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Directed Three-Dimensional Patterning of Self-Assembled Peptide Fibrils

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ABSTRACT

Molecular self-assembly is emerging as a viable ''bottom-up'' approach for fabricating nanostructures. Self-assembled biomolecular structures are particularly attractive, due to their versatile chemistry, molecular recognition properties, and biocompatibility. Among them, amyloid protein and peptide fibrils are self-assembled nanostructures with unique physical and chemical stability, formed from quite simple building blocks; their ability to work as a template for the fabrication of low resistance, conducting nanowires has already been demonstrated. The precise positioning of peptide-based nanostructures is an essential part of their use in technological applications, and their controlled assembly, positioning, and integration into microsystems is a problem of considerable current interest. To date, their positioning has been limited to their placement on flat surfaces or to the fabrication of peptide arrays. Here, we propose a new method for the precise, three-dimensional patterning of amyloid fibrils. The technique, which combines femtosecond laser technology and biotin–avidin mediated assembly on a polymeric matrix, can be applied in a wide variety of fields, from molecular electronics to tissue engineering.

Fibrous nanostructured objects are promising for their integration in future generations of micro and nano devices with possible industrial applications. Carbon and inorganic nanotubes have been most investigated in these fields.[1] Nanofibers and nanotubes of biological origin offer the advantage of synthesis under mild, physiological conditions; DNA fibers are the first nanoscale bio-materials that were demonstrated to function as building blocks in nanotechnology setups.[2,3] Another class of fibers are protein-based, such as natural fibrous proteins (silk fibroins, spider silks and viral fibers) and fibers formed through self-assembly of proteins and peptides.[4,5] The latter include the so-called amyloid fibers that form following protein misfolding and misassembly events and result in pathological states associated with human diseases.[6]

These families of proteins and peptides share common features, such as controlled assembly from repetitive building blocks and exceptional resistance to extreme conditions such as high and low temperature, detergents, and denaturants. This resistance to extreme conditions makes them attractive candidates for applications in nanotechnological settings, since it allows their interfacing with the world of "hard materials". Furthermore, the possibility of introducing site-specific changes at the sequence level offers the big advantage of tailor-made modifications.

In the past decade, there has been extensive research activity and progress in the chemistry of these materials. These studies allowed the identification of small amyloid building blocks (as small as hexa, tetra, or even dipeptides) that can be relatively inexpensive to produce by chemical peptide synthesis.[6] Pioneering work was done on their use as bio-templates for metal nanowires and scaffolds for tissue engineering;[7−9] furthermore, original reports start to appear on their controlled patterning and alignment.[10,11] However, much remains to be done in developing techniques for their accurate positioning on microscale surfaces and objects. In this letter, we propose a new method for the precise, three-dimensional patterning of amyloid fibrils. The technique, which combines laser technology and avidin−biotin mediated patterning of the self-assembling peptide fibers, could potentially be applied in a wide variety of fields, from molecular electronics to tissue engineering.

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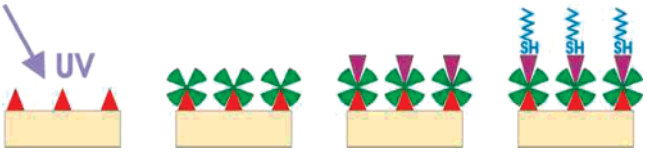

Figure 1. Functionalization of ORMOCER for peptide fibril growth. A thin layer of photobiotin (red triangles) is deposited on the ORMOCER surface and exposed to UV light (a) before being further functionalized further with avidin (green crosses, b) and iodoacetamide-functionalized biotin (purple triangles, c). The final step is the attachment of the cysteine-containing peptide through the SH-iodoacetamide reaction (d).

