Biosensors

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Fabrication of a Structure-Specific RNA Binder for Array Detection of Label-Free MicroRNA**

*Jeong Min Lee, Hyunmin Cho, and Yongwon Jung**

MicroRNAs (miRNAs) are a class of small noncoding RNAs (19–25 nucleotides) that regulate gene expression in viruses, plants, and animals.[1] Over 700 miRNAs have been identified in humans, and thousands more are expected to be found.[2] Accumulated studies have uncovered distinct miRNA expression patterns in various human diseases and thus shown the great potential of miRNA profiling for clinical applications, such as diagnosis and drug-efficacy evaluation.[3] Microarray-based detection offers an efficient method for profiling large numbers of miRNA expressions simultaneously. Numerous studies have demonstrated novel probe designs and strategies for miRNA labeling, as well as successful array-based miRNA profiling.[4] The use of these assays in the clinical and research fields, however, has been restricted by technical challenges to standardization, and especially by the excessive variation between protocols.[5] In particular, the miRNA-labeling process has been one of critical factors responsible for experimental variation.

In most miRNA-labeling methods, direct or indirect enzymatic labeling reactions against target miRNAs prior to (or sometimes after) hybridization are used to chip surface probes.[6] Attempts to provide valid, reliable analyses have focused on the performance of these reactions, which are primarily polymerization and ligation reactions of miRNAs, with better consistency.[7] On the other hand, the development of an miRNA array detection method free from labeling and amplification reactions would clearly simplify the process and greatly bolster the credibility of miRNA-profiling studies, particularly for diagnostic purposes. This goal could be attained by the development of a specific antibody for surface-bound miRNAs and the use of various antibody detection strategies. Until now, however, commonly recognized challenges, such as the unstable nature of RNA, have made the development of antibodies against specific RNA structures extremely difficult. Only one example of the development of anti-RNA antibodies has been reported, and in that case, the target RNA was already highly structured.[8]

Herein we describe the construction of a novel structure-specific RNA-binding protein that stably and specifically binds to double-stranded RNAs (dsRNAs) with a two-nucleotide (nt) 3′ overhang. This RNA binder acted like an antibody and enabled us to universally detect hybridized miRNAs on array surfaces without the need for enzymatic amplification or labeling reactions (Figure 1). This one-step capture method was used to detect non-amplified target miRNAs at a concentration as low as 10 pM in an array format: a detection limit comparable to that of a reported enzymatic labeling technique.[7a] Or was the method itself similar to a reported enzymatic labeling technique? The expression patterns of several human miRNAs in total RNA samples from human cells were also determined reliably with this RNA binder.

Nature utilizes many protein motifs that recognize specific RNA structures. We envisioned that a strong protein binder against surface-bound miRNAs could be fabricated from these RNA-binding motifs. A dsRNA molecule with a 2 nt 3′ overhang is a key structural feature that is formed during the processing of endogenous miRNA as well as small interfering RNA.[9] Capture RNA probes can be designed such that complementary miRNA hybridization results in the formation of dsRNA with a 2 nt 3′ overhang on chip surfaces (Figure 1). Detection of this RNA structure would enable the strict identification of surface-hybridized miRNAs. An abundant protein domain named PAZ, an RNA-binding module found in Argonaute and some Dicer proteins, specifically recognizes this RNA structure.[10] In this study, we first examined several forms of the PAZ domain from the human Dicer and Argonaute proteins. The partially truncated PAZ domain from human EIF2C1 Argonaute (see the Supporting Information) demonstrated the highest specificity for dsRNA with a 2 nt 3′ overhang and the weakest binding to the single-stranded-RNA capture probe; these features suggest excellent potential for a novel RNA binder (Figure 2a). However, the binding pattern of PAZ was not ideal for chip-based miRNA profiling. Although it displayed fast association with the target RNA structure, the PAZ domain dissociated from the RNA too quickly, which hampered the stable detection of surface RNAs (see the Supporting Information).

