Fast Affinity Induced Reaction Sensor Based on a Fluorogenic Click Reaction for Quick Detection of Protein Biomarkers

Jingxin Liu,† Mohammed A. A. Abdullah,‡ Liwei Yang,† and Jun Wang*,†

†Multiplex Biotechnology Laboratory, Department of Biomedical Engineering, State University of New York at Stony Brook, Stony Brook, New York 11788, United States
‡Department of Chemistry, State University of New York, University at Albany, Albany, New York 12222, United States

Supporting Information

ABSTRACT: Despite numerous biosensors currently available, the routine biomarker detection still largely relies on traditional ELISA and Western blot. Those standard techniques are labor intensive and time-consuming. Herein we introduce a fast affinity induced reaction sensor (FAIRS) that overcomes a few limitations of traditional and emerging biosensors. FAIRS is a general, one-step method and is naturally specific in detection. FAIRS probes are composed of a sandwich ELISA antibody pair that is conjugated with two fluorogenic click chemicals. This technology leverages significant differences of antibody affinity and chemical reaction rate, which are characterized to guide probe design. The stability, sensitivity, detection range, and response time are fully characterized. Application to IL-6 detection using blood serum and cell culture medium demonstrates that FAIRS can quantify IL-6 with high sensitivity in one step. With the unique features, FAIRS probes may find broad applications in medical sciences and clinical diagnostics, where quick detection of biomarkers is demanded.

Simple, high-performance sensing technologies for the detection of protein biomarkers are critical for disease diagnosis. Conventional protein detection techniques largely rely on an antibody-based, multistep, enzyme-linked, immunosorbet assay (ELISA) and Western blotting, which are usually time- and labor-consuming. In recent years, many emerging biosensors are introduced that feature a combination of high sensitivity and specificity, one step without washing, low-end instrument or device free, and fast response. Among them nanotechnology-based biosensors power the field to a new level. Surface plasmon resonance (SPR) in gold films with localized SPR and microring resonators has been developed to sense cytokine in real time.1–3 Plasmonic nanohole array further achieves label-free detection of biomarkers with large field of view for high-throughput assays.4–6 Nanoparticles and graphene have also been demonstrated for one-step detection of proteins when coupled with various mechanisms of surface enhanced Raman scattering, chemiluminescence, light scattering, and Förster resonance energy transfer (FRET).7–11 Many of those sensors need assistance of special instruments that a typical biomedical laboratory does not have or they are only applicable in limited circumstances. A more general biosensor possessing multiple merits is highly demanded to fit the needs of clinical diagnosis, drug discovery, and biomarker screening. Proximity response has been exploited to detect biomolecules simply in one step. For example, when DNAs are brought closely in space, the thermodynamics of DNA hybridization can trigger binding or separation of DNA strands selectively. This property has been widely used to design DNA devices for molecular diagnostics, sensing, and imaging applications.12–16 However, the sensor design generally involves a few DNAs, and thus, the unprocessed biological samples may interfere with the assay accuracy. FRET occurs when fluorophores or quantum dots are in proximity due to the affinity between biomolecules.17–19 But, the FRET-based sensors usually have a limited signal-to-noise ratio. The commercially available alpha bead-based proximity immunoassay overcomes that limitation. It requires a special signal reading instrument that is not available in most research institutions.20 There are other

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proximity triggered biochemical reactions that have been applied in DNA detection. Split fluorescent proteins become fluorescent after reassembly from two nonfluorescent fragments driven by additional DNA hybridization. The restoration of fluorescence takes only a few minutes when the tagged DNAs find their complementary parts. The similar idea for DNA detection is proximity fluorescent click reaction. Click chemistry is fast, simple to use, stable in aqueous solution, and orthogonal to biochemical processes. It has shown remarkable value in molecular imaging, medicinal chemistry, drug development and discovery, and chemical biology. Fluorophore can be inactivated by click chemistry on the same molecule, and the fluorescence is restored after click reaction. Therefore, no washing step is required for sensing biomolecules.

Here we introduce a wash-free, fast-response biosensor that takes advantage of differential kinetics of antibody—antigen affinity and click reaction. Tetrazine (TZ)-BODIPY is covalently linked with capture antibody via long chain polyethylene glycol (PEG) and azabenzonorbornadiene (AN) is conjugated with detection antibody via the same PEG linker. This fast affinity induced reaction sensor (FAIRS) respond rapidly with $t_{1/2} = 6.5$ min, once IL-6 as antigen is present in a solution. Without IL-6 recombiant protein, the FAIRS probes can be stable for $>24$ h without significant change of background or signal. Sensitivity and response time have been thoroughly studied. We have investigated the kinetics of the sensor and conclude the general design rule for proximity sensing. The FAIRS probes have been applied to detect IL-6 in human blood serum as well as in supernatants of stimulated microglial cells. Through quantification, we found the IL-6 concentrations detected by FAIRS probes are consistent with those by spiking and conventional ELISA.

