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A shear-enhanced CNT-assembly nanosensor platform for ultra-sensitive and selective protein detection

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ABSTRACT

Detection and quantification of low-concentration proteins in heterogeneous media are generally plagued by two distinct obstacles: lack of sensitivity due to high dissociation equilibrium constant K_D and non-specificity due to an abundance of non-targets with similar K_D. Herein, we report a nanoscale protein-sensing platform with a non-equilibrium on-off switch that employs dielectrophoretic and hydrodynamic shear forces to overcome these thermodynamic limitations with irreversible kinetics. The detection sensitivity is achieved with complete association of the antibody-antigen-antibody (Ab-Ag-Ab) complex by precisely and rapidly assembling carbon nanotubes (CNT) across two parallel electrodes *via* sequential DC electrophoresis and AC dielectrophoresis (DEP), and with single-CNT electron tunneling conductance. The high selectivity is achieved with a critical hydrodynamic shear rate between the activated dissociation shear rates of target and non-target linkers of the aligned CNTs. We are able to reach detection limits of 100 attomolar (aM) and 10 femtomolar (fM) in pure samples for two ELISA assays with low and high dissociation constant: biotin/streptavidin (10 fM) and HER2/HER2 antibody (0.44 ± 0.07 nM), respectively. For both models, irreversible capture and shearing allow us to tune the dynamic range up to 5 decades by increasing the CNT numbers. We also demonstrate in spiked serum sample high selectivity towards target HER2 proteins against non-target HER2 isoform of a similar K_D. The detection limit for HER2 in serum is lower than 100 fM.

1. Introduction

A sensitive, selective, rapid and affordable detection of proteins has potential applications in early detection (Kohno et al., 2011; Bruno and Njar, 2007; Anderson, 2005) and monitoring disease progressions (Kitano, 2002; Srinivas et al., 2001). Conventional gold standard assay for protein detection is the enzyme-linked immunosorbent assay (ELISA) (Jia et al., 2009; Findlay et al., 2000). But the assay lacks the requisite detection sensitivity for many clinical samples due to high dissociation constant K_D of most antibody-antigen pairs, rendering the antibody-antigen (Ab-Ag) complex thermodynamically unfavorable at target concentration lower than K_D. Since most antibodies have K_D in the low μM to nM range (Hu et al., 2007; Glaser, 1993), with a few high-affinity antibodies in the pM range, detections lower than pM are hence considered to be beyond reach. Furthermore, the detection dynamic range of ELISA is often 2–3 orders of magnitude as a result of target saturation at equilibrium (Gam, 2012). This low dynamic range of assays is incompatible with physiological protein concentrations that vary over 4 orders of magnitude in serum, blood or urine (Lilja et al., 2008; Rusling et al., 2010). Lack of selectivity is another problem for the ELISA assay that leads to false positives. For instance, false positive detection of bladder cancer urinary protein biomarker can be as high as 31% (Budman et al., 2008). False positives are caused by non-targets with similar K_D, which cannot be removed by conventional washing steps during the assay. Even if there is a significant difference in K_D between targets and non-targets, the low-abundant targets cannot compete for the antibodies when the concentrations of the non-target proteins exceed that of the target by orders of magnitude (Hortin and Sviridov, 2010). Therefore, development of a generic protein detection platform that has high sensitivity, selectivity and large dynamic range would then allow detection and quantification of arbitrary protein targets in untreated physiological samples by current commercial antibodies.

The technical obstacles of ELISA assay for early disease detection, accurate prognosis and highly reliable predictions are pushing the biosensing community to come up with novel detection technologies to enhance detection sensitivity and selectivity (Kokkinos et al., 2016; Pei et al., 2013). The nanoscale electrochemical immunoassay opens new horizons for highly sensitive yet simple and robust detection of biomarkers (Zhu et al., 2015; Chikkaveeraiah et al., 2012; Privett

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et al., 2010; Grieshaber et al., 2008). In particular, CNT sensors provide a wide electrochemical window, fast electron transfer kinetics, and biocompatibility, which make them a good candidate for electrochemical molecular recognition (Balasubramanian and Burghard, 2006). A CNT-FET label-free protein biosensors was reported by Maehashi et al. (2007) with a detection limit down to 250 pM. The detection is quantified by measuring source-drain current of CNT-FET as it changes with protein docking on the antibodies or aptamers functionalized surface. More recently, Gomes-Filho et al. (2013) overcome the detection limitation for an antibody-functionalized CNT ELISA sensor by using an enzymatic amplification technique with horseradish peroxidase (HRP) conjugated detection antibodies. The amperometric signals are thus amplified under optimum pH and buffer concentration with a detection limit of 0.033 ng/ml (~1 pM). Pandiaraj et al. (2014) on the other hand, doped the system with redox species Fe (III)/Fe (II), which decreases the charge-transfer resistance R_{ct} to improve the sensitivity.

However, the CNT-FET charge sensor can only detect charged proteins within one Debye length from the surface of the CNT and is hence sensitive to the sample ionic strength (Stern et al., 2007). Even though enzymatic/redox-reporter amplifies current signal, those electroactive species activities are strongly influenced by the medium pH (Zhang et al., 2016), which may affect the conformations of target proteins and thus limits the detection sensitivity. Moreover, the stability of enzyme functionality and thermodynamic affinity of the enzyme-conjugated antibodies can potentially compromise the high CNT sensitivity. Therefore, a robust and selective CNT-ELISA platform that does not depend on medium pH, ionic strength and extensive sample pretreatment would significantly enhance the CNT platform.

