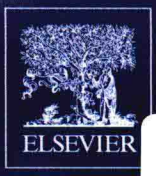

Edited by
Jaime Castillo-León
Winnie E. Svendsen

MICRO AND NANOFABRICATION USING SELF-ASSEMBLED BIOLOGICAL NANOSTRUCTURES

Micro & Nano Technologies Series

Micro and Nanofabrication Using Self-Assembled Biological Nanostructures

Edited by

Jaime Castillo-León
Winnie E. Svendsen

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PREFACE

Micro and Nanofabrication Using Self-Assembled Biological Nanostructures is a result of the research activities of a group of scientists worldwide who wrote the chapters. This book is dealing with the possibilities and challenges when using self-assembled peptide nanostructures for the fabrication of cell scaffolds, drug-delivery systems, biosensing platforms, and new nanostructures. In a very didactic way this work presents the state of the art in the use of self-assembled biological nanostructures, and its advantages and challenges.

We would like to thank all the authors for their effort in helping us to put together this work. We also would like to thank Elsevier for inviting us to publish this book and all the support during its preparation.

We hope that this book will motivate a broad audience of students and researchers interested in self-assembled biological nanostructures so as to consider these technologies as an alternative in their research resulting in new and exciting applications.

Jaime Castillo-León
Winnie E. Svendsen
2014

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CHAPTER 1

Self-Assembled Biological Nanofibers for Biosensor Applications

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1.1 INTRODUCTION

The use of nanomaterials in analytical systems has been gradually growing, due to the promise of increased precision and sensitivity offered by the nanoscale dimensions. When used as nanoscaffolds, the high surface-to-volume ratios provided by nanomaterials offer an increase in immobilization of functional sensing components, resulting in the ability to detect lower analyte concentrations [1].

Bionanomaterials, such as biological nanofibers, offer particular advantages over their inorganic counterparts: the laboratory conditions for their self-assembly and synthesis are mild when compared with harsh solvents and environments found in inorganic nanofabrication techniques; there are available primary sources for biological nanofibers originating from low-value coproducts of the pharmaceutical and biotech industry, offering cost-effectiveness and an environmentally friendly approach to the production of nanomaterials [2–5]; the biological nature of bionanofibers adds a biocompatibility element to their applications, as well as a versatility in functionalization due to the variety of chemical moieties provided by the amino-acid residues in their protein and peptide building blocks. Because of these benefits, biological nanofibers have been gathering considerable attention over the past decades by the scientific community, expanding the field by studying this bionanomaterial and showing its potential to be applied not only in biosensor systems, but also in a variety of bionanotechnological applications such as drug delivery systems, bioelectronics, and tissue reparation [6].

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Although bionanofibers are a promising material and there are continuous advances in their integration with analytical detection systems, there are still major challenges that have to be overcome before being able to fully commercialize their use in biosensing. Even lab-bench approaches of their use result in issues and limitations – mostly a function of the same characteristics that make this material attractive. Their stability in extreme temperatures, solvents, and conditions, required for their commercial use, renders some types of bionanofibers unusable [7–13]. The variety in surface moieties available for functionalization, one of the most attractive features of bionanofibers, is on the other hand also a hurdle to commercialization, since a specific functionalization strategy is needed for different systems, depending both on the type of bionanofiber, the primary source, and the functional biosensing element [14–18]. The precise manipulation and characterization of bionanofibers is an additional challenge, and novel nanotechnological methods must be developed to achieve this [13,19–23]. Finally, the poor conductivity of bionanomaterials requires additional consideration, as charge-transfer is a major parameter in biosensing systems.

1.2 TYPES OF SELF-ASSEMBLED BIOLOGICAL NANOFIBERS

Via self-assembly, biological building blocks, such as peptides and proteins, can yield a variety of nanofibers, each with specific characteristics reflecting the source material and the self-assembly conditions. Natural proteins tend to form wire-like, flexible nanostructures with high internal structural order, resulting in stability in natural environments. Artificially designed biological building blocks, on the other hand, will self-assemble into a variety of well-defined nanofibers such as nanotubes, nanowires, or nanofibrils, each with specific properties and advantages.

1.2.1 Natural Protein Nanofibers

There are several protein sources that naturally self-assemble into fibrous nanostructures with mechanical and chemical properties that render them useful in biosensors applications. From collagen and actin to cellular microtubules, nature has provided us with a myriad of biological building blocks that, under the right conditions, can be made to self-assemble into bionanofibers *in vitro* [24].

