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Highly sensitive nanomechanical assay for the stress transmission of carbon chain

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ABSTRACT

Here, we report the first quantitative experimental study into the molecular basis of the transmission of mechanical signal that originates from biochemical reaction focusing on the length of carbon chain. We designed an experiment by using n-alkanethiols with a same carboxyl group and different chain lengths (n=1, 5, 10 and 15) to immobilize a same receptor molecule on the gold surface of a microcantilever, and detected the nanomechanical response of biochemical reaction. The sensitivity of the microcantilever was found to be greatly influenced by the chain length of linker that is between the receptor molecule and the microcantilever surface. The efficiency of stress transmission increases significantly with decreasing length of carbon chain. At the same time, we develop a label-free microcantilever sensor for highly sensitive detection of Glycyrrhizic acid (GL). The detection limit of the microcantilever sensor for GL is found to be as low as 20 pg/mL for the shortest linker (n=1), which is 500 times lower than the longest linker (n=15) and 50 times lower than that of the corresponding iELISA. These findings will provide new insights into the fundamental mechanisms of stress transmission, which may be exploited for biochemical sensor and nanoactuation applications.

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1. Introduction

The microcantilever sensor has attracted considerable attention in recent years. Using immobilized receptor molecules, such as nucleic acids, proteins, and lipids, as well as cells and microorganisms, microcantilevers have been applied to a variety of problems to detect molecular interactions [1–8]. Furthermore, microcantilever arrays are extremely sensitive and can detect picomolar amounts of analyte in a complex background [8]. The principle behind the microcantilever sensing mechanism is the transduction of biomolecular interactions into a nanomechanical force. Analytes bind to receptor molecules that are immobilized on the surface of the microcantilever, and this causes changes in surface stress. This, in turn, generates a nanomechanical force that bends the microcantilever. An optical laser, focused on the microcantilever apex, is deflected as the microcantilever bends, allowing for direct measurement of receptor–analyte binding. Microcantilevers have advantages over other sensor techniques, including the ability to measure binding interactions in real time. A unique microcantilever feature is that measurements can be conducted both in air and solution, which may be particularly useful for detecting microbes. Furthermore, in contrast to most comparable technologies, small amounts of receptor and analyte are needed, and molecular labels are not required.

The stress originates from intermolecular interaction in the film of receptor molecules and transmits through the linker between the receptor molecule and microcantilever surface, and then ultimately, arrives at microcantilever surface and causes the bending. For microcantilever sensor based on surface stress effect, how to maximize the stress transmission from the molecular interaction to microcantilever surface is an important way of increasing the detection sensitivity of microcantilever sensor. The linker size between the surface and the receptor may play a key role in the stress transmission. Antibody immobilization on the surface is known as an important factor of the assay sensitivity. Different reagents used in receptor molecule (antibody) immobilization result in differences in the linker molecule sizes. Various reagents suitable for antibody immobilization have been described in literatures for different molecules detections. These reagents include homobifunctional cross-linkers dithiobis

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(succinimidylundecanoate) (DSU) [9], sulfo succinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (sulfo-LC–SPDP) [10], N-succinimidyl S-acetyl thioacetate (SATA) [11] and 2-iminothiolane hydrochloride (2-IT) [12–14], and monothiol linkers 3-mercaptopropionic acid [15], 11-mercaptopoundecanoic acid (MUA) [16], cysteamine [17] and aminoethanethiol [18]. In these sulfydrylation reagents, the linker molecules are straight alky chains, and there are 2-carbon chain length between the surface and the antibody for SATA, 3-mercaptopropionic acid, cysteamine and aminoethanethiol; 3-carbon chain length for 2-IT; 9-carbon chain length for sulfo-LC–SPDP; 10-carbon chain length for DSU and MUA. In these different detections (or sulfydrylation reagents), the microcantilevers show different sensitivities. For straight carbon chain, the carbon chain length may be critical to stress transmission, and ultimately affects the assay sensitivity. However, because of different affinities of antibody used in these detections, it cannot be evaluated how the chain length affects the stress transmission and the sensitivity. A same receptor molecule should be used.

