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Label-free, PCR-free chip-based detection of telomerase activity in bladder cancer cells

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ABSTRACT

Bladder cancer is one of the most common cancers in Worldwide. The determination of urinary telomerase activity is a promising tool for the diagnosis of bladder carcinoma owing to the high rate of expression of telomerase in cancer cells. Typical assay for telomerase activity is the telomeric repeat amplification protocol (TRAP) based on polymerase chain reaction (PCR) amplification. However, TRAP assay is susceptible to PCR-derived artifacts and requires time-consuming procedure, expensive equipments and reagents. To develop a new method for telomerase activity assay that is fast, simple, and cost-effective, we have examined a silicon-based microring resonator biosensor to detect label-free, PCR-free telomerase activity using telomerase extracted from two bladder cancer cell lines, J-82 and HT-1376, in a buffer solution and spiked urine. With telomerase primer immobilized microring resonator sensor system, we successfully demonstrate the detection of telomerse extracted from as little as 10 cells/μL and 100 cells/μL in buffer and urine, respectively. Especially, the results represented here is the first demonstration of the detection of telomerase activity in human urine on the chip-based system. From the results, we expect that the silicon photonic microring resonator system can provide a powerful tool for cost-effective and sensitive telomerase activity detection in urinary bladder cancer.

1. Introduction

As incidence of bladder cancer has constantly increased from 2006 to 2010, bladder cancer has become the fifth most common cancer and annually 60,000–70,000 cases have been reported in the US alone (Wang and Choudhary, 2011). The patients who are diagnosed with or have a medical history of bladder cancer are estimated at approximately 2.7 million in Worldwide (Falke and Witjes, 2011). Generally, over 90% of bladder cancers are transitional-cell carcinomas and about 60% among them are low-grade or non-muscle invasive tumors. A prognosis of non-invasive tumors is not severe if early endoscopic and intravesical treatments would be undergone. However, about 70% of cases will recur and up to 30% will progress to muscle invasive tumors within 5 years, necessitating vigilant lifelong surveillance for bladder cancer patients (Prasad et al., 2011). Patients with bladder cancer usually are observed on a 3-month to 6-month surveillance schedule. Mostly used approaches for the detection of bladder cancer are periodic cystoscopy and urine cytology. However, cystoscopy is relatively expensive and invasive which causes substantial patient's discomfort. In addition, urine cytology has limited sensitivity, especially for low-grade superficial lesions, and its accuracy depends on the pathologist's experience (Bravaccini et al., 2007; Fedriga et al., 2001; Sanchini et al., 2005). Therefore, the development of inexpensive, non-invasive, reliable, and simple test for bladder cancer detection is of utmost importance and would be a major advance in cancer screening and monitoring recurrence. In particular, the development of a point-of-care (POC) assay that can achieve a low-cost, easy to use, and portable device system is desirable because the diagnosis can be made immediately in a physician's office.

Various candidate bladder cancer biomarkers have been investigated and identified in order to ensure an initial cancer diagnosis, monitor the recurrence of patients and confirm treatment response (Kim and Bae, 2008; Vrooman and Witjes, 2008). Telomerase is one of the promising cancer biomarkers since it is believed to play a critical role in tumorigenesis (Kim et al., 1994). Telomerase is a ribonucleoprotein enzyme for catalyzing the addition of repeated DNA sequences TTAGGG, telomeres, at the end of chromosomal DNA. In normal somatic cells, the telomerase expression is repressed and telomeres shorten progressively with each cell division, which leads to cellular senescence (Greider, 1996). In contrast to somatic cells, cancer cells and germ cells are

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able to divide indefinitely and maintain their telomere lengths by expressing telomerase. Indeed, telomerase activity can be identified in over 80% of tissues samples derived from bladder carcinomas regardless of tumor stage or degree of differentiation (Müller, 2002).

