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Bioelectrochemical Switches for the Quantitative Detection of Antibodies Directly in Whole Blood

Alexis Vallée-Bélisle,[†,‡,○,◆] Francesco Ricci,[∥,⊥,◆] Takanori Uzawa,[#] Fan Xia,[†,▽] and Kevin W. Plaxco*[,†,‡,§]

[†]Department of Chemistry and Biochemistry, [‡]Center for Bioengineering, and [§]Interdepartmental Program in Biomolecular Science and Engineering, University of California, Santa Barbara, California 93106, United States

[∥]Dipartimento di Scienze e Tecnologie Chimiche, University of Rome, Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy

[⊥]Consorzio Interuniversitario Biostrutture e Biosistemi "INBB", Rome, Italy

[#]Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

[▽]School of Chemistry and Chemical Engineering, Huazhong University of Science and Technology, Wuhan 430074, China

Supporting Information

ABSTRACT: The development of rapid, low-cost point-of-care approaches for the quantitative detection of antibodies would drastically impact global health by shortening the delay between sample collection and diagnosis and by improving the penetration of modern diagnostics into the developing world. Unfortunately, however, current methods for the quantitative detection of antibodies, including ELISAs, Western blots, and fluorescence polarization assays, are complex, multiple-step processes that rely on well-trained technicians working in well-equipped laboratories. In response, we describe here a versatile, DNA-based electrochemical "switch" for the rapid, single-step measurement of specific antibodies directly in undiluted whole blood at clinically relevant low-nanomolar concentrations.

The development of rapid, low-cost point-of-care approaches for the quantitative detection of antibodies would drastically impact global health by allowing more frequent testing, narrowing the delay between diagnosis and treatment, and improving the penetration of molecular diagnostics into the developing world.[1] The primary obstacle to achieving these ends, however, is that existing *single-step* methods for the quantitative detection of antibodies generally fail when deployed directly in complex clinical samples. For example, because of the confounding effects of nonspecific adsorption to the sensor surface, approaches that monitor adsorbed mass or refractive index, such as microcantilevers and surface plasmon resonance (SPR), are not selective enough to work directly in undiluted blood serum, much less in whole blood.[2]

In response to the selectivity problem encountered by adsorption-based approaches, we and others have recently described electrochemical platforms that, in approximate analogy to fluorescence polarization assays,[3] detect antibody binding to a DNA-attached epitope[4] or to an epitope only,[5] reducing the efficiency with which an attached redox reporter collides with (and thus exchanges electrons with) an underlying electrode. The selectivity of this collision-based mechanism is not perfect, however; and while such sensors perform reasonably well in undiluted blood serum, they fail when challenged in undiluted whole blood.[6] Since dilution requires operator intervention and ultimately reduces the detection limit (by the dilution factor), it would be best to develop a signaling mechanism that is selective enough to transduce the presence of antibodies directly in whole blood, an effort that we explored in this work.

Inspired by the impressive selectivity of naturally occurring chemosensory systems, recent work has resulted in the emergence of a new class of synthetic biosensors that mimic nature by coupling target recognition with a robust structure-switching mechanism.[7−12] These typically employ nucleic acids or proteins re-engineered to undergo a large-scale, binding-induced conformational change that separates (or brings together) two reporter moieties to produce an optical or electrochemical output.[13−18] Of particular note, electrochemical "switch-based" sensors have proven to be selective enough to be employed directly in complex clinical samples.[16] They are also rapid, reagentless, and easily multiplexed.[19] Motivated by these arguments, we have developed a versatile electrochemical switch that supports the rapid, quantitative detection of antibodies directly in whole blood at clinically relevant low-nanomolar concentrations.

The design of our switch takes advantage of the occurrence of two antigen-binding sites on each antibody,[11] which are separated by ∼12 nm[20] (Figure 1). Specifically, we used DNA to engineer a switch that brings into close proximity (<4 nm) two copies of an antigen, epitope, or hapten (hereafter called "antigen" for simplicity), via the formation of a stem-loop structure. The two antigens, both linked at the extremities of the DNA strand are thus located in the middle of the two strands of the stem (Figures 1 and 2). Upon binding of the antibody to one of these antigens, the high effective concentration of the second antigen provides the driving force to open the switch (more favorable bidentate binding.[21]) and thus separate the reporter elements. Our motivation for using DNA as the scaffold for our switch is threefold. First, the chemistry of DNA supports the addition of a variety of antigens ranging from small molecules to polypeptides and proteins, either during its automated chemical

