Nanomechanical sensor for rapid and ultrasensitive detection of tumor markers in serum using nanobody

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ABSTRACT

Early cancer diagnosis requires ultrasensitive detection of tumor markers in blood. To this end, we develop a novel microcantilever immunosensor using nanobodies (Nbs) as receptors. As the smallest antibody (Ab) entity comprising an intact antigen-binding site, Nbs achieve dense receptor layers and short distances between antigen-binding regions and sensor surfaces, which significantly elevate the generation and transmission of surface stress. Owing to the inherent thiol group at the C-terminus, Nbs are covalently immobilized on microcantilever surfaces in directed orientation via one-step reaction, which further enhances the stress generation. For microcantilever-based nanomechanical sensor, these advantages dramatically increase the sensor sensitivity. Thus, Nb-functionalized microcantilevers can detect picomolar concentrations of tumor markers with three orders of magnitude higher sensitivity, when compared with conventional Ab-functionalized microcantilevers. This proof-of-concept study demonstrates an ultrasensitive, label-free, rapid, and low-cost method for tumor marker detection. Moreover, interestingly, we find Nb inactivation on sensor interfaces when using macromolecule blocking reagents. The adsorption-induced inactivation is presumably caused by the change of interfacial properties, due to binding site occlusion upon complex coimmobilization formations. Our findings are generalized to any coimmobilization methodology for Nbs and, thus, for the construction of high-performance immuno-surfaces.

KEYWORDS

nanobody-based biosensor, stress enhancement, early cancer detection, tumor markers, adsorption-induced inactivation

1 Introduction

Cancer is a leading cause of death, and poses difficult challenges to the whole world. The average 5-year survival rate is approximately 26% for advanced cancer patients, primarily due to a lack of effective therapies that restrict invasive distant cancer cell metastases. However, when cancer is diagnosed at an early stage, curable rates can be improved to levels exceeding 90%, by strategic surgical resection and drug therapies [1, 2]. Early diagnosis is therefore critical for clinical treatment of cancer; however, traditional diagnostic approaches such as biopsy and imaging still have limitations [3]. For example, computer tomography only detects tumors with a minimum diameter of 1 cm, which unfortunately occurs at a later stage of cancer. Hence, there is an urgent need to identify effective diagnostic technologies to complement traditional morphological methods for the early diagnosis of cancer.

Secretory proteins in the circulation can relay information from different tissue and cell types in the body [4, 5]. As a result, refined proteomic technologies have been used to predict and identify protein biomarkers used for the diagnosis and prognosis of specific diseases [6]. For instance, prostate specific antigen (PSA) levels are elevated at early prostate

cancer stages, providing important diagnostic and prognostic information, whereas histopathological examinations and imaging cannot reliably guide diagnosis and treatment [7]. Therefore, a promising strategy for the early diagnosis of cancer is to detect early tumor markers in blood. However, tumor marker concentrations are often ultralow for early-stage cancers. In addition, other serum protein concentrations may be several orders of magnitude higher, e.g., albumin, which causes elevated noise during detection [8]. Consequently, ultrasensitive sensing methods are required for these situations.

Conventional sensors, i.e., enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC), have inherent disadvantages, including limited sensitivity, expensive instrumentation, time-consuming procedures, and complicated labeling steps [9]. Fortunately, nanotechnology has provided biosensors with unprecedented sensitivity for cancer diagnosis and monitoring [10–13]. Recently, microcantilever biosensors have gathered considerable merit as emerging technologies [14–16]. The fundamental principle is that interactions between target and receptor molecules on the sensor surface are transduced to a surface stress, which mechanically bends the microcantilever [17–20]. Studies have shown that microcantilever sensors are sensitive, label-free,

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rapid, and real-time [21, 22]. Such attractive advantages make microcantilever biosensors widely exploited in proteomics, drug screening, and environmental monitoring [23–25]. However, for disease diagnosis, especially early cancer diagnosis, there is a long way to go in term of developing microcantilever sensing method to resolve the sensitivity bottleneck.

For microcantilever sensors based on mechanical principles, their inherent sensitivity is associated with stress effects. In terms of stress generation and transmission, receptor molecule size and orientation on the sensor surface are important factors which determine response levels from biochemical interactions [26, 27]. For sensors with gold-coated surfaces, receptor molecules are often immobilized on the surface through self-assembled monolayers (SAM) of thiolated molecules. However, antibody (Ab) (receptor molecule) orientation is therefore random, leading to antigen-binding site occlusion mediated by steric hindrance, and a loss in binding capabilities [28]. In contrast, for uniformly oriented Abs, increased antigen-binding sites are exposed and, thus, bind more antigens, thereby producing a larger deflection signal [29].

