

Customization of Protein Single Nanowires for Optical Biosensing

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In recent years, nanowire- or nanofiber-based biosensors have been attracting great attention and efforts in many fields.^[1–8] Besides their fast response and high sensitivity resulted from their nanoscale sizes and large surface-to-volume ratios, nanowire-based biosensors have the versatility for physical, chemical, and biological detection through various sensing approaches including electrical, optical, and photonic methods.^[1–5,7] Especially, optical or photonic nanowire biosensors are becoming increasingly important because of the high sensitivity, fast response, and high detecting spatial resolution, which arise from the fact that the large portion of the propagating field is pushed into evanescent field and is therefore exposed to environment.^[5–7] As optical sensors, they are immune to electromagnetic interference, safe for combustible or explosive specimens, and offer more options for signal retrieval (e.g., optical intensity, wavelength, polarization, phase, and fluorescence).^[5] So far, diverse preparing methods including vapour–liquid–solid (VLS) growth, drawing processing by local heating of ready fibers or pull from melt phase, and electrospinning have been developed to obtain optical or photonic nanowire devices including sensors from a variety of materials, such as semiconductors, silica, polymers, ceramics, and organic-inorganic composites.^[1–12] However, there are still open problems that remain unsolved, typically, (i) the difficulty of precise design of the fiber parameters like cross-sectional diameter, length, and tapers, and (ii) the difficulty of nanofiber integration with other devices, i.e., the issue of positioning, shaping and connection, due to the limitation of the currently available fiber

fabrication technologies; (iii) for biosensors, either harsh preparing conditions are needed which may be deleterious to detecting probe molecules, or cumbersome modification processes of probe molecules have to be taken for specific biodetection. These problems may be partially solved by femtosecond laser direct writing (FsLDW) as an intrinsic digital nano-prototyping technology:^[13–23] (i) fiber devices of different geometries are modeled with computer-aided design (CAD) and then converted to real-world matter structures by FsLDW with reasonably high spatial resolution down to sub-20-nm resolution,^[13,14,17,18] and the nature of CAD guarantees the designability and reproducibility of fabrication;^[15–21] (ii) some devices of different optical functions and structures to be integrated with the fiber sensor may be designed as a part of a global model and are created in one-run laser fabrication; (iii) functional components are doped into the precursory materials of FsLDW,^[22,23] forming an effective portion of nanobuilding blocks, as eliminates laborious surface modification process of nanofiber surfaces. Importantly, low collateral damage of FsLDW can be achieved due to high space restriction of laser energy and fs-scale pulse width that is much shorter than thermal diffusion time.^[13–23] So the bioactivity of functional molecules (e.g., proteins) can be adjustably retained in FsLDW.^[24]

On the other hand, as a wide class of natural product material with various characteristics and functions, good biocompatibility and typical environment-friendship, proteins have been widely studied and utilized in biomedical and environment-friendly applications until now.^[25–28] In this research, we particularly chose protein-based biosensing molecules for FsLDW-construction of nanowire sensor architectures. As a noncontact, maskless and versatile 3D micro/nano-processing technology with nanometric resolution (<20 nm),^[13,17,18] FsLDW has been reported to be successfully used in the fabrication of 3D micromechanics,^[29] catalytic microdevices,^[30] neuronal development real-time guiding microsystems,^[31] protein-based micro-optics,^[32,33] etc. Nevertheless, to our knowledge, neither the appropriate utilization of FsLDW for optical nanowire biosensors, nor functionalized all-protein-based single-nanowire optical biosensors have been reported to date. Therefore, here we demonstrate a simple, general, and biocompatible FsLDW approach to obtain all-protein single-nanowire optical biosensors from probe protein's aqueous ink. In this work, with or without mixture of “inert” proteins (e.g., bovine serum albumin, BSA), biosensing probe proteins (e.g., avidin,

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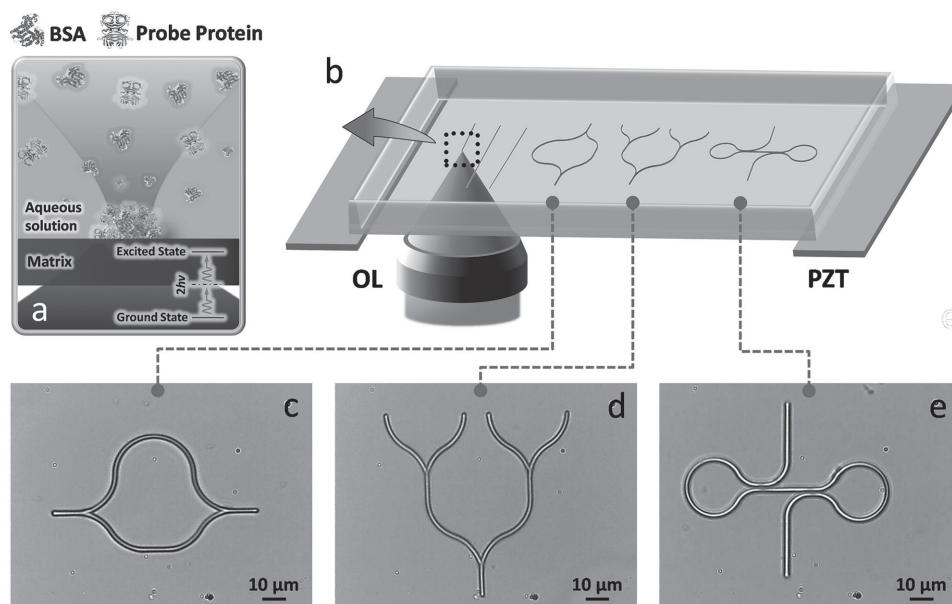


