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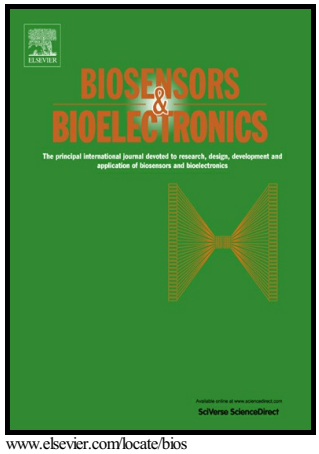

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Label-free detection of liver cancer cells by aptamer-based microcantilever biosensor

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Abstract

Liver cancer is one of the most common and highly malignant cancers in the world. There are no effective therapeutic options if an early liver cancer diagnosis is not achieved. In this work, detection of HepG2 cells by label-free microcantilever array aptasensor was developed. The sensing microcantilevers were functionalized by HepG2 cells-specific aptamers . Meanwhile, to eliminate the interferences induced by the environment, the reference microcantilevers were modified with 6-mercapto-1-hexanol self-assembled monolayers. The aptasensor exhibits high specificity over not only human liver normal cells, but also other cancer cells of breast, bladder, and cervix tumors. The linear relation ranges from 1×10^3 to 1×10^5 cells/mL, with a detection limit of 300 cells/mL (S/N=3). Our work provides a simple method for detection of liver cancer cells with advantages in terms of simplicity and stability.

Keywords: Microcantilever; HepG2 cells; TLS11a aptamer; Detection

1. Introduction

There were about 12.7 million cancer cases and 7.6 million cancer deaths in 2008 according to the worldwide cancer statistics released by the International Agency for Research on Cancer (GLOBOCAN) 2008 estimates(Jemal et al. 2011). Every year over 14 million cancer cases occur worldwide, but the global figure is projected to 22 million by 2030.(Jemal et al. 2014) Liver cancers are the third most deadly cancers, and most of them are associated with hepatitis B infection(Yang and Roberts 2010). Early diagnosis is of great significance for treatments of patients suffered from liver cancers. Unfortunately, many cases are diagnosed at a later stage when surgical resection or liver transplantation are not efficient. Straightforward and simple methodologies of diagnosing liver cancers in an early stage are essential for effective treatments of liver cancer cases. In many methods,α-fetoprotein (AFP) works as a liver cancer biomarker to diagnose liver cancer. However, due to the high blood level of AFP in other liver diseases, measuring the serum level of AFP lacks specificity for monitoring liver cancers.(El-Aneed and Banoub 2006)

