Sensors

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# A Mechanically Controlled Indicator Displacement Assay**

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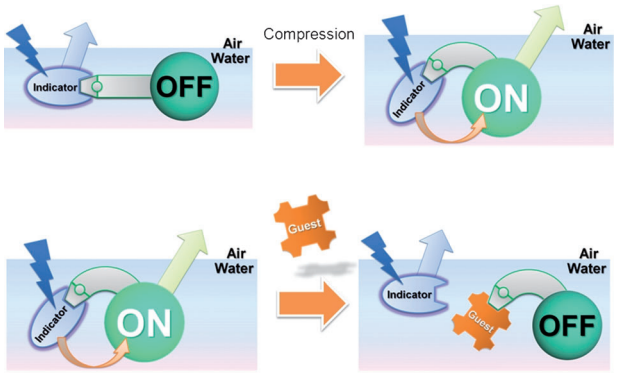
Detection and quantification of biologically active analytes is of paramount importance. These substances, whether therapeutic or harmful, are routinely evaluated for a range of properties, such as pharmaceutical activity and chemical toxicity. Such analyses are routinely performed in a variety of scientific disciplines, including drug discovery, environmental pollution monitoring, medical testing, and food safety evaluation. To this end, many studies aim to control the selectivity of a binding event such that the energy of binding between a receptor and an analyte is maximized.[1] To translate binding into detection and quantification, colorimetric or fluorescent molecular sensors are regarded as a very promising approach.[2] One sensing strategy is the indicator displacement assay (IDA). This approach uses a competitive binding event, between an indicator and a guest, to a complimentary site on a host.[3] This method allows the determination of total guest (or analyte) concentration accurately, leading to the broad use of this approach for developing multicomponent optical sensors[4] and enantioselective assays.[5]

To further develop the IDA strategy, we sought methods to increase the binding between host and guest molecules. The high effective concentration observed for molecular interfaces allows accentuated binding events, as compared to similar solution-based studies.[6] The binding that occurs between a host in one layer and a guest in another layer provides a conceptual mimic to the binding that occurs in living cells. One promising interfacial sensing environment is the air–water interface. Here, the position between the host and guest in the two-phase boundary facilitates and enhances binding.[7] Furthermore, a variety of monolayer assemblies have been prepared in an attempt to facilitate molecular recognition of biologically important substances.[8] One of the more promising strategies is mechanically controlled molecular recognition at interfaces, which enables facile control of molecular conformation. This control has allowed for reversible capture and release of guests, enantioselective binding of amino acids, and discrimination of nucleobases differing only by a single methyl group, all by using a simple and versatile stimulus, namely mechanical force.[9]

Herein, we introduce what we call a mechanically controlled indicator displacement assay (MC-IDA). This strategy utilizes a deformable monolayer assembly of fluorescently tagged host molecules formed at the air–water interface. This monolayer acts as a mechanically controllable signal-emission unit. External compression and expansion of monolayers in lateral directions can alter molecular conformations, resulting in control of molecular recognition (Scheme 1). We demonstrate that the fluorescent signaling between a host and an indicator can be switched on by surface compression, and the displacement of this indicator in response to an analyte can be enhanced to give very sensitive molecular sensing.

The goal of the present study was to demonstrate the application of mechanically controlled molecular recognition at interfaces to create novel applications of the IDA. For this task, we designed and synthesized an amphiphilic dilysine peptide host **1** (Figure 1 a; see the Supporting Information for details of the synthesis).[10] The host was designed to contain three important motifs: a phenylboronic acid, a cholesterol moiety, and a carboxyfluorescein indicator at the N-terminus of the peptide. The phenylboronic acid was chosen because it allows for reversible covalent bond formation with vicinal diols, as found in carbohydrates, glycopeptides, and α-hydroxycarboxylic acids.[11] The cholesterol group provides a hydrophobic functionality, imparting compatibility with organic media. The carboxyfluorescein was chosen as a fluo-




***Scheme 1.*** Illustration of the mechanically controlled indicator displacement assay (MC-IDA). The fluorescence resonance energy transfer (FRET) between the host and indicator was switched on by compression. Addition of guest caused the indicator to be displaced, effectively quenching the FRET process (switch off).

