

A Multivalent Structure-Specific RNA Binder with Extremely Stable Target Binding but Reduced Interaction with Nonspecific RNAs

Jeong Min Lee⁺, Ahreum Hwang⁺, Hyeongjoo Choi, Yongsang Jo, Bongsoo Kim,^{*} Taejoon Kang,^{*} and Yongwon Jung^{*}

Abstract: By greatly enhancing binding affinities against target biomolecules, multivalent interactions provide an attractive strategy for biosensing. However, there is also a major concern for increased binding to nonspecific targets by multivalent binding. A range of charge-engineered probes of a structure-specific RNA binding protein PAZ as well as multivalent forms of these PAZ probes were constructed by using diverse multivalent avidin proteins (2-mer, 4-mer, and 24-mer). Increased valency vastly enhanced the binding stability of PAZ to structured target RNA. Surprisingly, nonspecific RNA binding of multivalent PAZ can be reduced even below that of the PAZ monomer by controlling negative charges on both PAZ and multivalent avidin scaffolds. The optimized 24-meric PAZ showed nearly irreversible binding to target RNA with negligible binding to nonspecific RNA, and this ultra-specific 24-meric PAZ probe allowed SERS detection of intact microRNAs at an attomolar level.

Multivalent interactions between two or more assembled biomolecules (or simply multivalency) play a fundamental role in many biological actions by offering extremely strong but reversible interactions.^[1] Although the underlying principles of these dynamic interactions are far from understood, the use of multivalency has shown remarkable potential in diverse fields, ranging from drug discovery to vaccine development.^[2] Numerous highly effective inhibitors have been developed by carefully designing multivalent ligands or binders against diverse pathological targets.^[3] Multivalent display of antigens has also become a highly potent new

vaccine platform.^[4] Furthermore, it has been demonstrated that multivalent probes can be endowed with enhanced selectivities (not just affinities) to target surfaces depending on the densities of surface binding sites.^[5]

Multivalency can also be an influential concept for biosensing as the binding characteristics between target biomolecules and receptor probes are major determining factors for sensing ability. Multiple studies have demonstrated that increased valency can strengthen binding affinities by several orders of magnitude, potentially offering nearly irreversible binding stability of receptor probes against target molecules.^[3b,c,6] However, there is a general concern that even weak interactions between receptor probes and other nonspecific molecules can be also enhanced by multivalency. This increased non-specificity will significantly lower the signal-to-noise ratios of biosensors, largely eliminating enhanced target signals by multivalent probes. To apply multivalency to biosensors to attain the highest possible sensitivity, valency-dependent interactions of receptor probes against both specific and nonspecific targets must be carefully evaluated, and multivalent probes should be optimized to have maximal target binding and minimal nonspecific interactions. At present, however, precise evaluation of multivalent binding and resulting target sensing is highly limited by the complexity of multivalent biomolecules and their interactions.^[7]

Herein, PAZ, a small RNA binding protein that specifically binds to double-stranded RNA (dsRNA) with a 2-nucleotide (nt) 3' overhang,^[8] was used as a monomeric probe against surface-bound, double-stranded microRNA (miRNA) targets (dsRNA; Scheme 1). Like most RNA binding proteins, however, PAZ has a noticeable degree of interaction to nonspecific single-stranded RNA (ssRNA),

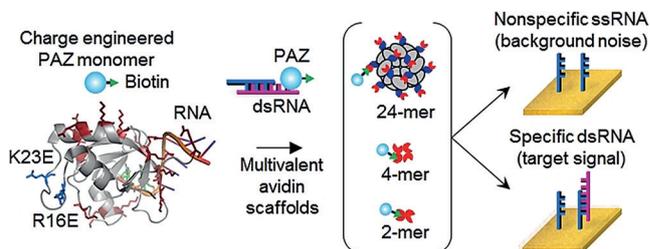
[*] Dr. J. M. Lee,^[†] A. Hwang,^[†] H. Choi, Y. Jo, Prof. B. Kim, Prof. Y. Jung
Department of Chemistry, Korea Advanced Institute of Science and Technology
Daejeon 34141 (Korea)
E-mail: bongsoo@kaist.ac.kr
ywjung@kaist.ac.kr

A. Hwang,^[†] Dr. T. Kang
Hazards Monitoring Bionano Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB)
Daejeon 34141 (Korea)
E-mail: kangtaejeon@kribb.re.kr

Dr. T. Kang
BioNano Health Guard Research Center, KRIBB
Daejeon 34141 (Korea)
Department of Nanobiotechnology, KRIBB School of Biotechnology, UST
Daejeon 34113 (Korea)

[†] These authors contributed equally to this work.