Within this range of naturally occurring bionanofibers, silkworm and spider silk are of particular interest to the scientific community, due to their simple molecular design and the impressive mechanical strengths that these materials exhibit [25–27]. The primary proteins present in silk are fibroin and sericin, although there is a wide variation between silk-producing species, and therefore also in the structural conformation of the silk nanofibers produced [28].

Silk nanofibers have been extensively integrated into nano- and microtechnology, and micropatterned silk proteins have been used to develop biocompatible nanoscale biosensor platforms [29], utilizing a metallization method to create free-standing silk/metal composite nanofibers [30]. The photonic properties of silk nanofibers have also been exploited for the development of optical biosensor platforms [31,32], and the specific biocompatibility of this nanomaterial has moved the field toward implantable biosensor systems [33].

1.2.2 Amyloid Fibrils

Amyloid fibrils are insoluble nanostructures resulting from the self-assembly of unfolded protein monomers. They hold a distinctive quaternary structure predominantly characterized by a rich, hydrogen-bonded, β-sheet conformation [34,35], a configuration with a rigid internal order that provides nanostructures with high strength, stability, and high-morphological aspect ratios [12,36]. It is perhaps their stability and insolubility in aqueous media that renders amyloid fibrils favorable in biosensor applications [17,37–39] with advantages over other biological nanofibers such as peptide nanotubes [11].

Because of the versatility of protein monomers as molecular building blocks, the source proteins that have been shown to undergo fibrillation under controlled environmental conditions are abundant and span a wide range of biological materials. The list includes insulin [15,40–42], considered a historical standard amyloid fibril source for bionanotechnological applications, fungal hydrophobins [43], ovalbumin [2], and lysozyme [44]. Important additions to this list have been proteins that are considered industrial waste materials, such as whey proteins [4,5,45–48] and eye lens crystallins [3,36,49].

1.2.3 Peptide Nanotubes and Nanowires

Inspired by Nature's great success in forming self-assembled fibrous materials, researchers have synthesized peptide monomers able to recreate protein-like interactions, by focusing on the same amino-acid repeat units that play a key role in the self-assembly of proteins [50,51].

One peptide in particular has attracted much attention from researchers in the field over the past decade: diphenylalanine. This aromatic dipeptide is known for being the core recognition motif of the Alzheimer's disease β-amyloid polypeptide, and its use in bionanotechnology has been encouraged by the mild environment (room temperature, aqueous conditions) needed for its self-assembly. An additional advantage of using peptides as molecular building blocks is their ability to form several different nanostructures depending on the environmental conditions used during the self-assembly [52], such as nanotubes [53,54] and nanowires [55–57].

Peptide nanotubes, hollow nanofiber structures formed by, among others, the diphenylalanine peptide, have been extensively considered for the application of self-assembled nanostructures in biosensor platforms. The amyloid-like aromatic stacking that allows the self-assembly of peptide nanotubes confers an unusual strength to this nanomaterial [58,59]. This property, along with an ease in functionalization offered by the chemistry available on their surface, has allowed for the creation of several biosensor platforms based on the use of peptide nanotubes [60–68].

Diphenylalanine nanowires, solid rod-like bionanofibers, have been gaining scientific interest because they offer most of the positive attributes of their tubular nanostructure counterparts, but with an additional chemical and thermal stability [10] that the nanotubes do not possess [11]. The nanowires' surface-dependent growth mechanism is also one of their advantages, especially for surface-based biosensor platforms. Although their synthesis involves aniline vapor and does require higher temperatures than for nanotube the self-assembly [55,56], covering a surface with peptide nanowires creates a biocompatibility useful especially for biosensor platforms tailored toward biological cell analysis [69–73] (Table 1.1).

Table 1.1 Examples of the Variety of Biological Nanofibers That Have Been Used in Biosensor Platforms

Bionanofiber	Source Biomolecule	Sensor Target	Biosensing Technique	References
Silk nanofibers	Silk proteins	Refractive index	Optical	[31,32]
Amyloid fibrils	Whey proteins	Glucose	Electrochemical	[17]
	β-Lactoglobulin	Humidity, enzyme activity	Mechanical	[37]
	β-Lactoglobulin	Humidity, pH	Electrical	[38]
Peptide nanotubes	Diphenylalanine	Glucose, NADH, H_2O_2, and ethanol	Electrochemical	[60–63]
	Naphthylalanine-naphthylalanine	Phenols	Electrochemical	[62]
	Cyclic peptides	*Escherichia coli*	Electrochemical	[66]
	Bola-amphiphilic peptides	Pathogens	Electrochemical	[67]
Peptide nanowires	Diphenylalanine	H_2O_2	Electrochemical	[69,72]
	Ionic-complementary peptides	Glucose	Electrochemical	[65]