The most well-known assay for telomerase activity is a PCR-based TRAP method (Bakalova et al., 2007; Sanchini et al., 2005). However, TRAP assay is time-consuming, susceptible to polymerase inhibition by cell-extract and requires expensive equipment and reagents (Sharon et al., 2010). Thus, many research groups have investigated various sensing technologies for direct detection of telomerase activity including optical (Ding et al., 2010; Ma et al., 2012; Patolsky et al., 2003; Pavlov et al., 2004b; Sharon et al., 2010; Xiao et al., 2004; Zhang et al., 2012), magnetic resonance (Grimm et al., 2004), electrochemical (Gabourdes et al., 2004; Li et al., 2010; Pavlov et al., 2004a; Sato et al., 2005; Shan et al., 2010; Sharon et al., 2010; Wu et al., 2012; Yang et al., 2011; Zheng et al., 2005), and surface plasmon resonance (SPR) (Maesawa et al., 2003; Sharon et al., 2010) methods. Although these techniques demonstrated the detection of telomerase activity without PCR process, most of the methods require additional labeling step or need expensive and sophisticated instruments, which are not suitable as a POC assay.

For the assay of telomerase activity, a silicon-based photonic microring device has been used in the present study. Silicon microring resonators are used for sensing by monitoring a shift in the resonant wavelength cause by a change in effective refractive index on the sensor surface (Luchansky and Bailey, 2012). They offer many advantages as a biosensing device, including highly sensitive, label-free, real-time, multiplexed detection within a single device, and high quality, low-fabrication cost due to complementary metal oxide semiconductor (CMOS) compatibility. The microring resonators have been demonstrated for label-free detection of various biomolecules and cells including proteins (De Vos et al., 2007; Iqbal et al., 2010; Park et al., 2013; Washburn et al., 2009, 2010), oligonucleotides (Qavi et al., 2011; Shin et al., 2013), and viruses (McClellan et al., 2012; Shang et al., 2012).

Here, we demonstrate a silicon microring resonator-based biosensor for the detection of telomerase activity without any labeling and PCR process (Scheme 1a). We used cell-extracts from two bladder cancer cell lines, J-82 and HT-1376, and examined their telomorization response in the buffer as well as spiked urine. Particularly, the reported results here represent the first example of the detection of the telomerase activity in human urine on the chip-based system. The developed telomerase assay based on the microring resonator system could detect telomerase activity extracted from 10 cells/μL in a buffer solution and 100 cells/μL in the spiked urine.