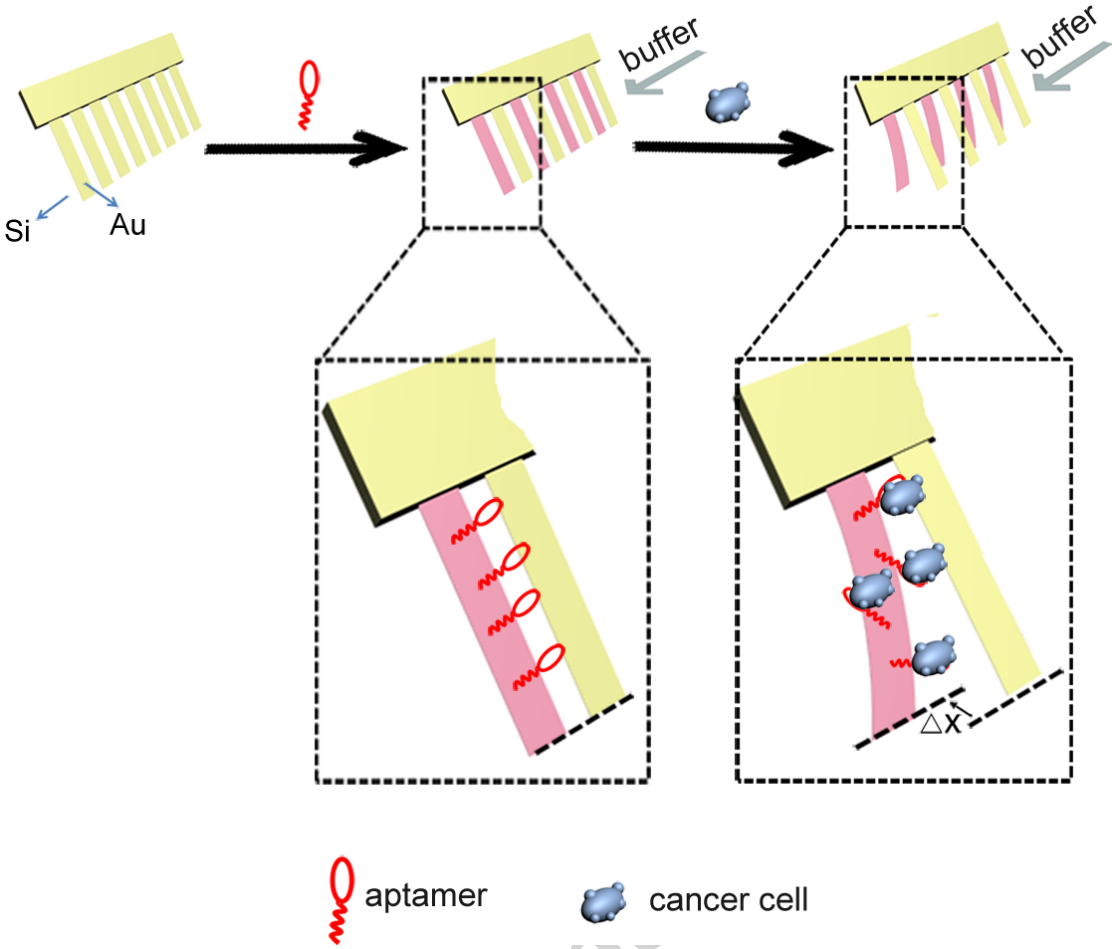
Aptamers, DNA or RNA ligands, have emerged as novel molecule probes in the past decade. Selected by SELEX methodology(Ellington and Szostak 1990; Tuerk and Gold 1990), aptamers can recognize from small molecules(Huizenga and Szostak 1995; Kawakami et al. 1998; Lauhon and Szostak 1995; Lorsch and Szostak 1994; Patel et al. 1997) to proteins and receptors(Bock et al. 1992; Hicke et al. 1996; Weiss et al. 1997; Xu and Ellington 1996). A liver cancer cells specific molecular probe， TLS11a atpamer, has been identified to recognize and diagnose liver

cancer(Shangguan et al. 2008) by binding to the membrane surface of hepatocellular carcinoma cells specifically(Kashefi-Kheyrabadi et al. 2014). Electrochemical methods have been developed to detect HepG2 cells using TLS11a aptamer(Kashefi-Kheyrabadi et al. 2014; Sun et al. 2015) with good specificity and sensitivity. However, these approaches are time consuming for they need complex procedures to prepare the experiments.

Nanomechanical systems have been developed due to the recent advances in nanotechnology(Eom et al. 2011). Mechanical interactions are fundamental to biology in nanomechanical sensors(Arlett et al. 2011). By monitoring the deflection or the changing frequencies of microcantilevers, the cantilever-based sensing is expanding in terms of technology development(Boisen et al. 2011), and has been emerging as a promising sensing technique with high specificity and sensitivity(Buchapudi et al. 2011). Microcantilever sensor enables label-free analyte determination without complicated labeling procedures and monitoring the interaction between probes and analytes *in-situ*. This technology has been applied in the determination of toxic chemicals(Karnati et al. 2007; Yan et al. 2004; Yang et al. 2010), heavy metal ions(Cherian et al. 2003; Velanki et al. 2007), biochemicals(Arntz et al. 2003; Baker et al. 2008; Subramanian et al. 2002) and microorganisms(Gunter et al. 2003; Li et al. 2006; Nugaeva et al. 2007). Microcantilever sensors have also applied in cancer researches based on investigating cancer-relevant molecules or proteins, such as genes, transcription factors and cellular antigens(Huber et al. 2015).

