



A smart probe for detection of sugar markers for applications in gastrointestinal barrier dysfunction

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ARTICLE INFO

Keywords:

Fluorescent probe
Gold nanoparticles
Sensing principle
Gastrointestinal barrier dysfunction
And sugar markers

ABSTRACT

Gastrointestinal (GI) barrier dysfunction is an early pathogenic event in many complex diseases. Despite the routine applications of invasive tests, saccharide molecules are used noninvasively for assessing GI tract mucosal barrier function. However, currently available methods for quantification of saccharides molecules are costly and laborious. Simplified, reliable, and high-throughput methods are desired so that GI permeability testing can become routine and widely used. Here, we have developed a one-component system comprising of a naphthyl-pyridine core coupled to a boronic acid receptor, which can be used for early detection of saccharide biomarkers (i.e., lactulose) for applications related to GI barrier dysfunction. For quantitation of lactulose as a model biomarker, we have designed gold nanoparticle decorated surfaces in a highly scalable 96-well format to enable sensitive testing of lactulose within a broad range of concentrations. To tackle current challenges in saccharide biomarker sensing, we developed a hybrid sensing principle integrating two optical modalities (plasmonics and fluorescence) with a synthetic smart-probe (naphthyl-pyridinium) for monitoring GI permeability. This technology can be further developed as an affordable and portable diagnostic tool for GI permeability screening for routine use, facilitating early detection of various diseases affecting the GI tract.

1. Introduction

Enabling technologies have been integrated into our daily lives for tracking health status *via* sensors and wearables, and some of them are being implemented as diagnostic and therapy monitoring tools (Gambhir et al., 2018). Despite umpteen efforts on monitoring debilitating maladies, adapting these tools into the point-of-care (POC) settings is still challenging since existing platforms are expensive,

low-throughput, unreliable, and limited in detection range (Gambhir et al., 2018; Inci et al., 2015b). As an example, gastrointestinal (GI) barrier dysfunction is now recognized as an early event in the pathogenesis of several diseases (Jones et al., 2014), such as inflammatory bowel disease (Detzel et al., 2015; Pastorelli et al., 2013), Crohn's disease (Michielan and D'Inca, 2015), cancer (Yang et al., 2016), and celiac disease (Camilleri et al., 2012; Paterson et al., 2007). Blood/stool tests, imaging of upper/lower GI tract, colonoscopy and biopsy are used for

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diagnosis of these diseases. However, many patients do not present any symptoms, where bleeding and infection are late indicators. Further, existing imaging strategies are expensive and cannot be deployed to POC settings (Yang et al., 2019). This leads to an intolerable delay in initiating therapy, and if not diagnosed and treated on time, the disease can progress to a debilitating phase that becomes difficult or impossible to treat and can be potentially fatal. There is a critical need to develop ways to effectively map and quantify leaky regions of the GI tract and use that to guide strategies for early detection (Bischoff et al., 2014; König et al., 2016). Although GI permeability can be assessed noninvasively by analyzing saccharide biomarkers in urine such as sucrose for upper GI permeability (Frias et al., 2012; McOmber et al., 2010), lactulose and mannitol for small intestine permeability, and the synthetic sweetener sucralose for colon, analyzing these markers needs costly and time-consuming tools, e.g., high performance liquid chromatography/mass spectrometry (HPLC/MS) (Anderson et al., 2004) that are impractical at the POC settings. Hence, there is a critical need to develop tools to guide strategies with low-cost, high-throughput, reliable, and sensitive fashions for early detection.

The integration of chemical probes with sensors such as synthetic materials-based platforms (Durmus et al., 2012; Inci et al., 2015a), electrical sensing (Akceoglu et al., 2021; Jonsson et al., 2016; Shafiee et al., 2015), photonic crystals (Erdem et al., 2021; Galian et al., 2006; Shafiee et al., 2014), and plasmonic sensors (Ahmed et al., 2020; Askim et al., 2013; Inci et al., 2013, 2020a; Mataji-Kojouri et al., 2020) are

necessary to create simple assays for detection of GI permeability at POC settings. Briefly, in earlier work (Resendez and Malhotra, 2019), we described the smart-probe that is a fluorescent molecule utilizing a boronic acid-appended naphthyl-pyridinium that acts dually as a modular internal charge transfer (ICT) fluorescent probe or donor-/acceptor pair ensembles where the receptor compound can act as a quencher for an anionic dye. As an ICT one-component probe, the boronic acid acts as a receptor for its sugar biomarker and upon interaction generates a fluorescent response. At physiological conditions ($\sim$ pH 7.4), the cationic boronic acid receptor has a high intrinsic affinity for cis-diols, which upon binding, partially neutralizes the charge of the quarternized nitrogen, disrupting the electron accepting ability for the excited singlet electron and alter the emissive properties of naphthalene. The increase in fluorescence is directly proportional to the concentration of the saccharide molecules. However, this standalone probe had limited detection ranges and is not practical to implement into a POC device for sensitive quantification of GI permeability.

To tackle the aforementioned challenges, here, we have developed a hybrid sensing principle integrating two optical modalities (plasmonics and fluorescence) with a synthetic smart-probe (naphthyl-pyridinium) for monitoring GI permeability (Fig. 1). For quantitating sugar concentrations, we have combined our smart-probe with localized surface plasmon (LSP) modality. First, AuNPs are immobilized via poly-L-lysine (PLL) on the surface of 96-well plate (Fig. 1a). When lactulose (used here as a model sugar) is added to the LSP surface in the absence of smart-