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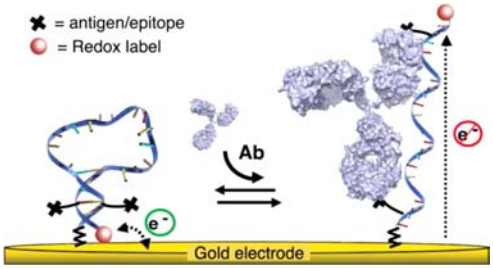

Figure 1. Antibody (Ab)-activated electrochemical "switch". The switch is composed of a single-stranded DNA probe containing two identical, covalently linked antigens (black crosses) and modified at one end with a redox label (methylene blue) and at the other end with a thiol group for convenient attachment to a gold electrode surface. In absence of the target antibody, the DNA adopts a stem−loop conformation that brings the two antigens in close proximity, and the redox label near the gold electrode, thus enabling rapid electron transfer. Upon binding of the relevant cognate antibody, the DNA probe opens, which moves the redox label far from the electrode, thus resulting in a lower electron transfer rate and Faradaic current. By coupling the recognition to a large-scale conformational change, we have created a sensor that is largely insensitive to the nonspecific adsorption that takes place when the sensor is deployed directly in complex biological samples.[2]

synthesis or through postsynthesis conjugation.[22] Second, DNA-based switches are robust (i.e., they are not triggered by nonspecific interactions) and relatively stable against degradation.[16,19] Finally, the base-pairing code of DNA renders it easy to tune the switch's thermodynamics to ensure that the sensor achieves optimal detection limits.[23]

As an initial proof-of-principle validation of these new molecular switches, we first fabricated and tested a switch employing the well-characterized hapten 2,4-dinitrophenol (DNP) (thus targeting anti-DNP antibodies[24]), which can be easily conjugated to DNA during commercial chemical synthesis [Figure 2; methods are described in the Supporting Information (SI)]. We designed the stem−loop sequence to remain largely closed in the absence of the antibody without being overly stabilized, which would harm affinity[23] and possibly favor the binding of two antibodies to a single switch. As described previously, to optimize the detection limit of such switch sensors, a switching equilibrium constant (K_S) of ∼0.5 (66% closed) represents the optimal tradeoff between low background and high affinity.[23] We achieved this equilibrium constant by linking a stem containing four Watson−Crick base pairs and a T−T mismatch to an unstructured, 18-base polythymine loop (∼10 nm) (Figure S1 in the SI). This linker is long enough to span the ∼12 nm distance between the two antigen binding sites on one antibody[20] (with each DNP linker providing an extra ∼1.5 nm) but short enough to ensure the cooperative binding of a single antibody to a single switch.[21]

For this "first-look" proof-of-principle demonstration, we converted the above switch into an optically reporting sensor by modifying its two termini with fluorescein (FAM) and Black Hole Quencher (BHQ-1), a well-characterized Förster resonance energy transfer pair with a convenient 5 nm Förster radius.[25] Consistent with its design principles, this optical switch responded to anti-DNP antibodies within seconds (Figure S2), producing a 3-fold increase in fluorescence at saturating target and achieving a subnanomolar detection limit (Figure 2a). Of note, this gain is lower than that seen for molecular beacons,[7] an analogous optical sensor for the detection of specific oligonucleotides; to ensure that one switch would not bind two antibodies simultaneously (and thus remain closed), we employed a stem with lower stability than is optimal for molecular beacons, raising the background and reducing the observed gain. In support of the proposed sensing mechanism, the binding of anti-DNP antibodies to the switch was inhibited by free DNP (Figure S2).

While the fluorescent readout described above is convenient for laboratory studies, the reduced equipment overhead of electrochemistry and its convenience for measurement directly in whole blood[26] renders electrochemical readouts better suited for point-of-care diagnostic applications.[16,19] To adapt the