In this work, cancer cells were determined by the microcantilever array sensor in

static mode. As shown in Scheme 1, HepG2 cells specific TLS11a aptamers were immobilized on the gold side of four microcantilevers (pink) in an array to sense cancer cells. The other four were backfilled by 6-mercapto-1-hexanol (MCH) and served as reference microcantilevers (yellow) to eliminate the interference induced by the environment (the backfill procedure is not shown in Scheme1). After introduction of HepG2 cells, TLS11a aptamers functionalized on sensing microcantilevers bound to the protein on the surface of HepG2 cells. The binding of aptamer-cell caused a change in the surface stress of the microcantilevers, forcing the microcantilevers bending towards the silicon side. As a result, a difference in defection (ΔX) between sensing cantilevers and reference ones was produced. The aptamer based microcantilever array sensor has potential in early diagnosis of liver cancer cells and provides a promising tool in the therapy of tumors.



Scheme 1. Schematic illustration of mechanism of HepG2 cells determined by microcantilever array sensor. Four microcantilevers were modified with aptamers as sensing microcantilevers (pink), and the other four worked as reference ones (yellow). ΔX indicates the differential signal (the deflection of sensing microcantilevers minus that of reference ones) induced by the interaction between aptamers and HepG2 cells. The gray arrow indicates the flow direction of binding buffer.

2. Experimental

2.1. Chemicals and materials

Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was obtained from Alfa

Aesar(USA). MCH was bought from Sigma-Aldrich (St. Louis, MO., USA). Other reagents were of analytical grade and used as received without any further purification. Deionized water (18.2 MΩ cm^{-1}) from a Milli-Q-water purification system (Millipore) was used in all the experiments. TLS11a aptamer(Shangguan et al. 2008) (5'-(SH)-$(CH_2)_6$-ACA GCA TCC CCA TGT GAA CAA TCG CAT TGT GAT TGT TAC GGT TTC CGC CTC ATG GAC GTG CTG-3') was synthesized by Sangon (Shanghai, China). The phosphate buffer saline (PBS, 10 mmol/L, containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na_2HPO_4 and 1.8 mmol/L KH_2PO_4, pH 7.4) served as binding buffer. And sterilized PBS was used to wash cells. Aptamers were dissolved in immobilization buffer (10 m mmol/L PBS containing 100μmol/L TCEP, pH 7.4). Backfill buffer was 2 mol/L MCH in 10 mmol/l Tris-HCl at pH 7.4. All of the solutions used in the experiments were filtered by 0.22 μm syringe filters to avoid blocking the pipe system in the instrument.

2.2. Cell line and culture

HepG2 (human liver cancer cells), Hela (human cervix cancer cells), MCF-7 (human breast cancer cells) and HL7702 (human normal liver cells) were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). T24 cell line was from American Type Culture Collection (ATCC, Wesel, Germany). HL7702 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Dingguo Company, China). HepG2, Hela, MCF-7 and T24 cells were cultured in Dulbeco's modified eagle's medium (DMEM, Dingguo Company, China), added with 10% fetal bovine serum (FBS, Dingguo Company, China), 100 U/mL

penicillin and 100 μg/mL streptomycin (Sangon Biotech Shanghai Co Ltd, China) in a humidified atmosphere of 5% CO_2 at 37℃. The cells were collected and separated from the medium after culturing for 48 hours, and then washed by sterile PBS and centrifuged at 1000 rpm for 5 minutes at least three times. The supernatant in the last centrifuge were applied in the control experiments. At last, the washed cells were suspended in PBS as sample. The cell number was determined using a blood counting chamber.

