Sandwich type biosensor of ε-subunit of F_{o}\textsubscript{1}-ATPase for ultrasensitive-detection of bladder cancer cell

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Abstract: It was an important challenge for bladder cancer diagnosis that to design a sandwich-type biosensor was used for ultrasensitive detection of bladder cancer cell. This inspiration was from that ε subunit of F_{o}\textsubscript{1}-ATPase could be regarded as a switch of the F_{o}\textsubscript{1}-ATPase biosensor and its application on the ultrasensitive detection. There was only one ε subunit in one F_{o}\textsubscript{1}-ATPase, and this character could be used to design a real single antibody sandwich-type biosensor. Due to ε subunit of F_{o}\textsubscript{1}-ATPase as an intramolecular rotation switch in the transition interchangeable motion, it was found that ε subunits can be interchanged between “rotor” and “stator” and remained its different activation at each status. Furthermore, Tip-chip device detection, which combined both a double antibody sandwich immunoassay and an intra-rotation switch of ε subunit F_{o}\textsubscript{1}-ATPase which plays a key role of the “rotor” and “stator” exchange hypothesis showing a signal amplification at space scale and a signal accumulation at time scale, was a powerful method. There were two states of the rotation switch model, one was the switch close state when the γ and ε subunits of F_{o}\textsubscript{1}-ATPase bound as one complex, and the other was the switch open state when the γε complex divided, and the intramolecular rotation coupling model based on the “binding change mechanism.”

Keywords: bladder cancer cell; F_{o}\textsubscript{1}-ATPase; sandwich-type biosensor; tip-chip device

1. Introduction

Bladder cancer is a common malignancy that more prevalent in developed countries; of note, 70% of bladder cancer are superficial at initial presentation, 30% of patients are invasive usually treated by surgical resection and intravesical chemotherapy; however, approximately 20% of these patients will recur and as many 10–30% will progress to invasive cause after treatment\textsuperscript{[1-3]}. Located in the plasma membrane of bacteria, the thylakoid membrane, and the mitochondrial inner membrane, F_{o}\textsubscript{1}-ATPase is a high-content membrane enzyme. In vivo, F_{o}\textsubscript{1}-ATPase could exchange electrochemical energy (protons potential, ΔμH\textsuperscript{+})
into chemical energy (ATP phosphoanhydride bonds). F$_a$F$_{1}$-ATPase has two different complexes F$_i$ and Fo which were coupled through two “stalks.” The soluble complex F$_i$ of simplest bacterial enzyme that is composed of five types of subunits in stoichiometry α$_3$, β$_3$, γ$_1$, δ$_1$, and ε$_1$ and extends 100 Å from the membrane and catalyzes ATP synthesis or hydrolysis. The membrane-bound complex Fo of most bacteria that is composed of three types of subunits in stoichiometry α$_1$, b$_2$, and c$_{10–15}$, which plays proton-transporting role. The center part of the stalk is the γε-c subunits complex, which bound to the ring-shaped oligomer of c subunits. The a subunit has two proton channels; it binds to one of c subunits and also connects to the α$_i$β$_i$δ-complex through the b$_2$-dimer and δ subunit. Six subunits of α$_i$β$_i$ are arranged alternately and formed a hexamer. On the hexamer, there are six nucleotide-binding sites, in which, three are catalytic and three are noncatalytic. The noncatalytic sites are related to the regulation of the F$_a$F$_{1}$-ATPase. At the molecular level, the γεc$_{10–15}$ subunit complex which is called as the rotor, and rotated relative to the rest of the enzyme which is called as the stator. The rotation realizes ATP catalytic mechanism. During ATP synthesis, the energy of the ion transport through F$_i$ drives the rotation of the ring-shaped c-oligomer, and the energy is transmitted to F$_i$ through γε-complex, which changes the catalytic sites affinity by driving the conformations transitions of the α$_i$β$_i$ hexamer.$^{[4-7]}$

In this paper, we build an ultrasensitive biosensor method that combines the sandwich-type enzyme immunoassay and the intra-rotation switch of ε subunit F$_a$F$_{1}$-ATPase which plays a key role of the “rotor” and “stator” exchange hypothesis as a signal amplification at space scale and a signal accumulation at time scale. The amplification ratio of the signal at space scale is one to one, at the same time, the signal at time scale could be accumulated over time.