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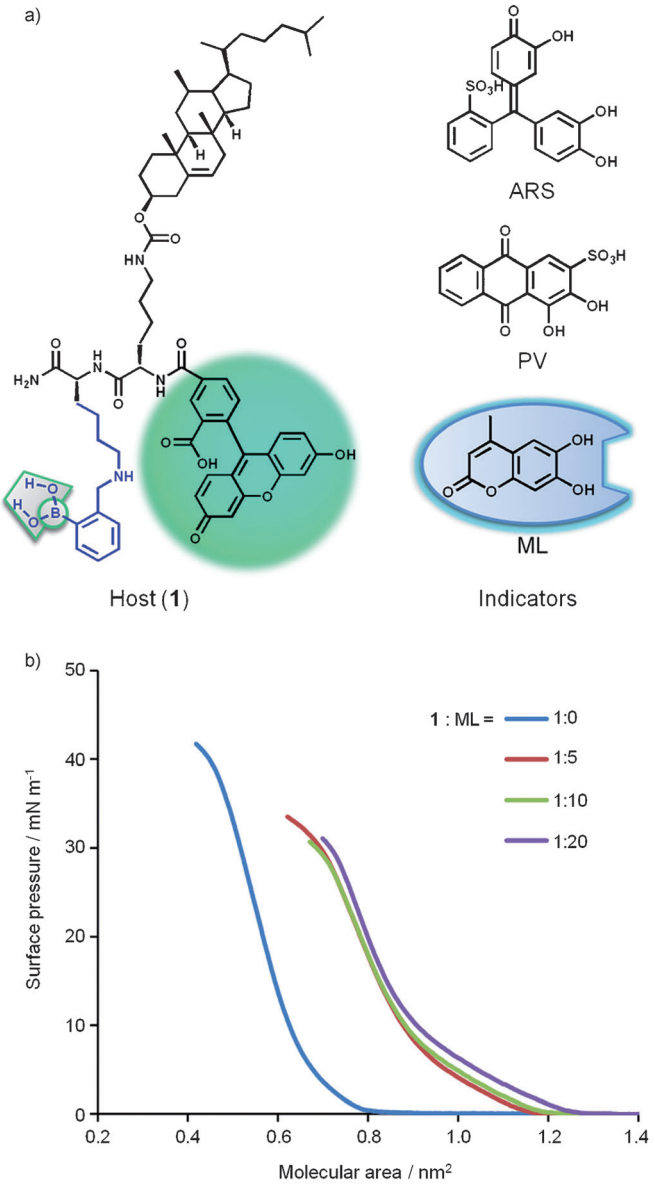


***Figure 1.*** a) Chemical structures of the host **1** and the indicators ML, ARS, and PV. b) $\pi$–$A$ isotherms of mixed monolayers of **1** and ML at the indicated molar ratios at 20 °C.

rescent probe because it is known to serve as a fluorescence resonance energy transfer (FRET) acceptor for coumarin-based indicators, such as 4-methylesculetin (ML).[12] The process of FRET involves a radiationless energy transfer between the excited state of a donor fluorophore and the ground state of an acceptor, provided that there is sufficient spectral overlap between these chromophores.[13]

The binding between **1** and ML was first confirmed through conventional optical measurements in solution (Supporting Information, Figure S1). ML shows a blue emission with a maximum of around 436 nm, whereas **1** has broad absorption with three maxima at 435, 460, and 492 nm. Upon addition of **1** into ML, the coumarin emission decreases with concomitant increase in the fluorescein emission at 522 nm (Supporting Information, Figure S1). These data are indicative of a FRET mechanism involving energy transfer from the excited ML to ground state **1**. In solution, it is expected that the amphiphilic nature of **1** could lead to any number of higher-order aggregates, such as vesicular-like or microtubular structures.[14] However, the balance between the hydrophobicity of the cholesterol moiety and amphiphilic nature of the peptide made this molecule a good candidate for monolayer formation at the air–water interface.

To investigate the ability to form a monolayer at the air–water interface, a solution containing host **1** was spread onto a water surface. The host was dissolved in a chloroform/methanol (4:1, v/v) mixed solution, and the surface pressure–area ($\pi$–$A$) isotherm was measured at 20 °C after the spreading solution had evaporated (Figure 1b). As expected, host **1** formed a stable monolayer at the air–water interface. This monolayer had a high collapse pressure of ca. 40 mN m$^{-1}$, and was not accompanied by a phase transition. The limiting molecular area of the monolayer was estimated to be ca. 0.61 nm$^2$ per molecule, which is larger than the reported value of 0.4 nm$^2$ per molecule for cholesterol.[15] This indicates that the chemical structure of the hydrophilic head groups, both carboxyfluorescein and phenylboronic acid, has an influence on the molecular packing in the monolayer. The large difference between the observed molecular area and the literature value is likely to be due to the charge state and the steric bulkiness of these head groups.

