ACS MEASUREMENT SCIENCE Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

pubs.acs.org/measureau

Article

Electrochemical Cellulase-Linked ELASA for Rapid Liquid Biopsy Testing of Serum HER-2/*neu*

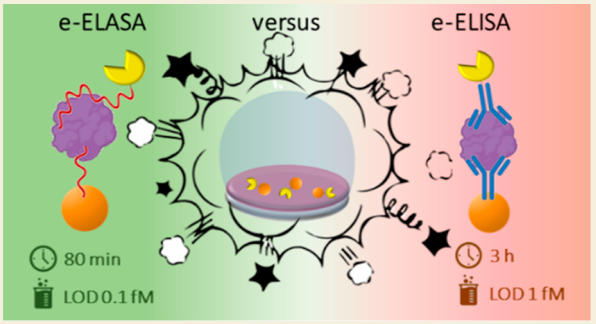
Ana Díaz-Fernández, Alexey Ferapontov, Mikkel Holm Vendelbo, and Elena E. Ferapontova*

Cite This: *ACS Meas. Sci. Au* 2023, 3, 226–235

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Non-invasive liquid biopsy assays for blood-circulating biomarkers of cancer allow both its early diagnosis and treatment monitoring. Here, we assessed serum levels of protein HER-2/*neu*, overexpressed in a number of aggressive cancers, by the cellulase-linked sandwich bioassay on magnetic beads. Instead of traditional antibodies we used inexpensive reporter and capture aptamer sequences, transforming the enzyme-linked immuno-sorbent assay (ELISA) into an enzyme-linked aptamer-sorbent assay (ELASA). The reporter aptamer was conjugated to cellulase, whose digestion of nitrocellulose film electrodes resulted in the electrochemical signal change. ELASA, optimized relative aptamer lengths (dimer vs monomer and trimer), and assay steps allowed 0.1 fM detection of HER-2/*neu* in the 10% human serum in 1.3 h. Urokinase plasminogen activator and thrombin as well as human serum albumin did not interfere, and liquid biopsy analysis of serum HER-2/*neu* was similarly robust but 4 times faster and 300 times cheaper than both electrochemical and optical ELISA. Simplicity and low cost of cellulase-linked ELASA makes it a perspective diagnostic tool for fast and accurate liquid biopsy detection of HER-2/*neu* and of other proteins for which aptamers are available.



KEYWORDS: *HER-2/neu, electrochemical enzyme-linked aptamer-sorbent assay (ELASA), cellulase, magnetic beads, aptamer, antibody, electrochemical sandwich assay*

1. INTRODUCTION

Recent progress in tumor detection and treatment largely improved cancer survival rates,[1] but for many cancers they are still low,[2] being less than 20% for aggressive forms of breast, liver, and esophagus cancers.[3] A non-invasive liquid-biopsy detection of tumor-specific molecular biomarkers, circulating in biological fluids, allows both timely diagnosis of early-stage tumors, prognosis of cancer state, and treatment monitoring.[4] Among those, blood-circulating cancer-specific proteins are considered as potent biomarkers of both the cancer type and state.[5] However, just a few of them are currently approved by the FDA for liquid biopsy screening of cancer: serum prostate specific antigen as a biomarker of prostate cancer[6] and serum alpha-fetoprotein as a biomarker of hepatocellular carcinoma (liver cancer).[7,8]

Human epidermal growth factor receptor-2 (HER-2/*neu*) is another promising and intensively studied predictive oncogene biomarker of human breast cancer,[9] being itself the target of a specific therapy.[10,11] Its overexpression and dimerization occur in up to 30% of primary breast cancers[9,12] and can be correlated with the particularly aggressive development of tumors, with patients tending to suffer from relapse and a shorter overall survival.[10,13] The poor prognosis of such cancers and their directed treatments with a monoclonal antibody, trastuzumab, targeting HER-2/*neu*,[10] require constant monitoring of the HER-2/*neu* state in response to treatments, currently performed by poorly suited for continuous monitoring techniques, PCR and FISH assays, using solid tumor biopsies.[14,15]

Structurally, HER-2/*neu* is a 185 kDa transmembrane glycoprotein belonging to the epithelial HER1-HER4 family, composed of the growth factor-binding extracellular domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain.[16] Therewith, the extracellular domain (ECD) can be cleaved from the cell surface and released into the blood-stream.[17] Serum ECD of HER-2/*neu* is detected in 15 to 40% of breast cancer patients[17−19] and liquid biopsy assaying of ECD released from the tumor cells offers a more convenient way of cancer treatment monitoring.

Detection of serum ECD (commonly referred to as "serum HER-2/*neu*"[20] and further referred in the text as HER-2/*neu* when discussing its serum determinations) can be performed by the enzyme-linked immunosorbent assay (ELISA), and

Received: November 30, 2022
Revised: April 5, 2023
Accepted: April 5, 2023
Published: April 14, 2023

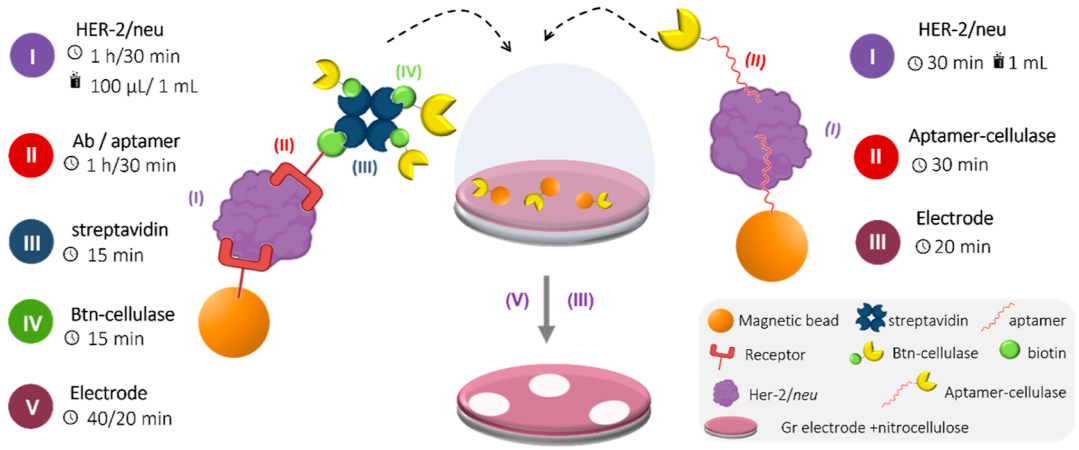


Figure 1. Scheme of e-ELISA (left) and e-ELASA (right) for HER-2/*neu* detection. Left panel: Ab-modified MBs capture the protein, further reacting with second biotinylated Ab linked via streptavidin to biotinylated cellulase; Right panel: aptamer-modified MBs capture the protein, further reacting with the second aptamer bioconjugated to cellulase. Cellulase-labeled sandwiches on MBs are applied onto the nitrocellulose-modified graphite electrode, and cellulase digestion of insulating nitrocellulose film results in the electrochemical signal change proportional to the concentration of the protein in a sample.

Table 1. List of the Aptamer Sequences Used in This Work

name	sequence (5′ → 3′)
trimer	biotin-GCAGCGGTGTGGGGGCAGCGGTGTGGGGGCAGCGGTGTGGGGTTTTT
dimer	biotin- TTTTTTGCAGCGGTGTGGGGGCAGCGGTGTGGGG
monomer	biotin-GCAGCGGTGTGGGG
monomer-T	biotin- TTTTTTTTTGCAGCGGTGTGGGG
trimer-NH_2	C_6-amino-GCAGCGGTGTGGGGGCAGCGGTGTGGGGGCAGCGGTGTGGGGTTTTT
dimer-NH_2	C_6-amino-TTTTTTGCAGCGGTGTGGGGGCAGCGGTGTGGGG

several ELISA kits are available in the market, for research purposes only. Their sensitivity and selectivity varies and may compromise precise detection of basal levels of HER-2/*neu*, valued between 10 pM−0.1 nM, with a cancer cut off of 0.2 nM (37 ng mL^{-1}) HER-2/*neu*.[15] Also, ELISA needs lab-running equipment, and its overall duration approaches 5 h, being the practical standard for clinical analysis,[15] while the cost per single assay ranges between 5 and 9 Euros (500−900 Euros per plate), primarily due to the use of expensive antibodies (Abs). All that makes routine optical ELISA less attractive for rapid and inexpensive screenings, such as at point-of-care (POC) sites.