Microcantilever nanomechanical motion ultimately arises from the transmitted stress, which is produced by molecular interactions between the antigen and Ab, across the carbon backbone of the Ab to the sensor surface. Consequently, stress transmission efficiency is also another important factor in determining microcantilever immunosensor sensitivities [17, 30]. In our previous study [31], we observed that shortened distances between antigen-binding regions and the sensor surfaces greatly improved stress transmission, and significantly increased microcantilever immunosensor sensitivity.

Ideally, receptors for microcantilever sensors must realize the highly oriented immobilization and shortened distance of stress transmission, in the meantime, tend to be small in size (to generate a denser receptor layer). Thus, the identification of ideal receptors and a well-established immobilization

method are key to achieving the ultrasensitive microcantilever immunosensors based on stress effects.

In the early 1990s, in camelid serum, scientists discovered an exception to the conventional Ab structure, i.e., the heavy chain antibody (HCAb) without light chains. At the N-terminus, the heavy chain contains a dedicated variable domain of nanometer (nm) dimensions, i.e., variable domain of heavy chain of heavy-chain antibody (VHH), also known as a nanobody (Nb). Nbs with a molecular weight (M_w) of ~ 15 kDa are the minimal Ab entities which retain full antigen-binding capacity and high affinity [32]. Phage display techniques have enabled the screening of high-affinity Nbs against virtually any disease biomarker by using huge, well-established VHH libraries [33]. In combination with protein engineering, Nbs can be labeled with tags, including biotins and cysteine residues. Therefore, Nbs have unique advantages over intact Abs, i.e., minimal size, expected dense receptor layers, increased orientated immobilization capabilities on sensor surfaces, short linkers for stress transmission, and their ease of production.

In this study, we propose a novel sensing methodology based on Nbs, aiming to develop the next generation microcantilever-based immunosensor, with ultrahigh sensitivity (Fig. 1). Owing to the inherent thiol group at the C-terminus, Nbs were covalently immobilized in directed orientation to the gold surface of microcantilevers via one-step reaction (Fig. 2(b)). ELISA and X-ray photoelectron spectroscopy (XPS) were performed to assess Nb activity on the microcantilever surfaces. To investigate the feasibility of our technique, we tested the sensitivity of microcantilever modified by Nbs against carcinoembryonic antigen (CEA) compared with intact Abs functionalized microcantilever (Fig. 2(c)). To further demonstrate technical adaptability and utility, Nbs against human epidermal growth factor receptor 2 (HER2) were used to biofunctionalize the microcantilever. Furthermore, we investigated the size effects of blocking reagents, which can influence the binding of Nbs

Figure 1 Overall schematic of the nanomechanical sensing platform for tumor marker detection, from sampling to therapy. (a) Patient blood sample collection and processing, (b) tumor marker detection using nanobody-functionalized microcantilevers, (c) early cancer diagnosis based on sensor responses, (d) targeted cancer therapeutic strategies and, and (e) increased therapeutic effects from cancer treatments.

Figure 2 Nanomechanical sensors based on Nbs. (a) Distinguishing features of conventional Abs, camelid HCABs, and Nbs structure, size, and M_w . The single VHH-domain is the minimal fragment in HCABs containing an entire antigen-binding site, i.e., the Nb. (b) Nb preparation by protein engineering (red arrows 1–2) and the oriented immobilization of Nbs on gold-coated microcantilever surfaces owing to inherent thiol group via one-step binding (black arrow 1). (c) Preparation of conventional Abs using hybridoma technology (red arrows 1–4), and the random immobilization of conventional Abs onto gold-coated microcantilever surfaces via self-assembled alkanethiol monolayers (black arrows 1–3). (d) Schematic representation of a photodetection device. PSD: position sensitive detector.

to antigens, thus affecting detection sensitivity.

2 Results and discussion

2.1 Nb characterization

Protein gel electrophoresis was used to characterize the M_w of anti-CEA Nb. The stained protein bands analysis demonstrated high Nb purity (Fig. 3(a)). When compared with protein standard markers, the M_w s of bovine serum albumin (BSA), CEA, and anti-CEA Nb were 75, 110, and 15 kDa, respectively.

The M_w of intact Ab was 150 kDa (Fig. 2(a)) [34]. Thus, the M_w of the Nb is equivalent to one-tenth of the corresponding conventional Ab. Similarly, this small Nb M_w agreed with the ideal receptor size for microcantilever sensors.