Very similar isotherms were observed when monolayers were formed using different concentrations of **1** in the spreading solution (Supporting Information, Figure S2). This observation indicates that known aggregation processes between boronic acid molecules[16] do not occur to any appreciable extent. This conclusion was also supported by the surface morphology of this monolayer. The monolayer was found to have a smooth surface, which is indicative of the formation of a quasi-ideal monolayer (Supporting Information, Figure S3).

It was postulated that the boronic acid group, which is hydrophilic in nature, would be directed toward the water subphase. Binding was expected to occur between this moiety and catechol-containing indicators that had been dissolved in the subphase, thus impacting the shape of the $\pi$–$A$ isotherm. First, we prepared a mixed monolayer of host **1** and a matrix lipid, methyl stearate (MS), for dilution of the host. Next, a suite of pH indicators, namely 4-methylesculetin (ML), alizarin red S (ARS), and pyrocatechol violet (PV), were individually added into the subphase. These indicators lead to the formation of covalent complexes, thereby causing a significant shift in molecular area (Supporting Information, Figure S4). This result arises from the bulkiness of the indicators attached to **1**. Binding constants $K$ were calculated from the molecular area values at a certain surface pressure using a Langmuir-type equation (see the Supporting Information),[9f,g] revealing the association of the host **1** monolayer and two indicators ML ($K = 5.5 \times 10^5$ L mol$^{-1}$) and ARS ($K = 1.1 \times 10^5$ L mol$^{-1}$) at a surface pressure of 5 mN m$^{-1}$ (Supporting Information, Figure S5). These binding constants are accentuated at the air–water interface, and are enhanced by one to two orders of magnitude over the corresponding solution systems.[5b] Although almost complete binding

between the host and ML was achieved, one of the drawbacks was the lack of solubility of the indicator in the water.

A strategy was devised to overcome the insolubility problem. The indicators were first mixed with **1** in the stock solution and then this mixed solution was spread over the water subphase. The $\pi$–$A$ isotherms recorded for this approach are shown in Figure 1b. The boronate ester formation in the spreading solution was deemed to form the complex (**1**:ML) quantitatively, because the deviation in molecular area saturated at molar ratios between 1:2 and 1:5. The presence of the complex caused large deviations in the molecular area of the monolayers. The robust character of the monolayer was not affected, as it retained its collapse pressure of ca. 30 mN m$^{-1}$. The AFM images of the resulting monolayer showed relatively smooth and homogeneous surfaces (Supporting Information, Figure S6).

We then studied fluorescence behavior of the **1**:ML complexes. Figure 2a shows the fluorescence spectra of the **1**:ML (1:5) monolayer formed at different surface pressures at the air–water interface. At a $\pi$ value of 10 mN m$^{-1}$, excitation at 373 nm produced a blue fluorescence emission around 450 nm. This emission arises from the excited ML chromophore. As the surface pressure increased to 20 mN m$^{-1}$, the same 373 nm excitation produced a new green fluorescence around 530 nm. The presence of this peak signaled an energy transfer between excited ML and ground state fluorescein. Figure 2b shows the dependence of the fluorescence of the monolayer as a function of ML concentration, with a constant surface pressure of 20 mN m$^{-1}$. As the molar ratio of ML in the stock solution increased, the emission band from the excited ML dominated. The fluorescein signal is likely to be still present, but is completely overwhelmed by the emission of the excess ML. In contrast, the lower mixing ratio of 1:2 (**1**:ML) gave the most fluorescein emission, relative to the ML peak. Generally, FRET efficiency depends on both the distance and conformation of the fluorophores, as well as overlap efficiency of donors and acceptors. In this study, the donor and acceptor are connected covalently through the dipeptide chain. The distance between these moieties will dictate the extent of the FRET signal, which is lowest at a surface pressure of 10 mN m$^{-1}$. However, the donor and acceptor units will have a closer arrangement as the occupied molecular area is decreased (Figure 2c). As a result, an optimal switch-on sensing results at a mixing ratio of **1**:ML of 1:5 and a surface pressure of 20 mN m$^{-1}$.

By using this optimized set-up, the displacement of the indicator by an aqueous analyte was anticipated to occur. We chose D-glucose (Glc) as a model for displacement, as it is routinely studied as a target in human blood.[17] Addition of a small amount of Glc to the water subphase beneath the **1**:ML monolayer led to a decrease in the magnitude of the green fluorescence observed for the monolayer, with little change in the ML peak (Figure 3a). This trend continued with increasing concentration of Glc in the subphase. Thus, the resonance energy transfer from the excited ML to the fluorescein moiety turned off in a ratiometric fashion as the analyte concentration in the subphase was increased. This result clearly indicates the displacement of the indicator (ML) from the host by the analyte (Glc).