1.3 PRACTICAL LABORATORY CONSIDERATIONS

The mild conditions for self-assembly vary depending on the protein or peptide source used as building block, and some systems require specific pH or temperature conditions during the assembly, and a different set of conditions for stability and storage. The effect of postassembly conditions on the nanofibers' structural integrity and stability are especially important when utilizing the nanofibers in biosensor platforms that require extreme conditions such as high voltages, temperatures, or extreme pHs.

1.3.1 Temperature Stability

Amyloid fibrils are known for their stability, which includes a resistance to thermal stress up to 100°C [74], due primarily by their highly ordered internal structure [75]. It is important to note that when working with amyloid fibrils, a temperature-related instability occurs at the lower temperature extremes instead of the higher ones. Domigan et al. have investigated the effect of storage temperatures on the morphology of amyloid fibrils prepared from bovine insulin [36]. The fibrils' morphology was unchanged upon long-time storage at room

temperature, but subjecting amyloid fibrils to freezing/thawing cycles resulted in fibril fracturing, yielding very short fibrils with lengths in the range of 1/10 of their original dimensions. Although the amyloid fibril structure remained intact, validated by fluorescence-based techniques, the integrity of the nanofibrils' morphological properties was lost. This important effect needs to be taken into consideration when utilizing amyloid fibrils in biosensor platforms, especially those requiring biocompatibility, since fibril fragmentation is known for increasing cytotoxicity [76]. Additionally, the freezing/thawing effect on fibril fragmentation is important to consider when shipping the material via airfreight, where the material is subjected to extreme low temperatures.

Peptide nanotubes, although used extensively in electrochemical biosensor platforms, encounter some issues in stability when compared with other biological nanofibers. High temperatures have been found to affect the peptide nanotubes' structural stability, and structural degradation has been observed when diphenylalanine nanotubes have been exposed to temperatures higher than 100–150°C [8,9,21]. A phase change has been observed in this temperature range, corresponding to a decrease in polarization and a corresponding phase transition at ~140°C [77]. This degradation at high temperatures is particularly cumbersome when wanting to utilize these nanofibers in biosensor platforms that require autoclave-based sterilization. As an alternative, peptide nanowires have proven to be much more robust in terms of temperature stability. Their self-assembly already requires temperatures above 100°C [55,56], and the nanofibers can withstand temperatures as high as 200°C, with a thermal decomposition only observed at ~250–300°C [10].

1.3.2 Solvents and Solubility

The stability of biological nanofibers in liquid environments is essential to their application in biosensor platforms, especially in systems where the nanofibers act as nanoscaffolds for a functional component such as enzymes or other active biomolecules. Degradation of the nanoscaffolds can result in a leakage of those components, and ultimately in an instability and lack of reproducibility of sensorial parameters.

Although diphenylalanine peptide nanotubes have been used to create proof-of-principle biosensor platforms, these nanofibers have been found at a later date to solubilize in aqueous solutions and methanol [11]. This limitation renders them unemployable in liquid-based systems, and research has moved toward utilizing other insoluble nanofibers for this purpose, like peptide nanowires from the same source dipeptide [10] or amyloid fibrils, which are insoluble by definition and have been specifically tested for solubility in common aqueous biological solvents and solvents used in microfabrication processes such as ethanol, isopropanol, methanol, and acetonitrile (with only the last one having a severe degradation effect onto the nanofibrils) [13].

1.4 FUNCTIONALIZATION APPROACHES

The majority of applications of biological nanofibers in biosensor platforms involve the use of the nanostructures as scaffolds for sensing components such as enzymes, antibodies, or other relevant biomolecules. The attachment of those components onto the nanofibers can rely on several mechanisms and interactions (see Figure 1.1), and is mainly dependent on the activity and stability of the functional components.