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Experimental Section

Chemicals and Reagents. tert-Butyl 11-azatricyclo[6.2.1.02,7] undeca-2,4,6,9-tetraene-11-carboxylate (Chemical and Reagents) is obtained by a 100 K centrifugal filter and washed 3 times with acetone.

Preparation of TZ-PEG3400-Capture Antibody. Synthesis of fluorescent BODIPY-TZ-NHS ester is assisted by triethylamine and clicked 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Pierce), N-hydroxysulfosuccinimide (Sulfo-NHS; Thermo Fisher Scientific), aliphatic amine latex beads (2% w/v, 0.4 μm) Thermo Fisher Scientific, phosphate-buffered saline tablets (PBS; MP Biomedicals), 0.2 M carbonate buffers (Alfa Aesar), ELISA MAX Standard Set Human IL-6 (BioLegend), TMB reagents (BioLegend), Amicon 100 K carboxyl centrifugal filter Unit (Thermo Fisher Scientific), Zeba 7K MWCO Spin Desalting Columns (Thermo Fisher Scientific), HyClone Iscove’s Modified Dulbecco’s Medium (IMDM; GE Healthcare Bio-Sciences), Fetal Bovine Serum (FBS; GE Healthcare Bio-Sciences), triluorooacetic acid (Sigma-Aldrich), d-octol human serum (ZenBio), and Dulbecco’s Modified Eagle Medium/Ham’s F-12 (DMEM/F12; GE Healthcare Bio-Sciences), 1% penicillin/streptomycin (Thermo Fisher Scientific), LPS (E. coli K12, 1 μg/mL; Invitrogen).

Cell Culture and Stimulation. Human C20 microglial cell line is a gift from David Alvarez-Carbonell at Case Western Reserve University. C20 cells were cultured in DMEM/F-12 medium supplemented with 10% FBS at 37 °C in humidified 5% CO2 incubator. Before collection of supernatants, the cells were plated at a density of 2 × 105 cells/well on a 96-well plate. They were either stimulated by 1 μg/mL LPS for 24 h or kept in medium without stimulation as control. Culture medium from wells were collected and centrifuged at 3000 rpm for 10 min to remove debris. The supernatants were obtained for measurement of IL-6 concentration by various approaches.

Preparation of AN-PEG3400-Detection Antibody. tert-Butyl 11-azatricyclo[6.2.1.02,7] undeca-2,4,6,9-tetraene-11-carboxylate (200 mg) was deprotected first in trifluoroacetic acid (TFA; 2 mL) to generate 7-azabenzonorbornadiene (AN). The reaction mixture was stirred for 1 h at RT, and then the solvent was evaporated in vacuum. The crude product was purified by crystallization with ethyl acetate. To prepare AN-PEG3400-Biotin, the purified AN (50 mM in anhydrous DMF, 100 μL) was reacted directly with NHS-PEG3400-Biotin (10 mM in anhydrous DMF, 100 μL) for 1 h at room temperature, catalyzed by triethylamine (0.25 μL). The product was filtered with a 7 K Zeba spin column to remove excess AN.

A total of 200 μL of biotinylated detection antibody at 0.5 mg/mL was conjugated with 1 mg/mL streptavidin at the molar ratio of 1:1 for 0.5 h. The excess streptavidin was removed by a 100 K centrifugal filter (Amicon). Then 5 μL of AN-PEG3400-Biotin at 1 mM was added to streptavidin-detection antibody conjugate, and the mixture was incubated for 1 h at room temperature. The excess AN-PEG3400-Biotin was removed by 2 μm microbeads coated with streptavidin. The AN-conjugated detection antibody was further purified and concentrated by a 100 K centrifugal filter. To validate the final product, TZ (17.7 μM, 100 μL in DMSO) was added in the final product (1 mg/mL, 5 μL) and 100 μL of PBS on 96-well plates. The enhancement of fluorescence was used to determine whether the final conjugate was correctly produced. Fluorescence intensity was read by a microplate reader (Synergy H1 Hybrid Multi-Mode; BioTek) with an excitation at 488 nm and an emission at 525 nm.