Next, we measured the binding affinity of anti-CEA Nb and monoclonal antibody (mAb) against CEA (anti-CEA mAb) to CEA, using surface plasmon resonance (SPR) (Figs. 3(b) and 3(c)). By analyzing the binding kinetics for respective antigen-binding sites, we found that both receptors bound to their targets with high affinity, establishing a molecular basis for sensitive CEA detection. The fitted data showed that anti-CEA

Figure 3 Receptor molecule properties. (a) The M_w s of anti-CEA Nb, CEA, and BSA using gel electrophoresis. The binding affinities of (b) anti-CEA Nb and (c) anti-CEA mAb to CEA using SPR.

Nb bound to CEA with an affinity of 8.05×10^{-10} M, whereas anti-CEA mAb has a slightly higher affinity of 3.59×10^{-10} M. In addition, the binding affinities of anti-HER2 Nb and anti-HER2 mAb to HER2 were also measured using SPR (Fig. S1 in the Electronic Supplementary Material (ESM)).

2.2 Nb activity on the microcantilever surface

To confirm Nbs were correctly immobilized on the microcantilever surface, we evaluated the surface morphology of the microcantilever gold-coated surfaces in different modification states, using an atomic force microscopy (AFM). The root-mean-square roughness (R_q) values of the bare gold surface and CEA-Nb-coated gold surface were 1.2 and 1.5 nm (Fig. 4(a)), respectively. This latter increase in R_q indicates that anti-CEA Nbs were successfully immobilized to the gold surface. Similarly, we observed a further increase in R_q value for the CEA-mAb-

coated gold surface (R_q 4.6 nm, Fig. 4(a)) which may be due to their larger molecular size.

To further verify that anti-CEA Nbs were successfully immobilized to the microcantilever surface, we performed a designed ELISA with anti-Myc mAb (Myc is a polypeptide consisting of 10 amino acids, which functions as a protein tag) conjugated with horseradish peroxidase (anti-Myc mAb-HRP) added to the microplate well containing a microcantilever functionalized with anti-CEA Nbs (tagged by Myc tag). The absorbance (450 nm) B reached 0.743 after the addition of 3,3',5,5'-tetramethylbenzidine (TMB) (middle panel in Fig. 4(b)). A naked microcantilever served as a negative control; its absorbance B was 0.017 (left panel in Fig. 4(b)). These large differences in absorbance verified the successful functionalization of microcantilevers with anti-CEA Nbs. After the verification of Nb immobilization at the molecular and microstructure

Figure 4 Verification of immobilization and holding activity of anti-CEA Nb on the microcantilever surface. (a) AFM topography of the microcantilever alone, the CEA-Nb-functionalized microcantilever, and the CEA-mAb-functionalized microcantilever. (b) Immobilization and activity of anti-CEA Nbs on the microcantilever as characterized by ELISA. (c) Representative XPS spectra of a microcantilever coated with anti-CEA Nb before (dark green) and after (red) CEA binding. High-resolution XPS spectra of (d) Au 4f, (e) S 2p, and (f) O 1s core level.

level, we wanted to confirm whether the anti-CEA Nbs on the microcantilever surfaces maintained CEA-binding capacity. To confirm this, anti-CEA mAb-HRP was added after the binding of CEA to anti-CEA Nbs on the microcantilever surface (right panel in Fig. 4(b)). The absorbance B was 0.286 and much larger than negative control, which exhibited a holding CEA-binding capacity of anti-CEA Nbs on the sensor surface.

To further validate anti-CEA Nb activity on the sensor surface, XPS was used to directly characterize the sensor surface before and after antigen binding (Fig. 4(c)). When compared with the CEA-Nb-functionalized microcantilever, upon CEA addition, we observed that the binding energy (BE) switched from 83.91, 162.01, and 532.31 to 83.45, 161.54, and 532.71 eV for Au 4f, S 2p, and O 1s, respectively (Figs. 4(d)–4(f)). This is likely due to the chemical environment changes around Au, S, and O elements. When CEA bound to anti-CEA Nb on the sensor surface by hydrogen bonds, Au element electrons transferred inward, S element electrons transferred inward, and O element electrons transferred outward. With respect to the atomic concentration (AC) on the sensor surface, we observed a significant difference between the two modification states. There were varying degrees of increase in S, N, and O atomic concentrations, while a 2.55% decrease in C atomic concentration was also recorded. These BE and AC differences were presumably affected by the variations of chemical bonds and surroundings, suggesting that CEA had successfully bound to anti-CEA Nbs on the sensor interface. Hence, we inferred that anti-CEA Nbs on the microcantilever surface still maintained an active state, thus laying the foundation for robust biomarker detection. Overall, the data from AFM, ELISA, and XPS studies correlated well with each other; thus, the Nb layer was successfully immobilized to the sensor surface, and more importantly, it was active.