Figure 1. Schematic of protein FsLDW and optical microscopy images of protein micro/nanowaveguide devices fabricated by FsLDW. a) Illustration of two-photon polymerization of proteins in core of the focal area of a focused Fs-laser beam. b) The schematic of aqueous programmable FsLDW of various protein micro/nanowaveguide devices with complex geometries. c) Mach-Zehnder interferometer. d) Tree-form power divider. e) Sagnac resonator.

antibodies) were covalently photo-crosslinked by FsLDW into protein-nanowire biosensors for specific biotin-detection without laborious chemical modifying procedures. Excellent designability and repetitiveness were sufficiently demonstrated with satisfactory morphology (roughness average ≤ 5 nm), various designed sizes (≥ 150 nm) and complex geometries. The as-formed protein nanowires were proved experimentally to be adequate as nanowaveguides for evanescent optical biosensing (transmission windows for 700-nm diameter, around ≈ 500 and ≈ 680 nm; transmission loss in air, ≈ 0.05 dB μm^{-1} at 532 nm and ≈ 0.06 dB μm^{-1} at 633 nm). As a result, good biocompatibility, adjustable high biosensitivity (0.2 ppb or sensitivities even higher) can be readily realized for all-protein-based single-nanowire optical biosensors, which might be of wide and great uses for novel environment monitoring and biomedical applications.

In the experiment, protein single nanowires and nanowire devices could be readily fabricated with a set of home-made FsLDW system was utilized to directly write out nanowire devices as designed from aqueous solution of proteins (BSA, 450 mg mL^{-1} ; avidin, 50 mg mL^{-1}) and methylene blue (MB, 0.6 mg mL^{-1}) as a photosensitizer (see **Figure 1**, experimental details of fabrication are described in the Supporting Information). The amount of the introduced probe protein could be arbitrarily prepared as needed and be larger than that by surface modification on nanowires with similar size. For proper refractive index matching (refractive index of substrates < refractive index of waveguides) and then applicable waveguiding performance, MgF_2 slices with lower refractive index (≈ 1.39) were chosen as the substrate here. Processing parameters were carefully optimized. For good morphology and optical performances, relatively more laser energy accumulation was needed (larger laser power density and exposure time, smaller scanning step).^[32,33] However, on the

contrary, relative low laser energy accumulation was good for maintaining bioactivity of functional proteins as proved in Figure S1, Supporting Information. In Figure S1 (Supporting Information), for avidin here, the bioactivity can be characterized by the specific binding ability of biotin.^[24,29,31] By confocal microscopy, fluorescein-biotin was used for conveniently measuring fluorescence intensity to characterize the binding ability of biotin of avidin/BSA microhydrogels obtained by FsLDW (BSA, 450 mg mL^{-1} ; avidin, 50 mg mL^{-1}). As illustrated in Figure S1a,b (Supporting Information), avidin/BSA microsquares (Figure S1c, Supporting Information) fabricated with different processing parameters exhibited adjustable specific binding ability of biotin (laser power density, 27.5–45.8 $\text{mW } \mu\text{m}^{-2}$; scanning step, 100–200 nm; exposure time, 1000 μs ; laser power was measured before the objective). That is, bioactivity of crosslinked avidin and therefore biosensitivity of protein nanowires here could be tailored by tuning the processing parameters of FsLDW (more details are given in the Supporting Information). Therefore, in consideration of both optical performances of protein nanowires and proper bioactivity of crosslinked probe protein (avidin here), the laser power density, scanning step and exposure time were set as 36.6 $\text{Mw } \mu\text{m}^{-2}$, 100 nm, and 1000 μs , respectively. Under these parameters, acceptable waveguiding performances and high biosensing activity of protein single nanowires could be simultaneously realized.

Excellent designability and reproducibility of this approach were experimentally demonstrated for the fabrication of protein nanowires (see **Figure 2a**; linewidth of optimized protein nanowires, from ≈ 150 to ≈ 650 nm). The smallest linewidth of high-quality protein nanowires achieved in this work was ≈ 150 nm as proved in Figure 2b. Specially, in Figure 2c,e, several parallel identical protein single nanowires were easily fabricated (designed diameter, 700 nm), which