2.3. Functionalization of microcantilever array

Eight identical microcantilevers in an array are 500 μm long, 90 μm wide, 1 μm thick, and with 20 nm thick gold on one side (Micromotive GmbH, Mainz, German). When the probes interact with targets, microcantilevers bending towards the gold side is identified as bending up (positively), and bending towards the opposite side refers to bending down (negatively). To remove organics, microcantilever array was washed by piranha (98% H_2SO_4 : 30% H_2O_2, 7:3.) for 30 seconds, followed by ultraviolet ozone cleaning about 30 minutes. (Caution: piranha solution is a very strong oxidant and reacts violently with many organic materials, it must be handled with extreme care). After careful cleaning, four microcantilevers in the array were functionalized with 1μmol/L TLS11a aptamers by inserted into capillaries filled with immobilization buffer containing probes for 3 hours at room temperature to serve as sensing microcantilevers, as shown in Figure 1A. Then the whole microcantilever array was immersed in backfill buffer for 1 hour. As a result, the active sites on the remaining four microcantilevers were blocked to work as reference microcantilevers, meanwhile,

non-specific adsorptions would be prevented on the sensing microcantilevers. To obtain actual coverage information, we have investigated the morphology of the aptamers (1μmol/L) immobilized on the gold surface in binding buffer by atomic force microscope (AFM). The functionalization and backfilled procedures were identical to that of microcantilevers. AFM imaging was carried out on NanoScope Multimode 8 (Digital Instruments, Veeco, USA). The images were captured in Tapping Mode, using commercial Si_3N_4 tips (DNP-S10, 0.06 N/m, Veeco, USA). The result was shown in Figure 1B.

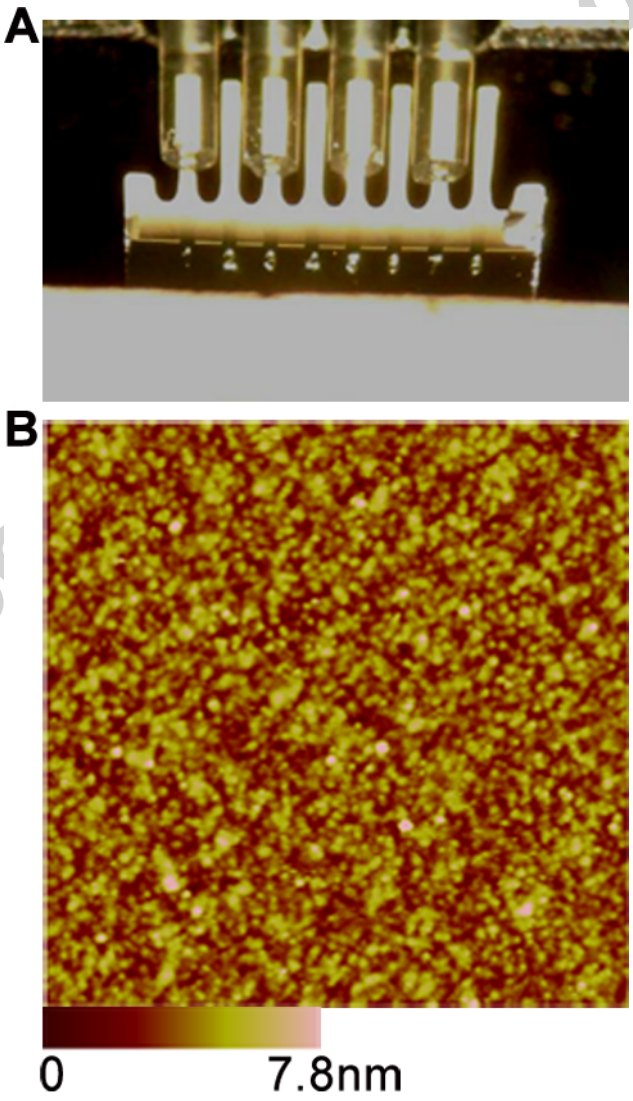


Figure 1. (A) Picture of functionalization procedure of microcantilevers by immersing into capillaries containing of 1μmol/L TLS11a aptamers. (B) AFM topography image of aptamers (1μmol/L) on gold surface (2μm×2μm).