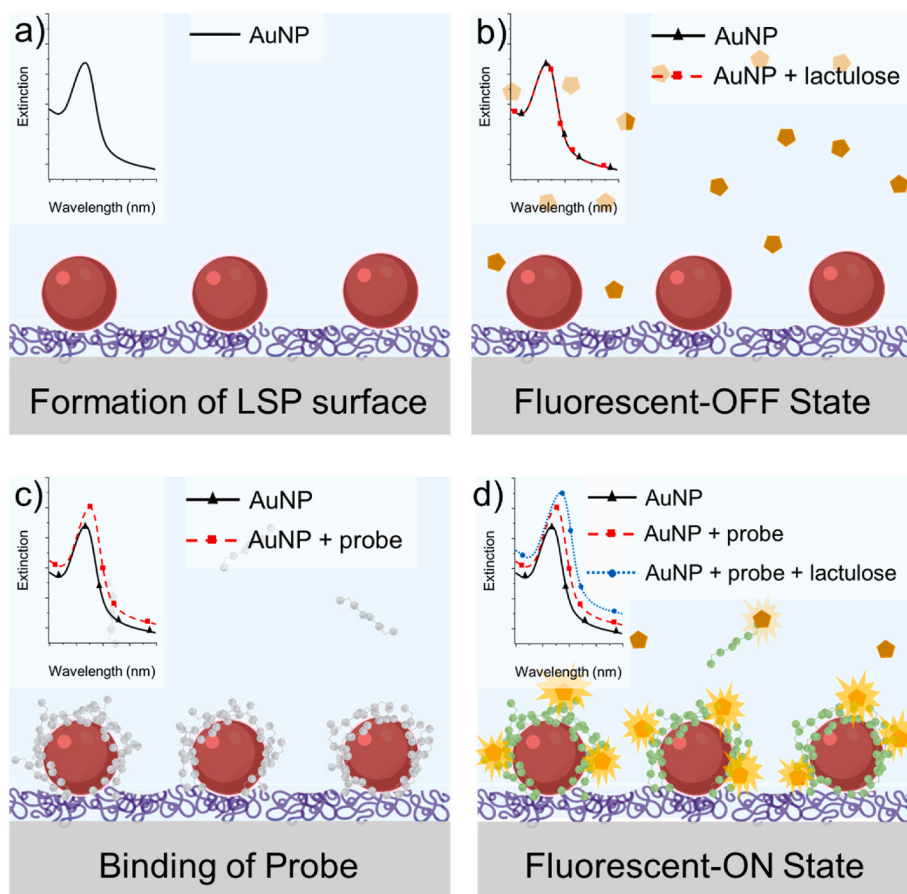


Fig. 1. Schematic illustration of the assay. a) AuNPs are immobilized via poly-L-lysine (PLL) to provide localized surface plasmon (LSP) resonance. Inset shows extinction spectrum for the configuration (b) When lactulose is applied in the absence of smart-probe molecules, there is Fluorescent-OFF state in the assay. Addition of lactulose does not change the LSP background signal due to a slight variation of background refractive index. Extinction spectrum and resonance wavelength remains the same after addition of lactulose (inset). c) After adding smart-probe to the LSP surface, it forms a layer on the AuNPs, causing wavelength shifts and intensity changes (inset). d) Combined interactions of smart-probe/lactulose mixture lead to Fluorescence-ON state. Addition of smart probe to immobilized AuNPs shifts the resonance wavelength (inset). When lactulose is introduced to this solution, its interaction with probe molecules around the AuNPs results in a further red shift of the resonance wavelength. At the same time a fluorescence signal is generated which is enhanced by the localized plasmons of AuNPs.

probe molecules, there is Fluorescent-OFF state (Fig. 1b). In this state, lactulose molecules, which are water soluble, do not vary the refractive index of aqueous solution significantly. Hence, no resonance shift is visible in the extinction spectrum of the LSP surface. On the other hand, when smart-probe is applied to the LSP surface, it forms a layer on the AuNPs (Fig. 1c). Formation of this layer shifts the resonance wavelength. By addition of lactulose to the LSP surface (Fluorescence-ON state (Fig. 1d)), the plasmonic resonance wavelength shifts further due to the reaction of fluorophores with lactulose molecules and the local increase in the refractive index near AuNPs. At the same time, at this step the reaction between the marker sugar and the smart probe enables the fluorescence behavior which is enhanced due to the excitation of localized plasmons in AuNPs. Therefore, this system holds a hybrid plasmonic-fluorescent sensing mechanism from a biosensing perspective that could be potentially implemented into the detection of GI permeability at POC settings.

2. Materials and methods

2.1. Materials

All the reagents and solvents were obtained commercially from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Hampton, NH, USA) and were used without further purification. A CombiFlash Rf + Lumen chromatography system (Teledyne ISCO, Lincoln, NE, USA) was used to purify the compounds. An Agilent 6490 iFunnel Triple Quadrupole Mass Spectrometer from Agilent Technologies Inc. (Santa Clara, CA, USA) was used to for LC-MS analysis. The ^1H (400 MHz) and ^{13}C (101 MHz) NMR spectra were taken on an Agilent 400-MR NMR spectrometer from Agilent Technologies Inc. (Santa Clara, CA, USA). Chemical shifts (δ) are expressed in ppm, coupling constants (J) are expressed in Hertz, and splitting patterns are described as follows: d = doublet; m = multiplet; dd = doublet of doublets.

2.2. Synthesis of the smart-probe

The smart-probe was designed via the synthesis of two compounds (*i.e.*, 2,6-dipyridinyl naphthalene and 4,4'-(naphthalene-2,6-diyl)bis(1-(2-boronobenzyl)pyridinium). The chemical structure of these compounds were illustrated in Fig. S1. **Synthesis of 2,6-dipyridinyl naphthalene (2):** To an oven dried 3-neck round bottom flask (25 mL) purged with argon, was added Pd(OAc)₂ (0.018 g, 0.08 mmol, 4 mol%), XPhos (0.046 g, 0.096 mmol, 4.8 mol%), 2,6-dibromonaphthalene (0.570 g, 2 mmol), 4-pyridinyl boronic acid (0.540 g, 4.4 mmol), and 1,4-dioxane (11.2 mL). After the mixture pre-stirred at 25 °C for 30 min, a degassed aqueous solution of NaOH (2.8 mL, 10.2 mmol, 1.2 M) was added to the mixture and vigorously stirred at 95 °C for 48 h. At the end of the reaction, the organics were extracted with ethyl acetate (2 x 10 mL) and subsequently washed with deionized water (10 mL x 2). The organic dried with MgSO₄ and concentrated on a rotary evaporator. A silica slurry was made and was purified by silica gel flash chromatography with a mixture 3:2 of Hex:EtOAc/EtOH(3:1) as eluent to give the title compounds as a white crystalline solid (0.338 g, 59% yield). ^1H NMR was performed under 400 MHz and Chloroform-*d*, and resulted in δ 8.78–8.70 (m, 4H), 8.19–8.14 (m, 2H), 8.05 (d, J = 8.5 Hz, 2H), 7.82 (dd, J = 8.5, 1.7 Hz, 2H), 7.69–7.62 (m, 4H). Similarly, ^{13}C NMR was performed under 101 MHz and CDCl₃, and resulted in δ 150.42, 147.88, 136.37, 133.45, 129.49, 126.21, 125.43, 121.80. MS-ESI experiment showed a m/z value for C₂₀H₁₄N₂ [M+H]⁺: 283.119, and found as 283.2 **Synthesis of 4,4'-(naphthalene-2,6-diyl)bis(1-(2-boronobenzyl)pyridinium (3):** To a solution of 2-bromomethylphenyl boronic acid (0.451 g, 2.1 mmol) in DMF (7 mL) was added, 2,6-dipyridinyl naphthalene (0.282 g, 1 mmol), and the reaction was stirred at 65 °C for 48 h. The reaction mixture was cooled to room temperature and cold acetone (25 mL) was added to induce further precipitation of a pale yellow solid. The precipitate was centrifuged, washed with acetone (3 x 20 mL) and dried

under a stream of nitrogen (0.571 g, 79% yield). ^1H NMR and ^{13}C NMR were performed. Accordingly, ^1H NMR (400 MHz, DMSO-*d*₆) provided a result as δ 9.16–9.09 (m, 4H), 8.86 (d, J = 1.8 Hz, 2H), 8.73–8.64 (m, 4H), 8.52 (s, 4H), 8.32 (d, J = 8.7 Hz, 2H), 8.25 (dd, J = 8.7, 1.8 Hz, 2H), 7.86–7.78 (m, 2H), 7.53–7.39 (m, 4H), 7.32 (dd, J = 7.7, 1.4 Hz, 2H), 6.05 (s, 4H). ^{13}C NMR (101 MHz, DMSO-*d*₆) resulted in δ 154.68, 145.48, 135.86, 133.51, 131.07, 130.91, 129.80, 129.39, 128.82, 126.03, 125.40, 63.29. After MS-ESI experiment, MS-ESI m/z was calculated for C₃₄H₃₀B₂N₂O₄ [M+H]⁺: 276.120, and found as 276.200.