2.3 Nanomechanical detection of tumor markers

In this study, we have developed a Nb-based microcantilever immunosensor. Conventional Abs are typically immobilized

onto gold surfaces via thiol self-assembly monolayers by the reaction of NHS ester with active amino groups of auto-antibodies, which can be inefficient due to the conjugation mode of point to point. However, the amino groups are distributed throughout the Ab domain, which can result in the random orientation of conventional Abs (Fig. 2(c)); this process is complex, time-consuming, and even worse, produces a random orientation. However, we bioengineered the simultaneous synthesis of Nbs and attached a thiol group, without any additional manipulation and manufacturing cost. On the basis of the native thiol group at the C-terminus, Nbs can be covalently and directionally immobilized onto microcantilever gold surface through Au–S linkage via one-step functionalization. Concurrently, the one-step immobilization shows high immobilization efficiency based on the conjugation mode of point to plane (Fig. 2(b)).

Now that we have presented the basic element of our novel biosensor, we analyzed the mechanical responses for CEA detection, a typical marker of colon cancer, ranging from 0.1 to 500 ng·mL^{−1}. After being functionalized with anti-CEA Nbs, the microcantilever was installed into a flow chamber, and its deflection was monitored in real time using an optical lever (Fig. 2(d)). After a stable baseline of deflection was achieved, a CEA sample diluted in phosphate buffered saline (PBS) was sequentially injected into the chamber. For a 50 ng·mL^{−1} CEA sample, a notable trend of fast downward deflection (bending toward the silicon side) was observed within the first 5 min after sample addition (Fig. 5(a)). Final deflection of the stable equilibrium state was achieved at ~ 45 nm after ~ 1 h. Furthermore, to investigate and quantify the relationship between microcantilever deflection and CEA concentration, the deflection responses for various concentrations (0.1–50 ng·mL^{−1}) were detected (Fig. 5(a)). Equilibrium-bending signals of ~ 41, 25, 18, and 8 nm were obtained for 50, 10, 1, and 0.1 ng·mL^{−1} CEA samples, respectively. We observed a clear trend in decreasing bending signals concomitant with reducing CEA concentration, indicating a significant positive correlative

Figure 5 Nanomechanical responses of microcantilevers for CEA detection. Real-time deflections of microcantilevers caused by (a) the binding of CEA to anti-CEA Nbs on the sensor surface, and (b) the binding of CEA to anti-CEA mAbs on the sensor surface. (c) Equilibrium deflections of microcantilevers for various CEA concentrations using anti-CEA Nbs (purple) and anti-CEA mAbs (black) as receptor molecules, respectively. The gray box represents three-fold of noise. The inset shows the linear response range (0.1–50 ng·mL^{−1}) for CEA-Nb-functionalized microcantilever. Error bars represent the standard deviations of three repeated determinations. (d) Detection data for spiked CEA in serum using microcantilevers functionalized with anti-CEA Nbs.

relationship between signals and CEA concentrations. To discriminate bending signals from nonspecific interactions, we performed two control experiments. Firstly, we replaced the capture Nb by anti-HER2 Nb which was nonspecific to CEA. When 500 ng·mL⁻¹ CEA was added, we detected no deflection signal. Similarly, minimal bending signals were detected after 500 ng·mL⁻¹ BSA was injected into the flow chamber loaded with CEA-Nb-functionalized microcantilevers. These control experiments highlighted that considerable deflection was caused by the specific binding of anti-CEA Nb to CEA. Bending toward silicon side may be caused by increased intermolecular repulsion between antigen-Nb complexes on the microcantilever surface, resulting in differences in surface stress between the Nb coated gold surface and the silicon nitride surface of the microcantilever. The CEA concentration as low as 0.1 ng·mL⁻¹ was detectable, superior to the resolution required for CEA-based diagnosis of colon cancer (5 ng·mL⁻¹). These data demonstrated that our Nb-based microcantilever immunosensor can rapidly and quantitatively detect CEA in real time with a high sensitivity, as an example of tumor marker detection. Simultaneously, the feasibility of our technique was confirmed.

