Fluorescence Polarization-Based Rapid Detection System for Salivary Biomarkers Using Modified DNA Aptamers Containing Base-Appended Bases

Hirotaka Minagawa,‡ Akihisa Shimizu,‡ Yuka Kataoka,‡ Masayas Kuwahara,*,‡ Shintaro Kato,† Katsunori Horii,† Ikuo Shiratori,*‡ and Iwao Waga†

†NEC Solution Innovators, Ltd., 1-18-7, Shinkiba, Koto-ku, Tokyo 136-8627, Japan
‡Graduate School of Integrated Basic Sciences, Nihon University, 3-25-40 Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan

Supporting Information

ABSTRACT: The field of care testing toward the analysis of blood and saliva lacks nowadays simple test techniques for biomarkers. In this study, we have developed a novel nucleobase analog, Ugu, which is a uracil derivative bearing a guanine base at the 5-position. Moreover, we attempted the development of aptamers that can bind to secretory immunoglobulin A (SIgA), which has been examined as a stress marker in human saliva. It was observed that the acquired aptamer binds strongly and selectively to the SIgA dimer (Kd = 13.6 nM) without binding to the IgG and IgA monomers of human serum. Reduction of the aptamer length (41 mer) successfully improved 4-fold the binding affinity (Kd = 3.7 nM), compared to the original, longer aptamer (78 mer). Furthermore, the development of a simple detection system for human saliva samples by fluorescence polarization was investigated, using the reported human salivary α-amylase (sAA) and the SIgA-binding aptamer. Comparison of the present method with conventional enzyme-linked immunosorbent assay techniques highlighted a significant Pearson’s correlation of 0.94 and 0.83 when targeting sAA and SIgA, respectively. It is thus strongly suggested that a new simple test of stress markers in human saliva can be quantified quickly without bound/free (B/F) separation.

Saliva is a biological sample that can be noninvasively collected, and its biomarkers can be used to detect changes in biological stress systems, in order to easily investigate the effect of acute and chronic stress to physical health and illness.1–3 Current research focuses on the stress status estimation and the timely diagnosis of various systemic and local diseases, by measuring salivary biomarkers.4 Among them, salivary α-amylase (sAA) is secreted due to the direct nerve action of the sympathetic nervous system and the regulation of norepinephrine, and its concentration rises due to acute stress. Therefore, sAA is a sensitive biomarker affected by physical stress-related changes that reflect the nervous system activity.5,6 Furthermore, salivary secretory immunoglobulin A (SIgA) has been investigated as a decline biomarker of the immune system function,7,8 while its association with stress and upper respiratory tract infection established SIgA as a reliable biomarker for identifying infection risk in athletes.9,10

Although the ELISA assay is mainly applied to measure saliva markers, its utilization at the point of care is difficult, because the operation is complicated and time-consuming. Furthermore, the development of an evaluation system that could simultaneously measure multiple markers, at low cost, is strongly desired in large-scale cohort studies.11 A simple method for measuring the sAA concentration based on the amylase activity has been developed and commercialized,12 but it is widely influenced by pH and salt concentration, whereas it cannot be measured along with other markers. Therefore, this method cannot be used as a general-purpose platform. Additionally, there is no other method available for measuring SIgA except for ELISA, reinforcing the need for novel, simple techniques to determine the salivary SIgA concentration.

Aptamers are single strand DNA or RNA molecules,13,14 and a large number has already been reported in the literature.15–17 Chemical modifications or additions/extensions of additional functions are more feasible in aptamers compared to antibodies, while they can be employed as switching sensors through structural changes, because of their stability at room temperature and resistance to thermal denaturation. Therefore, they have attracted attention as diagnostic agents and molecular elements of simple sensors, also due to their high detection sensitivity in combination with the polymerase chain reaction (PCR).18,19 Despite numerous reports of successful aptamer acquisition and the production of aptamer sensors for the detection of various targets, not many cases of successful
detection of targets in actual samples have been reported so far.20