On the other hand, electrochemical ELISA (e-ELISA) offers sensitive, selective, and fast (30−70 min) tools for HER-2/*neu* monitoring, though, often at the expense of complex assay designs and cumbersome electrode/probe modification protocols, or inconvenient detection techniques, obscuring their potential POC use.[21−23] Simpler and more effective e-ELISA on magnetic beads (MBs), with an alkaline phosphatase label, detected 85 pM HER-2/*neu* in the diluted serum in 2 h,[24] while the O_2-dependent DNAzyme-linked immunoassay detected 1 fM HER-2/*neu* in the 20% human serum in 1 h.[25] Most robust cellulase-linked e-ELISA on MBs offered an exceptional interference-free 1 fM detection of HER-2/*neu* in the human serum in a 3 h assay[26] (Figure 1, left).

Quite expensive Abs used in these immunoassays are generally considered as providing better specificity and stronger binding than any alternative biorecognition elements. However, using Ab is not only expensive but can introduce data deviations due to variations between batches. Ab's versatility in electrochemical assay designs is also restricted to just a few based either on the increased charge-transfer resistance due to protein binding or ELISA-like sandwich constructions.[27] The Ab replacement for inexpensive, easily synthesized, and chemically modified, yet selective and stable aptamers is the most practical way to simplify and standardize the assays and, eventually, to make them cheaper.[28,29] In particular, electrochemical aptasensors were shown to provide excellent specificity and sensitivity of protein analysis.[30−32] However, despite the decades of studies, it is still uncertain how efficiently Ab can be replaced by aptamers in clinical assays. A handful of works suggested that using aptamers might eventually lead to the lesser sensitivity and increased limits of detection (LOD) due to the aptamers' lower affinities for proteins.[25,33]

Here, we explored the Ab replacement for a HER-2/*neu*-specific aptamer (its homotrimer, homodimer, and monomeric forms) in the cellulase-linked sandwich e-ELISA on MBs, with this transforming ELISA into the enzyme-linked aptamer-sorbent assay (e-ELASA). To make it suitable for POC testing, we minimized the assay time by optimizing sandwich assembly steps and their duration (Figure 1, right). As a result, e-ELASA appeared 300 times cheaper than e-ELISA (aptamer vs Ab cost), while maintaining the selectivity and sensitivity for HER-2/*neu*. 1.3 h e-ELASA detected from 0.1 fM to 100 pM HER-2/*neu* in 10% human serum samples and showed excellent selectivity against serum proteins.

2. MATERIALS AND METHODS

2.1. Materials

Specific HER-2/*neu* DNA aptamers[34] (Table 1) were produced by Metabion (Germany) and delivered as a desalted lyophilized powder. The monomer and trimer aptamers were selected against the native

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235

glycoprotein, isolated from human gastric cancer cells that overexpress HER-2/*neu*, the monoclonal Ab-immobilized protein being a selection target.[34] Biotinylated sequences were used for the modification of streptavidin-covered MBs (capture aptamers), and amino-modified sequences were used for conjugation to cellulase (reporter aptamers). Biotinylated Ab against ErbB2 (a.k.a. HER-2/*neu*) (BAF1129; Source: Polyclonal Goat IgG) was purchased from Biotechne (Denmark). Recombinant Human HER-2/*neu* protein expressed in HEK293 cells (contains 636 amino acids corresponding to the extracellular domain ECD of HER-2/ErbB2, glycosylated, MW 110 kDa) was from SinoBiological Inc. (Beijing, P.R. China). Human serum albumin (HSA), bovine serum albumin (BSA), human serum from male AB plasma, sterile-filtered (Lot 121K16972; stored at −20 °C) components of buffer solutions, cellulase from *Aspergillus niger*(4% nitrocellulose solution in ethanol/diethyl ether), BiotinTag Micro Biotinylated Kit, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and BiotinTag MicroBiotinylated Kit were obtained from Sigma-Aldrich (Denmark). Streptavidin-coated magnetic beads DynabeadsTM MyOne T1 (Invitrogen) and Invitrogen ErbB2 (HER2) Human ELISA kits were from Fisher Scientific (Denmark). All aqueous solutions were prepared with ultrapure water (18 MΩ cm) from a Millipore Milli-Q Reference A+ system.

2.2. Cellulase Conjugation to the Aptamer or Biotin

Cellulase was covalently linked to the amino group of either trimer or dimer aptamer sequences using the EDC/NHS COOH-activation and amine coupling reaction[35] by following the previously described protocol.[25] For this, a 50 μL mixture of 13.5 μL of a 100 μM aptamer solution in 10 mM PBS (7.54 mM Na_2HPO_4, 2.46 mM NaH_2PO_4, and 0.154 M NaCl, pH 7.4), 5.15 μL of a 48.7 mM NHS in H_2O, 10 μL of 500 mM EDC in H_2O, 2.52 μL of a 19.8 mM cellulase solution in PBS, and 18.83 μL PBS, to have a final concentration of 27 μM, 5, 100, and 1 mM, respectively, were incubated for 2 h at room temperature (rt) and further stored in a freezer until used in the assay when needed. Conjugation of cellulase to biotin was performed following the reported protocol[36] using the BiotinTag Micro-Biotinylated Kit. For this, 100 μL of a 20 mg mL^{-1} cellulase solution were mixed with 10 μL of a 5 mg mL^{-1} biotin-amido-hexanoic acid 3-sulfo-*N*-hydroxysuccinide ester (BAC-sulfoNHS) solution in 10 mM PBS under gentle stirring, at rt. Then, the mixture was column-spun for 2 min at 2100 rpm, and four fractions containing the biotinylated cellulase were collected. The concentration of biotinylated cellulase was determined using the Bradford method. For this, 1.5 mL of the Bradford reagent was mixed with 50 μL of the blank, the standard solutions (0.01−10 mg mL^{-1} BSA) and the collected fractions were allowed to react for 5 min; after that the absorbance was read at 595 nm.

2.3. Modification of Magnetic Beads

Streptavidin T1-MBs were modified with a biotinylated either aptamer or Ab. For modification, 200 μL of the MB suspension (10 mg mL^{-1}) were washed 3 times with 200 μL of 0.1% BSA in PBS and then incubated with 200 μL of either 8 μM aptamer or 200 μg mL^{-1} Ab solutions in PBS for 1 h at rt, under agitation at 300 min^{-1}. After 4 times washing with 0.1% BSA in PBS and magnetic separation with DynaMag 2 (Fisher Scientific), the modified beads were re-suspended in 200 μL of a solution 0.1% BSA in PBS and stored at 4 °C.

2.4. Sandwich Assay

For analysis of HER-2/*neu*, a sandwich assay was performed according to the reported protocol,[26] where the concentration of the MB suspension was optimized to avoid any MB aggregation or multilayer formation on the electrode surface. 40 μL of the aptamer- or Ab-modified MBs were incubated for 30 min in 960 μL samples containing increasing concentrations of HER-2/*neu*. Serum samples were diluted 10 times with PBS, and 40 μL of the modified MBs were added to 960 μL of such sample. Then, MBs were washed 3 times with 0.1% BSA in PBS and then incubated with 100 μL of 1 μM biotinylated aptamer or 600 μg mL^{-1} biotinylated Ab for 30 min. After two washing steps with 0.1% BSA in PBS, MBs were incubated with 250 pM streptavidin for 15 min and, after the supernatant was discarded, with 250 pM biotinylated cellulase for 15 min. Finally, MBs were washed 4 times and re-suspended in 200 μL of PBS, pH 5, in which the activity of cellulase is optimal. For the aptamer-cellulase conjugate, MBs (after incubation with HER-2/*neu* and washing steps) were incubated with a 1 μM aptamer-cellulase bioconjugate for 30 min. Then, MBs were washed 3 times with 0.1% BSA in PBS and re-suspended in 200 μL of 0.1 M PBS, pH 5. All incubations with MBs were performed at rt, under agitation at 300 min^{-1}. The volumes and incubation times described here refer to the optimized conditions.

