Preparation of F_0F_1-ATPase Nanoarray by Dip-Pen Nanolithography and Its Application as Biosensors

Zheng Li, Xiaolong Liu*, and Zhenxi Zhang*

***Abstract*—Dip-pen nanolithography (DPN) is a widely used technique to create nanoscopic patterns of many different materials. F_0F_1-ATPase is a nanoscale rotary molecular motor, and could be used as a biosensor or an ideal motor device in a micro-/nanosystem. In this paper, the DPN technique was used to create nanoarrays of F_0F_1-ATPase within chromatophore on a gold surface. The feature size of our F_0F_1-ATPase patterns was an average of 130 nm, and mathematically, there were no more than ten F_0F_1-ATPases in each dot. The biological activity of patterned F_0F_1-ATPase was demonstrated by its adenosine triphosphate synthesis, which was indicated by the fluorescence change of labeled F1300. The patterned F_0F_1-ATPase nanoarrays were further constructed as biosensors to detect H9 influenza A virus. The results showed that the biosensor arrays had a remarkable *S/N* ratio and excellent specificity. This type of biosensor arrays can be further used in high-throughput, high-sensitive detection in future. Meanwhile, the precise patterning of F_0F_1-ATPase with desired size, position, and biological activity would accelerate its application in many fields.**

***Index Terms*—Chromatophores, dip-pen nanolithography (DPN), F_0F_1-ATPase, microarray.**

I. Introduction

PRECISION nanoscale deposition of a biomolecule on suitable surfaces is a fundamental requirement for much of the current nanoscience research and promises to facilitate exciting industrial applications. Dip-pen nanolithography (DPN), a method of depositing molecules from an ink-coated atom force microscopy (AFM) tip onto a substrate of interest, is a powerful tool for creating nanoscale patterns of many types of active biomolecules, including deoxyreboneculeic acid (DNA) [1]–[3], proteins [4]–[8], and membrane protein complexes [9].

F_0F_1-ATPase is composed of two functional linked parts—F_0 and F_1. This complex catalyzes endergonic synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Pi) using a transmembrane proton-motive force (PMF) generated by oxidative phosphorylation or photosynthesis [10]–[12]. In *Escherichia coli*, F_0 and F_1 are composed of ab_2c_n and $\alpha_3\beta_3\gamma\delta\varepsilon$, respectively. These two parts are structurally linked by two stalks; a central stalk that links to the c-ring composed of γ and ε subunits, and an outer stalk that links $\alpha_3\beta_3$ to a subunit composed of δ and b_2 subunits. The downhill proton flux through F_0 drives rotation of the c-ring and hence γ and ε, forcing a conformational change in F_1 that results in ATP synthesis from ADP and Pi. Conversely, ATP hydrolysis in F_1 causes reverse rotation of the rotor that drives F_0 to pump protons in the reverse direction.

F_0F_1-ATPase would be an ideal nanomotor for driving autonomous nanodevices, wherein light energy could be converted to ATP, which then serves as a fuel source for the motor. It may also enable the creation of a new class of sensors, mechanical force transducers, and actuators [13]. Montemagno *et al.*. have fabricated the nanoarrays of F_1-ATPase, through patterning F_1-ATPase on electron beam lithography fabricated nickel nanofeatures [14], then they further constructed the hybridized nanomotor that was powered by F_1-ATPase [13]. However, F_0F_1-ATPase is actually combining the function of F_0 and F_1 together, which can not only act as a motor, but can also act as an energy supply machine or a biosensor. Therefore, the spatially controlled immobilization of intact F_0F_1-ATPase on suitable surfaces at the nanoscale with desired position, size, shape, and biological activity is much more important. This is an enabling technology that is critical to the long-term goal of integrating biomolecular motors with nanoelectromechanical systems. However, the fabrication of the F_0F_1-ATPase nanoarray on a suitable surface has not been reported yet.