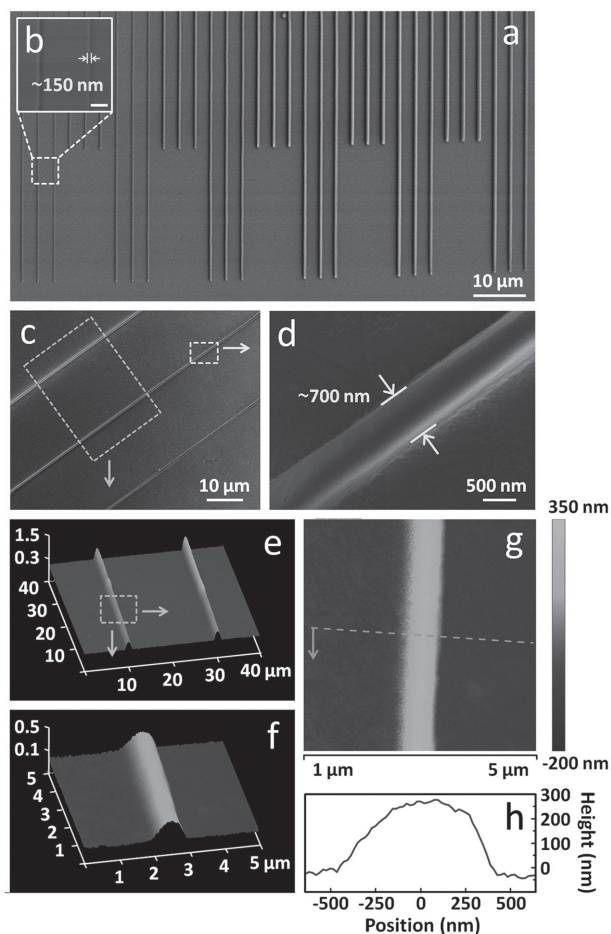


Figure 2. Characterizations of the morphology of protein (avidin/BSA) single nanowires on the MgF_2 slice by SEM and AFM. a) SEM image of parallel protein single nanowires with linewidth from ≈ 150 to ≈ 650 nm in good agreement with design. From right to left, diameter of nanowires 1–6, ≈ 650 nm; 7–12, ≈ 550 nm; 13–18, ≈ 450 nm; 19–24, ≈ 350 nm; 25–30, ≈ 250 nm; 31–33, ≈ 150 nm. b) The enlarged SEM image of high-quality protein nanowires with the smallest linewidth of ≈ 150 nm in a). Scale bar, $1\ \mu\text{m}$. c) The SEM image of three parallel protein single nanowires with identical geometries as designed. Optimized processing parameters: laser power density, scanning step, and exposure time were set as $36.6\ \text{mW}\ \mu\text{m}^{-2}$, $100\ \text{nm}$, and $1000\ \mu\text{s}$. d) Enlarged view showing details (≈ 700 -nm diameter and good surface quality) of the protein nanowire in the yellow dashed box in c). e) The oblique view of AFM characterization of the region in cyanic dashed box in c). f) The enlarged oblique view of AFM characterization of the region in cyanic dashed box in e) for visually exhibiting the morphology of the protein nanowire. g) The planform AFM image of f) showing a roughness average of ≈ 5 nm or even less. h) The cross section curve of the protein nanowire at the position of the green dashed line in g).

would be applied in the followed experiments of optical waveguiding and biosensing. Figure 2d–g of characterizations by scanning electron microscope (SEM) and atomic force microscope (AFM) showed the regional details of protein single nanowires in Figure 2c. The width of the protein nanowaveguides was measured to be ≈ 700 nm by SEM and AFM, which was in agreement with the designed value (see Figure 2d,g,h). The cross section of the protein nanowire at the position of the green dashed line in Figure 2g was shown in Figure 2h. The geometry of the cross section is similar

to a half round with a radius of ≈ 350 nm (see Figure 2f,h). By AFM characterization, the roughness average (R_a) was determined to be ≈ 5 nm or even less, so that the surface quality was good enough for an optical nanowaveguide. Because of the soft quality of protein microhydrogel (elastic modulus from 0.03 to 3 MPa for albumin),^[34] the half-round cross section and nanometric R_a of protein nanowires might be due to so-called self-smoothing behavior caused by local surface tension during the rinsing step in water. As the voxel size of two-photon polymerization is quadratically dependent on the laser light intensity, slight fluctuation of the laser pulse energy may result into obvious unevenness of the surface.^[32,33] For this reason, in order to obtain this good morphology (especially surface quality) of protein nanowires, the FsLDW system should work in stable environment with constant air relative humidity (RH, 30%) and temperature ($23 \pm 0.2\ ^\circ\text{C}$) to ensure its steady operation. Besides, the core part of the gauss beam from the femtosecond laser was intercepted and used in FsLDW for its better beam quality.

Based on good morphology and surface quality, protein nanowires worked well as optical nanowaveguides. Here, with an evanescent coupling method,^[5–7] incident light was efficiently launched into a protein single nanowire using nanoscale fiber tapers (see Figures 3a, and 4a–c). In Figure 3, a broadband supercontinuum (Fianium Ltd., SC450) was launched into an MgF_2 -supported 700-nm-diameter protein single nanowire in air. Transmission spectra were detected by a spectrometer (Ocean optics, QE65000) to study the waveguiding properties. The central wavelength of the supercontinuum was ≈ 600 nm, as proved by curve 1 in Figure 3b. With the increase of propagation distance of incident light, the transmission spectrum changed as shown in Figure 3b. Due to the guiding loss of the protein nanowire, output intensity of light went down along with the propagation in the protein nanowire (Figure 3b).

In previous reports,^[5–7] green or blue light with shorter wavelengths showed smaller loss during the propagation in subwavelength nanowaveguides and was supposed to be output at the end of a sufficiently long nanowaveguide because of the so-called “filtering effect.” But the experimental results in this work revealed that waveguiding windows of protein nanowires were at wavelengths of ≈ 500 and ≈ 680 nm in air (two transmission peaks in Figure 3b). And smaller guiding loss was proved at ≈ 680 nm in air, which was caused by the higher absorption of BSA/avidin/MB composite material at shorter wavelengths (see Figure S2a, Supporting Information; composite films were obtained by ultraviolet (UV) polymerization). So, more red light with longer wavelength was output at the end of a protein nanowire with enough long propagation distance (see Figure 3c and insets of Figure 5d,e,f). The dip of transmission spectra with the biggest loss was at ≈ 560 – ≈ 580 nm in air in Figure 3b, of which the most probable reason might be the introduction of MB to the material system. Compared to flat and relative low absorption spectrum of BSA/avidin/1173 (a colorless and transparent UV photosensitizer) composite, BSA/avidin/MB composite film had absorption peaks at ≈ 600 and ≈ 660 nm in air as shown in Figure S2a, Supporting Information (high concentration of MB of $\approx 20\ \text{mg}\ \text{mL}^{-1}$ for