The technique is based on the selective attachment of photosensitive biotin (photobiotin) on surfaces and 3D structures and exploits thiol chemistry and self-assembly of peptide fibrils. These structures consist of ORMOCER, a photostructurable organic−inorganic hybrid, on which photobiotin can be irreversibly attached when exposed to UV light from a laser or a lamp.[12] However, the technique is not limited to ORMOCER; photobiotin can be photolytically attached on a variety of organic and inorganic materials such as silicon,[13] glass,[14,15] and PDMS;[16] this gives this technique a large flexibility and applicability. Once biotin has been immobilized on ORMOCER, then it is first incubated with avidin and subsequently with the iodoacetamide-functionalized biotin *N*-(biotinoyl)-*N*′-(iodoacetyl) ethylenediamine. Finally, the 3D structures are immersed in an aqueous solution of peptides that contain a cysteine residue. The peptide solutions have been "aged" so that self-assembled fibrils are already formed in solution. It is generally accepted that formation of amyloid fibrils in solution follows a classical nucleation and growth mechanism; small oligomers first form slowly and subsequently serve as nuclei for fibril elongation and development.[6] The term "aged" refers to solutions where, according to previously determined kinetic conditions,[17] mature amyloid fibrils reaching the order of microns in length have been formed. The self-assembly of the peptide fibrils into bridges on the structures is initiated through the controlled evaporation of water. However, in order for this to occur between two specific positions, there is a seed, or "anchoring point", required. This is provided by the covalent bond formation between the iodoacetamide group in the biotin derivative and the thiol group in the cysteine. The requirement for the "seeding", and therefore the selectivity of the technique, is demonstrated with the use of peptides that do not contain cysteine. In this case, self-assembled peptide fibrils are formed in solution, as shown in Figure 2b, but there is no peptide fibril attachment on the functionalized structures.

For the 3D patterning, first three-dimensional structures are made using multiphoton lithography of ORMOCER. Nonlinear optical micro-stereolithography based on multiphoton polymerization of polymeric mixtures allows the fabrication of three-dimensional structures with submicron resolution. When the beam of an ultrafast infrared laser is tightly focused into the volume of a photosensitive material, the polymerization process can be initiated by nonlinear

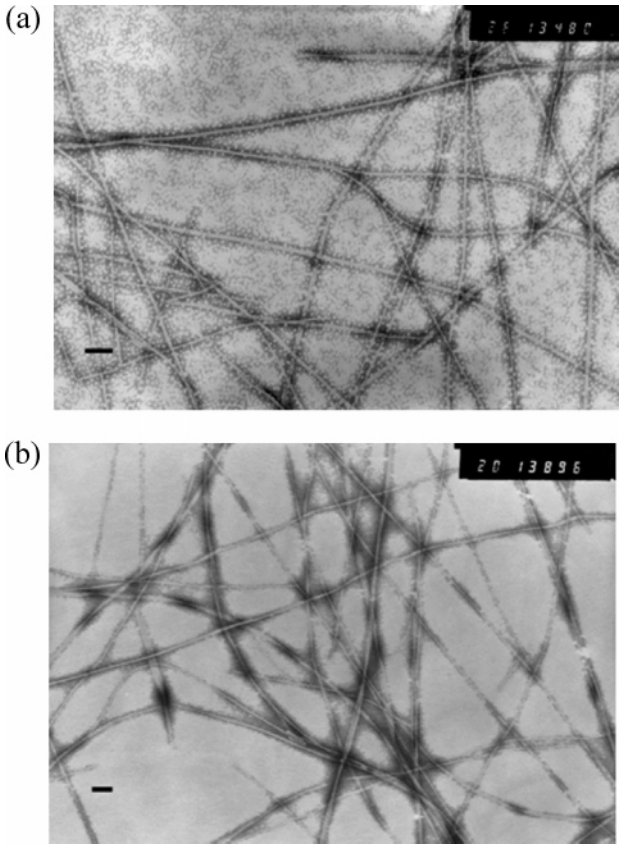

Figure 2. (a) TEM image of fibrils of the cysteine-containing peptide (CNGAITIG, bar 150 nm) and (b) TEM image of fibrils of the peptide that does not contain cysteine (NSGAITIG, bar 150 nm).

absorption within the focal volume. By moving the laser focus three-dimensionally through the resin, 3D structures can be fabricated. The technique has been used with a variety of acrylate and epoxy materials, and several components and devices have been fabricated such as photonic crystal templates,[18] mechanical devices,[19] and microscopic models.[20] The highest resolution reported to date is 65 nm;[18] the real advantage however of nonlinear optical micro-stereolithography over other lithographic technologies lies in its intrinsic ability to directly write three-dimensional structures of millimeter size with submicron resolution, by directly converting computer-designed patterns into structures without the need of mask, mold, or stamp.

