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Research Article

A Novel Biosensor to Detect MicroRNAs Rapidly

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δ-free F_0F_1-ATPase within chromatophore was constructed as a novel biosensor to detect miRNA targets. Specific miRNA probes were linked to each rotary β subunits of F_0F_1-ATPase. Detection of miRNAs was based on the proton flux change induced by light-driven rotation of δ-free F_0F_1-ATPase. The hybridization reaction was indicated by changes in the fluorescent intensity of pH-sensitive CdTe quantum dots. Our results showed that the assay was attomole sensitivities (1.2×10^{-18} mol) to target miRNAs and capable of distinguishing among miRNA family members. Moreover, the method could be used to monitor real-time hybridization without any complicated fabrication before hybridization. Thus, the rotary biosensor is not only sensitive and specific to detect miRNA target but also easy to perform. The δ-free F_0F_1-ATPase-based rotary biosensor may be a promising tool for the basic research and clinical application of miRNAs.

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1. Introduction

MicroRNAs (miRNAs) are small noncoding RNA molecules 20–22 nucleotides(*nt*) in length, which function to regulate gene expression. Recent studies have found that some miRNAs have altered expression in cancer cells, and specific alterations in miRNA expression may be an important biomarker for toxins which have serious effects on human health [1–4]. The effective detection is crucial for better understanding the roles of miRNAs in cancer cells, the response of cells to stress, and the process of cellular growth, proliferation, and metabolism. Therefore, many different methodologies have been used to profile miRNA expression, including Northern blotting with radio-labeled probes [5, 6], oligonucleotide microarrays [7, 8], and quantitative PCR-based amplification of precursor or mature miRNAs [9, 10]. However, the normal detection approaches require many steps such as enrichment, reverse transcription, amplification, labeling, and clean-up [11]. Most of these methods are time consuming due to ovenight hybridization reaction and complicated fabrication. The most popular approaches to hybridization analysis such as Northern blotting and microarrays take a long time for hybridization of probes and their target molecules because of the static hybridization kinetics on a solid phase. To solve this problem, microfluidics technology has been used to stir the hybridization solution to increase the rate of diffusion for rapid identification [12, 13]. For instance, the hybridization time of Surface Acoustic Wave (SAW) microagitation chips can be reduced and signal intensities increased more than 6 fold [14]. Thus, methods for rapid measuring the expression profiles of miRNAs will considerably accelerate the field in addition to the sensitivity and specificity.

Recent advances in nanotechnology have enabled the biosensor design and production of a variety of nanodevices for biological purpose. It is well known that F_0F_1-ATPase is a complex of two parts, F_0 ($a_1b_2c_n$) and F_1 ($\alpha_3\beta_3r_1\delta_1\varepsilon_1$). It is connected by a central stalk composed of γ and ε subunits and a peripheral stalk made of the δ and b subunits. The proton translocation through F_0 drives rotation of the c-ring oligomer together with $r\varepsilon$ complex, forcing conformational changes in F_1, which result in ATP synthesis from ADP and Pi [15, 16]. It is also known that the δ-subunit plays a switch role between the F_0 and F_1 in the ATP synthesis [17]. The δ-free F_oF_1-ATPase is constructed with a_3, b_3, ε, and γ as well as c_n subunits as rotator and a, b_2 as stator. Based on this, Su et al. constructed a nanomotor by using δ-free F_oF_1-ATPase within chromatophores [18].

Here, we developed a rapid method to detect miRNAs using the rotary nanoscale biosensor based on δ-free F_0F_1-ATPase. The miRNA probe system, which was linked to the rotary β subunit of F_0F_1-ATPase, was used to detect their target miRNAs. CdTe quantum dots- (QDs-) labeled chromatophores were responding to change of proton concentration of outside surface. In our experiments, miRNA probes linked to β subunit act as a rotary propeller. By this procedure, it is possible to monitor a real-time hybridization between the immobilized miRNA probes and the stirred target miRNAs. In addition, the procedure does not require enrichment, reverse transcription, amplification, and hybridization before detection. In this study, the method is sensitive to attomole of *mir-145* (1.2×10^{-18} mol) and is capable of discriminating a single base difference among *let-7* family members.