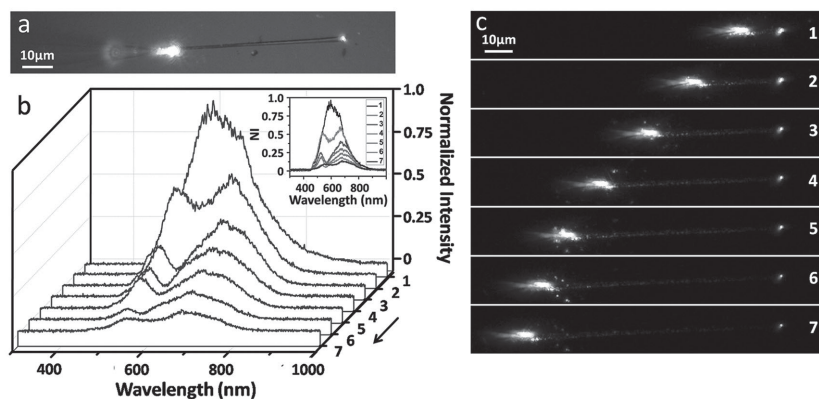


Figure 3. Waveguiding tests of the MgF_2 -supported 700-nm-diameter protein nanowire with a broadband supercontinuum as incident light and the evanescent coupling method using a nanoscale fiber taper. a) The optical microscopy image of the waveguiding test with a broadband supercontinuum. b) With the increase of propagation distance from test 1 to test 7, transmission spectra changed and output intensities went down, exhibiting the transmission windows at ≈ 500 and ≈ 680 nm (exhibited by a “3D waterfall” form). (Inset) 2D form of the collection of transmission spectra of b). c) Dark field optical microscopy images of the waveguiding tests with a broadband supercontinuum (coupled in from the left) and increasing propagation distance. The images of (1)–(7) correspond to spectra from (1) to (7) in b), respectively.

UV polymerization). This agreed with the previous reports on interaction between proteins and MB.^[35] For avidin/BSA microhydrogels obtained by FsLDW, little MB was left inside the hydrogel network after sufficiently rinsing (almost colorless in Figures 1c–e, 3a, 4a–c, and 5b by optical microscope). The blue shift of absorption for BSA/avidin/MB microhydrogels by FsLDW might be caused by the low concentration of MB ($<0.0032 \text{ mg mL}^{-1}$) and the interaction between MB and proteins as reported.^[35] Here, it is particularly worth emphasizing that the transmission window of protein nanowires at red and near-infrared wavelengths (from ≈ 600 to ≈ 700 nm) is precisely the important biological optical window for various tissues and organs, for example, skin and adipose tissue. This further implies that the protein single nanowire biosensors may be of great significance and potential for biomedical applications.

Additionally, monochromatic incident light with wavelength of 532 or 633 nm from semiconductor lasers was evanescently coupled into the MgF_2 -supported 700-nm-diameter protein single nanowire with a fiber taper (see Figure 4a–c). In Figure 4d, propagation-distance-dependent output intensities were characterized with incident light from a 532-nm laser for instance. By changing the coupling position and increasing the propagation distance, the output intensity of the guided monochromatic light gradually decreased at the

end of the 100- μm long protein single nanowire. Similar phenomenon could be observed for incident light of 633 nm or other wavelengths. According to the results in Figure 4d, output intensities dependent on propagation distance were calculated and exhibited in Figure 4e for 532-nm incident light. The output intensity of each nanowire decreases with propagation distance exponentially, which is attributed to the exponential absorption of the light propagated along the nanowire obeying Lambert–Beer law. The fitted curve of the experimental data yielded a waveguiding loss of $\approx 0.05 \text{ dB } \mu\text{m}^{-1}$. By the same method, the curve of 633-nm incident light was obtained in Figure 4f. The waveguiding loss for 633 nm was calculated to be $\approx 0.06 \text{ dB } \mu\text{m}^{-1}$. These results agree well with the transmission spectra in Figure 3b. In this work, although the guiding loss is relative large possibly because of the material characteristics of proteins, the waveguiding performance of the protein single nanowire is acceptable for the application as nanobiosensors.

Owing to the extremely high affinity of specific interaction (dissociation constant, $K_d \sim 10^{-15} \text{ m}$),^[36] avidin–biotin technology has been widely applied in biological nanofabrication,

