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主　論　文　の　要　旨

論文題目　Oxide Nanowire Microfluidics for the Analysis of Extracellular Vesicles
(酸化物ナノワイヤマイクロ流体による細胞外小胞の分析)

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論　文　内　容　の　要　旨

Extracellular vesicles (EVs) have shown promising features as biomarkers for early cancer diagnoses and prognoses. The ability to analyze EVs could strongly impact cancer-related research studies. The outer layer of cancer cell-derived EVs consists of organotropic metastasis-induced membrane proteins and specifically enriched proteoglycans, and these molecular compositions determine EV surface charge. Although many efforts have been devoted to investigating the correlation between EV subsets obtained through density-, size-, and immunoaffinity-based captures and expressed membrane proteins, understanding the correlation between EV subsets obtained through surface charge-based capture for effective downstream analysis of EVs is lacking. Here, we propose a methodology to isolate EV subsets obtained

through surface charge-based capture using oxide nanowire microfluidic devices. First, we fabricate oxide nanowires with different surface chemical properties providing diverse surface charges based on isoelectric points of the materials. And, with the ZnO nanowire device, whose use does not require any purification and concentration processes, we demonstrated the correlation between negatively charged EV subsets and expressed membrane proteins of colon cancer cells for cancer detection. We have shown the potential of ZnO nanowires to capture EVs that is rapid and simple compared to conventional methods. Nevertheless, key parameters including crystallinity and morphology of ZnO nanowires to maximize the EVs capture performance had not yet been investigated. Thus, we subsequently present a comprehensive investigation that highlights the correlation of ZnO nanowire physicochemical properties to the capture performance of breast cancer cell-derived EVs. Finally, we adapted our findings in tunable nanowire properties with highly efficient label-free capturing of EVs for other two alternative applications rather than cancer detection, including an EV classification device for specific sorting of EV subpopulations based on surface charge and a nanowire-based blood purification model for eliminating of cancer-derived EVs to suppress the progression of breast cancer via EV-induced metastasis inhibition.

Extracellular vesicles (EVs) are spherically structured, cell-derived membrane vesicles and they play a crucial role in cell-to-cell communication. These micro- and nano-sized vesicles carry molecular messages, including proteins, lipids, DNAs, RNAs, and metabolites. The molecules reflect the physiological and pathological conditions of parent cells and provide useful information for clinical applications including early detection, diagnosis, and prognosis of diseases as well as the utilization in monitoring surgery and treatment responses. EVs have been identified in many different biological fluids like whole blood, plasma, serum, urine, saliva, breast milk, *etc*. Among these fluids, urine stands out as a non-invasive sample fluid that is easy and inexpensive to obtain and can be collected over time to monitor changes. The urinary extracellular vesicles (UEVs) were first isolated by Wiggins *et al.*. Consequently, the work of Pisitkun *et al.* had a major impact of raising interest in UEVs by demonstrating proteomic analysis of urinary exosomes associated with renal diseases (kidney disorders). From that, the potential for clinical applications of UEVs became established. Recent reports have shown the presence of such molecules as RNAs, microRNAs, and proteins, which can be used as potentially as biomarkers in renal diseases (*e.g.*, diabetic kidney disease), urological malignancies (*e.g.*, prostate, bladder and kidney cancers), non-urological

malignancies (*e.g.*, lung, liver, pancreas cancers), and arterial hypertension in UEVs.

However, there is a concern in application of UEVs to analysis due to the great variability among isolation techniques that have been used; this has resulted in it being difficult to apply the most effective protocol that is convenient and universal in a clinical setting. Thus, the lack of a standardized protocol remains a significant limitation in the field of EV analysis, especially, when all analytical validations of the assay have to be considered including accuracy, precision, specificity, limits of detection and quantification. Comprehensive standardized guidelines for isolation of EVs and over all analytical processes have been provided that suggest all experimental details as well as critical evaluations in the EV analysis procedures. Some researchers have discussed the development of a variety of EV isolation and interrogation approaches, and highlighted potentials and limitations. Others have addressed the whole range of methods that are used for human EV isolation and characterization.