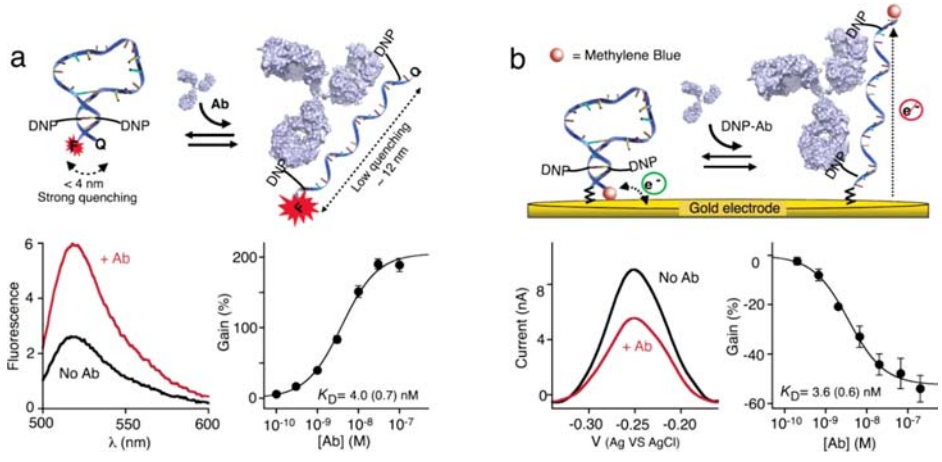

Figure 2. As an initial validation of the antibody-activated switch architecture, we used the well-characterized hapten DNP,[24] thus targeting anti-DNP antibodies (DNP-Ab). To do so, we designed a tetramodified DNA stem−loop structure containing two DNP moieties attached in the middle of the five-base-pair double-stranded stem and an 18-base polythymine loop (∼10 nm). The stability of the stem was "tuned" to slightly favor the closed state (switching equilibrium constant of ∼0.5; see Figure S1), as this represents the optimal tradeoff between low background (linked to higher stem stability) and high affinity (achieved at lower stem stability).[23] Using either (a) fluorescent reporters (5′-FAM and 3′-BHQ-1) or (b) an electrochemical readout (5′-C6-thiol group for attachment to the gold electrode and a 3′-methylene blue redox reporter), we observed robust responses to the addition of the sensor's specific antibody target (∼200% fluorescence gain and ∼50% signal current reduction). The two sensors exhibited similar detection limits (0.3 and 0.6 nM, respectively, at a coefficient of variation of 3). The titrations shown in this figure represent averages of measurements conducted with three sensors (independently fabricated in the case of the electrochemical sensor), and the error bars reflect the standard deviations.

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antibody switches to this more promising mechanism, we attached the DNA stem−loop to a gold electrode by replacing the 5′-FAM with a 5′-C6-thiol group (enabling easy attachment via the formation of a self-assembled sulfur monolayer upon codeposition with 6-mercaptohexanol) and the 3′-BHQ-1 with the redox reporter methylene blue (Figure 2b). As expected, in the absence of its target antibody, the switch produced a significant Faradaic current. In the presence of a saturating amount of anti-DNP antibody (30 nM), the current fell by ∼50% within a few minutes (Figure 2b). The electrochemical switch displayed a subnanomolar detection limit similar to that of the optical switch, suggesting that surface attachment did not significantly alter the binding-induced structural change that takes place upon antibody binding.[27] Of note, this detection limit is well below clinically relevant antibody concentrations.[31,32]

A potential limitation of the switch architectures described above is that they require the synthesis of a tetramodified DNA strand containing the two antigens and the anchor moiety and redox label (or the fluorophore and quencher in the case of the optical switch). In response, we also designed a modular DNA switch that reduces the cost and complexity of the synthesis by enabling attachment of the two antigens via hybridization of two copies of a single antigen-modified oligonucleotide (Figure 3 top). To test this modular design, we used the well-characterized hapten digoxigenin (Dig) as our antigen (Figure 3) and found that the gain (∼45%) and detection limit (∼1 nM) compared closely to those of the tetramodified DNA strand switch described above (Figure 2b). The modular switch was also highly specific, remaining "unactivated" even upon the addition of a 100-fold higher concentration of random human antibodies (Figure 3).

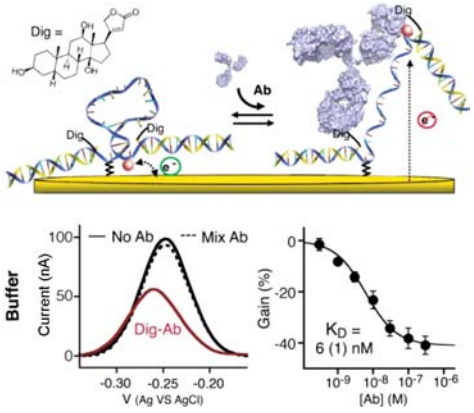

Figure 3. To simplify the switch synthesis (i.e., to avoid the synthesis of a tetramodified DNA probe), we designed a modular architecture in which the two antigens are attached via hybridization of two copies of a 17-base DNA strand (shown in yellow) modified only via the addition of the antigen (here the hapten Dig). The modular stem−loop scaffold contains a frame inversion near one end to allow for symmetrical labeling with two copies of the same modified recognition strand, reducing the fabrication cost and complexity (Figure S1C). The gain and affinity of this modular switch compared closely to those of the nonmodular electrochemical sensor (Figure 2b). The modular sensor showed high specificity, as it was insensitive to the addition of a 3 μM mixture of human antibodies (dashed line). The titrations shown in this figure represent averages of measurements conducted with three independently fabricated sensors, and the error bars reflect the standard deviations.