2.3. Fluorescence characterization

Stock solutions of the probe compound were initially prepared in DMSO (2 mg/mL) and then diluted to the desired initial 0.5 mM concentration before adding to the wells. A lactulose solution was prepared in 0.1 M sodium phosphate buffer and then diluted at 2-fold of the desired final concentration. Using a 96-well plate (Corning #3694, Corning, NY), to each well received 20 μL of probe compound and sugar concentration (each data point recorded in triplicates) and the fluorescence measurements were performed using a standard plate reader (Tecan Spark 10 M) (gain 100, flashes 30, z-position 1.8 cm). Plates were also shaken for 30 s (2 mm orbital amplitude) prior to reading.

2.4. Synthesis of gold nanoparticles

Sodium citrate (60 mg) in DI water (90 mL) was heated in a 250 mL two-necked an Erlenmeyer flask for 10 min under vigorous stirring. Then, 6 mg of hydrogen tetrachloroaurate (III) hydrate (HAuCl₄·3H₂O) were added to the solution, and the mixture was boiled for 30 min. At the end of this period, the solution color turns into grey within 10 min, and then, gradually turns into red. AuNP solution was cooled immediately until the temperature of the solution reached to 90 °C. Later, 25 mM sodium citrate (6 mL) and 10 mM HAuCl₄·3H₂O (6 mL) were added to the solution. Again, the mixture was boiled for 30 min, and cooled down to room temperature. For 30 nm diameters of AuNPs, the reaction was stopped and their size characteristics were evaluated as indicated below. For 40 nm diameters of AuNPs, after the cooling-down step, the mixture was boiled again for 10 min. Then, the solution was cooled down to room temperature and allowed to incubate for 24 h. The diameter and size distribution of synthesized gold nanoparticles were analyzed using dynamic light scattering (DLS). Gold nanoparticle size distribution analysis was performed in a standard DLS cuvette (25 μL AuNP solution/2975 μL DI water in cuvette) at 25 °C.

2.5. Developing LSP layer on the 96-well plate surface

A 100 μL of PLL hydrobromide (Mw: 150,000–300,000) (0.25 mg/mL in DI water) was added into each well, and incubated for 24 h at 4 °C for ensuring its attachment on the well surface. After the incubation, the PLL solution was discarded and the plate surface was washed with DI water (200 μL) using pipetting. The AuNP nanoparticle solution (30 nm or 40 nm of diameters; 100 μL per well) was added to the PLL-coated surface and were allowed to incubate for 24 h at 4 °C. AuNP solution was then discarded and washed once with sodium phosphate buffer (PB) (0.1 M; pH 7.4).

2.6. Measurements of signal

The extinction intensity (scanning wavelength range: 350–800 nm) and fluorescence intensity (excitation wavelength: 340 nm; emission wavelength: 435 nm) were measured using a standard plate reader (Tecan Spark 10 M). The plasmonic data was determined as the wavelength shift at the resonance, and all data for wavelength and extinction intensity measurements were indicated as the mean of wavelength changes $\pm$ standard deviation (Taylor and Thompson, 1998). Considering the resolution of the plate reader instrument (wavelength accuracy

and repeatability are ≤ 0.8 nm and ≤ 0.5 nm, respectively), the data was stated with one decimal digit in the results, and the errors from the finite resolution of the reader were considered in the analysis.

2.7. Preparation of solutions

A 2 mM of smart-probe was prepared in DMSO as a stock solution. A 100 mM of lactulose was prepared in sodium phosphate buffer (PB) (0.1 M; pH 7.4) as a stock solution, and it was then diluted with PB to adjust the concentrations. All solutions were prepared fresh before applying to the LSP sensing surface or to the plain wells for fluorescence measurements. In the experiments, 200 μ L of lactulose-probe mixture was applied to the wells (either LSP sensing surface or plain wells), and incubated in the dark for 30 min at room temperature. Each experimental set was performed with six replicates. After incubation, the extinction intensity (scanning wavelength range: 350–800 nm) and fluorescence intensity (excitation wavelength: 340 nm; emission wavelength: 435 nm) were measured using a standard plate reader.

2.8. Numerical modelling

Electromagnetic Waves module of COMSOL Multiphysics was used for analysing plasmonic response. Periodic boundary conditions were used to model the AuNP-coated glass surface. AuNPs of various dimensions were studied. Broadband light (400–800 nm) and normal illumination were used for excitation from the top. The total computation time was about 3 min and 30 s. Mesh convergence test was performed for accurate results. E-field distribution and wavelength of peak values of extinction spectra were calculated for AuNPs with different diameters.

2.9. Statistical analysis

We performed student's t-test using GraphPad Prism (La Jolla, CA). The statistical significance threshold was set at 0.05 ($p < 0.05$).

3. Results and discussions

3.1. Synthesis of smart-probe (naphthyl-pyridinium) compound

The probe was obtained in two-steps by first synthesizing the naphthyl-pyridine scaffold through the Suzuki-Miyaura coupling method with known procedures (Yang et al., 2012). To install the sugar receptor moiety, the pyridine nitrogens were quarternized by nucleophilic alkylation with 2-bromomethyl phenyl boronic acid which yielded the probe compound (3) (Fig. 2a). Briefly, 2,6-dipyridinyl naphthalene was synthesized first, and confirmed with standard 1-D nuclear magnetic resonance (NMR) and mass spectrometry/electrospray ionization (MS/ESI). Later, 2,6-dipyridinyl naphthalene was reacted with 2-bromomethylphenyl boronic acid solution as explained in Materials and Methods. The final product, i.e., 4,4'-(naphthalene-2,6-diyl)bis (1-(2-boronobenzyl)pyridinium), was obtained and confirmed with NMR (Fig. 2b) and MS/ESI.

3.2. Fluorescence characterization of naphthyl-pyridinium probe

Having naphthalene as the core scaffold of the probe compound provides an intrinsic fluorescent property that allows the use of this compound as a standalone probe for sugars. This relies on having the sp^2 -hybridized boron atom directly attached to the naphthalene chromophore, which can form a conjugated system and act as an electron acceptor. This type of boronic acid-based sugar probe relies on a quaternary nitrogen that is separate from the fluorophore unit and is primarily responsible for the internal charge transfer mechanism. Upon recognition of a sugar molecule with the boronic acid moiety, the boron's atom hybridization changes from sp^2 -to sp^3 -hybridization, resulting in an overall net neutral state of the probe switching off the internal charge transfer mechanism and inducing the fluorescent properties (Arimori et al., 2002; Resendez and Malhotra, 2019).