Both are a powerful method for detecting low concentration antigens. There were two states of the rotation switch model, one was the switch close state when the γ and ε subunits of F$_a$F$_{1}$-ATPase bound as one complex, and the other was the switch open state when the γε complex divided the intramolecular rotation coupling model based on the “binding change mechanism”.$^{[8]}$

2. Materials and Methods

2.1. Cell and reagents

*Thermomicrobium roseum* wa0073 (ATCC27502) was purchased from ATCC (USA). NeutrAvidin, BNHS (biotin N-hydroxysuccinimide ester), adenosine diphosphate (ADP), and ATP were purchased from Sigma-Aldrich (St. Louis, USA). The Ni-NTA Superflow Cartridge was purchased from QIAGEN (German). The ATP detection kit (luciferase/luciferin) was purchased from Promega (USA). The single-chain antibody of ε subunit (6× His tag) was customized from ABT Genetic Engineering Technology Corporation (Beijing, China). The 200 μl silylated-Tips were customized from Jinmaike Technology Development Co., Ltd. (Beijing, China).

2.2. The F$_a$F$_{1}$-ATPase chromatophores preparation

*T. roseum* was cultured at 60°C in a shaker incubator (200 rpm) for 24 h and then the cell cultures were centrifuged at 5000 g for 20 min at 4°C. Collected the pellets and resuspended in 20 buffer A (pH 8.0, 0.02 mol/L Tris–HCl, 0.1 mol/L NaCl, 0.02 mol/L MgCl$_2$, and 10% glycerin). The cells were homogenized twice by high-pressure homogenizer JNJ-02C (JINBIO, China) at 4°C. After the centrifugation at 5000 g for 30 min, the precipitate was removed, and the supernatant extract was collected to centrifuge at 180,000 g for 90 min at 4°C. After the final centrifugation, the F$_a$F$_{1}$-ATPase chromatophores, which were in the precipitate extract, were homogenized in 5 ml buffer A (the concentration of glycerin was increased to 50%) and then were stored at −80°C for use at a later date.$^{[9]}$

2.3. The ε subunit single-chain antibody preparation

According to a previously published method,$^{[10]}$ the ε subunit was expressed and purified. The single-chain antibody of ε subunit (6× His tag) was customized from ABT Genetic Engineering Technology Corporation (Beijing, China). We express single-chain antibody from the custom made E.coli, and homogenized and collected the extractive of bacteria. Then, the single-chain antibody was purified using a Ni-NTA Superflow Cartridge (QIAGEN) according to the handbook and stored at −20°C for use at a later date.

2.4. The antibody and bladder cancer cell ligand biotinylation

The BCMab1 antibody was prepared as described elsewhere.$^{[11]}$ The concentration of antibody and bladder cancer cell ligand was 1 mg/ml in carbonate buffer (0.2 mol/L CB pH 9.6) and added BNHS 10 μg/ml (final concentration) for 4 h at 25°C and then dialyzed within PBS (pH 8.0) over night at 4°C to remove the disconnected biotin. The dialyzed sample was added 20% glycerol and stored at −20°C for use at a later date.

2.5. The bladder cancer cell biosensor preparation

The equimolar Ab-NTA and BNHS were mixed in CB (0.2 mol/L, pH 9.6) for 4 h at 25°C and then the equimolar NiSO$_4$ was put into the Ab-NTA-biotin mixture and reacted for 10 min. The Ni-NTA-biotin was mixed with single-chain ε subunit antibodies for 20 min at 25°C and then dialyzed within PBS (pH 8.0) over night at 4°C to remove the disconnected Ni-NTA-biotin. The complexes were incubated with chromatophores for 2 h at 25°C and
then were centrifuged 100,000 g 3 times for 20 min at 4°C to wash the pellet. The chromatophores ε subunit antibody-Ni-NTA-biotin complex was connected with the avidin and the bladder cancer cell ligand orderly. Then, the complex was homogenized in a store buffer (pH 8.0, 0.05 mol/L Tricine–NaOH, 0.05 mol/L MgCl₂, 0.05 mol/L KH₂PO₄, and 20% glycerol) and stored at –20°C for use at a later date.

