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Nanomechanical detection of cholera toxin using microcantilevers functionalized with ganglioside nanodiscs

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Abstract

The label-free detection of cholera toxin is demonstrated using microcantilevers functionalized with ganglioside nanodiscs. The cholera toxin molecules bind specifically to the active membrane protein encased in nanodiscs, nanoscale lipid bilayers surrounded by an amphipathic protein belt, immobilized on the cantilever surface. The specific molecular binding results in cantilever deflection via the formation of a surface stress-induced bending moment. The nanomechanical cantilever response is quantitatively monitored by optical interference. The consistent and reproducible nanomechanical detection of cholera toxin in nanomolar range concentrations is demonstrated. The results validated with such a model system suggest that the combination of a microcantilever platform with receptor nanodiscs is a promising approach for monitoring invasive pathogens and other types of biomolecular detection relevant to drug discovery.

1. Introduction

Membrane associated macromolecules are very important drug targets involved in drug metabolism, signal transduction and the formation of ion channels. The study of membrane associated systems is, however, challenging as they are difficult to express and purify in a functionally active form. In particular, membrane bound proteins are inherently insoluble or prone to aggregation and oligomerization in aqueous solution. There have been substantial advances in the development of techniques that allow the analysis of membrane associated ligand receptors in a model native-like lipid bilayer environment [1–3]. Recently, nanodiscs have been used to solubilize and functionally stabilize several membrane proteins [4–7]. A nanodisc is a small discoidal phospholipid bilayer surrounded by an amphipathic protein belt. The liposomes traditionally used for solubilizing membrane associated macromolecules [8] form large complexes which are heterogeneous, unstable and can skew the phenomena observed on the surface. Instead, nanodiscs provide homogeneous discs with controlled stoichiometry of the incorporated protein. Moreover, nanodiscs offer access to both sides of the bilayer structure, obviating the problem of the orientation of the embedded membrane protein as can be the case in vesicles and supported membrane mono- or bilayers. Furthermore, the encircling membrane scaffold protein can be used to anchor the membrane macromolecule containing nanodisc to different surfaces while keeping the membrane protein receptor intact and free to execute its functionally important motions.

The stability of membrane proteins in nanodiscs has been utilized for several applications such as solid state NMR [9], redox potentiometry [10] and steady-state kinetics experiments [11]. Nanodiscs also have been shown previously to be a viable platform to stabilize membrane proteins to maintain full functionality for surface-based applications using SPR [12], LSPR [13] and SAMDI (self-assembled monolayer MALDI) [14] as well as mechanistic studies of ligand binding. Herein, we present the efficacy of nanodisc receptors for the nanomechanical detection of cholera.

Cholera is an infectious gastroenteritis caused by enterotoxin-producing strains of the bacterium *Vibrio cholerae* whose action on the mucosal epithelium lining of the small intestine is responsible for causing exhaustive diarrhea. The cholera toxin (CT, MW = 87 kDa) consists of two subunits, subunit A that is responsible for ADP ribosylating catalysis and subunit B that binds to ganglioside GM1 (monosialotetrahexosylganglioside) present on the intestinal epithelium cell. Various approaches have been employed for the detection of CT including enzyme-linked immunosorbent assays (ELISA) [15], antibody-based microarrays [16] and fluoroimmunoassays [17]. These methods, however, generally require direct or indirect labeling of the CT for detection. Furthermore, the affinity of the interaction between cholera toxin and GM1 has been well defined and explored using multiple techniques [18–22]. The ganglioside–liposome immunoassay [23] and ganglioside microarray [24] have been reported for detecting CT, but these techniques also require labeling the CT with either dye or a fluorescent tag and subsequently measuring the intensity of the signal. It is useful, therefore, to develop a label-free method to directly detect the binding of CT to GM1 or, in general, the interaction of a macromolecule with membrane associated receptors that are important drug targets.

Nanomechanical sensors based on microcantilevers have emerged and gained increasing attention as a sensitive and label-free detection platform for a wide range of chemical and biological interactions [25–30]. Microcantilevers with a layer of high affinity receptors on one side can transduce the binding of target molecules into a nanomechanical response of the cantilever. For the cantilever signal transduction platform operating in the static deflection mode, the cantilever's response to such a binding event is deflection in the range of a few to hundreds of nanometers as a result of the adsorption-induced surface stress and consequently the development of a bending moment. The cantilever deflection can be measured optically using lasers [31–33] as well as electrically by piezoresistive [34, 35] or MOSFET-embedded microcantilevers [36, 37].

