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Article

Rapid, Cost-Effective Peptide/Nucleic Acid-Based Platform for Therapeutic Antibody Monitoring in Clinical Samples

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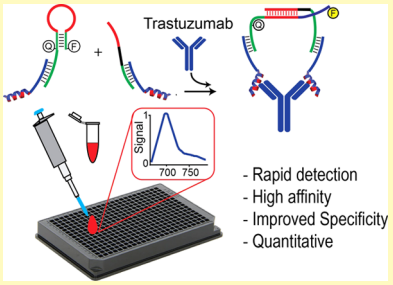
ABSTRACT: We demonstrate here a homogeneous assay, named NanoHybrid, for monoclonal antibody quantification directly in serum samples in a single-step format. NanoHybrid is composed of both synthetic peptide nucleic acids (PNAs) and nucleic acid strands conjugated to recognition elements and optical labels and is designed to allow fast fluorescence quantification of a therapeutic antibody. More specifically, we have characterized our analytical assay for the detection of trastuzumab (Herceptin), a monoclonal antibody (mAb) drug used for breast cancer treatment and for tumors overexpressing the HER2/neu protein. We show here that NanoHybrid is capable of performing fast drug quantification directly in blood serum. The results obtained with a pool of samples from breast cancer patients under trastuzumab treatment are compared with CE-IVD ELISA (enzyme-linked immunosorbent assay) showing a good agreement (Cohen's K = 0.729). Due to the modular nature of the NanoHybrid platform, this technology can be programmed to potentially detect and quantify any antibody for which a high-affinity recognition element has been characterized. We envision the application of NanoHybrid in a point-of-care (POC) drug monitoring system based on disposable kits for therapeutic drug management.



KEYWORDS: *serological test, trastuzumab, immunotherapy, optical biosensor, DNA nanoswitches*

The use of monoclonal antibodies (mAbs) had a huge positive impact, representing an additional therapeutic option for the treatment of several diseases, including cancer and autoimmune disorders, radically ameliorating the prognosis.[1] The therapeutic mAb market has skyrocketed in the past decades, and since the approval of Orthoclone in 1986, administered as prevention for kidney transplantation rejection, the interest in the development of these drugs has increased.[1] It is worth noting that therapeutic mAbs are expensive biological drugs, which present a variable percentage of nonresponder patients.[2−4] Recent studies reviewed the importance of mAb theranostics, concluding that clinical care could greatly benefit from therapeutic drug monitoring (TDM) of different mAb classes,[5,6] to adjust the drug dose, to reach major effectiveness, and to promptly modify the therapy, in the case of nonresponder patients.

A lack or loss of response to mAb treatment can be due to inadequate blood concentrations of the drug, to the presence of antidrug antibodies (ADA) raised against the drug, or to the sequestering effect that is exerted by free unbound portions of the targeted antigen.[7,8] Despite recent findings showing that the fluctuation of the antibody concentration in the blood can affect the therapeutic efficacy and therefore the prognosis, the traditional clinical practice does not include yet an individual pharmacokinetic-based adjustment of the therapeutic dose. Currently, most of the techniques for mAb quantification are ELISA-based assays,[9] a technique that is sensitive and robust but time-consuming and labor-intensive. Despite immunoassays representing the gold standard in mAb quantification, they do not provide a rapid response and are not suitable for medical clinics nor applicable for self-testing devices. Lateral flow immunoassays are noninstrumental and rely on visual detection of colored lines for results, enabling easy portability and allowing testing at any time and at any place by nontechnical personnel; however, they are qualitative and not quantitative, and this impedes their application in therapeutic monitoring.