2.6. Tip-bladder cancer cell capture chip device built

First, the 200 μl silylated-Tips were incubated with 100 μl BNHS (1 μg/tip) in CB (0.2 mol/L, pH 9.6) for 4 h at 25°C and then washed with PBS (0.1 mol/L, pH 8.0) for 5 times. Second, the tips were incubated with 100 μl avidin (2 μg/tip) in PBS for 20 min at 25°C and then washed with 100 μl PBS for 5 times. Finally, the tips were incubated with the 100 μl biotinylated BCMab1 antibody (2 μg/tip) in PBS for 20 min at 25°C and then washed with 100μl PBS for 5 times.

2.7. Bladder cancer cell capture

Add 1 ml each the blank, negative control and positive sample into a 96 deep well plates (1.2 ml/well) orderly. Used the tip automatic load-wash device to load these simples (load-blow 30 cycles) and then the bladder cancer cells were captured by the BCMab1 antibody binding to the tip.

Add 1 ml/well PBST into the 96 deep well plates, used the tip automatic load-wash device to wash the tips (load-blow 30 cycles) twice. In the same way, PBS was used to wash the tips 3 times.

2.8. Bladder cancer cell biosensor connected with the bladder cancer cells

Add 1ml/well bladder cancer cell biosensor (30 μg/ml) into the 96 deep well plates, used the tip automatic load-wash device to load the biosensor simples (load-blow 30 cycles) and then the bladder cancer cells biosensor connected with the bladder cancer cells captured by the tip. As described in above, the tips were washed with PBST and PBS to remove the unconnected biosensor.

2.9. Detected bladder cancer cells

Using the tip automatic load-wash device to load 100 μl synthesis buffer (pH 8.0, 0.05 mol/L Tricine-NaOH, 5 mol/L KH₂PO₄, 0.005 mol/L MgCl₂, 20% glycerol, and 0.3 mmol/L/ADP), reacted for 30 min at 25°C. The reacted synthesis buffer was injected into a 96-well detection plates, the luciferase/luciferin reagent was added 20 μl per well at the same moment and then the ATP concentration was measured, in which, the bladder cancer cell biosensor synthesized.

2.10. Statistical analysis

The paired t-test was applied for statistical analysis in this paper. Compared with the control or the negative group, *P < 0.05 was statistically significant. The standard deviation measurements that were represented by error bars were obtained from at least five independent experiments.

3. Results

3.1. Operation processing and biosensor principles

3.1.1. Bladder cancer cells are enriched by the tip-capture chip device

Tips were fabricated by a new enrichment method to improve their throughput and selection to isolate cancer cells based on tips’ surface marker. In the first stage, tips’ surface was coated with the capture antibody (BCMab1) linking by biotin-avidin system that the tip-capture chip device was built and then used the tip automatic load-wash device to load simples (load-blow 30 cycles). Due to its high affinity and selectivity of the bladder cancer cells, BCMab1 of the tip-capture chip device could capture and enrich the bladder cancer cells from the positive samples such as bladder cancer cell line and patient’s blood [Figure 1].

3.1.2. Sandwich tip-bladder cancer cell biosensor to detect bladder circulating tumor cells (CTCs)

After captured the bladder cancer cells, the bladder cancer cell biosensor (F₀F₁-ATPase linking another antibody of the bladder cancer cell) was added, and it could bind to the bladder cancer cell’s surface antigen through another antibody. There were many antigens on one bladder cancer cell’s surface, so that the sandwich structure of tip-antibody-cell-F₀F₁-ATPase could dramatically amplify the signal. Each step of the process, the tips should be washed by PBST and PBS and then the ATP synthesis is initiated resuspension the biosensor-target complex in reaction buffer (pH8.0). At the end of the reaction period, the complex is isolated by pull down, and the supernatant containing the synthesized ATP was transferred into 96-well plates to react with luciferin–luciferase and detected [Figure 2].