Various methods have been utilized to create a sensing layer on the microcantilever surface including antibodies [31, 33], single-chain antibody fragments [38], thiolated single-stranded DNA oligonucleotides [39, 40] and DNA aptamers [41]. Nevertheless, creating a robust and efficient receptor layer on the cantilever surface remains as one of the paramount challenges that the cantilever-based sensor platform must overcome to meet the requirements as a universally applicable detection platform capable of providing consistent and reproducible results [42, 43]. Nanodiscs allow immobilization of the membrane associated protein receptors on the cantilever surface while maintaining a stability and bioactivity otherwise difficult to achieve using currently available methods. In this regard, integrating receptor-incorporated nanodiscs to microcantilevers may offer new avenues to create a stable receptor layer of important membrane proteins for a functioning sensor platform based on the nanomechanical signal transduction approach.

To validate and evaluate the efficacy of the nanodisc–microcantilever approach, we investigate the detection of cholera toxin as a model system using ganglioside GM1-incorporated nanodiscs immobilized on the static mode microcantilevers. We show that the label-free detection of cholera toxin is possible by measuring the stress-induced deflection

of the cantilever, which confirms the potential of the system combining the advantages of nanodiscs and the nanomechanical sensor platform for applications in detecting toxins and investigating the association between macromolecules and membrane proteins in nanodiscs.

2. Materials and methods

2.1. Preparation of nanodiscs

Nanodiscs with ganglioside GM1 (Axxora, USA) were prepared by mixing the desired ratios of Dimyristoylphosphatidylcholine (DMPC, solubilized in chloroform, Avanti Polar Lipids) and GM1 (solubilized in a 1:1 mixture of chloroform/methanol), keeping the total GM1 + DMPC molar amount constant. The ratio of GM1:DMPC was 5:95. The phospholipids were quantitated by inorganic phosphate analysis. The GM1/DMPC mixture was then dried under a gentle stream of argon and placed under vacuum overnight to remove traces of organic solvents. The lipid film was then resolubilized in a standard buffer (100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4) with 20 mM sodium cholate by sonication for 10 min at 60 °C. His-tagged MSP1T2 [44] was added to obtain a final MS1D2/(lipid + GM1) ratio of 1:85. One nanodisc is formed by two MSP1D2 molecules and ~170 DMPC molecules. Once the total reconstitution mixture was added at the appropriate ratios, it was incubated for 45 min on ice for 1 h. To remove the detergents and concomitantly initiate the self-assembly process, an excess of Amberlite was added (1g of amberlite per ml of reconstitution solution). This complex mixture was then incubated at 25 °C with shaking for an additional 4 h. The formed nanodisc assemblies were separated from the Amberlite and filtered through a syringe filter (Millipore Corp.). The nanodisc mixture was then injected onto a calibrated Superdex 200 HR 10/30 size exclusion column (GE Healthcare) equilibrated with 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl and connected to a Waters HPLC system with a photodiode array detector at a flow rate of 0.5 ml min⁻¹. The nanodisc fraction was pooled, concentrated and buffer exchanged into a 100 mM phosphate buffer and frozen and stored at -80 °C. The concentration of the GM1 nanodisc was determined by using ϵ_{280} for MSP1T2 which is 21 000 M⁻¹ cm⁻¹.

2.2. Immobilization of the nanodiscs on a DTSSP-functionalized microcantilever gold surface

The rectangular tipless microcantilevers (500 $\mu\text{m} \times 100 \mu\text{m} \times 1 \mu\text{m}$, $k = 0.03 \text{ N m}^{-1}$, Nanosensors) used for immobilizing the nanodiscs were coated with 25 nm of gold with 5 nm of Ti adhesion layer by electron beam evaporation (BOC Edwards), and cleaned with a UV-ozone cleaner for 25 min immediately before the functionalization process.