2.5. Modification of Graphite Electrodes

Working electrodes were spectroscopic graphite rods (Gr, Electron Microscopy Sciences, Hatfield, USA, catalogue number 70200, 3 mm diameter) fitted into Teflon holders (Ø 1.1 cm). First, they were polished on emery paper (SIC paper #1000, HV 30−800, Struers, Denmark) for 30 s and then on A4 paper for another 30 s to a mirror luster. Then, they were modified with a layer of nitrocellulose by placing a 10 μL drop of a 0.5% nitrocellulose solution in ethanol over the working electrode and the Teflon holder and dried in the air for at least 15 min.

2.6. Electrochemical Measurements

Electrochemical measurements were performed with a μAutolab type III potentiostat (Methrom, Netherlands) run under GPES software (v. 4.9.007), in a three-electrode cell with the working Gr electrode, an Ag/AgCl (3 M KCl) reference electrode, and a Pt wire as a counter electrode. For HER-2/*neu* detection, 5 μL of the MB suspension (after sandwich labeling) were placed on the nitrocellulose-modified Gr electrode and incubated for 20 min. Then, the electrode was washed with water and placed in the electrochemical cell. Chronocoulometry (CC) was performed at 0 V, with a pulse duration 10 s and a pulse period 0.1 s, and the data for construction of calibration plots were collected at the final 10 s measurement point. All measurements were performed at rt in 0.1 M PBS, pH 7.4, in a 40 mL cell, inside the faradic cage representing a routine lab set-up. Background "blank" experiments were done with MBs incubated in solutions with no HER-2/*neu*, following the rest of the protocol and thus the recorded CC signal was a background signal subtracted from the signals in the presence of the proteins. In serum interference analysis, the "blank" signals were corrected for the signal from the same electrode recorded in PBS prior the "blank" experiments. Error bars are the standard deviation calculated from three independent measurements from three different electrodes.

2.7. Serum Sample Preparation and ELISA Measurements

Human serum samples were taken after informed consent and approved by the Regional Ethics Committee for Science in the Central Region of Jutland, Denmark (approval number: 1-10-72-168-22). ELISA measurements were performed with SpectraMax ABS Plus (Molecular Devices) controlled by a computer with SoftMax Pro Software. For HER-2/*neu* analysis in human serum samples with the ELISA kit, serum samples, buffer, and reagents were prepared following manufacturer instructions. More specifically, the ELISA sandwich immunoassay protocol involved the following steps. First, 100 μL of the blank (PBS), standards for calibration with increasing and known concentrations of HER-2/*neu*, and the samples diluted two times with PBS were added to different wells of the microplate already modified with the capture Ab and incubated of 2.5 h. Next, the liquid was removed, and the wells were washed 4 times with washing buffer and 100 μL of the biotinylated detection Ab was added to each well and incubated for 1 h. Then, the wells were washed 4 times with washing buffer and 100 μL of streptavidin-HRP solution were added and incubated for 45 min. Next, the wells were washed 4 times and 100 μL of (3,3′,5,5′-tetramethylbenzidine) TMB were added and incubated for 30 min in the dark. Finally, 50 μL of a stop solution were added, and the absorbance was measured at 450 nm. All incubations were performed under gentle agitation at 200 min^{-1} with IKA KS 260 basic shaker, and at rt.

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235

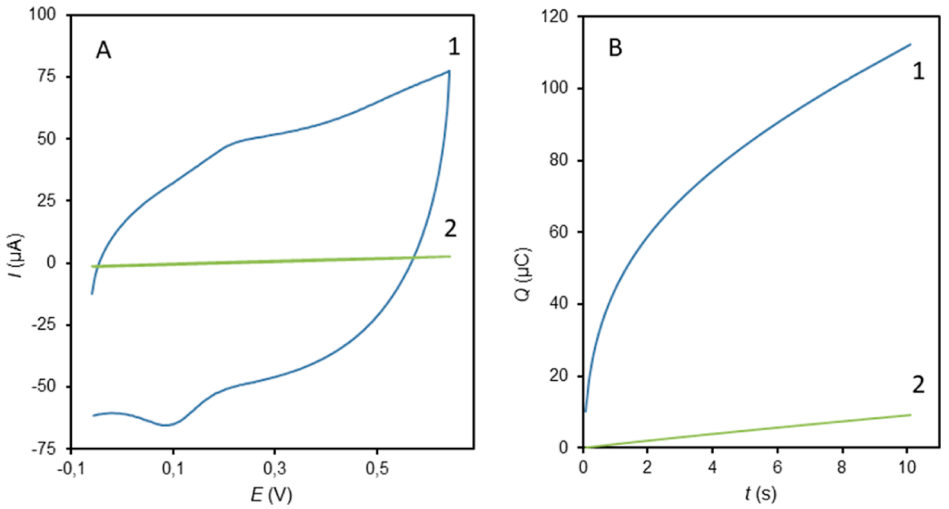


Figure 2. Representative (A) CVs (scan rate: 100 mV s^{-1}) and (B) CC recordings ($E_{detection}$ = 0 mV) recorded with (1) bare and (2) nitrocellulose-modified Gr electrodes in 0.1 M PBS, pH 7.4. A redox couple at 100−200 mV in CVs recorded with (1) bare Gr electrode correlates with redox transformation of surface quinones. These quinoid groups disappeared after electrode modification and did not contribute to the sensor response.

3. RESULTS AND DISCUSSION

We interrogated the performance of the electrochemical cellulase-linked ELISA on MBs and of its analogue e-ELASA, by replacing the capture and reporter Ab with the aptamers (Figure 1). The e-ELISA is based on the Ab-(protein)-Ab sandwich assembly on MBs, and its labeling with a hydrolytic enzyme cellulase.[26] Cellulase-labeled sandwiches are applied onto the insulating nitrocellulose film-modified Gr electrode (Figures 1, 2), digestion of which results in the dramatic capacitive charge changes,[26,37] proportional to the concentration of the protein in the analyzed sample. The reporter aptamer was finally directly conjugated to cellulase, whose digestion of the nitrocellulose film electrodes resulted in the electrochemical signal change. To keep the assay's exceptional analytical performance but reduce the assay's cost, we studied three aptamer sequences: a highly specific 42 nts long homo-trimer, 28 nts homo-dimer, and the original 14 nts monomer. We aimed at the system that allowed similar specificity of the assay and LOD as the immunoassay. To improve the assay performance, we also minimized its time by optimizing the sandwich assembly and washing steps and the biocatalyst action time.

3.1. Optimization of the Assay Time

The original 3 h 10 min long Ab−Ab assay[26] was faster than the available commercial ELISAs (>4 h) yet slow for POC testing. The assay time included (strategy 1): 1 h HER-2/*neu* capturing on Ab-modified MBs, 1 h sandwich assembly with a biotinylated reporter Ab, a 2-step labeling of the assembled sandwich with a biotinylated cellulase, through streptavidin, ensuring 3 cellulase labels per sandwich, and, finally, 40 min digestion of nitrocellulose-modified electrodes by the cellulase-labeled assembly on MBs.

The assay time was reduced by minimizing MB incubation times with HER-2/*neu* (step I) and secondary Ab (step II), and the time cellulase was allowed to digest the electrode (step V) (Table 2). First (strategy 2), HER-2/*neu* and secondary Ab were allowed to react with MBs for 30 min only, while the cellulase action time was kept at 40 min, as in the original assay (strategy 1). Then (strategy 3), the incubation time was kept as in strategy 2, while the cellulase action time decreased to 20 min. With both strategies, the analytical signal for 1 pM of HER-2/*neu* differed from the blank signal, being 68 ± 6 μC (strategy 2) and 44 ± 7 μC (strategy 3), which were 50.4 and 67.8% lower than the analytical signals of the original immunoassay (137 μC) (strategy 1). Obviously, the sensitivity of e-ELISA suffered from the diminished times of HER-2/*neu* capturing and sandwich labeling (Figure 3A). To overcome that (strategy 4), the sample volume in strategy 3 was increased from 100 μL to 1 mL, ensuring more HER-2/*neu* molecules were present in the sample. Then, the analytical signal increased to 125 ± 8 μC, becoming only 9% lower than that in the original assay (Figure 3A). With this, the assay time reduced to 1 h 50 min, the analytical performance remaining almost the same as in the original assay (strategy 1).