We report such a platform here by significantly enhancing both the thermodynamic sensitivity and selectivity of the ELISA complex with non-equilibrium and irreversible phenomena related to CNTs. The CNTs are used as capturing, reporting and selectivity enhancement agents in conjunction with a precise but rapid assembly technique. The high aspect ratio of the CNT endows them with large induced dipoles (Zhou et al., 2007), large dielectrophoretic mobility (Zhou et al., 2006) and large hydrodynamic drag (Hölzer and Sommerfeld, 2008). After targets are introduced to bind with polyclonal capture antibodies on the parallel Au electrodes that are 1 μm separated, a solution containing the monoclonal detection antibodies functionalized CNTs with wrapped DNAs are driven by DC electrophoresis and AC dielectrophoresis (DEP) to assemble across the electrode pair and form antigen-antibody-antigen (Ab-Ag-Ab) ELISA complex. The rapid and irreversible DC and AC DEP trapping not only captures more targets than is allowed by molecular association at equilibrium, but also significantly reduces the overall assay time compared to diffusion-limited assays. Zhou et al. (2006), Cheng et al. (2010) and Basuray et al. (2009) have proven that the high AC field at assembled CNTs across two parallel electrodes can rapidly isolate bacteria and long DNAs in a flowing solution by dielectrophoresis. Next, a cross-flow passes across the assembled CNTs to irreversibly shear off non-target-CNT complexes, thus enhancing the selectivity of the platform. Since the hydrodynamic drag force of CNTs is inversely proportional to the nature log of CNT's aspect ratio, the force is 2 orders of magnitude higher than that of a normal wash without CNTs. Thus, the force can be precisely tuned with shear rate to achieve the optimal selectivity for an Ab-Ag pair, as the CNTs are perfectly aligned in the assembly. Only target-CNTs complexes remain after shear, and the number of detectable targets is governed by the CNT number that can be easily tuned to produce a large dynamic range. As a result, we have developed a new robust CNT protein nanosensor platform that is simple and rapid, with high sensitivity and specificity over a 5-decade dynamic range.

2. Material and methods

2.1. Reagents and chemicals

Streptavidin, 11-Mercaptoundecanoic acid, 1X PBS (pH 7.4), EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), human serum, and gold etchant were obtained from Sigma Aldrich. EZ-link amine-PEG_2-Biotin was obtained from VWR, MES sodium salt was purchased from Fischer Scientific. HER2 protein, HER2 capture antibody, detection antibody, HER2 partial recombinant protein were purchased from Novus Biologicals. Single strand DNA was obtained from IDT (Integrated DNA Technologies) with a sequence of 5′ TGG TTC TCT CCG AAA TAG CTT TAG GG of molecular weight is 8898.8 g/mol. COOH-SWCNTs was purchased from Carbon Solution. A dialysis kit was obtained from Thermo Scientific with a MWCO value of 7000. 54.59 mg of 11-mercaptoundonaic acid was dissolved in 50 ml of 95% of alcohol solution to make a 5 mM of thiol solution, and the pH was adjusted to 2 with 0.1 M HCl. 0.1 M of MES (pH 5.5) was prepared by dissolving 10.861 g MES sodium salt in 500 ml of DI water, and the pH is adjusted with 0.5 M of NaOH. 100 mM of EDC solution was made by dissolving 19 mg EDC in 1 ml of MES solution, and 50 mM of biotin solution was prepared with 19 mg biotin in 1 ml of MES solution. Different concentrations of streptavidin, HER2 and negative control HER2 isoform were dissolved in 1X PBS. 10 μl of 0.08 μg/μl of HER2 was spiked into 490 μl of human serum to make a 1 nM sample. The biotin solution was stored in a freezer at 4 °C until ready for use, and all streptavidin and HER2 samples were stored at −20 °C. The samples were slowly dissolved at 0 °C before use.

2.2. Fabrication of chip pattern with 1 μm and 3 μm gaps

Patterns were fabricated on glass slides that were first soaked in base piranha for 3 min and washed thoroughly with DI water. Detailed fabrication steps for chips with 3 μm and 1 μm gaps are discussed in the supplementary section (S.1a and S.2a).

2.3. Functionalization of biotin/capture HER2 antibody on gold electrode

Chips with 1 μm wide gap were incubated in 5 mM thiol solution for 24 h after N_2 gas purging for 5 min to remove oxygen. Detailed techniques and results are discussed in the supplementary section (S.2b and Fig. S-2).

2.4. Dispersion of COOH-SWCNTs and functionalization of DNA and biotin/detection HER2 antibody on CNT surface

4 mg of COOH-SWCNTs with a length of 1–3 μm were added into 6 ml of DI water and ultra-sonicated with 1 s pulse and 1 s rest for 5 min under 40% power (10 W) using Qsonica sonicators. The upper 1 ml solution was collected after ultra-centrifugation for 90 min at 18334*g*. Then, 40 μl of the solution was mixed with 40 μl of 1 mM ssDNA solution in 1X PBS under a light sonication at 20% power with 1 s pulse and 1 s rest for 5 min. After DNAs have non-specifically bound to CNTs, 10 μl of EDC in MES solution was added to react with the DNA wrapped COOH-SWCNTs, and the solution was lightly sonicated at 20% power with 1 s pulse and 1 s rest for 5 min. To functionalize biotin onto COOH-SWCNTs, 10 μl of 50 mM of biotin was then added to covalently bind with CNTs for 2 h and the solution was gently mixed with a rotator. At last, the solution was dialyzed to remove any non-bounded biotin and EDC for 6.5 h, and the collected solution was used as a biotin functionalized CNTs stock solution. To functionalize detection (monoclonal) HER2 antibody onto COOH-SWCNTs, 10 μl of 0.05 mg/ml of monoclonal HER2 antibody in 1XPBS solution was added to covalently bind with CNTs for 2 h, and the solution was mixed with a rotator. At last, the solution was centrifuged at a speed of