We employed a second RNA-binding motif to further stabilize the RNA–PAZ complex. PAZ binds the 2 nt 3′ overhang side of dsRNA and covers approximately seven base pairs (bps) of the remaining dsRNA (structures are depicted in the Supporting Information).[11] The introduction

[*] J. M. Lee, H. Cho, Prof. Dr. Y. Jung
BioNanotechnology Research Center
Korea Research Institute of Bioscience and Biotechnology
P.O. Box 115, Yuseong, Daejeon 305-600 (Korea)
Fax: (+82) 42-879-8594
E-mail: ywjung@kribb.re.kr
and
Nanobiotechnology, School of Engineering
University of Science and Technology (UST)
P.O. Box 115, Yuseong, Daejeon 305-333 (Korea)

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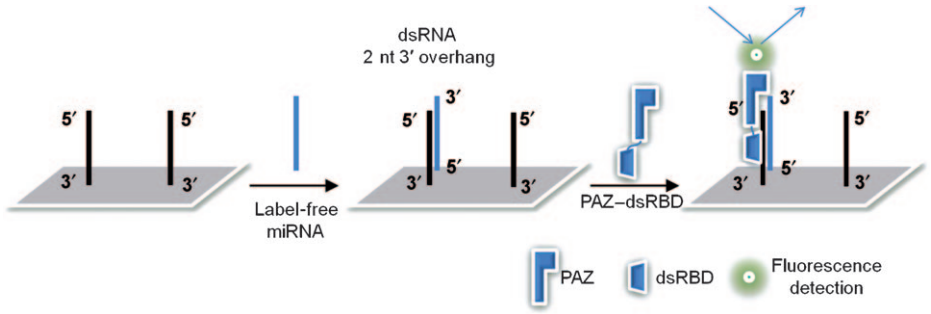

Figure 1. Schematic representation of miRNA detection by the structure-specific RNA-binding protein PAZ–dsRBD. Single-stranded capture RNA probes are immobilized on chip surfaces through their 3′ ends. Hybridization of label-free miRNAs on the surface results in the formation of dsRNA with a 2 nt 3′ overhang. This dsRNA (with a 2 nt 3′ overhang) is specifically recognized and detected by PAZ–dsRBD.

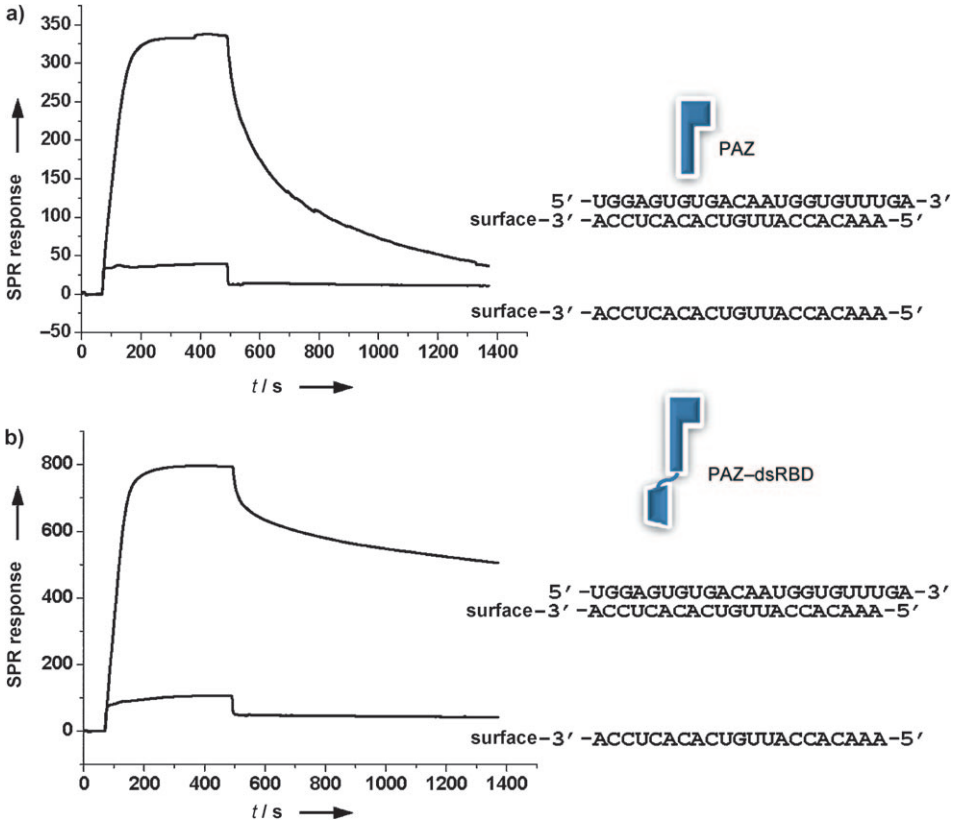

Figure 2. SPR sensorgrams of the binding of a) the PAZ domain and b) the PAZ–dsRBD construct to the single-stranded RNA capture probe and the miRNA-hybridized dsRNA structure (the SPR response is given in response units). The SPR gold chip surface was first covered with the single-stranded RNA capture probe, and the protein constructs (PAZ or PAZ–dsRBD) were applied for 7 min; the surface was subsequently washed for 15 min, and protein dissociation was monitored. After the removal of bound proteins, the same chip was treated with complementary miRNA followed by the PAZ and PAZ–dsRBD protein constructs. The sequences of the RNA capture probe and miR122b are shown.