Motivated by these concerns, we have recently developed a fluorescence-based programmable nucleic acid nanoswitch, here named as NanoHybrid, capable of detecting clinically relevant antibodies directly in human plasma in a one-step and no-wash format.[10] The NanoHybrid platform is composed of both synthetic peptide nucleic acids (PNAs) and nucleic acid strands conjugated to recognition elements and optical labels, designed to allow fast fluorescence quantification of a target IgG antibody. To demonstrate the possibility to use such a novel analytical platform for immunotherapy monitoring, we

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selected trastuzumab (or Herceptin), a growth-inhibitory humanized monoclonal anti-HER2/neu, as the antibody target, currently used for passive immunotherapy in the treatment of breast cancer.[11] Such an antibody is usually infused and the concentration is kept between 35 μg/mL (240 nM) and 123 μg/mL (845 nM) for a determined period of time, depending on the treatment.[12] To achieve the correct therapeutic efficacy, Herceptin levels must be maintained above 20 μg/mL (137 nM) in blood serum.[12] Although trastuzumab has largely had clinical benefits in early and advanced HER2+ (human epithelial growth factor receptor 2) breast cancer, many patients fail to respond to it.[13,14] Furthermore, circulating shed HER2 extracellular domain (ECD) is able to bind to free trastuzumab in the bloodstream acting as an inhibitor,[15,16] thus enhancing trastuzumab clearance.[17] Closely monitoring and consequently adjusting trastuzumab blood levels thus aim to reduce drug side effects and increase therapy effectiveness.

RESULTS AND DISCUSSION

Modular Design of the DNA Nanoswitch. Our fluorescence-based NanoHybrid nucleic acid platform is composed of three key elements. The first element (strand #1, Figure 1A) is a synthetic DNA strand that is designed to adopt a stem−loop conformation with a 20 nt (nucleotide) tail. The stem−loop spontaneously forms, thanks to two self-complementary portions of 5 nucleotides, and displays a loop of 15 nucleotides (red portion). Strand #1 is also labeled with a fluorophore/quencher pair placed at the end of the stem portion so that in the stem−loop conformation the proximity of the quencher to the fluorophore guarantees an optical off state (low fluorescence signal). The second element (strand #2, Figure 1A) is a synthetic DNA strand containing three motifs: a 15 nt domain (light red portion in strand #2, Figure 1A) fully complementary to the loop of strand #1, a poly-T linker for flexibility purposes (20 nucleotides), and a 17 nt tail. The third element (strand #3, Figure 1A) is a PNA−peptide chimera sequence composed of a 17-residue PNA domain (blue portion, strand #3, Figure 1A) conjugated to a synthetic peptide (helical-like motif, Figure 1A) that acts as the recognition element for the target antibody. In this specific case, the recognition element is a 12-aa-long peptide (sequence QLGPYELWELSH) that has been reported to be specifically recognized with high affinity by trastuzumab.[18,19] The PNA domain in strand #3 (blue strand #3, Figure 1A) is designed to be complementary to the tails of strand #1 and strand #2, allowing an efficient hybridization to these strands and the formation of, respectively, the reporter module (strand #1 + strand #3) and the input module (strand #2 + strand #3). Instrumental for our strategy is the thermodynamic optimization of these synthetic modules that are designed so that in the absence of the target antibody no hybridization between strand #1 and strand #2 will occur.

