BeNatural publishable executive summary - Project objectives and major achievements during the entire duration of the project

BeNatural aimed to translate the fundamental understanding drawn from self-assembling mechanisms of natural nanofibers and nanotubes into a fabrication strategy of nano-scale devices in a rational manner. The overall requirement for reaching this point was a thorough understanding of the relation between the assembly mechanisms, the crystalline structure of assembled structures, the physical and chemical properties of the structures, and finally how these aspects will perform when integrated for instance in a system.

The **main objectives** of the project were grouped in 4 major areas, namely;

1. To develop new self-assembling nanowires and nanotubes from building blocks inspired from natural folding and assembly motifs of proteins.
2. To use these nanowires and nanotubes as templates for the growth of inorganic/nano/materials.
3. To develop techniques to manipulate, assemble and position these materials in a controlled manner, in view of their integration in future generations of nanoscale devices.
4. To use the self-assembling nanostructures in biosensing and tissue engineering applications.

These objectives were realised through 9 workplans (WP), each having a responsible scientist and several other contributing partners. These workplans were dedicated to the following main activities (brief description):

1. Design and crystallography of fibrous folds
2. Design of building blocks for nano-assemblies
3. Nanoscale characterization and assessment of building blocks
4. Diagnostic structural and aggregation analysis of self-assembled nanostructures
5. Modification of building blocks for specific binding and recognition
6. Integration of nano-assemblies into Microsystems
7. Biosensing applications of developed nanostructures
8. Nerve axonal regeneration by 3D nanostructure scaffolds
9. Management and dissemination

The key findings and achievements of the Project are summarized below.

The main objectives for WP1 were to design, express and purify novel fibrous constructs, to asses correct folding and physico-chemical stability of the proteins and to crystallise and determine the structure of the fibrous proteins. Several constructs of natural fibrous proteins were designed, expressed and purified to homogeneity by P4- USDC and crystallization trials were carried out. The crystal structure of the avian reovirus fibre sigmaC construct has been determined at 0.17 nm resolution and has been delivered ahead

of time. New peptides based on the structure have been designed and studied for their ability to form self-assembling nanostructures in solution.

The main objectives of WP2 were to design and optimise new aromatic peptide building blocks based on the structural paradigm of -amyloid-based nanotubular structures (deliverable D2.1) and to design and optimise peptide building blocks based on the structural features of naturally occurring self-assembled fibrous proteins. Short aromatic dipeptides containing the Fmoc group were designed and synthesized by P2 (TAU-MMB). Several fibrous building blocks have been identified by P1 (UoC) based on information provided by P4 (USDC).
Both aromatic and fibrous building blocks were studied for their ability to self-assemble efficiently and readily into well-ordered structures at the nano-scale by using TEM (Transmission Electron Microscopy) and SEM (Scanning Electron Microscopy). Based on further detailed structural information provided by WP3 and WP4, iterative cycles of optimization were carried out.

The main objectives for WP3 were to provide nanoscale information on the morphology, chemical, physical and mechanical properties of both the building blocks resultant assembled bionanostructures and devices. Nanoscale screening protocols have been well established by P6-UNOTT. Assessment of the bionanostructures for chemical and thermal stability was quantified using the atomic force microscopy (AFM) by *ex situ* imaging, thermal heating and nanothermal techniques. Morphological investigations of bionanostructures was also performed under a range of experimental conditions, using a variety of substrate chemistries, and where relevant incorporation into end application devices has been assessed by AFM and scanning electron microscopy (SEM). The mechanical properties of the nanostructures were investigated using a modification to the conventional tapping mode AFM, HarmoniX. The HarmoniX mode generates a mechanical map of the sample enabling the stiffness and adhesive nature of the material to be quantified. The information generated was continually fed into the consortium to aid development of building blocks with idealized properties dependent on end application requirements.

The main WP4 objectives were to provide structural characterization using high resolution X-ray and neutron fiber diffraction for the self-assembled fibers and tubes formed by the peptide building blocks identified in WP2. High resolution fiber diffraction analysis was carried out for the self-assembling building blocks by P5 – ILL at the ESRF (European Synchrotron Radiation Facility). Fiber diffraction work on the aromatic building blocks designed by P2 (TAU-MMB) systems showed that (i) all of them adopted structures that have many features in common with amyloid structures (ii) they are remarkably stable and change little even when the humidity of their immediate environment is changed very significantly (Delivered 4.1). All fibrous building block peptides designed by P1 (UoC) studied by fiber diffraction to greater or lesser extent of sample ordering were found to adopt the cross β structure associated with amyloid type molecule.