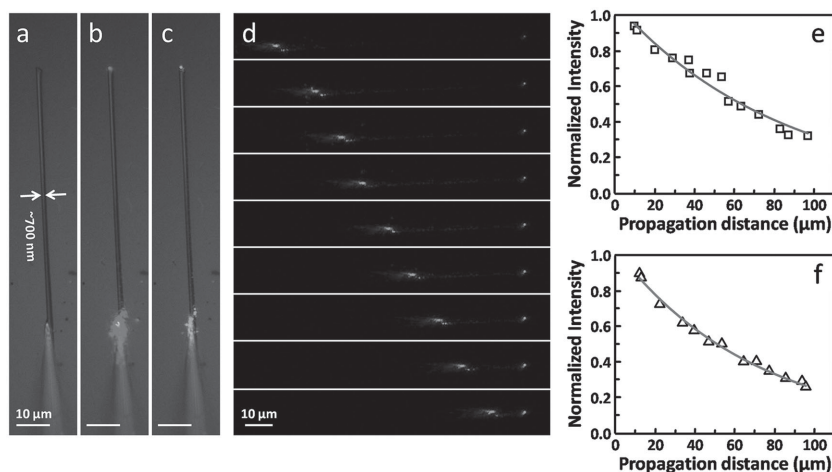


Figure 4. Waveguiding tests of the MgF_2 -supported 700-nm-diameter protein nanowire with monochromatic incident light of 532 or 633 nm laser and the evanescent coupling method using a nanoscale fiber taper. a) The optical microscopy image of the protein nanowire coupled with a nanoscale fiber taper without incident light for clearly exhibiting the details of the evanescent coupling. b) The optical microscopy image of the protein nanowire evanescently coupled with 633-nm laser (exactly the same set of the protein nanowire and the fiber nanotaper in a). Scale bar, 10 μm . c) The optical microscopy image of the protein nanowire evanescently coupled with 532-nm laser (exactly the same set of the protein nanowire and the fiber nanotaper in a). Scale bar, 10 μm . d) Dark field optical microscopy images (from top to bottom) of the waveguiding tests with the increasing output intensities and correspondingly decreasing propagation distance by using 532-nm incident light (coupled in from the left). e) The propagation-distance-dependent output intensities with 532-nm incident light and their fitted curve by first-order exponential decay fitting that obeys the Lambert–Beer law. f) The propagation-distance-dependent output intensities with 633-nm incident light and their fitted curve by first-order exponential decay fitting that obeys the Lambert–Beer law.

