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Aptamer-NASBA LOC as a Prospective Tool for Systemic Therapy of Cancer: Quantitative Detection on Signaling Molecular Profiling

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Abstract— **As the present technology of cancer treatment cannot cure the diseases, a prospective therapy, named 'systemic therapy', brings forth a new trend in cancer treatment. The aptamer-NASBA-based lab-on-a-chip (LOC) for systemic therapy was designed, fabricated and tested as an ultra-sensitive tool to monitor signaling molecular profiling in serum samples. The chip is divided into four parallel functional areas, corresponding to four groups of signaling molecules (i.e. hormones, neurotransmitters, cytokines and tumor biomarkers). The results can help doctors fully understand the body of patients. The chip is modeled on a 384-well microplate, which is completely compatible with common microplate readers in a biological laboratory. It can distinguish 24 signaling molecules in the same blood sample quantitatively and simultaneously. The chip was made of PDMS and silicon with a deposited gold layer, which was coated by aptamers before bonding; then, the LOC was operated by external valves and a vacuum pump. Its performance was demonstrated by detecting the presence of a synthetic peptide, GnRH (gonadotropin-releasing hormone) in artificial samples. The results indicated that the LOC has the potential to quantify traces of biomarkers even at subfemtomolar levels. Compared with our previous immuno-NASBA LOC, the aptamer-NASBA LOC showed an increased sensitivity and better repeatability.**

I. Introduction

Cancer is a worldwide threat to mankind. Although many diagnosis methods and therapy are continually invented to treat the diseases, it still cannot be cured. Doctors found that using only one kind of method to treat cancer is not enough. Therefore, systemic therapy becomes a new trend in cancer treatment.[1,2] Since the majority of anti-cancer drugs or treatments have side effects, if they irreversibly damage the cancer patients' immune system or metabolic system, the therapy results will turn negative. The critical of systemic therapy is to maintain the balance within the system of human body during anti-cancer treatment, see Fig 1. Based on the patient's physical condition, doctors can choose different types of drugs or treatments to regulate the balance of the patient's body so as to establish the maximum inhibition force against tumors. This strategy has the potential to turn cancer into a chronic disease, which can make the patients survive for many years with tumors inside. For example, the study of using arsenic trioxide therapy in patients with acute promyelocytic leukemia (APL) indicates the promising prospect of the strategy.[3,4] However, the fragile balance in systemic therapy is not easy to maintain, even for experienced doctors. The demand of systemic therapy calls for a precise method or tool to simplify and standardize the assessing process of the different systems in the patients' body. In the human body, three major systems cooperate with each other, including the immune system, the nervous system and the basic metabolic system. When cancer cells appear, the relationship of all systems in the body will be bothered by the 'invader'. Since signaling molecules of each system can indicate each status, the signaling molecular profiling is a good tool to disclose the condition inside the body.

In this study, a Lab-on-a-chip (LOC) based on the aptamer-NASBA assay (aptamer-nucleic acid sequence-based amplification) was designed, fabricated and verified to assess the systems within patients' body. Being a disposable chip, it was made by common materials and free of any valve or sensor. The dimensions of the LOC device and the position of each chamber are exactly matched with a 384-well microtiter plate. The LOC device was based on a new strategy of immuno-NASBA assay, which is named as 'Aptamer-NASBA', see Fig 2. 1st antibodies are all replaced by aptamer DNAs, which has good ability to recognize a specific target biomarker.[5] To DNA aptamers, one of its advantages is easy to be synthesized and modified. The aptamers were connected with a –SH group that can couple to the gold atom chemically.[6] Since Au atoms will be sputtered on the surfaces of every reaction chambers in the chip during the fabrication process, aptamers could easily bind on the chambers' surface. The 2nd antibodies and barcode DNA tags was connected by the biotin-streptavidin-biotin bridge as described as before.[7] As a result, the new protocol could increase the specificity of the measurement. The preliminary tests have verified the purpose. The LOC device here is simple, but it represents a trend of development, which the LOC devices could be designed as an explorative tool for scientists in the front line of biomedical research.