To study the sensitivity, microcantilevers functionalized with intact anti-CEA mAbs via thiol self-assembly monolayers were prepared for CEA detection (Fig. 2(c)). Figure 5(b) illustrates microcantilever deflection as a function of time for various CEA concentrations, with 500 ng·mL⁻¹ BSA as control. Similar to the anti-CEA Nb based microcantilever immunosensor, we observed a steady decline in deflection signal concomitant to decreasing CEA concentrations. For 50 ng·mL⁻¹ CEA in PBS, the equilibrium deflection of CEA-mAb-functionalized microcantilever was only 4 nm. In contrast, the equilibrium deflection of CEA-Nb-functionalized microcantilever was 10 times larger (Fig. 5(a)). Similarly, the significant deflection differences were observed at other CEA concentrations especially at low concentrations. These data suggested a much higher sensitivity of CEA-Nb-functionalized microcantilevers and indicated a much better sensing capability of Nb. More importantly, it came to our attention that the Nb had a worse affinity for CEA binding than the intact Ab (Figs. 3(b) and 3(c)), suggesting the critical role of Nb in the stress enhancement for nanomechanical sensor.

Furthermore, Fig. 5(c) illustrates variations in equilibrium deflections in microcantilevers functionalized with anti-CEA Nb (purple points) and anti-CEA mAb (black points) respectively versus CEA concentrations. With a background noise of ~ 1 nm, the lowest CEA concentration detected, above three times noise level, was 50 ng·mL⁻¹ using intact Abs. However, when Nbs served as receptor molecules, CEA concentration as low as 0.1 ng·mL⁻¹ was detectable. Closer inspection revealed that equilibrium deflections scale linearly with the logarithm of the CEA concentrations in the range of 0.1–50 ng·mL⁻¹ (inset in Fig. 5(c)). The regression equation is $D = 11.045 \log c + 19.195$ with a correlation coefficient (R^2) of 0.978, where D denotes the deflection value and c is CEA concentration. The detection limit of CEA-Nb-functionalized microcantilever is 0.03 ng·mL⁻¹ (or 2.73×10^{-12} M in equivalence) estimated from three-fold of noise, which is three orders of magnitude better than that obtained by using conventional Ab functionalized microcantilever and is also significantly superior to the threshold in clinical diagnosis (5 ng·mL⁻¹). These data demonstrated our technology can be applicable to the ultrasensitive detection and quantification of tumor markers even at an extremely low concentration, which paves the way for the construction of next generation microcantilever immunosensor with ultrahigh sensitivity.

Proper functionalization of a sensor interface with receptor molecules is important but highly challenging [35]. We would also like to note that efforts are also focused on the creation of densely packed homogeneous receptor layers on sensor surfaces [28]. More importantly, short linker distances between interactive regions and sensor interfaces are key to enhancing stress transmission for microcantilever-based immunosensor in surface stress mode [29, 30]. Therefore, Nbs clearly outperformed conventional antibodies thanks to their small-size format and facile decoration with a C-terminus thiol group. Thus far, microcantilever immunosensors in static mode achieved a limit of detection (LOD) of 1–5 ng·mL⁻¹ for tumor marker detection using intact Abs as receptor molecules [24, 25], some of which only slightly improved the sensitivity, even when using a sandwich assay [23]. However, the LOD observed here, without complicated labellings and additional manipulations, was far superior to these reported values and was based on the unique mechanical properties of Nbs.

Our data indicated that sensitivity was improved by maximizing the orientation degree of the antigen-binding site, and minimizing the receptor molecule size. Owing to the native thiol group, Nbs may be immobilized in a site-directed manner, allowing more exposed active sites to enable increased antigen-binding capacities. Furthermore, shorter distances between antigen-binding regions and microcantilever surfaces could be achieved due to their small size. In the meantime, denser receptor layers could also be obtained. Consequently, uniformly oriented and denser receptor molecules could theoretically help bind more antigens, thus generating larger stresses. More importantly, greatly shortened transmission distances could also generate more efficient stress transmission by significantly decreasing stress losses. In these instances, Nb-functionalized microcantilevers could induce greater stresses and larger responses, resulting in much higher sensitivities for microcantilever immunosensors.

Similar to Nb, it was previously shown that half Ab fragment based microcantilever immunosensors demonstrated improved sensitivities when compared with that using conventional antibodies as receptor molecules. However, half Ab fragments failed to increase receptor density on the sensor surface, and only reduced the distance between the antigen-binding region and the sensor surface by half. Also, the half-Ab preparation process is complicated; complex chemical treatments can generate receptor activity losses of more than 25% [29]. Another example is the application of single-chain Fv (scFv) Ab fragment, and the LOD of microcantilever functionalized with scFv was increased to $\sim 1 \times 10^{-9}$ M. However, sensor stability, particularly under acidic condition, was unsatisfactory. It was therefore difficult to reuse and recycle scFv-functionalized microcantilevers [28]. In addition, the scFv size was twice that of Nb, which contributed to the poor presentation in receptor density and stress transmission efficiency.

