Supplementary Materials: Design of an Affibody-Based Recognition Strategy for Human Epidermal Growth Factor Receptor 2 (HER2) Detection by Electrochemical Biosensors

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1. 1-Naphthol Calibration Curve

An enzyme-amplified detection scheme based on the coupling of secondary biotinylated bioreceptor with streptavidin-alkaline phosphatase enzyme conjugate was applied. The enzyme catalyzed the hydrolysis of the electro-inactive 1-naphthyl-phosphate to the electro-active 1-naphthol, which was detected by means of differential pulse voltammetry (DPV). Electrochemical response to 1-naphthol of the graphite screen-printed arrays was evaluated (Figure S1).

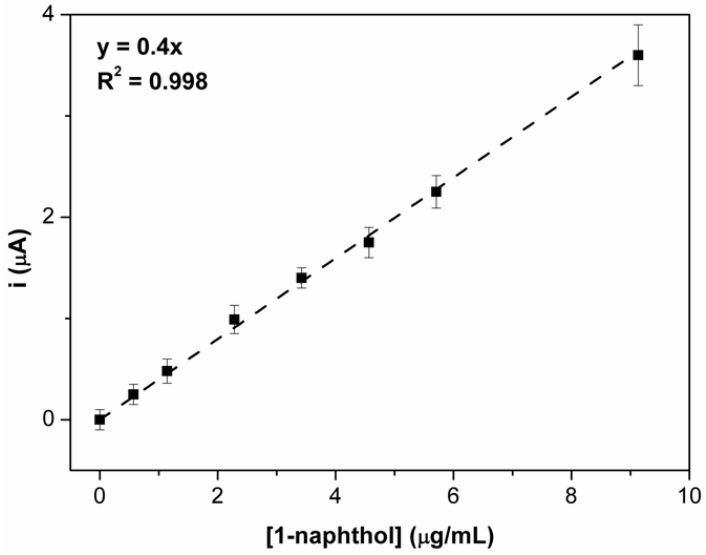

Figure S1. Calibration curve of 1-naphthol.

As shown in Figure S1, the detection of 1-naphthol concentration was achieved in the linear range between 0.6 and 9 μg/mL (y = 0.4x, coefficient of determination (R^2) = 0.998) with a limit of detection of 0.75 μg/mL (calculated as 3 times standard deviation of blank divided the slope of calibration curve ($3S_{blank}$/Slope).

2. Optimization of Experimental Parameters of Affibody/Affibody Assay

The optimization of the experimental parameters in the case of dual affibody sandwich assay was performed in order to find the best conditions for human epidermal growth factor receptor 2 (HER2) binding and detection. The suitable experimental conditions were chosen in accordance with the current difference value (ΔI) obtained in the presence of 10 ng/mL HER2 (I_{HER2}) and the blank (0 ng/mL HER2, I_{Blank}), and the percentage Relative Standard Deviation (%RSD) values. The Figure S2 shows the optimization of Af concentration as signaling bioreceptor.

The Figure S3 shows the optimization of the incubation time of the affibody (Af) with HER2 protein.

The Figure S4 shows the choice of several blocking agents.

The Figure S5 shows the incubation time optimization for biotin 1 mM added with BSA 1% w/v used as blocking agent.

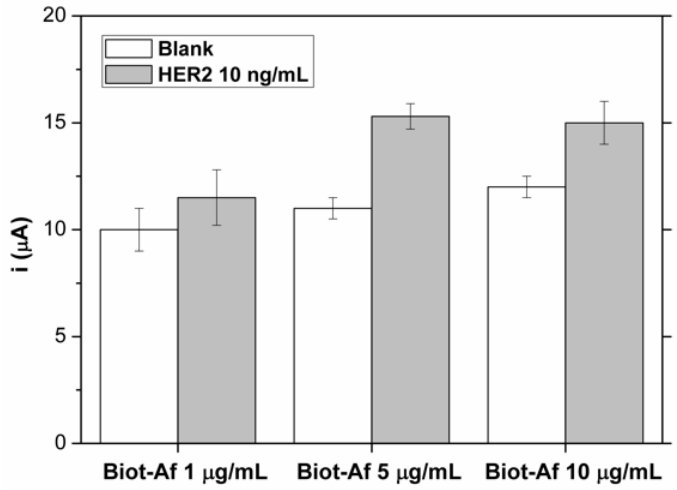

Figure S2. Optimization of Af concentration as signaling bioreceptor. The concentration of HER2 is 10 ng/mL and the concentration of secondary biotinylated affibody (Biot Af) is 5 μg/mL.