The construction of F_0F_1-ATPase as a biosensor has been reported for the application in the detection of avian flu H9A virus at a single molecular level [15]. Microarray is an ideal tool in high-throughput detection. A decrease of the feature size from micrometers to nanometers will further improve the detection throughput and sensitivity, and decrease the sample consumption. Therefore, the fabrication of the nanoarray of the F_0F_1-ATPase-based biosensors will accelerate its application as a high-throughput detection tool in future.

In this paper, we created nanopatterns of F_0F_1-ATPase within a chromatophore on a gold surface using the DPN technique. A chromatophore is a vesicle of the lipid–protein complex, which only has one F_0F_1-ATPase in addition to other proteins [16], [17]. The biological activity of F_0F_1-ATPase was demonstrated by its ATP synthesis, which was indicated by the fluorescence intensity change of labeled F1300. Then, the patterned nanoarray of F_0F_1-ATPase was further constructed as biosensors to detect H9 influenza A virus.

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II. Materials and Methods

A. Chemicals and Materials

Chemicals 16-mercaptohexadecanoic acid (MHA), 1-octadecanethiol (ODT), streptavidin, and ADP were purchased from Sigma. Fluorescein reference standard (FRS) (Catalog Number: F1300) was purchased from Molecular Probes. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) and N-hydroxysuccinimide (NHS) were purchased from Fluka. Biotin–hydrazide was purchased from Dojindo (Japan). Lipid–biotin was purchased from Avanti. All other analytically purified reagents were purchased domestically.

The avian H9 influenza A virus, H9 influenza A antibody, and avian H5 influenza N1 were gifts from Dr. Ni Zhiqian (Harbin Veterinary Research Institute).

Tricine–NaOH buffer (0.1 mM Tricine, 5 mM $MgCl_2$, 5 mM KCl, pH8), ATP synthesis buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 10% glycerol, pH8.5), phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, and 25 mM Na_2HPO_4, pH7.4, 1% glycerol), and twin-bit silicon-oxide-nitride-oxide-silicon memory (TSM) buffer (0.25 M sucrose, 4 mM $MgCl_2$, 50 mM tricine–NaOH, pH8.0).

B. Methods

1) Preparation of Gold Substrates: The gold substrates with 4 nm thickness on a glass surface were prepared according to [8] through thermal evaporation by Prof. G. Yu (Institute of Chemistry, Chinese Academy of Sciences). Before deposition, the glass surface was cleaned in a 1:1 mixture of H_2O_2 and H_2SO_4 for 2 h. Then it was extensively rinsed by dd-water and dried in N_2.

2) Preparation of Chromatophores and F_0F_1-ATPases-Based Biosensors: Chromatophores were prepared from the cells of R. rubrum according to [16] and [17]. In brief, the harvested R. rubrum cells (5 g) were resuspended in 50 ml ice-cold TSM buffer. Then, the resuspended cells were broken up through sonication (25% intensity, turn on 5 s, with 5 s interrupt) for 15 min on ice. The cell lysate was washed through centrifugation (25 000 g, 4 and 30 min), and the precipitates were discarded. The supernatants were further centrifuged (145 000 g, 4 °C, and 90 min). Then, the supernatants were discarded, and the resulting wine pellets were chromatophores. The concentration of chromatophores was determined by OD880.

The preparation of the biotin-labeled fluorescence chromatophores was as follows: F1300 was labeled into chromatophores through sonication for 5 min on ice, and the free fraction was washed three times by centrifugation (15000 r/min, 30 min, and 4 °C). The pellets were resuspended in a tricine–NaOH buffer. The fluorescence chromatophores were labeled with biotin by adding the lipid–biotin ethanol solution into the F1300-labeled chromatophores (100/5 V:V). After incubation at room temperature for 30 min, the free fraction was washed by centrifugation (15000 r/min, 30 min, and 4 °C).