2. Materials and Methods

2.1. Materials. The water-soluble and pH-sensitive CdTe quantum dots (QDs) were gifts from Professor Tang Fangqiong (Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, China). In this study, we used the QDs with maximum emission wavelength at 535 nm. ATP and streptavidin were purchased from Sigma-Aldrich (USA). Biotin-(AC5)2Sulfo-OSu was purchased from Dojindo (Japan). Lipidbiotin was purchased from Avanti. The δ-free ATPase within chromatophores was prepared as the previous study [18, 19]. The β-subunit of F_0F_1-ATPase was expressed and purified as described previously [20]. The antibody was prepared according to the procedure [21]. *Mir-145* probe sequence is 5′-GGGGAUUCCUGGAAACUGC-3′. The other probe sequences follow as *let-7a* (5′-AACTATACAATCTACTACCTTATCCT-3′), *let-7a* (5′-UGAGGUAGUAGGUUGUAUAGUU-3′), *let-7b* (5′-UGAGGUAGUAGGUUGUGUGGUU-3′), *let-7c* (5′-UGAGGUAGUAGGUUGUAUGGUU-3′), and *let-7d* (5′-AGAGGUAGUAGGUUGCAUAGU-3′), respectively. Total RNA was extracted from MCF-7 human breast cancer cells by using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. All other analytically purified reagents are of analytical grade.

2.2. Cell Culture. The MCF-7 human breast cancer cell was kindly provided by Professor Jianwen Chen. Cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (PAA) at 37°C in a humidified 5% CO_2 incubator.

2.3. Biosensor Assembly and miRNA Detection. The surface of chromatophores was labeled by QDs according to [22]. Briefly, the chromatophores (100 μL) were resuspended in buffer A (50 mM tricine–NaOH, 5 mM $MgCl_2$, 10 mM KCl, pH 6.5) and incubated for 3 hours at room temperature with 100 μL CdTe QDs ($1 \times 10^{15}/\mu$L, dissolved in water). Free QDs were washed away by centrifuging at 13 000 rpm for 30 minutes at 4°C in three times. The precipitate (QD-labeled chromatophores) was re-suspended in 100 μL of 50 mM tricine buffer (pH 6.5). Meanwhile, 2 μL of 2 μM biotin was added in 20 μL β subunit antibody at room temperature for 30 minutes, followed by adding 2 μL of 2 μM streptavidin at room temperature for 30 minutes. The streptavidin-biotin-labeled β-subunit antibody was incubated with 5 μL QDs-labeled chromatophores fixed on the glass slips at 37°C for 1 hour. Redundant free biotin-streptavidin-labeled β-subunit antibody was rinsed with 50 mM TSM buffer (50 mM Tricine-NaOH pH 7.0, 0.25 M sucrose, and 4 mM $MgCl_2$). Then 100 μL 10 μM miRNA probe labeled with biotin was added and incubated at room temperature for 30 minutes. Free probes were washed out by 50 mM TSM buffer. The δ-free F_0F_1-ATPase with chromatophore was immobilized onto the glass surface through the biotin-streptavdin-biotin attachment. MiRNA probe system was hybridized with miRNA target in 100 μL formamide hybridization solution at 37°C. Before the detection, the sample was exposed under the cooled light source with a 570 nm filter for one hour to initiate the rotation of the F_0F_1-ATPase. During illumination, the buffer containing 2 mM NaN_3 and 2 mMATP was infused into the chamber to inhibit the hydrolysis activity of the F_0F_1-ATPase and hold force between the β_3 and γ to tightly band them together [18, 19, 23]. The hybridized reaction was recorded by our detection platform (BPCL) (see Supplementary Materials available online at doi: 10.1155/2009/671896). Fluorescence was excited at 471 nm and emitted at 535 nm.

