



In situ single step detection of exosome microRNA using molecular beacon



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ABSTRACT

In situ single step detection of microRNAs (miRNA) in a whole exosome has been developed as a novel diagnosis method that can be utilized for various diseases. Exosomes are small extracellular vesicles that contain biomarker miRNAs produced from their originating cells and are known to travel through the circulatory system. This makes exosomal miRNAs from the body fluids an attractive biomarker that can lead to a paradigm shift in the diagnosis of disease. However, current techniques, including real-time PCR analysis, are time-consuming and laborious, making them unsuitable for exosomal miRNA detection for diagnosis. Thus, the development of alternative methods is necessary. Herein, we have demonstrated that exosomal miRNAs can be detected directly using a nano-sized fluorescent oligonucleotide probe, molecular beacon. MiRNA-21 in exosomes from breast cancer cells were detected successfully by molecular beacons in a quantitative manner. Permeabilization by streptolysin O treatment further enhanced the delivery of molecular beacons into exosomes, giving significantly increased signals from target miRNAs. In addition, we selectively detected cancer cell-derived exosomal miRNA-21 among heterogeneous exosome mixtures and in human serum. The method developed in the article is simple, fast, and sensitive, so it will offer great opportunities for the high-throughput diagnosis and prognosis of diseases.

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1. Introduction

Exosomes are small extracellular vesicles with sizes of 30–100 nm that are produced and secreted by a wide variety of different cells in the body [1–4]. They are known to enter the circulatory system, and, therefore, they are found in most of body fluids, including blood, urine, semen, saliva, bile, and breast milk [5–7]. In the past, it has been recognized that exosomes have a key role in the elimination of unnecessary biomolecules, such as proteins, from cells. However, current views on exosomes are drastically different, and their physiological and pathological significance has been increasingly highlighted. They are now thought of as extracellular organelles that contain important cellular biomolecules and that mediate intercellular communications among cells and tissues in the human body by conveying information to

distant tissues, thereby regulating, for example, the proliferation, differentiation, death, and immune response of cells [5,6,8–10]. Similar to cells, exosomes are encompassed with lipid bilayer and composed of proteins, peptides, lipids, carbohydrates, and nucleic acids derived from their originating cells [11]. Thus, detection of exosomal biomolecules from the body fluids offers great opportunities for the diagnosis of diseases.

MicroRNAs (miRNAs) are small non-coding RNAs of 19–25 nucleotides in length, and they have key roles in regulating cellular processes, such as development, differentiation, proliferation, and apoptosis in the human body [12]. There has been extensive research on utilizing miRNAs as biomarkers for the identification of diseases, because miRNAs reflect an important pathogenic process [13–21]. For example, the high expression level of miRNA-21 (miR-21) is known to be associated with low survival rate and poor therapeutic outcome of colon adenocarcinoma [22]. Recent studies have shown that miRNAs also are present in circulating exosomes and transferred to other cells, altering the function of those target cells [1,23]. These findings provide evidence that exosomal miRNAs can be used for the early prediction of disease signature in the body

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and, in this context, specific exosomal miRNAs have proven to be important biomarkers of diseases [24]. Different miRNAs are involved in and can be used as diagnostic biomarkers of various cancers, i.e., lung, breast, pancreatic, colon, metastatic gastric, prostate, ovarian, and esophageal cancers [23,25–31]. In most cases, the current methods for the detection of miRNA from the exosomes require a series of procedures, even after isolation of the exosomes, ranging from sample treatment to detection, such as exosome lysis, miRNA isolation, cDNA synthesis, and real-time PCR analysis. In addition, due to its small size, mature miRNA must be elongated with a specific oligonucleotide, such as a poly (A) tail, before the cDNA synthesis step to ensure that the size is large enough to be detected in the PCR product. Thus, it is essential to have an easy, simple, accurate, fast, and inexpensive technique for the detection of specific miRNAs in exosomes.

The molecular beacon (MB), nano-sized oligonucleotide probe is dual-labeled oligonucleotide hairpin probe with a fluorophore and a quencher at each end. This unique structure makes the MB a suitable probe for the imaging of RNAs in living cells, because it has high specificity and low background fluorescence [32–38]. Unbound MBs that are not interacting with the target RNAs in the living cells do not have to be removed from the cells due to their self-quenching ability [39]. Recently, miRNAs in solution were successfully quantified by using MBs, and MBs that targeted mature or premature miR-21 had high specificity and also distinguished the small fold changes of miRNA expression similar to real-time PCR analysis [40].

Herein, we used MBs for the *in situ* detection of specific miRNAs from exosomes. In this study, we isolated exosomes from MCF-7, i.e., breast cancer cells, and we detected exosomal miR-21 as a target miRNA, because it is known to be a key player in many diseases, including cancer of the lung, breast, and colon (Fig. 1). We tested exosomes that originated from different types of cells to determine whether MBs bind to miR-21 with high specificity. We also investigated whether MB is delivered into exosomes by going through the exosomal membrane and discussed whether permeabilization treatment can be used to improve the delivery of MBs inside the exosome, giving a high level of hybridization.