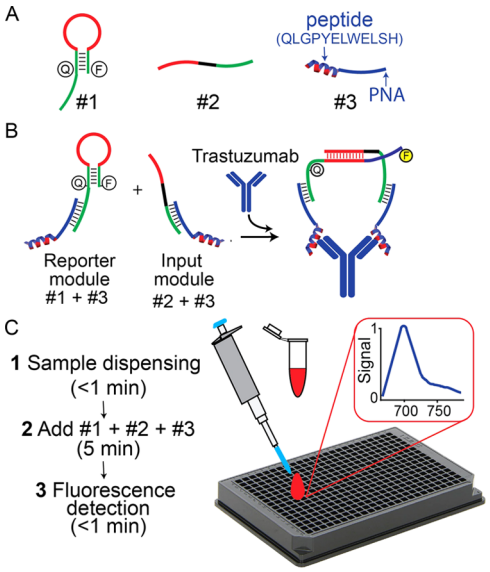

Figure 1. Fluorescence-based NanoHybrid nucleic acid platform for antibody detection. (A) NanoHybrid components. (B) Antibody binding to the reporter and input modules induces the colocalization of the components in a confined volume. This brings strand #1 and strand #2 to close proximity, thereby dramatically increasing their local concentration and allowing efficient hybridization between the two modules. As a result, the fluorescence signal intensity increases as a function of the concentration of the antibody in the sample solution. (C) NanoHybrid allows rapid, single-step fluorescent detection of the target antibodies directly in bodily fluids by performing three steps (sample dosing, the addition of the reporter and input modules, and fluorescence readout).

Under these conditions, a low fluorescence signal will be observed. In the presence of the specific target antibody, the binding of the peptide of the two modules to the two binding sites of the target antibody will bring strand #1 and strand #2 to close proximity (colocalization effect, Figure 1B). As a result, this will dramatically increase their local concentrations and allow their efficient hybridization, generating an increase in the fluorescent signal. The high specificity and signal gain of the NanoHybrid platform, due to the specific antibody-induced conformational change, are the core mechanisms that make this sensor selective and sensitive enough to be utilized directly in complex biological samples as blood serum.[10]

NanoHybrid Optimization. We first performed several experiments to optimize NanoHybrid components and their relative concentrations. Initially, we studied the ability of the 12-aa peptide to be employed as an efficient recognition element for trastuzumab.[19] To do so, we designed three variants of the PNA−peptide strand (strand #3 in Figure 1) with different orientations of the PNA strand linked to either the N-term or the C-term of the peptide (Figure 2A). We then employed these variants as recognition elements in a standard ELISA platform to evaluate their ability to bind trastuzumab (Figure 2A). The PNA probe designed with the peptide exposing the C-term portion recognizes trastuzumab in a dose-dependent manner (#3, Figure 2A),[18] whereas the other two variants (V1 and V2, Figure 2A) having different PNA−peptide sequence orientations show lower reactivity. We then selected this PNA−peptide variant (#3) for our next experiments with the NanoHybrid platform. First, we proved the formation of both reporter (#1 + #3) and input (#2 + #3) modules (Figure SI1). Since we envisaged the NanoHybrid application for trastuzumab quantification in serum samples, all of the experiments for the platform design and optimization were performed in 10% v/v standard serum to be immediately compatible with the desired application. As a standard serum, we used pooled serum collected from the clot of healthy human donors.

We initially tested the effect of the concentration of the PNA−peptide chimera probe (strand #3) on the signal output. The highest fluorescence signal gain was obtained using the PNA probe at a concentration of 750 nM (Figure SI2). Then,

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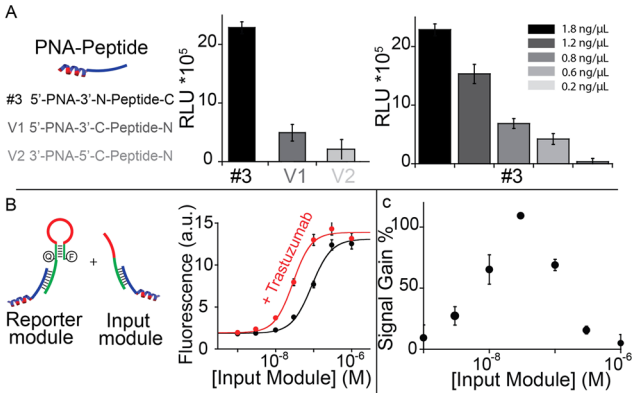