The process of functionalization of ORMOCER for peptide fibril growth is illustrated in Figure 1. First a thin layer of photobiotin is deposited on the ORMOCER surface. When this layer is exposed to UV light (Figure 1a), it is immobilized irreversibly on the ORMOCER surface.[12] The samples are washed thoroughly to remove the nonimmobilized photobiotin, and they are subsequently functionalized further with avidin (Figure 1b) and finally with the iodoacetamide-functionalized biotin (Figure 1c). The next step is peptide attachment on the functionalized surfaces (Figure 1d); this is achieved through the thiol group of the peptide that will react with the biotin-conjugated iodoacetamide group. A thin layer of peptides will form on the structures;

this layer will act as an "anchoring point", which, under the right conditions, will trigger the further assembly of the peptide nanostructured fibrils into micron-sized "bridges".

The peptides used in this work derive from sequences of a natural beta-structured fibrous protein, the adenovirus fiber. The fibrous stalk domain of this protein is made up from repetitive amino acid sequences that correspond to beta-strand motifs joined by loops and turns at the three-dimensional structure of the protein.[21] There is accumulating evidence that amino acid repeats might have evolved as optimized self-association motifs in biological assembly processes, including amyloid fibril formation.[6] Short synthetic peptides corresponding to the building blocks of the adenovirus fiber protein self-assemble into amyloid-type fibrils in solution.[17] The octapeptide NSGAITIG (arginine-serine-glycine-alanine-isoleucine-threonine-isoleucine-glycine) corresponds to a short beta strand-and-loop region of the natural protein and has been identified by previous biochemical studies as an elementary self-assembling building block.[17] It has therefore been chosen as the basic building block used in this study. We have recently replaced the serine residue by cysteine in order to exploit the possibility of selective attachment of the cysteine to the iodoacetamide-conjugated biotin. The NCGAITIG peptide forms amyloid-type fibrils in solution, as well as its isoform, CNGAITIG. In all cases, the ability of the peptides to form fibrils in solution was confirmed using all the structural criteria that apply to amyloid fibril structural characterization. Transmission electron microscopy (TEM) images of fibrils of cysteine-containing as well as the fibrils of the serine-containing peptide are shown in Figure 2, panels a and b, respectively.

Peptide fibrils were visualized either with scanning electron microscopy (SEM) or using the well-established diagnostic test of fluorescence emission of the dye Thioflavin T that binds specifically to amyloid fibrils and gives blue fluorescence when excited at 440 nm. A thioflavin fluorescence image and a SEM image of the directed 3D assembly of the peptide fibrils is shown in Figure 3, panels a and b, respectively. In this case, a series of 3D columns is fabricated using multiphoton polymerization; the columns are subsequently functionalized as described earlier. Then the structures are immersed in the cysteine-containing peptide solution and they are left to dry slowly. The peptide bridges form during the drying process. They appear to form over the shortest distance, as it can be seen in the SEM image of Figure 4a. The length and the diameter of the peptide fibrils will depend on the design of the 3D structures, i.e., the distance between them and the diameter of the fibril support (Figure 4, panels b and c). Once the fibril bridges form, they remain at their position even when the sample is immersed in water for as long as 24h. However, for the fibril bridges to form, it is essential that there is a trigger; we believe is provided by a thin layer of peptides formed on the 3D structures, due to the iodoacetamide-thiol reaction. This thin layer is clearly visible in Figure 5a. When the peptide used does not contain cysteine, then there is no formation of peptide thin layer of fibrils as shown in Figure 5b and subsequently no bridges.