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Scheme 1. Representation of the binding evaluation for monovalent and multivalent (charge engineered) PAZ probes against specific dsRNA and nonspecific ssRNA on gold surfaces. The complex structure of the PAZ domain and RNA is shown with positive surface residues, four of which (including blue K23 and R16) are systematically mutated to negative Glu.

which is primarily due to its overly positive surface charges.^[9] We quantitatively evaluated monovalent and multivalent interactions of charge engineered PAZ probes to both target dsRNA and nonspecific ssRNA by surface plasmon resonance (SPR) analysis. Multivalent PAZ probes were assembled by various natural and artificial multivalent avidin scaffolds (Scheme 1). Effects of the binding features of these PAZ probes on sensing ability were subsequently examined with surface-enhanced Raman scattering (SERS)-based miRNA detection. The binding stability of PAZ to target dsRNA was vastly increased by increased valency, resulting in highly enhanced target SERS signals. More importantly, nonspecific binding to surface ssRNA by multivalent probes could be minimized by increased negative charges on the probes, while maintaining enhanced binding to target dsRNA. The optimized multivalent PAZ probe allowed attomolar SERS detection of miRNAs, an unprecedented achievement with often poorly selective RNA binding protein probes.^[10]

To vary the RNA binding specificity of PAZ, positive surface residues that are distant from the RNA binding region were systematically mutated to negative Glu (Supporting Information, Figure S1). The constructed PAZ probes, ranging from wild-type PAZ (net surface charge +6, PAZ+6) to a PAZ with a -3 net surface charge (PAZ-3), were then subjected to the nonspecific capture ssRNA and miRNA-bound specific dsRNA surfaces (Figure 1a; Supporting Information, Figure S2). RNA binding profiles of PAZ clearly display specific but rather unstable binding to target dsRNA and visible levels of nonspecific binding to ssRNA. Furthermore, both specific and nonspecific interactions were corre-

spondingly weakened as surface positive charges were decreased. We next examined the RNA binding specificity of multivalent forms of these PAZ probes. Site-specifically biotinylated PAZ was assembled onto the previously developed 24-meric avidin scaffold,^[11] which can cluster over 20 monomeric PAZ probes on the spherical cage scaffold (Supporting Information, Figure S3). As shown in Figure 1b, the target binding stability was dramatically improved by multivalency, leading to nearly irreversible binding. Association speeds were proportionally slowed by reduced positive charges from 24mer-PAZ+6 to 24mer-PAZ-3. Binding specificity (dsRNA binding/ssRNA binding) of multivalent wild-type PAZ (24mer-PAZ+6) was comparable to that of monomeric PAZ+6 (Figures 1a vs. b). Interestingly, however, nonspecific ssRNA binding levels of 24mer-PAZ+2 and 24mer-PAZ-3 probes were dramatically reduced to a near background level (Figure 1b), offering extremely high binding specificities for these probes (particularly 24mer-PAZ+2; Supporting Information, Figure S4). The data indicate that multivalency affects specific dsRNA binding differently from nonspecific ssRNA binding, and multivalent probes therefore can be optimized to have highly enhanced binding specificity.

We next applied the ultra-specific 24mer-PAZ+2 and wild-type 24mer-PAZ+6 to a single-crystalline nanowire (NW)-based SERS sensor platform.^[12] The gold (Au) NWs are single-crystalline and have a diamond-shaped cross section, diameters of about 150 nm, lengths of 10–20 μm , and atomistically flat facets.^[13] Similar to our SPR experiments, capture ssRNAs were immobilized on an Au NW, and the resulting NW was placed on an Au film for Raman signal enhancement. Upon miRNA hybridization, resulting dsRNAs were recognized by SERS-dye-labeled multivalent PAZ probes (Figure 2a). The laser spot (diameter of 1 μm) was focused at the center of the Au NW, and the polarization was perpendicular to the long axis of NW. SERS signals with

