microRNA Detection with an Active Nanodevice F_oF_1-ATPase

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Keywords: Molecular Motor, F_oF_1-ATPase, Chromatophore, Processive Rotating, Ultrasensitive Biosensor.

Abstract: A novel nanodevice was constituted with a rotary motor and a "battery", F_oF_1-ATPase and chromatophore. The former can processively rotate at about 10^3 r.p.m for more than one hour once the latter was recharged by shine. If the nanodevice is captured by a target such as miRNA and processively rotates for 30 minutes, the number of targets will be amplified by 10^5 ATP molecules. The sensitivity of detection was lower than 1.0 pM. Therefore, this method has potential to be developed into an ultrasensitive biosensor to detect low expressed targets such as miRNA.

1 INTRODUCTION

F_oF_1-ATPase is the ubiquitous enzyme that uses the transmembrane electrochemical potential to synthesize ATP in bacteria, chloroplasts and mitochondria. The holoenzyme can be divided into two rotary motors, F_o and F_1. F_1 motor consists of a crown type "stator" ($\alpha_3\beta_3$) and a eccentric "rotor" (γ), while F_o motor consists of a "stator" (a subunit) embedded in membrane and a ring channels "rotor" (c_n). The two "stators" are fixed by b_2 and δ subunits, while the two "rotors" are mechanically coupled by ε subunit. The membrane embedded F_o unit converts the proton motive force(p.m.f) into mechanical rotation of the "rotor", thereby causing cyclic conformational change of $\alpha_3\beta_3$ crown ("stator") in F_1 and driving three ATP molecules synthesis for each rotation at nearly 100% efficiency(Boyer, 1997; Noji et al., 1997; Yasuda et al., 1998; Abrahams et al., 1994; Diez et al., 2004; Toyabe and Muneyuki, 2015; Shu et al., 2010).

In vitro, however, F_oF_1-ATPase must be reconstituted in polymersome and coupled with Bacteriochlorophyll to maintain its ATP synthesis, where the Bacteriochlorophyll converted the light energy into transmembrane p.m.f(Choi and Montemagno, 2005). The polymersome with Bacteriochlorophyll was named chromatophore. The combination of F_oF_1-ATPase and chromatophore is a sophisticated nanomachine, in which chromatophore function as a "battery" to power the rotary motor F_oF_1-ATPase, as well as the "battery" can remotely be recharged by shine. Furthermore, the combination of F_oF_1-ATPase and chromatophore can conveniently be prepared by the phototrophic bacterium, in which the cells were disrupted by sonication on ice. Each chromatophore vesicle of 100 nm diameter contains on average one F_oF_1-ATPase(Feniouk et al., 2002).

Additionally, molecular simulation(Shu and Lai, 2008) and nanoporous membrane experiment(Dong et al., 2011) have demonstrated that the motor can achieve about 10^3 r.p.m at saturated substrate concentration and the chromatophore can processively power the motor for more than one hour once the "battery" was recharged by shine(Zhang et al., 2005; Deng et al., 2007; Cheng et al., 2010), which means one motor can generate about 10^5 ATP molecules during 30 minutes. On the other hand, microRNA(miRNA) is often the marker of early diagnoses of cancer. Thus, there is an urge to have a high sensitive detection of miRNA due to its low expression in early cancer cell. Here, we constituted a nanodevice with the combination of F_oF_1-ATPase and chromatophore to detect the miR26a, a marker of hepatocarcinoma.

2 EXPERIMENTAL RESULTS

As Fig.1 shows, when target microRNA was base-paired between capture probe and detection probe, the motor embedded in chromatophore can be linked to the magnetic beads surface, while the free motors can be washed away as shown in Fig.2. The number of captured motors is equal to that of microRNAs because of there is only one ε subunit in F_oF_1-ATPase motor. The amount of ATP generated by F_oF_1-ATPase, thus, is in direct proportion to the num-

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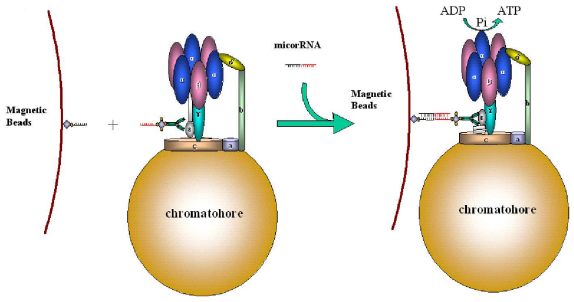

Figure 1: Cartoon of the biosensor based on ε subunit conjugation(not to scale). The hybridized motors are captured by the magnetic beads, while the free motors will be washed away. Thus, the number of the hybridized miRNA is proportion to that of motors.

ber of motors and time of synthesis based on the same storage energy in chromatophore. Here, the motor function as an amplifier in which the number of target microRNA is amplified to 10^5 times ATP molecules during 30 minutes. The detector is able to be sensitive to 1.0 pM of miR26a. Therefore, the active nanodevice has a potential to be developed into a dynamic biosensor.

