in electrical current conductivity. After the functionalization step, the functionalized nanostructures can be integrated with transducers and used for the detection of compounds of biomedical relevance. Following changes in current, potential or capacitance as a result of the interaction between the analyte and the biorecognition element the presence and quantity of these samples can be confirmed.

Scheibel et al. fabricated conducting nanowires thanks to a controlled self-assembly of amyloid fibers and selective metal deposition (Scheibel et al., 2003). Nanowires containing cysteine residues were used to covalently link colloidal gold nanoparticles on the surface of these nanostructures. And additional metal was then deposited by chemical enhancement of the colloidal gold by reductive deposition of metallic silver and gold from salts. The final biotemplated metal wires showed an increase in conductivity from the pA- to the mA-range, figure 11.
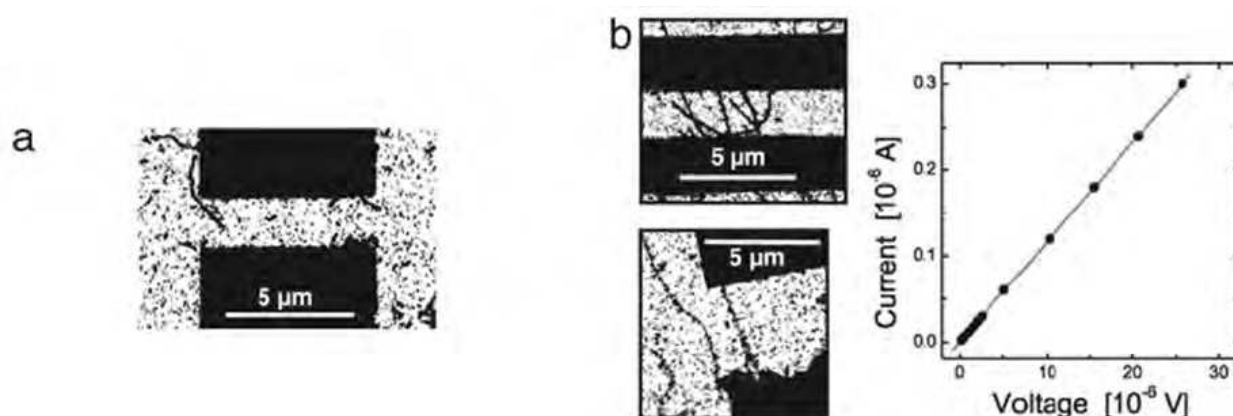

Fig. 11. Electrical behavior of NM-templated metallic fibers. (a) Gold nanowires not bridging the gap between two electrodes did not conduct. (b) Gold nanowires bridging the gap between two electrodes (*Left*) exhibited linear I–V curves (*Right*), demonstrating ohmic conductivity with a low resistance of R =86 Ω (the same for each). Such an ohmic response is indicative of continuous, metallic connections across the sample. Reprinted from Scheibel, T. et al. PNAS, 100, 2003, 4527-4532. Copyright (2003) National Academy of Sciences, U.S.A.

In a different approach, Sasso et al. used a conductive polymer, polypyrrole, in order to increase the conductivity of self-assembled peptide nanofibers. The presence of the conductive polymer on the surface of the nanofibers increased their conductivity and rendered possible their use as the biorecognition element in a dopamine biosensor. By employing this sensor, dopamine detection was possible with a detection limit of 3.1 μM; a value close to the dopamine concentration in in-vivo systems (Sasso et al., 2011).

Enzyme-modified peptide nanotubes have been utilized for the detection of compounds of biomedical relevance such as glucose, ethanol, or hydrogen peroxide as demonstrated by several groups. The enzymes were connected to the self-assembled peptide nanostructures through different matrices such as poly(allylamine hydrochloride) (Cipriano et al., 2010) or glutaraldehyde (Yemini et al., 2005, Yemini et al., 2005). Finally the modified peptide nanotubes were immobilized on the surface of metallic electrodes using polymer matrices like polyethyleneimine. Amperometric detection was used to detect and quantify the presence of the previously mentioned compounds. Based on a similar approach, Yang and co-workers developed a glucose sensor using glucose oxidase functionalized peptide

nanofibers. The developed biosensor was able to electrochemically detect glucose in a concentration range required for clinical applications (Yang et al., 2009).

In a different approach, de la Rica et al. employed anti-E. coli and IgG antibodies to functionalize peptide nanotubes against *E. coli* and *S. typhi* cells, which are involved in several diseases. Using these functionalized nanostructures, they fabricated a reusable pathogen biochip able to detect analyte cells in the range from 10^2 to 10^4 cells within one hour. The capturing of the cells by the antibodies was monitored by following changes in the capacitance (de la Rica et al., 2010).