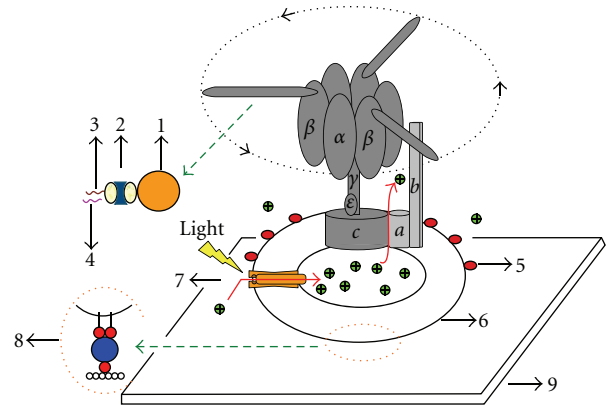

FIGURE 1: Schematic diagram of the biosensor based on F_0F_1-ATPase within chromatophore. 1, 2, 3, 4, 5, 6, 7, 8, and 9 represent the antibody against β subunit, the linking system composed of [biotin-AC5-sulfo-OSu]-streptavidin-[biotin-AC5-sulfo-OSu], miRNA probe, target miRNA, 535 nm QDs, chromatophore, bacteriorhodopsins (BRs), the linking system of lipidbiotin-streptavidin-[biotin-AC5-sulfo-OSu]-Polylysine, and the glass surface, respectively.

2.4. Data Analysis. The data were presented as means ± standard deviation of the mean. All experimental data were from average of at least 4–6 independent tests.

3. Results

3.1. Rotary Biosensor Design. Figure 1 showed the scheme of a novel rotary biosensor based on F_0F_1-ATPase within

a chromatophore. Chromatophores were immobilized to glass surface by lipidbiotin-streptavidin-biotin-Polylysine system [18]. Each β subunit of F_0F_1-ATPase was linked with a miRNA probe, which was used to detect target miRNA. Thus, three target miRNAs can bind specifically to a single F_0F_1-ATPase. After illumination, the chromatophores can maintain the proton electrochemical gradient across the plasma membrane for a long time (see Supplementary Materials). The light-driven rotation of F_0F_1-ATPase l resulted that the protons are pumped out of chromatophores and the proton concentration around chromatophores increased [15, 16], which leads to an enhancement of fluorescent intensity of CdTe quantum dots (QDs). The pH-sensitive QDs labeled on the outer surface of chromatophores are used as a proton sensor to detect proton flux [22]. Based on this, the changes of fluorescent intensity can indicate the alteration of H^+ concentration ($[H^+]$). When target miRNAs bind to the capture probes, the rotation of δ-free F_0F_1-ATPase slows down, which leads to a decrease in fluorescent intensity. In the course of detecting target miRNAs, miRNA probes linked to β subunits function as a rotary propeller to stir the sample solution, which results in dynamic hybridization reaction. The rotation of δ-free F_0F_1-ATPase-based biosensors enables one to monitor hybridization reactions in real time. In our experiment, the hybridization reaction is detected by recording the changes of fluorescence intensity. Using this method, the results can be obtained in 500 seconds.