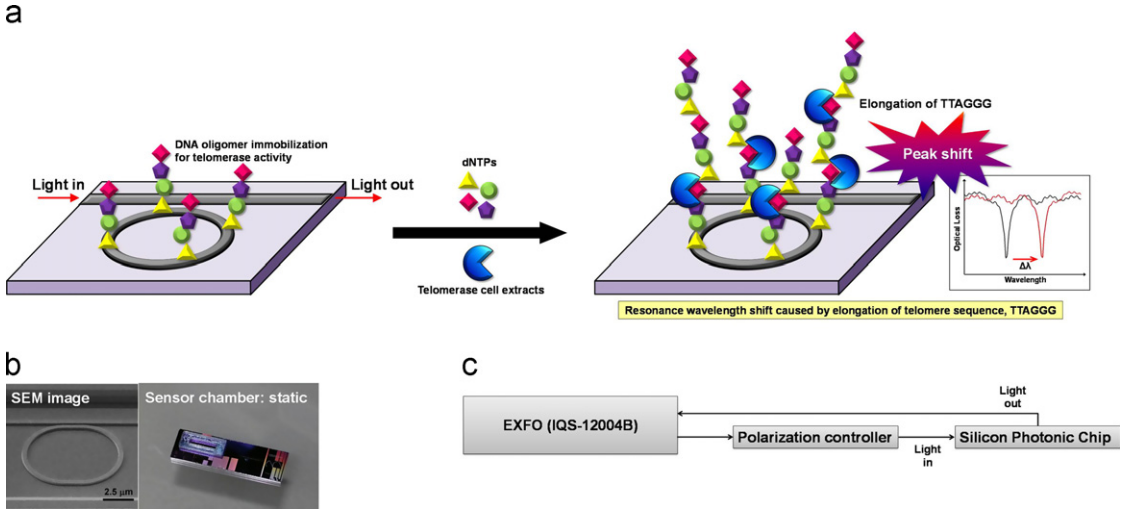
2. Experimental section

2.1. Materials

All oligonucleotides were purchased from Integrated DNA Technologies (IDT), Inc. (Coralville, IA, USA). The oligonucleotides were HPLC-purified and freeze-dried products. The stocks of the oligonucleotides were diluted to 100 μM in nuclease-free deionized water and kept at 4 °C. The sequences of the oligomers were 5′-NH_2-$(CH_2)_6$-AATCCGTCGAGCAGAGTTAGGGTTAGGG-3′ (Positive control primer) and 5′-AATCCGTCGAGCAGAGT-TAGGGTTAGGG-$(CH_2)_6$-NH_2-3′ (Negative control primer). Mixed deoxyribonucleotide triphosphate (dNTP) set was purchased from Qiagen, Inc. (Valencia, CA, USA). TRAPeze® telomerase detection kit was purchased from Merck Millipore (Billerica, MA, USA). Bladder cancer cell lines, J-82 and HT-1376, were obtained from American type culture collection (ATCC) (Manassas, VA, USA). 3-aminopropyltriethoxysilane (APTES), sodium cyanoborohydride, glutaraldehyde (GAD), 100 mM CHAPS solution and mineral oil were purchased from Sigma Aldrich (St. Louis, MO, USA). Normal human urine was obtained from Innovative research, Inc. (Novi, MI, USA) and stored at 4 °C until used. Other chemicals were analytical reagent grade, and used as received. All samples and buffers were prepared using deionized (DI) water obtained from a Milli-Q water purification system.

2.2. Preparation of cell-extracted telomerase

J-82 and HT-1376 were transitional cell carcinoma cell lines of urinary bladder. From the both cell lines, telomerase was extracted by the CHAPS method (Piatyszek et al., 1995). Cells were grown in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum at 37 °C. In the exponential phase of growth, the cells (40×10^6 cells) were washed two times using PBS, pelleted by centrifugation for 5 min at 400g, and resuspended in 200 μL of ice cold lysis buffer containing 0.5% CHAPS, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol,



Scheme 1. (a) Schematic representation of detection of telomerase activity on the microring device. (b) a SEM image of a race-track style ring with radius 5 μm, coupling length 2.042 μm (the linear waveguide dimension is 220 nm × 500 nm and the gap between linear waveguide and ring is 220 nm) and a photo image of sensor chip with static plastic chamber. (c) Sensing diagram of silicon microring system.

0.1 mM benzamidine, 10% glycerol, and 10 mM Tris–HCl, pH 7.5. The resuspension was incubated in ice for 30 min to entirely lyse cells. After cell lysis, the lysate was centrifuged at 12,000*g* for 30 min at 4 °C. The supernatant was collected using pipette carefully and aliquoted to the tubes with 20 μL each. The extracted telomerase was stored at −80 °C before use or used immediately.

2.3. Confirmation of telomerase extracts using TRAP kit

The confirmation of telomerase extracts from bladder cancer cell lines was performed by a commercial TRAP kit from Merk Millipore (Billerica, MA, USA). 2 μL of cell extracts or controls were added to 48 μL of reagent mixtures including 10× TRAP reaction buffer, 50× dNTP mix, primer mix, Taq polymerase (5 units/μL) and nuclease free dH_2O into each PCR tube. The tubes were placed in the thermocycler block and then incubated at 30 °C for 30 min. After incubation, 3-step PCR was performed at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min with 35 cycles. The resulted PCR products with 5 μL of loading dye were loaded and run on 4% agarose gel with ethidium bromide in 1× TBE buffer for 30 min at 120 V. After electrophoresis, the gel was determined by the ImageQuant (LAS4000, GE).

2.4. Immobilization of telomerase oligomers on the chip

The design and fabrication method of the silicon microring sensor chip as well as the measurement procedure have been previously described (Park et al., 2013; Shin et al., 2013). Briefly, the silicon microring is a race-track style ring with a radius of 5 μm and coupling length is 2 μm (Scheme 1b). In addition, the waveguide dimension is 220 nm × 500 nm and the gap between linear waveguide and ring is 220 nm. First of all, the sensor chip was treated by oxygen plasma. The plasma-treated chip was immersed into 2% of APTES solution in a mixture of elthanol/deionized water (95%/5%, v/v) at room temperature for 2 h. Then, the chip was washed three times thoroughly using ethanol and deionized water. The chip was dried under nitrogen gas and heated at 120 °C for 15 min. 50% GAD solution and 50 mM borate buffer were mixed well with 10 mM sodium cyanoborohydride solution using deionized water. The APTES-functionalized chip was then incubated with 50 μL of GAD mixture solution at room temperature for 1 h and then washed three times using deionized water, followed by drying with nitrogen gas. Next, 100 μM of telomerase oligomers were diluted to 2 μM using 20 mM sodium cyanoborohydride solution in 50 mM MES buffer, pH 6.0. Finally, 50 μL of telomerase oligomer solutions were applied to each microring chip and incubated at 4 °C overnight. After overnight, the chip was washed three times using 50 mM MES buffer, pH 6.0 and deionized water followed by drying with nitrogen gas. An acrylic static chamber ($6 \times 2 \times 1$ mm^3) was then pasted onto the chip to enclose the microring sensor area (Scheme 1b). At this time, the chips were considered ready for optical measurement. After applying telomerase extract samples to the chamber, a mineral oil was used for preventing evaporation.

