New Label-Free & Labeled Technology for Protein Characterization and Quantitation

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We present a new label-free platform for protein characterization and quantitation based upon Localized Surface Plasmon Resonance or LSPR on a nanostructured metallic film. With recent advancements in manufacturing techniques, reproducibility of nanostructured thin films has allowed the transition of LSPR from purely academic interest into the first commercialization of systems for practical use in research, bioprocess and diagnostic applications. Similar to gold colloidal solutions, the nanostructured gold films exhibit a distinct color due to the preferential absorption of certain wavelengths within the spectrum of a white light. The color of the film changes as biomolecules or other chemicals come into contact with the LSPR surface, enabling precise and sensitive quantitation of biomolecular interactions. This article reviews the sensitivity and dynamic range of LSPR, which is achieved at a fraction of the cost and with higher throughput than traditional surface plasmon resonance (SPR). It also introduces the technique of amplification of LSPR signals through enzymatic reactions to achieve sensitivities greater and faster than published results for ELISA end-point analyses.

Since Medieval times to the present day, stained glass is in prominent display throughout the windows of churches all across Western Europe. It was only in 1857 though that Faraday[1] and later Rayleigh and Mie[2] formulated a scientific explanation regarding the magnificent coloration of these enduring stained glass marvels. As it turns out, the origin of the beautiful stained glass colors is based upon the selected sands used by the ancient artisans. Unbeknownst to them, sands from different locals contained various quantities of metal salts or minerals (Gold chloride, Gold oxides, Cobalt & Silver compounds, etc ...) which when melted into glass resulted in distinct colors ranging from reds and blues to yellow. We now understand that the metal minerals decomposed in the melt and the metal ions aggregated into nanometer size inclusions. These fabulously enduring glass colors are based upon an absorption of certain wavelengths of light by the electronic oscillation modes of the metal nanoparticles, called localized surface plasmons.[3]

A wealth of basic studies using colloidal solutions showed that colors/wavelengths absorbed can be tailored by the nature of the metal, the size and the local environment surrounding the metal colloids.[4] The use of colloidal solutions to monitor binding events for label-free bioanalyses has become routine.[5] However, the transition from the academic lab to commercialization has been awaiting manufacturing techniques that, in a large and cost-effective scale, can replicate what researchers have actually achieved in Eppendorf tubes.[6] With the emergence of powerful metrology tools able to explore the nanoworld, the relationship between the nano and the macro realm could finally be mastered. Today, stable metal films, with precise nanostructuring and hence with tunable absorption (Fig. 1), can be manufactured on a routine basis using a wide range of surfaces. These films are at the core of a new commercial technology, termed LSPR or localized surface plasmon resonance. The aim of this article is to introduce the salient features of LSPR technology and indicate how LSPR is useful for protein characterization and quantitation. A companion article that focuses on specific applications in bioprocessing will follow in a future publication.

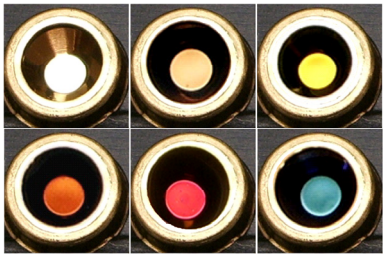

Figure 1
Composite image of nanostructured gold film with various colors; the top left image is bulk gold as a reference. The five other films, with colors from beige to blue, are gold LSPR surfaces with the same amount of gold but with various degrees of nanostructuring. Diameter at the bottom of the wells: 3 mm.

