Detection of Chilli Leaf Curl Virus using Attenuated Total Reflectance (ATR) mediate Localized Surface Plasmon Resonance Based Optical Platform

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Supplement 1:

Experimental details. The experimental setup consists with a tungsten halogen bulb (Philips: 6V, 10 W) as a broadband light source, a high refractive index right-angle prism (N-SF11, \( n_c = 1.7786@632.8 \text{nm} \), base dimensions 20 mm × 18 mm), a plano-convex lens (\( f = 50 \text{ mm}, \theta = 12.5 \text{ mm} \)) and objective lens (0.65 NA, 30X). The incident light intensity from tungsten halogen bulb was collimated (3 mm beam diameter) using plano-convex lens and incidence on the prism surface at 68° angle. The reflected light from the prism base surface was focused onto input of fiber spectrometer (AvaSpec-ULS2048CL-EVO) using objective lens.

Supplement 2:

Functionalization of the substrate. The chemical cleaning improves the surface quality. First the substrate was cleaned with isopropyl alcohol solution followed by sonication for 3 minutes, then the substrate was dipped in 1.0M NaOH solution for 30 minutes to remove any surface impurity. After repeatedly washing with DI water, the samples were kept in 1:1 ratio of methanol and concentrated HCl solution for 45 minutes. Then the sample was again thoroughly washed with DI water and incubated in concentrated H2SO4 for 15 minutes to hydroxylate (-OH) the surface of the substrate. The hydroxylated substrates were incubated in 2% APTES (prepared in absolute ethanol and acetic acid with volumetric ratio 10:4) solution for 15 minutes. Then these substrates were washed with ethanol for 2 to 3 times and heated in oven at 110°C for 2 hours to improve the condensation process in ambient condition. The surface functionalization process is given in Figure S1. This APTES treatment generates amine functional groups (-NH2) on the surface which are suitable for gold nanoparticles immobilization.
**Synthesis of gold nanoparticles (AuNPs).** The gold nanoparticles were synthesized in solution phase according to the procedure described by Anton et.al.¹ A stock solution of auric chloride (HAuCl₄·3H₂O) of 50 mM was prepared in DI water. From the prepared stock, 0.2 ml solution was added in 80 ml DI water and heated until boiled. As boiling starts, 0.645 ml of 5 mg/ml concentration sodium citrate trihydrate solution was added dropwise. The solution was further heated until it was turned purple red in colour. Once the colour of the required gold nanoparticle solution was achieved, the solution was removed from the heat bath and allowed to cool down to room temperature for further used. The LSPR absorption spectrum of this synthesized gold nanoparticle was measured by UV-Vis spectrometer (Perkin Elmer Lamda 950) and LSPR peak was found at 535 ± 2 nm wavelength (Figure S2). The average particle diameter was confirmed from the Scanning electron microscopy (SEM; JSM-7600F) image analysis and found between 50 nm to 55 nm as presented in inset of Figure S2.

**Gold nanoparticle immobilization.** The gold nanoparticles were immobilized on functionalized coverslip surface by ATR configured evanescent wave based LSPR absorption method. For the data
acquisition during experiments, the spectrometer (AvaSpec-ULS2048CL-EVO) integration time was set to 35 milliseconds (ms) where number averaging was set for 20 data points. This was confirmed that the data recording interval between two measurement was 700 ms in continuous running mode.

During the immobilization, visually was observed the change of purple-red color of gold nanoparticle solution that indicate the reduction of nanoparticle density in colloidal solution only because of binding of nanoparticles (Figure S3). At starting, the immobilization on active amine (-NH$_2$) functional surface was initiated via coulombic attraction between AuNPs and -NH$_2$ groups but with time gravity assisted immobilization was dominated.

![Figure S3](image_url) Gold nanoparticles solution inside sample cavity on ATR-LSPR prism surface and sequential change of solution color during the binding.