2.5. Measurement of telomerization on the microring chip

The telomerization activity was performed on the telomerase primer-immobilized microring chip. We applied 10 μL of telomerase solution in the presense of 20 mM dNTP mix (5 mM of each dATP, dTTP, dGTP, and dCTP) to the acrylic chamber using a micro-pipette. The telomerase solution consisted of 20 mM Tris–HCl buffer, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20 and telomerase extracted from bladder cancer cell lines, J-82 and HT-1376. The resonant spectrum was immediately obtained for the baseline measurement. The microring chip was then kept at 37 °C for 1.5 h. Then the chip was carefully washed using 10 mM PBS buffer, pH 7.4 in order to eliminate non-reacted dNTPs and chemicals in reaction buffer that might affect the effective index change. In addition, the human urine spiked with the extracted telomerase was used to examine the telomerase activity at the same condition. For negative control experiments, heat-treated telomerase extracts at 85 °C for 10 min and negative control primer that had an adverse sequence in the comparison with telomerase primer were used. Transmission loss spectrum was measured with EXFO IQS-12004B DWDM passive component test system (Q=24,000 and FSR=17 nm) and sensing flow diagram was shown in Scheme 1c.

3. Results and discussion

3.1. Confirmation of telomerase extracts from bladder cancer cell lines

Prior to examine the telomerization on the microring device, we confirmed the existence of telomerase in the cell extracts from bladder cancer cell lines, J-82 and HT-1376, using commercially available TRAP assay kit. For the negative controls, cell extracts from J-82 and HT-1376 was heated at 85 °C for 10 min in a thermocycler. Telomerase is known as a heat-sensitive enzyme and thereby easily inactivated by heat shock. Another telomerase-cell extract that should be shown at 50-bp band included the assay kit was used as a positive control. In Fig. 1, we proved that the presence of telomerase from the cell extracts of J-82 (lane 3) and HT-1376 (lane 5) by gel electrophoresis. They showed the same band at the position of 50-bp band with the positive control obtained from the kit (lane 2). For negative controls given by heat-shock, both cases did not present any band of telomerase (lane 4 and 6).

3.2. Detection of telomerase activity in reaction buffer

Silicon photonic microring resonators have emerged as sensitive label-free biosensors that allow the detection of chemical reactions or biomolecular recognition events near the sensor surface (Qavi and Bailey, 2010). In order to test the telomerization on the chip, telomerase primer-immobilized silicon photonic microring device was first prepared. The resonance wavelength of the microring resonator is sensitive to alteration in effective refractive index of the microring caused by an elongation of telomere sequence (TTAGGG) at the end of the immobilized primer in the presence of telomerase extracts from bladder cancer cell lines and dNTPs (Scheme 1a). The amine group (NH_2)-terminated telomerase primer was conjugated with the aldehyde functional group activated by APTES and GAD treatment on the surface of the microring. Also, we showed the resonance wavelength shift upon the surface functionalization from

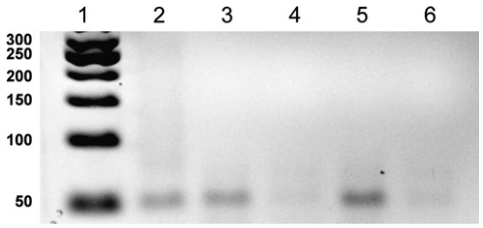


Fig. 1. Agarose gel electrophoresis of telomerase extracts of J-82 and HT-1376. M: size marker (50-bp), (1) positive telomerase cell extract from TRAP kit; (2) cell extract of J-82; (3) heat-shocked cell extract of J-82; (4) cell extract of HT-1376 and (5) heat-shocked cell extract of HT-1376. Heat-shock was performed at 85 °C for 10 min.