3.1.3. Biosensor principles

To the feature of F₀F₁-ATPase, the sandwich-type biosensor used one antibody on tip to capture the cell and the other antibody bind ε subunit of the ATPase, as signal amplification effect [Figure 3].

Based on the feature of F₀F₁-ATPase, one type of rotary biosensor was designed on the bases of rotary ε subunit regulation to F₀F₁-ATPase activity. Figure 2 showed that this type of biosensor is constructed by binding the biotinylated ε subunit single-chain antibody to ε subunit of F₀F₁-ATPase.
on chromatophore and then the biotinylated probe was linked to ε subunit antibodies through NeutrAvidin. The targets can be macromolecular antigen or small molecules. When the specific probe captures the target, the rotary speed of ε subunit was changed and the ATP synthesis activity was influenced and then ATP concentration can be detected by luciferase–luciferin system to indicate the existence of targets.

Furthermore, sandwich-type immunoassay is a type of assay method with high sensitivity and is one of the most popular immunoassays used in the normal enzyme immunoassay. However, the limitation of this detection is always owing to the equimolar ratio of the enzyme and signal antibody. The inspiration of signal amplification was from the $F_{o}F_{1}$-ATPase. Due to one ε subunit in one chromatophore and this character can be used as one antibody to one antigen as a signal amplification at space scale first, next such signal amplification at time scale can improve the sensitivity greatly and make it more easily to reach ultra-high-sensitive detection. Due to the reason was that three ATP can be synthesized per circle for one $F_{o}F_{1}$-ATPase, and the rotation rate of $F_{o}F_{1}$-ATPase is about 300 circles/s; hence, one ATPase will produce 54,000 ATP molecules per minutes$^{5,12}$ and $F_{o}F_{1}$-ATPase on the chromatophore can produce ATP signal continuously. When ATP synthesis activity continues to 1 h, the ATP molecules can be reached to about $3 \times 10^6$, such signal amplification. For the purpose, approach is used immunoassays methods design sandwich-type biosensor base on ε subunit intrarotation switch of $F_{o}F_{1}$-ATPase for ultrasensitive detection of bladder cancer cell.

As showed in Figure 4, (a) switch of ε subunit $F_{o}F_{1}$-ATPase closed state (b) linking on the cell switch of ε subunit closed state (c) after the binding the cell, added the ADP molecular, ATP synthesis is initiated resuspending the biosensor-target complex in reaction buffer (pH8.0). (6) The synthesized ATP reaction buffer was separated and transferred into 96-well plates to react with luciferin–luciferase and then detected the bladder cancer cells.

3.2. BCMaB1, a specific and high affiliative monoclonal antibody against bladder cancer

It was well known that for ultrasensitive detection of bladder cancer cell, a specific monoclonal antibody was very important. We had screened and identified a specific and high affiliative monoclonal antibody BCMaB1 against bladder cancer cell; this would be a very interested biomarker to bladder cancer of detection. The BCMaB1 only have immunoreactivity against bladder cancer$^{13}$. The specificity and affinity of BCMaB1 are suitable for us to design a bladder cancer cell capturer binding to the tip.

3.3. Standard detection of bladder cancer cell and specifically

Based on the above, the monoclonal antibody specifically with high affinity for the bladder cancer cell, in order to test
the proof of principle, first the cultured bladder cancer cell line T24 was detected using the tip-bladder cancer cell biosensor. Different concentrations of cells were detected using our novel biosensor. Relative ATP synthesis activity was detected when different concentrations of cells were added, starting from 0, 5, 10, and 100 cells (each group includes eight independent samples). As shown in Figure 5, the bladder cancer cells could be detected even at the lowest concentration (5 cells/sample).

Second, the specificity of $F_{o}F_{1}$-ATPase biosensor between T24 cell line (positive cell) of the bladder cancer and HCV29 cell line (negative cell) of the normal bladder was tested. The cell concentration of the control group is 0, the cell concentrations of sample groups were the same as above, such as 5, 10, and 100 cells (at each cell level, the positive group, and the negative group have the same cell concentrations), and each group includes six independent samples. Exhilaratingly, we found that designed $F_{o}F_{1}$-ATPase biosensor with the specific antibody of the bladder cancer cell exhibits a significantly higher (by at most 3-fold) chemiluminescence signal other than normal bladder cells sequences 1.2, 2, and 3 times, respectively ($P < 0.05$). However, in the negative groups with different cell levels, the difference was not significant [Figure 6].