Table 2. Summary of Each Strategy Experimental Conditions Used for Optimization of Assay Duration

			incubation time with	
strategy	sample volume	HER-2/*neu*	antibody/aptamer	nitrocellulose digestion
1	100 μL	1 h	1 h	40 min
2	100 μL	30 min	30 min	40 min
3	100 μL	30 min	30 min	20 min
4	1 mL	30 min	30 min	20 min

Thus, by minimizing the duration of individual steps of the original Ab−Ab assay and increasing the sample volume to 1 mL, the assay time reduced from 3 h 10 min to 1 h 50 min, with practically no loss of assay's sensitivity, and this optimized immunoassay was further adapted to the e-ELASA format.

3.2. Replacement of Ab for the Aptamer

In the optimized 1 h 50 min protocol (strategy 4), we replaced Ab for a homotrimer aptamer characterized by the high affinity toward HER-2/*neu*[34] and evaluated the performance MB-(Ab)-aptamer and MB-(aptamer)-aptamer assays (brackets denote the MB-modifying biorecognition element). For both, responses to HER-2/*neu* linearly scaled with the logarithmic concentration of the protein (Figure 3B). 1 fM HER-2/*neu* detected by the e-ELASA showed the response approaching that of the immunoassay (only 1.25−1.5 times lower), the sensitivity of both assays being 24 ± 2 μC fM^{-1} and 22 ± 3 μC

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235

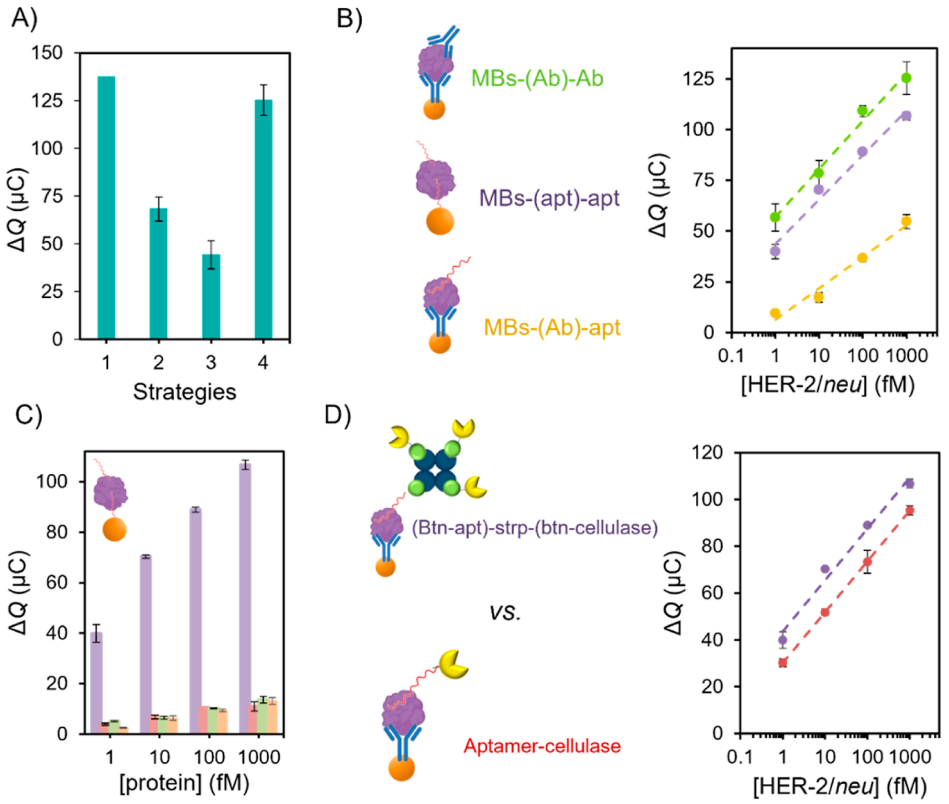


Figure 3. E-ELISA and e-ELASA for HER-2/*neu* detection in 0.1 M PBS, pH 7.4, by CC at 0 mV, 10 s pulse. (A) Time and sample volume optimization of e-ELISA (MBs-(Ab)-Ab): (1) 1 h with 1 pM HER-2/*neu* in 100 μL, 1 h with secondary Ab and 40 min of electrode digestion; (2) 30 min with HER-2/*neu* in 100 μL, 30 min with secondary Ab and 40 min on electrodes; (3) 30 min with HER-2/*neu* in 100 μL, 30 min with secondary Ab, and 20 min on electrodes; (4) 30 min with HER-2/*neu* in 1 mL, 30 min with secondary Ab, and 20 min on electrodes. (B) Responses of sandwich assays: (green) MBs-(Ab)-Ab, (purple) MB-(aptamer)-aptamer, and (yellow) MBs-(Ab)-aptamer. (C) Selectivity of the e-ELASA using the biotinylated aptamer as a reporter for (purple) HER-2/*neu*; (red) HSA, (green) UpA, and (orange) thrombin. (D): Response of e-ELASA with (purple) biotinylated aptamers reacting with biotinylated cellulase via streptavidin (max. 3 cellulase molecules per sandwich) and (red) aptamer-cellulase bioconjugate (1 cellulase per sandwich). In e-ELASA, a homo-trimer aptamer sequence was used. In MB-(apt)-apt and MB-(Ab)-apt notations, MBs are modified with the receptor in brackets.

fM^{-1} for e-ELISA and e-ELASA, correspondingly. The response of the MB-(Ab)-aptamer sandwich assay was however 6-fold lower. It reflects the competitiveness in HER-2/*neu* binding sites for the aptamer and polyclonal Ab receptors, either having the same or closely situated preferential binding sites on the HER-2/*neu* surface. It becomes even more intriguing if we consider the possibility of asymmetrical homodimerization of HER-2/*neu* in the "back-to-head" conformation, triggered by the binding to Ab/Ab fragments.[38,39] Homodimerization during HER-2/*neu* binding to Ab-modified MBs explains the effective formation of the Ab-HER-2/*neu*/HER-2/*neu*-Ab sandwiches. To address the lower efficiency of the aptamer binding to such homodimers, we should assume that dimerization "hides" the aptamer binding sites, impeding the sandwich assembly.

The selectivity of HER-2/*neu* biorecognition by the aptamer, in e-ELASA, was impressively high (Figure 3C). At four concentrations of serum proteins (1, 10, 100, and 1000 fM), non-specific responses from the HSA, the most abundant protein in the human serum,[40] always present in serum thrombin,[41] and UpA, overexpressed in most human cancers,[42] were negligibly small compared to the signal from 1 fM HER-2/*neu*, making the e-ELASA interference free. The concentration dependence of the non-specific binding signal for all interferents was extremely weak, being 10, 12, and 13% for 1 pM HSA, thrombin, and UpA, respectively (Figure 3C). Thus, both the sensitivity and selectivity of e-ELASA were conserved when Ab was replaced for the aptamer as both a capturing and reporting biorecognition element.

With this, we showed that the replacement of both the capture and reporter Ab for the aptamer couple provided the high sensitivity and selectivity of e-ELASA, approaching that of e-ELISA, with only a minute drop of the sensor response signal and the same LOD.

3.3. Replacement of the Biotinylated Aptamer-Streptavidin-Biotinylated Cellulase Labeling by the Aptamer-Cellulase Bioconjugate

The number of sandwich assembly steps was minimized, to further decrease e-ELASA duration. Cellulase was directly conjugated to the amine-functionalized aptamer via EDC/NHS chemistry. With this, two cellulase labels per sandwich were potentially lost, but two labeling steps of the original assay (reactions with streptavidin and biotinylated cellulase, step III and step IV, Figure 1) were annulled. To make bioconjugation handier, it was performed with a large excess of cellulase, and the formed cellulase-aptamer conjugate was not isolated from the reaction mixture. No non-specific binding of unreacted cellulase or EDC/NHS effects on the assay's response was detected (Figure S1, ESI). The MB-(aptamer)-aptamer-cellulase bioconjugate sandwich response to 1 pM of HER-2/*neu* was only 11% lower than that observed with