II. Material and Methods

A. Chip design

The previous prototype of 6-channel immuno-NASBA chip was designed according to 96-well microtiter plate.

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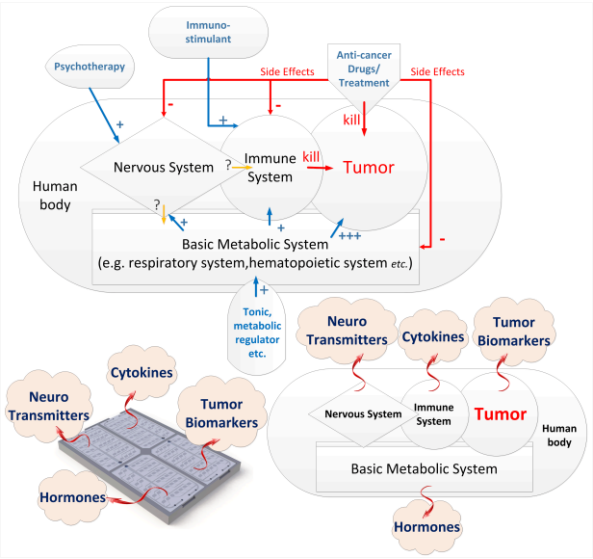

Figure 1 Aptamer-NASBA chip for Signal Molecular profiling in the systemic therapy for cancer patients. The aptamer-NASBA chip was designed specifically for the systemic therapy of cancer patients. It can quantify hormones, neurotransmitters, cytokines and tumor biomarkers from one blood sample simultaneously, which provide reference data for the comprehensive assessment of the balance inside human body.

Therefore, the LOC can be read by a common microplate reader. Besides the plate mentioned above, there are 384-well and 1536-well microtiter plates for the instrument. Here, the 384-well one was chosen as the model, which could be still compatible with the same microplate reader. Derived from the previous chip, the linear dimension is reduced by 2 times, and then the chip will turns into 1/4 of its original size. After minor modification, 4 reduced chips pieced together on a specific underplate to form a 24-channel device of the original size. Thus, the chamber density increased 4 times. Relevantly, the cassette of the aptamer-NASBA chip has to be redesigned. See Fig 3. The 4 pieces of small chips were used to detect four groups of signaling molecules, which are hormones, neurotransmitters, cytokines and tumor biomarkers. Although 4 pieces of small chips are separated on the underplate, their inlets and outlets could be connected by the channels in the external cassette to realize synchronous operations. Totally, there are 2 air-bleed ports, 2 inlets and one outlet on the top of cassette, see Fig 3. The new LOC device can quantify 24 kinds of biomarkers at the same time. In addition, since the linear dimensions shrink to 50% of the origin, the volume of each reaction chamber is actually reduced to 1/8, and then the reagent consumption of each immuno-NASBA test could be decreased sharply.

B. *Device Fabrication*

The chip was fabricated using MEMS fabrication technologies. The brief process flow is diagrammed in Fig 4. Bonding of the two layers is also shown in the same figure. The bottom layer was made from a silicon wafer and using standard fabrication processes. A 500μm depth of microfluidic chamber was etched by RIE at the first step of silicon wafer process.[8,9] Then a 50 angstroms Ti was sputtered onto the silicon wafer as an adhesive layer for the

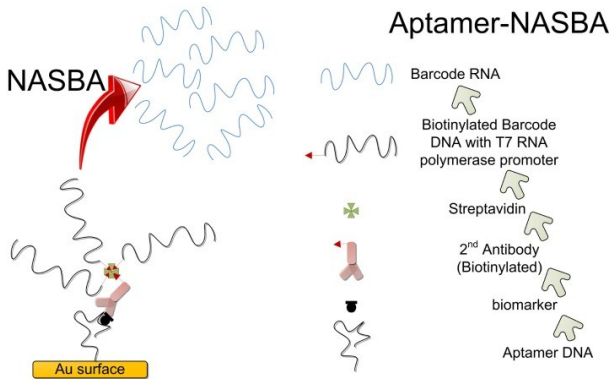

Figure 2 Amplification mechanism of Aptamer-NASBA assay. Aptamer DNAs replace 1st antibodies in the sandwich immunoassay. Streptavidin has 3 additional binding sites to biotin, so the streptavidin on the antibody could catch biotinylated barcode DNAs which contained a T7 RNA polymerase promoter. The barcode DNA plays a role as a bridge to connect immunoassay and NASBA amplification.