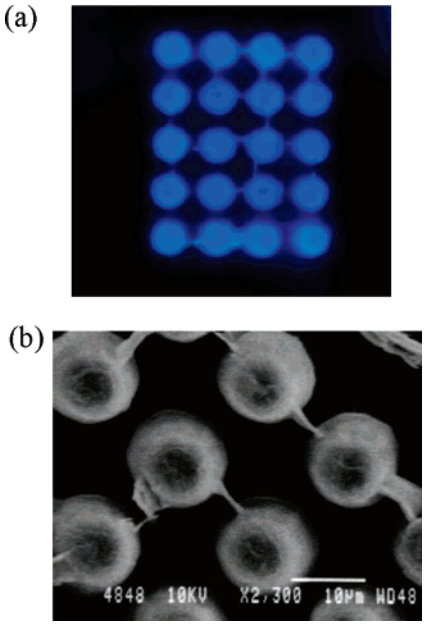

Figure 3. (a) Thioflavin fluorescence image and (b) SEM image of a 3D column array, with peptide bridges self-assembled between the columns.

To conclude, we have demonstrated a new method for the controlled self-assembly of peptides in three dimensions. The method is based on biotin−avidin and thiol chemistry and ORMOCER is used as a substrate. Thiol-functionalized biomolecules can be easily produced in the laboratory scale and can even be commercially available, so this method should be applicable not only to peptides, but to other self-assembling biomolecules as well. Amine-reactive biotinylation reagents are also commercially available and can be used as an alternative to the thiol-reactive biotinylation reagent used in this work. Furthermore, as photobiotin can be easily attached to a variety of materials, this technique is suitable for a variety of applications, from molecular electronics to biosensors and tissue engineering.

The use of carbon nanotubes and inorganic nanowires in gas sensor,[22] biosensor,[23,24] and NEMS[25] applications has been demonstrated. Peptide fibrils, nanotubes, and nanoassemblies have already been employed in biosensors[26] and could potentially prove more useful, as their chemistry can be easily tailored to provide certain functionality such as specific biomolecule recognition. In addition, their fabrication is considerably less costly and does not involve extreme conditions such as high temperature or vacuum, and the use of chemicals.

In molecular electronics, of particular interest is the use of peptide fibrils as templates for the growth of inorganic materials, such as metals (silver, gold, and platinum), ferromagnetic metals (cobalt and nickel), and semiconducting materials.[8,27] In nature, the organization of inorganic matter is often precisely controlled through templating mechanisms mediated by fibrous proteins. Well known examples include the epitaxial growth of nacre in oysters and silicate spicules in sponges. In a pioneering work, metal and semiconductor binding peptides were displayed at the surface of filamentous