Figure 2. NanoHybrid design and optimization. (A) ELISA assays to select the optimal PNA–peptide chimera probe for trastuzumab detection. PNA–peptide #3 showed trastuzumab recognition activity, whereas V1 and V2 do not have significant binding affinity. Increasing concentration of PNA–peptide #3 in the coating step of the ELISA assay directly correlates with a higher luminescence intensity (n = 3). (B) Fluorescent binding assays obtained by adding increasing concentrations of the input module to a fixed concentration of reporter module (10 nM) in the absence (black curve) and presence (red curve) of trastuzumab (200 nM). (C) Signal gain % as a function of the input module concentration. Maximum signal gain of NanoHybrid is achieved at 30 nM input module. Fluorescence experiments have been performed in 10% blood serum containing the reporter module (strand #1, 10 nM; strand #3, 750 nM) by adding increasing concentrations of strand #2 in the presence (200 nM) and absence of trastuzumab at 25 °C.

to identify the optimal input strand (strand #2) concentration, we performed binding curves at increasing concentrations of input modules added to a solution containing the reporter module in the absence and presence of trastuzumab antibody (200 nM) (Figure 2B). As expected, we observed an improved affinity toward the input module in the presence of the target antibody due to the colocalization effect upon target binding. More specifically, we observed a change in $K_{1/2}$ (the concentration at which we observed 50% of maximum signal) from 100 ± 10 to 30 ± 5 nM in the absence and presence of trastuzumab, respectively (Figure 2B). The % signal change observed in the presence of the antibody at different input module (strand #2 + strand #3) concentrations can thus give a good estimate of the optimal concentration to be used in the next experiments. We selected a concentration of 30 nM that leads to an average signal change of 105 ± 5% (n = 3) (Figure 2C).

Analytical Performance of Trastuzumab NanoHybrid. To test the analytical performance of the NanoHybrid platform, we initially tested the platform by adding increasing concentrations of trastuzumab and measured the fluorescence output to obtain a dose–response curve (Figure 3A). Statistical analysis shows a linearity range between 10 and 75 nM trastuzumab with R_2 = 0.9918 (Figure 3B), while the plateau is reached approximately at 200 nM trastuzumab (Figure 3A). To validate the analytical performance of the system, we performed a spike and recovery assessment through quantification with the standard addition method.[20] We prepared spiked sera (10% v/v dilution) by adding trastuzumab to achieve four standard concentrations (0, 10, 30, and 50 nM). The experiment was performed by preparing the serum samples testing each concentration with four standard addition points of 0, 20, 30, and 40 nM of trastuzumab (n = 17). For each experiment, a linear regression analysis was performed and the initial concentration of trastuzumab was calculated. Figure 3C shows the distribution of the data points independently, calculated with the standard addition method by spiking the known concentration of the antibody and calculating the initial concentration. Every quantification was performed 17 times by calculating the average of two values.

We successfully identified the four concentrations with the following recoveries: 99% for the samples with 0 nM trastuzumab initial concentration, 102% for the samples with 10 nM trastuzumab, 121% for the samples with 30 nM trastuzumab, and 105% for the samples with 50 nM trastuzumab. Results were shown to be statistically significant using a one-sided analysis of variance (ANOVA) test. In Figure 3C, the data are represented with bars indicating the first and third quartiles and the median values. The confidence interval (C.I.) values for the spiked concentrations are the following: 0 nM, C.I. 95% = −0.3 to 0.1 nM; 10 nM, C.I. 95% = 8.9–11.7 nM; 30 nM, C.I. 95% = 32.8–39.9 nM; and 50 nM, C.I. 95% = 48.7–56.4 nM. The NanoHybrid platform demonstrated noteworthy potential for the detection of specific antibodies in about 15 min (Figure SI3) with no signal amplification after relatively simple sample preparation.