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235

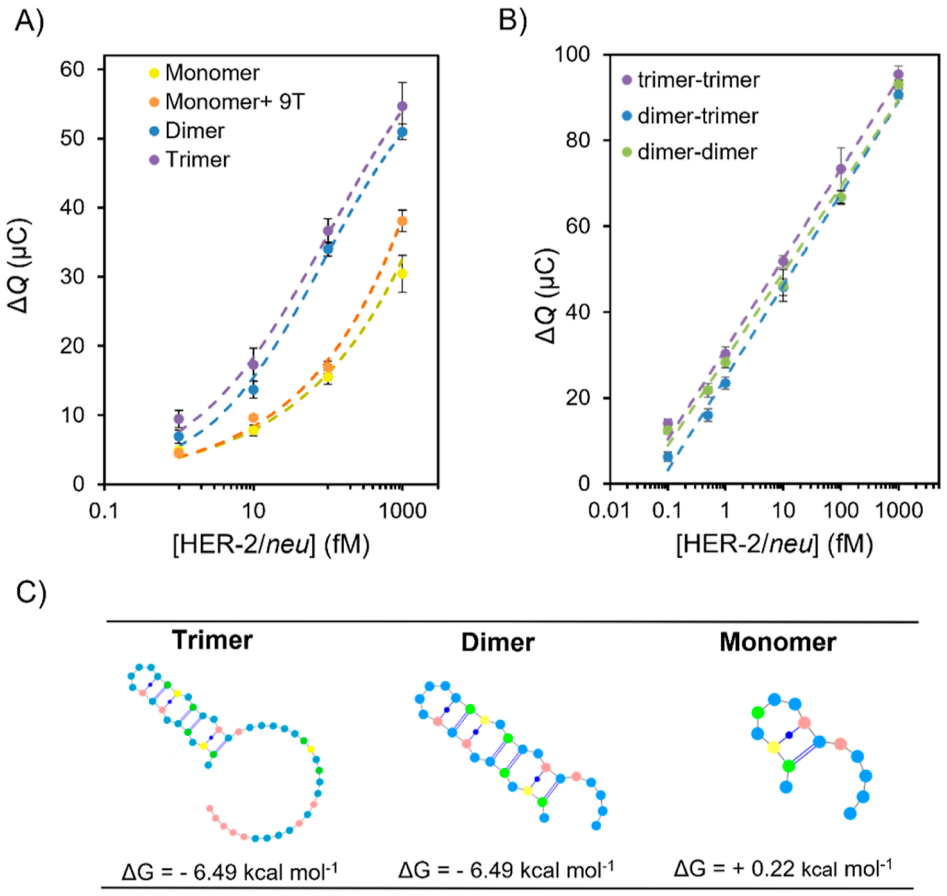


Figure 4. (A) Responses of the MB-(Ab)-aptamer assays with different reporter aptamers, recorded by CC at 0 mV in 0.1 M PBS, pH 7.4: (purple) trimer, (blue) dimer, (orange) monomer-9T, and (yellow) monomer. (B) Responses of e-ELASA to the increasing concentration of HER-2/*neu* for the (purple) MB-(trimer)-trimer and (green) MB-(dimer)-dimer and (blue) MB-(dimer)-trimer sandwich. (C) Secondary structures of the trimer, dimer, and monomer aptamer sequences specific for HER-2/*neu* predicted by mfold software in 0.154 M NaCl at 25 °C; drawn with VARNAgui 3−9 version.[45] The mfold generated structures are the first approximation of oligonucleotide structures but not their accurate resolution.

streptavidin-biotinylated cellulase labeling. For both, the increase in the response signal with the HER-2/*neu* concentration showed identical sensitivities of 22.0 ± 3.0 μC fM^{-1} (the biotinylated aptamer) and 21.7 ± 0.1 μC fM^{-1} (the aptamer-cellulase bioconjugate) (Figure 3D). It can be seen that the replacement of three biotinylated cellulase labels, envisioned for the biotin/streptavidin linkage, for just one label present in the cellulase-aptamer bioconjugate did not result in any dramatic decrease of the sensitivity or the amplitude of the CC response, which may be attributed to the higher residual activity of cellulase in the bioconjugate. As shown earlier, cellulase activity after biotinylation dropped 3.5 fold.[36] Thus, direct bioconjugation of the reporter aptamer to cellulase further reduced the total e-ELASA time to 1 h 20 min, with no loss in the assay's sensitivity.

3.4. Sensitivity of HER-2/neu Detection with Homo-Trimeric and Truncated Aptamer Versions

Here and in other works,[22,23,25,26] the aptamer sequence designed as a homotrimer, by triple repetition of the original monomeric sequence obtained by the SELEX, was used.[34] The homo-trimer showed the highest affinity for native HER-2/*neu* (versus the monomer) and was shown to bind strongly to the cell surface-exposed ECD of this complex protein.[34] However, the cost of its synthesis and modification increases with its length, and, in our experience, a high G content makes its synthesis additionally challenging, due to the tendency of long G-rich sequences to self-fold, which interferes with oligonucleotide synthesis and modification and, finally, results in low product yields and thus high costs of such aptamers. To reduce the system complexity and cost, the applicability of shorter aptamers for HER-2/*neu* was tested in e-ELASA; affinities of a monomer (14 bases), a monomer with a 9T spacer at the cellulase attachment site, added to increase the aptamer binding availability (23 bases), a homodimer (28 bases), and a homotrimer (42 bases) were compared (Figure 4C). In the affinity assay, Ab-modified MBs reacted with HER-2/*neu* and then with either of the biotinylated aptamers labeled with cellulase via streptavidin. All aptamers showed typical affinity binding curves relative to the HER-2/*neu* concentration (Figure 4A). Dissociation constants K_d were estimated by fitting these data to the electrochemical version[42] of the Scatchard equation[43]

$$\Delta Q = \Delta Q_{max} \times [\text{protein}]/(K_d + [\text{protein}]) \quad (1)$$

where ΔQ is the electrochemical signal change, K_d is the apparent dissociation constant, and [protein] is the protein concentration. The K_d values were 26.1 fM for the trimer, 37.4 fM for the dimer, 85.0 fM for the monomer-9T, and 121.0 fM for the monomer. The homotrimer showed the highest affinity for the protein, with the homodimer having a close K_d. The original monomeric aptamer exhibited the lowest, more than 4.6-fold and 3-fold lower affinity for HER-2/*neu* compared to

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235

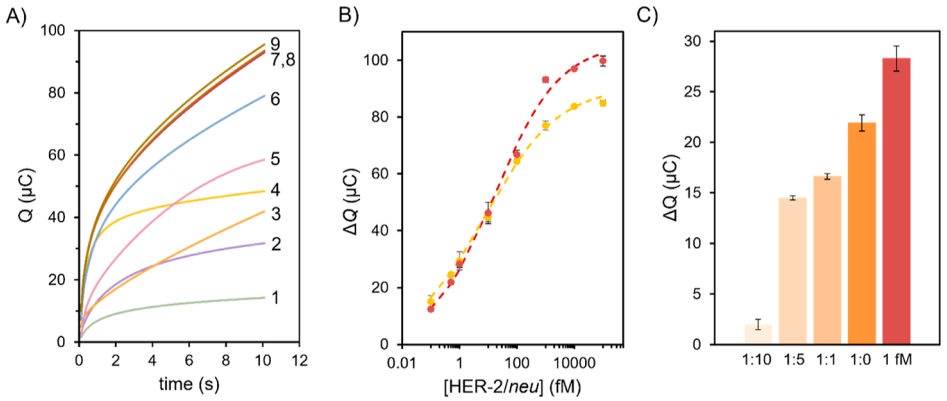


Figure 5. E-ELASA for HER-2/*neu* detection in the human serum performed by CC at 0 V, in 0.1 M PBS pH 7.4. (A) Representative CC responses of nitrocellulose modified Gr electrodes (1) in non-spiked 10% human serum and (2−9) after the exposure to MB-(dimer)-dimer sandwich assembled in 10% human serum containing (2) 0.1 fM, (3) 0.5 fM, (4) 1 fM, (5) 10 fM, (6) 100 fM, (7) 1 pM, (8) 10 pM, and (9) 100 pM HER-2/*neu*. (B) Calibration curves for HER-2/*neu* detection constructed with CC data for MB-(dimer)-dimer sandwich assembly in (red) PBS and (yellow) 10% human serum. (C) Background signals corresponding to different blank dilutions of human serum (pale orange) 1:10, (light orange) 1:5, (orange) 1:1, (dark orange) undiluted and (brick red) 1fM HER-2/*neu* detection in PBS obtained for the MB-(dimer)-dimer sandwich assembly in the absence of HER-2/*neu*. ΔQ was calculated as a Q response in the assay performed in the media not-containing the analyte ("blank") minus Q recorded with the same nitrocellulose-modified electrode prior the "blank" assay.

the dimeric and trimeric variants, correspondingly. A similarly pronounced yet moderate improvement in binding affinities of homodimeric aptamers was ascribed to the aptamer binding either to homodimeric proteins, providing two structurally similar binding sites or protein monomers possessing two or more structurally identical surface domains.[44] Independent of the reason, the aptamer binding sites should neighbor each other to allow both dimer and trimer binding.