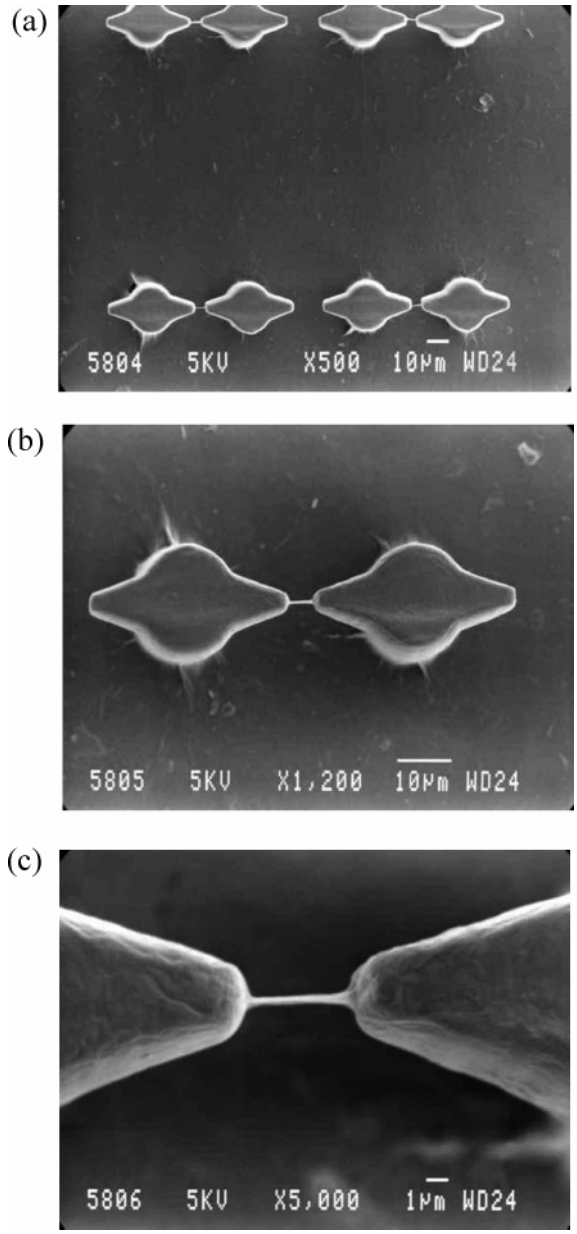

Figure 4. (a) SEM image of a series of 3D ORMOCER columns, with peptide fibril bridges self-assembled between them. (b) One pair of 3D ORMOCER columns, with peptide fibril bridges self-assembled between them. (c) Detail of the self-assembled fiber bridge.

bacteriophages and were used for the fabrication of conductive and semiconductive nanowires[27,28] A natural continuation of the work presented here will be the use of mineralized peptide fibers, since cysteine is a metal-binding amino acid. The use of peptide building blocks functionalized with semiconductor-binding sequences can also be foreseen. This will enable the direct self-assembly of nanoscale electronic circuits and devices.

Another domain where our methodology would be particularly applicable in tissue engineering. Peptide networks are already investigated as cell supports in the form of injectable hydrogels.[29,30] A combination of larger scaffolds with well-defined biodegradable peptide supports in a "scaffold on scaffold" format could possibly be used as a

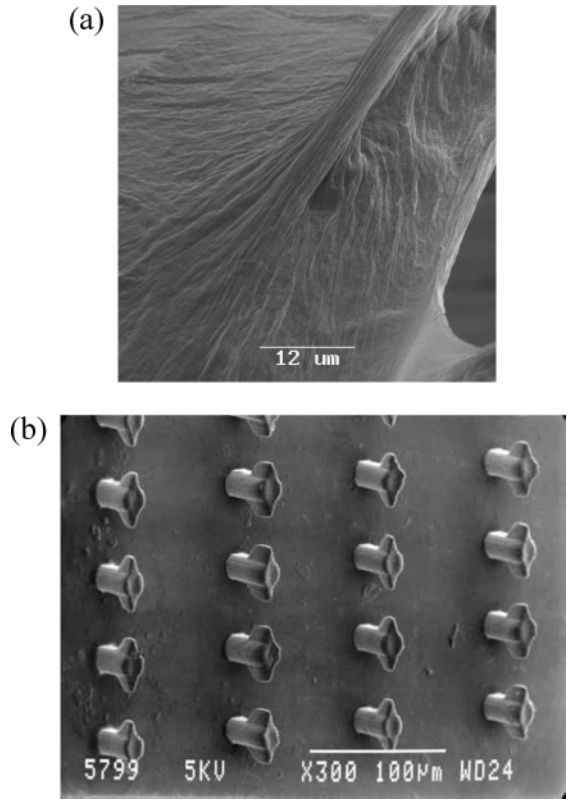

Figure 5. (a) Thin layer of peptide fibrils embedded on the ORMOCER structure. (b) ORMOCER structures functionalized as described in the text; the peptide used does not contain cysteine and therefore no bridges are formed.

support to allow the directed growth of several cell types into ordered arrays of functional biological units.