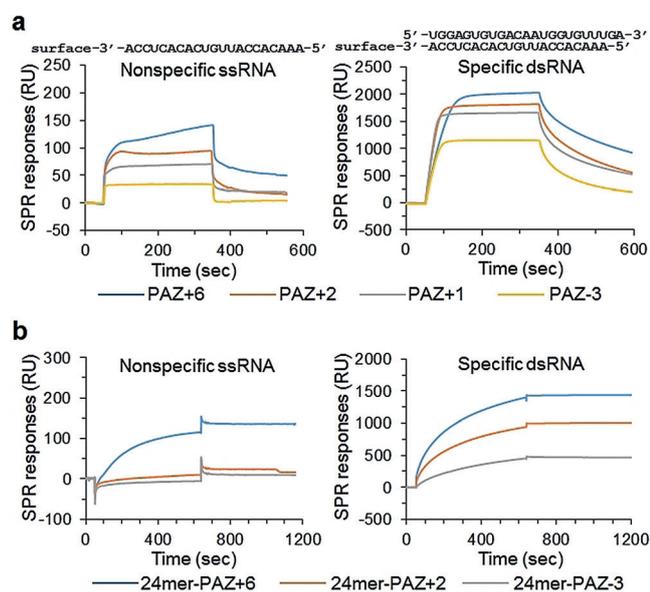


Figure 1. Specific RNA binding properties of charge engineered PAZ probes. a) SPR sensorgrams of monomeric PAZ probes binding to the nonspecific capture ssRNA (left) and the specific miRNA hybridized dsRNA (right). The ssRNA and dsRNA sequences are indicated. A surface bound miRNA is ca. 1100 RU. b) Nonspecific (left) and specific (right) RNA binding of 24-meric PAZ variants. A surface bound miRNA is ca. 150 RU, and a binding buffer contains additional 2.5% glycerol.

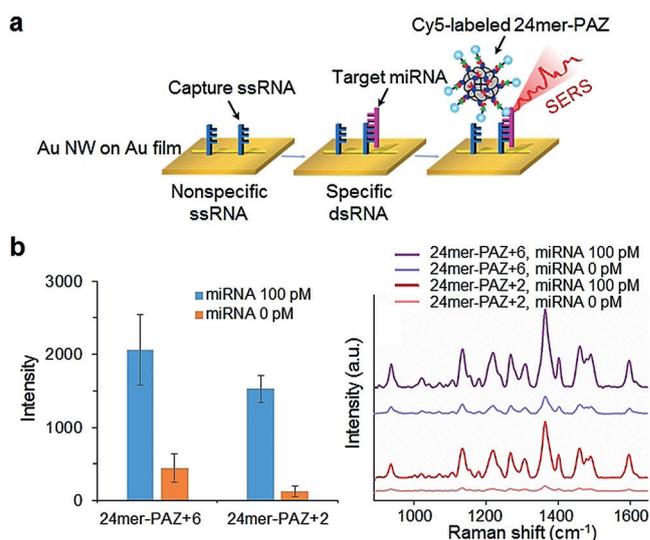


Figure 2. MiRNA detection by the single-crystalline Au NW-based SERS sensor. a) Representation of miRNA detection. b) SERS detection of miRNA with 24mer-PAZ+6 and 24mer-PAZ+2. Plots of 1580 cm^{-1} band intensities (left) and SERS spectra of Cy5 measured from NW-based SERS sensors (right) are shown. Error bars, 1 s.d. ($n=10$).

or without target miRNA indicate dsRNA target signals or nonspecific background noise, respectively. Although 24mer-PAZ+6 provided slightly higher miRNA signals, 24mer-PAZ+2 showed significantly higher signal-to-noise ratios with minimal background signals (Figure 2b), which correlates well with the SPR binding data (Figure 1b). The binding specificity of PAZ probes examined by SPR was successfully translated to the sensing ability of these probes measured by the NW-based SERS sensor.

To further investigate how the RNA binding specificity of PAZ probes is affected by multivalency, two other multivalent PAZ+2 probes were assembled with naturally dimeric rhizavidin (RA, 2-mer) and tetrameric streptavidin (STA, 4-mer; Supporting Information, Figures S5–S7). As shown in Figure 3a, RNA binding profiles of monomeric PAZ+2 were different from Figure 1a, likely due to an increased PAZ concentration and a reduced surface RNA density (Supporting Information, Figure S8 Note). Nonetheless, the target binding stability of PAZ+2 was clearly increased as the valency increased. On the other hand, all three multivalent PAZ+2 probes showed significantly lower nonspecific ssRNA binding than that of the PAZ+2 monomer. These binding characteristics of the PAZ+2 monomer and multimers were again explained well with the miRNA SERS detection results (Figure 3b; Supporting Information, Figure S8). A possible explanation for the reduced nonspecific binding of the multimeric PAZ probes is net negative surface charges of all avidin scaffolds (protein sequences in the Supporting Information). Repulsive forces between avidins and RNAs might be strong enough to negate relatively weak nonspecific interactions but too weak to notably influence specific interactions. Furthermore, compared to PAZ monomers, large sizes of multimeric PAZ probes might hamper their binding to ssRNA and dsRNA, which are immobilized on a dextran-coated SPR chip with a coating thickness up to 100 nm.