Often, selective receptors are immobilized on the microcantilever surface using alkanethiol linkers, which form self-assembled monolayers (SAMs) on the gold-coated surface of the microcantilever. These thiolated molecules are chosen due to the strong affinity of sulfur head groups with the gold surface of the microcantilever. Not much information is available on the mechanical properties of SAMs, especially regarding the nature of stress transmission of alkanethiol. Berger et al. showed that, during vapor-phase n-alkanethiol adsorption on microcantilever surface, the difference in surface stress response correlates well with differences in chain length of n-alkanethiol, and longer-chain alkanethiol on the cantilever surfaces results in larger surface stress values [19]. Desikan et al. investigated, in the liquid phase, the effect of chain length on the nanomechanical stress generation on microcantilever surfaces during the adsorption of n-alkanethiols of different chain lengths and showed that adsorption of shorter-chain alkanethiols on the cantilever surfaces results in larger surface stress values [20].

Both Berger and Desikan study the effect of chain length at the step of the alkanethiols adsorption on the microcantilever, due to the binding of sulfur head groups of alkanethiols to the gold surface of the microcantilever. However, there is no report on the effect of chain length of alkanethiols when it is used as a linker that links the receptor to the microcantilever surface.

Licorice root is an herbal medicine in China for several thousand years. It is commonly used in Chinese herbal medicines (CHMs) and Chinese proprietary medicines (CPMs). Glycyrrhizic acid (GL) (Fig. 1) is a major active compound and a quality control marker of licorice root. It has anti-viral [21], anti-inflammatory [22], anti-carcinogenesis [23] and anti-hepatitis [24] activities. Its antiviral activity against severe acute respiratory syndrome (SARS)-associated coronavirus has been demonstrated in vitro [25]. GL is also used as a food additive and masking agent in pharmaceutical products because of its sweet taste (170 times sweeter than sucrose) [26].

The content of GL in the licorice roots varies considerably with strains, cultivars, growing regions, climatic conditions, and harvest age. The quality of the raw herbs used in the CPMs affects the final therapeutic outcomes and consumer safety. An effective method is needed to screen large numbers of licorice root samples for the quality control. Existing methods for the determination of GL include high-performance liquid chromatography (HPLC) [27], liquid chromatography–ion trap mass spectrometry (LC–ITMS) [28], capillary electrophoresis [29]. However, these methods of detection of herbal ingredients are complex, time consuming, and require costly and bulky instrumentation. Immunoassay based detection techniques are being developed as an alternative to detect these GL in samples. There are reports of enzyme-linked immunosorbent assays (ELISAs) based on monoclonal antibody (MAB) against GL [26]. The ELISA reported by Mizutani et al. had a detect range of 20–200 ng/mL. Such assays are highly specific and exhibit the necessary sensitivity and accuracy for the detection of these low
molecular weight active compounds of CHMs. Although having individual strength, each assay currently suffers from the inability to identify or quantify the GL for quality control marker of licorice root. True universal label-free detection of these herb ingredients for highly sensitive and specific detection in a high-throughput fashion is not yet a reality.

In order to study the chain length effect on stress transmission and the sensitivity of microcantilever sensor, here we designed an experiment by using n-alkanethiols with a same carboxyl group and different chain lengths (Fig. 1, n = 1, 5, 10 and 15) to immobilize a same receptor molecule on the gold surface of microcantilever, and detected the nanomechanical response of biochemical reaction. At the same time, in order to development a label-free sensor for highly sensitive GL detection, a monoclonal antibody (mAb) against GL and GL were used as the receptor molecule and analyze, respectively. The surface concentrations of immobilized antibodies were estimated using surface plasmon resonance (SPR).

2. Experimental details

2.1. Reagents and solutions

Thioglycolic acid HS(CH₂)₅COOH, 6-mercaptohexanoic acid HS(CH₂)₆COOH, 11-mercaptoundecanoic acid HS(CH₂)₁₀COOH, 16-mercaptohexadecanoic acid HS(CH₂)₁₅COOH, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glycyrrhizic acid (95% purity) was purchased from Tauto Biotech Co. Ltd. (Shanghai, China). All other chemicals were obtained from Beijing Chemical Reagents Co. (Beijing, China). The anti-GL monoclonal antibody (anti-GL mAb) was available from our previous studies [30]. Buffers and solutions used include coating buffer (0.05 M carbonate buffer, pH 9.6), phosphate buffered saline (PBS) (0.1 M phosphate buffer containing 0.9% sodium chloride, pH 7.5), PBS with 0.1% (v/v) Tween-20 (PBST), PBST containing 0.5% (w/v) gelatin (PBSTG), citrate-phosphate buffer (0.01 M citric acid and 0.03 M monosodium phosphate, pH 5.5).