From a structural perspective, aptamers' affinities agreed with their secondary structures predicted by the mfold software[46] (Figure 4C). Though such theoretical predictions should be considered with caution, as a very first approximation of true oligonucleotide structures, the pronounced similarity of the self-complementary regions of the dimer and trimer sequences can be correlated with a similarity of their binding affinities and suggest that these regions are responsible for binding to the protein.

Consistently, the trimer and dimer assays showed very close and pronounced responses to HER-2/*neu*, while the signal from the monomer assay was essentially lower (Figure 4A). It is worth noting that the introduction of a 9T spacer in the cellulase-binding terminus of the monomer improved the response and can be recommended for the functionalization of other short aptamer sequences in order to conserve their binding ability after the bioconjugation reaction.

Because the homodimer showed the affinity close to that of the homotrimer, in further experiments, we used the dimer in the sandwich construction, both as a capture and reporter element. In all cases, the e-ELASA response to HER-2/*neu* approached that observed within the trimer−trimer assay and was linear within the 0.1 fM−1 pM logarithmic concentration range (Figure 4B). At 0.1 fM of HER-2/*neu* (the LOD in the buffer solution), the dimer-trimer signal was 44% and the dimer−dimer was 88% of the signal detected in the trimer−trimer assay, indicating better performance of the dimer−dimer sandwich assay. Its sensitivity of 20.1 ± 0.7 μC fM^{-1} approached that of the trimer−trimer assay, 21.7 ± 0.1 μC fM^{-1}.

Accordingly, among the studied monomer, homodimer, and homotrimer combinations, the dimer couple showed the best performance, and the dimer-dimer-based e-ELASA was further used as the optimal, fast, and least expensive assays for the detection of HER-2/*neu* in the serum.

3.5. Performance of the Dimer−Dimer e-ELASA in PBS and Serum

The performance of the dimer−dimer assay was evaluated by CC in PBS and serum spiked with HER-2/*neu* (Figure 5A). Digestion of the organic film by cellulase-labeled dimer−dimer sandwich resulted in the capacitive charge of the electrode linearly increasing with the logarithmic concentration of HER-2/*neu* (Figure 5B). The analytical working range was between 0.1 fM and 100 pM HER-2/*neu* and the LOD was 0.1 fM HER-2/*neu*, according to the IUPAC definition as "*the smallest amount of concentration of analyte in the sample that can be reliably distinguished from zero*". The linear range of the calibration plot, between 0.5 fM and 1 pM, followed the dependence ΔQ (μC) = (22.1 ± 0.3) log[HER-2/*neu*] + (29.4 ± 0.5), with a correlation factor of 0.9998.

To test assay applicability for liquid biopsy analysis, the e-ELASA performance was verified in the human serum. First, effects of human serum proteins (albumins and other) were evaluated with the human serum differently diluted by PBS (10, 20, 50, and 100%). The 10% dilution gave the minimal background signal (2.0 ± 0.6 μC versus 1.7 ± 0.5 μC in PBS, 16% from the lowest 0.1 fM concentration of HER-2/*neu*), not interfering with HER-2/*neu* detection (Figure 5C) and further analysis was performed in 10% serum solutions. The largest background signal observed in the undiluted serum might be related to nonspecific binding of such serum proteins as HSA, whose micromolar concentration in the serum could yield the response of such amplitude (extrapolation of the interference data in Figure 3C to μM HSA concentrations). The presence of residual levels of HER-2/*neu* in any human serum sample should also be taken into account, sample dilution minimizing its effect. The response of HER-2/*neu*-spiked serum samples showed almost the same analytical pattern as that of spiked PBS (Figure 5B), maintaining the same LOD and the working range. However, at 100 pM HER-2/*neu* and higher concentrations, some matrix effects, partially impeding binding of the active form of HER-2/*neu* to the aptamer, started to

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235

interfere with the response. The linear working range in the 10% serum was the same as in PBS and followed the dependence $\Delta Q(\mu C) = (16.3 \pm 0.7) \log[\text{HER-2/}neu] + (29 \pm 1)$, with a correlation factor of 0.9970.

With this, we have showed that robust analytical determination of HER-2/*neu* in serum samples required 10-fold serum dilutions providing the minimized background signal close to the one observed in PBS, and this sample preparation protocol was further used for analysis of human samples.

3.6. Analysis of Human Serum Samples with Optical ELISA and e-ELASA

e-ELASA was used for HER-2/*neu* detection in human serum samples, and the results were compared with those obtained with the commercial ELISA kit. HER-2/*neu* levels in human serum samples were determined by both methods, and the results of e-ELASA matched well the results of the optical assay (Figure 6). Therewith, ELISA required almost 5 h for analysis

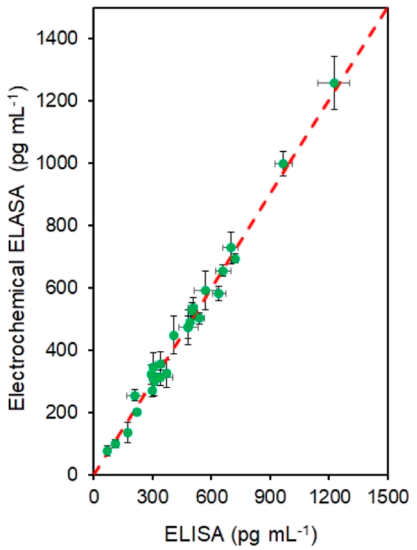


Figure 6. Concordance between the ELISA and e-ELASA detection of HER-2/*neu* in human serum samples (N = 28) showing background levels of HER-2/*neu*. The dashed red line represents a 100% concordance. Error bars correspond to 3 independent measurements from 3 different electrodes.

(Table 3) and lab-running plate-reading equipment, while e-ELASA took 1 h 20 min, and the electrochemical set-up used can be easily miniaturized for portable POC applications. Not the least, based on the price of basic reagents used in the assays, such as Ab and aptamers and cellulase, the price of e-ELASA was essentially, 300 times lower than that of ELISA.

In comparison with other electrochemical approaches for HER-2/*neu* detection, both antibody- and aptamer-based (Table 3) e-ELASA offer the most attractive combination of the assay's time and such analytical characteristics as LOD, sensitivity, and selectivity, including performance in the serum. In its performance, it competes with FDA-approved ADVIA centaur chemiluminescence technology, enabling 2.7 fM HER-2/*neu* detection in the serum in just 18 min; however, due to bulky and expensive lab-running equipment, this assay is perfect for clinical/hospital laboratories but is less suitable for POC testing (Table 3). As can be seen, the overall performance of e-ELASA allows us to consider that it may be suitable for clinical analysis of HER-2/*neu* as a POC alternative to commercial ELISA and other optical assays.

4. CONCLUSIONS

Here, we showed that electrochemical cellulase-linked ELASA on magnetic beads, obtained by replacing e-ELISA's capture and reporter antibodies for aptamers, allows fast and reliable detection of HER-2/*neu* in the serum, with binding affinities of dimeric and trimeric aptamers rivaling those of antibodies. Replacing the antibodies by the aptamers, directly bioconjugated to the enzymatic labels, reduced both the cost and duration of analysis. As a result, based on the reagents' cost, e-ELASA was estimated 300 times less expensive than e-ELISA (or optical ELISA), while maintaining the selectivity and sensitivity of HER-2/*neu* detection. 80 min long e-ELASA enabled the 0.1 fM to 100 pM HER-2/*neu* detection in 10% human serum samples with no interference from human serum albumin, urokinase plasminogen activator, and thrombin, which competes with the best approaches for HER-2/*neu* detection. The aptamer-based assay was analytically validated by human sample analysis that showed results matching those obtained with optical ELISA, making the cellulase-linked e-ELASA an accurate analytical tool for liquid biopsy analysis of HER-2/*neu*, and eventually of other proteins for which the aptamer-protein couples are available.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmeasuresciau.2c00067.