2.4 Serum detection of tumor markers

Our technique has exhibited excellent sensitivity for CEA detection in standard samples, however, whether it was sufficiently sensitive to detect CEA in routine clinical samples was unclear. Blood has a complex composition and is quite different to standard samples; its complexity may cause severe cross-reaction and high background noise. Therefore, to further evaluate our immunosensor in detecting tumor markers in blood, we sought to detect CEA in serum. The real-time deflection profile for different concentrations of CEA spiked in human serum is shown in Fig. 5(d). The stable microcantilever

deflections at 70 min were approximately 13, 21, 39, and 82 nm for 5, 10, 50 and 100 ng·mL⁻¹ CEA spiked in serum, respectively. These data were slightly higher than the same CEA concentrations in PBS. A slightly upward deflection signal was observed when the serum without CEA was injected into the chamber (see control in Fig. 5(d)), which served as a reference. This response may be caused by increased effects of non-specific physical adsorptions and cross-reactions, thereby contributing to the increased background noise at ~ 2 nm. However, even at a low CEA concentration (e.g., 5 ng·mL⁻¹), the deflection signal of CEA-Nb-functionalized microcantilever was remarkably higher than 3-fold of background noise, indicating that the detection limit of CEA in serum is much lower than the threshold of clinical diagnosis (5 ng·mL⁻¹) using our technique.

These data showed that Nb-based microcantilever sensor can easily, rapidly, sensitively, and quantitatively detect CEA in serum that contain complex protein components with a sufficient sensitivity. Thus, our technique may be used for on-site CEA detection for early-stage colon cancer screening in medical system. Furthermore, an additional important advantage of our method is the simplicity and convenience. Serum required no complicated sample processing including biomarker enrichment and labelling, and similarly, complex data processing was not required.

2.5 Selectivity, stability of Nbs, and reusability of Nb-coated microcantilevers

To analyze method selectivity, the microcantilever functionalized with anti-CEA Nb was exposed to four proteins (HER2, PSA, BSA, and CEA) dissolved in PBS at the same concentration (100 ng·mL⁻¹). A deflection curve versus time was recorded (Fig. 6(a)). We found that CEA was the only protein to induce a significant deflection response of the microcantilever, whereas the others induced negligible effects. This indicated that Nb-based microcantilever immunosensors in surface stress mode had excellent selectivity, and specifically recognized

CEA from other serum proteins. The huge capacity of the phage display library ensured the screened Nbs against unique conformation sites of CEA protein, which minimized cross-reaction and thus assured the high specificity of our immunosensor.

Having performed immunosensor sensitivity and selectivity analyses, we also focused on Nb stability on the microcantilever surface. We stored a CEA-Nb-functionalized microcantilever at 4 °C in the refrigerator for a week. We observed a 15% drop in the deflection signal when compared with the fresh microcantilever modified by anti-CEA Nb upon addition of 10 ng·mL⁻¹ CEA in PBS (Fig. 6(b)). These observations indicated our immunosensor had good stability, and Nb layers on the sensor surface retained adequate binding capacity. Our results were consistent with previous studies that compared Nb stability with conventional Abs in the electrochemical sensing and drug screening [36, 37], suggesting Nbs are promising molecular diagnostic tools for clinical settings.

Reusability is an important feature of immunosensors, but still a challenge for high-performance immunosensor construction. In SPR experiments, we observed a slow dissociation of anti-CEA Nb from CEA after rinsing chips with PBS (Fig. 3(b)). Because of this slow off-rate, it was impossible to completely wash off and eliminate CEA from microcantilever surfaces in a timely manner. Thus, we regenerated Nb layers by rinsing the chamber with glycine 2.0 and then equilibrated it with PBS until the baseline became stable again. The subsequent injection of 1 ng·mL⁻¹ CEA in PBS resulted in an approximately 70% response level (Fig. 6(c)), seen before regeneration. This result indicated that regeneration was partially successful, receptor layers were still active and microcantilever reusability was verified. In the presence of robust structure [33], Nbs may tend to remain their antigen-binding capacities on the sensor surface under acidic conditions during washing steps. It is conceivable that our finding shapes the way that Nb-based immunosensor can be reused.