3.2. Sensitivity and Detection Rang of miRNA Assay with Dynamic Hybridization Reaction. To determine the sensitivity of the method with dynamic hybridization property, we used miRNA (*mir*-145) probes to capture their target miRNAs in a complex RNA background and the change of fluorescence intensity was detected. The low limit of detection for the assays was found to be 1.2×10^{-18} mol (0.72×10^6 molecules) (see Supplementary Materials). Figure 2(a) showed the fluorescence intensity changes of biosensors with the different amounts of loads at 37°C, respectively. The no miRNA loading as control shows the low limit of detection. Serial 10-fold dilution of total RNA extracted from MCF-7 cells was used to analyze the detection range of the biosensors. The rate of fluorescence changes in 500 seconds were approximately 563.94 ± 9.68 U/s, 554.08 ± 9.95 U/s, 531.73 ± 10.97 U/s, 517.06 ± 4.19 U/s, 501.29 ± 10.05 U/s, 487.66 ± 6.95 U/s and 471.27 ± 5.94 U/s for biosensor hybridized with miRNA probe, miRNA from 10 cells, 10^2 cells, 10^3 cells, 10^4 cells, 10^5 cells and 10^6 cells, respectively. Using this novel rotary biosensor, we were able to detect not only amounts of relatively abundant miRNAs but also low level miRNAs because we observed a broad range of miRNAs expression levels from 1.2×10^{-18} mol (0.72×10^6 molecules) to 1.2×10^{-13} mol (0.72×10^{11} molecules). Compared to changes in fluorescence intensity of miR-145 from 10^6 cells, more miRNAs did not lead to the further changes because of the quantitative limitation of chromatophores. Increase of quantity of chromatophores can obtain a broader detection range. As shown in Figure 2(b), the rate of fluorescence changes in 500s of the different molecules is linear, and the detection range is about 5 orders of magnitude, which implies that this method can be used to quantify miRNAs with a wide range.

3.3. Specificity of miRNA Assay with Dynamic Hybridization Reaction. Figure 3 showed the Specificity of miRNA assay with dynamic hybridization reaction by the four let-7 family members. It is well known that Most human miRNAs differ by three or more nucleotide bases, but function as the different roles in cancer cells and the process of cellular growth, proliferation, and metabolism. In the let-7 family, the members differ by only one or two nucleotide base. To determine whether our miRNA assay is capable of discrimination among family members under the condition of dynamic hybridization, we attempted to use *let-7a* probes to capture *let-7a*, *let-7b*, *let-7c,* or *let-7d* miRNA targets in 1 pM. The fluorescence intensity changes of *let-7a* binding to the *let-7a* probes (the rate of fluorescence change in 500 seconds: 471.36 ± 10.17 U/s) was relatively slight compared with that of the biosensor only loaded with miRNA probe (the rate of fluorescence change in 500 seconds: 559.96 ± 7.05 U/s), which indicated a specific binding to *let-7* miRNA target (Figure 3). In contrast, the fluorescence intensity changes of *let-7b*, *let-7c,* and *let-7d* binding to the *let-7a* probes (the rate of fluorescence change in 500 seconds: 553.14 ± 10.07 U/s, 549.42 ± 9.97 U/s and 552.99 ± 8.94 U/s) were more dramatic than that of *let-7a* binding to the *let-7a* probes and were similar to that of biosensor only loaded with miRNA probe. These results suggest that the rotary biosensor can be used to discriminate among the four *let-7* family members.

3.4. Comparative Analysis of Dynamic and Static Hybridization Reaction. The normal hybridization procedure fails to move solution volumes, and so this static hybridization reaction requires incubation over night. To demonstrate utility of the static hybridization, Figure 4 shows that the fluorescence intensity changes of biosensors were hybridized with miRNA target in 100 μL formamide hybridization solution at 37°C for 1 hour, 6 hours, and 12 hours, respectively. Before the detection, the sample in the buffer containing 2 mM NaN_3 and 2 mMATP was exposed to the cooled light source with a 570 nm filter for 1 hour to initiate the rotation of the F_oF_1-ATPase. Then, the results of static hybridization were recorded by our detection platform. Compared to the control only loaded with miRNA probe (565.89 ± 5.19 U/s), no significant binding signal was observed when hybridized with miRNA target for 1 hour (555.57 ± 8.15 U/s). These data suggested that the static hybridization was strongly correlated with the incubation time, which is consistent with previous study. The biosensor with dynamic hybridization (469.87 ± 8.01 U/s) can capture more target miRNAs than the biosensor with static hybridization for 6 hours (511.47 ± 10.01 U/s). But, upon static hybridization for 12 hours (451.90 ± 7.61 U/s), the rate of fluorescence changes was just slighter than that in of dynamic hybridization (469.87 ± 8.01 U/s). The activity of δ-free F_0F_1-ATPase did not change because of the overnight hybridization reaction (data not shown). In comparison with