The F_0F_1-ATPase-based biosensor was constructed according to [15]. In brief, the H9 influenza A antibody was served as a capture unit by linking to the β-subunit of F_0F_1-ATPase by β-subunit antibody–biotin–streptavidin–biotin linking system.

3) Fabrication of the F_0F_1-ATPase Nanostructures on the Gold Surface Through DPN: We patterned F_0F_1-ATPase within the chromatophore on the gold surface through an indirect DPN method. A silicon nitride tip was coated with MHA molecules by dipping the cantilever into the saturated MHA ethanol solution four times (1 min of each); then, the tip was dried in N_2. The DPN experiments were carried out on the Nanoscope III system (NanoInk, USA). The MHA self-assembled monolayer (SAM) arrays were formed by holding the MHA-coated tip in contact with the gold surface for 0.3 s in room temperature at a relative humidity of 45%. The surrounding areas were passivated with ODT by immersing the substrate into a saturated ODT ethanol solution for 30 min, then it was followed by extensively rinsing with ethanol and dd-water, and dried in N_2. The terminal COOH groups in the MHA SAM were activated by reaction with NHS (0.2 M) and 1-ethyl-(dimethylamino)propyl carbodiimide (0.1 M) water solutions for 1 h, then it was extensively rinsed with dd-water and dried in N_2. After this, 400 μL droplet of a 10 mM biotin–hydrazide solution was dropped onto the patterned areas and incubated for 30 min at room temperature, then it was followed by extensively rinsing with dd-water. Then, 400 μL droplet of a 2 mM streptavdin solution in the PBS buffer was dropped onto the patterned surface and incubated for 10 min at room temperature, then it was followed by extensively rinsing with dd-water. The areas surrounding the streptavidin features were passivated by 5% bovine serum albumin (BSA) at 37 °C for 2 h, and then, it was extensively rinsed with phosphate buffered saline tween-20 (PBST) buffer (0.05% Tween-20). At last, 200 μL biotin-labeled fluorescence chromatophores were dropped onto the patterned areas and incubated for 10 min at room temperature, then it was extensively rinsed with the PBST buffer (0.05% Tween-20). The F_0F_1-ATPase-based biosensors were patterned on the gold surface in the same way.

Because the gold film on the glass surface was very thin, it could permeate and can permeate 80% of the lights. The patterned chromatophores and biosensors were imaged by a fluorescence microscope (Olympus IX71) immediately and recorded by a cooled digital charge-coupled device (CCD) camera (Princeton Scientific) with excitation at 485 nm and emission at 538 nm. The recorded pictures were analyzed by the software of Winview/32 (Princeton Scientific).

The schematic view of patterning F_0F_1-ATPase within the chromatophore through DPN is shown in Fig. 1.

III. Results and Discussion

A. Patterning the Nanoarray of F_0F_1-ATPase Within Chromatophore Through DPN

MHA is a widely used "ink" molecule for patterning on the gold surface. The COOH terminal of the MHA molecule can easily couple to other functional molecules through covalent bonds or static forces. Then, the interested biological molecules can be further linked to those patterned features through molecular recognition. This indirect method can pattern nearly all