Basics of LSPR technology

Unlike SPR, a well-known sister technology which utilizes flat gold thin films surrounded by sophisticated instrumentation,[7] LSPR technology resides in the sensor itself and requires only elementary components.[8] A schematic of a LSPR instrument is shown in Fig. 2A. White light from a tungsten halogen bulb is directed onto the nanostructured LSPR film. The light interacts with the localized surface plasmons so that some wavelengths are absorbed by the film. The reflected light therefore has certain wavelets missing and is analyzed by a spectrometer. Figure 2B compares the absorptions of a gold nanostuctured LSPR film (red) and a regular gold thin film (dark yellow). The insets are camera images of both films. The regular gold thin film absorption is featureless and can be described by the Rayleigh scattering (i.e. ~1 / λ^4, where λ is the wavelength of light). In contrast, the nanostructured LSPR film exhibits a strong absorption near 550 nm that is the linear superposition of the Rayleigh scattering and the Localized Surface Plasmon absorption. The Localized Surface Plasmon Resonance has a natural width of ~ 80-100 nm, but its maximum position or λ_{max} can be tracked in real-time with a resolution of a few picometers (Fig. 2C).

The position of the LSPR absorption λ_{max} is sensitive to the nature of the interface between the nanostructured gold surface and its environment. As an example, in Fig.2C, the LSPR surface is used for the label-free and real-time detection of a human IgG antibody binding to immobilized protein A. Prior to the injection of the antibody, the LSPR signal λ_{max} is constant, i.e. $\Delta\lambda_{max} = 0$. Upon injection of the antibody, the resonance starts to red-shift and reaches 3200 pm after 3 min. After a brief rinse with PBS, the pH of the solution is lowered to pH 2 so that the interaction between the antibody and the

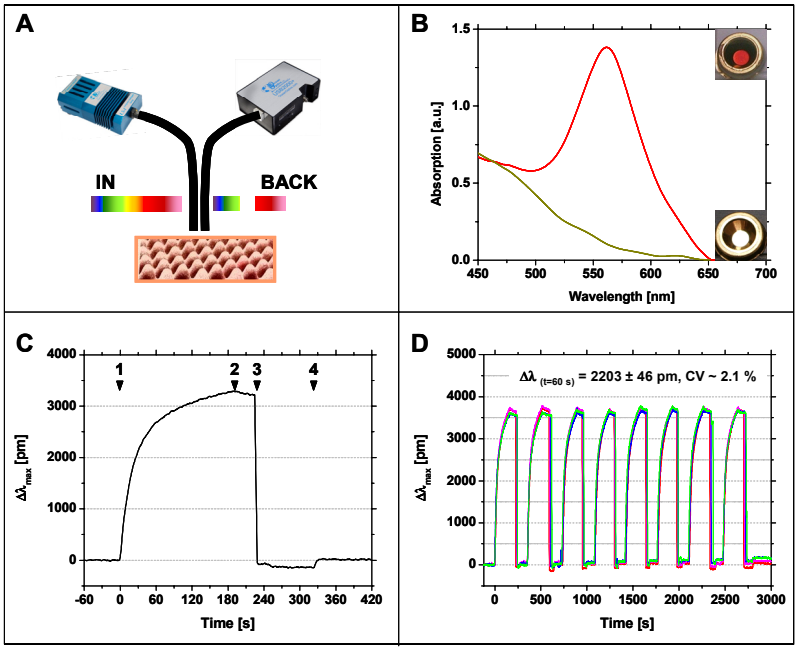

Figure 2

Description of the LSPR technology. **A)** An instrument is composed of a source of white light, the LSPR film and a spectrometer to read the reflected light. Unlike in SPR, the impinging light can couple to the localized surface plasmons under any angle. For convenience, in this configuration of an instrument, the light arrives at 90° through optical fibers. The reflected light is sent to the linear array CCD spectrometer (Ocean Optics, USB 2000, with SONY 2048 pixels chip that cover 430-730 nm range) using optical fibers for analysis. **B)** Absorption spectra of a regular flat gold surface (dark yellow) and the LSPR nanostructured film (red), along with a true color of both films. Absorption is related to the reflected signal through Abs = $-\log_{10}$(Ref / Normalization). The peak position λ_{max} is computed in real-time by a proprietary algorithm. With current hardware, the standard deviation on the peak position is ~ 6 pm. **C)** Sensorgram showing the binding and elution of human IgG on a protein A surface in real-time at 3 Hz. Prior to the IgG injection, the plasmon position is stable. Upon injection (1), the plasmon red-shifts by over 3200 pm. After a brief rinse with PBS (2), the IgG is eluted from the surface with 2 mM HCl, pH ~ 2-3 (3). The plasmon position recovers its initial value when PBS replaces HCl (4), thus completing the binding-elution cycle. **D)** To test the robustness and the reproducibility of the LSPR technology, the same experiment is performed 8 times on 4 surfaces simultaneously. By computing the shift of the IgG injection after 60 sec., the CV of the 32 readings is ~ 2.1%.