To immobilize the nanodiscs on a gold-coated microcantilever, the cantilevers were first functionalized with 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP, Thermo Scientific) as a linker molecule. DTSSP contains two terminal *N*-hydroxysuccinimide esters (NHS groups) reactive towards to primary amines, joined by a disulfide linkage that binds to a gold surface. When DTSSP is immobilized on the gold surface, the NHS esters remain available for further interaction with amines to form covalent amide bonds. The nanodiscs bind to the DTSSP by taking advantage of the primary amine functional groups present in lysine (Lys), one of the component amino acids, in the membrane scaffold protein (figure 1(a)).

The gold-coated microcantilevers were first immersed in 5 mM of DTSSP in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) for 30 min at room temperature. After immobilizing the DTSSP, the cantilevers were washed with PBS and then immersed in 130 μM of GM1 or 30 μM of empty nanodiscs for 1 h at room temperature. To remove the unbound nanodiscs, the cantilevers were again washed with

PBS and this was followed by immersing in 1% (10 mg ml⁻¹) BSA blocking buffer (Thermo Scientific) for 30 min at room temperature to reduce the nonspecific binding of molecules on both the front and back sides of the cantilever. The functionalized cantilevers were finally washed with PBS and inserted to the fluidic cell to be exposed to cholera toxin in various concentrations prepared in PBS for binding measurements.

2.3. Optical detection of microcantilever deflection

The MultiMode AFM (Veeco) with a fluidic cell was used to optically monitor the nanomechanical response of the microcantilever as a result of the receptor–analyte interaction (figure 1(b)). The nanodiscfunctionalized microcantilevers were stabilized in the fluidic cell for approximately one hour before introducing the cholera toxin (Sigma) for detecting the binding induced cantilever deflection. The measurements were conducted at the static exposure condition without a continuous flow of the buffer solution, and the fluidic cell was temperature-controlled to maintain 24 °C.

3. Results and discussion

To validate the binding of cholera toxin to the receptors present on the cantilever surface, the deflection of a microcantilever functionalized with ganglioside-incorporated nanodiscs and nanodiscs without GM1 receptors are compared as shown in figure 2(a). The changes in the vertical deflection signal directly measured from the position sensitive detector (PSD) of the AFM demonstrate the distinct trends of the two different cases over the period of the binding event.

The measurable deflection occurs only on the GM1 nanodisc immobilized microcantilever when it is exposed to 100 nM cholera toxin, while a cantilever with empty nanodiscs shows a negligible difference in deflection when exposed to the same concentration of cholera toxin. The increase in the vertical deflection signal indicates that a compressive stress is generated when the CT binds to the GM1 nanodiscs, which is sufficient to bend the cantilever away from the functionalized gold surface as observed in the cases of DNA hybridization [40, 45] and antigen–antibody binding (table 1) [33]. Such a surface stress is considered to originate from a combined effect of various forces, especially for biomolecules that involve complex interactions, when receptor–analyte binding occurs on the cantilever surface [46]. Considering the isoelectric point (pI) of CT among other factors, the toxin molecules having a pI of 6.6 will be negatively charged in PBS of pH 7.4. Thus, the CT molecules binding to the GM1 nanodiscs on the cantilever surface will likely increase the electrostatic interaction that contributes to the repulsive force producing the compressive surface stress. The maximum cantilever deflection of ~12 nm is measured when the CT binding to the GM1 nanodisc–cantilever reaches a steady-state (figure 2(b)).

However, no deflection is observed after the equivalent binding time is allowed for the cantilever with empty nanodiscs, indicating the absence of the binding reaction to the immobilized nanodiscs without GM1 receptors. The results first confirm that the binding of the target analyte, cholera toxin, occurs only on the cantilever functionalized with ganglioside-incorporated nanodiscs. It is also suggested that the specific conjugate chemistry employed allows the immobilization of nanodiscs on the gold-coated cantilever surfaces and the nonspecific binding or physisorption of the CT on the front or back side of microcantilevers has a negligible influence on the cantilever deflection and subsequent detection measurements.

It is noteworthy that the detection of membrane protein and ligand interaction on a microcantilever array sensor has recently been demonstrated by measuring the changes in the resonant frequency with a virus binding to receptor-containing liposomes immobilized

on the resonating cantilevers [47]. The detection of CT–GM1 binding in a fluid using the current approach provides evidence that the nanomechanical signal transduction based on measuring the deflection of the cantilever is likewise capable of detecting the specific target molecule binding to an active membrane protein incorporated in nanodiscs, without the possible damping effect of the media on resonating cantilevers that may lead to reduced sensitivity [48, 49].