In this process, the corresponding LSPR absorbance peak evolution with time was monitored compared to baseline (DI) spectrum. The spectra were recorded for every 100 seconds interval till the absorbance reaches to 0.43 units (data acquisition limit was decided by looking at absorbance at 0.43 units before any cluster formation of nanoparticles on substrate surface). Thus, the sensor response was optimized by maximizing the LSPR absorbance peak 0.43 units for 45 minutes. The unbounded nanoparticles were removed from the sample chamber and then cleaned with DI water. The nanoparticles immobilized surface was observed under SEM (Figure S4) and nanoparticle density was calculated with the help of open-source image processing software (Image J).
Figure S4. Schematic representation of nanoparticle immobilized surface and scanning electron microscope image of immobilized gold nanoparticles on the functionalized surface.

Supplement 3:

Antibody-Antigen (HIgG-GaHIgG) immobilization process. After preparing the LSPR probes, the immobilized gold nanoparticle surface was further treated with linkers as shown in process II and process II in Figure S5. First, in process I, the nanoparticle surface was treated with cystamine. As seen in process II, the glutaraldehyde (25% aqueous solution, diluted to 5% in DI water at room temperature) was used as secondary linker that rapidly reacts with the positively charged amine (-NH$_2$) groups and formed chemically stable crosslink surface. This aldehyde widely used for biological enzymes immobilization.

Figure S5. After preparing the LSPR surface, series of experimental processes were followed to detect the target sample, like in process II and process III for cystamine and Glutaraldehyde treatment, process IV for human IgG (HIgG) immobilization to prepare for receptor surface, process VI is the detection of specific Goat-anti human IgG (GaHIgG) target sample.
**Viral and non-viral sample preparation.** The virus infected leaf was collected from the Chilli plants and sap was extracted from this plant tissue as shown in Figure S6. The extracted sap was filtered and centrifuge (3000rpm) to remove any suspended tissues for further use. Similarly sap was prepared from the healthy plant.

**Figure S6.** Top tender samples of ~100 mg was drawn from diseased and healthy plants and total plant DNA was isolated and quantified for preparation of standard samples for testing with ATR-nanobiosensor, (a) Chilli plants showing leaf curl symptoms in the greenhouse following transmission of chilli leaf curl virus through whitefly inoculation from the field samples, (b) Healthy chilli plants raised from seeds in the controlled environment.

**Supplement 4:**

**Refractive index solutions preparation.** The sucrose (Sucrose GR; MERCK) solution was prepared by mixing weight/volume percentage concentration (w/v) in DI water to get various R.I solution. The total volume of solution was adjusted to 5 ml vials (TARSONS). Different concentrations of the sucrose solution (5 % - 40%) was prepared in DI water to get various refractive index (1.3418–1.3999) solution. The sucrose powder was added to DI water in steps of 5% (w/v) increment. The refractive index values of these sucrose solutions were measured using handheld digital refractometer (Reichert AR200, accuracy ± 0.0001 at 27 °C) and found varied linearly from 1.3418 to 1.3999 refractive index unit (RIU) with the sucrose concentration (Figure S7).
**HlgG-GaHlgG binding results.** The binding results during all process are shown in **Figure S8.** The dynamic of LSPR peak during the incorporation of each analyte confirmed the binding.

Figure S7. Sucrose solution concentration vs measured refractive index.

**Redshift in LSPR peak.** After completing each binding process, the unbound molecules were removed for the sample chamber by cleaning with DI water. This cleaning will ensure the stable binding of molecules on the surface. After binding shift in LSPR peak was observed (**Figure S9**) that
indicates that RI of near the nanoparticles surface changed during the incubation of each process step.

![Image](image_url)

**Figure S9.** The change in LSPR peak position after incubation of each process like Cystamine, Glutaraldehyde, H1gG, BSA and GaHlgG compared to immobilized gold nanoparticle peaks in DI water.

**Absorption rate (K_a) calculation.** The absorption rate was calculated after rescaling the time-resolved absorbance response of GaHlgG from 100 seconds to 250 seconds at different concentration as shown in **Figure S10.**

![Image](image_url)

**Figure S10.** Linear fitting of time-resolved absorbance response GaHlgG data at different concentration in the association phase.