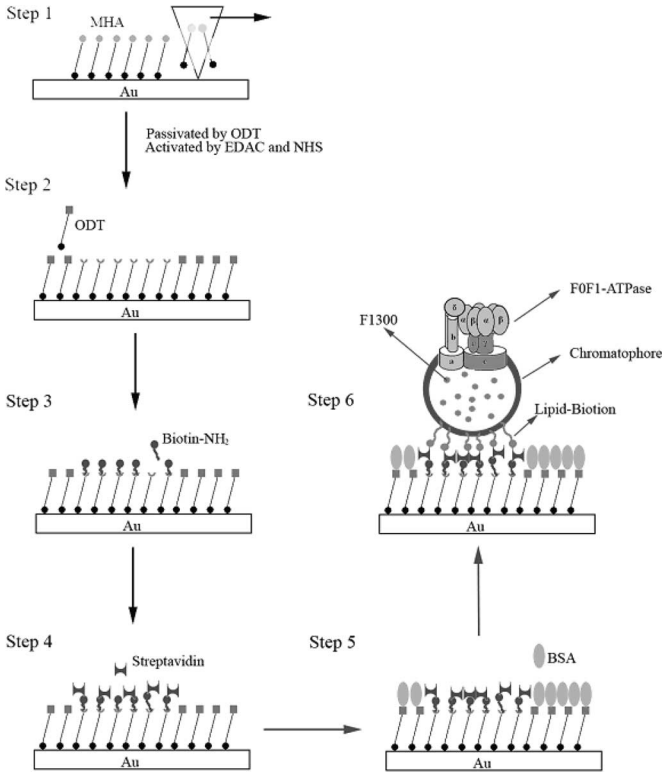

Fig. 1. Schematic view of patterning F_0F_1-ATPase within the chromatophore through DPN.

types of biological molecules on the gold surface without losing their biological activity.

Lateral force microscopy (LFM) is a secondary contact AFM mode that detects and maps relative differences in the frictional forces between the probe tip and the sample surface. Fig. 2(A) shows the LFM image of MHA patterns on a gold surface. The feature size of the MHA patterns is an average of 130 nm. The naked gold surfaces surrounding the MHA patterns were passivated by ODT. Fluorescence-labeled chromatophores were linked to the MHA patterns through molecular recognition of biotin–streptavidin–biotin as described in Section II-B. Fig. 2(B) shows the fluorescence image of formed chromatophore patterns. The non-specific absorption of chromatophores was prevented by further passivation of the surface with 5% BSA. The chromatophore is a vesicle of lipid–protein complex with an average diameter of 45 ± 15 nm. The detailed structure of the chromatophore vesicle has been determined by Geyer *et al.* [18]. One chromatophore only has one F_0F_1-ATPase, as well as LH_1, LH_2, bc_1, and reaction center (RC) complexes. Our results showed that the chromatophore patterns had an average diameter of 130 nm. Taking the average diameter of chromatophores into account, this showed that there were less than ten chromatophores (F_0F_1-ATPases) in each dot of the chromatophore patterns mathematically.

The average fluorescence intensity of a single F1300-labeled chromatophore in solution is 18 ± 15 on the gold surface (the data were averaged from 25 single F1300-labeled chromatophores). Taking these data into account, the maximum fluorescence intensity of each spot should be 180 on average