The main objectives for WP5 were to modify the identified in WP2 self-assembling building blocks with amino acids targeted for metal binding and cell recognition. Three cysteine –containing peptides were designed by P1 (UoC), studied and were found to successfully template the growth of silver, gold and platinum nanoparticles. Moreover, three aromatic peptides were designed by P2 that contain the Arg-Gly-Asp (RGD) motif, a cell attachment site of a large number of adhesive extracellular matrix, blood, and cell surface proteins. The ability of the peptide to self assemble into hydrogel were analyzed. In addition, a cysteine peptide modified with the cell-binding motif RGD at the opposite end was specifically designed by P1 in order to attach to gold surfaces/electrodes with the cysteine end and support cell attachment at the other end). These peptides were supplied to partners P6, P7 and P2 for testing their positioning and integration in MEAs as well as eventual scaffolding role in nerve regeneration.

The main goal of WP6 was to investigate manipulation methods that would allow to integrate the biological nanostructures supplied by partners P1 (UoC) and P2 (TAU) with Microsystems.
A Field Effect Transistor (FET) with attached nanobioassemblies was fabricated and presented to the consortium by P3-DTU Nanotech. The FET microchip was fabricated using standard photolithography and allowed the manipulation, immobilization and electrical characterization of the peptide self-assembled nanostructures. A second FET microarray with attached nano-assemblies was fabricated using a similar technique but in this case having the option of parallel assembly. The manipulation and immobilization of the peptide self-assembled nanostructures was done by dielectrophoresis (DEP) and microfluidics. Using this type of FET microchip, empty peptide nanotubes and silver filled peptide nanotubes were manipulated, immobilized and an I-V curve was build to characterize the conductivity of these biological samples. In the I-V curve for a silver filled peptide nanotube a big increase in the current is obtained due to the presence of silver inside the nanotube; compared with the current obtained passing though an empty peptide nanotube the increase is about 10^9 times. These results constitute a huge step in the development of a Field Effect Transistor using biological fibres. **A common P1-P2-P3 US patent on this subject was submitted on December 28, 2009**. Another objective for WP6 was to report on existing literature and know-how concerning properties of the assemblies and methods for combining them. The report was expanded into a review article ([1] Castillo, J., Dimaki, M., and Svendsen, W. (2009) Integ. Biol. 1, 30-42) and will be further expanded in a shape of a handbook which is on preparation and will be published in 2011 by Taylor & Francis. A second report on initial electrical, structural and electrochemical measurement of the peptide nanotubes and nanofibers was circulated between the consortium describing the potential of these nanostructures for their future integration in biosensing devices.

The main objectives for WP7 were to incorporate the nanostructures being developed from the other work packages on surface of Micro-Electrode Arrays biochips for purposes of improving device characteristics and demonstrating potential concrete applications with high market potential in the area of life sciences and to develop a biosensor platform for applications in clinical diagnostics and drug discovery. Partner 7 (AYANDA) has selected the peptide nanofibrils that had produced the best results in

relation to adhesion and stability on MEA microelectrodes. Experiments using peptide fibrils based on building block from the adenovirus fibre shaft (GAITIG) have resulted in selective coating of the MEA electrodes with good electrode coverage when using high concentration peptide solutions. The obtained coatings can be used for cell monitoring applications using the MEA technology. Cellular cultures with cysteine peptide fibrils designed by P1 (UoC) with or without the cell attachment motif RGD show good biological activity, especially due to the fact that there is a better coverage of such electrode versus non-coated electrodes. The biosensor platform has been validated with experiments using Gabazine, a $GABA_A$ receptor antagonist, where expected biological results have been achieved. These first positive results indicate that peptide fibrils are a valuable add on for MEA technology, improving the chances to get recordings from more electrode sites without affecting cell culture viability and biological response to drugs. Furthermore, three reports on diagnostic platform using clinical–like samples, on biological assays with ion channel drugs on cells, and on market feasibility assessment of biosensors for diagnostics, were delivered.

The main objectives for WP8 were to study the degenerative process following optic nerve injury and to test the eventual effect of hydrogels formed by the self-assembled building blocks on optic nerve regeneration. Partner 2 (TAU –NBC) has developed a protocol for studying the degenerative process in the optic nerve. Several self-assembling peptides provided by P1 (UoC) were found to form implantable hydrogels and were tested on axotomized optical nerves to test if they can act as 3D scaffolds for nerve regeneration. Very encouraging results were obtained with hyaluronic acid and one GAITIG –based peptide. Another goal was to use implantable electrodes to allow nerve stimulation at pre-determined periods in order to examine whether electrical activity could assist axonal regeneration and survival. After AYANDA (P7) tested several types of puff electrodes made out of several different materials they delivered to P2 (TAU) an electrode which could be implanted and wrapped the optic nerve. MRI analysis showed that the electrode wraps around the optic nerve and does not cause any inflammation or any sings of stress to the implanted animal.

The management (WP9) supported the progress of the project, the consortium and the coordinator in the daily activities, including administrative, financial and human resources management. Communication and Dissemination activities gained high priority, and the project website http://www.materials.uoc.gr/~BeNatural/ presents the most important public activities. The obtained results have been largely disseminated using classical ways, such as conferences, publications, public lectures, etc.