Representative CC responses of nitrocellulose-modified Gr electrodes in "blank" experiments (PDF)

Table 3. Selected Examples of Most Efficient Biosensors for HER-2/*neu*[a]

assay format	technique/equipment	LOD/detection range	medium	time (min)	ref.
e-ELASA on MBs with a cellulase label	CC/potentiostat	0.1 fM/0.1 fM−0.1 nM	10% serum	80	this work
e-ELISA on MBs with an DNAzyme-aptamer label	CC/potentiostat	10 fM/10 fM−1 nM	20% serum	60	24
ADVIA Centaur sandwich immunoassay	CL/luminometer	2.7 fM/2.7 fM−1.9 nM.	serum	18	20
invitrogen ErbB2 Human ELISA Kit	absorbance/plate reader	43 fM/43 fM−11 nM	serum	4 h 45 min	47
direct immunoassay on Fe_3O_4@TMU-21/MWCNT SPCE	amperometric/potentiostat	1.62 fM/	buffer	45	48
direct immunoassay on CND-chitosan SPCE	ECL/spectro ECL	110 fM/	buffer	30	49

[a]CL: chemiluminescence; CNDs: carbon nanodots, ECL: electrochemiluminescence; ErbB2: HER-2/*neu* protein; Gr: graphite electrode, MBs: magnetic beads, NPs: nanoparticles, and SPCE: carbon screen printed electrodes concentrations reported in the original papers in g mL^{-1} were recalculated in fM for the MW of HER-2/*neu* protein complex of 185 kDa (if recalculated for ECD MW, the LODs/detection ranges would be twice higher). The LOD is cited in accordance with the IUPAC definition as "the smallest amount of concentration of analyte in the sample that can be reliably distinguished from zero".

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235

AUTHOR INFORMATION

Corresponding Author

Elena E. Ferapontova − *Interdisciplinary Nanoscience Center (iNANO), Faculty of Natural Sciences, Aarhus University, 8000 Aarhus C, Denmark;* orcid.org/0000-0003-1177-3204; Email: elena.ferapontova@inano.au.dk

Authors

Ana Díaz-Fernández − *Interdisciplinary Nanoscience Center (iNANO), Faculty of Natural Sciences, Aarhus University, 8000 Aarhus C, Denmark;* orcid.org/0000-0002-1867-5925

Alexey Ferapontov − *Interdisciplinary Nanoscience Center (iNANO), Faculty of Natural Sciences, Aarhus University, 8000 Aarhus C, Denmark*

Mikkel Holm Vendelbo − *Department of Nuclear Medicine & PET Centre, Aarhus University Hospital, DK-8200 Aarhus N, Denmark; Department of Biomedicine, Aarhus University, 8000 Aarhus C, Denmark*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsmeasuresciau.2c00067

Author Contributions

CRediT: **Ana Díaz-Fernández** data curation (lead), formal analysis (lead), investigation (equal), methodology (equal), validation (equal), visualization (equal), writing-original draft (equal); **Alexey Ferapontov** data curation (equal), investigation (supporting), validation (equal); **Mikkel Holm Vendelbo** conceptualization (supporting), funding acquisition (equal), investigation (supporting), project administration (supporting), resources (supporting), validation (equal), writing-review & editing (supporting); **Elena E. Ferapontova** conceptualization (lead), data curation (supporting), formal analysis (supporting), funding acquisition (lead), investigation (lead), methodology (equal), project administration (lead), resources (lead), supervision (lead), validation (supporting), visualization (supporting), writing-original draft (equal), writing-review & editing (lead).

Notes

The authors declare the following competing financial interest(s): The basic principle of the cellulase-linked bioassays on MBs studied here is patented by one of the authors; the current work was performed independently and by no means was affected by that fact. All authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge NovoNordisk Foundation for funding of the project "Validating Serum Tests for Human Epidermal growth factor Receptor-2 for Precise Diagnosis and Stratification of Breast and Gastro-Oesophagal Cancers", grant reference number NNF20OC0065428.

REFERENCES

(1) https://www.cancerresearchuk.org/. 2023. Cancer Research UK. Last access 5 March, 2023.

(2) Coleman, M. P. F.; Forman, D.; Bryant, H.; Butler, J.; Butler, J.; Rachet, B.; Maringe, C.; Nur, U.; Tracey, E.; Coory, M.; Hatcher, J.; et al. Cancer survival in Australia, Canada, Denmark, Norway, Sweden, and the UK, 1995−2007 (the International Cancer Benchmarking Partnership): an analysis of population-based cancer registry data. *Lancet* **2011**, *377*, 127−138.

(3) Crew, B. Worth the cost? A closer look at the da Vinci robot's impact on prostate cancer surgery. *Nature* **2020**, *580*, S5−S7.

(4) Soda, N.; Rehm, B. H. A.; Sonar, P.; Nguyen, N.-T.; Shiddiky, M. J. A. Advanced liquid biopsy technologies for circulating biomarker detection. *J. Mater. Chem. B* **2019**, *7*, 6670−6704.

(5) Dragani, T. A.; Matarese, V.; Colombo, F. Biomarkers for Early Cancer Diagnosis: Prospects for Success through the Lens of Tumor Genetics. *BioEssays* **2020**, *42*, 1900122.

(6) Filella, X.; Fernández-Galan, E.; Fernández Bonifacio, R.; Foj, L. Emerging biomarkers in the diagnosis of prostate cancer. *Pharmgenomics Pers. Med.* **2018**, *11*, 83−94.

(7) Gish, R. G. Early detection of hepatocellular carcinoma through surveillance using biomarkers. *Gastroenterol. Hepatol. (N Y)* **2014**, *10*, 121−123.

(8) Lok, A. S.; Sterling, R. K.; Everhart, J. E.; Wright, E. C.; Hoefs, J. C.; Di Bisceglie, A. M.; Morgan, T. R.; Kim, H. Y.; Lee, W. M.; Bonkovsky, H. L.; Dienstag, J. L. Des-gamma-carboxy prothrombin and alpha-fetoprotein as biomarkers for the early detection of hepatocellular carcinoma. *Gastroenterology* **2010**, *138*, 493−502.

(9) Wang, S.; Saboorian, M. H.; Frenkel, E.; Hynan, L.; Gokaslan, S. T.; Ashfaq, R. Laboratory assessment of the status of Her-2/*neu* protein and oncogene in breast cancer specimens: comparison of immunohistochemistry assay with fluorescence in situ hybridisation assays. *J. Clin. Pathol.* **2000**, *53*, 374−381.

(10) Weigel, M. T.; Dowsett, M. Current and emerging biomarkers in breast cancer: prognosis and prediction. *Endocrine-Related Cancer* **2010**, *17*, R245−R262.

(11) Seung, E.; Xing, Z.; Wu, L.; Rao, E.; Cortez-Retamozo, V.; Ospina, B.; Chen, L.; Beil, C.; Song, Z.; Zhang, B.; et al. A trispecific antibody targeting HER2 and T cells inhibits breast cancer growth via CD4 cells. *Nature* **2022**, *603*, 328−334.

(12) Slamon, D. J.; Clark, G. M.; Wong, S. G.; Levin, W. J.; Ullrich, A.; McGuire, W. L. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/*neu* Oncogene. *Science* **1987**, *235*, 177−182.

(13) Yarden, Y.; Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 127−137.

(14) Perez, E. A.; Cortés, J.; Gonzalez-Angulo, A. M.; Bartlett, J. M. S. HER2 testing: Current status and future directions. *Cancer Treat. Rev.* **2014**, *40*, 276−284.

(15) Ross, J. S.; Slodkowska, E. A.; Symmans, W. F.; Pusztai, L.; Ravdin, P. M.; Hortobagyi, G. N. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *Oncologist* **2009**, *14*, 320−368.

(16) Yarden, Y.; Sliwkowski, M. X. Untangling the ErbB signalling network. *Nature Rev. Mol. Cell Biol.* **2001**, *2*, 127−137.