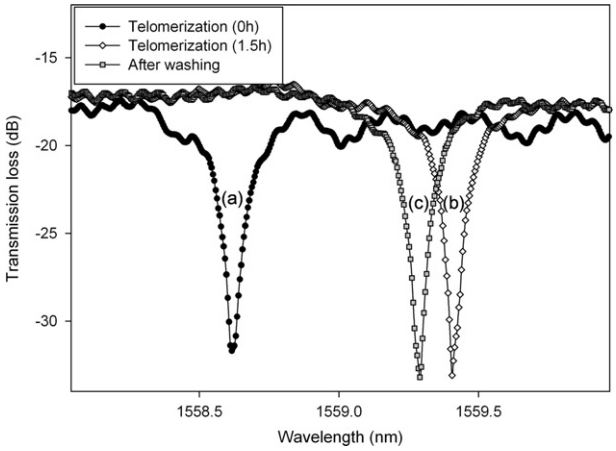


Fig. 2. Resonant wavelength shift induced by telomerization on the silicon photonic microring resonator biosensor functionalized with telomerase primers telomerase extract from J-82 cells (1000 cells/μL) (a) immediately after the addition of telomerase extract and 20 mM of dNTP mix (5 mM dNTPs each) in the reaction buffer, (b) after the incubation at 37 °C for 1.5 h, (c) after washing with 10 mM PBS buffer, pH 7.4.

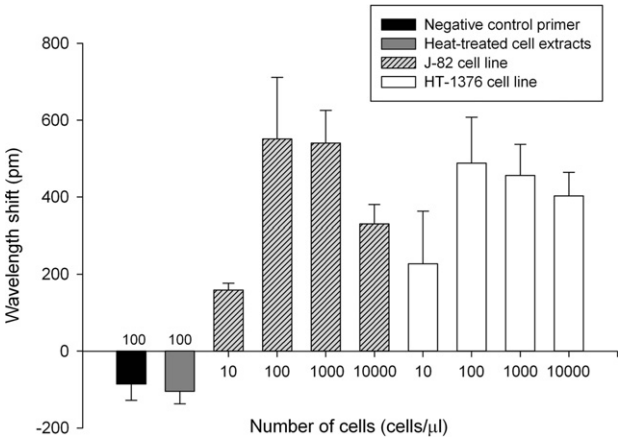


Fig. 3. Microring resonator sensor responses of telomerase activity corresponding to various concentrations of telomerase extracted from bladder cancer cell lines, J-82 and HT-1376, in the reaction buffer at 37 °C for 1.5 h. The heat-inactivated telomerase and non-telomeric primer immobilized microring device in the existence of dNTPs used as control experiments.

APTES/GAD to the primer treatment (see Supplementary Fig. S1). Then, the primer-functionalized microring chip was reacted with telomerase extract and 20 mM of dNTP mix (5 mM dNTPs each) in the reaction buffer and its resonant spectrum was immediately measured using an optical measurement system as a baseline for monitoring peak wavelength shift due to the telomerization (Fig. 2a). Fig. 2 shows the resonant spectrum of the primer functionalized microring sensor in telomerase extract from J-82 cells (1000 cells/μL) after incubation at 37 °C for 1.5 h (Fig. 2b) and followed by washing with 10 mM PBS buffer, pH 7.4 (Fig. 2c). The washing step insures the elimination of non-reacted dNTPs and chemicals in reaction buffer that might affect the effective index change. The incubation time was determined by monitoring the resonant wavelength shift in every 20 min (see Supplementary Fig. S2). The resonant wavelength shows red-shift with time as the telomerization process continues and it levels off after 60 min due to the gradual denaturation of telomerase in the non-native environments of the reaction buffer. In order to insure the completion of telomerization, we set the incubation time to be 1.5 h for subsequent experiments. The resonant wavelength increases ~790 pm after 1.5 h of incubation due to the elongation of the immobilized primer in the presence of telomerase. Since the microring device is only sensitive to the refractive index change within the confines of the evanescent field extended from the sensor surface, the shift in the resonance wavelength of the microring sensor occurs when the dNTPs in buffer solution are added to the end of the surface immobilized oligomer by telomerization. In other word, the telomerase-induced telomerization of the primer changes the effective refractive index of the microring, and thus telomerase activity could be determined. After the washing step, the peak wavelength moved to shorter wavelength (~115 pm), therefore the total change in resonant wavelength due to the telomerization was obtained to be ~675 pm (Fig. 2c). All results in this paper were obtained from the data obtained after washing step and the experiments were repeated at least three times for each case.