Preparation of TZ-PEG3400-Capture Antibody. Synthesis of fluorescent BODIPY-TZ-NHS ester is assisted by WuXi AppTec (Figures S2 and S3). A total of 300 μL of this chemical at 16.3 mM in anhydrous DMSO was mixed with 0.16 mg NH2-PEG3400-COOH at a molar ratio of 25 to 1, and the mixture was stirred for 1 h at room temperature. The product was purified by a 10 μL cellulose filter and washed 3 times with MES (pH = 6) buffer. The solution was centrifuged at 10000 rpm three times to remove excess insoluble Tz-NHS ester, and the concentration of TZ-PEG3400-COOH was adjusted to 0.166 mM.

A total of 2 mg EDC and 5 mg of sulfo-NHS in MES buffer (1 mL, pH = 6) were mixed and incubated for 15 min, before taking 100 μL of the mixture into 50 μL of TZ-PEG3400 COOH at 0.1 mM. After a 1 h incubation, 1.2 μL of mercaptoethanol was added to quench the unreacted EDC. The solution was desalted by a 7 K zeba desalting column to remove small chemicals, including byproducts, and to collect TZ-PEG3400-NHS.

IL-6 capture antibody was concentrated to 1 mg/mL in 20 mM carbonate buffer, measured by UV–vis Spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific). A total of 100 μL of IL-6 antibody was mixed with 100 μL of freshly
Figure 1. Synthesis of FAIRS conjugates and fluorogenic click reaction. (a) Synthesis of AN conjugated detection antibody with PEG<sub>1400</sub> spacer arm. (b) Synthesis of TZ conjugated capture antibody with PEG<sub>1400</sub> spacer arm. (c) Schematic of fluorogenic click reaction with AN conjugated detection antibody and Tz conjugated capture antibody in the presence of antigen.

made TZ-PEG<sub>1400</sub>-NHS for 1 h, before purification by fast protein liquid chromatography (FPLC; BioRad) equipped with a Superdex 200 10/300 GL column (GE Healthcare). The collected conjugate was further concentrated by a 10 K centrifugal filter, and the concentration was measured by a UV–vis spectrophotometer. To validate the final product, a total of 100 μL of AN at 10 μM in PBS was added to 5 μL of the conjugate. The fluorescence was monitored in real time, and a >20× increase of signal passed the validation.

Calculation of Reaction Rate. AN-PEG<sub>1400</sub>-detection antibody and TZ-PEG<sub>1400</sub>-capture antibody were mixed directly with IL-6 recombinant protein in PBS for determination of the reaction constant. The measurements were performed under pseudo first order condition, using fixed amount of IL-6 protein (8000 pg/mL) and an excess of antibody conjugates at 0.5, 0.1, 0.05, and 0.01 mg/mL. Once the mixtures were loaded to wells of a 96-well plate, fluorescence signals were collected intermittently at select time points (0, 10, 20, 30, 40, 50, and 60 min). Fluorescence intensity versus time was fitted to a first order exponential equation $y = y_0 + A_1 \exp \left(-\frac{t}{t_1}\right)$, where the pseudo first order rate constant ($k_{obs}$) equals 1/$t_1$. The obtained $k_{obs}$ values were then plotted against antibody conjugate concentrations. The slope of the fitted straight line corresponds to the second order rate constant ($k_2$). Each measurement was repeated in triplicate.

One-Step Cytokine Detection. AN-PEG<sub>1400</sub>-detection antibody and TZ-PEG<sub>1400</sub>-capture antibody as FAIRS probes were diluted 2 and 3 times for FAIRS detection and conventional ELISA assay.

For conventional ELISA assay, capture antibody at 10 μg/mL in PBS was incubated in a 96-well plate overnight at 4 °C. After three washes with 3% BSA in PBS, various concentrations of IL-6 recombinant proteins were added to wells and incubated for 2 h. The unbound proteins were washed three times, detection antibody, with 100 times dilution, was added to each well, and they were incubated for 1 h. The wells were washed another three times, followed by incubation with diluted streptavidin-HRP conjugate with 500 times dilution for 30 min. TMB reagents were transferred to wells to develop colors for the reading of optical density with a microplate reader.

Statistical Analysis. Fluorescence intensity data were analyzed using GraphPad Prism-6 (GraphPad Software). The same program was used to fit curves and find rate constants. Data points on plots were expressed as mean ± S.D., with each repeated for at least three times experimentally. Significant differences between groups were determined using a one-way analysis of variance (ANOVA) with Dunnet’s multiple comparisons test, considering $P < 0.05$ as significant differences.