2.2. Apparatus

The 96-well polystyrene microtiter plates were purchased from Costar (Corning, NY). Silicon nitride microcantilevers (Veeco Instruments, Plainview, NY) were used. The dimensions of the V-shaped microcantilevers were 0.6 μm × 20 μm × 200 μm. One side of the microcantilever had a thin film of chromium (15 nm) covered with a 60 nm layer of gold deposited by e-beam evaporation and this has been done by Veeco Instruments before buying from them. The experimental setup (Fig. 1) used for microcantilever sensor detection was home-built as described in our previous studies [12–14,31].

2.3. Antibody immobilization

The antibody immobilization procedures on the microcantilever were all in the microplate well that was replaced to a new one after each step. Each microcantilever was pretreated with “piranha dip” (H₂O₂/H₂SO₄ = 1:3, 100 μL) for 15 min and washed with deionized water for three times. The microcantilever was dried under a gentle stream of nitrogen gas.

The procedure of antibody immobilization was illustrated in Fig. 1. After washed, the microcantilever was immersed in 10 mM alkanethiol solution in ethanol for 24 h at room temperature. Afterward the alkanethiol modified microcantilevers were washed with ethanol for 2 times and deionized water for 3 times and dried. Functionally active NHS-esters were obtained by the reaction of alkanethiol carboxyl group with a fresh mixture of 0.2 M EDC and 0.05 M NHS in water for about 30 min at ambient temperature. After washed with deionized water for 3 times and dried, the microcantilevers were immersed into the 200 μL of 4.0 μg/mL antibody solution diluted by PBSTG and incubated for about 2 h at 37 °C. The antibody functionalized microcantilevers were washed with PBST for three times and dried. The functionalized microcantilevers were ready to use.

2.4. Microcantilever sensor detection

The functionalized microcantilever was mounted in a fluid cell (0.6 mL) (Fig. 1). PBS was circulated through the cell by a peristaltic pump. After the air bubble was drained off, the flow rate was controlled at 4 mL/h and kept constant during each experiment. The temperature of fluid cell was controlled at 310.00 ± 0.01 K while the room temperature was maintained at 301 ± 0.5 K. The deflection of the microcantilever was measured by monitoring the position of a laser beam reflected from the apex of microcantilever onto a position sensitive detector (PSD). The cantilever was equilibrated under these conditions for several hours until a constant drift was achieved. And after that, the analytes dissolved in PBS were added into the fluid cell and microcantilever deflection was monitored in situ.

Licorice sample with four-year-old cultivated was prepared and extracted as described in our previous studies [30]. The root samples of Glycyrhiza uralensis Fisch were collected to a maximum depth of 91 cm. The resultant residue was dissolved in 4.0 mL of PBS. The dilution ratio was 500 for microcantilever detection. The diluted sample was detected with antibody immobilized using the shortest alkanethiol (HSCH₂COOH) as described above.

2.5. Surface plasmon resonance (SPR) measurements

Before the immobilization of the antibody, bare sensor chip was cleaned using “piranha dip” (H₂O₂/H₂SO₄ = 1:3) for 5 min. It was then rinsed in pure ethanol and deionized water. The SPR measurements were performed with the precleaned gold chip on a Biacore 3000 device (Biacore AB) using PBS. A running solution and reaction solutions were performed at a flow rate of 5 μL/min. The protocols and concentration of reagents were the same as those used for the immobilization of antibody on gold surface of microcantilever (Fig. 1).