Considering our earlier study (Resendez and Malhotra, 2019), we examined a range of probes and showed their applications in measuring various of sugars (as a single unit and/or combination of sugars) under various conditions, including different pH values and temperatures. Since the optimum condition for this probe was observed at pH 7.4,

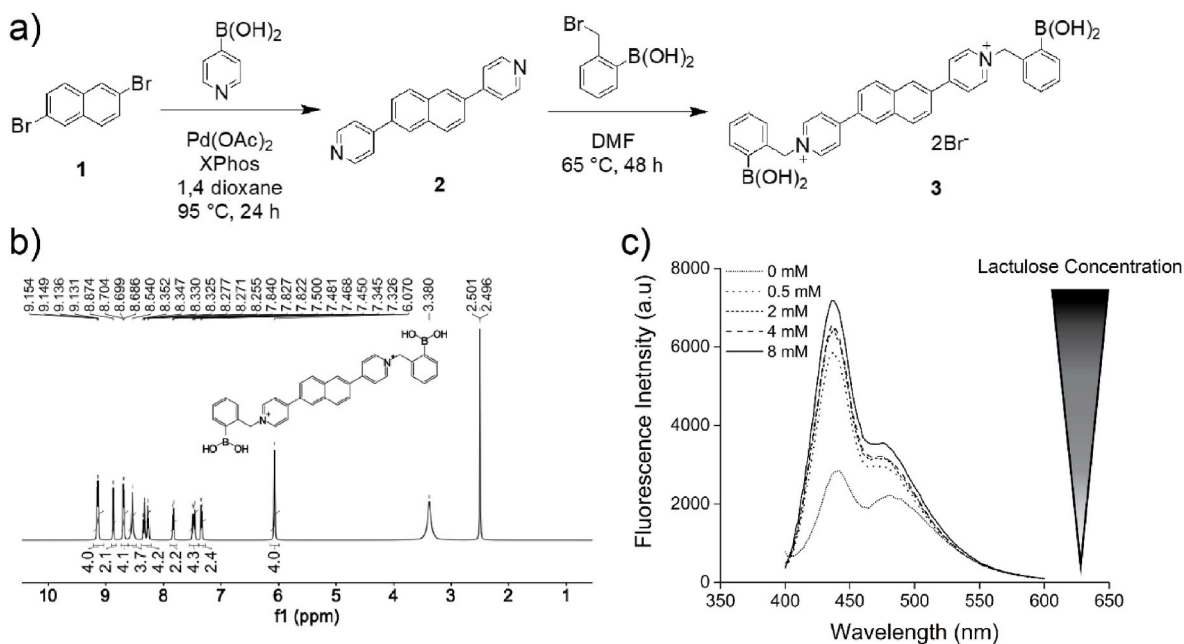


Fig. 2. Synthesis and characterization of the smart-probe. a) Scheme for the preparation of smart-probe (naphthyl-pyridinium) compound. b) NMR bands demonstrates the successful synthesis of smart-probe. The chemical structure is indicated as inset. c) Fluorescence characterization spectra of the probe compound (250 μ M) is plotted in the presence of lactulose spanning from 0 to 8 mM prepared in 0.1 M sodium phosphate buffer (pH 7.4 and λ_{ex} = 340 nm).

which is also in the pH range of urine (pH 4.5 – pH 8.0), we first applied different concentrations of lactulose (prepared in 0.1 M phosphate at pH 7.4) to the probe solution (250 μ M) for evaluating the performance of fluorescence formation during this reaction (Fig. 2c). The emission profile was evaluated by exciting the probe compound at 340 nm in the absence of lactulose and upon introduction of lactulose, provided an increase in fluorescence response up to 3-fold increase in signal at 8 mM lactulose concentration (Fig. 2c). Although this probe gave a decent increase, its dynamic range was limited as minimal response is observed (Resendez and Malhotra, 2019). Thus, we envisioned using the enhancement of this response by the localization of LSP layer to improve the dynamic detection limits of the probe.

3.3. Numerical simulations of LSP enhancement

We performed numerical simulations to describe the working principle of the designed assay. Our simulations support that formation of a thin layer of probe molecules, or probe-lactulose mixture around the AuNPs would result in a large shift of the resonance wavelength. Fig. 3a shows a top view of electric field intensity at resonance on the equator plane of AuNPs for bare AuNPs, AuNPs covered with probe molecules, and AuNPs covered with a mixture of probe molecules and lactulose, respectively. Fig. 3b shows the calculated extinction spectrum for each case. According to the simulation, the addition of probe-lactulose mixture shifts the resonance to larger wavelengths and also enhances the intensity of electric field (Brolo, 2012; Russell et al., 2012). In our simulations, the resonance wavelength (λ_{peak}) was found to be $\sim$ 530 nm for the LSP surface, and this value shifted to 542.7 nm after for AuNPs surrounded by a layer of probe-lactulose molecules. In the experiments, we found resonance wavelengths at 529.7 ± 0.2 nm and 543.5 ± 0.5 nm, respectively. Similarly, in both experimental and numerical

simulation results, we observed an increase in the extinction intensity values after applying the probe-lactulose mixture to the LSP surface (Fig. 3b).

3.4. Measuring fluorescence-enhanced LSP signals

To explore the effect of presence of a gold surface forming an LSP layer, we designed an assay, where the smart-probe measurements described earlier was performed on top of this LSP surface. Here, we evaluated different lactulose concentrations (500 μ M–100 mM) when applying a fixed concentration of probe (200 μ M) and measured the changes in fluorescence intensity on the plasmonic surface (w/LSP sensing surface) and the plain well (w/o LSP sensing surface) (Fig. 3c). The signal was increased as proportional to the concentration of the lactulose under both conditions. The presence of the plasmonic surface significantly amplified the measured fluorescent sensitivity (except in 500 μ M of lactulose) in comparison to the measurements done in parallel without such a plasmonic layer ($n = 3-6$, $p < 0.05$). Overall, these unique interactions between the fluorescence output caused by probe-lactulose mixture and LSP on the surface enhanced both the overall fluorescent signal intensity and plasmonic signal shift.

3.5. Characterizing the smart-probe concentrations

After obtaining the signals in the presence of probe-lactulose complexes, we designed an experimental set to evaluate various probe concentrations ranging from 10 μ M to 400 μ M. Control was designed as probe without lactulose. In addition to the fluorescence experiments that assess the effects of different lactulose concentrations (500 μ M–100 mM) in the presence of a fixed probe concentration (200 μ M) (Fig. 3c), we measured the wavelength changes when applying different