(17) Mori, S.; Mori, Y.; Mukaiyama, T.; Yamada, Y.; Sonobe, Y.; Matsushita, H.; Sakamoto, G.; Akiyama, T.; Ogawa, M.; Shiraishi, M.; Toyoshima, K.; Yamamoto, T. In vitro and in vivo Release of Soluble erbB-2 Protein from Human Carcinoma Cells. *Jpn. J. Cancer Res.* **1990**, *81*, 489−494.

(18) Krainer, M.; Brodowicz, T.; Zeillinger, R.; Wiltschke, C.; Scholten, C.; Seifert, M.; Kubista, E.; Zielinski, C. Tissue Expression and Serum Levels of HER-2/neu in Patients with Breast Cancer. *Oncology* **1997**, *54*, 475−481.

(19) Molina, R.; Jo, J.; Filella, X.; Zanón, G.; Farrus, B.; Muñoz, M.; Latre, M. L.; Pahisa, J.; Velasco, M.; Fernandez, P.; Estapé, J.; Ballesta, A. M. C. erbB-2, CEA and CA 15.3 serum levels in the early diagnosis of recurrence of breast cancer patients. *Anticancer Res.* **1999**, *19*, 2551−2555.

(20) Lftner, D.; Cheli, C.; Mickelson, K.; Sampson, E.; Possinger, K. ADVIA Centaur® Her-2/Neu Shows Value in Monitoring Patients with Metastatic Breast Cancer. *Int. J. Biol. Markers* **2004**, *19*, 175−182.

(21) Emami, M.; Shamsipur, M.; Saber, R.; Irajirad, R. An electrochemical immunosensor for detection of a breast cancer biomarker based on antiHER2-iron oxide nanoparticle bioconjugates. *Analyst* **2014**, *139*, 2858−2866.

(22) Salimian, R.; Kékedy-Nagy, L.; Ferapontova, E. Specific picomolar detection of a breast cancer biomarker HER-2/*neu* protein in serum: Electrocatalytically amplified electroanalysis by the aptamer/PEG-modified electrode. *ChemElectroChem* **2017**, *4*, 872−879.
(23) Zhu, Y.; Chandra, P.; Shim, Y.-B. Ultrasensitive and selective electrochemical diagnosis of breast cancer based on a hydrazine−Au nanoparticle−aptamer bioconjugate. *Anal. Chem.* **2013**, *85*, 1058−1064.
(24) Al-Khafaji, Q. A. M.; Harris, M.; Tombelli, S.; Laschi, S.; Turner, A. P. F.; Mascini, M.; Marrazza, G. An electrochemical immunoassay for HER2 detection. *Electroanalysis* **2012**, *24*, 735−742.
(25) Díaz-Fernández, A.; Ferapontova, E. E. Covalent Hemin/G4 complex-linked sandwich bioassay on magnetic beads for femtomolar HER-2/*neu* detection in human serum via direct electrocatalytic reduction of oxygen. *Anal. Chim. Acta* **2022**, *1219*, 340049.
(26) Malecka, K.; Pankratov, D.; Ferapontova, E. E. Femtomolar electroanalysis of a breast cancer biomarker HER-2/*neu* protein in human serum by the cellulase-linked sandwich assay on magnetic beads. *Anal. Chim. Acta* **2019**, *1077*, 140−149.
(27) Liu, G.; Lin, Y. Nanomaterial labels in electrochemical immunosensors and immunoassays. *Talanta* **2007**, *74*, 308−317.
(28) Hermann, T.; Patel, D. J. Adaptive recognition by nucleic acid aptamers. *Science* **2000**, *287*, 820−825.
(29) Dunn, M. R.; Jimenez, R. M.; Chaput, J. C. Analysis of aptamer discovery and technology. *Nature Rev. Chem.* **2017**, *1*, 0076.
(30) E Ferapontova, E.; V Gothelf, K. Recent advances in electrochemical aptamer-based sensors. *Curr. Org. Chem.* **2011**, *15*, 498−505.
(31) Willner, I.; Zayats, M. Electronic aptamer-based sensors. *Angew Chem Int Ed Engl.* **2007**, *38*, 6408−6418.
(32) Malecka, K.; Mikuła, E.; Ferapontova, E. E. Design Strategies for Electrochemical Aptasensors for Cancer Diagnostic Devices. *Sensors* **2021**, *21*, 736.
(33) Arshavsky-Graham, S.; Heuer, C.; Jiang, X.; Segal, E. Aptasensors versus immunosensors-Which will prevail? *Eng. Life Sci.* **2022**, *22*, 319−333.
(34) Mahlknecht, G.; Maron, R.; Mancini, M.; Schechter, B.; Sela, M.; Yarden, Y. Aptamer to ErbB-2/HER2 enhances degradation of the target and inhibits tumorigenic growth. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 8170−8175.
(35) Hermanson, G. T.. In *Bioconjugate Techniques*; Hermanson, G. T., Ed., Second Edition; Academic Press: New York, 2008, pp 213−233.
(36) Fapyane, D.; Nielsen, J. S.; Ferapontova, E. E. Electrochemical enzyme-linked sandwich assay with a cellulase label for ultrasensitive analysis of synthetic DNA and cell-isolated RNA. *ACS Sensors* **2018**, *3*, 2104−2111.
(37) Fapyane, D.; Ferapontova, E. E. Electrochemical assay for a total cellulase activity with improved sensitivity. *Anal. Chem.* **2017**, *89*, 3959−3965.
(38) Hu, S.; Sun, Y.; Meng, Y.; Wang, X.; Yang, W.; Fu, W.; Guo, H.; Qian, W.; Hou, S.; Li, B.; Rao, Z.; Lou, Z.; Guo, Y. Molecular architecture of the ErbB2 extracellular domain homodimer. *Oncotarget* **2015**, *6*, 1695−1706.
(39) Lobner, E.; Humm, A. S.; Goritzer, K.; Mlynek, G.; Puchinger, M. G.; Hasenhindl, C.; Ruker, F.; Traxlmayr, M. W.; Djinovic-Carugo, K.; Obinger, C. Fcab-HER2 Interaction: a Menage a Trois. Lessons from X-Ray and Solution Studies. *Structure* **2017**, *25*, 878−889.e5.
(40) Álvarez-Martos, I.; Møller, A.; Ferapontova, E. E. Dopamine binding and analysis in undiluted human serum and blood by the RNA-aptamer electrode. *ACS Chem. Neurosci.* **2019**, *10*, 1706−1715.
(41) Malecka, K.; Ferapontova, E. E. Femtomolar detection of thrombin in serum and cerebrospinal fluid via direct electrocatalysis of oxygen reduction by the covalent G4-hemin-aptamer complex. *ACS Appl. Mater. Interfac.* **2021**, *13*, 37979−37988.
(42) Jarczewska, M.; Kekedy-Nagy, L.; Nielsen, J. S.; Campos, R.; Kjems, J.; Malinowska, E.; Ferapontova, E. E. Electroanalysis of pM-levels of urokinase plasminogen activator in serum by phosphorothioated RNA aptamer. *Analyst* **2015**, *140*, 3794−3802.
(43) Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **1949**, *51*, 660−672.
(44) Manochehry, S.; McConnell, E. M.; Li, Y. Unraveling Determinants of Affinity Enhancement in Dimeric Aptamers for a Dimeric Protein. *Sci. Rep.* **2019**, *9*, 17824.
(45) https://varna.lri.fr/index.php?lang=en&page=home&css=varna. 2023 Last access 5 March 2023.
(46) Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucl. Acids Res.* **2003**, *31*, 3406−3415.
(47) https://www.thermofisher.com/elisa/product/ErbB2-HER2-Human-ELISA-Kit/EHERBB2. 2023 Last access 5 March 2023.
(48) Ehzari, H.; Samimi, M.; Safari, M.; Gholivand, M. B. Label-free electrochemical immunosensor for sensitive HER2 biomarker detection using the core-shell magnetic metal-organic frameworks. *J. Electroanal. Chem.* **2020**, *877*, 114722.
(49) Guerrero-Esteban, T.; Gutiérrez-Sánchez, C.; García-Mendiola, T.; Revenga-Parra, M.; Pariente, F.; Lorenzo, E. Bifunctional carbon nanodots for highly sensitive HER2 determination based on electrochemiluminescence. *Sens. Actuat. B: Chem.* **2021**, *343*, 130096.

https://doi.org/10.1021/acsmeasuresciau.2c00067
ACS Meas. Sci. Au 2023, 3, 226−235