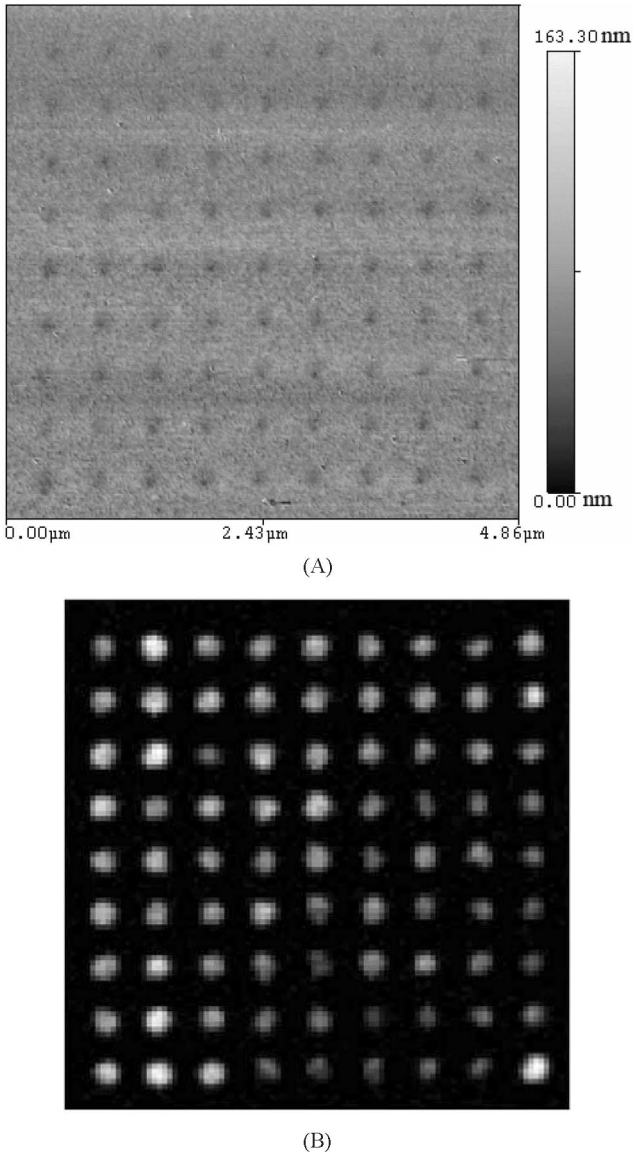

Fig. 2. (A) LFM image of MHA patterns on the gold surface. (B) Fluorescence image of patterned chromatophores through an indirect DPN process.

(ten chromatophores maximum could bind to the MHA spot). Actually, the average fluorescence intensity of each spot on the fabricated chip is just 68. Although the orientation of the molecular assembly would cause a little bit decrease of the fluorescence intensity, it also clearly means that there were less than ten chromatophores (F_0F_1-ATPases) in each spot of the nanoarray.

This provides a new route to research the basic properties and novel applications of F_0F_1-ATPase at nanoscale.

B. Biological Activities of Patterned F_0F_1-ATPase Nanoarrays

To further confirm the biological activities of F_0F_1-ATPase patterned on the gold surface, ADP with a final concentration of 2 mM in the ATP synthesis buffer was added onto the pattern areas to initiate ATP synthesis. It is well known that F_0F_1-ATPase is a reversible ATP synthesis/hydrolysis molecular machine

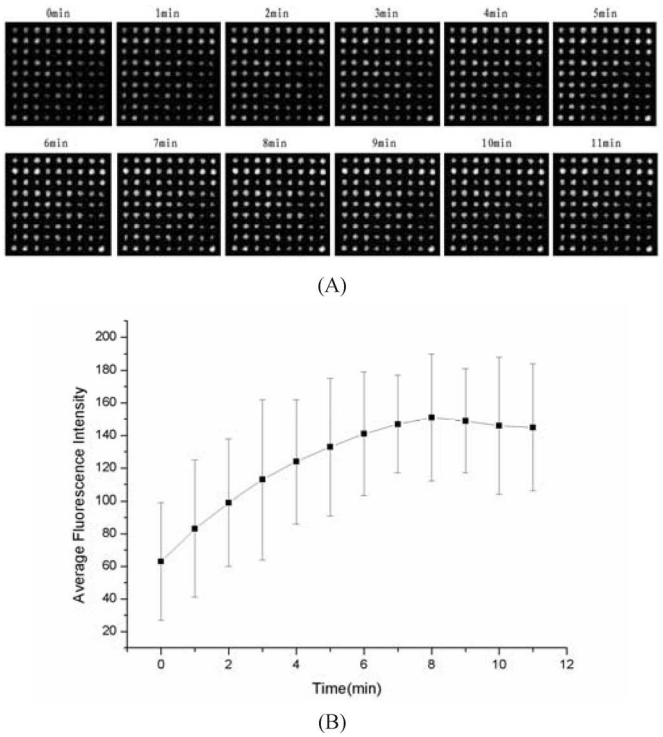

Fig. 3. (A) Fluorescence images of intensity increase of F_0F_1-ATPase patterns during the ATP synthesis. (B) Fluorescence intensity curve corresponding to (A).