gold sputtering.[8] Photoresist was deposited and patterned to expose the chamber. Au was sputtered and a lift-off process was done to form the gold pads. For the PDMS cover, it also started with a silicon wafer. PDMS was spun onto a silicon wafer and several plastic sticks with open holes were used to form the inlet and outlet. Then the prepared PDMS cover was taken off from the silicon wafer. Before bonding the cover on the chambers of the chip, the reagents of specific aptamer DNAs and standard controls were transferred into the uncovered chambers by robots or pipettes manually. Then a room-temperature plasma was applied for the bonding of these PDMS cover and microfluidic chamber wafer. The cassette was fabricated by common methods described before.[10-14]

C. *Protocol of Aptamer-NASBA assay*

The aptamer-NASBA assay can be regarded as a combination of a sandwich immunoassay and NASBA assay. In the chip, all the immunoassays took place on the surface of gold inside the reaction chamber. All reagents are prepared from DEPC-treated water. First, the surface of chamber was coated by aptamer DNA. Then the blocking buffer solution blocked the residual reaction sites. After that, the chambers were interacted with target biomarkers in samples or calibration solutions. Unbound molecules had been washed away by Phosphate Buffered Saline Tween-20 (PBST) and PBS. Then the chambers were incubated with the biotinylated antibodies. After wash, the immune complex interacted with streptavidin, which can bind the biotin on the antibody. Since a streptavidin has 3 additional binding sites to biotin, the streptavidin on the antibody could catch biotinylated barcode DNAs which contained a T7 RNA polymerase promoter. Finally, NASBA system was introduced and barcode DNAs were amplified in the cycles of NASBA assay.

To test the sensitivity and specificity of the aptamer-NASBA LOC, a well-studied short peptide, Gonadotropin-releasing hormone (GnRH), was selected as the model to measure. The GnRH peptide was obtained from Boster™ Bio-Engineering Ltd. The 3'SH-DNA aptamer was synthesized by Shanghai Generay™ Biotech Co., Ltd

according to the published sequence of anti- GnRH aptamer.[15] The antiserum of GnRH was obtained from immune mouse via traditional methods. The antibody was biotinylated by Biotin labeling kit-NH2 of Dojindo's™ Company and purified before use by the AllPrep™ protein Procedure kit. Streptavidin was obtained from ProSpec™ Company. Barcode DNA was obtained from the PreTect™ HPV-Proofer kit (Norchip®). The amplification product of positive controls contained a large number of DNA tags with the T7 RNA polymerase promoter, which were purified and biotinylated by the Biotin 3' End DNA labeling kit. All the NASBA assays are carried out as described in the user manual of the PreTect™ HPV-Proofer kit (Norchip®).

The standard sample of GnRH was diluted in every 3 times by DEPC-treated water and used to the pre-treatment of aptamer-NASBA chips. All the NASBA assays were incubated at 41℃ in Biotek® Synergy® 2. The Data was analyzed by Microsoft Excel® and OriginPro®.

D. Operation of Aptamer-NASBA LOC device

Driven by a vacuum pump, all the different reagents can flow accurately into the paralleled chambers and react simultaneously via the external valve system as described as before.[7] The most complicated procedure has been greatly simplified in high-throughput biochemical assays, during which the reagent was added into the reaction wells in parallel.

III. RESULTS AND DISCUSSION

Calibration curve of aptamer-NASBA assay are shown in Fig. 5, inset A.