Pilot Clinical Study. We aimed to test the optimized nanoswitch in a pilot study on breast cancer patients treated with trastuzumab-based therapy in the clinical practice. We employed 19 samples from 12 different patients obtained from

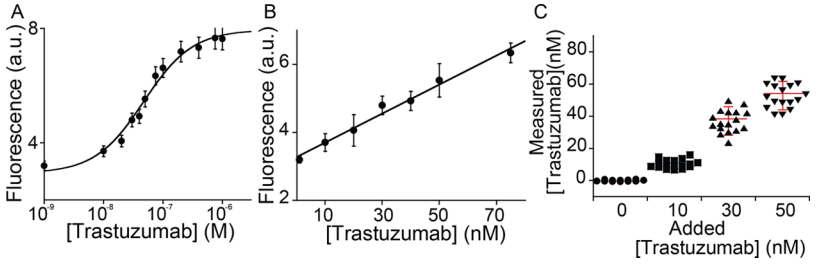

Figure 3. Trastuzumab quantification in serum samples. (A) Fluorescence binding assay performed by adding concentrations of trastuzumab in blood serum samples (n = 5). (B) Linear dynamic range of trastuzumab detection is between 10 and 75 nM (R^2 = 0.9918, n = 5). (C) NanoHybrid accuracy of the antibody quantification was assessed by spiking sera samples with different concentrations of trastuzumab (0, 10, 30, and 50 nM). The experiments have been performed in 10% blood serum containing the reporter and input modules (strand #1, 10 nM; strand #2, 30 nM; strand #3, 750 nM) at different spiked concentrations of trastuzumab at 25 °C. Each fluorescence value is the mean value of four replicates performed on different serum samples (serum samples = 5).

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a 2012 cohort. Among these samples, four were from patients not treated ("AAC, receiving adjuvant therapy without trastuzumab"). The remaining 15 samples were from 8 patients treated with trastuzumab. These samples were divided into two groups: "C" samples were collected from patients receiving four doses of trastuzumab as a neoadjuvant therapy before surgery for the removal of the solid tumor, while "D" samples were collected from the same patients 1 month after surgery. Between groups C and D, each number indicates one patient (Ex: 1C and 1D are samples collected from the same patient in two different visits to the hospital). Trastuzumab concentration in patient sera was quantified using both a commercial ELISA kit for the detection of free antibodies (red bars, Figure 4A), according to manufacturer's instructions, and the

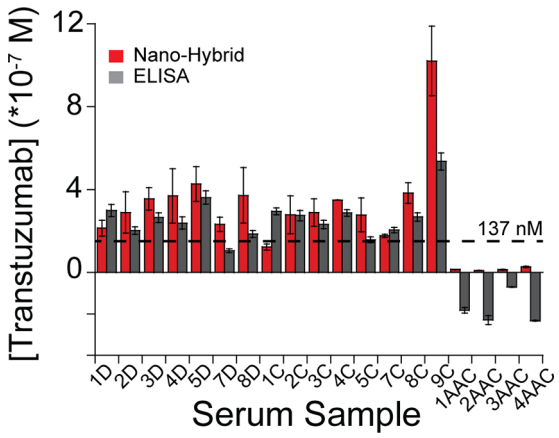

Figure 4. Comparison between the NanoHybrid platform and the ELISA assay. Comparison between the quantification values obtained with ELISA (red bars) and with Nanohybrid using the standard addition method (gray bars).

nanoswitch platform (gray bars, Figure 4A). In this latter case, we calculated the concentration of each sample independently by preparing four standards at the concentrations of 0, 200, 300, and 400 nM, respectively, and applying the standard addition method. Fluorescence measurements demonstrated that our platform is able to detect the presence of trastuzumab with high specificity in 10% human blood serum solution of breast cancer patients. All of the negative samples were under the 20 μg/mL threshold (137 nM mAb) suggested for minimum trastuzumab efficacy.[12] Moreover, our single-step platform allowed us to discriminate the positive samples from the negative ones in about 10 min. The ELISA results gave comparable results in terms of positive/negative discrimination (Cohen's kappa = 0.729) although with some significant difference in the quantified values when compared with those obtained with the nanoswitch platform, likely due to a difference in the Ab-recognizing epitopes employed by the two techniques.