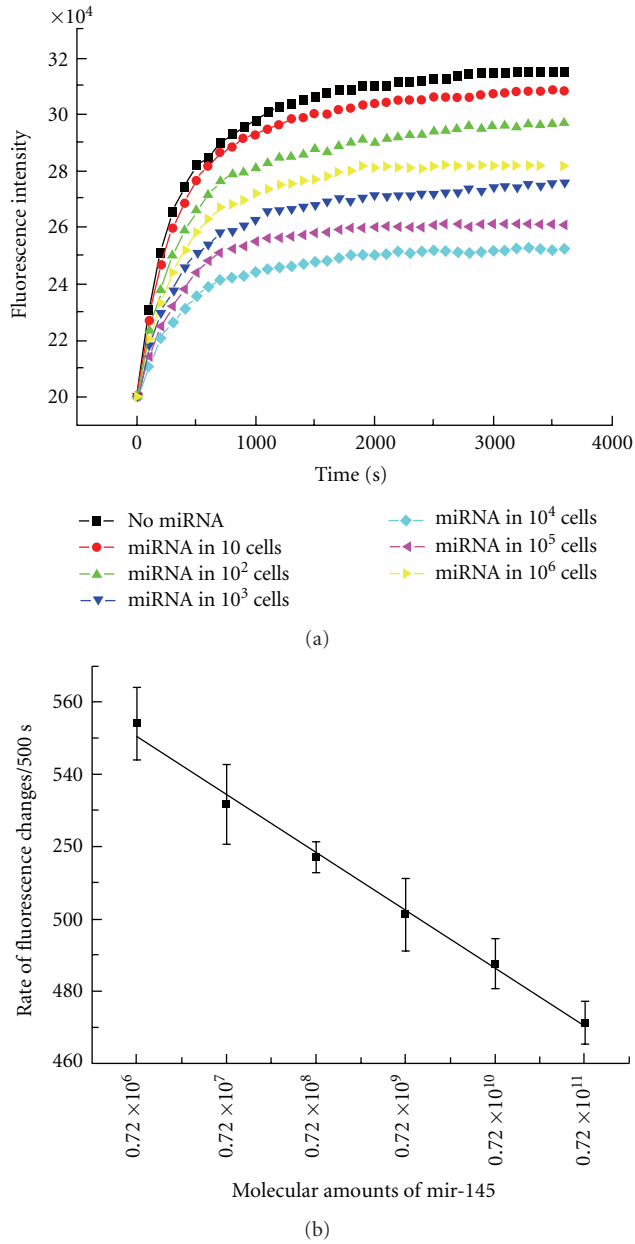

Figure 2: Detection range of the miRNA biosensor assay with dynamic hybridization. (a) Total RNA extracted from MCF-7 cells was serially 10-fold diluted. Different amounts of miR-145 could be detected by the biosensor loaded without or with target miRNAs derived from 10 cells, 10^2 cells, 10^3 cells, 10^4 cells, 10^5 cells, and 10^6 cells reaction at 37°C, respectively. (b) Correlation between the rate of fluorescence changes and molecular amounts of miRNAs. The mean value was calculated from three independent detection experiments. Error bars show variations among the experiments as the standard deviation of the mean.

the static hybridization, the dynamic hybridization using the rotation of miRNA probe system will greatly reduce the time to detect target miRNAs.

4. Discussion

Here we present a novel method for the rapid detection of target miRNA. The miRNA probe system linked to rotary β subunit of δ-free F_0F_1-ATPase was used to detect target miRNA. The change of proton flux was recorded through the changes of fluorescence intensity of QDs, which can monitor real-time hybridization state [22]. In this study, the biosensor has function as a rapid, sensitive, and specific tool to detect miRNAs.