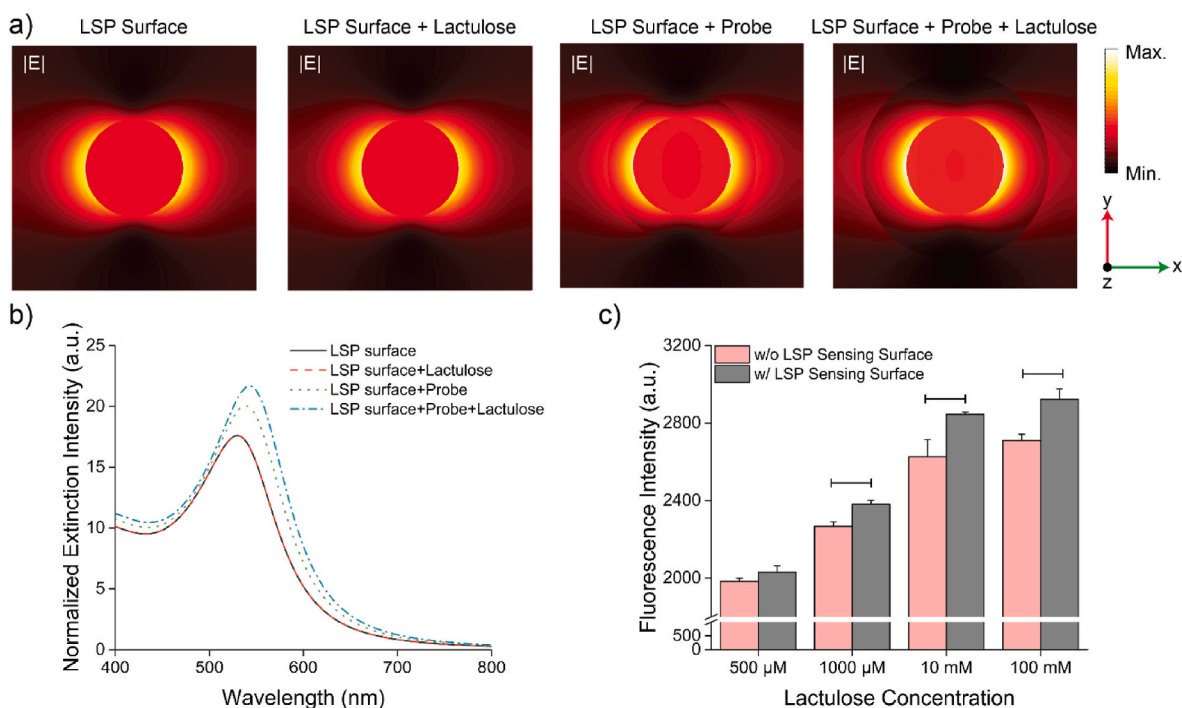


Fig. 3. Numerical simulation and experimental results of assay. a) Numerical simulations demonstrate the working principle of assay. The top view of electric field intensity at resonance on the equator plane of AuNPs is demonstrated (from left to right) for LSP surface immersed in phosphate buffer solution, LSP surface with lactulose introduced to the phosphate buffer solution, LSP covered with probe molecules, and LSP surface-covered with a mixture of probe molecules and lactulose, respectively. b) The calculated extinction field spectrum for each case is plotted. Adding probe-lactulose mixture causes shifts in the resonance to larger wavelengths and also enhances the intensity of electric field. c) In the presence of different lactulose concentrations, the fluorescence signals are collected from the surfaces with LSP and without LSP sensing surface. For statistical analysis, we used non-parametric Mann-Whitney test for comparisons with the statistical significance threshold set at 0.05 ($p < 0.05$). Horizontal brackets represent statistically significant differences between the groups. Data is represented with average value $\pm$ standard deviation ($n = 3-6$).

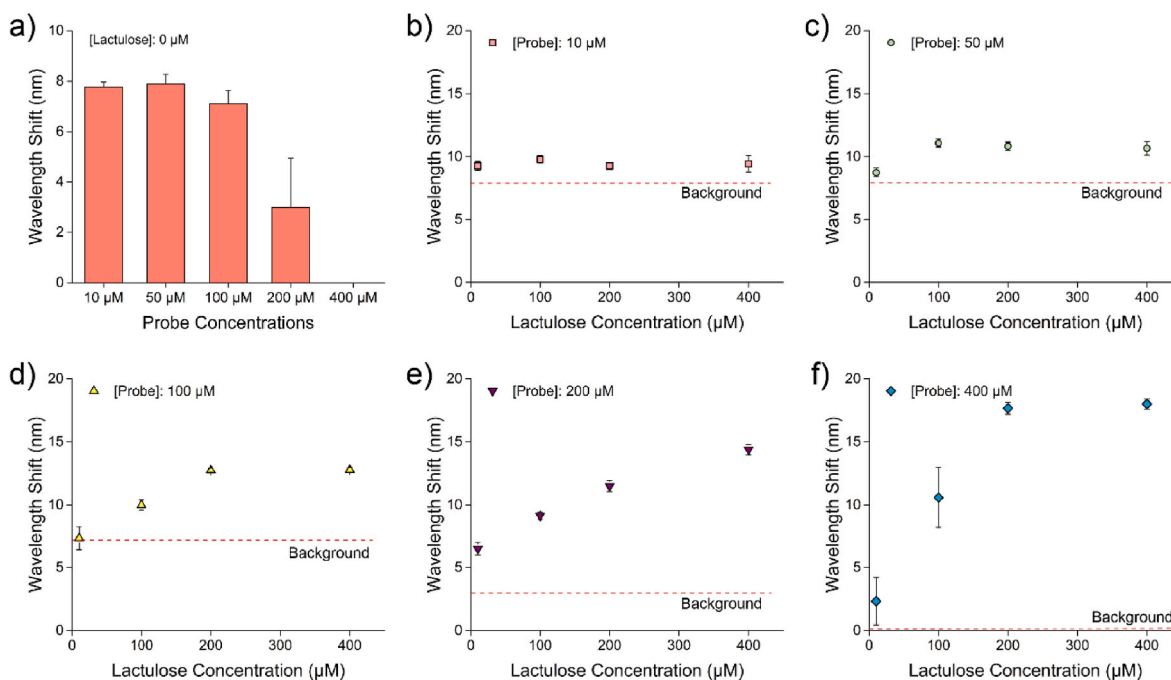


Fig. 4. Evaluating impact of smart-probe concentration on the LSP response. a) Control experiment (background) is performed when the assay is designed with LSP surface and different probe concentrations whereas lactulose is not applied to the surface. Higher concentrations (200 and 400 μM) of probe compound provided lower background than that of other groups. b-f) The assay is then tested with various lactulose concentrations from 10 to 400 μM. Different concentrations of probe compound ranging from 10 μM to 400 μM are evaluated in terms of wavelength shift, linearity, and background signal. As a result, 200 μM of probe provide more linear range with high wavelength shifts and low background signal. Data is represented with average value $\pm$ standard deviation ($n = 6$).