Aptamers with affinity for a desired target are selected from a library of random sequences with a primer region, using the systematic evolution of ligands by exponential enrichment (SELEX) method.13,14 Disclosed aptamers with high binding affinity and slow off-rate use, apart from natural nucleic acids, modified nucleic acids that introduce highly hydrophobic amino acids to the bases.21,22 In our previous study, we have developed a DNA aptamer with a base-extended base (BAB) modification and adenine derivatives at the 5-position of uracil, which displayed high affinity for small molecules and proteins.23,24

Moreover, we have successfully developed recently an aptamer with a high binding affinity for sAA, using a library containing the nucleobase analog Uad.25 It is known that this sAA-binding aptamer exhibits a more diverse complex structure than the same oligomer consisting of natural bases. The uniqueness of the three-dimensional structure is considered to contribute to its high binding affinity. The binding of sAA-binding aptamers to salivary α-amylase has been confirmed by pull-down experiments, using aptamer beads, and by lateral flow devices using gold colloids that could be a simple detection method for salivary sAA. Furthermore, we examined aptamers as simpler detection systems for SlgA, which is a stress biomarker in saliva. Similar to sAA-binding aptamer, the use of a library containing the nucleobase analog Uad disclosed some clones showing binding to SlgA. However, the clone with the strongest binding affinity exhibited cross-reactivity to IgG.

Hence, in this study, we developed and applied a novel analog, U8, which features a new BAB modification and bears a guanine base at the S-position. Thereby, the interaction between the nucleic acid bases is strengthened compared to analog Uad, enhancing thus its structural specificity. As a result, it was possible to obtain a clone, which can bind specifically to SlgA, without cross-reactivity to IgG, and develop a detection system for stress biomarkers in saliva based on a fluorescence polarization (FP) assay, using the SlgA-binding aptamer in addition to the sAA-binding aptamer.

■ EXPERIMENTAL SECTION

Materials. The selection target, SlgA, was purchased from MP Biomedicals (Santa Ana, CA, USA). Dynabeads MyOne Carboxylic Acid magnetic beads for the target immobilization and Dynabeads MyOne SA C1 magnetic beads for the recovery of biotinylated DNA were purchased from Invitrogen (Carlsbad, CA, USA). KOD dash polymerase for PCR and the incorporation of base-modified oligonucleotide was obtained from Toyobo Co., Ltd. (Osaka, Japan). Primer, 5′-biotinylated reverse (Rv) primer. The amplification of the target beads at 25 °C for 15 min, and the beads were washed with SB and eluted with 7 M urea to yield ssDNA. The elution was then amplified by PCR, using the Fw primer and the S′-biotinylated reverse (Rv) primer. The amplified dsDNA was incubated with Dynabeads MyOne SA C1 magnetic beads, treated with 0.02 M NaOH (aq) to elute the ssDNA library containing Uad or U8. After washing the target immobilized beads, and dUadTP, and eluted with 0.02 M NaOH (aq).

Pull-Down Beads Assay. The pull-down assay with magnetic beads was conducted based on reported procedures. After 400–450 pmol of the synthesized clones (IgAα1, IgAα2, IgAγ1, or IgAα1γ1), using nonlabeled DNA
template, and biotinylated Fw primer were mixed with 3 mg of Dynabeads MyOne SA C1 magnetic beads in SB at room temperature for 30 min, the magnetic beads were washed in triplicate with SB.

The DNA template was then eluted with 0.02 M NaOH (aq), and the beads were washed with SB three times and suspended in SB. Clone beads (200 μg), i.e., SlgA capture beads or primer-immobilizing magnetic beads, i.e., control beads, were mixed with 5 μg of SlgA or 90% human saliva in SB at 25 °C for 60 min. After removing the supernatant and washing with SB in triplicate, the bound proteins were eluted after treatment with 0.1% SDS at 95 °C for 10 min. The eluted samples were electrophoresed on PAGE L C520L (ATTO, Japan), according to the manual.