After calculating the slope (binding rate) from the Figure S10, again slope values were plotted as a function of GaHlgG concentration as shown in **Figure S11.** A linear co-relation was observed between binding rate and concentration.
Figure S11. All calculated slope values were further fitted linearly and $K_a$ was calculated.

**Table S1**: Calculation of refractive index for every set of experimental procedure during R.I study with sucrose solution, GaHlgG detection process and GaHlgG concentration variation study.

| Experiment set 1 | Refractive index calculation |
|------------------|-------------------------------|
| **R.I study with sucrose solution (From figure 2b)** | Sample | Time | $A_{540}$ Absolute | $\Delta A_{540}$ relative | $\Delta A_{540}$ % | Absolute value | Relative |
| 0(DI)            | 10    | 0.4262 | 0.0624 | 6.24 | 1.3568 | 1.3328 |
| 5                | 257   | 0.4971 | 0.2312 | 23.12 | 1.3615 | 1.3439 |
| 10               | 438   | 0.556  | 0.3714 | 37.14 | 1.3654 | 1.3532 |
| 15               | 679   | 0.6051 | 0.4883 | 48.83 | 1.3686 | 1.3609 |
| 20               | 933   | 0.6649 | 0.6307 | 63.07 | 1.3726 | 1.3703 |
| 25               | 1181  | 0.7123 | 0.7436 | 74.36 | 1.3757 | 1.3778 |
| 30               | 1425  | 0.7599 | 0.8569 | 85.69 | 1.3788 | 1.3852 |
| 35               | 1695  | 0.8116 | 0.9800 | 98.00 | 1.3823 | 1.3934 |
| 40               | 1924  | 0.86   | 1.0952 | 109.52 | 1.3854 | 1.4010 |

| Experiment set 2 | Analytes | Time | $A_{540}$ Absolute | $\Delta A_{540}$ relative | $\Delta A_{540}$ % | Absolute value | Relative |
|------------------|----------|------|--------------------|--------------------------|----------------|----------------|----------|
| **GaHlgG detection (From figure 3a)** | AuNps    | 3167 | 0.4253             | 0.0602                    | 6.02 | 1.3567 | 1.3326 |
|                  | Cys      | 3780 | 0.4741             | 0.1764                    | 17.64 | 1.3600 | 1.3403 |
|                  | DI       | 3935 | 0.4674             | 0.1605                    | 16.05 | 1.3595 | 1.3393 |
|                  | GadH     | 4835 | 0.5515             | 0.3607                    | 36.07 | 1.3651 | 1.3525 |
|                  | DI+PBS   | 4956 | 0.4895             | 0.2131                    | 21.31 | 1.3610 | 1.3427 |
|                  | HlgG     | 6684 | 0.5109             | 0.2640                    | 26.40 | 1.3624 | 1.3461 |
|                  | DI+PBS   | 6626 | 0.5048             | 0.2495                    | 24.95 | 1.3620 | 1.3451 |
|                  | BSA      | 7790 | 0.5155             | 0.2750                    | 27.50 | 1.3627 | 1.3468 |
|                  | DI+PBS   | 7985 | 0.5078             | 0.2567                    | 25.67 | 1.3622 | 1.3456 |
|                  | GaHlgG   | 9246 | 0.5454             | 0.3462                    | 34.62 | 1.3647 | 1.3515 |
|                  | (30μg/ml)| 9430 | 0.5193             | 0.2840                    | 28.40 | 1.3630 | 1.3474 |