Unlike previously reported single-step antibody measurement methods,[4,5,11,28−30] the structure-switching mechanism underlying this new antibody sensor architecture is selective enough to be employed directly in whole blood (Figure 4). Indeed, the gain and detection limit observed when the sensor was deployed directly in whole blood (Figure 4 top) were very close to those observed when the sensor was employed in simple buffer solutions. This close similarity suggests that the electrochemical switch should remain relatively insensitive to sample-to-sample variations other than those associated with a change in the specific antibody concentration (e.g., changes in pH, ionic strength, etc.).

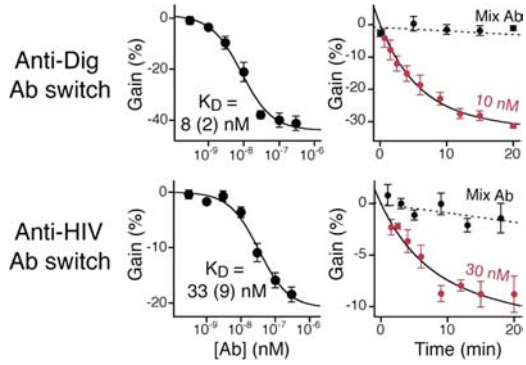

Figure 4. Antibody switches support the rapid detection of various specific antibodies directly in whole blood with response time constants of less than 5 min. (top) The anti-Dig antibody sensor performed similarly well when challenged directly in whole blood, producing a rapid decrease in the current signal in blood containing 10 nM anti-Dig antibody (with a response time of <5 min) but not in blood doped with a mixture of 30 nM anti-HIV antibody (see below) and a 100-fold higher concentration of random, pooled human immunoglobulin G (dotted line). We also tested a switch containing a 14-residue polypeptide epitope taken from the HIV-1 protein gp41 that is specifically recognized by the clinically relevant anti-HIV antibody AF5.[36] The switch performed robustly in whole blood with a gain (20%), detection limit (10 nM), and response time comparable to those of the anti-Dig antibody switch. The titrations and kinetics shown in this figure represent averages of measurements conducted with three independently fabricated sensors, and error bars reflect the standard deviations.

The modular switch improves the ease with which we can attach and employ more complex and clinically relevant antigens, including polypeptide epitopes. To demonstrate this, we fabricated and tested a switch presenting a 14-residue epitope from the HIV-1 protein gp41 that binds the clinically relevant anti-HIV antibody AF5.[36] Again, the switch performed robustly in whole blood (gain ∼20%, detection limit ∼10 nM) with a response time similar to that of the anti-Dig antibody switch (Figure 4 bottom). The origin of the smaller gain displayed by the anti-HIV antibody sensor is unclear, but it could be due to the larger recognition element (∼18 amino acids) employed in this sensor, as this could destabilize the stem−loop via steric repulsion, which in turn would increase K_S and reduce the population shift seen upon antibody addition. Alternatively, the average distance between the epitope binding sites may differ from antibody to antibody, which could affect the extent to which the open, target-bound state is "off".

Here we have described a new class of bioelectrochemical switches for the detection of specific antibodies. They respond rapidly (<10 min) and sensitively to their targets at low nanomolar concentrations and perform well even when

challenged directly in undiluted whole blood. Moreover, this new class of electrochemical switches is versatile, as they support the use of both small-molecule haptens and polypeptide epitopes for antibody detection. We believe that they can likely be engineered to support the detection of even non-antibody targets as long as the targets present two or more recognition sites spaced far enough apart to induce the required stem opening.

As the basis for sensors, the switches described here may provide significant advantages relative to existing antibody detection technologies. Despite the recent development of innovative homogeneous immunoassays,[33] for example, current methods for the quantitative detection of antibodies still mostly rely on ELISAs and Western blots, which are multistep, wash- and reagent-intensive processes that require specialized technicians and take several hours. While immunochemical dipsticks are far more convenient and well-suited for point-of-care applications, they are qualitative.[34] Switch-based sensors, in contrast, are as quantitative as laboratory based assays but likely can also be adapted to convenient point-of-care formats.[35] Finally, because of the high selectivity of their structure-switching signaling mechanism, the antibody-activated switches are relatively insensitive to nonspecific absorption of interferents, even when challenged with samples as complex as undiluted whole blood. In view of their advantages, the antibody-activated switches presented here appear to be well-positioned for adaptation as, for example, point-of-care diagnostics.

■ ASSOCIATED CONTENT

Supporting Information

Supporting methods, figures, and references. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

kwp@chem.ucsb.edu

Present Address

○Département de Chimie, Université de Montréal, Montréal, Québec, Canada H3C 3J7.

Author Contributions

◆A.V.-B. and F.R. contributed equally.

Notes

The authors declare no competing financial interest.

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