**FP Assay.** The FP assay was conducted according to the improved methodology of Gokulrangan et al.27 Clones of the fluorescent-modified aptamer were prepared using a 5′-fluorescent (TYE665) labeled primer. TYE665 is an alternative fluorescent dye for Cy5, which has an excitation/emission wavelength at 645 nm/665 nm, and other information is not publicized. Fluorescent-labeled clones (10 nM) were denatured at 95 °C for 5 min, folded at 4 °C for 5 min, and then incubated with the target samples in a selection buffer 2 (SB2; 40 mM HEPES pH 7.5, 125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 0.1 mM dextran) at 25 °C for 5 min. After incubation, samples were measured at 25 °C using FP spectroscopy on TECAN infinite M1000PRO (TECAN, Switzerland), according to the manual (N = 2) with 635 nm fluorescent dye for Cy5, which has an excitation/emission wavelength at 645 nm/665 nm, and other information is not publicized.

### RESULTS AND DISCUSSION

The aptamer selection was realized using a library of known, modified nucleic acids (U₄d), to acquire SlgA-binding aptamers (Figure S1).24,25 After eight rounds of SlgA aptamer selection, five sequences, each of which occupies more than 5% of the enriched library, were chosen as aptamer candidates (Table S1).

Measurement of the binding affinities of five sequences for SlgA on a SPR instrument, indicated that all five clones were binding to SlgA (Figure S2). Furthermore, we measured the binding affinities for several types of IgG and IgA from human serum by SPR to point out IgA₄d clones with the highest affinity for SlgA. The IgA human serum exists predominantly in the form of monomers, whereas the majority of IgA (SIgA) in external secretions, e.g., saliva, is present in the form of dimers.28 As a result, IgA₄d showed cross-reactivity to full-size IgG and IgA from human serum (Figure S3).

Therefore, we prepared a fresh library containing modified nucleobase U₄° (Figure 1) and conducted aptamer selection. After eight rounds of SlgA aptamer selection, three sequences, each of which occupies more than 5% of the enriched library, as shown in Table 1, were chosen as aptamer candidates.

The evaluation of the binding affinities of these three sequences to U₄° clone by SPR indicated that all candidates bind to SlgA. Among the tested clones, IgAgu1 had the highest binding capacity to SlgA, based on the K_d values and the value of the SPR resonance units for SlgA at 200 nM (Figure 2). The binding of IgAgu1 clone to human serum IgG and IgA was measured by SPR, revealing that the IgAgu1 clone did not bind to IgG or serum IgA, whereas it showed high binding specificity to SIgA (Figure 3). SlgA has not been reported to bind to SIgA. The IgAgu1 clone binds to SIgA specifically.

IgAad1, IgAad2, IgAad3, IgAad4, and IgAad5 clones were used to generate magnetic beads, able to bind to SlgA. A pull-down assay was then conducted for SlgA from human saliva in SB, utilizing the generated beads. It was observed that all the clones bind to SlgA in SB (Figure 4a), but binding to SIgA in human saliva could be confirmed only for 2 clones, IgAgu1 and IgAad2 (Figure 4b).

In general, IgAad4 showed broad binding specificity for antibodies, whereas IgAgu1 showed high specificity only for the dimer IgA. IgAad4 strongly bound to SlgA (K_d = 1.14 nM, Figure S2), and binding to SlgA in saliva was also confirmed, as displayed in Figure 4. Considering the binding specificity of the examined clones to SlgA, IgAgu1 was selected as a SlgA-binding aptamer candidate. In view of the salivary SlgA concentration range (0.6–1.2 μM) that has been reported so far, the high affinity of IgAgu1 for SlgA (K_d = 1.36 nM) can be sufficiently used as a detection element for SlgA in saliva.