Materials and Techniques. *Materials.* The material used for the fabrication of the 3D nanostructures is the organic−inorganic hybrid ORMOCER (Micro Resist Technology), a biocompatible material that shows high transparency in the visible and near-infrared ranges. ORMOCER contains a highly crosslinkable organic network as well as inorganic components leading to high optical quality and mechanical as well as thermal stability.

Photobiotin (100 μg/mL, Sigma Aldrich) solution and Avidin (100 μg/mL, Sigma Aldrich) solution were used for the functionalization of ORMOCER structures and films as previously described.[12] The structures and the films used for experiments were sterilized before use for at least 1h in UV (Fluoarc, UV lamp). Photobiotin has a wide area of applications in patterning proteins, ligands, and other species onto solid substrates or for nonspecific labeling of proteins, DNA and RNA probes or other molecules due to its composition: biotinyl group, a linker group and the photoactivatable part: the nitrophenyl azide group. When the epoxy rings on ORMOCER are opened, photobiotin can bind to the ionized oxygen and carbon atoms when exposed to UV light and also the aryl azide groups of biotin are converted to an aryl nitrene, which is extremely reactive. In plus, biotin has an extremely high affinity for avidin; their interaction occurs rapidly and is the strongest noncovalent interaction known in nature. These characteristics are the main factors for the

functionalization of the ORMOCER structures and further binding of *N*-(biotinoyl)-*N*′-(iodoacetyl) ethylenediamine. The photobiotin−avidin functionalized ORMOCER structures were subsequently incubated with 100 μg/mL of *N*-(biotinoyl)-*N*′-(iodoacetyl) ethylenediamine (Molecular Probes) for 40 min in the dark at room temperature and were washed three times with double-distilled (dd) water. The cysteine-containing peptides were further attached to *N*-(biotinoyl)-*N*′-(iodoacetyl) ethylenediamine via the well-known interaction of thiols with the iodoacetamide moiety.

The octapeptides used had the following sequences: NSGAITIG (arginine-serine-glycine-alanine-isoleucine-threonine-isoleucine-glycine), NCGAITIG (cysteine instead of serine in the second position), and CNGAITIG. The peptides were purchased from Eurogentec (Belgium) and had a degree of purity higher than 95%. Peptide solutions in dd water at a concentration of 2 mg/mL and aged for 12 days were used for the positioning experiments. The peptide solutions were incubated on the ORMOCER structures for 40 min at room temperature. They were subsequently washed a first time first by leaving dd water on the sample for 3−5 min, and were then followed by two more washes.

Transmission Electron Microscopy (TEM). The peptide solutions used for positioning were diluted to a final concentration of 0.6 mg/mL in ddH_2O and 8 μL were placed on a 300 mesh Formvar-coated copper grid. After 2 min excess fluid was removed with a filter paper and the samples were negatively stained with 8 μL of 1% uranyl acetate for 2 min. They were subsequently observed with a JEOL JEM-100C microscope operating at 80kV.

Scanning Electron Microscopy (SEM). Samples were covered with 10 nm gold using the sputtering technique and were observed with a JSM-840 SEM microscope operating at 5 kV.

Thioflavin T Fluorescence. Thioflavin T (5 μM dye solution, Sigma) freshly prepared solutions were used for visualization of the amyloid fibrils with fluorescence. After incubation with Thioflavin T for 30 min in the dark, the samples were rinsed with dd water. Fluorescence was detected using a Zeiss fluorescence microscope equipped with a Laser Scanning System Radiance 2100 (400−700 nm) and with a Carl Zeiss Axio Camera HR.

Multi-Photon Polymerization. The setup for the fabrication of three-dimensional microstructures by multiphoton microstereolithography has been described elsewhere.[20,31] The structures were fabricated layer-by-layer bottom up with the last layer attached to the coverslip. After the completion of the component build process, the sample was developed for 3 min in a 50:50 solution isopropanol: 4-methyl-2-pentanone and rinsed in isopropanol.

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