### 3.4. Clinical blood samples detection

To evaluate the application of this biosensor detection for the bladder cancer patient, we detected two groups of human blood samples, one group was from the bladder tumor patients, and the other group was from the normal person. Measured with the same method, we found that the results exhibit very significant difference between the two groups [Figure 7]. It indicates that the designed biosensor can be used for the detection of clinical human blood samples, and this method may be employed to diagnose bladder cancer in the future.

### 4. Discussion

This is a method that combines the sandwich-type enzyme immunoassay and the intra-rotation switch of $\varepsilon$ subunit $F_{o}F_{1}$-ATPase which plays a key role of the “rotor” and “stator” exchange hypothesis as a signal amplification at space scale and a signal accumulation at time scale. The amplification ratio of the signal at space scale is one to one, at the same time, the signal at time scale could be accumulated over time. This ultrasensitive detection of bladder cancer cell may be applied to detect CTC without pre-enrichment.

Although the existence of CTC has been known for over a hundred of years, it has get more and more attention on the use of CTCs as a real-time liquid biopsy during the last few decades$^{[14,15]}$. CTC occurs as a very low concentration in blood of the vast majority of cancer instances, which is a tough challenge for any detecting systems$^{[13,16,17]}$. Recently, to enrich CTCs, many systems had got large improvement using microfluidic devices$^{[18,19]}$, each method has their own advantages and disadvantages$^{[20-24]}$. Until recently, the novel whole cell-based technologies have enabled significant progress in deleting EpCam-positive cells are enriched by immunomagnetic separation particles; CTCs are finally counted using a semiautomated fluorescent microscope. This is an expensive diagnostic system with some problems, such as after the assay, CTCs cannot be used for the subsequent cell foundations study, as well as the high-throughput screening, so that to build a sensitive, accurate, specific, and quick detection is a serious challenge for determining effective treatment of cancer.

![Figure 3. Sandwich-type biosensor of $\varepsilon$ subunit of $F_{o}F_{1}$-ATPase $\varepsilon$ subunits role stator](image)

![Figure 4. Sandwich-type biosensor of $\varepsilon$ subunit of $F_{o}F_{1}$-ATPase $\varepsilon$ subunit as role interchangeable switch of $\varepsilon$ subunit. (a) Switch of $\varepsilon$ closed state; (b) switch of $\varepsilon$ closed state (c) switch of $\varepsilon$ opened state when the adenosine diphosphate was added and then the ATP synthesis was begin](image)
The model of the intra-molecular switching based on the γε-complex conformational change between close and open state, is coupled with ATP synthesis according to the “binding change mechanism”. When the γε-complex conformation is in close state, the F\textsubscript{o}F\textsubscript{1}-ATPase motor rotates slow and the ATP synthesis decrease; when the γε-complex conformation is in open state, the F\textsubscript{o}F\textsubscript{1}-ATPase motor rotates fast and the ATP synthesis increase. However, some puzzles still exist such as how the ε subunit works during the ATP syntheses and how it regulates F\textsubscript{o}F\textsubscript{1}-ATPase.

The experiments had designed a molecular motor biosensor, which was demonstrated that this method has potential to be developed into an ultrasensitive rapid molecular motor to detect very low concentration targets such as CTCs. Although, other methods such as real-time liquid biopsies and microfluidic devices are powerful methods for detecting lower concentrate positive cells, especially for the CTCs, the molecular motor biosensor as a low-cost method would be particularly helpful in developing countries.

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6. Author’s Contributions

Xu Zhang and Jinku Zhang did biosensor design; Xu Zhang and Yuhan Yan did data analysis; Xing Kang, Jinku Zhang, and Xuejia Zhu did cell culture and antibody preparation; Zhao Yang and Chong Li discussed and interpreted data; and Xu Zhang and Chong Li conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

No potential conflicts of interest were disclosed.

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