CONCLUSIONS

The current gold standard diagnostic method for the detection of antibodies is the ELISA technique, which allows precise estimation of biomarkers in a sample. Due to the several incubation and washing steps, ELISA is often not appealing for point-of-care (POC) applications due to the requirement of trained technicians or special machinery, high cost, and a long time for execution. In this work, we successfully applied the NanoHybrid platform for the development of a rapid homogeneous assay, capable of detecting trastuzumab directly in blood serum without washing steps, reducing expenses and time. We confirmed the results through a small pilot study on 19 clinical samples derived from breast cancer patients upon trastuzumab treatment or treatment-naive patients, whereas the amount of trastuzumab was assessed quantitatively by measuring the fluorescence intensity 15 min after the addition of the patients' sera. One of the major advantages of the NanoHybrid platform is its modularity and versatility: it allows us to potentially detect any antibody through the simple expedient of changing only the PNA−peptide strand (strand #3).[21] We demonstrated here the capability of the sensor to react in the presence of the specific antibody, and we were able to discriminate whether the antibody levels in patients were above the minimum concentration of trastuzumab required for the therapy (137 nM). Moreover, mAb concentration in the blood serum perfectly fits within the linearity range of the NanoHybrid-based assay. This system is amenable for point-of-care (POC) applications due to its rapidity and simplicity and the lack of washing steps.

Despite the NanoHybrid assay has been able to distinguish patients not reaching the minimum efficacy dose threshold, further improvements in NanoHybrid are necessary to achieve trastuzumab quantification through a single fluorescence measurement and a standard calibration curve. In addition, taking into account that NanoHybrid is a homogenous assay, and so intrinsically different when compared to ELISA, and considering also that the NanoHybrid system requires the simultaneous binding to both antibody paratopes to be activated, we can speculate that such a technique could be differentially affected by the presence of the free receptor unbound portion or ADA. Indeed, it has been demonstrated that trastuzumab-binding soluble receptors or antidrug antibodies can be present in the bloodstream of some patients, conditions that influence the overall prognosis.[15,16,22,23] However, many mechanisms behind trastuzumab resistance or partial response are yet to be studied and demonstrated.[24] Further studies are needed to clarify whether NanoHybrid provides additional clinical information relative to the mAb integrity in the bloodstream and the presence of the free unbound receptor.

In conclusion, NanoHybrid allows the detection and quantification of specific antibodies without requiring specialized labs or trained personnel. The next step is to further develop and clinically validate our NanoHybrid drug detection system, embedding it into a POC diagnostic system. The results obtained in this pilot clinical study will pave the way for future wider clinical trials.

EXPERIMENTAL SECTION

Oligonucleotide Sequences, Trastuzumab, and Structure Predictions. DNA strands modified with AlexaFluor 680 and Black Hole Quencher 2 were purchased from IBA GmbH, Germany and resuspended in TE buffer (pH 8.0) at a final concentration of 100 μM before being aliquoted and stored at −20 °C protected from light. Peptide nucleic acid (PNA)−peptide chimeric probes were purchased from Panagene, South Korea and resuspended in a solution of 10% acetonitrile in molecular-grade water before storage at −20 °C. Antihuman ErbB2 therapeutic antibody (Trastuzumab) was purchased from Aurogene (Rome, Italy) and Evidentic (Berlin, Germany), aliquoted, and stored at −80 °C. Unmodified DNA sequences were purchased from IDT and Eurofins Genomics (Ebersberg, Germany), purified either by standard desalting or

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high-performance liquid chromatography (HPLC), and, upon arrival, resuspended to 100 μM with molecular-grade water.

Nucleic acid sequences are the following:

Alexa680/BHQ2-modified DNA stem−loop (strand #1): 5′-GTC ACC GCA AAA TAA GAT CT (BHQ2) C GCA CCT GAG TGG TAA TCT AGT GCG T (Alexa680)−3′.