Generally, long sequences are likely to form various secondary structures that destabilize the conformation of the target aptamer’s binding site.30 Therefore, we considered that the binding specificity could be improved by suppressing the

![Figure 1. Chemical structures of the 2′-deoxynucleoside-5′-triphosphate analogs dU₄°TP and dU₄°TP, bearing the modified bases U₄° and U₄°, respectively.](Image 324x658 to 564x749)

| Table 1. Ten Most Common Sequences Derived from the U₄° Modified ssDNA Library |
|-----------------------------|-----------------------------|-----------------------------|
| **Clone name**              | **Sequence ratio**          | **Random region sequence**  |
| IgAgu1                    | 7.1%                       | tGCtGtGtGtttCtAtttAttAGCCGCA |
| IgAgu2                    | 5.7%                       | tAAAtAAttCtAtCtCCtGCGGttAGAGAG |
| IgAgu3                    | 5.1%                       | tAGCAAtAAtAAGGtGtACACCGGtGtGtG |
| IgAgu4                    | 4.7%                       | tCAACtCAAtCtACtGACtACtCACtCt |
| IgAgu5                    | 4.6%                       | tCCtCtCtGtACtCtCtCtCtCtCtCt |
| IgAgu6                    | 3.6%                       | tGCCAttCtACtCtCtCtCtCtCtCtCt |
| IgAgu7                    | 3.5%                       | tAAttAAttttCtACtCtCtCtCtCtCt |
| IgAgu8                    | 2.7%                       | tCtGtACtCtCtCtCtCtCtCtCtCt |
| IgAgu9                    | 2.6%                       | tACtCtACtCtCtCtCtCtCtCtCt |
| IgAgu10                   | 2.3%                       | tAAttttCtACtCtCtCtCtCtCtCt |

“Sequence ratio was defined as the ratio of the sequence to the total number of sequences, which was generated by a next generation sequencer. bctd indicates U₄°."
Figure 2. Representative SPR sensorgrams illustrating the interaction between the SIgA and the aptamer candidates of (a) IgAgu1, (b) IgAgu2, and (c) IgAgu3. Measurements were performed with multicycle kinetics, and various concentrations of SIgA (12.5–200 nM) were injected over the respective aptamer-immobilizing sensor chip for 120 s at a flow rate of 50 μL/min. The black dotted line represents the measured curve, and the red line represents the fitting curve.

Figure 3. Binding of the IgAgu1 clone to the SlgA or other antibodies: (a) SPR response curve of the interaction between the SlgA or other types of IgG and the aptamer IgAgu1. SlgA, IgG1 kappa, IgG, or IgG-Fc (each of 400 nM) were injected over the respective aptamer-immobilizing sensor chips for 120 s at a flow rate of 50 μL/min. The red line represents the SlgA measured curve, and the black and blue lines represent the IgG1 kappa and IgG measured curves, respectively. The green and the orange lines represent the IgG-Fc1 and IgG-Fc2 measured curves, respectively. (b) SPR response curves of the interaction between the SlgA or serum IgA and the aptamer IgAgu1. SlgA or serum IgA (each of 50 μg/mL) was injected over the respective aptamer-immobilizing sensor chips for 120 s at a flow rate of 50 μL/min. The red line represents the SlgA measured curve, and the gray lines represent the serum IgA measured curves, respectively.

Structural instability, which could be achieved by minimizing the sequences. In this case, the sequence minimization was examined using IgAgu1 as a SlgA-binding aptamer candidate and the SPR methodology as an index for binding recordings (Figure 5a,b). As a result, we achieved the minimization by 41 bases and improved the $K_d$ value to 3.7 nM (Figure 5c).

Our next goal was to develop a simple detection system of sAA and SlgA by the FP assay. This system has the great advantage of simple detection, which does not require B/F separation, while the measurement of the sample concentrations requires only mixing of the fluorescent-labeled aptamer with the examined sample. It is generally considered that the sensitivity of FP is affected by the difference in molecular weight between the target molecule and the molecule bearing the fluorophore and by the local fluorophore movement.