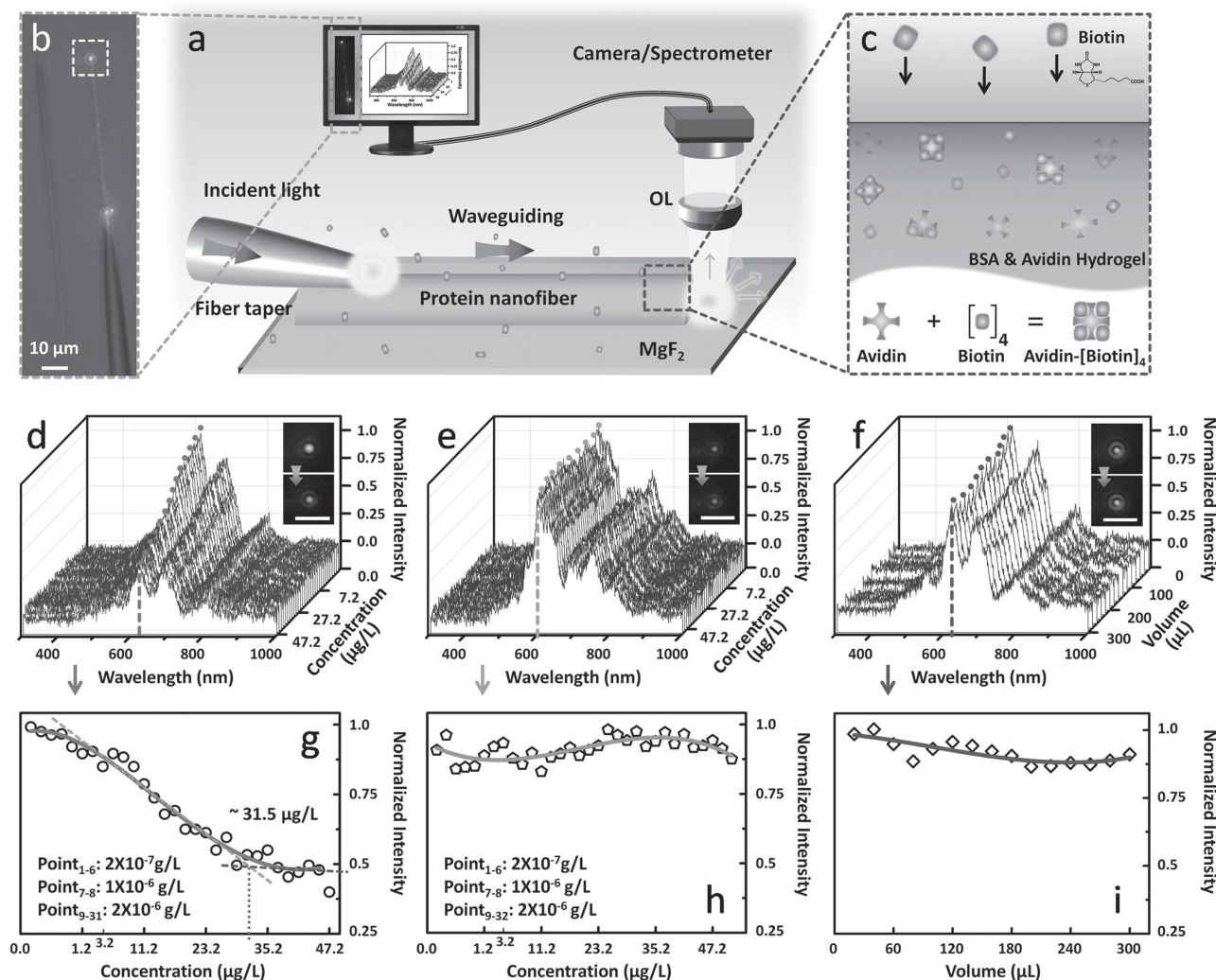


Figure 5. The biotin optical sensing with high specificity and sensitivity (0.2 ppb or even lower concentration) of the protein (avidin/BSA) single nanowire. a) The schematic of the biosensing of biotin using an avidin/BSA single nanowire. b) The optical microscopy image of the 700-nm-diameter avidin/BSA single nanowire evanescently coupled with a broadband supercontinuum for biosensing of biotin. c) The illustration of the interaction between biotin in aqueous surrounding and avidin crosslinked in the hydrogel network of the protein nanowire. d) Transmission spectra of an avidin/BSA nanowire arranged in the form of “3D waterfall” with increasing biotin concentration (from 0.0 to $\approx 47.2 \mu\text{g L}^{-1}$). (Insets) The optical microscopy images of output light before (top) and after (bottom) the changing process of biotin concentration. Scale bar of insets, 10 μm . e) Transmission spectra of a BSA nanowire arranged in the form of “3D waterfall” with increasing biotin concentration (from 0.0 to $\approx 47.2 \mu\text{g L}^{-1}$). (Insets) The optical microscopy images of output light before (top) and after (bottom) the changing process of biotin concentration. Scale bar of insets, 10 μm . f) Transmission spectra of an avidin/BSA nanowire arranged in the form of “3D waterfall” with addition of pure water (total volume up to 300 μL , as the same as total volume of added biotin solution in d,e). (Insets) The optical microscopy images of output light before (top) and after (bottom) the adding process of pure water. Scale bar of insets, 10 μm . g) The biotin-concentration-dependent peak intensities of transmission spectra in d) and their third-order polynomial fitted curve. h) The biotin-concentration-dependent peak intensities of transmission spectra in e) and their third-order polynomial fitted curve. i) The peak intensities of transmission spectra in f) dependent on volume of added water and their third-order polynomial fitted curve.

synthesis, biosensing, protein biochips, etc.^[37–40] In general, biotin itself is frequently used as an important detecting target for nutrition, diagnosis, labeling, and signal amplification in biosensing as well.^[36–40] In this work, as an example, crosslinked-avidin-based microhydrogels were used to specific detection of biotin (see Figure S1, Supporting Information). Herein, we should mention that BSA microhydrogels do not possess the specific biotin-binding property (just non-specific absorption in hydrogel network; see the Supporting Information with more details), as shown in the results in Figure S3 (Supporting Information). So, by proper selection

and mixture with the “inert” proteins, well-tailored protein single-nanowire optical biosensors could be produced using a certain amount of biosensing probe proteins as needed (avidin, antibodies, etc.). Also, much more varied biosensing probes may be facily modified on the protein nanowire biosensors by avidin–biotin interaction for multifarious detecting specimens.^[36,40]

As shown in Figure S1 (Supporting Information) of supporting information, numerous avidin/BSA nanowires had been fabricated by FsLDW with different processing parameters. In this work, the 700-nm-diameter avidin/

BSA nanowire, which has been exhibited and studied in Figures 2–4, were demonstrated of biotin optical sensing with high specificity and sensitivity (see Figure 5). As shown in the schematic of Figure 5a and the optical microscopy image of Figure 5b, the broadband supercontinuum was evanescently coupled into the avidin/BSA single nanowire on the MgF_2 slice. The set of the fiber taper and the protein nanowire was positioned and immersed in pure water at the beginning of the tests. After $\approx 60\text{-}\mu\text{m}$ propagation in the avidin/BSA nanowire, the output light at the end (region in the dotted box in Figure 5b) was caught and metered by a camera and a spectrometer (Ocean optics, QE65000). During adding biotin aqueous solution with a certain concentration into the surrounding (not fluorescein–biotin here and $10\text{ }\mu\text{L}$ every time, that is, every data point), the images and spectra of the output light (the dotted box region in Figure 5b) were collected and processed for biosensing (Figure 5d–f). When added into the aqueous surrounding of the protein nanowire, biotin molecules could be specifically bound to the avidin molecules crosslinked in the hydrogel network of protein nanowire both on the surface and inside (experimentally proved in Figure S4 (Supporting Information) and schematically illustrated in Figure 5c). The material characteristics of the protein nanowire might be changed after the interaction of avidin and biotin, especially optical properties such as refractive index. Because of the specific biotin absorption of avidin-containing hydrogel, the original local distribution of biotin molecules was probably altered in the surrounding. As a consequence, the evanescent field of the light guided in the protein single nanowire would change, which could influence evanescent power leakage of the waveguiding nanowire, and further the guiding performances and the output light (guiding loss, output spectrum and intensity, etc.).

In Figure 5d, by arranging and formatting the transmission spectra in the form of “3D waterfall,” the regularly decreasing peak intensities were intuitively exhibited along with increasing biotin concentration (from 0.0 to $\approx 47.2\text{ }\mu\text{g L}^{-1}$). There was a spacing interval of 10 min between every two spectra in Figure 5d, that is, every two dots in Figure 5g, for the sufficient diffusion after the addition of biotin. In the insets of Figure 5d, the huge difference, mainly a variation of light intensity, between the images of output light can be visually observed before (top picture) and after (bottom picture) the change of biotin concentration. The biotin-concentration-dependent peak intensities of transmission spectra were shown in Figure 5g. As shown by intensity data and fitted curve in Figure 5g, from the concentration of $\approx 31.5\text{ }\mu\text{g L}^{-1}$, the avidin/BSA nanowire biosensor became saturated with biotin, which was probably the top limit of detection. As for the lowest limit of detection, the concentration shift with steps as low as $2 \times 10^{-7}\text{ g L}^{-1}$ could be detected, as shown in the first six dots of data in Figure 5g. But the slope of the fitted curve of the first six dots is bigger than that of the followed data, which indicates that the lowest limit of detection might be still not reached in our existing data at present. (Here, although the decrease of intensity of the first six points was smaller than one of the followed data, the slope of fitted curve was calculated to be still much larger for the small concentration changing steps.) Even so, the detectable

biotin concentration as low as $2 \times 10^{-7}\text{ g L}^{-1}$ proved fairly high sensitivity of the protein single-nanowire biosensors here. Furthermore, in Figure 5g, obvious and approximately linear decrease of output intensity was exhibited during the increase of biotin concentration with $0.2\text{-}\mu\text{g L}^{-1}$ titration step from point 1 to point 6. Titration step smaller than $0.2\text{-}\mu\text{g L}^{-1}$ might be meaningless for considering the system errors and fluctuations of present data. Although it was a little less-conservative and optimistic, it might be still of considerable validity to estimate $0.2\text{ }\mu\text{g L}^{-1}$ (0.2 ppb) as the highest biotin-detection sensitivity reached in our present data.

