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Self-Assembly of Collagen Building Blocks Guided by Electric Fields

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Abstract

Protein building blocks are programmed to assemble hierarchically and yield a defined fiber morphology of micrometric length and precise nanometric diameter. The key step of this method is to align the building blocks with an AC field prior to assembly. The resulting protein nanofibers are straightforwardly integrated with the circuitry for potential applications in bionanotechnology.

Introduction

The self-assembly of nanoscale building blocks is a ubiquitous phenomenon in biology that has been essential for cell function since the origin of life. The final size and shape of these biological assemblies is determined by the spatial arrangement of nanometric building blocks, which are positioned with exquisite precision.[1] This observation has become a central topic in bionanotechnology because biological self-assembly has the potential to bridge the dimensional gap between nanoscale objects and microscale devices, a key step for integrating the fascinating physical properties of nanomaterials with common microprocessors and circuits.[2] In vivo, these self-assembly processes are guided by an intricate network of biomolecular signals orchestrated by cells.[3] However, the incommensurable complexity of cell regulation is still poorly understood, and therefore it is extremely difficult to replicate biological self-assembly conditions in vitro. Consequently, attempts to control self-assembly in vitro rarely yield a single structure with defined size and shape, thus impeding its application as a reliable fabrication method. Therefore, it is desirable to find an external stimulus that guides self-assembly in vitro to generate objects with unique morphology determined by the spatial arrangement of constituent biomolecules. [4] Here, we guide the assembly of collagen building blocks with electric fields to obtain protein nanofibers of micrometric length and precise nanometric diameter at a desired

location on a chip. This method could bridge the gap between nanoscale objects and microscale devices, a central issue in bionanotechnology.

In this communication, we apply AC electric fields to guide the self-assembly of protein building blocks and obtain micrometric structures with desired morphology and precisely controlled nanometric dimensions. This is achieved by applying a non-uniform AC field of desired frequency and peak-to-peak potential with a pair of planar electrodes separated by a micrometric gap (Figure 1). At a particular frequency, this AC field polarizes the protein building blocks and pulls them towards points of maximum electric field, in this case, the electrode tips and the gap between the electrodes.[5] The accumulation of building blocks in these regions triggers their self-assembly and yields structures with different morphology and dimensions depending on the duration of the AC field stimulus. The application of AC fields is ideal for manipulating biomaterials because it is non-destructive and does not change the composition of the solution, which are both common issues related to the application of direct currents. It will be shown that the combination of top-down photolithography and bottom-up self-assembly results in the facile fabrication of microstructures containing nanometric features and fully integrated with microfabricated parts. Therefore, we propose that protein self-assembly could be an effective tool in nanofabrication and an alternative to other costly techniques such as electron beam lithography or AFM lithography, which require sophisticated equipment and highly trained personnel.

We studied the incorporation of protein self-assembly in nanofabrication by guiding the self-assembly of collagen building blocks with AC fields generated by microfabricated electrodes. Collagen is a highly abundant structural protein that exemplifies the benefits and difficulties of biological self-assembly. On the one hand, collagen can assemble to grow myriads of structures ranging from 1D nanofibers to 3D hydrogels,[6] making it a versatile building block in the fabrication of many advanced materials such as nanowires,[7] bioscaffolds[8] and supracrystals.[9] On the other hand, in vitro, it is difficult to control collagen self-assembly to yield assemblies with defined size and shape and at a desired place on a chip. Figure 2a shows a micrograph of the arrow-shaped microelectrodes separated by a gap of $\approx 2 \mu\text{m}$. The presence of collagen on the chip was determined by performing a specific immunoassay with fluorescently labelled polyclonal antibodies against collagen type I (Methods Section can be found in the Supporting Information online). We observed that collagen accumulated at the tips and at the gap between the electrodes after applying an AC field of frequency 10 kHz and 10 V peak-to-peak potential (Figure 2b). No fluorescence was observed at higher or lower frequencies, which agreed well with previous reports that the accumulation of dielectric objects with AC fields occurs only in a narrow frequency range.[10] Remarkably, collagen was not found randomly distributed on the chip but was only detected at the gap between the electrodes, which demonstrated the ability to selectively position our biological self-assembling unit at the region of interest by using alternating currents.

To demonstrate that the observation of fluorescence was related to the presence of collagen and not due to non-specific interactions, we corroborated the outcome of the immunofluorescence experiment with label-free Raman spectroscopy. In Figure 2c, the

following signals specific to collagen were detected at the gap between the electrodes, which further demonstrate the presence of collagen: C–C stretching of proline (855–856 cm⁻¹), [11] phenylalanine in collagen (1004 cm⁻¹), [11, 12] CH₃CH₂ collagen twisting mode (1313 cm⁻¹), [13] CH₃CH₂ wagging (1343 cm⁻¹), [11] CH₃CH₂ deformation (1343 and 1442 cm⁻¹), [11] as well as other peaks indicative of the presence of proteins: amide I (region 1600–1650 cm⁻¹). [14]