combined with reversible proton pump out/in. When the F_1-ATPase rotates clockwise, the ATP is synthesized from ADP and phosphorus, and the protons are pumped out of the chromatopore. This causes an increase of the pH value in the interior environment of the chromatophore. F1300 is an extensively used pH-sensitive fluorescence probe, which has been used for detecting the proton flux in chromatophores [15], [19]. The fluorescence intensity of F1300 is increasing with the increase of the pH value. If the ATP synthesis of F_0F_1-ATPase is initiated successfully, the fluorescence intensity of F1300, which has been labeled into the inner surface of chromatophores, will increase. Fig. 3(A) shows the increase of fluorescence intensity of F1300-labeled chromatophores. Fig. 3(B) shows the fluorescence intensity curve corresponding to Fig. 3(A). In Fig. 3(B), the fluorescence intensity increase was drastic at the beginning. After 8 min of adding ADP, the fluorescence intensity reached its maximum. Then, the fluorescence intensity began to decrease slightly, which was mainly due to the fluorescence quench. From Fig. 3(A) and (B), it is clear that the synthesis of ATP was initiated successfully by F_0F_1-ATPase. It is demonstrated that F_0F_1-ATPase can pattern on a suitable surface with the nanoscale feature, but without losing its biological functions.

Here, we hypothesized that the fluorescence intensity plateau presented in our results was mainly because of F_0F_1-ATPase consuming all of the ADP surrounding it. To prove this theory, we varied the amounts of substrates in order to observe whether the time to plateau changes. The maximum ATP synthesis activity of our F_0F_1-ATPase was in the condition of 2 mM ADP concentration in our study. Therefore, the volume of ATP synthesis buffer was varied, but the ADP concentration was

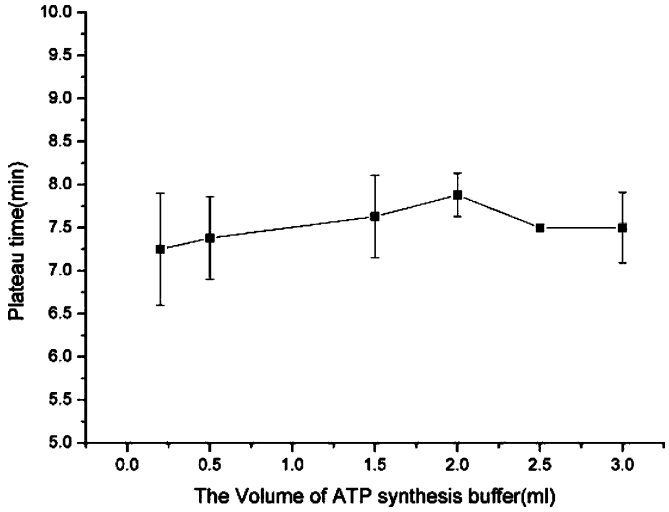

Fig. 4. Time to plateau corresponding to different amounts of ATP synthesis substrates. The F_0F_1-ATPase nanoarrays were 400 × 400, and the average feature size of the nanoarrays varied from 100 to 150 nm. We took photos at each 0.5 min time interval and repeated each experiment four times independently. The results show that the time to plateau changed from 6 to 8 min stochastically.

left unchanged in our experiments. Fig. 4 shows the time to plateau corresponding to different amounts of substrates. However, it seems that there was not a large difference of the time to plateau under different amount of substrates. The time to plateau changed stochastically from 6 to 8 min in each case. This means that the fluorescence intensity plateau was not caused by emptying its substrates. There were another two possibilities about the fluorescence intensity plateau presented here. In one case, the F_0F_1-ATPase was still working, but the pH value in the interior of the chromatophore had exceeded the response range of F1300; in another case, the F_0F_1-ATPase stopped working because of the high pH value in the interior of the chromatophore or other unknown reason.