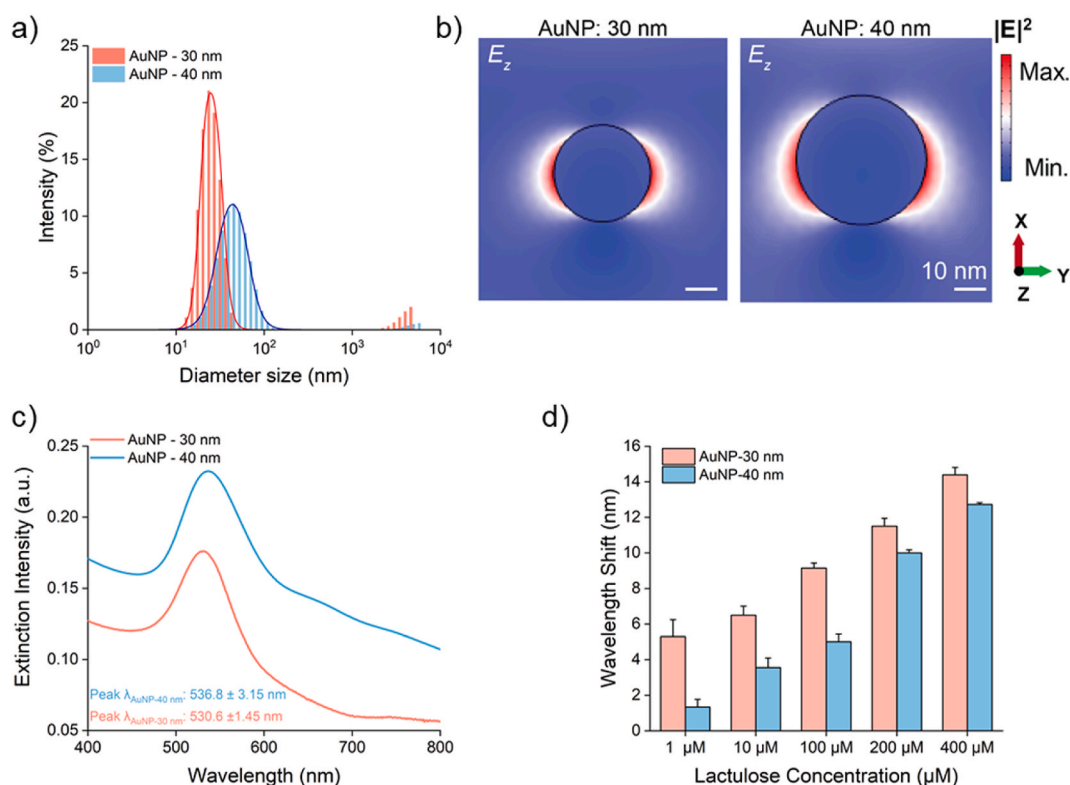


Fig. 5. Experimental and numerical characterizations of AuNP and evaluating its effect on the assay performance. a) Dynamic light scattering (DLS) measurements are presented for 30 nm and 40 nm of AuNP diameters. b) Electric field distribution around AuNPs with different diameters (30 nm and 40 nm) is depicted. c) Extinction intensity plots are presented with their wavelength values as ~ 530.6 nm and ~ 536.8 nm for 30 nm and 40 nm diameter of AuNP, respectively. d) Different diameters of AuNP are evaluated with a range of lactulose concentrations (1–400 μM) in the presence of 200 μM probe compound. Overall, 30 nm of AuNP-coated surface provides higher wavelength shifts when applying different lactulose concentrations. Data is represented with average value $\pm$ standard deviation ($n = 3-6$).

concentrations of probe (10 μM –400 μM) and lactulose (10 μM –400 μM). We therefore benchmarked the probe concentration effect over different lactulose concentrations in terms of wavelength shifts. The lower probe concentrations provided higher background signals at the ranges of 7.8 nm–7.1 nm in the absence of lactulose (Fig. 4a). At 200 μM of probe, the background signal drastically dropped down to 3 nm, whereas the signal was minimum at 400 μM of probe due to visual aggregation of probe in the absence of lactulose. When applying different lactulose concentrations, we observed concentration dependent LSP signal increase due to the formation of fluorescence signal. The detection linearity for each probe concentration also showed different characteristics. At both 10 μM and 50 μM of probe (Fig. 4b and c), the assay yielded LSP signal shifts between 9.3 nm and 10.7 nm. At probe concentrations beyond 100 μM , we observed a more linear detection range that can differentiate the lactulose concentrations (Fig. 4d–f). Although 400 μM of probe yielded higher LSP signal up to 18 nm, the detection curve linearity was saturated after 250 μM of lactulose concentrations. In the assay, different concentrations of probe compound ranging from 10 μM to 400 μM are evaluated in terms of fluorescence signal, linearity, and background signal. As a result, 200 μM of probe provide a broad linear range with high fluorescence signals and low background signal. Among these concentrations, 200 μM probe provided linearity spanning from 10 μM to 400 μM of lactulose concentrations with LSP signals ranging from 6.5 nm to 14.4 nm. Moreover, when applying higher lactulose concentrations (500 μM –100 mM) to the LSP surfaces, we observed linear increments in fluorescent signals that were proportional to the lactulose concentrations (Fig. 3c).

Considering the fluorescence measurements, 10 μM of probe compound provided higher background than that of other groups (Fig. S2).

The detection linearity was observed between 50 μM and 200 μM of probe concentrations whereas the fluorescence signal was quenched in higher probe concentration (400 μM), leading to lower linearity in the detection (Fig. S2). Considering outcomes from both wavelength and fluorescence measurements, we employed 200 μM of probe concentration for our following experiments.

3.6. Modulating LSP signals

We then evaluated the effect of gold nanoparticle (AuNP) diameters over the LSP signal. We first synthesized to two batches of AuNPs, and obtained two types of AuNPs with 30 nm and 40 nm diameter sizes, as confirmed by the dynamic light scattering (DLS) measurements (Fig. 5a). In the first batch, AuNP size resulted in a sharp peak around 30 nm albeit both particles provided monodispersed characteristics. During the synthesis of gold nanoparticles, there might be some larger particles (or aggregates) in the solution, which are very small portion ($\sim 5.9\%$) of all nanoparticles. This is a characteristics of gold nanoparticle synthesis. Through numerical simulations, we observed an alteration in the E-field distribution for each diameter (Fig. 5b). The measured extinction intensity spectra as a function of wavelength was measured as 530.6 ± 1.5 nm and 536.8 ± 3.2 nm for 30 nm and 40 nm of AuNPs, respectively (Fig. 5c). The numerical simulations resulted in similar wavelength peak values as 530.8 nm and 536.2 nm, respectively (Fig. S3). We also assessed the assay performance with these two types of AuNPs when measuring their enhanced LSP signal with different lactulose concentrations and fixed probe concentrations (200 μM) (Fig. 5d). As a result, AuNP with 30 nm diameter size provided higher LSP response up to 14.4 ± 0.4 nm compared to that of AuNP with 40 nm. AuNP with 30 nm