The recent miRNAs detection method has called for the rapidity of the hybridization reactions and real-time detection. In the normal hybridization reaction, the vicinity of the corresponding probe spot will take long time to finish, especially in case of low concentrated miRNA molecules. In our experiments, the rotation of F_0F_1-ATPase-induced fluid

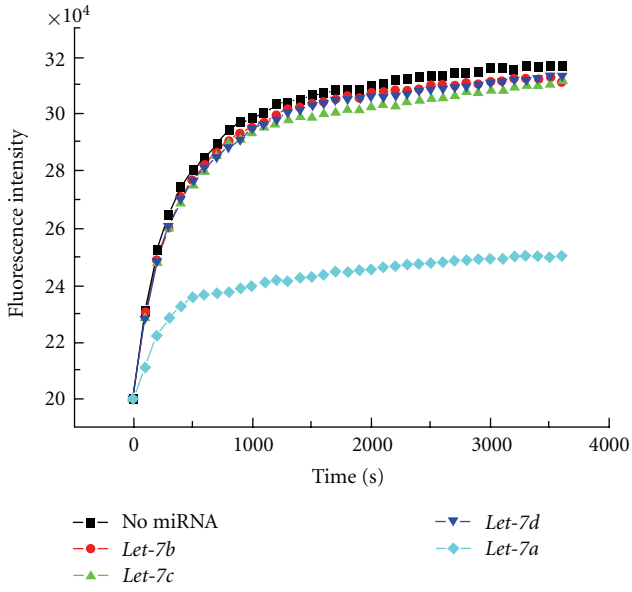

Figure 3: The miRNA biosensor with dynamic hybridization can be used to specifically detect the target miRNAs. The *let-7a* probes were used to capture their target miRNAs. The biosensor was only hybridized without any miRNAs or with incompletely complementary target *let-7b*, *let-7c*, *let-7d* or complete complementary target *let-7a* at 37°C, respectively.

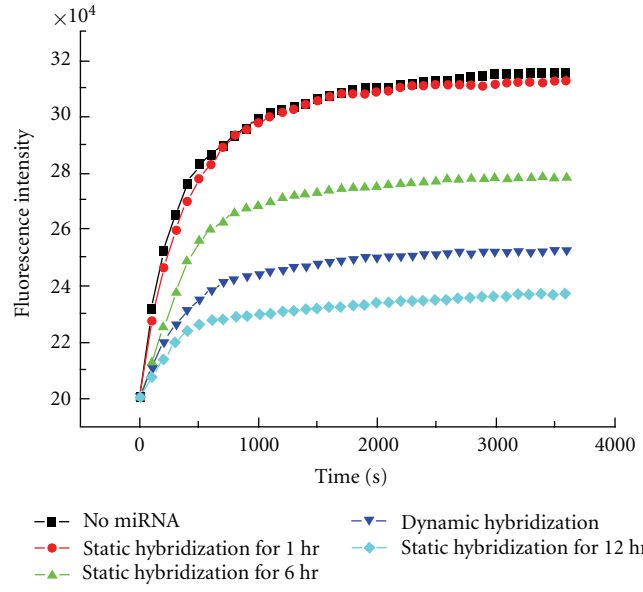

Figure 4: The dynamic hybridization resulted in a decrease in hybridization time. In the static hybridization, the biosensor was hybridized without or with miRNAs extracted from MCF-7 cells in the hybridization solution at 37°C for 1 hour, 6 hours, and 12 hours before detection, respectively. For the dynamic detection, the hybridization of biosensor and target miRNAs was monitored in real time without the preincubation with target miRNAs.

movement of nanoliter volumes could increase in collision between the probe and the target, thereby accelerating the hybridization kinetics [24, 25]. The efficiency of biosensor with dynamic hybridization efficiency was more than that of static hybridization for 6 hours (Figure 4). Meanwhile, the rotation of δ-free F_0F_1-ATPase makes it possible to monitor the real-time hybridization, which can yields data during the hybridization reaction. Furthermore, there is no need to reverse transcription and amplification before hybridization, thereby reducing the overall time of the detection.