C. Application of the Fabricated F_0F_1-ATPase Nanoarray as Biosensors to Detect H9A Virus Specifically

It has been reported that F_0F_1-ATPase could work as a biosensor at the single molecular level [15]. To further demonstrate that its nanoarray has the potential of working as a high-throughput biosensor tool, a drop of H9 influenza A or H5 influenza N1 virus was added onto the pattern of F_0F_1-ATPase-based biosensors and incubated at room temperature for 20 min. Then, the ATP synthesis buffer was added onto the pattern to initiate the ATP synthesis of F_0F_1-ATPase-based biosensors. Fig. 5(A) shows the fluorescence images of the nanoarrays of F_0F_1-ATPase-based biosensors undergoing ATP synthesis, when these nanoarrays were preincubated with tricine buffer only (a), H5N1 virus (b), and H9A virus (c); Fig. 5(B) shows the fluorescence intensity change curves of these nanoarrays corresponding to Fig. 5(A); the statistics results of fluorescence intensity increase times of these nanoarrays at 6 min compared to its beginning, as shown in Fig. 5(C). As shown in Fig. 5, the ATP synthesis of these nanoarrays was initiated successfully (a, b, c). But the fluorescence intensity of the nanoarray, which was incubated with H9A

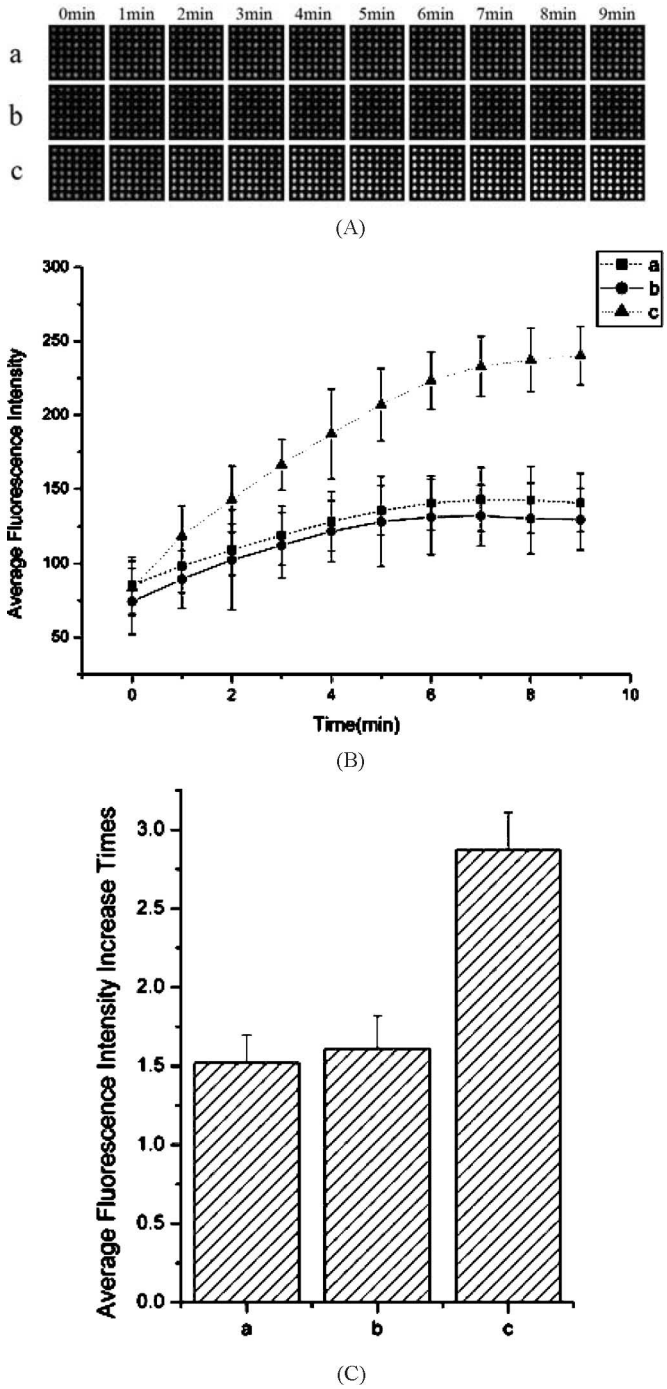

Fig. 5. (A) Application of the F_0F_1-ATPase nanoarray as biosensors to detect H9 influenza A virus specifically. The F_0F_1-ATPase-based biosensor was constructed through [15]; the avian H9 influenza A virus antibody was adopted as a capture unit. The fluorescence images of the chromatophore nanoarray intensity increase during its ATP synthesis were shown. (a) ATP synthesis of the nanoarray was initiated without incubation with any virus; the feature size of this nanoarray was 160 nm on average. (b) Nanoarray was incubated with H5 influenza N1 virus for 20 min at room temperature, then its ATP synthesis was initiated by the ATP synthesis buffer; the feature size of this nanoarray was 140 nm on average. (c) Nanoarray was incubated with H9 influenza A virus for 20 min at room temperature, then its ATP synthesis was initiated by the ATP synthesis buffer; the feature size of this nanoarray was an average of 170 nm. (B) Fluorescence intensity curves of a, b, and c corresponding to (A). (C) Statistics results of fluorescence intensity increase times at the 6 min of a, b, and c compared to its beginning in (A).