of an RNA binder specific for the remaining uncovered dsRNA, such as the dsRNA-binding domain (dsRBD),[12] might slow the dissociation of PAZ from the hybridized miRNA. Of the four different dsRBD proteins tested (see the Supporting Information), dsRBD from *Aquifex aeolicus* RNase III showed the lowest nonspecific binding to the chip surfaces. The selected dsRBD motif was linked to the C terminus of the PAZ domain, since N-terminal linking yielded a protein with a slightly lower specificity against miRNA-hybridized RNAs (see the Supporting Information). The resulting RNA binder, PAZ–dsRBD, demonstrated highly improved binding stability to the surface-bound miRNA, as determined by surface plasmon resonance (SPR) analysis (Figure 2b). Even in the presence of a large excess of tRNAs, PAZ–dsRBD stably and specifically bound to the surface-bound miRNA with a 2 nt 3′ overhang (see the Supporting Information). The affinity constant (K_d) of PAZ–dsRBD for surface-bound miRNA was measured to be approximately 7.3 nM (see the Supporting Information), which is comparable to the affinity constants found for antibody–antigen interactions. The binding efficiency of PAZ–dsRBD to surface-bound miRNA was affected by the presence of various mismatches, particularly those near the protein-binding region (see the Supporting Information). Moreover, capture-probe modifications, such as O-methylation and 5′ phosphorylation, clearly hindered PAZ–dsRBD binding to surface-bound miRNAs, as did the use of a DNA probe (see the Supporting Information). The binding, however, was not altered by locked nucleic acid (LNA) modifications on the RNA capture probe (see the Supporting Information). LNA modifications have been widely used to improve detection limits and mismatch discriminations of various methods for the array detection of miRNA.[13]

We subsequently attempted the array detection of synthetic human miRNAs on a glass slide with the fabricated RNA binder PAZ–dsRBD. Capture RNA probes were spotted in a microarray format on glass slides through their 3′ ends, and miRNAs were applied to the glass surfaces at varying concentrations. Surface-bound miRNAs were directly identified by treatment of the slides with singly biotinylated

PAZ–dsRBD (biotin–PAZ–dsRBD; the protein construction is described in the Supporting Information) and subsequently with streptavidin labeled with carbocyanine 3 (Cy3). A commonly used fluorescence scanner was used for profiling. Detection can also be performed in one step without a decrease in the signal-to-noise ratio by using a mixture of biotin–PAZ–dsRBD and Cy3–streptavidin. Highly specific fluorescence signals were observed for the target miRNA, miR96, with this miRNA-detection system (Figure 3). As

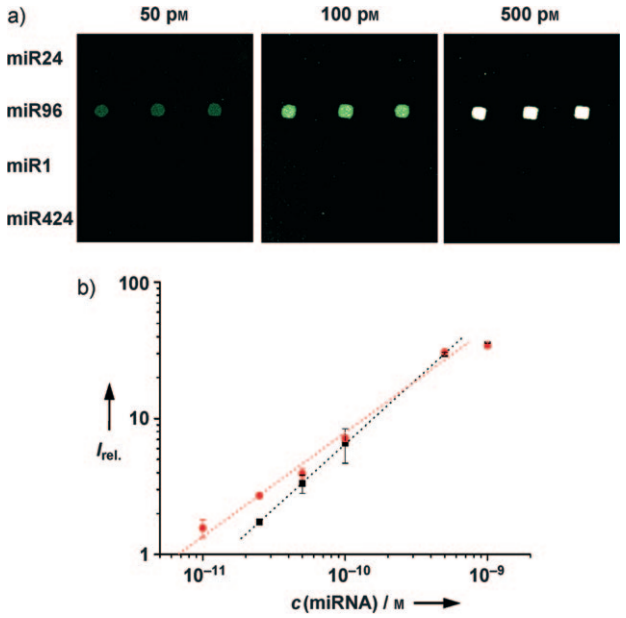

Figure 3. Microarray detection of synthetic miRNAs. a) Fluorescence images for miR96 detection. Four RNA capture probes (against miR24, miR96, miR1, and miR424) were spotted on a glass slide, and miR96 was applied to the surface in varying concentrations. Hybridized miR96 was detected with biotinylated PAZ–dsRBD/Cy3–streptavidin as described in the text. b) Concentration-dependent relative fluorescence intensities of miR1 (red circles) and miR96 (black squares). In each case, the mean fluorescence intensity of the target-miRNA spot was divided by that of the same capture-RNA spot without miRNA treatment to give the relative signal intensity. The plots were drawn on the basis of three independent experiments.