2.3 Measurement of deflection curves

The measurement of deflection was performed on the commercial Cantisens sensor platform (Concentris GmbH, Switzerland). After functionalization by TLS11a aptamers and blocked by MCH, the microcantilever array was inserted into the measurement cell of equipment and fixed on a holder. The binding buffer flowed through the measurement cell at a low speed of 0.42μL/s, and the flow direction relative to the microcantilever array was shown in Scheme 1. The temperature of cell was maintained at 25.00±0.01℃ by an automatic calibration system in the equipment. The deflection of each microcantilever was measured *in-situ*, and the differential signal was the average deflection of sensing microcantilevers minus that of reference ones. The microcantilever array was equilibrated in the binding buffer until a stable differential signal was obtained. Then HepG2 cells at different concentrations suspended in 250μL PBS were injected into the measurement cell to interact with TLS11a aptamers immobilized on the sensing microcantilevers. The deflection of all the microcantilevers including the stable baseline and deflection induced by the interaction of aptamers and cancer cells were recorded.

3. Results and discussion

3.1 Deflection of microcantilevers induced by the interaction of aptamers and cells

As shown in Figure 2A, after recording baseline for 200 seconds, 2500 HepG2 cells in 250 μL PBS (10000 cells/mL) were injected into the measurement cell, so aptamers were exposed to HepG2 cells. The shaded area in Figure 2 represents the period of HepG2 cells flowing through the measurement cell. The injection of HepG2 cells induced the sensing microcantilevers to bend down greatly (black, Figure 2A), and there were slight negative bending of reference ones (red, Figure 2A). Negative differential deflection was obtained and shown in Figure 2B. The specific interaction between HepG2 cells and aptamers induced a change in surface stress to transduce into nanomechanical response of microcantilevers. The weak deflection of reference microcantilevers was ascribed to the inference of environment. As shown in Scheme 1, the size of HepG2 cells are far bigger than that of aptamers, causing the steric repulsive force and impelling the sensing microcantilevers to bend negatively. Besides, the negative charges on the cell membrane generated electrostatic repulsion to induce compress stress of the microcantilevers.