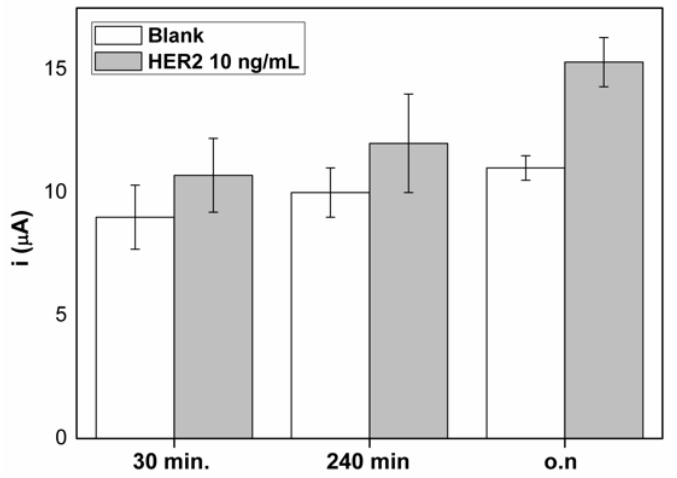

Figure S3. Optimization of the incubation time of the affibody (Af) with HER2 as a signaling bioreceptor. The concentration of the HER2 is 10 ng/mL, the concentration of Biot-Af is 5 μg/mL.

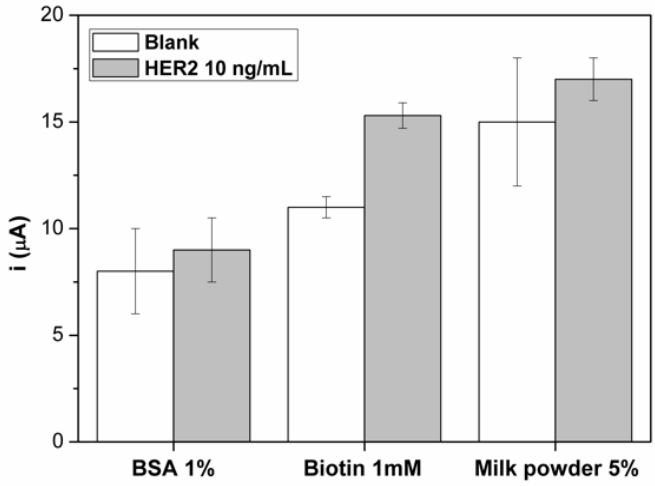

Figure S4. Blocking agent optimization: bovine serum albumin (BSA) 1% w/v, milk powder 5% w/v and biotin 1 mM containing BSA 1% w/v. The concentration of HER2 is 10 ng/mL, the concentration of Biot-Af is 5 μg/mL.

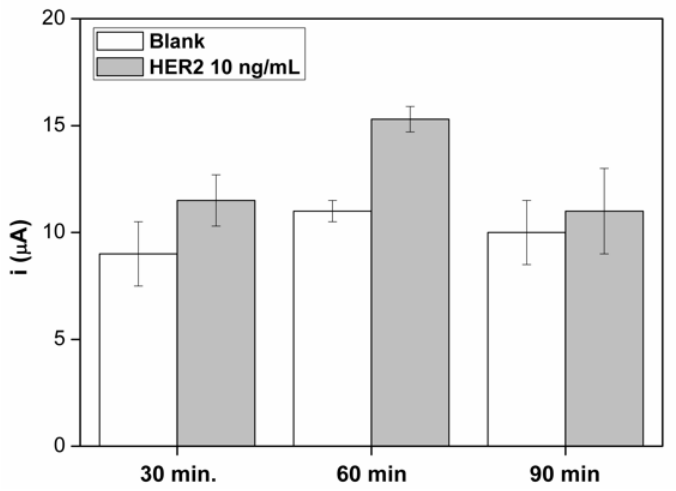

Figure S5. Incubation time optimization for biotin 1 mM added with BSA 1% w/v as blocking agent. The other experimental conditions are the same as that of Figure S3.

3. Optimization of Experimental Parameters of Antibody/Affibody Assay

The schematic representation of antibody/affibody-based sandwich assay procedure for HER2 detection was reported in Figure S6.