discussed above, however, RNA detection with the PAZ domain alone was highly inefficient (see the Supporting Information), probably as a result of the rapid dissociation of PAZ from the dsRNA during washing steps. Relative signal intensities for target miRNAs were calculated by dividing the fluorescence intensity of each miRNA spot by that of the capture RNA alone. Concentration-dependent signals were measured for two different miRNAs (miR96 and miR1), and a linear signal dependence was observed over a concentration range of 10–500 pM (Figure 3b).

We next examined the expression patterns of four miRNAs (miR24, miR96, miR1, and miR424) in human tissue. Total RNA extracts (5 μg) from human tissue were applied directly to the capture-probe-spotted microarray chips in hybridization solution (0.1 mL). Hybridized miRNAs were then detected with biotin–PAZ–dsRBD/Cy3–streptavidin proteins as described above. The resulting array data clearly indicated that human liver and heart tissues contain different levels of miR24 and miR1 (Figure 4). As

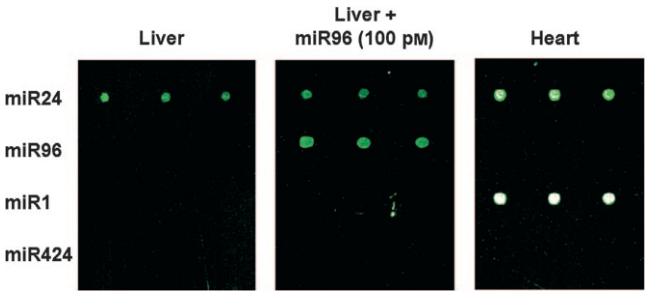

Figure 4. Fluorescence microarray detection of four human miRNAs from tissue-specific total RNA extracts. Human liver (left-hand image) or heart (right-hand image) total RNA extract (5 μg) was applied directly to the probe-spotted glass slide. Hybridized miRNAs were universally detected by biotinylated PAZ–dsRBD/Cy3–streptavidin as described in the text. Spike-in miR96 (100 pM) was added to the liver RNA extract (middle image).

previously reported,[14] miR1 is highly abundant in heart tissue (the concentration calculated in this study is approximately 380 pM: about 38 fmol in 5 μg of total RNA), whereas liver tissue contains an extremely low level of miR1. The expression pattern of miR24 in these two tissue types (ca. 65 pM in liver tissue; ca. 205 pM in heart tissue) also correlated well with the results of a previous real-time PCR study.[14] Furthermore, the spike-in detection of synthetic miR96 (100 pM) in liver total RNA was effectively demonstrated (Figure 4). Extremely low levels of miR424 and miR96 are expressed in both tissues, as shown in this study and previously.[14,15]

In summary, we have synthesized a novel structure-specific RNA-binding protein and used it successfully for the array detection of miRNAs without enzymatic labeling or amplification reactions. The synthesized protein displayed high binding specificity for the target RNA structure (dsRNA with a 2 nt 3′ overhang) and, more importantly, exemplary stability with the bound RNA: a characteristic that is essential for chip-based RNA detection. The present strategy of constructing a highly specific RNA binder by blending multiple native RNA binding motifs offers great promise for the future development of new RNA binders. Our study is also the first example of antibody-like protein-based array detection of miRNA. This detection method can simplify miRNA profiling and may improve standardization processes. It should thus greatly aid the use of miRNA profiling for diagnostic purposes. This method is also compatible with many currently employed tools, such as oligonucleotide microarrays, fluorescence scanning, and ELISA-based detection methods. It will, however, clearly be necessary to lower the detection limit of the assay to enable the observation of less abundant miRNAs. LNA-modified RNA capture probes could be used for this purpose. We are currently investigating possibilities in the further engineering of the PAZ–dsRBD construct, including the addition of signal-amplifying enzymes for ELISA-like detection with improved sensitivity.

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