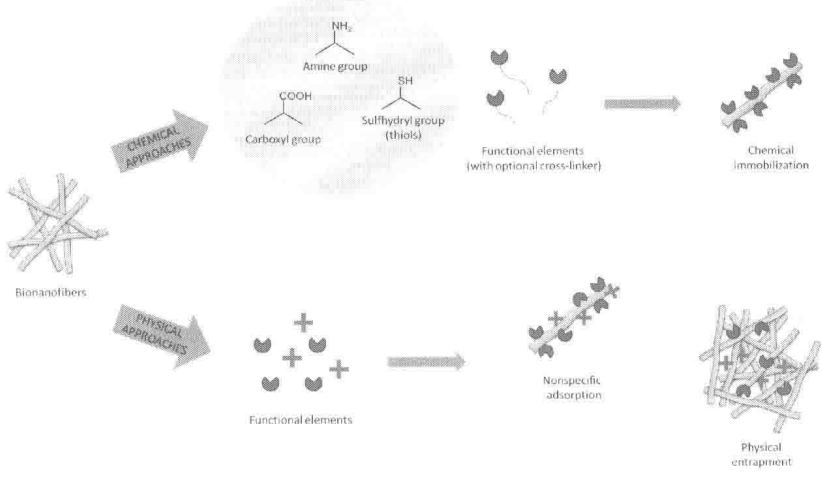

Fig. 1.1. Schematic of common bionanofiber functionalization approaches.

1.4.1 Chemical Approaches

Chemistry-based approaches are preferred for nanofiber functionalization because of the many advantages they offer, including stronger nanofiber/biomolecule attachment and long-term system stability. Additionally, the variety of chemical moieties present on the nanofiber surface offers a favorable approach in exploiting protein chemistry and other biological interactions, using the amino acid residues on the surface of the functional components (e.g., enzymes and antibodies).

Cross-linking is a functionalization approach based on strong covalent bonds, and for this reason is considered the most stable immobilization method [78]. Cross-linker molecules can act as a bridge in functional component/nanofiber interactions with free moieties that are not necessarily compatible. This functionalization mechanism has alternative advantages, due to the flexibility in cross-linking conditions, spacer arm, and specificity in chemical orientation of the functional component. This latter concept is extremely important in biosensing systems that require a specific activity of the functional component, such as enzymatic biosensing mechanisms, where a loss of enzymatic activity can occur due to conformational restrictions [79].

The active functional groups that are primary targets for the functionalization are primary amines (present in lysine residues) [14,17], thiols (cysteine residues) [80], and carboxylic acids (aspartic and glutamic acid residues) [65,66]. These groups are commonly used in protein chemistry, and their operational chemistry is well understood and characterized.

1.4.2 Physical Approaches

For cases where a specific functionalization is not possible, either because of poor nanofiber surface chemistry, lack of specificity of cross-linker interactions, or high costs of functionalization reagents, other nonspecific functionalization approaches can be used.

Physical adsorption is the most cost-effective method for nanofiber functionalization, and its simplicity is an attractive advantage over more

specific techniques. Functional components can be attached to a nanofiber scaffold by simple physical interactions such as hydrogen bonding, Van der Waals forces, or ionic attractions [81–83]. Adsorption can be achieved in mild conditions, and as a noninvasive method it rarely affects the functionality of the immobilized component, e.g., enzymatic activity. Being nonspecific, adsorption also allows for a facile multifunctionalization of the nanofibers. There are issues though with this method, and often the attachment is weak and can result in functional component dissociating and therefore leakage over long periods of time or under changes in environmental conditions such as ionic strength, temperature, or pH [84].

An additional nonspecific, but stronger and more stable approach is network physical entrapment. When used in relatively high concentrations, biological nanofibers can act as a polymeric network that, when assembled onto a surface, can encapsulate functional components. The greatest disadvantage of physical entrapment is the mass transfer limitations that can arise depending on the porosity of the obtained nanofiber networks. An optimization of entrapment parameters should be carried out to allow analytes to pass through the network while retaining the encapsulated functional components to avoid their leakage (Table 1.2).

1.5 COMMON CHALLENGES IN BIOSENSOR PLATFORMS

The major factors that are limiting the application of bionanofibers in biosensor platforms are caused by the small dimensions of the individual nanofibers as well as their biological nature. The individual manipulation of biological nanostructures is crucial when developing biosensor platforms that require single nanofibers to be placed in a specific location (see Figure 1.2). Fortunately, nanotechnological tools are in constant development, and there are several techniques that can be used for achieving precise handling of bionanofibers. An additional common challenge in bionanofiber-based biosensor development is the poor electrical properties of biological materials, a trait that must be kept under consideration especially in electronic and electrochemical detection systems. Mindful consideration of these attributes, though, can aid in optimizing conditions to ultimately overcome these charge-transfer limitations.