Invading strand (strand #2): 5′-GTC ACC GCA AAA TAA GAT TTT TTT TTT TTT TTT TTT TTA GAT TAC CAC TCA G−3′.

PNA−peptide chimera probe (strand #3): 5′-TCT TAT TTT GCG GTG AC−3′−N-term−QLG PYE LWE LSH−C-term.

V1: N-term−QLG PYE LWE LSH−C-term−5′−TCT TAT TTT GCG GTG AC−3′.

V2: 3′-CAG TGG CGT TTT ATT CT-5′-N-term−QLG PYE LWE LSH−C-term.

Enzyme-Linked Immunosorbent Assay (ELISA) of Trastuzumab. PNA−peptide probe stock solutions were heated to 90 °C for 10 min and diluted to a final concentration of 500 ng/μL. The solution was then diluted with carbonate buffer (pH 9.4) to a final concentration of 5 ng/μL and 100 μL was added per well (500 ng per well) in a 96-well white microplate (Optiplate, PerkinElmer) and incubated O/N at 4 °C for 16 h. After coating, the buffer was removed, and the plate was blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 37 °C. In the meantime, primary antibody solutions (trastuzumab) were prepared. Regarding trastuzumab, several dilutions were prepared in a blocking buffer (from 1.8 to 0.2 ng/μL). The blocking buffer was removed from the plate, and trastuzumab solutions were added and incubated for 1 h at 37 °C. Washing with PBS−Tween 0.05% was repeated three times before the addition of secondary antibody antihuman IgG (1 mg/mL) at the final dilution of 1:10000 in the blocking buffer. A 1 h incubation at 37 °C followed. Finally, the plate was washed three times before the addition of the Femto ELISA Super Signal (Thermo Scientific) substrate for the luminescence reaction and the luminescence was read in a luminometer after 3 min of incubation. A standardized pool of normal human serum (Sigma-Aldrich) has been used to perform optimization studies and as background control. Quantification of trastuzumab in real samples has been performed using a commercial ELISA kit for quantitative analysis of free trastuzumab in serum and plasma samples (Matrix Biotech).

Ethics Committee Approval and Patient Consent. Clinical samples were obtained from 12 patients treated in 2012 at Fondazione IRCCS Istituto Nazionale dei Tumori by Dr. Elda Tagliabue (Ethical Committee approval 28/09/2018 Prot. INT 148/18 Istituto Tumore di Milano, amended December 2019 no. 562 DG). In detail, plasma was collected from eight patients after a treatment protocol consisting of three or four cycles of AT (adriamycin plus taxotere) followed by four cycles of CMF (cyclophosphamide, methotrexate, and fluorouracil) and trastuzumab. Samples were collected at two time points: at the end of the treatment protocol (sample C) and after surgery, approximately 1 month later (sample D). Moreover, sera were obtained from four additional patients after treatment with three or four cycles of adjuvant AT but before treatment with trastuzumab. Samples were anonymized, shipped to Ulisse BioMed, aliquoted upon arrival, and stored at −80 °C until use.

Quantification of Unbound Trastuzumab in Patient Serum. The concentration of trastuzumab was assessed by SHIKARI Q-TRAS (Martiks Biotech, Gölbaşı, Ankara, Turkey). Nineteen serum samples (15 positive and 4 negative) were diluted 100 times in assay buffer, added to the precoated plate, and incubated for 30 min at room temperature. The plate was washed three times with 300 μL of wash buffer, and 100 μL of HRP-conjugated probe was added to each well. Another incubation and wash step followed as described above. Finally, 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to each well and after 10 min of incubation at room temperature the reaction was blocked with 100 μL of stop solution. The optical density at 450 nm was measured after 15 min of incubation at RT.