RESULTS AND DISCUSSION

The 2nd order kinetics of antibody–antigen affinity is normally above $1 \times 10^6$ M<sup>-1</sup> s<sup>-1</sup>,<sup>30</sup> while even the fastest TZ-trans-cyclooctene (TCO) click reaction can barely reach that rate.<sup>43</sup> Such a large discrepancy of reaction rates underlies the design of FAIRS probes for one-step detection. The sandwich ELISA antibody pair that is purchased from Biolegend and is validated by conventional well-plate-based ELISA has been conjugated with their respective fluorogenic click chemicals to be FAIRS probes. When the probes are mixed together in a low concentration, click chemicals will not react significantly due to the relatively slow reaction rate. Once the antigen is present, the antibodies rapidly form a sandwich structure due to fast binding kinetics, which physically move click chemicals in

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proximity, leading to higher local concentration and click reaction to restore fluorescence.

Synthesis of FAIRS probes is shown in Figure 1. AN is conjugated with detection antibody through long chain PEG₃₄₀₀ mediated with streptavidin–biotin interaction. Possible steric hindrance has not been found to influence the binding affinity of detection antibody (Figure S4). Such a long arm is designed to ensure AN is approachable by TZ from capture antibody. TZ chemical contains BODIPY dye, which fluorescence is suppressed by the unreacted TZ. AN as a strained dienophile reacts with TZ through irreversible inverse-Alder reaction and release dinitrogen and release the product.TZ and capture antibody are also spaced by the same PEG₃₄₀₀ to free TZ from spatial hindrance. The conjugation protocol has been optimized to have 3–5 PEG₃₄₀₀ spaced click chemicals on both capture antibody and detection antibody. Such ratio for capture antibody is controlled by the stoichiometric chemical modification of antibody and is validated by FPLC spectrum (Figure S5), and the ratio for detection antibody is managed by the amount of streptavidin and biotinylated chemicals. In our previous studies, this ratio of modification with a long chain oligonucleotide is the optimal one, without significantly impairing antibody binding capabilities.

The long chain PEG₃₄₀₀ is a hydrophilic, flexible polymer that facilitates solubility and exposure of AN and TZ in aqueous solutions or culture media. It is chemically inert and does not have nonspecific binding to most biomolecules. Once the PEG₃₄₀₀ is stretched, it can reach a length between 25 and 35 nm, which is longer than a typical monoclonal antibody at 10–15 nm. Thus, even if AN and TZ are on the far ends of the formed antibody sandwich structure, they still have a chance to physically contact and react to each other.

We have fully characterized the FAIRS probes in terms of turn-on fold, stability, response time and kinetics. Figure 2a shows 23-fold fluorescence turn-on signal when TZ-BODIPY is reacted with AN. The peak emission wavelength is measured to be 535 nm. Excellent stability has been found for both reacted and unreacted FAIRS probes. The control sample without antigen IL-6 marginally increases the signal by 5.1% over 24 h, and the signal after detection of IL-6 keeps stable for the same period. When IL-6 recombinant protein is added to the FAIRS probes, they immediately respond by increasing fluorescence signal within minutes (Figure 2b,c). Various probe concentrations have been tested to characterize the response time and second reaction kinetics (Figure S1). All the curves completely reach plateaus after ~20 min. The fastest response was observed at 0.5 mg/mL (3.3 μM) concentration of probes; however, this condition is not recommended for a practical application due to the high cost of reagents. Despite different probe concentrations used, the characteristic t₁/₂ of half height is uniformly at about 6.5 min, with standard deviation of 1.0 min, which is comparable to other available one-step technologies for protein detection.

The second order rate constant of FAIRS detection is calculated to be 5.263 × 10⁻¹² M⁻¹ s⁻¹ (Figure 2d). It is lower than the rate constant of most antibody–antigen binding, possibly because the aforementioned 10⁶ M⁻¹ s⁻¹ rate corresponds to single antibody–antigen binding instead of two antibody binding to the same antigen, and chemical modification of the antibody may also influence the binding affinity to certain degree. We have also measured and obtained the second order rate constant of AN-TZ click reaction only at 5.15 M⁻¹ s⁻¹. This indicates the proximity brought by antibody–antigen binding that enhances the AN-TZ reaction by ~10²² times. Theoretically, the FAIRS probes at 0.05 mg/mL are equivalent to 200 molecules/μL. When in proximity, 3–5 molecules with an average 25 nm separation distance result in between 1.9 × 10⁸ to 3.2 × 10⁹ molecules/μL³, assuming all chemicals are accessible for the reaction. Or, the local concentration is raised to ~10²³ molecules/μL³.
324 concentration of AN or TZ has been theoretically boosted by 325 $1022\times$ increase of reaction rate. Therefore, a significant 326 increase in local concentration is the main factor contributing 327 to the fast response of FAIRS.