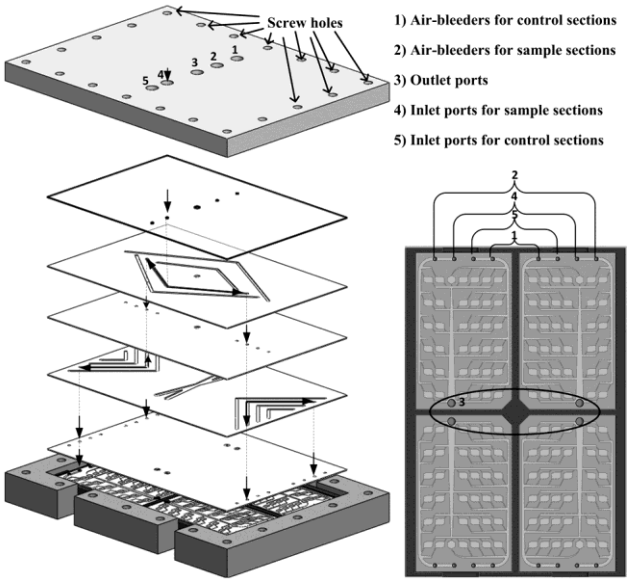

Figure 3 The channel networks inside the Aptamer-NASBA LOC. Except the channels inside aptamer-NASBA chip,.the channels in the cassette connected different inlet ports and outlet ports of all chips. A typical fluid pathways are shown in the dashed lines. For rapidly and inexpensively prototyping, the casstte was designed with a series of laminated structure. When the chips and cassette are assembled correctly and fixed by screws, there are only 5 exchange ports left on the top of the cassette, including 2 air-bleeder, 2 inlet and one outlet. The four inner chips can be operated as one microfluidics device.