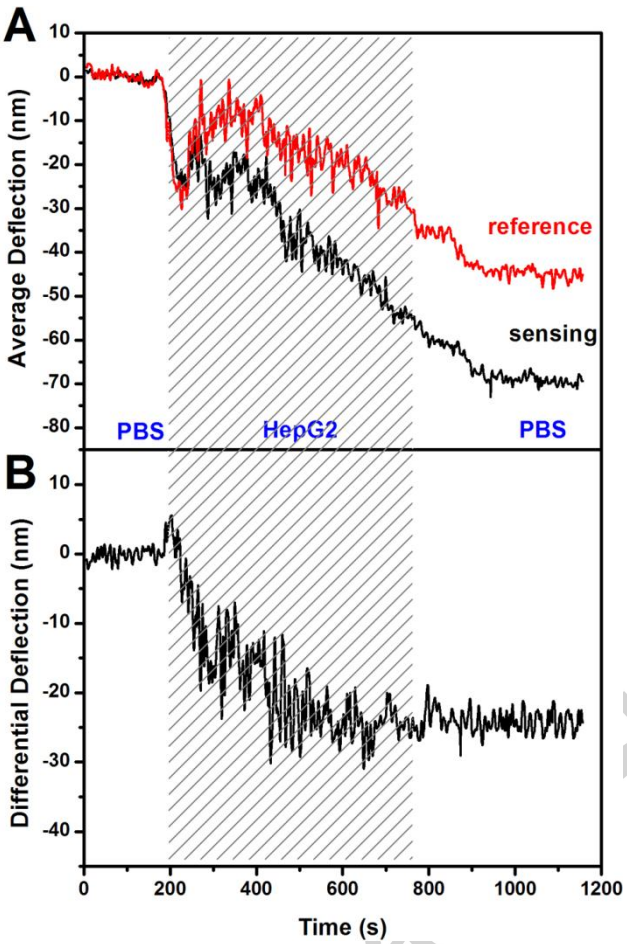


Figure 2.

Figure 2. Deflection of microcantilevers modified by aptamers by injecting of HepG2 cells (10000 cells/mL) into the measurement cell. (A) Average deflection of sensing (black) and reference (red) microcantilevers, respectively. (B) Differential deflection between sensing and reference microcantilevers. The shaded area was the period of HepG2 cells flowing through the measurement cell.

3.2 Relationship of amplitude of deflection and concentration of HepG2 cancer cells

To investigate the relationship between deflection of microcantilevers and concentration of HepG2 cells, we injected different concentration of HepG2 cells

into the measurement cell to interact with aptamers. The specific transduction of aptamers-HepG2 cells binding into a direct nanomechanical response via surface stress. As shown in Figure 3A, the higher the cell concentration, the greater the microcantilevers bending. The linear relationship between amplitude of microcantilever bending and logarithm of cell concentration ranges from 1×10^3 to 1×10^5 cells/mL, with a correlation coefficient of 0.9956. The limit of detection (LOD) of the HepG2 cancer cells by the aptamers modified on the gold side of the microcantilever array is 300 cells/mL (S/N=3). Three experiments were conducted at each concentration of cells in the same conditions. The error bar in the Figure 3C represents the standard deviation of three replicates. The comparison of microcantilever sensors with other methods for the detection of HepG2 cells was shown in Table 1. As the experiments were performed in the flow liquid system, there would be a certain number of cells can not interact with aptamers, but flow away with the buffer.