Additionally, with the same methods and procedures but different material combinations, the control experiments were done and the results were illustrated in Figure 5e,f,h,i. A BSA single nanowire with diameter of 700 nm was fabricated on the MgF_2 slice by FsLDW with the identical processing data and parameters as avidin/BSA nanowires discussed above for similar morphology, size and performances (from 450 mg mL^{-1} BSA aqueous ink; laser power density, $36.6\text{ mW }\mu\text{m}^{-2}$; scanning step, 100 nm ; exposure time, $1000\text{ }\mu\text{s}$). The BSA single nanowire experienced the same experimental procedures and biotin-concentration change as the avidin/BSA nanowire in Figure 5d,g did. With the broadband supercontinuum evanescently coupled into the BSA nanowire, the transmission spectra and their peak intensities were, respectively, exhibited in Figure 5e,h, which remained approximately the same during the change of biotin concentration (similar changing process with Figure 5d,g). There was also no obvious difference between the output images before (top inset) and after (bottom inset) the biotin concentration change (see insets of Figure 5e). Meanwhile, the same procedures were implemented for another avidin/BSA nanowire (as the same as the one used for Figure 5d,g) using pure water instead of biotin solutions. The volume of added water was equal to that of the added biotin solution for experiments of Figure 5d,e. As shown in Figure 5f,i, the spectra and their peak intensities, and the inset images of output light were not influenced obviously during or after the addition of water up to only $300\text{ }\mu\text{L}$. It means that the addition of a very small amount of water did not bring about unacceptable error (partly because of the large size of the container possibly, a glass petri dish with diameter of 10 cm was used here). Based on these results of experiments above, it was proved that the output intensities went down regularly in specific response to the increase of biotin concentration in the aqueous surrounding, enabling the specific biosensing of the avidin/BSA nanowire (Figure 5).

During the biotin sensing processes, the variation of biotin concentration (from 0 to $73\text{ }\mu\text{g L}^{-1}$ and even higher) was experimentally proved of not inducing discernible change of equilibrium swelling state (including geometry, volume and water content) of protein (avidin/BSA) nanowire hydrogel (see Figure S5, Supporting Information). This experimental phenomenon agrees well with the explanation and discussion proposed by Lyon and co-workers.^[41] As proved and discussed in Lyon's work, biotin-modified microhydrogels immersed in water would shrink in response to binding of avidin, which was caused by the ability of avidin to bind up to 4 equiv of biotin and therefore an increase in the network

crosslink density. Importantly, the “multivalent” binding is required to form “crosslinking” to increase hydrogel network crosslink density (at least 2:1 for biotin: two-biotin-bound avidin). So, if the biotin-modified microhydrogels nonspecifically adsorbed four-biotin-bound avidin or were monovalently bound with three-biotin-bound avidin, no discernible swelling or shrinkage would happen.^[41] For the situation here, the bond of free biotin molecules with crosslinked avidin molecules would not induce the increase of hydrogel network crosslink intensity. On the other hand, monovalent bond and nonspecific absorption of even large molecules like avidin did not change the geometry or volume of microhydrogels in Lyon’s work,^[41] let alone the introduction of small molecules like biotin. As a result, the equilibrium swelling state of avidin/BSA micro/nano-hydrogels were not changed discernibly in response to biotin bond (swelling or shrinkage of protein nanowires), which was experimentally demonstrated and theoretically explained reasonably.

Further in Figure S6 (Supporting Information), BSA/avidin-hydrogel microsquares (Figure S6a, Supporting Information) and nanowires (Figure S6b, Supporting Information) were fabricated with the same structure-design and optimized FsLDW parameters (laser power density, $36.6 \text{ mW } \mu\text{m}^{-2}$; scanning step, 100 nm; exposure time, 1000 μs). BSA/avidin-hydrogel micronanostructures in Figure S6a3,b3 (Supporting Information) experienced the same biotin introduction procedures as tests in Figure 5g (from 0.0 to $\approx 47.2 \mu\text{g L}^{-1}$). Compared with those smooth BSA/avidin-hydrogel micronanostructures without biotin-absorption (Figure S6a1,a2,b1,b2, Supporting Information), surfaces of BSA/avidin-hydrogel microsquares and nanowires were obviously rough with a morphology of granular structure with uneven grain sizes after biotin-absorption (Figure S6a3,a4,b3,b4, Supporting Information). In addition, all of the BSA/avidin-hydrogel microsquares in Figure S7 (Supporting Information) experienced the same biotin-absorption processes as mentioned above, and exhibited similar roughened morphology of granular structure with uneven grain sizes without exception. This demonstrates the repeatability, and excludes other fortuitous causes including inappropriate FsLDW parameters and fluctuations of FsLDW system. In consequence, the surface-roughening phenomenon of as-prepared BSA/avidin hydrogels is proved to be probably induced by biotin-absorption. Therefore, it is indicated that biotin-absorption onto BSA/avidin hydrogels might induce the formation of morphology with granular structure, that is, originally smooth surface of BSA/avidin hydrogels may become rough gradually and responsively to biotin-absorption. Surface quality is an important factor to determine protein nanowaveguides’ transmission loss. So biotin-absorption-induced protein nanowaveguide surface-roughening might cause obvious transmission loss increase and output intensity decrease, based on which probably, protein single-nanowire optical biosensors for special biotin-detection were achieved.