In order to enhance the method’s detection sensitivity, it is important to minimize the size of the fluorescent-labeled aptamer, i.e., increase the molecular weight ratio with the target. Therefore, we analyzed in detail the minimized sequences of the sAA-binding (AMYad1-2) and SlgA-binding (IgAgu1-3) aptamers and then sought for sequences that increase the fluorescence anisotropy (mP) during the target binding. Specifically, various sequences, labeled with a fluorescent dye (Tye665) at the 5’-end, were prepared.

Then, a sequence with substantial changes in the mP values was mixed with each target molecule, while AMYad1-2 and IgAgu1-3 aptamers were selected as detection clones for sAA and SlgA, respectively (Figures S4 and S5). The binding of AMYad1-2 and IgAgu1-3 to the targets was also confirmed by SPR (Figure S6).

AMYad1-2 had almost the same $K_d$ value in SPR with the original minimized clone (AMYad1-2), but IgAgu1-3 exhibited an about 3-fold higher $K_d$ value in SPR than the original minimized clone (IgAgu1-3) (Figures 5c and S6b). Because of the minor alteration of the fluorescence anisotropy, when IgAgu1-3 binds to 1 μM SlgA (Figures S5b), IgAgu1-3 cannot be used for the detection of SlgA by the FP method. Instead, considering the actual concentration of SlgA in human saliva (0.6–1.2 μM), we assessed that IgAgu1-3 could be sufficiently used as a candidate aptamer for FP assays.

Our study confirmed that the mP values changed in a target concentration-dependent way, when the binding of AMYad1-2 and IgAgu1-3 to each target (sAA and SlgA) was examined in SB2 (Figure 6). We also confirmed target specificity of AMYad1-2 and IgAgu1-3 between sAA and SlgA in FP measurement (Figure 6). Thus, those aptamers enable to measure sAA and SlgA with high specificity using FP measurement.

A good regression line could be obtained by fitting the change in fluorescence anisotropy using a 4-parameter logistic nonlinear regression model, and the inflection point molar concentrations were estimated at 3.1 nM for sAA (Figure 6a) and 287 nM for SlgA (Figure 6b). These values are about 7-fold greater for sAA and about 27-fold greater for SlgA than the $K_d$ values calculated from the SPR method (Figures 6 and S6). The difference in binding activity between different methodologies...
was reported previously, and the difference in the binding activity between FP and SPR in this study could be related to the immobilization status of the binding assay. The 3′ tail of aptamers and free aptamers were used for SPR and FP, respectively.

In addition, we prepared a DNA oligo in which all the Ugu in IgAgu1-3 were substituted with natural T (designated as IgAgu1-3N) and checked the binding affinity using the SPR and FP assay (Figure S7a,b). The result clearly showed that IgAgu1-3N was unable to bind to SIgA, suggesting that the BAB modification was important for the recognition of SIgA.

To compare the difference in target specificity between IgAad4 and IgAgu1, we predicted the secondary structure of the IgAad4 and IgAgu1 sequence that substituted Uad or Ugu to natural base (T) using the VALFold program with the general DNA parameters (Figure S8). Though we could not explain the reason for the high specificity of IgAgu1 judging from the comparison of the second structure between IgAad4 and IgAgu1 (Figure S8), we speculated that guanine bases of IgAgu1 are likely to contribute various conformations through hydrogen bonds and stacking interactions, as noticed in guanine quadruplex structures.

Though the maximal fluorescence anisotropy (FA) variation observed between unbound state aptamer and protein binding state aptamer is supposed commonly ranging from 50 to 100 mP, a smaller FA variation (20 to 40 mP) in our study is significant to monitor the concentration change of sAA or SIgA (Figure 6). Because the magnitude of FA variation was
reported to be influenced by selecting fluorophore, changing TYE665 to other fluorophore, for example, tetramethylrhodamine, could probably increase the range of FA values. Furthermore, using bivalent aptamer could be effective to enhance the target detection sensitivity.

The human saliva samples were measured by the FP method, as well. Particularly, both sAA and SIgA exhibited significant correlation between the FP and the ELISA method, when the measured mP values of the same saliva sample were compared with the absorbance (monitoring at 450 nm) recordings of the ELISA method (Figure 7). Especially in the case of sAA, Pearson’s correlation coefficient showed a high correlation of 0.94 between the FP and the ELISA method (Figure 7a).