To investigate the roles of avidin scaffold charges, two negative residues (D37 and E102) on weakly acidic STA (pI ca. 6.5) were mutated to positive Lys, and various 4mer-PAZ+2 charge variants were assembled (Supporting Information, Figure S9). Enhanced RNA binding by reduced negative charges on STA was higher for nonspecific ssRNA than for specific dsRNA (Figure 3c; Supporting Information, Figure S10), supporting our rationale for reduced nonspecific ssRNA binding by negative charges on avidin scaffolds. More dramatic changes of the RNA binding specificity were observed with deglycosylated, neutralized native avidin (avidin pI ca. 10.5) from egg whites (Neutravidin, NA). Tetrameric PAZ+2 assembled on NA (NA-PAZ+2) showed significantly higher nonspecific ssRNA binding than even monomeric PAZ+2, while specific dsRNA binding was comparable to those of other 4mer-PAZ+2 variants (Figure 3c). The presence of basic (positive) forms of NA likely contributes to this high nonspecific ssRNA binding of NA-PAZ complexes (Supporting Information, Figure S9). Multivalent 4mer(NA)-PAZ+2, with the worst specificity, clearly demonstrated an aforementioned concern of how multivalency can also drastically increase nonspecific binding (Supporting Information, Figure S10).

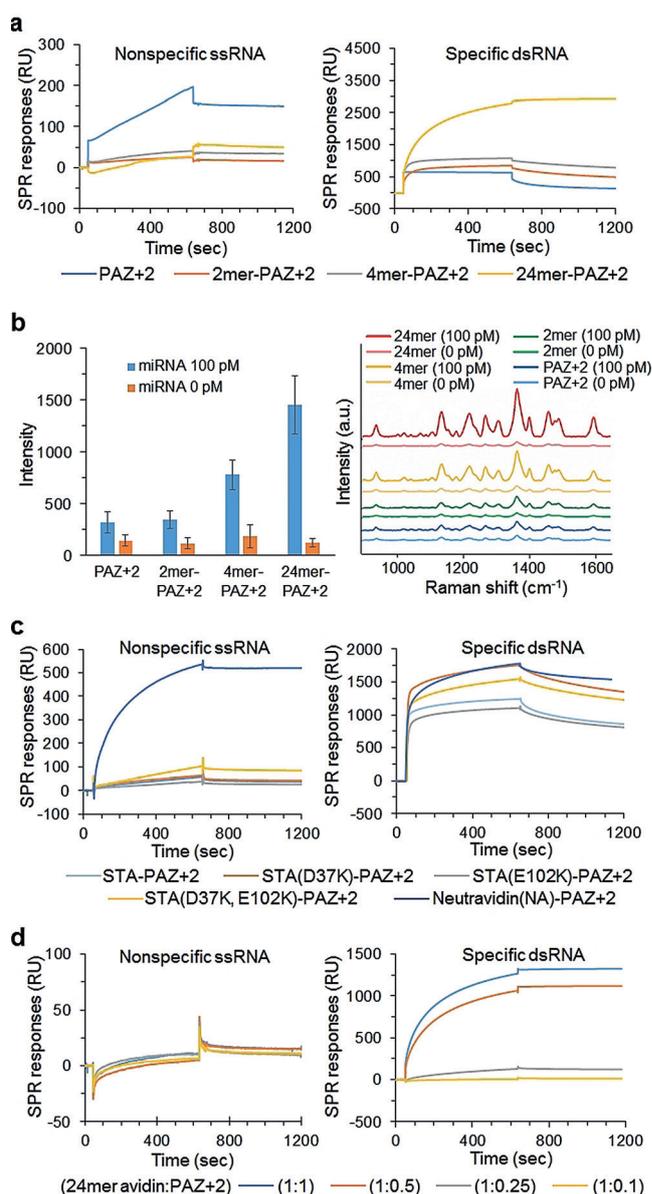


Figure 3. Specific RNA binding properties of multivalent PAZ probes. a) SPR sensorgrams of monomeric and multimeric PAZ+2 probes binding to the nonspecific capture ssRNA (left) and the specific miRNA hybridized dsRNA (right). A surface bound miRNA is ca. 300 RU, and a binding buffer contains 2.5% glycerol. b) SERS detection of miRNA with monomeric PAZ+2 and multimeric PAZ+2. Error bars, 1 s.d. ($n = 10$). c) Nonspecific (left) and specific (right) RNA binding of 4mer-PAZ+2 charge variants with a surface bound miRNA ca. 150 RU. d) Nonspecific (left) and specific (right) RNA binding of valency controlled 24mer-PAZ+2 probes with a surface bound miRNA ca. 300 RU and a binding buffer containing 2.5% glycerol.