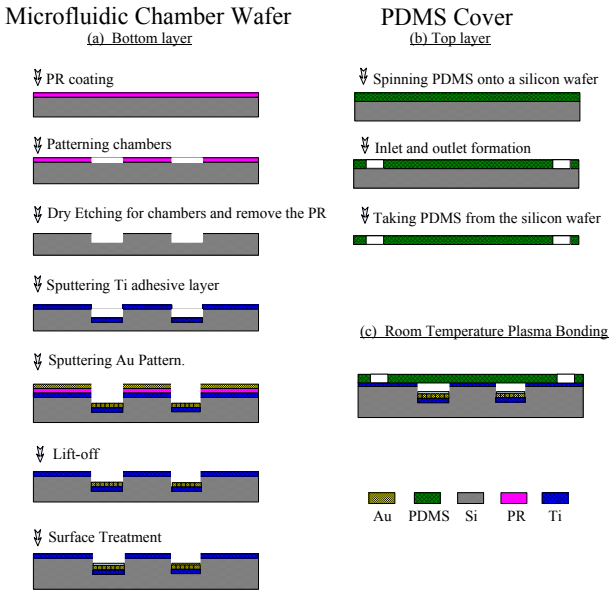

Figure 4 Microfabrication of Aptamer-NASBA chip

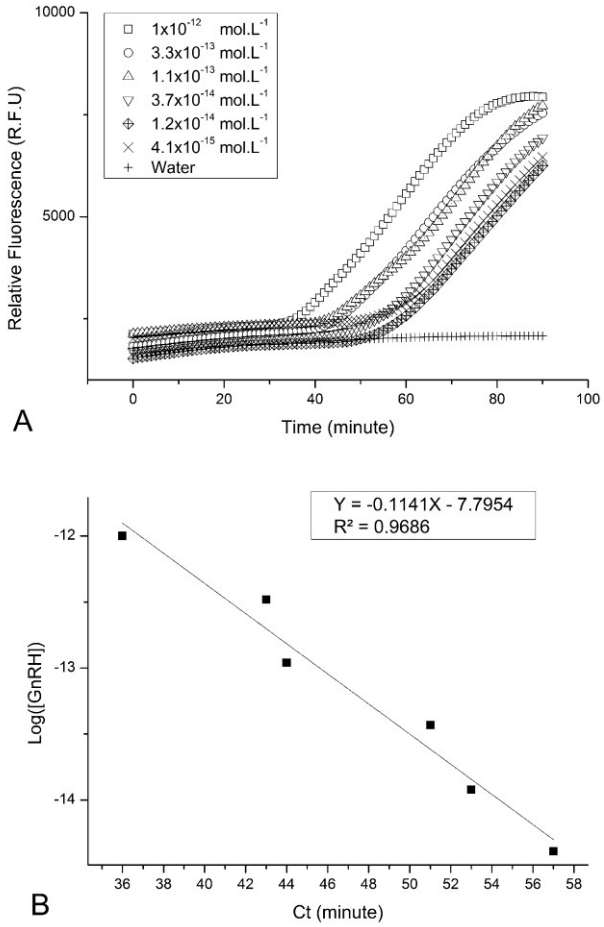

Figure 5 Determination of Quantitative detection assays on Aptamer-NASBA chips using GnRH.

The plot of kinetic curves using the series of gradient standard solutions were shown in inset A; all the solutions presented classical amplification curves except the blank control. the Ct value of each kinetic curve was calculated using 'moving average threshold' as the reference; inset B shows the result of linear regression analysis on these Ct values and relative logarithms of their contact concentration.

When the concentrations of GnRH were between 4.1×10^{-15} mol·L^{-1} and 1×10^{-12} mol·L^{-1}, the aptamer-NASBA assays presented very standard amplification curves; the negative control solution (water) had no positive signal. Actually, the GnRH solution at 2×10^{-16} mol·L^{-1} still presented a weak positive signal, which Ct value was nearly 1.5 hours. In order to quantify amplification signals, C_t, the threshold time is defined artificially as the concept of cycle threshold (C_t) value in the real-time fluorescence quantitative PCR assay, which presents the time required for the fluorescent signal to exceed background noise level. But the signals of different amplification assays showed different noise levels, so the artificial threshold for C_t values lacked a uniform standard. Through trial and error method, we found a good way to specify the moving average threshold, which is defined as 105% of the average fluorescence value in 5 minutes before each point:

$$Moving\ Average\ threshold\ (n) = 105\% \times \frac{\sum_{n-5}^{n-1} Fluoresence\ value(i)}{5}$$

When the point goes beyond its moving average threshold for the first time, its amplification time will be recorded as C_t, which was plotted into Fig 5. Inset (B). Finally, we gained a good linear curve about Ct and Log [GnRH concentration] in Fig 5. Inset B, which indicated that Aptamer-NASBA assays can be performed in a common microplate reader, but the correlation coefficient of data was not perfect. Perhaps, the present aptamer-NASBA LOC have reached the utmost ability of a common microplate reader. Further development of the chip requires improving the precision of fluorescent readers.

Obviously, the strategy of replacing 1st antibody by aptamer DNA in the chip was successful. The leading advantage may be not the improvement of affinity, but the higher selectivity of inner surface in chambers. From the previous analysis of the immuno-NASBA chip, we confirm that the problem of non-specific absorption obstructs the further progress of immuno-NASBA chip. Here, the gold layer in the chamber can absorb 3'-SH-modified aptamer DNAs by chemical reactions, not by physical adsorption. Thus, the high selective surface reduces non-specific absorption of proteins naturally.

From the preliminary test of aptamer-NASBA LOC, we confirmed that signaling molecular profiling could be investigated by the 24-channel LOC. Although some high-throughput microarray chips for proteins have been developed, redundant data may be not helpful to the judgment of doctors. Since most signaling molecules have been studied intensively, the data of these molecules are more valuable than other proteins. For the patients with cancer or chronic diseases, variation laws of signaling molecules in one patient are easier to be summarized than those in all people. The LOC device for signaling molecular profiling is suited to be custom-built for each chronic patient, which would play an important role in the long-term therapy. However, this LOC device is just a prototype at the present. Since it is still far from clinical application, further development of the aptamer-NASBA LOC requires the assistance knowledge of applied statistics and medical science.

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