Figure 3 shows a sensor response at various concentrations 328 of recombinant IL-6 proteins. Except for 10 pg/mL, all 329 fluorescence intensities rise with time and reach plateau 330 between 12.5 and 20 min. The fluorescence data at 30 min 331 were used to generate calibration curve (Figure 3b), which 332 shows linearity over $10^2$ dynamic range (the higher 333 concentration was not measured due to significant deviation 334 from the physiological condition). The limit of detection 335 (LOD) is calculated to be 29 pg/mL, higher than LOD by 336 standard sandwich ELISA at <10 pg/mL. This trade-off is 337 saved by the fast response and simple procedure of our sensor. 338 Better sensitivity could be achievable by other design of 339 fluorogenic chemicals to improve signal-to-noise ratio and 340 brightness.

The FAIRS probes have been applied to measure cytokine 341 IL-6 in human blood samples that were spiked with 100 pg/ 342 mL IL-6 recombinant proteins. IL-6 is an important marker for 343 diagnosis of inflammatory diseases such as sepsis where IL-6 344 level in blood can be elevated to a range of $\sim 150$ to >1000 pg/ 345 mL. Blood serum was diluted 2 or 3× to lower autofluorescence background before FAIRS detection. In both diluted serum samples, fluorescence signals are increasing 350 over time, while they are significantly higher than background noise. After quantification using calibration curves, signals are 352 converted to the diluted concentration, which is further multiplied by the dilution times to calculate the original concentration. The sample of 2× dilution was calculated to 355 contain 111.0 pg/mL IL-6 before dilution, and the 3× dilution sample has 72.8 pg/mL IL-6 before dilution. When the 3× dilution sample was analyzed, the real IL-6 concentration was 358
only 33.3 pg/mL close to detection limit. That may cause the significant deviation from the actual amount. Nevertheless, there is no statistical difference of IL-6 concentration measured by these two dilutions.

363 The Fairs probes have been applied to the medium supernatant of cultured cells without stimulation (control) and the supernatant after lipopolysaccharide (LPS) stimulation for 24 h. C20 cell line is derived from immortalized human primary microglia, and it maintains microglial morphology, surface markers, and immune responses of microglia.\textsuperscript{33,36} Upon stimulation, C20 cells produce an array of inflammatory factors, including IL-1\beta, IL-6, CCL2, and CXCL10.\textsuperscript{37} Our results in Figure 4c show IL-6 is detectable in the stimulated samples, while the control sample has a negligible amount of IL-6. The quantified concentrations of IL-6 by Fairs method and by conventional well-plate based method were compared in Figure 4d. In both control samples and LPS stimulated samples, the quantified IL-6 is consistent between the two methods, as they do not have significant differences statistically.

### CONCLUSION

380 In summary, we have developed a differential kinetics driven Fairs technique for rapid, one-step detection of antigens. The Fairs detection is based on fast affinity of antibody and antigen and slow reaction of fluorescent click chemistry. The sensor has been fully characterized, and the response time is found to be 6.5 ± 1.0 min. Our study discovered that the significant increase of local concentration of click chemicals due to targeted antibody–antigen binding quantitatively match the differences of the intrinsic second order rate constants. This provides a guideline for further design of such sensors, taking advantage of a kinetics discrepancy. The Fairs probes have been applied to real samples and are found accurate in measuring IL-6 concentrations. With the multiple values including simple procedure of detection and high specificity and sensitivity, we envision that Fairs can find broad applications in the diagnosis of inflammatory diseases, drug and biomarker discovery, and those in a field setting.

### ASSOCIATED CONTENT

385 386 Supporting Information
387 The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b04502.
388 Curve fitting for kinetics calculation; synthesis route of chemicals; NMR spectrum of chemical; influence of steric hindrance; FPLC and nanodrop result of conjugation (PDF)

### AUTHOR INFORMATION

390 Corresponding Author
391 *E-mail: jun.wang.5@stonybrook.edu.
392 ORCID
393 Liwei Yang: 0000-0001-7834-0249
394 Jun Wang: 0000-0002-8781-8248

### Notes
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### REFERENCES

(37) Davis, R. L.; Buck, D. J.; McCracken, K.; Cox, G. W.; Das, S. Neuroimmunol. Neuroinflammation 2018, 60, 1–11.