Next, telomerase cell extracts from J-82 and HT-1376 cell lines at concentrations ranging from 10^1 to 10^4 cells/μL were prepared in the telomerase reaction buffer. In addition, non-telomeric primer-immobilized microring device and heat-treated both cell extracts were prepared as negative controls. The resonant wavelength shift corresponding to different concentrations of telomerase extracts was examined in Fig. 3. For both cell lines, their resonance wavelength shifted to longer wavelength in regard to telomeric primer-immobilized microring device and the lowest detectable telomerase activity was telomerase extracted from 10 cells/μL, corresponding to total 100 cells (the reaction buffer ~10 μL). At the lowest concentration, the peak wavelength shifts were 158 ± 18 pm and 227 ± 135 pm in telomerase cell extracts of J-82 and HT-1376, respectively. In contrast, control experiments with the heat-inactivated telomerase as well as non-telomeric primer immobilized microring device in the existence of dNTPs showed blue-shifts in the resonant wavelength. Since the baseline measurement was performed after the addition of telomerase reaction buffer, the blue-shift of resonant wavelength (~100 pm) corresponds to the removal of non-reacted dNTPs and chemicals by the washing procedure. Interestingly, the resonance peak shift seemed lower at high concentrations (> 1000 cells/μL) than low concentrations. In our experimental procedure, we took the first measurement of the resonance wavelength just after adding telomerase solution and used as a baseline to obtain the shift in the resonance wavelength after the telomerization. We can assume that the telomerization happened instantly for high cell concentration after the addition of the reaction solution, resulting in apparent smaller wavelength shift. Label-free detection of telomerase activity based on silicon microring resonator showed significantly improved sensitivity (~10 cells/μL) compared to previous studies based on label-free, PCR-free, chip-based assay such as SPR (Detection limit ~180 cells/μL), field-effect-transistor (Detection limit ~65 cells/μL) (Sharon et al., 2010), and impedance spectroscopy (Detection limit ~1000 cells) (Yang et al., 2011).

3.3. Detection of telomerase activity in human urine

Human voided urine is one of the best sources of bodily fluids for non-invasive bladder cancer detection since it is easier to collect than other bodily fluids (i.e. blood, cerebrospinal fluid) and abundant biological components from adjacent organs such as bladder and prostate are excreted into urine. Several studies on the telomerase activity in urine for bladder cancer using TRAP assay have shown good sensitivity (> 90%) and specificity (> 88%) (Sanchini et al., 2004, 2005). However TRAP assay is time-consuming, susceptible to polymerase inhibition by cell-extract and requires expensive equipment and reagents, which make unsuitable as an inexpensive and simple to use POC diagnostic method. Until now, most of chip-based direct telomerase activity detection has been demonstrated with telomerase