As regards the slight activity loss of anti-CEA Nb, unfolding and denaturation upon harsh surface regeneration may be

Figure 6 Performance metrics (other than sensitivity) of our immunosensor using Nbs as receptor molecules, including selectivity, stability, reusability, and adaptability. (a) Deflection curves of CEA-Nb-functionalized microcantilevers with injection of 100 ng·mL⁻¹ HER2, PSA, BSA, and CEA, respectively. (b) Stability of the immunosensor for CEA (10 ng·mL⁻¹) detection using fresh (black) and 8-day-stored (red) microcantilevers. (c) Sensograms of CEA binding to immobilized anti-CEA Nbs before (black) and after (red) surface regeneration with 1 ng·mL⁻¹ CEA injection. (d) Real-time microcantilever nanomechanical responses caused by the specific binding of HER2 to anti-HER2 Nbs immobilized on the sensor surface.

major contributors. To improve it, the protection of gold surface with small organic molecules should be considered [38]. From this perspective, Nb-functionalized microcantilevers can easily be recycled and reused after regeneration treatments, which will minimize the apparatus costs. In the meantime, in contrast to the difficult acquisition and complex immobilization procedures of conventional Abs, the ease of Nb production significantly reduces the material costs, and likewise, one-step immobilization lowers the threshold for technical trainings. These attractive advantages ultimately benefit affordability for the patient, bring down costs, and widen the broad application of the technology to healthcare systems. These attributes pave the way for large-scale roll-out of our technique into medical practice.

2.6 Adaptability and generalizability of the presented sensor

Having analyzed the CEA levels using Nb-coated microcantilevers, we also focused on technique adaptability and generalizability; therefore, a microcantilever immunosensor for HER2 detection was also developed. HER2 is a tyrosine kinase receptor, and a significant early diagnostic marker and therapeutic target for breast cancer [24]. Figure 6(d) shows the real-time deflection curves of HER2-Nb-functionalized

microcantilevers for different concentrations of HER2 buffered in PBS. Microcantilever deflection signals exhibited significant correlations with HER2 concentrations, indicating the successful establishment of a simple, rapid, highly sensitive and quantitative HER2 detection method. With a background noise of approximately 1 nm, the LOD for HER2 detection was considerably lower than $2 \text{ ng}\cdot\text{mL}^{-1}$ estimated from three-fold of noise, thus meeting the criteria for clinical settings. Similar to CEA detection, the presented sensor achieved a much lower limit for HER2 detection when compared with HER2-mAb-functionalized microcantilevers ($50 \text{ ng}\cdot\text{mL}^{-1}$, Fig. S2 in the ESM). These data also suggested the universality and reliability of our technique.

2.7 Adsorption-induced inactivation of Nbs on the sensor surface

To prevent nonspecific adsorption, blocking reagents are typically used to block unbound sites on the sensor surface after receptor immobilization. We initially chose BSA, a common blocking reagent, to block microcantilevers when using Nbs as receptors. Surprisingly, at the initiation of this trial, deflection signals of CEA-Nb-coated microcantilevers exhibited very small (Fig. 7(a)), which were similar to microcantilevers modified by intact Abs (Fig. 5(b)). The significantly inferior outcomes

Figure 7 Inactivation of Nbs surrounded by coimmobilized macromolecule layers on the surface-based immunosensor. (a) Real-time nanomechanical deflections of CEA-Nb-functionalized microcantilevers blocked by BSA and MCH respectively, upon $100 \text{ ng}\cdot\text{mL}^{-1}$ CEA injection. (b) Equilibrium deflections of CEA-Nb-functionalized microcantilevers for 100, 50, and $10 \text{ ng}\cdot\text{mL}^{-1}$ CEA, using MCH (red) and BSA (blue) as blocking reagents respectively. Error bars were determined as the standard deviation of deflection signals from three separate microcantilevers. (c) Height changes in CEA-Nb-functionalized gold surfaces by two-dimensional AFM imaging. AFM measurements were carried out for microcantilevers coated with Nb only, blocked by MCH, and BSA. (d) Schematic outlining possible Nb inactivation mechanisms.

were somewhat at odds with the expected result analyzed in introduction. Thus, we conjectured there may be some potential issues in binding of CEA to anti-CEA Nbs. Simultaneously, we noted that BSA had a M_w of 75 kDa (Fig. 3(a)), which was 4 times greater than Nb (~ 15 kDa), and twice as small as intact Ab (~ 150 kDa). Hence, we speculated that BSA occluded Nb binding sites due to size-induced steric hindrance. CEA is a considerable macromolecule in terms of M_w (110 kDa), thus normal binding of CEA to anti-CEA mAbs/Nbs may require sufficient binding site exposure on receptor molecules.