In addition, the trend and magnitude of the cantilever deflection during the period of the binding reaction as well as the initial binding rate show a dependence on the CT concentration, suggesting the viability of the nanodisc–cantilever platform to quantitatively detect and differentiate the target molecule. The microcantilever deflection for the duration of the CT binding to the GM1 nanodiscs was measured for various CT concentrations in the 1–100 nM range (figure 3(a)), useful for the sensitive detection of cholera toxin in clinical applications. The difference in the vertical deflection signal from the PSD increases with increasing CT concentration, indicating a larger cantilever deflection for a higher analyte concentration. The deflection signal measured in volts is converted to deflection in nanometers based on the predetermined cantilever deflection sensitivity of 80 nm V⁻¹. The cantilevers show an observable response immediately after the injection of the CT, enabling the real-time monitoring of the analyte–receptor binding events. The deflection reaches a steady-state within ~20 min when the functionalized cantilever is continuously exposed to a solution containing CT, which is comparable to the time that the steady-state binding is reached in the biomolecular detection previously reported using microcantilevers [40, 45].

The initial rate of the cantilever deflection as a result of CT binding to the GM1 nanodiscs is also determined by the initial concentration of the analyte CT injected to the fluidic cell as shown in figure 3(b). To estimate the initial rate, the cantilever deflection over the first 200 s from the binding curve in figure 3(a) is fitted linearly for each CT concentration, and the slopes of the initial rate line fits are plotted [50]. Approximately a 125% increase in the initial binding rate is observed with a ten fold increase in the CT concentration in the nanomolar range.

The consistency and reproducibility of the nanodisc–cantilever detection system are validated by determining the average maximum cantilever deflection for various analyte concentrations. The maximum cantilever deflection values in figure 4 show the average of the deflection values from duplicated experiments as the cantilevers reach the steady-state deflection during the CT–GM1 binding interaction. The corresponding stress values are estimated using the relationship derived from Stoney's equation (equation (1)) [28]

$$\Delta\sigma = \frac{Et^2}{3l^2(1-\nu)}\Delta d \quad (1)$$

where $\Delta\sigma$ is the differential surface stress, E is the elastic modulus of the silicon cantilever (~120 GPa), ν is Poisson's ratio (~0.27), t is the cantilever thickness (1 μ m), l is the length (500 μ m) and Δd is the deflection of the cantilever. The calculated surface stress is comparable to previously reported values from the hybridization of single-stranded DNA [40] and antigen–antibody molecular recognition [51] (table 1). The receptor–analyte binding induced surface stress leading to the cantilever deflection increases linearly in proportional to the CT concentration for the measured detection range and begins to reach saturation at higher concentrations, which can be correlated with the Langmuir adsorption isotherm often observed in biomolecules binding to receptors immobilized on surfaces from their aqueous solution [52].

The effect of a small variation in the elastic modulus of each cantilever on the measured deflection can be estimated by using the equation (2) derived from Euler–Bernoulli beam theory for a cantilevered rectangular beam, where w is the width of the cantilever and k is the cantilever spring constant that can be calculated based on the widely available thermal tune method [53–55].

$$E = k \frac{4l^3}{wt^3}. \quad (2)$$

The spring constant of the gold-coated microcantilevers is calculated to be $0.038 \pm 0.003 \text{ N m}^{-1}$. The variation in the spring constant affecting the elastic modulus is determined to induce less than 0.5 nm of cantilever deflection when the differential surface stress ($\Delta\sigma$) is $\sim 2 \text{ mN m}^{-1}$, suggesting that the small difference in the stiffness of the microcantilevers has a negligible effect on the magnitude of the deflection. Thus, once the cantilever deflection is calibrated with known concentrations of target molecules, quantitative detection of unknown concentrations of analytes would also be possible without evaluating the stiffness of the microcantilever for each measurement.

The consistent increase in the maximum cantilever deflection and the surface stress with increasing CT concentration demonstrate the potential of label-free CT detection based on monitoring the nanomechanical response of microcantilevers with membrane protein-incorporated nanodiscs.