virus (c), was increased more rapidly and strikingly than others. The fluorescence intensity of each dot in the nanoarray, which was incubated with H9A virus (c), was increased nearly three times at 6 min compared to its beginning [see Fig. 5(C)]. However, the fluorescence intensity of each dot in other nanoarrays, which was not incubated with any virus (a) or incubated with H5N1 virus as control (b), was very similar and both of them were only increased less than 1.5 times at 6 min compared to its beginning [see Fig. 5(C)]. This means the capture unit (H9A antibody) has recognized and captured its target [15] specifically. These data suggest that the nanoarray of F_0F_1-ATPase would be a promising high-throughput biosensor in future.

F_0F_1-ATPase will be an ideal motor or energy-providing system for the micro-/nanomachine because of its small size, smart and perfect structure, and ultrahigh energy transfer efficiency [20]. F_0F_1-ATPase has also been demonstrated that it could be used as a biosensor. Therefore, patterning F_0F_1-ATPase on a suitable surface with desired size, position, and feature is the basis of developing some F_0F_1-ATPase powered nanodevice, or using it as a real high-throughput biosensor in some biomedical applications. Developing the nanoscale microarray or precise orientating of F_0F_1-ATPase will accelerate its application in many fields.

IV. Conclusion

In this paper, we used the indirect method to pattern F_0F_1-ATPase within the chromatophore on the gold surface. The pattern feature of our nanoarray was 130 nm on average, and there were less than ten F_0F_1-ATPases in each dot mathematically. The ATP synthesis activity was indicated by the fluorescence intensity increase of F1300. It was demonstrated that the patterned F_0F_1-ATPase could preserve its biological activity on the gold surface at nanoscale. The potential of using this nanoarray as a high-throughput biosensor tool was demonstrated by its specific detection of H9A virus.

F_0F_1-ATPase is a nanoscale rotary molecular motor, and its force generation and size scale are compatible with nanofabricated devices. Theoretically, the chromatophore patterning method used in our paper can be further improved to the single molecule level. This means that there would be only one F_0F_1-ATPase molecule in each dot. Precise patterning of a single F_0F_1-ATPase at a desired position will create its potential applications as a nanomotor for driving the nanoelectromechanical devices, or as an energy supply/storage device in a micro-/nanosystem. Meanwhile, the nanoarrays of F_0F_1-ATPase-based biosensors presented here can be further used as a high-throughput, high-sensitivity tool in future biodetection applications.

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