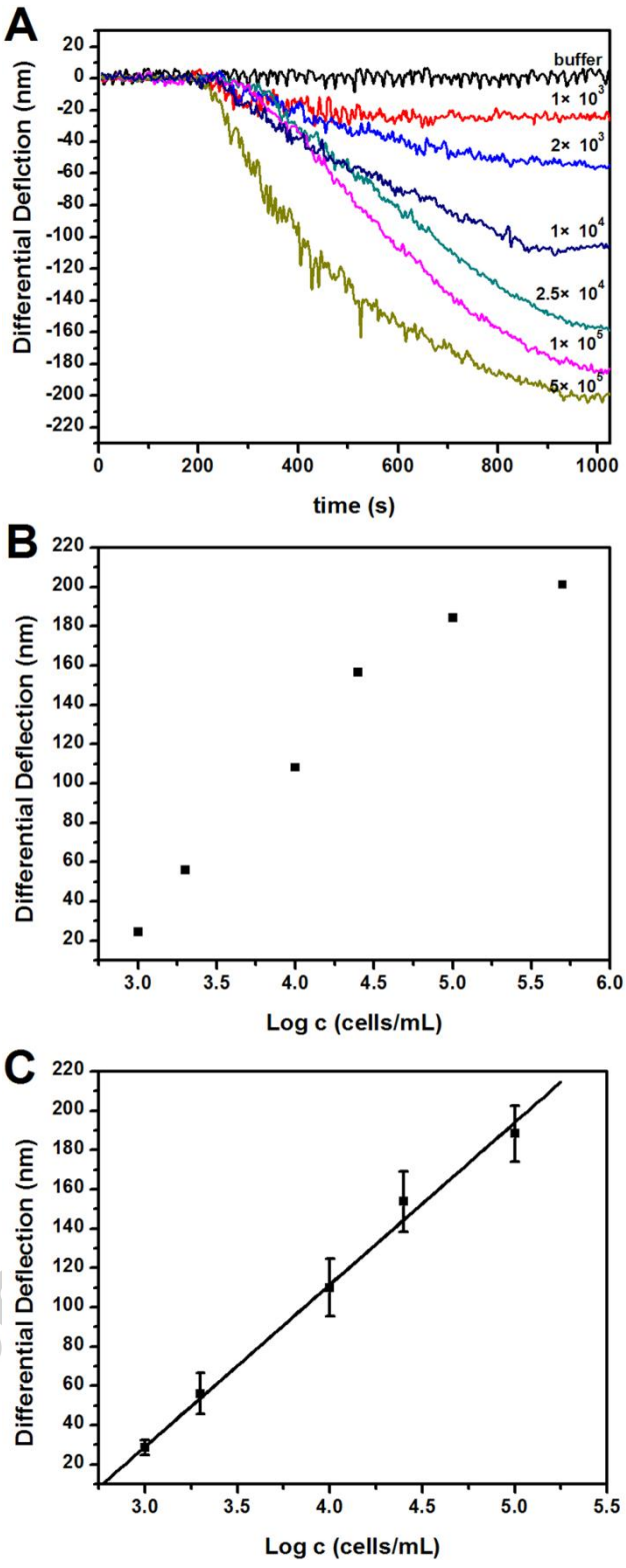


Figure 3. (A) Differential deflection curves of microcantilevers as a function of time with injection of different concentrations of HepG2 cells. (B) Differential deflection of microcantilevers (1000 s) as a function of HepG2 cells concentration in solution.

(C) Calibration curve of the linear response of differential deflection (1000 s) versus HepG2 cells concentration. The error bars represent the standard deviation of data (n=3).

Table 1 Detection sensitivity of various sensing methods for HepG2 cells

Analytical method	Immobilized receptor	Concentration range	Detection limit	References
Electrochemical method	TLS11a aptamer	1×10^2-1×10^6 cells/mL	2 cells/mL	(Kashefi-Kheyrabadi, et al. 2014)
Electrochemical method	TLS11a aptamer	1×10^2-1×10^7 cells/mL	15 cells/mL	(Sun et al. 2016)
Electrochemical method	TLS11a aptamer	1×10^2-1×10^7 cells/mL	30 cells/mL	(Sun et al. 2015)
ECL immunosensor	Antibody	3×10^2-1×10^4 cells/mL	256 cells/mL	(Liu et al. 2015)
Microcantilever assay	TLS11a aptamer	1×10^3-1×10^5 cells/mL	300 cells/mL	This work
ECL immunosensor	Antibody	4×10^2-1×10^4 cells/mL	396 cells/mL	(Wang et al. 2013)
liquid crystal microdroplet assay	β -galactose	1.0±0.01 cells/μm^2	_	(choi et al. 2015)

3.3 Specificity of the aptamers detecting HepG2 cells

The specificity of the mirocantielver array depending on the probes that modified on it. Probes modified on the microcantilever array play an important role as sensing element. To investigate the specificity of TLS11a aptamer recognizing HepG2 cells by microcantilever array sensor, control experiments were performed with cell supernatant, normal liver cells (HL7702) and other type cancer cells (Hela, T24 and MCF-7). Figure 4A has shown the differential deflection of microcantilevers when exposed to cell supernatant and other cells at concentration of 10000 cells/mL. The