3 MATERIALS AND METHODS

3.1 Cell Lines and Reagents

Thermomicrobium roseum wa0073 (ATCC27502) was purchased from ATCC (USA). The luciferase/luciferin ATP detection kits were purchased from Promega Corporation (USA). ADP, (+)-biotin N-hydroxysuccinimide ester and NeutrAvidin were purchased from Sigma-Aldrich (St. Louis, USA). The microRNA extraction kits were purchased from Tiangen, and the RNA probes were synthesized by Sangon Biotech (Shanghai) Co., Ltd.. The miR26a capture probe sequence was 5'-biotin-AAAAAAAAAAAGCCTATCCT-3', the detection probe sequence was 5'-GGATTACTTGAAAAAAAAAAAA-biotin-3'
and 2′Ome modified miR26a RNA sequence (UUCAAGUAAUCCAGGAUAGGCU) was synthesized by Gene Pharma company (Shanghai). Streptavidin magnetic beads (1.0 μm, Dynabeads®MyOneTM streptavidinC1) was purchased from Life Technologies, Inc. The microplate luminometer was a Centro XS3 LB 960 (Germany).

3.2 Preparation of Chromatophore Containing the F_oF_1-ATPase

The chromatophore containing the F_oF_1-ATPase was isolated and purified according to our previous published protocols(Cheng et al., 2010; Shu and Ou-Yang, 2012). *Thermomicrobium roseum* was cultured at 60 °C for 24 h, and the cells were collected by centrifugation at 4,000 rpm for 20 minutes. The pellets were resuspended in 20 ml buffer A (pH 6.0), containing 20 mM Tricine-NaOH (pH 6.0), 2 mM $MgCl_2$, 100 mM NaCl, and 10% glycerin (v/v), and sonicated for 3 minutes. The lysate was centrifuged at 8000 rpm with R20A2 rotor for 30 minutes at 4 °C, and the supernatant was collected and centrifuged at 40,000 rpm for 90 minutes at 4 °C. The precipitate containing the chromatophores was resuspended in buffer B (pH 8.0), with 20 mM Tricine-NaOH (pH 8.0), 2 mM $MgCl_2$, 100 mM NaCl, and 10% glycerin (v/v), and stored at −80 °C for further use.

3.3 Preparation of Monoclonal Antibodies of ε-subunit

The ε-subunit was expressed and purified as in Ref.(Su et al., 2006). The ε-subunit monoclonal antibodies were prepared according to the method for monoclonal antibody production procedure in Ref.(Hanly et al., 1995), purified by precipitation with 33% $(NH_4)_2SO_4$ at 4 °C for 12 h, and the IgG parts were separated using Sephadex G-200 and stored at −20 °C before use.

3.4 Preparation of Detection Probe-conjugated Motor

The motor with miR26a detection probe were prepared by conjugating the streptavidin to the ε subunit of F_oF_1-ATPase embedded in chromatophore through biotinylated ε subunit monoclonal antibody(shown in Fig.1). Then the biotinnylated miR26a detection probe was bound to streptavidin. Specifically, 15 μl of chromatophores (50 mg/ml) and 8 μl (0.5 mg/ml) of biotinylated ε-subunit monoclonal antibodies were mixed, diluted with 1 ml with PBS buffer, and then incubated at 37 °C for 60 minutes. The free ε subunit monoclonal antibodies were washed away by centrifugation at 40,000 rpm for 20 minutes at 4 °C. The precipitate was resuspended in 500 μl of PBS buffer. Then, 7.5 μl (0.1 mg/ml) of NeutrAvidin was added and diluted into 1 ml with PBS buffer, and incubated at 37 °C for 10 minutes. The free neutravidin was then washed away by centrifugation at 40,000 rpm for 20

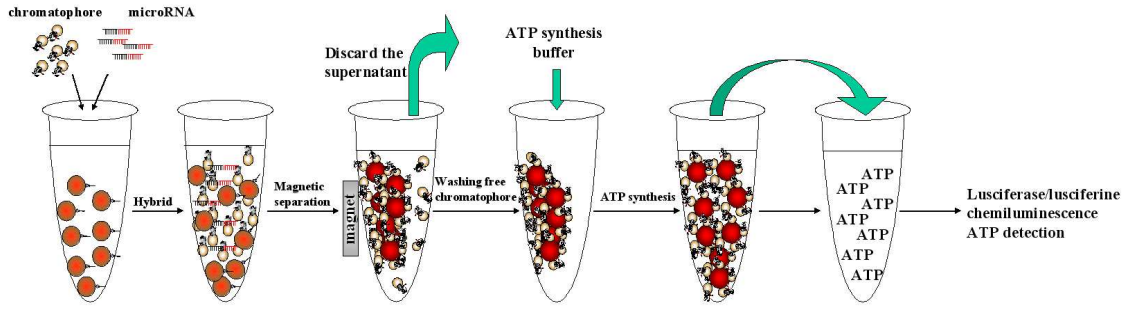

Figure 2: The procedure of miRNA detection. The first step is hybridization of target microRNA; The second step is separation between the captured motors and free ones. The free motors will be wash away, while the number of captured motors is equal to that of microRNAs because of there is only one ε subunit in F_oF_1-ATPase motor; The third step is ATP synthesis in the captured motors during 30 minutes; The final step is measure of ATP concentration with luciferase-luciferin.