Fluorescence Measurements. Nanoswitch components were resuspended and diluted to 1 μM in a saline buffer (150 mM NaCl, 50 mM Na_2HPO_4, pH 7.4 or 150 mM NaCl, 20 mM HEPES, pH 7.4). Quantification was performed by measurements using a Nanodrop1000 spectrophotometer (Thermo Scientific) by repeating each measurement three times and calculating the average absorbance at 260 nm. The absorbance and the molar extinction coefficient were used to calculate the concentration empirically with the Lambert−Beer law. The components were then diluted in the saline buffer to a 10× stock solution and finally added to the wells at their final concentration. Moreover, most of the experiments were performed in a solution containing 10% final v/v of commercially available standard human serum (Fisher Scientific).

Measurements were performed using an EnVision (PerkinElmer) microplate reader, set up with an excitation filter at 660 and 40 nm bandwidth, an emission filter at 720 and 40 nm bandwidth, both with 90% transmittance, and a cutoff mirror at 585 nm. Measurements were performed at 25 °C (room temperature) in black 96- or 384-well no-binding microplates purchased from Greiner Bio-One International GmbH (Germany). Data was collected after 30 min of incubation to ensure the stabilization of the components.

Binding Assays and Data Analyses. Binding curves reported in Figure 2 were obtained from 10% v/v human serum using 10 nM strand #1 and 750 nM strand #3 by sequentially increasing the concentration of strand #2 in the presence (200 nM) and absence of trastuzumab. Several dilutions of input strands were prepared to 10× of their final concentration. This solution was dispensed in the plate, and a 10× solution of the input strand was added to each well. The final concentrations of the input strands tested were 0, 1, 3, 10, 30, 100, 300, and 1000 nM. Binding curves reported in Figure 3 were achieved in 10% v/v human serum containing the reporter and input modules (strand #1, 10 nM; strand #2, 30 nM; strand #3, 750 nM) and different spiked concentrations of trastuzumab at 25 °C. For each concentration, the fluorescence signal was recorded every 10 min until it reached equilibrium. Binding curves were obtained by plotting the fluorescence signal vs concentration of the target (i.e., strand #2 in Figure 2; antibody in Figure 3) in solution and fitting the data with the following simplified Langmuir-type equation

$$F = F_0 + \Delta F \times \frac{[\text{target}]}{[\text{target}] + K_D}$$

where F_0 is the fluorescence background, ΔF is the fluorescence signal change, and K_D is the concentration of the target at half-maximum signal change.

The fluorescence intensity (F.I.) gain percentage (signal gain) was calculated by comparing the value of fluorescence obtained at the same concentration of the input module in the presence and absence of the antibody using the following formula

$$\text{gain\%} = \frac{(\text{F. I. (+Ab)} - \text{F. I. (without Ab)})}{\text{F. I. (without Ab)}} \times 100$$

where F.I. (+Ab) and F.I. (without Ab) represent the intensities of fluorescence emission in the presence and absence of trastuzumab, respectively.

Trastuzumab Quantification Using the Standard Addition Method. To quantify trastuzumab directly from the patients' sera, we used the standard addition method. This method allows us to precisely quantify an analyte in a sample by adding specific amounts of the same analyte. Using the standard addition method, it is possible to determine the concentration of the target molecule in the solution through linear regression analysis and by tracing the intercept on the x-axis a value corresponding to the initial concentration of the analyte. We performed fixed additions of trastuzumab to serum containing an unknown concentration of trastuzumab, and we extrapolated the initial amount by applying the aforementioned method. Finally, the recovery value can be calculated.

Statistics. Results were represented as the mean ± SD (standard deviation). Statistical differences were expressed as Student's t-test with the calculation of the p-value score. Differences between spiked serum concentrations were calculated with a one-sided ANOVA test. Cohen's K was used to calculate the score between tests when

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imposing a clinically significant threshold. Schemes and calculations have been performed with Prism—GraphPad.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.0c01046.

Optimization of the PNA−peptide chimera probe (strand #3) and kinetic experiments (PDF)

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⊥M.M. and A.P. contributed equally. The manuscript was written through the contribution of all authors. All authors have given approval to the final version of the manuscript.

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