The detection sensitivity was based on two lines of evidence. First, dynamic hybridization reaction might improve assay sensitivity. Peytavi et al. demonstrated that the microfluidic system increased the hybridization signal by 10-fold compared with a static hybridization system [13]. Other groups have demonstrated that inducing a microagiation in the fluid layer on a microarray can reach the signal amplification by 6-fold [14, 26]. Our method was sensitive enough to detect the amounts of miRNAs in 10 MCF-7 cells. Especially, *mir-145* is considered act as tumor suppressor genes or oncogenes and down-regulated in MCF-7 breast cancer cell line. Second, QDs have unique optical properties and exceptional photostability according to the previous study [27, 28]. In our experiments, more than two hundred QDs were attached on one chromatophore, which can enhance sensitivity in the process of miRNA detection (see Supplementary Material).

In terms of specificity, our method is capable of discrimination of a single base change in the target miRNAs even under particularly permissive binding condition. In the experiments, we observed good discrimination among *let-7a*, *let-7b*, *let-7c*, and *let-7d* using *let-7a*-specific probes (Figure 3). During the hybridization reaction, the rotation of δ-free F_0F_1-ATPase might decrease the binding of unspecific miRNAs. The results showed that rotary miRNA probe system tend to bind the target miRNA complementary completely to the probe sequence.

In conclusion, our results demonstrated that δ-free F_0F_1-ATPase-based rotary biosensor is a promising research tool that can be applied to large-scale miRNAs detection combined with the lab-on-a-chip technology.

Acknowledgments

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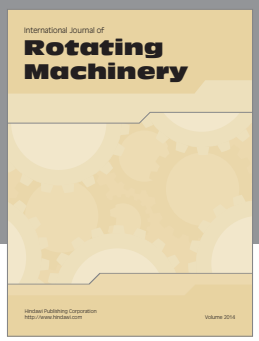

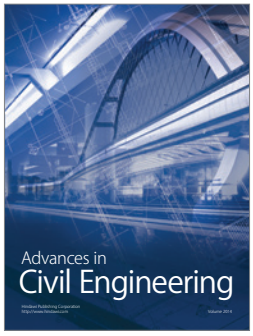

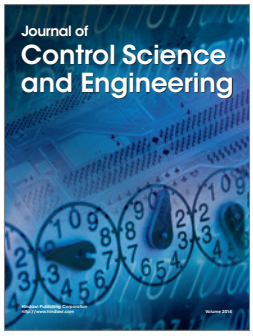

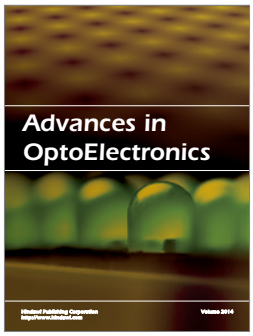

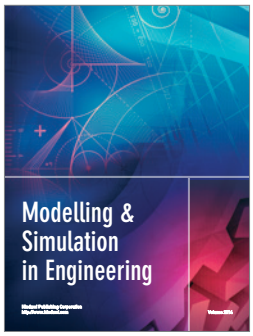

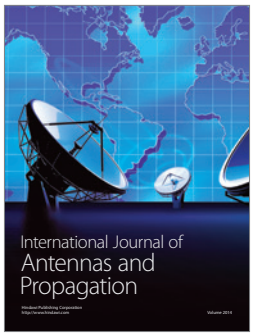

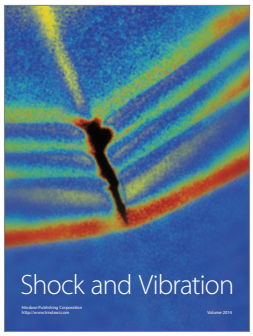

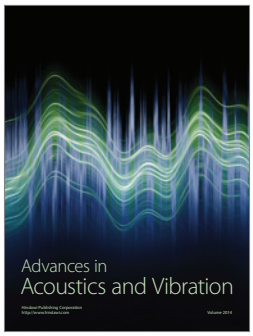

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