In general, ELISA is a time-consuming method and needs two kinds of antibodies to detect the target molecules on the basis of the sandwich method. For example, sAA measurement using a human amylase AssayMax ELISA kit required about 4 h and eight experimental steps, while the FP aptamer assay needs only one kind of aptamer and could measure sAA concentration within about 5 min with two experimental steps. Therefore, the FP aptamer assay takes great advantage for the practical application of monitoring sAA or SIgA in real time.

Figure 6. FP measurement comparison of the fluorescent-labeled sAA-binding aptamer (AMYad1-2-3) and the fluorescent-labeled SIgA-binding aptamer (IgAgu1-3-3). (a) The AMYad1-2-3 were incubated with 0.12–2000 nM of the sAA (closed circles) or 15.6–2000 nM of SIgA (open circles) in SB2 and measured. The changes of the fluorescence anisotropy for sAA were fitted by a 4-parameter logistic nonlinear regression model. (b) The IgAgu1-3-3 were incubated with 0.12–2000 nM of the SIgA (open circles) or 15.6–2000 nM of sAA (closed circles) in SB2 and measured. The changes of the fluorescence anisotropy for SIgA were fitted by a 4-parameter logistic nonlinear regression model.

Figure 7. Correlation between the outcome of the FP and ELISA methods on human saliva samples. (a) The outcome of the FP using the fluorescent-labeled sAA-binding aptamer (AMYad1-2-3). sAA-ELISA: the saliva was diluted to 1/30,000; sAA-FP: the saliva was diluted to 1/1,000. (b) The outcome of the FP using the fluorescent-labeled SIgA-binding aptamer (IgAgu1-3-3). SIgA-ELISA: the saliva was diluted to 1/1,000; SIgA-FP: the saliva was diluted to 1/100. Because of the difference of detection sensitivity between FP and ELISA, the dilution factor of saliva was optimized for FP and ELISA, respectively. The mP values of the sAA and SIgA-binding aptamers using the FP method were compared with the absorbance recording (monitoring at 450 nm) from the ELISA kit. Sixteen saliva samples were collected twice from eight people (30–50 years old) on different days.
the ELISA method. However, in this case aptamers should feature high binding affinity and specificity.

■ CONCLUSIONS

In the current study, we developed a new BAB modification by introducing a guanine base on uracil, and acquired a SlgA-binding aptamer that can selectively bind to SlgA, a highly specific salivary marker for immune stress. To our knowledge, this is the first report of an aptamer that binds to SlgA in human saliva. The sequences of sAA and SlgA-binding aptamers were also minimized and optimized, while a new simple detection system, capable of quantifying sAA and SlgA in saliva through the FP method, without B/F separation, was developed. The recorded values of sAA and SlgA in human saliva samples showed significant correlation with the results of sAA and SlgA recordings of the predominant ELISA method. These results strongly suggest that a new simple test system for salivary stress markers can emerge, with anticipated applications on future large-scale cohort studies that require sAA and SlgA recordings. Future plans aim also to the development of novel aptamers of high affinity and specificity that could selectively target various salivary biomarkers, through diverse BAB modifications.

■ ASSOCIATED CONTENT

* Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b03450.

Materials and additional text showing detailed experimental procedures, characterization of all compounds, and Supplementary Scheme S1, Table S1, and Figures S1–S8 (PDF).

■ AUTHOR INFORMATION

Corresponding Authors
*Phone: +81 3 5317 9398. E-mail: mkuwa@chs.nihon-u.ac.jp.
*Phone: +81 3 5534 2619. Fax: +81 3 5534 2620. E-mail: iku-shiratori@cx.jp.necc.com.

ORCID
Masayasu Kuwahara: 0000-0002-0810-4627
Ikuo Shiratori: 0000-0003-3713-4910

Notes
The authors declare no competing financial interest.

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