The overall workflow procedures for studying EVs consist of three main steps: pretreatment, isolation, and downstream analysis. The key to successful determination of EVs mostly relies on the sample preparation processes of the pre-analysis and isolation since they directly determine the qualitative and quantitative

information of EVs from that sample. Before carrying out any experiment, consideration must be given to what target information is expected to be obtained from UEVs in the downstream analysis, *e.g.* the morphology, variety of components, and proteomic and genomic information. The use of force in some techniques may lead to break-up or deformation of the EV structure. There are many techniques that have been developed for EV isolation. In this literature review, we focus on four principles that rely on specific properties of the EVs including size and density, solubility, and surface affinity, and that can be realized in an integrated microfluidic system. Each technique has its own key potential and limitations. A number of recent studies have tried to combine two or more different techniques together to improve isolated matter purity, yield and richness of the biomolecule types, *e.g.* ultra-centrifugation with ultra-filtration (UC-UF), ultra-centrifugation with size-exclusion chromatography (UC-SEC), and ultra-filtration with size-exclusion chromatography (UF-SEC). Still, all these techniques have intensive labor requirements.

Microfluidics techniques are integrated platform that have been developed within the lab-on-a-chip concept promoting high robustness, sensitivity, and selectivity of EV analysis. As noted earlier, the ideal analysis methodology is to

detect EVs directly without any extensive pretreatment steps from urine, and numerous studies have tried to find out whether realizing this is possible.

In 2017, we proposed a novel approach to UEV isolation microfluidics that enables EV collections at high efficiency and by in situ extractions of diverse miRNA molecules that significantly exceed the number of items being extracted by the conventional ultracentrifugation method and a commercially available kit.[37] This methodology moves researchers toward the goal of miRNA-based noninvasive and simple early disease diagnoses and timely medical checkups from urine. Although nanowires have shown great potentials for analyzing properties of cells or intracellular components, none of the previous studies have dealt with applications to collect EVs. We discovered utilization of distinct properties of nanowire, including the relatively small size as EV molecule, the large surface-to-volume ratio for high adsorption area, and the variations of surface charge, promoting highly sensitive interaction and the reduction of processing time and sample volume. Furthermore, oxide nanowires provide high flexibility in on demand application by tailoring physical property and surface chemistry in the fabrication process.

Herein, in this research, various oxide nanowire devices were fabricated for analysis of EVs and its contents, namely membrane protein markers and EV-

encapsulated miRNAs. This thesis provides the series of nanowire-based device development in order to improve performance and maximize the potentials of nanowire-based devices by manipulating surface physicochemical properties of oxide nanowires. In chapter 2, three types of metal oxide nanowires with distinct surface chemistry were used to evaluate and identify the most suitable candidate material for label-free capturing of EVs. In addition, this device was applied to realize membrane protein profiling of EVs obtained via charge-based capture for detection of colon cancer within a single device. Chapter 3 provides a comprehensive investigation of ZnO nanowire in terms of physical properties in EV capture performance. Various morphologies and crystallinities of nanowires were tailored using an ammonia-assisted hydrothermal method, then the correlation between device efficacy and physicochemical property of nanowires from different growth conditions were observed. Our discoveries in chapter 2 and 3 were subsequently utilized to promote specific adsorption of EVs on nanowires based on crystallinity. Moreover, to achieve releasability of the captured molecules for further downstream analysis, we additionally apply an elution essay promoting specific desorption of EVs based on ionic strength and surface charge. This classification concept to sort EV subpopulations is described in Chapter 4, we provide the analysis of distinct protein

membrane markers and EV-encapsulated miRNAs profiling in each EV fraction. Last but not least, in Chapter 5, an alternative utilization of the nanowire-based methodology in cancer therapeutic application, as a model of blood purification device, were proposed for the first time. Since the device application had been mostly focusing on cancer diagnosis and monitoring in previous studies. Growing of evidences indicated that EVs involve in metastasis and proliferation of cancers, but, thus far, how their elimination from biofluid in order to inhibit the abnormal growth of cell has been poorly understood. Here, we described an EV-elimination device using nanowires which can efficiently remove EV-miRNAs from cancer cell supernatant for inhibiting abnormal growth in recipient cell without toxicity to cell. Finally, Chapter 6 is dedicated to some concluding remarks and future perspectives.