minutes at 4 °C. The precipitate was resuspended in 500 μl of PBS buffer. Then, 10 μl (1μM) of biotinylated detection probe were added, diluted into 1 ml with PBS buffer, and incubated at 37 °C for 10 minutes. The free detection probes were then washed away by centrifugation at 40,000 rpm for 20 minutes at 4 °C. The precipitate was resuspended in 500 μl of PBS buffer.

3.5 Preparation of Capture Probe-conjugated Magnetic Beads

The magnetic beads conjugated with miR26a capture probe were prepared by conjugating the streptavidin magnetic beads with the 5′-biotin modified miR26a capture probe according to the manufacturer's procedure(shown in Fig.1). Specifically, 100 μl 10 mg/ml streptavidin magnetic beads were pipetted into a microtube and washed twice with 100 μl 1×B&W buffer (pH 7.5, 5 mM Tris-HCl, 0.5 mM EDTA, 1 M NaCl). The magnetic beads were resuspended in 100 μl 2×B&W buffer. Then 100 μl biotinylated capture probe in H_2O was added and incubated for 15 minutes at 25 °C using gentle rotation for the binding reaction. After the binding reaction, the conjugated magnetic beads were washed three times with 1×B&W Buffer. In the end, the capture probe-conjugated magnetic beads were resuspended in PBS buffer and stored at 4 °C for usage.

3.6 miRNA Hybridization with Capture Probe-conjugated Magnetic Beads and Detection Probe-conjugated Motor

30 μl of complementary miR26a (for sensitivity test) in appropriate concentrations (a series of concentrations ranging from 1 pM to 100 pM) was incubated with 30 μl of 10 mg/ml magnetic beads with capture probe in 20 μl hybridization buffer (final concentration is 5×SSC, 5× denhardt's solution) at 37 °C for 30 minutes. Then the magnetic separation was performed to remove the un-hybridized target microRNA and resuspended the magnetic beads with 50 μl hybridization buffer. For the binding of detection chromatophore, 30 μl detection probe-conjugated chromatophore was added into the magnetic beads and incubated for 10 minutes at 37 °C. After the hybridization was completed, the three times washing with PBST and PBS were performed respectively to remove the unbound chromatophore completely as shown in Fig.2.

3.7 ATP Synthesis Assay

The ATP synthesis activity of F_oF_1-ATPase within the chromatophores was determined using the luciferin-luciferase method. The the magnetic beads were resuspended with 100 μl ATP synthesis buffer (10 mM Tricine NaOH (pH 8.0), 5 mM $MgCl_2$, 5 mM Na_2HPO_4, 0.3 mM ADP 10% glycerol) and incubated at 37 °C for 60 minutes, then separated by magnetic separator. 30 μl supernatant was transported into the 96-well plate with three times, and 30 μl luciferase/luciferin working solution was added into, finally the chemiluminescence signals displayed in microplate luminometer were recorded immediately as shown in Fig.2.

The relative light density emitted from luciferase-luciferin at different miR26a concentration is shown in Fig.3, in which the light density of buffer is normalized. The results indicated that the measured value is in direct proportion to the miR26a concentration. The results means that the sensitivity of detection was lower than 1.0 pM.

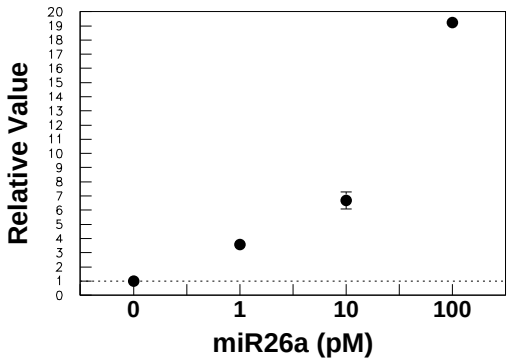

Figure 3: The relation between relative measured value(mean± s.e.m.) and miR26a concentration. "0" corresponds to the buffer, and its light density is normalized. Data points represent an average of 10-15 samples.

4 CONCLUSIONS

We have developed a novel nanodevice constituted with a rotary motor and a "battery", F_oF_1-ATPase and chromatophore. The former can processively rotate at about 10^3 r.p.m for more than one hour once the latter was recharged by shine. If the nanodevice is captured by a target such as miRNA and processively rotate for 30 minutes, the number of targets will be amplified by 10^5 ATP molecules. The sensitivity of the detection was lower than 1.0 pM. This method has potential to be developed into an ultrasensitive biosensor to detect low expressed targets such as miRNA.

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