4. Summary and conclusions

We have demonstrated the capability of the nanomechanical signal transduction platform integrated with specific receptor-incorporated nanodiscs as a sensitive and label-free technique to detect cholera toxin. The results presented herein confirm that the microcantilever-based nanomechanical sensing platform operating in the static deflection mode can be synergistically combined with nanodisc technology to directly measure the binding events between proteins and membrane associated receptors incorporated into the nanodiscs. The use of nanodiscs allows the immobilization of the GM1 ganglio-side on the microcantilever surface to create a stable receptor layer while maintaining the bioactivity of the incorporated membrane protein. The detection of cholera toxin in nM range concentrations, useful for the sensitive detection of cholera toxin in clinical applications, is confirmed by the consistent and reproducible deflection of the microcantilevers with CT binding specifically to the GM1 nanodisc immobilized cantilevers.

Although antibodies have been shown to provide high sensitivity and selectivity for the target analytes against which they are specifically generated, it may not be always possible or efficient to generate antibodies against all the proteins that naturally bind to membrane protein receptors. In contrast, nanodiscs can be considered as a universal platform to directly incorporate, solubilize and immobilize the membrane protein receptors for investigating the protein and membrane receptor interactions. When combined with the unique multiplexing capability of the microcantilever platform, such a detection approach may potentially be utilized in evaluating the specific binding of various macromolecules and membrane proteins in nanodiscs without labeling the target or requiring antibodies, and may find useful applications in drug discovery and pathogen monitoring.

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Figure 1.

(a) Schematic of a GM1 nanodisc immobilized on the cantilever gold surface using DTSSP and (b) the experimental setup used for the microcantilever-based detection of cholera toxin. The cholera toxin prepared at various concentrations was injected to the GM1-nanodisc-functionalized microcantilevers in the temperature-controlled AFM fluidic cell for the binding experiments. The optical method was used to monitor the nanomechanical response (deflection) of a microcantilever.

Figure 2.

(a) The cantilever deflection as a result of the binding of the cholera toxin (CT) to GM1-incorporated nanodiscs and nanodiscs without a GM1 receptor. Cantilevers functionalized with nanodiscs without GM1 receptors do not show consistent deflection with CT binding as observed in the GM1 nanodisc immobilized cantilevers. (b) The maximum cantilever deflection with 100 nM CT binding to GM1 and empty nanodiscs. The deflection of the cantilever without the GM1 receptor is negligible as CT does not bind to empty nanodiscs.

Figure 3.

(a) The time-dependent cantilever deflection with binding of the cholera toxin (CT) at concentrations in the nM range to GM1-incorporated nanodiscs immobilized on the microcantilevers. The deflection of cantilevers functionalized with GM1 nanodiscs increases with increasing CT concentration, demonstrating the capability to quantitatively detect and differentiate target molecules. The conversion of the deflection signal in volts measured from the AFM PSD to nanometers is based on the predetermined cantilever deflection sensitivity of 80 nm V^{-1} . (b) The initial rate of CT–GM1 binding depending on the CT concentration. To estimate the initial rate, the cantilever deflection over the first ~200 s from the plot A is fitted linearly for each CT concentration, and the slopes of the initial rate line fits are plotted.

Figure 4.

The maximum cantilever deflection and corresponding surface stress versus analyte (CT) concentration. The increase in the surface stress with increasing CT concentration leads to a consistent increase in the cantilever deflection, validating the label-free detection of cholera toxin based on the nanomechanical response of a cantilever. The maximum cantilever deflection values are obtained by averaging the duplicated deflection measurements when the cantilevers reach a steady-state during the binding interaction. The estimated surface stress values are calculated using Stoney's equation.

Table 1

The type of induced surface stress and the estimated stress values for various biomolecular binding events measured using microcantilevers.

Binding system	Type of induced surface stress	Estimated surface stress (mN m^{-1})	Reference
Antibody–antigen (PSA)	Compressive	$\sim 1\text{--}50$ ($1\text{ ng ml}^{-1}\text{--}100\text{ }\mu\text{g ml}^{-1}$)	[33]
Antibody–antigen (myoglobin)	Compressive	$\sim 2\text{--}6$ ($20\text{--}100\text{ }\mu\text{g ml}^{-1}$)	[51]
DNA hybridization	Compressive	$\sim 1\text{--}5$ ($10\text{ pM--}1\text{ }\mu\text{M}$ complementary ssDNA)	[40]
DNA hybridization	Compressive	~ 5 (12-mer ssDNA hybridization)	[45]