As presented in this section, several alternatives are available in order to improve the conductivity of self-assembled peptide nanostructures and impulse their use as biorecognition elements in biosensors or diagnosis platforms. The permanent advances in surface chemistry and synthesis of new conductive polymers will accelerate the integration of biological self-assembled nanostructures in biosensing applications.

6. Applications

All the previously mentioned advantages make self-assembled peptide nanostructures a very promising biomaterial with huge potential in several biomedical applications. These applications have been reviewed in numerous articles (Aggeli et al., 2001, de la Rica & Matsui, 2010, Gao & Matsui, 2005, Gazit, 2007, Hauser & Zhang, 2010, Kyle et al., 2010, Kyle et al., 2008, Rajagopal & Schneider, 2004, Scanlon & Aggeli, 2008, Woolfson & Ryadnov, 2006, Yan et al., 2010), and a detailed description of some of these applications has already been given in this chapter. In order to provide a broader vision of the possibilities of using these biological nanostructures with biomedical purposes, a list of different self-assembled peptide nanostructures is presented in Table 2. The table lists the type of structure and their use.

Self-assembled nanostructure	Application	Reference
Hydrogel	3D scaffold to stimulate pre-osteoblast (MC3T3-E1) cell attachment and growth	(Zhang et al., 2009)
Hydrogel	Extracellular matrix to grow primary human dermal fibroplasts	(Kyle et al., 2010)
Hydrogel	Injectable delivery	(Branco et al., 2009)
Hydrogel	Therapeutic effects as a wound dressing for the treatment of deep second degree burns in rats	(Meng et al., 2009)
Hydrogel	Carrier for therapeutic proteins	(Koutsopoulos et al., 2009)
Hydrogel	Drug delivery of pyrene	(Li et al., 2009)
Hydrogel	Gene delivery	(Rea et al., 2009)
Hydrogel	Promoting axonal growth in the injured spinal cord	(Ueda et al., 2008)
Hydrogel	Cartilage tissue engineering	(Kisiday et al., 2002)
Hydrogel	Implantation of cardiac progenitor cells	(Tokunaga et al., 2010)
Nanotubes	Detection of neurotoxins	(Kim et al., 2011)

Self-assembled nanostructure	Application	Reference
Nanotubes	Pathogens detection	(de la Rica et al., 2008, de la Rica et al., 2010)
Nanotubes	Immunosensor	(Cho et al., 2008)
Nanotubes	Glucose, ethanol and hydrogen peroxide detection	(Yemini et al., 2005, Yemini et al., 2005)
Nanotubes	Antiviral agent	(Horne et al., 2005)
Nanotubes	Controlled drug release	(Chen et al., 2011)
Nanotubes	Hydrogen peroxide detection	(Cipriano et al., 2010)
Nanotubes	Drug delivery	(von Maltzahn et al., 2003)
Nanofibers	Copper detection	(Viguier et al., 2011)
Nanofibers	Dopamine detection	(Sasso et al., 2011)
Nanofibers	*Yersinia pestis* detection	(Men et al., 2010)
Nanofibers	Glucose detection	(Yang et al., 2009)

Table 2. Biomedical applications of self-assembled peptide nanostructures.

7. Conclusions

As presented throughout this chapter, self-assembled peptide nanostructures are biomaterials with numerous advantages making them excellent candidates for applications with biomedical purposes, including biosensing, diagnosis, drug delivery, and tissue reparation, among others. The biological nanostructures are synthesized under mild conditions, at low cost and rapidly, and can be easily functionalized with different functional compounds (antibodies, enzymes, quantum dots, fluorescent molecules, metallic and magnetic particles). However, it is important to be aware of the challenges involved when working with these self-assembled nanostructures. Several options to solve issues related to size control, manipulation, low conductivity and stability under liquid conditions have been presented. Despite these disadvantages, self-assembled nanostructures are already playing an important role in applications such as tissue reparation, drug delivery and biosensing. Future work should be directed towards a more complete study of the biocompatibility and immunogenicity of this biomaterial in order to clarify the consequences of an exposure to it.

8. Acknowledgements

9. References

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These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentials of different synthetic and engineered biomaterials. Contributions were not selected based on a direct market or clinical interest, than on results coming from very fundamental studies which have been mainly gathered for this book. This fact will also allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessarily imposed by industrial or profit concerns. The book collects 22 chapters related to recent researches on new materials, particularly dealing with their potential and different applications in biomedicine and clinics: from tissue engineering to polymeric scaffolds, from bone mimetic products to prostheses, up to strategies to manage their interaction with living cells.

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