To validate this conjecture, we next used 6-mercapto-1-hexanol (MCH), a low- M_w compound ($C_6H_{14}OS$, $M_w = 134.24$ Da), to block unbound sites on the sensor surface. Interestingly, following MCH blocking, we observed a significant increase in deflection (up to 45 nm) when compared with the slight deflection signal of the BSA-blocked microcantilever, upon $100\text{ ng}\cdot\text{mL}^{-1}$ CEA in PBS injection (Fig. 7(a)). Microcantilevers blocked by MCH only showed no obvious deflection responses (see control; Fig. 7(a)), demonstrating an excellent efficacy in reducing nonspecific adsorption. Consequently, the greatly increased deflection signal was induced by specific interactions between CEA and anti-CEA Nbs. The micromolecule blocking reagents may enable Nb binding sites fully exposed, contributing to the restoration of normal specific binding. This was an interesting and significant outcome for sensitivity improvement. Moreover, for different CEA concentrations (10 , 50 , and $100\text{ ng}\cdot\text{mL}^{-1}$), all MCH-blocked microcantilevers revealed dramatic increases in deflection signals (Fig. 7(b)). When CEA concentration was less than $10\text{ ng}\cdot\text{mL}^{-1}$, it was difficult to discriminate deflection signals from background noise using BSA blocking method. However, deflection signals can be distinguished readily by using MCH blocking technique, suggesting strong correlations between deflection signals and blocking reagent sizes.

The structure of monolayers on gold surfaces may provide important clues for an increased understanding of Nb functions and mechanisms on sensor interfaces [39]. Therefore, to investigate the intrinsic mechanism of adsorption-induced inactivation of Nbs on sensor surfaces, we characterized the microcantilever surface using AFM. As shown in Fig. 7(c), Nb-coated microcantilever had an average height of 4 nm (R_q 1.5 nm). After MCH blocking, no statistical increase in height profile was measured, indicating antigen-binding sites on Nbs were still exposed (middle panel in Fig. 7(d)). However, when BSA was used as a blocking reagent, we observed a significant increase in height, i.e., a maximum of 8.3 nm (R_q 4.3 nm). Thus, antigen-binding sites may have been occluded, and Nb binding capacities substantially weakened (bottom panel in Fig. 7(d)). Combining the worse sensing performance of BSA-blocked microcantilever, a possible explanation for these results may be the lack of active binding sites due to size-induced steric hindrance effects of blocking reagents. MCH, a micromolecule blocking reagent, adequately addressed this issue by retaining fully exposed Nb binding sites, thereby ensuring high detection sensitivity.

We further analyzed the sensing performance of microcantilever immunosensors using Ab fragments as receptor molecules. ScFv-coated immune-surfaces theoretically provide more antigen-binding sites thanks to the small-size format of scFv molecules. However, when BSA was used to block microcantilever surfaces in dynamic mode, we were somewhat surprised to find that the sensitivity of scFv-functionalized microcantilever [25] is substantially lower when compared with the reported value in another literature using intact Ab as receptor [24], which can be well explained by our finding and conclusion. Blocking reagents may prevent normal interaction

between Nbs and antigens on sensor surfaces via binding sites occlusion. Our findings about the prevention effects in the coimmobilization process may provide further understanding of macromolecular behaviors on the interface. More importantly, our results shed new light onto existing problems about how to improve surface-induced inactivation, and new strategies for the construction of high-performance bioinspired immuno-surfaces or functional films of other devices.

3 Conclusions

In summary, we have developed a novel microcantilever-based immunosensor in surface stress mode, using Nbs as receptor molecules. Oriented immobilization and dense layer of Nbs provided higher antigen-binding capacities, leading to increased surface stress. More importantly, greatly shortened distances between antigen-binding regions and sensor surfaces remarkably enhanced the stress transmission. For microcantilever immunosensors based on mechanical principles, these advantages dramatically elevated the sensor sensitivity. Hence, the LOD of CEA was as low as $0.03\text{ ng}\cdot\text{mL}^{-1}$, achieving three orders of magnitude higher sensitivity compared with CEA-mAb-functionalized microcantilevers ($50\text{ ng}\cdot\text{mL}^{-1}$). In addition, Nbs attached to gold surfaces retained activity after regeneration treatments, thus facilitating effective recycling and reuse. Our technique provides an ultrasensitive, simple, rapid, and low-cost detection method for tumor marker detection, after one-step immobilization without complicated labellings and additional manipulations. Further, interestingly, we observed Nb inactivation when using macromolecules to block microcantilevers, but this disappeared when using micromolecule blocking technique. Our AFM analysis suggested that Nbs were occluded within macromolecules. Thus, the adsorption-induced inactivation was presumably due to Nb binding sites occlusion, which rendered antigens inaccessible. These findings can be generalized to any coimmobilization methodology for Nbs and, thus, for the construction of high-performance immuno-surfaces, which profoundly shaped high sensor sensitivity. Overall, our technique may be a promising alternative to conventional methods for early-cancer detection in the clinic. Thus, in the future, we anticipate large-scale roll-out in populations that have a high- or medium-risk of cancer.

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