

Metal oxide nanowires for early disease diagnosis

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Abstract

Metal oxide nanowires recently show their great promise for analyzing cells or intracellular components due to the nanowires feature of a stealth effect against cell surfaces [1]. Here we showed a novel usability of the nanowires to achieve an efficient capture of extracellular vesicles, which surfaces is similar to the cell surfaces, and early diagnosis based on the vesicles microRNAs. Our methodology could achieve extracellular vesicles capture in urine over 95% within 40 min, and detect cancer- and type 2 diabetes-related microRNAs from urine samples.

1. Introduction

MicroRNAs extraction and analysis from body fluids is central to achieving early disease diagnosis, however, a methodology to perform microRNAs extraction and analysis is still difficult and unattainable situation due to ease of microRNAs degradation in body fluids. For demonstration of early diagnosis based on microRNAs analysis in body fluids, recently, researchers focus on extracellular vesicles. Conventionally, extracellular vesicles are recovered using ultracentrifugation or commercially available pretreatment kits, however, low recovery rate (less than 20%) and long recovery time (over 5 h) prevent researchers from demonstrating early diagnosis based on extracellular vesicles in body fluids. For early diagnosis via microRNAs detection in extracellular vesicles, here, we demonstrate a methodology of vesicles capture using nanowires and microRNAs extraction from the captured vesicles.

2. Experimental

For capturing extracellular vesicles, we decided to use metal oxide nanowires. So far, we integrated SnO₂ nanowires into fused silica microchannels by using photolithography process and vapor liquid solid technique (VLS) [2-4]. The special feature of the nanowire structure is the controllability of pore size (20-400 nm) by varying the number of nanowire growth as a cycle, consequently we obtained the hyper-branched of nanowire networks structure. Then we could separate DNA molecules within 13 s under the applied DC electric field 500 V/cm. Moreover, we demonstrated that highly dense nanowires served as a molecular filter could perform high throughput filtration of DNA molecules from a mixture of λ DNA and T4 DNA within 1 s [5]. As presented above, the surprising biomolecules separation and filtration was highlighted by using the outstanding properties of the nanowire structures integrated with the microfluidic channel.

In this paper, we prepared ZnO nanowires (100 nm in diameter and 2-3 μ m in length) on a desired area. Firstly, we made ZnO seed layer on glass substrates and baking 300 °C for 10 minutes. ZnO seed layer was recrystallized by heating at 550 °C for 1 hour and to be scaffold of ZnO nanowire growth. We patterned growing ZnO nanowires on a desired position by using lithography techniques, and then, dipped the patterned substrate in nanowire growing solution at 95 °C for 12 hours. For cleaning, the substrate was exposed with dimethylformamide and ultrasonication for 1 minutes.

Since surfactant for extraction process has a risk of peeling off nanowires from substrates, we anchored nanowires into PDMS, intentionally. For anchoring process, we pored uncured-PDMS to grown nanowires, cured PDMS, and peeled off the PDMS with nanowires; we could anchor nanowires into PDMS through this process. And then, we grew up ZnO nanowires self-assembly. SEM images showed that the nanowires were robustly anchored into PDMS. We bonded the nanowires-embedded PDMS

to herringbone-structured PDMS.

3. Results and discussion

We performed detecting fluorescent intensity with respect to extracellular vesicles concentration by staining membrane protein of the vesicles. After Alexa Fluor 488 conjugated CD63 antibody were applied to captured extracellular vesicles, we performed confocal microscope observation. The vesicles model sample was prepared by cell culture supernatant of HepG2 cultured for 4 days. Firstly, we introduced 1.5 μL of the prepared vesicles solution to nanowires using aspirator and incubated for 3 hours at ambient temperature. And then, we performed blocking process by incubating 1% BSA-PBS for 15 minutes at ambient temperature. Finally, we introduced antibody solution and observed fluorescent intensity of the nanowire area by confocal microscope. Comparison of fluorescence intensity using nanowires with that using conventional method showed that the nanowires could capture extracellular vesicles, efficiently. And also, the nanowires could detect the vesicles membrane proteins in response to the vesicles concentration. Surprisingly, nanowires take about 5 hours for whole detection process, which is much faster than conventional methods taking over 24 hours. This is because small structure size is suitable for the vesicles diffusion. From these results, we concluded that ZnO nanowires have a potential to capture extracellular vesicles.

Next, we introduced 1 mL of urine samples from healthy donor to demonstrate the vesicles capture onto nanowires. Since the ZnO nanowires has positively-charged surface and 200-nm mean spacings between nanowires, the vesicles with negatively-charged surface and 120-nm mean diameters were efficiently captured. Capturing rate, which was calculated from the vesicles concentrations before and after introduction into nanowires, was estimated to be over 95%. Next, we showed microRNAs extraction from the captured vesicles onto nanowires, and analyzed microRNAs expression using microarray. Scatter plot and histogram revealed that microRNAs expression level using nanowires is much higher than that using ultracentrifugation. Finally, we applied 1 mL of urine from prostate cancer, diabetes, and healthy donor into nanowires. MicroRNAs from the captured vesicles in them showed different expression levels. Venn diagrams highlighted that nanowires could detect prostate cancer- and type 2 diabetes-related microRNAs from 1 mL urine samples, which were previously found only in serum.

4. Conclusions

Since we could find disease related microRNAs from an early stage prostate cancer patient (stage T1C), our developed approach should allow researchers to find disease related microRNAs in extracellular vesicles for future medical applications.

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References

- [1] Rahong, S.; Yasui, T.; Kaji, N.; Baba, Y., Recent Developments in Nanowires for Bio-Applications from Molecular to Cellular Levels. *Lab Chip* **2016**, *16*, 1126-38.
- [2] Yasui, T.; Rahong, S.; Motoyama, K.; Yanagida, T.; Wu, Q.; Kaji, N.; Kanai, M.; Doi, K.; Nagashima, K.; Tokeshi, M., *et al.*, DNA Manipulation and Separation in Sublithographic-Scale Nanowire Array. *ACS Nano* **2013**, *7*, 3029-3035.
- [3] Rahong, S.; Yasui, T.; Yanagida, T.; Nagashima, K.; Kanai, M.; Klamchuen, A.; Meng, G.; He, Y.; Zhuge, F.; Kaji, N., *et al.*, Ultrafast and Wide Range Analysis of DNA Molecules Using Rigid Network Structure of Solid Nanowires. *Sci. Rep.* **2014**, *4*, 5252-5259.
- [4] Rahong, S.; Yasui, T.; Yanagida, T.; Nagashima, K.; Kanai, M.; Meng, G.; He, Y.; Zhuge, F. W.; Kaji, N.; Kawai, T., *et al.*, Three-Dimensional Nanowire Structures for Ultra-Fast Separation of DNA, Protein and Rna Molecules. *Sci. Rep.* **2015**, *5*, 10584.
- [5] Rahong, S.; Yasui, T.; Yanagida, T.; Nagashima, K.; Kanai, M.; Meng, G.; He, Y.; Zhuge, F.; Kaji, N.; Kawai, T., *et al.*, Self-Assembled Nanowire Arrays as Three-Dimensional Nanopores for Filtration of DNA Molecules. *Anal. Sci.* **2015**, *31*, 153-157.