| Experiment set 3 | Analytes | Time | $A_{540}$ Absolute | $\Delta A_{540}$ relative | $\Delta A_{540}$ % | Absolute value | Relative |
|------------------|----------|------|--------------------|--------------------------|----------------|----------------|----------|
| **GaHlgG (From figure 3c)** | DI+PBS   | 8000 | 0.5018             | 0.2424                    | 24.24 | 1.3618 | 1.3447 |
|                  | 0.5      | 8670 | 0.5047             | 0.2493                    | 24.93 | 1.3620 | 1.3451 |
|                  | 1        | 8670 | 0.5084             | 0.2581                    | 25.81 | 1.3622 | 1.3457 |
|                  | 10       | 8670 | 0.5116             | 0.2657                    | 26.57 | 1.3624 | 1.3462 |
|                  | 30       | 8670 | 0.5264             | 0.3010                    | 30.10 | 1.3634 | 1.3485 |
|                  | 50       | 8670 | 0.5475             | 0.3512                    | 35.12 | 1.3648 | 1.3518 |
Supplement 5:

Once the cDNA binding was confirmed on the surface, then the infected Chilli ssDNA was introduced on the surface and the real-time LSPR absorption change was observed at 540 nm. The complete detection process of viral ssDNA is shown in Figure S12 also each process is labelled.

![Figure S12](image)

Figure S12. The time-resolved absorbance response steps during viral ssDNA (1.5 μg/ml) binding on the cDNA probe

Table S2: Calculation of refractive index for every set of experimental procedure during the cDNA probe preparation and viral ssDNA detection with concentration.

| Experiment set 1 | Analytes | Time | $A_{540}$ Absolute | $\Delta A_{540}$ relative | $\Delta A_{540}$ % | Absolute value | Relative |
|------------------|----------|------|--------------------|--------------------------|-------------------|--------------|----------|
| cDNA probe preparation (From figure 5a) | AuNPs | 2426 | 0.4238 | 0.0567 | 5.67 | 1.3566 | 1.3324 |
| | Cys | 2900 | 0.4396 | 0.0943 | 9.43 | 1.3577 | 1.3349 |
| | DI | 2950 | 0.4338 | 0.0805 | 8.05 | 1.3573 | 1.3340 |
| | GadH | 3891 | 0.5109 | 0.2640 | 26.40 | 1.3624 | 1.3461 |
| | DI | 3900 | 0.4543 | 0.1293 | 12.93 | 1.3587 | 1.3372 |
| | TE | 3960 | 0.4588 | 0.1390 | 13.90 | 1.3589 | 1.3378 |
| | cDNA | 5470 | 0.468 | 0.1619 | 16.19 | 1.3596 | 1.3394 |

| Experiment set 2 | Analytes | Time | $A_{540}$ Absolute | $\Delta A_{540}$ relative | $\Delta A_{540}$ % | Absolute value | Relative |
|------------------|----------|------|--------------------|--------------------------|-------------------|--------------|----------|
| Viral DNA detection (From figure 5b) | TE buffer | 5600 | 0.46652 | 0.1584 | 15.84 | 1.3595 | 1.3391 |
| | 0.5 | 6190 | 0.46931 | 0.1650 | 16.50 | 1.3596 | 1.3396 |
| | 1.5 | 6190 | 0.47068 | 0.1683 | 16.83 | 1.3597 | 1.3398 |
| | 2.5 | 6190 | 0.47486 | 0.1782 | 17.82 | 1.3600 | 1.3404 |
| | 3.5 | 6190 | 0.47876 | 0.1875 | 18.75 | 1.3603 | 1.3410 |

After confirming the detection of ChiLCV, the selectivity of the developed sensor was examined by using healthy Chilli leaf ssDNA as unknown target DNA sequence. After incubating with healthy Chilli DNA on cDNA linked LSPR probe surface, no prominent change in LSPR absorbance was observed as
shown in Figure S13 (olive green curve) compare to baseline. Whereas, the infected ChiLCV DNA showed the measurable change in absorbance. The absorbance signal for the non-specific ssDNA binding was found to be lesser than specific ssDNA binding.

Figure S13. Real-time change in absorbance due to specific (viral ssDNA) binding and non-specific (healthy DNA) binding with cDNA immobilized LSPR probes where the change in absorbance for allowed and forbidden conjugation is represented in schematic.

Reference:
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