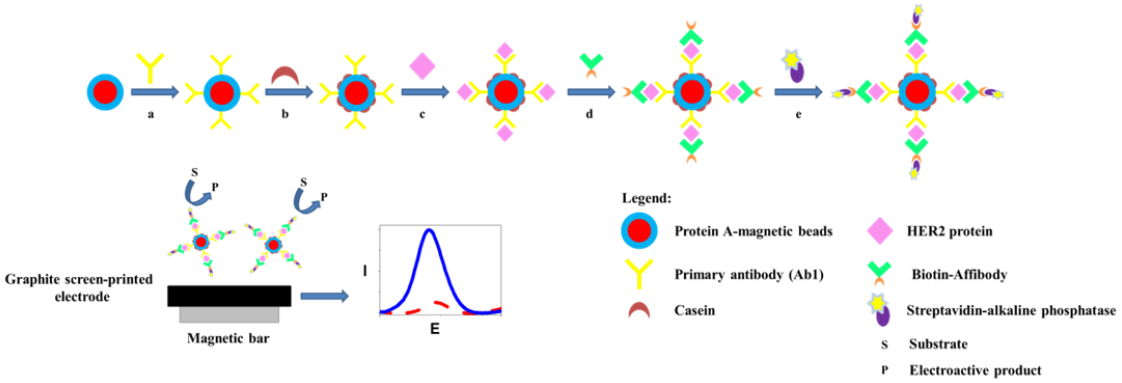

Figure S6. Schematic representation of antibody/affibody assay for HER2 detection: (**a**) functionalization of protein A-modified magnetic beads with the primary anti-HER2 antibody (Ab1); (**b**) blocking step with casein; (**c**) affinity reaction with HER2 protein; (**d**) incubation with the biotin-modified affibody; (**e**) addition of streptavidin-alkaline phosphatase conjugate. Electrochemical measurements were then performed in accordance with Section 2.3.5.

The protein A-modified (ProtA-MBs) magnetic beads were functionalized with 50 μg/mL of the primary anti-HER2 antibody (Ab1) in accordance with the procedure reported in literature [19]. Furthermore, a higher antibody (Ab) concentration (100 μg/mL) did not produce a significant increase of the sensibility of the assay (data not shown).

Various incubation times (30, 45 and 60 min) of the Ab with the ProtA-MBs were tested (Table S1, assay step a). The highest current difference (ΔI) was obtained using an incubation time of 45 min, which was thus selected for the further experiments.

In order to find the best blocking agent for the Ab-modified ProtA-MBs, various blocking agents were tested. In particular, the beads were incubated with 1% w/v BSA, 10 μg/mL rabbit Immunoglobulin G (rIgG) and 5% w/v casein for 30 and 60 min. Affinity reaction with HER2 (0 or 10 μg/mL), functionalization with secondary the biotinylated affibody and with streptavidin alkaline phosphatase enzyme were then conducted in accordance with Sections 2.3.3 and 2.3.4. Electrochemical measurements were finally carried out in accordance with Section 2.3.5.

The results of the optimization of the blocking agent were summarized in Table S1, assay step b. The optimal results both in terms of current difference ($\Delta I = I_{HER2} - I_{Blank}$) and of percentage Relative Standard Deviation (%RSD) were obtained using 5% *w/v* casein for an incubation time of 30 min.

Table S1. Experimental parameters optimization for antibody/affibody assay. Current difference (ΔI) represents the difference between the current using 10 ng/mL (I_{HER2}) and 0 ng/mL (I_{Blank}) HER2 buffered solution. The letters of assay step column are in accordance with Figure S1. %RSD values were calculated using at least 10 measurements obtained by different screen-printed arrays.

Assay step	Parameter		Current Difference (μA) ($\Delta I = I_{HER2} - I_{Blank}$)	%RSD
a	Ab incubation time	30 min	1.2	10
		45 min	2.2	11
		60 min	2.0	12
b	1% w/v BSA incubation time	30 min	1.3	10
		60 min	1.0	9
	10 μg/mL rIgG	30 min	1.5	11
		60 min	1.7	12
	5% w/v casein incubation time	30 min	2.2	11
		60 min	2.0	10

Ab1: primary anti-HER2 antibody.

4. Optimization of Experimental Parameters of Affibody/Antibody Assay

The schematic representation of affibody/antibody assay procedure for HER2 detection was reported in Figure S7.