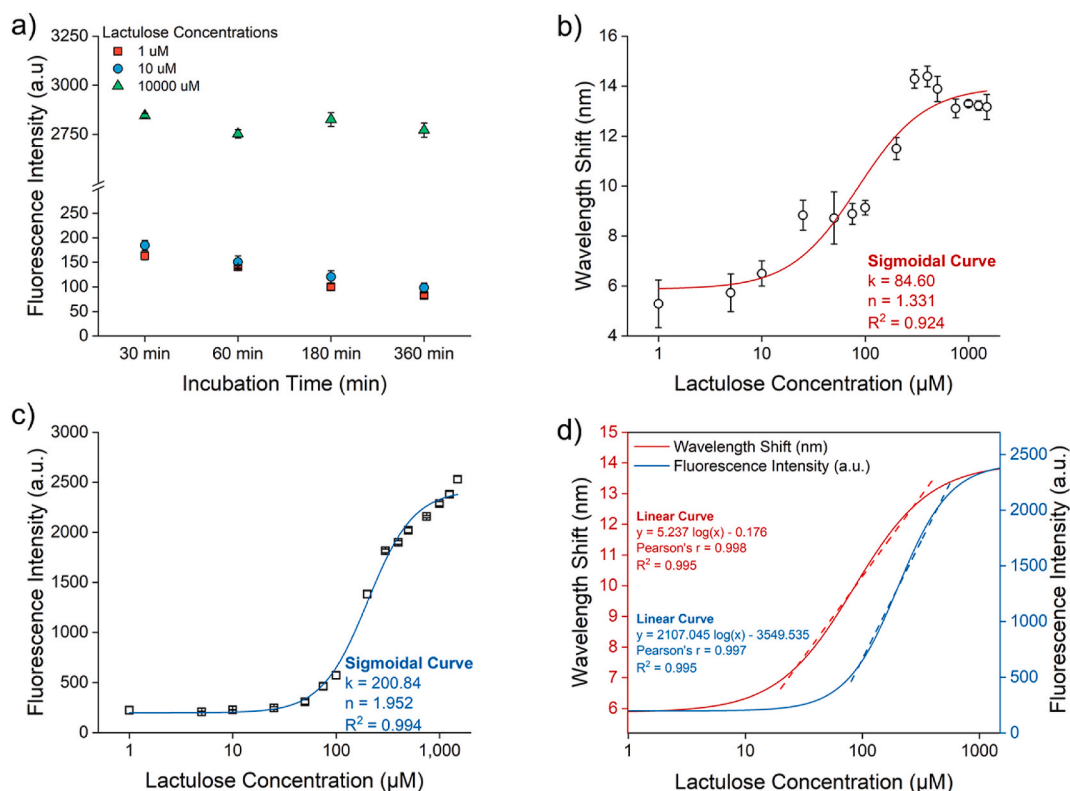


Fig. 6. Evaluating incubation time and lactulose concentrations. a) The incubation time is evaluated with a broad range of probe concentrations. Fluorescence intensity increases in higher probe concentrations. Between 30 and 60 min, the signals are constant in all lactulose concentrations, and then, signals decrease at higher incubation periods. The optimum assay incubation is therefore fixed as 30 min. After optimizing all parameters (probe concentration, AuNP diameter size, and incubation time), a broad range of lactulose concentrations is evaluated through sigmoidal curves for wavelength (b) and fluorescence (c) changes. (d) According to the exponential sections in the calculated sigmoidal curves, the wavelength measurements provided higher sensitivity at low-end concentrations of lactulose than that of fluorescence measurements, whereas fluorescence signals detected higher concentrations easily and also provided better sigmoidal curve characteristics compared to the wavelength measurements. Data is represented with average value $\pm$ standard deviation ($n = 6$).

diameter size presented LSP signal shifts across all lactulose concentrations tested ($n = 3-6$, $p < 0.05$). Through fluorescence measurements, we observed incremental signals proportional to lactulose concentrations, and there was no statistically significant difference in different AuNP diameters for same lactulose concentrations ($n = 3-6$, $p < 0.05$) (Fig. S4). Considering all these characterization and performance studies, we selected 30 nm diameter of AuNPs to implement the LSP layer.

3.7. Evaluating the assay incubation time

To understand the required time for assay with an efficient performance, we designed an experiment by altering lactulose concentrations (1 μM -10 mM) in different incubation time slots (30 min-6 h) (Fig. 6a). After 60 min, the fluorescence intensity dropped in all concentrations, especially this was more considerable at lower concentrations. The signal was similar at 30 min and 60 min of incubation time slots at all concentrations, and therefore, we selected the incubation time as 30 min for the following experiments.

3.8. Evaluating the assay performance

After optimizing all parameters, the assay was designed as 200 μM of probe concentration and 30 min incubation time slot (Fig. 6b-d). We then applied different lactulose concentrations (1-1500 μM) to the LSP surface, and measured the wavelength peak value for each condition (Fig. 6b). The increase in the LSP signal was majorly proportional to the concentration of lactulose due to the enhancement of signal capability. Briefly, we observed an LSP signal shift as 5.39 ± 1 nm at 1 μM of lactulose, and the signal yielded to 13.2 ± 0.5 nm at 1500 μM of lactulose. The binding-responses of both wavelength and fluorescence measurements were calculated by fitting a sigmoidal curve function to the data through Hill Slope calculations, a commonly employed equation for non-linear fitting dose-response equations in immunoassays (Eq. (1)):

$$f(x) = A + (B - A) \left(\frac{x^n}{k^n + x^n} \right) \quad (\text{Eq. 1})$$

where A represents the minimum asymptote, n is the Hill Slope (coefficient), k states the inflection point (Michaelis constant), and B is the maximum asymptote. Through this calculation, we plotted sigmoidal curves for both cases. Briefly, the sigmoidal plot for wavelength measurements resulted in 0.924 of R^2 , 84.60 of IC50, and 1.331 of Hill Slope (coefficient) (Fig. 6b), indicating positively cooperative binding between the receptor and the ligand. As demonstrated in numerical calculations (Fig. 3a and b), we experimentally confirm that the effect of reaction between the probe and lactulose molecules causes changes in LSP signal (i.e., wavelength shift). The control is defined as that lactulose is applied to the surface when there is no probe. In this case, we did not observe any signal changes on the resonance wavelength. When the probe is applied to the surface, there is a signal change due to the binding of probe to the LSP surface, which is not observed when lactulose is applied alone. When we start adding the lactulose to probe-loaded LSP surface, the fluorescent signal appears due to the reaction between the probe and lactulose and this binding generates local refractive index changes at the close vicinity of the surface. This effect is dependent on the lactulose concentration and becomes stronger as we applied higher concentrations (Fig. 6b).

In addition, we plotted the data of fluorescence measurements through the sigmoidal curve function, and observed 0.994 of R^2 , 200.84 of IC50, and 1.952 of Hill Slope (coefficient), and similarly, this result indicated the positively cooperative binding in the fluorescence measurements (Fig. 6c). In addition, we re-evaluated these sigmoidal plots, and the ranges within the exponential behavior were fitted to a linear trendline as shown in Fig. 6d. In these ranges, the wavelength measurements resulted in 0.995 of linearity along with 0.998 of Pearson's

correlation coefficient, whereas the fluorescence data provided 0.995 of linearity along with 0.997 of Pearson's correlation coefficient. Under the light of these calculated sigmoidal plots, we concluded three major points: (i) the wavelength peak measurements provided a lower detection limit (Fig. 6d) since a clear wavelength shift was visible well before of the fluorescence measurements in terms of lactulose concentrations, pointing out the improvements (nearly one order of magnitude) in the detection limit due to the LSP layer. (ii) According to the sigmoidal fit curves, the fluorescence measurements (Resendez and Malhotra, 2019) were capable of responding higher concentrations than that of the wavelength measurements. (iii) Considering the signals and their error bars (standard deviations) at each concentration, fluorescence signals provided better sigmoidal curve characteristics (Fig. 6c) compared to the wavelength measurements (Fig. 6b). Elaborating this result, there would be some reasons: (1) the LSP surface was constructed through the synthesized gold nanoparticles with 30 nm of diameter. As we observed in our experiments (Fig. 5a), although the nanoparticles were highly mono-dispersed, there were some non-uniformities in their sizes that might interfere with the uniformity of nanoparticles immobilized on the LSP surface. (2) The other reason might be that the less ordered distribution and not complete homogeneity in the nanoparticle dimensions within the LSP surface. Therefore, the performance of the wavelength measurements might be hampered by these two possible reasons, causing the fluctuations in the signals as we observed higher standard errors in Fig. 6b. Since these two possible reasons were related to the construction of the LSP surface, these factors would affect much more the wavelength measurements with respect to the fluorescence measurements. To address these challenges, one possible solution would be to measure any possible variations in the nanoparticle batches through DLS or TEM analysis. The second would be the visualization of the surface topography of LSP surface in order to evaluate the degree of uniformity and homogeneity of the nanoparticles on the surface. All these assessments would help and guide how the LSP surface would be improved in terms of sigmoidal curve characteristics and the detection of higher lactulose concentrations. Consequently, the presented platform provided dual detection modalities including (i) wavelength peak measurements for low-end concentrations (higher sensitivity); and (ii) fluorescence measurements for better sigmoidal curve characteristics and detection at higher concentrations.