Next, we measured the ratio of fluorescence peaks ($R = F_{525}/F_{450}$), and the results obtained are depicted in Figure 3b. For this purpose, Gaussian curve-fitting was conducted (Supporting Information, Figure S7). Apparently, the $R$ value gradually decreased up to 190 nM of the Glc concentrations (Figure 3b). This concentration corresponds to a 1:1 stoichiometric binding of **1**:Glc (Figure 3c). In other words, Figure 3b suggests that there is very tight binding of the guest to the host monolayer saturating at one equivalent. As the guest molecules diffuse toward the host–indicator monolayer, the displacement event occurs over time. Because the

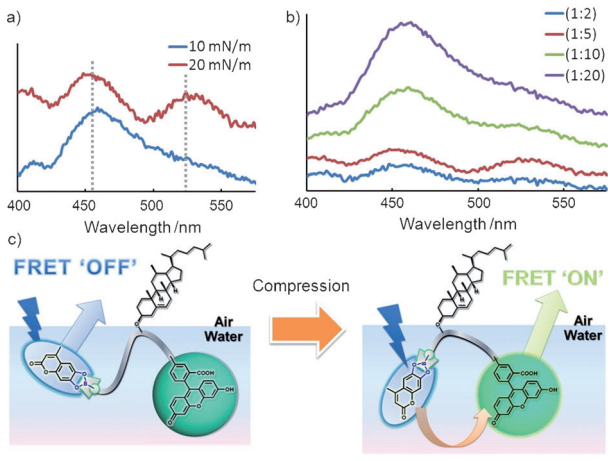


***Figure 2.*** Fluorescence spectra of monolayers of the host–indicator complexes (**1**:ML) at the air–water interface under different conditions: a) monolayers of **1**:ML (1:5) formed at a surface pressure of 10 and 20 mN m$^{-1}$; b) monolayers of **1**:ML with molar ratios of 1:2, 1:5, 1:10, and 1:20, at a surface pressure of 20 mN m$^{-1}$. c) Representation of the FRET-on mechanism.

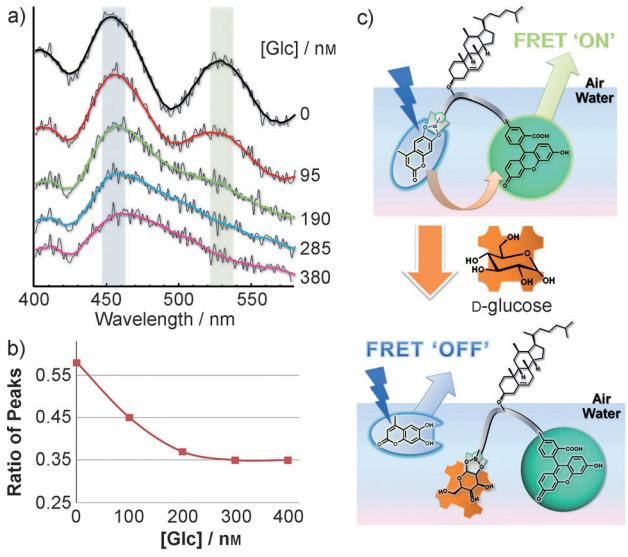


***Figure 3.*** a) Fluorescence spectral change of the monolayer of **1**:ML (1:5) at the air–water interface upon the addition of D-glucose. Concentration of added glucose [nM] is shown on the right side of each spectrum. Acquired data are smoothed (thick lines) using adjacent averaging procedure (enter number of points = 150). b) Plot of ratio of fluorescent peaks ($R = F_{525}/F_{450}$) versus glucose concentration. c) Displacement of ML from the host by D-glucose.

  

diffusion coefficient ($D$) of glucose in pure water is $6.73\times 10^{-6}\,cm^2\,s^{-1}$,[18] the mean square diffusion length ($\langle L^2\rangle^{1/2}$) of glucose traveled in the trough can be calculated. According to a well-known diffusion equation ($\langle L^2\rangle = 2Dt$), $\langle L^2\rangle^{1/2}$ was calculated to be 0.13 cm during 20 min after the injection of Glc into the subphase. This value is slightly lower than the subphase thickness (sub-cm level) in the Langmuir experiments. However, the injection of the guest solutions into the subphase may facilitate the guest moving to the interfacial region.

In summary, we have demonstrated that mechanically controlled molecular recognition at the air–water interface can be successfully applied to IDAs. The present method, using the host–indicator compexes at a specifically defined interfacial region, may further be applicable to biological and environmental analyses.

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