differential deflection of microcantilevers at 1000s are plotted in column chart (Figure 4B). Differential deflection of supernatant injection is equal to that of buffer only, which indicates that the cells wash procedure is effective to remove the interfering substance in culture. As a result, the deflection is surely induced by the cells. The slight microcantilever deflection induced by HL7702 cells, normal liver cells, indicates that this method can differentiate between normal and cancer cells of liver. Besides, the injection of other type cancer cells, Hela, T24 and MCF-7, demonstrate that the sensor can distinguish HepG2 cells from other tumors, such as cervical, bladder and breast tumors. In a word, this aptamer based microcantilever can recognize HepG2 cells from other tumors as well as liver normal cells. The experiments of mixing HepG2 with healthy cells as well as other cancer cells were performed. The differential deflection of microcantilevers induced by different proportions of mixing cells (10000 cells/mL) were shown in Figure S1 in Supplementary Material.

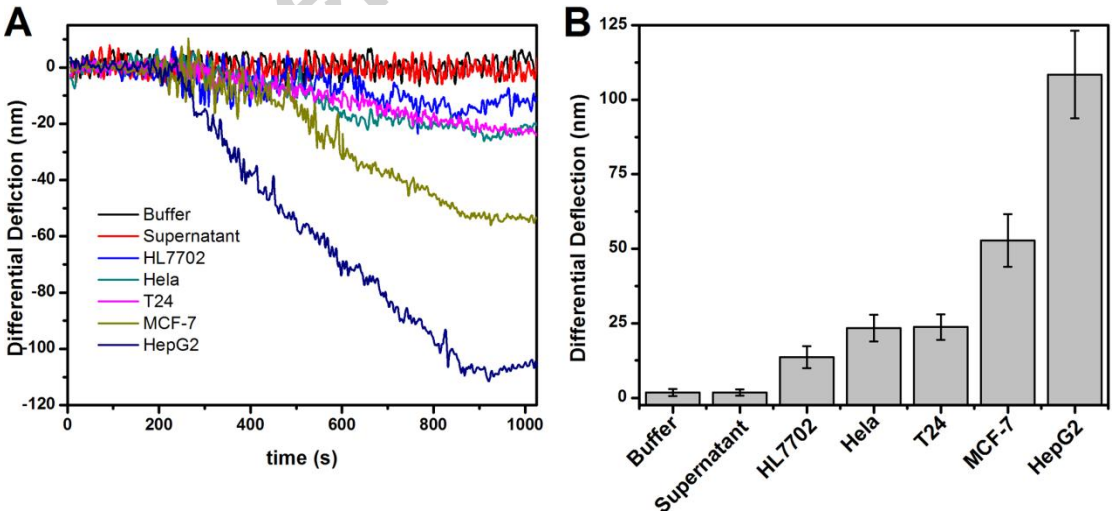


Figure 4. Control experiments to investigate the specificity of aptamer based

microcantilever array sensor. (A) Bending curves of microcantilevers exposed to cell supernatant, 10000 cells/mL HL7702, Hela, T24, MCF-7 and HepG2. (B) The deflection value of microcantilevers exposed to cell supernatant, 10000 cells/mL HL7702, Hela, T24, MCF-7 and HepG2 at 1000s. The error bars represent the standard deviation of data (n=3).

4. Conclusion

Diagnosing liver cancer cells in early stage remains a challenge in the biosensors engineering. A label-free and facile method using aptamer based microcantilever array sensor to detect liver cancer cells has been developed. TLS11a aptamers work as probes to recognize HepG2 cells. The microcantilevers deflection was linearly dependent on HepG2 cells concentration over the range from 1×10^3 to 1×10^5, with a correlation coefficient of 0.9965. The limit of detection is 300 cells/mL. The sensor exhibits good selectivity over several kinds of cancer cells as well as liver normal cells. It will be a promising tool in tumor diagnosis and therapy.

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Highlights

- A simple method of detection of liver cancer cells based on aptamer functionalized on microcantilever array sensor has been developed.
- Liver cancer cells were determined by a label-free method .
- The interaction between liver cancer cells with aptamer was screened in real time
- The method may also have potential for investigating other molecules.