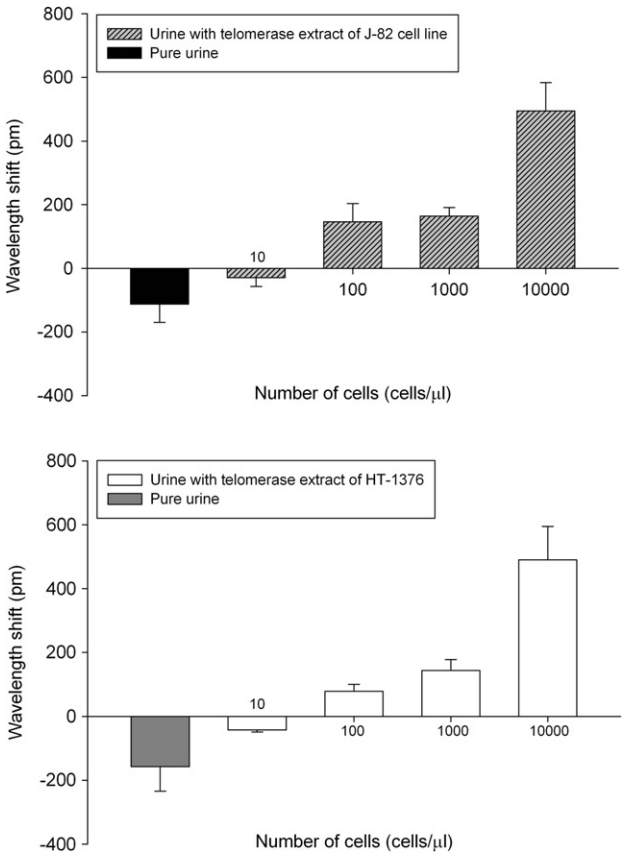


Fig. 4. Microring resonator sensor responses of telomerase activity corresponding to different concentrations of telomerase extract-spiked human urine including dNTPs. (a) J-82 cell line and (b) HT-1376 cell line. Both were incubated at 37 °C for 1.5 h. Pure urine indicates the human urine with dNTPs excluding telomerase cell extracts.

extracts from cancer cell lines in buffer solution. The telomerase ribonucleoprotein is known to be unstable in an aggressive medium like urine (acidic pH, proteases, RNases, salts and urea) (Müller et al., 1998), thus we set out to investigate the validation of the applicability of label-free, PCR-free silicon microring biosensor in the detection of telomerase in urine. Normal human urine samples spiked with telomerase extract at the concentrations ranging from 10^1 to 10^4 cells/μL from J-82 and HT-1376 bladder cancer cell lines in the presence of 20 mM of dNTPs were prepared. The measuring procedure was same as described in the previous section. To examine the resonant wavelength shift by telomerase activity, the spectrum was measured promptly after applying the telomerase mixture in the urine. The telomerase primer immobilized microring device was reacted with the spiked urine mixture at 37 °C for 1.5 h and then the peak wavelength shift was investigated using the optical test instrument. Finally, the spectrum of the resonance wavelength was measurement after washing. For both cell extracts from 10^2 to 10^4 cells/μL, they appeared a distinct resonance wavelength shift (Fig. 4a and b). However, 10^1 cells/μL exhibited wavelength change to a short wavelength in both cell lines. Pure human urine without telomerase extracts was also tested as a negative control. Control experiment revealed that pure urine without telomerase extracts did not lead to telomerization in the presence of dNTPs. Although the peak wavelength shift in the spiked-urine was smaller than the one in the reaction buffer, the discrimination of the resonance wavelength change corresponding to the different concentrations of telomerase extracts was obvious. Actually, human urine consists of many macromolecules including cell-free proteins, DNA, RNA, micro RNA, and various cells. Unlike the reaction buffer for telomerase activity, we assumed that many crude constituents and non-optimized reaction condition for enzyme reaction of telomerase in the urine affected to the telomerase activity on the device. As a result, the limit of detection for telomerase spiked urine was 100 cells/μL. For 100 cells/μL of telomerase extracts from J-82 and HT-1376, the peak shifts presented 146 ± 56 and 78 ± 22 pm, respectively. The results here represent the first example of telomerase activity detection in urine on the chip-based system and do not require labeling and purification steps. Therefore, we expect that the silicon microring resonator system can be useful as a potential diagnostic and monitoring tool for urinary bladder cancer.

4. Conclusion

In this work, we have developed a label-free, PCR-free sensor based on silicon microring resonators for telomerase activity detection in bladder cancer cells. Direct and sensitive detection of telomerase activity on the telomere primer immobilized sensor was successfully demonstrated using telomerase extracted from bladder cancer cell lines, J-82 and HT-1376, in a buffer solution as well as human urine. The label-free assay on the microring device provides the detection limit of 10 cells/μL and 100 cells/μL in a buffer solution and human urine, respectively, within an analysis time of 1.5 h. In comparison with previously reported methods, the telomerase assay using the microring resonator demonstrated significantly improved capabilities including high sensitivity, shorter analysis time, and inexpensive and simple procedure. We expect that the microring resonator system can provide a powerful tool for the detection of telomerase in urinary bladder cancer. Moreover, silicon microring resonator technology can allow cost-effective mass-production of the sensor chips. Such capability of the silicon microring resonator system can offer potential applications to extensive point of care (POC) systems for bladder cancer diagnostic and monitoring.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.02.001.

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