In summary, based on advantages of FsLDW approach, all-protein single nanowire optical biosensors and various nanowire devices could be readily constructed from proteins’ aqueous ink with outstanding designability and reproducibility. In comprehensive consideration of high quality of

protein nanowires and well-tailored bioactivity of crosslinked proteins, FsLDW system and processing parameters were carefully optimized to achieve applicable optical performances (waveguiding loss of $\approx 0.05 \text{ dB } \mu\text{m}^{-1}$ for 532 nm and $\approx 0.06 \text{ dB } \mu\text{m}^{-1}$ for 633 nm in air) and biotin-binding capacity of avidin nanowires for biosensing applications. Importantly, one optical window of the protein single nanowire waveguides was found to be at $\approx 680 \text{ nm}$ and coincided with the important red or near-infrared biological window of various tissues and organs, for example, skin and adipose tissue. Further, by directly using probe proteins (e.g., avidin) as “building blocks,” the evanescent optical biosensing of biotin with specificity was achieved with 0.2 ppb or even higher sensitivity for the avidin/BSA nanowire biosensors in aqueous surrounding. Consequently, the significant merits, such as versatility, facilely adjustable high sensitivity, good biocompatibility, satisfactory designability, and repeatability of nanowire-devices by FsLDW, etc., may endow the all-protein-based single-nanowire biosensors with potential in the biomedical detection, environment monitoring and homeland security, etc.

Experimental Section

FsLDW Experimental Procedures with Details: With a high-numerical-aperture ($\text{NA} = 1.42$) oil-immersion objective lens (OL, 60 $\times$), the beam from a femtosecond titanium:sapphire laser (Spectra Physics 3960-X1BB; repetition rate, 80 MHz; pulse width, 120 fs; central wavelength, 790 nm) was tightly focused onto the interface between the protein aqueous ink and substrates as needed. With the assistance of the photosensitizer, the polymerization of protein molecules was induced by two-photon absorption and highly confined to the core region of laser focal spot with laser energy density over the threshold (see Figure 1a). So the nanometric resolution ($< 200 \text{ nm}$ minimum width of lines with satisfactory topography^[32]) was guaranteed for protein hydrogel nanostructures produced by FsLDW. Then, as shown in Figure 1b, the programmable 3D scanning and fabrication were well achieved by the combination of a piezo stage (PZT; for sample’s vertical movements; PI P-622 ZCD; precision, 1 nm) and a two-galvano-mirror set (for horizontal scanning of laser beam). The geometries of nanowire nanodevices were designed by 3Ds Max to obtain corresponding computer processing data. Controlled by the processing data, various complicated protein nanowire nanodevices, for instance, of waveguide devices in Figure 1c–e (c, Mach–Zehnder interferometer; d, tree-form power divider; e, Sagnac resonator), would be readily written out on the substrate by FsLDW. As proved in our previous work,^[15,32,33] the refractive index of protein microhydrogel by FsLDW is ≈ 1.55 in air and ≈ 1.45 in water. After rinsing with water, the protein single nanowires or nanowire devices as shown in Figures 1 and 2 were obtained on the MgF_2 slices.

The Adjustment of Bioactivity of Crosslinked Avidin (Figure S1, Supporting Information): Avidin/BSA microstructures were immersed in aqueous solution of fluorescein-biotin (1.3 mg L^{-1}) for 10 min for saturated specific binding. And then the microstructures were rinsed thoroughly with pure water for 15 times to remove unbonded fluorescein-biotin. Then the adjustable bioactivity (the specific binding ability of biotin) of crosslinked avidin was characterized and studied by confocal microscopy.

The Biotin-Binding Ability of BSA Microsquares Obtained by FsLDW (Figure S3, Supporting Information): For BSA microhydrogels obtained by FsLDW with different processing parameters (from 450 mg mL⁻¹ BSA aqueous ink, scanning step from 100 to 200 nm), similar fluorescence intensities were observed after 10-min immersion in fluorescein-biotin aqueous solution (1.3 mg L⁻¹) and 5-time rinsing (see confocal microscopy images of Figure S3, Supporting Information). It means that different degree of laser action and crosslinking generated no difference of biotin absorption. After sufficient rinsing (15 times as the same as avidin/BSA microhydrogels in Figure S1, Supporting Information), there was hardly any fluorescein-biotin left as shown in Figure S3d,e (Supporting Information).

The Influence of Biotin-Avidin Binding Activity on Equilibrium Swelling State (Including Geometry, Volume and Water Content) of Avidin/BSA Micro/Nano-Hydrogels Obtained by FsLDW (Figure S5, Supporting Information): In Figure S5 (Supporting Information), the sample was immersed in a 10-cm-diameter petri dish with 50 mL pure water at first. The side lengths of a 10 × 10 × 4-μm³ avidin/BSA microsquare and a 2 × 2 × 4-μm³ one kept nearly constant under different biotin concentrations with just slight fluctuation probably caused by experimental error of characterization and measurement (see Figure S5, Supporting Information). The insets of Figure S5a (Supporting Information) were selected from the data group in Figure S5b (Supporting Information) which depicted the whole data group of optical microscopy images of the same 10 × 10 × 4-μm³ and 2 × 2 × 4-μm³ microsquares under different biotin concentrations. By infusing with 1 or 10 mg L⁻¹ biotin aqueous solutions, biotin concentration was changed step by step from 0 to 73 μg L⁻¹ and even higher (10 μL per time and total volume kept constant at 50 mL).

Here, the avidin/BSA microsquare were fabricated with the same protein ink and processing parameters as those for FsLDW of avidin/BSA nanowire optical biosensors. (Protein ink: avidin, 50 mg mL⁻¹; BSA, 450 mg mL⁻¹; methylene blue, 0.6 mg mL⁻¹. Processing parameters: laser power density, 36.6 mW μm⁻²; scanning step, 100 nm; exposure time, 1000 μs.)

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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