2. Materials and methods

2.1. Detection of miR-21 using MB in solution

The design of MB used in this study for targeting mature miR-21 was 5'-Cy3-GCGCGTCAACATCAGTCTGATAAGCTACGCGC-BHQ2-3' [40]. The random MB (R-MB) sequence, i.e., 5'-Cy3-GCGCGTCTCTGCCTAAGTGTACGCGC-BHQ2-3', was used as a negative control. The underlined bases indicate the stem sequence of MB. MiR-21 MB and R-MB were synthesized by Integrated DNA Technologies and CosmoGene, Inc. respectively. Sequences of synthetic target miR-21 DNA, random target DNA for solution assay, and blocking DNA were 5'-TAGCTTATCAGACTGATGTTGA-3', 5'-TCAACATCAGTCTGATAAGCTA-3', and 5'-CAACATCAGTCTGATAAGCT-3', respectively (CosmoGene, Inc.). Hybridization of MB with target miRNA in PBS solution was assessed using a Varioskan™ Flash Multimode Reader (Thermo Scientific, USA) by measuring the fluorescence signals with an excitation wavelength of 545 nm and an emission wavelength of 570 nm. For the reaction, the samples were incubated at 37 °C for up to 2 h in a black 384-well microplate until they were to be analyzed.

2.2. Cell culture and exosome-free FBS preparation

MCF-7 cells and NIH/3T3 cells were cultured in DMEM (Biowest, France), and CHO-K1 cells were cultured in IMDM (Life Technologies). All cell lines were maintained in a humidified atmosphere of 5% CO₂ at 37 °C, and all media were supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest) and 1% (v/v) penicillin and streptomycin (Life Technologies). For the production of exosomes from the cells, initially, the cells were grown in media that contained 10% FBS to 70% confluence, washed twice with PBS, and then maintained for an additional two days in media that contained 10% exosome-free FBS.

For the preparation of exosome-free FBS, the FBS was loaded into polycarbonate tubes and centrifuged at 4 °C at 120,000 g for 10 h using a TLA-100.3 fixed angle rotor in an ultracentrifuge (Optima TL-100, Beckman Coulter, U.S.). The supernatant was filtered using a 0.22-μm syringe-filter and stored at 4 °C.

2.3. Exosome isolation by Total Exosome Isolation™, ExoQuick-TC™, and ultracentrifugation

In our research, three different methods were used for exosome isolation, two of which were commercially available reagents from different companies. The exosomes were isolated from culture media after cell culture according to the manufacturer's instruction. Briefly, the conditioned media were centrifuged at 2000 g for 30 min to remove cell debris. Next, a volume of the supernatant was mixed with an equal volume of Total Exosome Isolation™ solution (Invitrogen) and mixed well. Then, the mixture was incubated at 4 °C overnight and then centrifuged at 10,000 g for 1 h. The supernatant was discarded, and the pellets that contained the exosomes were resuspended in PBS.

The exosomes also were isolated by a precipitation method using ExoQuick-TC™ (System Biosciences) according to the vendor's instructions although we did not show the results using exosomes isolated using ExoQuick-TC™ as they are similar to

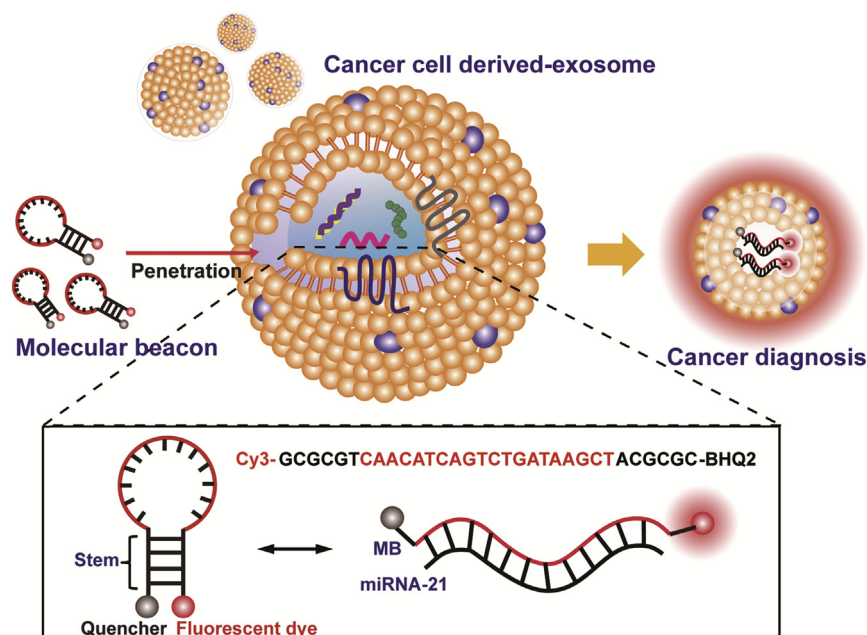


Fig. 1. Illustration for the *in situ* detection of exosomal miR-21 using MB for the diagnosis of diseases such as cancer.

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