We also varied the PAZ probe valency on the 24-mer avidin by assembling different ratios of PAZ+2 monomers to the scaffold. Again, the specific dsRNA binding was increased as the valency increased, but it was significantly reduced when less than 6 PAZ+2 monomers were clustered on the 24-mer avidin (1:0.25; Figure 3d). However, all these 24mer-PAZ+2 probes showed similarly low nonspecific ssRNA binding, indicating that the 24-mer avidin scaffold is dominantly

responsible for low multivalent, nonspecific binding. These binding patterns of 24mer-PAZ + 2 probes were also consistent with the miRNA SERS detection results (Supporting Information, Figure S11). The PAZ valency was similarly varied on tetrameric STA from four to one (Supporting Information, Figure S12). Although both nonspecific and specific interactions were lowered by reduced valency, overall nonspecific binding was very low, likely due to weakly negative STA. Taken together, we demonstrated that binding specificity of a monomeric PAZ, a multivalency degree, and the nature of assembling scaffolds collectively influence binding properties of multivalent probes. More importantly, specific and nonspecific interactions were differently affected by these factors, and thereby multivalent probes with strong target binding but reduced nonspecific binding could be devised for RNA targets.

The sensing ability of the 24mer-PAZ + 2 probe, which showed the highest signal-to-noise ratio against target miRNA, was next examined. For two different miRNAs, SERS detection with this multivalent probe offered dynamic ranges of nearly four orders of magnitude and detection limits of 10 fM, corresponding to 5 attomole in 500 μ L hybridization volume (Figure 4a; Supporting Information, Figures S13 and

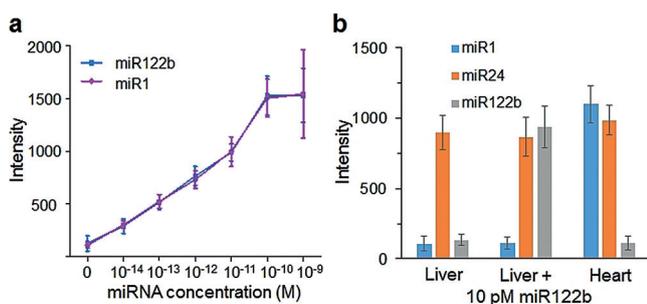


Figure 4. NW-based SERS miRNA sensing with 24mer-PAZ + 2. a) Plots of 1580 cm^{-1} band intensities versus the concentrations of two target miRNAs (miR 122b and miR1). b) SERS detection of three miRNAs (miR122b, miR24, and miR1) in total RNA extracts from liver and heart. Error bars, 1 s.d. ($n = 10$).

S14). This detection limit is significantly lower than those of previously reported protein probe-based miRNA sensors without amplification reactions.^[14] For instance, our previously developed dsRNA binder, which was fabricated with monomeric PAZ and a dsRNA binding protein, offered a detection limit of only 10 μM ,^[9] although the present NW-based SERS sensing strategy could contribute to enhanced sensitivity to some extent.^[12] SPR binding profiles of PAZ + 2 monomer and multimers against extremely low surface miRNA (ca. 20 RU) further indicate how 24mer-PAZ + 2 stably recognizes target dsRNAs even at a low density (simulating low target concentration; Figure S15). 24mer-PAZ + 2 also allowed a simultaneous multiplexed analysis of three miRNAs in total RNA extracts from the liver and heart (Figure 4b; Supporting Information, Figure S16). The calculated amounts of these miRNAs correlated well with previous reports (Supporting Information, Figure S17).^[9]

In conclusion, we demonstrated how dsRNA-specific multivalent probes can be generated to have extremely strong target binding and even reduced nonspecific binding from a monomeric probe. By using a well-defined target binder (PAZ) and versatile avidin scaffolds, the effects of monomer binding, interaction valency, and scaffold properties on multivalent probe specificity were quantitatively studied. We found that multivalency greatly enhanced target RNA binding, while relatively weak nonspecific binding can be selectively minimized by negative charges on both the PAZ monomer and the assembling scaffolds. The RNA binding properties of multivalent PAZ probes translated well to NW-based SERS sensing results. By developing an ultra-specific multivalent probe for a SERS sensor, we have begun to uncover the extraordinary potential of multivalency in biosensing. Furthermore, the present data will be valuable to elucidate multivalent biomolecular interactions, of which the fundamental principles are barely understood. Future work will focus on extending and modifying the present strategy of designing ultra-specific multivalent probes for other diverse target biomolecules and sensor platforms.

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Conflict of interest

The authors declare no conflict of interest.

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