3.9. Calculating binding constants

To determine the interaction model of the binding, we performed an equilibrium isotherm model for host-guest binding interactions in the LSP measurements (Dibekkaya et al., 2016; Fornstedt et al., 2013; Langmuir, 1916; Li and Husson, 2006; Rocha, 2015; Saylan et al., 2017). We therefore calculated the binding constants between the probe and lactulose for solution-based interactions and binding near the metal nanoparticles. Briefly, the isotherm model was demonstrated below (Inci et al., 2020b; Li and Husson, 2006) (Eq. (2)):

$$\Delta\text{WS}_{\text{ex}} / C = K_A (\Delta\text{WS}_{\text{max}} - \Delta\text{WS}_{\text{eq}}) \quad (\text{Eq. 2})$$

where ΔWS is described as the response value (wavelength shift) upon lactulose binding; C is lactulose concentrations (μM); and K_A (1/ μM) and K_D (μM) are forward and reverse equilibrium constants, respectively. Subscripts (ex, max, and eq) denotes experimental, maximum, and equilibrium, respectively.

We evaluated the plots with their entire experimental data points of wavelength and fluorescence measurements. For the wavelength measurements (Fig. 6b), the binding constants (K_A and K_D) were calculated as ~ 0.26 and ~ 3.91 , respectively. Further, the calculated $\Delta\text{RA}_{\text{max}}$ value of the model ($\Delta\text{WS}_{\text{max}} = \sim 12.8$) agreed with the experimental data ($\Delta\text{WS}_{\text{max}} = \sim 13.2$). By adapting this model to the entire experimental data of fluorescence measurements (Fig. 6c), we calculated the K_A and K_D constants as ~ 0.03 and ~ 40.82 , respectively. Similarly, the

calculated $\Delta RA_{\max}$ value in fluorescence interactions ($\Delta F_{\max} = \sim 2196.3$) agreed with the experimental data ($\Delta F_{\max} = \sim 2528.3$).

4. Conclusion

Here, we present a dual detection modality that includes only one recognition element (probe in our system) that binds to a target (lactulose), and this reaction is detected by plasmonic signals. The probe itself does not fluoresce in absence of the lactulose molecule. When the lactulose is present, the probe becomes fluorescent, and accordingly, the plasmonic readout mechanism uses this effect as a local enhancement on the LSP of AuNPs (Brolo, 2012; Russell et al., 2012). Therefore, the LSP surface acts as an indirect sensing tool to detect the binding events/reactions between the probe and the lactulose that causes a fluorescent signal to arise. This binding signal (probe-lactulose) is monitored by the changes in the resonance wavelength and the local increase in the refractive index at the close vicinity of LSP surface.

To broaden its applicability, throughput, and scalability, LSP modality is simply implemented in a 96-well plate surface using gold nanoparticles and PLL, thus avoiding expensive and time-consuming conventional lithographic production processes for LSP surface design and fabrication. Further, the platform chemistry is versatile, and therefore, different kinds of nanoparticles (e.g., Ag nanoparticles, nanocubes, and nanorods) could be easily integrated via amine-groups of PLL molecules. From the read-out perspective, intrinsic production of fluorescence signals proportional to saccharide concentrations, yields a larger LSP signal shift and allows to detect sugars in a wider range and at lower concentrations compared to the standard methods. The platform is also comprehensively evaluated with finite element (FE) simulations. Considering the detection of GI permeability, various types of sugars could be potentially tested on our model platform, and the probe would be designed accordingly. We have previously shown that the boronic acid-based probes can be utilized to determine the ratio of various carbohydrates (Resendez and Malhotra, 2019). As such, the method is certainly robust as a sensing strategy for detecting different sugars. On the other hand, the current study is a further advancement of this work and shows that miniaturized of boron-based reporter system on LSP chip, can be used effectively in measuring the concentration of a single sugar such as lactulose, and can potentially help in determining the inflammation the increased permeability in the GI tract. Therefore, the current manuscript presents a proof-of-concept study to evaluate whether the fluorescence and LSP signals can be mutually enhanced in the presence of target sugar unit, i.e., lactulose. We hence assessed this feature by detecting lactulose only in the experiments. The overall focus of this study is to present the potential of a new technology, which is derived from a boron-based receptor/reporter system that can successfully determine the increased permeability in the GI tract. The data strongly supports the potential of our approach and could be helpful in real world setting. Moreover, as shown in our earlier study, a number of probes and different sugar and their combinations would be tested in a future study.

Adapting current tools and techniques for monitoring GI diseases such as IBD, Crohn's disease, ulceritis, and colitis into POC settings remains challenging due to their high costs, low throughput, unreliability, and limited detection ranges. Current diagnostic strategies are further limited by asymptomatic cases where bleeding and infection manifest at later stages. Conventional imaging methods are also expensive and unsuitable for POC deployment, contributing to delayed treatment and severe disease progression. Therefore, there is a critical need for the development of cost-effective, high-throughput, and reliable tools that can map and quantify GI permeability, enabling early detection in POC settings before disease progression. For instance, non-invasive assessments of GI permeability using urinary saccharide biomarkers—such as sucrose for upper GI tract permeability, lactulose and mannitol for the small intestine, and sucralose for the colon—rely on expensive, time-intensive techniques like high-performance liquid chromatography/

mass spectrometry (HPLC/MS). Consequently, the development of affordable, sensitive, and scalable tools for early detection is essential. Integrating these tools into POC settings would enable individuals to monitor their GI health rapidly and conveniently, facilitating timely therapeutic intervention. Additionally, this strategy could be adapted to target various sugar biomarkers (e.g., sucrose, mannitol, sucralose) to monitor regions of increased GI permeability. Following this initial screening, further clinical interventions could be implemented to determine appropriate treatment strategies. In sum, this integrated sensing strategy is highly sensitive, high-throughput, and accurate for measuring GI permeability at the POC settings, and it would be potentially applicable for the noninvasive monitoring in the near future.

CRediT authorship contribution statement

Fatih Inci: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Angel Resendez:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Merve Goksin Karaaslan:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Mallesh Pandrala:** Writing – review & editing, Investigation, Data curation. **Amideddin Mataji Kojouri:** Writing – review & editing, Methodology, Formal analysis. **Rajib Ahmed:** Writing – review & editing, Methodology, Formal analysis. **Mehmet Giray Ogut:** Visualization. **Bakthan Singaram:** Writing – review & editing, Supervision. **Sanjay V. Malhotra:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Utkan Demirci:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: S. V.M. is a co-founder of (i) Arxeon, Inc; (ii) Discovery Biotech, and (iii) Scientific Advisor to Cadila Pharmaceutical Ltd. U.D. is a co-founder of and has an equity interest in: (i) Vetmotl Inc., (ii) LevitasBio, (iii) Hermes Biosciences. U.D.'s interests were reviewed and managed in accordance with his institutional conflict-of-interest policies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2024.117040>.

Data availability

Data will be made available on request.

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