After demonstrating the selective positioning of collagen at the gap between microfabricated electrodes, we studied the growth of structures with different morphology. To this end, we applied the AC field stimulus for different increments of time and visualized the position, orientation and state of aggregation of the collagen building blocks with atomic force microscopy (AFM). The size of the nanostructures was estimated from height measurements. Lateral dimensions are biased due to tip convolution artefacts. In Figure 3a, nanostructures with a mean diameter of 1.4 ± 0.2 nm ($n = 30$) were found at the gap between the electrodes after 90 s. This value agrees well with the reported diameter of tropocollagen, which is the natural building block of collagen nanostructures. [15] This observation demonstrates that the building block is not aggregated prior to assembly. After 120 s, the formation of collagen nanofibers is observed at the edge and the gap between the electrodes (Figure 3b). The average diameter of the nanofibers is 13 ± 1 nm ($n = 10$). It is remarkable that all the nanofibers show a very similar diameter, since collagen self-assembly usually yields structures of different morphology in the absence of a guiding stimulus. Similarly, in Figure 3c, the length and the diameter of the nanofibers increases when the AC field is applied for 180 s. In this case, the protein nanofibers show a diameter of 17 ± 2 nm ($n = 10$). However when the AC field is applied for 240 s nanofibers are not observed anymore and a continuous, unstructured collagen bridge is observed between the electrodes (Figure 3d). This could be due to the overheating of the solution after the application of the AC field for a prolonged time period. [16] All in all, the results shown in Figure 2 demonstrate that the judicious application of AC fields of adequate frequency and peak-to-peak potential can guide the assembly of collagen building blocks and yield protein nanofibers that are perfectly integrated with microfabricated electrodes. The generation of collagen nanofibers of controlled diameter shown in Figure 3b,c could be particularly useful in the fabrication of collagen-templated nanowires [7] or biosensors [17] with previously demonstrated methodologies.

The growth of collagen nanofibers of precise diameter in Figure 3b,c is an interesting phenomenon that is not usually observed when collagen building blocks are assembled in the absence of a stimulus. In these images we can see that the nanofibers are aligned with respect to the distribution of electric field lines between the electrodes. In Figure 3a the tropocollagen building blocks do not show a preferential orientation of the protein at the gap between the electrodes. However, these protein building blocks are clearly stretched and aligned following the distribution of electric field lines at the tip of the electrodes. Based on these observations, the following mechanism of nanofiber formation is proposed (Figure S2).

Collagen self-assembly can be considered as the formation of structural nuclei at the tips and then propagation (growth) of these structural nuclei. [18] In the absence of a guiding

stimulus, the self-assembly of collagen begins as a random process in which the protein building blocks collide with each other with arbitrary orientations. As a result, collagen nanofibers nucleate at different rates throughout the solution. Under these conditions, nanofibers of uncontrolled diameter are obtained because it is not possible to separate the nucleation of new assemblies from the growth of previously formed nanofibers (Figure S2a).

Upon application of the AC field the collagen building blocks are polarized and driven to the regions where the field is the strongest, that is, the electrode edges. This phenomenon is usually referred to as positive dielectrophoresis, and it has been previously used to accumulate objects such as nanoparticles, nanotubes and cells at specific locations on a chip. [19] Positive dielectrophoresis has three important effects on the collagen building blocks, namely it: 1) increases the concentration of tropocollagen at the gap between the electrodes, where the field is the strongest; 2) induces the formation of a dipole in each building block; [20] and 3) aligns the building blocks with respect to the distribution of electric field lines and currents.[21] These effects last as long as the AC field is applied. Collagens are assembled side by side by charge interactions and water-assisted hydrogen bonding. Since the AC field does not change the solvation layer around collagen, the main effect of positive dielectrophoresis on collagen self-assembly is related to electrostatic interactions between collagen building blocks. Indeed it has been previously reported that the objects of different origin assemble head-to-tail due to the formation of dipoles triggered by AC fields. For example, latex beads can form chains under positive dielectrophoresis conditions.[22] Nanotubes assemble head-to-tail and side-by-side in a staggered fashion.[23] This type of AC field assembly imitates the natural self-assembly of collagen, which also grows in the longitudinal direction by packing monomers head-to-tail in a staggered fashion.[18] In other words, the AC field generates very favorable conditions to trigger the self-assembly of collagen building blocks. As a consequence, nucleation and growth are not random processes, but happen homogeneously and simultaneously, which favors the growth of assemblies of precise diameter (Figure 2Sb, Supporting Information).

In conclusion, we were able to program the self-assembly of biological building blocks in vitro to obtain protein structures of defined dimensions. The key step in harnessing this biological self-assembly process was to control the concentration and orientation of the constituent proteins with AC fields generated by microfabricated electrodes. The resulting protein nanofibers are straightforwardly integrated with the circuitry, which makes this approach especially appealing for the fabrication of nanowires[7] and biosensors.[17] Since the distribution of electric field lines is determined solely by the geometry of the electrodes, it should be possible to guide the selective growth of other assemblies with as-desired size and shape by simply changing the electrode layout. Furthermore, other stimuli, such as magnetic fields, could be explored to align the building blocks prior to assembly. Finally, the results shown here pave the way for the utilization of AC fields to control other biological self-assembly processes relevant in bionanotechnology such as the formation of bacterial S-layers[24] and the assembly of amelogenin.[25]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Scheme of electric field-guided assembly of collagen building blocks with arrow-shaped microelectrodes; a) An AC field is applied between microelectrodes covered by a solution of tropocollagen; b) Scheme of the distribution of electric field lines at the gap between the electrodes (based on the simulation in Figure S1). At the correct frequency, protein building blocks accumulate at the electrode edges and the gap between the electrodes where the field is the strongest.

Figure 2.

Accumulation of collagen at the gap between micro-fabricated electrodes; a) Micrograph of the microfabricated electrodes; b) Fluorescence image after treatment with polyclonal anti-collagen type I and FITC-labeled secondary antibodies; c) Raman spectrum collected at the gap between the electrodes, indicated by arrows in (a).

Figure 3. AFM images and cross-sectional analysis of collagen nanostructures found at the gap between the electrodes when the AC field was applied for: a) 90 s; b) 120 s; c) 180 s; d) 240 s. Arrows indicate the cross-section lines.