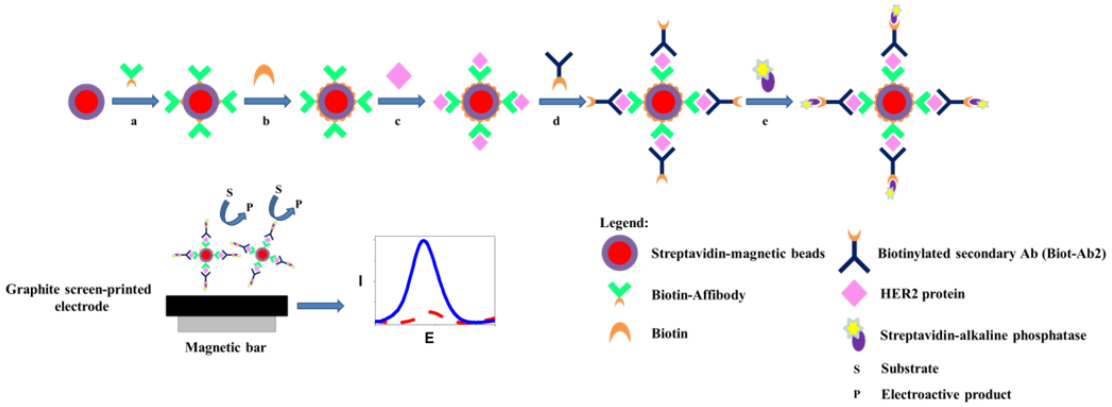

Figure S7. Schematic representation of affibody/antibody-based assay for HER2 detection: (**a**) functionalization of streptavidin-modified magnetic beads with the biotinylated affibody; (**b**) blocking step with biotin; (**c**) affinity reaction with HER2 protein; (**d**) incubation with the biotinytilated antibody (Biot-Ab2); (**e**) addition of streptavidin-alkaline phosphatase conjugate. Electrochemical measurements were then performed in accordance with Section 2.3.5.

The functionalization of the streptavidin-modified magnetic beads (Strept-MBs) with the biotinylated affibody (Biot-Af) and the blocking step with biotin, in the case of affibody/antibody sandwich assay, were conducted following the optimized procedure reported respectively in Sections 2.4.1 and 2.4.2 (5 μg/mL affibody concentration; overnight affibody incubation time; 1 μM biotin solution; 90 min biotin incubation time). The modified beads were then incubated with 10 or 0 (Blank) μg/mL HER2 for 20 min in accordance with Section 2.4.3.

The concentration and the incubation time of the secondary biotinylated antibody were optimized. In particular, the beads were incubated with 0.5, 1 and 5 μg/mL of biotinylated antibody solution for 30, 60 or 90 min, followed by the addition of streptavidin-alkaline phosphatase conjugate and the addition of the enzyme substrate. Electrochemical measurements were then performed in accordance with Section 2.3.5. Results, expressed as current difference (ΔI) between

the current obtained in the presence (10 μg/mL) and in the absence of HER2 (0 μg/mL) (respectively I_{HER2} and I_{Blank}) were reported in Table S2. %RSD values were calculated using at least 10 measurements obtained through different screen-printed arrays.

Table S2. Experimental parameters optimization for affibody/antibody assay. Current difference (ΔI) represents the difference between the current using 10 ng/mL (I_{HER2}) and 0 ng/mL (I_{Blank}) HER2-buffered solution. The letters of assay step column are in accordance with Figure S2. %RSD values were calculated using at least 10 measurements obtained by different screen-printed arrays.

Assay step	Parameter		Current Difference (μA) ($\Delta I = I_{HER2} - I_{Blank}$)	%RSD
d	Biot-Ab2 concentration	0.5 μg/mL	1.2	9
		1 μg/mL	2.4	10
		5 μg/mL	1.8	11
	Biot-Ab2 incubation time	30 min	1.3	8
		60 min	2.4	10
		90 min	1.9	12

Biot-Ab2: biotinylated secondary Antibody.

1 μg/mL Biot-Ab2 concentration and 30 min incubation time were not sufficient in order to bind the amount of the protein, while for higher Biot-Ab2 concentration and incubation time (5 μg/mL and 90 min respectively) a decrease of ΔI values was observed, probably due to its nonspecific adsorption on the surface of the beads. Therefore, the concentration of 1 μg/mL Biot-Ab2 of and the incubation time of 60 min were chosen for further experiments.