3. Results and discussion

The anti-GL mAb used is a mouse monoclonal antibody and an IgG1 isotype with κ light chain and has a high affinity constant of 9.96 × 10¹⁰ L/mol [30]. Fig. 2 shows the deflection profiles for antibody–antigen binding, where antibodies were immobilized using self-assembled monolayer (SAM), based on (a) thioglycolic acid (HSCH₂COOH), (b) 6-mercaptohexanoic acid (HS(CH₂)₆COOH), (c) 11-mercaptoundecanoic acid (HS(CH₂)₁₀COOH), and (d) 16-mercaptohexadecanoic acid (HS(CH₂)₁₅COOH) (corresponding to n = 1, 5, 10 and 15, respectively in Fig. 1). It can be seen that the functionalized microcantilevers rapidly bent, reaching an equilibrium bending signal. The bending occurs because the binding between GL antibody–antigen interactions produces a difference in surface stress between the functionalized surface on the top and the silicon surface on the back side of the microcantilever. The response of the microcantilever shows that the deflection increases significantly when the concentration of GL increases. It can be seen from Fig. 2(a) that the microcantilever bent rapidly for higher concentration (100 ng/mL), reaching an equilibrium bending signal Δz ~ 133 nm under constant flow conditions. For lower concentration (1 ng/mL), the bending of microcantilever was smaller (Δz ~ 64 nm). The other
charts where the antibodies were immobilized via SAMs of other n-alkanethiols (n = 5, 10 and 15) in Fig. 2 show the same trend. To evaluate the specificity of the technique, reference microcantilever without antibody was exposed to 1000 ng/mL of GL. No significant response was observed, indicating little interferences from nonspecific small molecules on the microcantilever sensor (Fig. 2). Licorice sample with four-year-old cultivated was detected with antibody immobilized using SAM based on the shortest alkanethiol (HSCH₂COOH). The concentration of GL detected with the microcantilever was approximately 8.6 ng/mL (dark black curve in Fig. 2(a)), and it was 10.16 ng/mL with icELISA [30]. The average recovery of GL determined with the microcantilever was 84.6%. Our investigation shows that microcantilevers based on the nanomechanical response have the sensitivity for label-free detection of GL and quantify the binding affinity of the GL antibody and GL as the antigen.

Intermolecular interactions described by the Lennard–Jones potential, as well as hydration forces, steric, van der Waals force and electrostatic forces between adsorbates have been proposed as mechanisms for the origins of the observed surface stresses [1,2,19,32]. The changes of surface hydrophobicity and conformation due to adsorbed molecules also play an important role [1,33]. Whatever the origin of the surface stress is, after originating from intermolecular interaction in the receptor molecules film, the stress transmits through the link between the receptor molecule and microcantilever surface, and then ultimately, arrives at microcantilever surface and causes the bending.

As can be seen from the deflection profile of the microcantilevers, upon injection of 100 ng/mL GL antigen, the microcantilever functionalized with antibody via n-alkanethiols of n = 1 and 5 displayed the deflection Δz ~ 133 nm and 115 nm, respectively, and the microcantilever functionalized via n-alkanethiols of n = 10 and 15 only had the deflection Δz ~ 70 nm and 29 nm, respectively. When the concentration of GL was 10 ng/mL, the microcantilever functionalized with antibody via n-alkanethiols of shortest chain (n = 1) displayed the maximum deflection (Δz ~ 99 nm) and the microcantilever functionalized via n-alkanethiols of longest chain (n = 15) had the smallest deflection (Δz ~ 10 nm). When the concentration was low to 1 ng/mL, the microcantilever functionalized with antibody via n-alkanethiols of n = 1 and 5 still had the deflection Δz ~ 64 nm and 45 nm, respectively, while there were almost no response for the microcantilever functionalized via n-alkanethiols of n = 10 and 15. These results indicate that chain length of the n-alkanethiol have an important impact on the sensitivity of the microcantilever sensor. To determine whether the different n-alkanethiols cause the difference in the surface concentrations of immobilized antibodies which may affect the sensitivity of the microcantilever sensor, surface plasmon resonance (SPR) was used. The procedure and condition of antibody immobilization on the SPR-chip were the same as those on microcantilever. The binding of antibodies to the layer of n-alkanethiols produced the SPR responses of 2482 RU (n = 1), 2586 RU (n = 5), 2694 RU (n = 10) and 2484 RU (n = 15) (Fig. 3), respectively. The results show that the surface concentrations of immobilized antibodies were almost equal for different n-alkanethiols used. Specifically, for n = 1 and n = 15, the SPR responses (2482 and 2484 RU) were equal, but the sensitivities of the microcantilever for n = 1 and 15 are very different. These indicate the difference in the sensitivity of the microcantilever sensor is not caused by different surface concentrations of immobilized antibodies but the chain length of n-alkanethiol.
Different chain lengths of n-alkanethiol result in difference in chain lengths of the linker that is between the surface and the antibody. The stress (or strain) originates from antigen–antibody binding and is transmitted through the linker to the microcantilever surface, and ultimately, causes the microcantilever bending. Thus it can be seen that the length of the carbon chain plays a key role in the stress transmission. Fig. 4 shows the steady-state microcantilever deflection as a function of different GL concentration and different chain lengths of linker (n = 1, 5, 10, 15) that is between the surface and the antibody. Each point represents the average for three determinations and the error bars represent the standard deviation of data. It is clear to see that the sensitivity of the microcantilever increases significantly with decreasing chain length of linker. These findings suggest that short carbon chain transmits stress (or strain) more efficiently. This result is, to our knowledge, the first direct proof that the carbon chain length is very important for the transmission of mechanical response that originates from biochemical reaction. Our findings are of substantive importance, not only for microcantilever studies but for all investigations of biological interfaces, where the linker is a carbon chain. Although the work presented herein is focused on biochemically well-defined interfaces, these findings will also be important for our understanding of mechanical signal transmission of biological interfaces and membranes.

Fig. 5(a) is still the steady-state microcantilever deflection as a function of different GL concentration and different chain lengths of linker. Here the abscissa axis is not the concentration, but the common logarithm of the concentration. It can be seen from the results that there is almost linear relationship between the deflection and the common logarithm of the concentration for every each chain length of linker. We can write a general product form for the microcantilever response,

\[ z = k \log C + A \]  

where \( k \) is a constant, \( C \) is the concentration of the antigen (GL), and \( A \) is related to the chain length of the linker and should be a function of the length.

Fig. 5(b) shows the steady-state microcantilever deflection as a function of chain length of linker when the antigen concentrations were 10 and 100 ng/mL, respectively. For every each concentration, the response of the microcantilever increases significantly with decreasing chain length of linker, exhibiting negative linear relationship. Thus, we can also write another form for the microcantilever response

\[ z = -Bn + D \]  

where \( B \) is a positive constant and does not change with the concentration of the antigen, \( n \) is the carbon number (CH\(_2\)) of linker chain,
and $D$ is a constant value for the same concentration of antigen and different antigen concentrations correspond to different values of $D$. Thus $D$ should be a function of the antigen concentration.

By synthesizing Eqs. (1) and (2), we obtain the microcantilever deflection expressed as the function of the chain length of the linker and the antigen concentration

$$z = k \log C - Bn + E$$  

(3)

where $E$ is a constant.

The constants $k$, $C$, and $B$ were constructed with deducing coefficients of least-square method. The comparison of fitting line and experimental microcantilever deflection data for each chain length is shown in Fig. 5(a). The limit of detection (LOD) of the GL was calculated as a signal 3 times the background noises. In Fig. 5(a), the LOD were the points at which the calculated lines and the lines of the 3 times the background noises intersected. The LODs for different chain length of linker were determined to be as low as 20 pg/mL ($n = 1$), 130 pg/mL ($n = 5$), 1.7 ng/mL ($n = 10$) and 10 ng/mL ($n = 15$), respectively, and the LOD for the shortest linker is 500 times lower than the longest linker. The LOD of conventional indirect competitive enzyme-linked immunosorbent assay (icELISA) for the antigen and antibody used here was 1.1 pg/mL [30]. The LOD of the microcantilever sensor (20 pg/mL) for the GL was 50 times lower than that of the corresponding icELISA. We can see that the chain length of linker has great influence on the LOD. By optimizing the chain length of linker, the LOD of the microcantilever sensor can be lowered significantly. This would be important for molecules detection at very low concentrations.

Eq. (3) shows that there is a linear relationship between deflection and common logarithm of the concentration. This relationship indicates that with the increase of antigen concentration, the increment of microcantilever deflection becomes smaller. But the microcantilever deflection has a linear relationship with the chain length of linker. Therefore, the carbon chain length of the linker plays a key role in the stress transmission and has greater impact on the microcantilever sensitivity than the antigen concentration does.

4. Conclusions

We have developed the first quantitative experimental model to evaluate the effect of the carbon chain length on the mechanism of signal which is generated by the biochemical reaction. We show that the microcantilever sensitivity is greatly influenced by the chain length of linker that is between the receptor molecule and the microcantilever surface. A label-free microcantilever sensor for highly sensitive detection of GL was developed. The detection limit of the microcantilever sensor for GL is found to be as low as 20 pg/mL for the shortest linker, which is 500 times lower than the longest linker and 50 times lower than that of the corresponding icELISA. Our findings suggest that the length of the carbon chain plays a key role in the stress transmission, and the efficiency of stress transmission increase significantly with decreasing length of carbon chain.

The main achievement of this work is identifying the transmission of mechanical signal at the molecular scale using a highly sensitive nanomechanical microcantilever. We also propose that, armed with a fundamental understanding of the key factors of stress transmission, one can optimize and propose innovative sensing strategies that are designed to maximize the induced surface stress signal. These findings will provide new insights into the fundamental mechanisms of stress transmission, which have broad implications in the study of biochemical interactions from molecular thin films to cellular membranes.

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