protein A surface is disrupted. This produces a sudden decrease of the LSPR signal indicating that the antibody has been removed from the surface. When the pH is re-established to its PBS value, the LSPR signal returns to its original value, i.e. $\Delta\lambda_{max} = 0$, an indication that the sensor has been regenerated. It is important to realize that traces such as this one, also called sensorgrams, represent raw LSPR data. Unlike SPR, LSPR does not require correction for bulk effect signals which occur during injections and rinses. This is the consequence of the localized nature of the plasmons and the fact that they extend and sense only 20-30 nm from the surface which is unlike regular surface plasmons of thin films that sense changes up to 200-1000 nm away from the surface.[7-8]

To illustrate the performance of current LSPR technology, a 4-channel biochip is used to monitor channels simultaneously in real-time when the same human antibody is injected. Figure 2D superimposes the sensorgrams when 8 cycles and binding-regeneration are performed. Analysis of reproducibility is performed by computing the shift $\Delta\lambda_{max}$ after an arbitrary time lapse of 60 seconds after the injection. For these 32 independent repeats, the shift reading yields a CV of ~ 2.1%. A CV of similar magnitude is measured across channels and across different sensors of the same batch. This underscores the apparent consistencies possible for both the manufacturing and biofunctionalization of the nanostructured films.

Selected Applications

Label-free techniques are considered powerful tools in bioprocess monitoring for quality management applications and in R&D for applications such as antibody

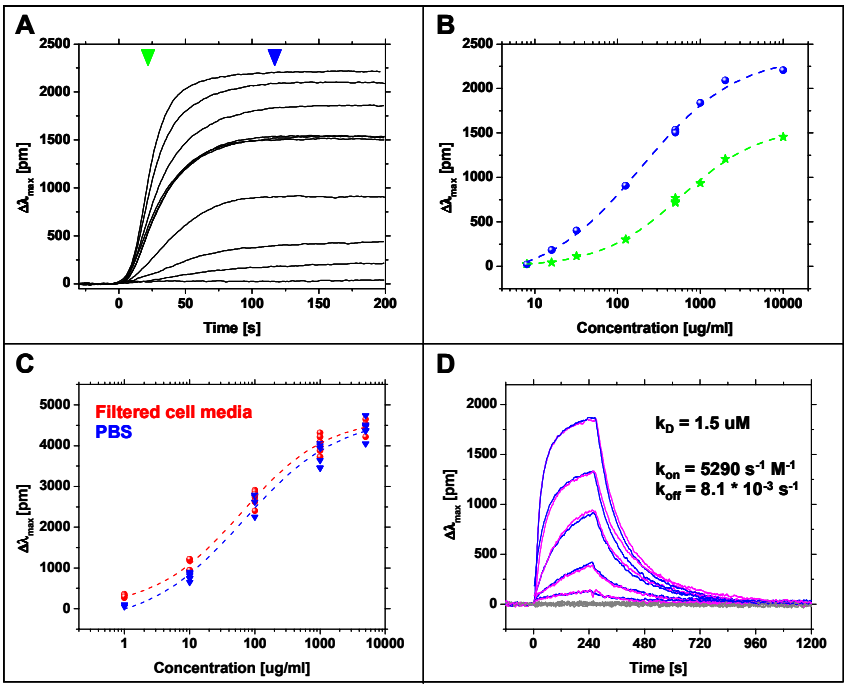

Figure 3

A few examples to illustrate the depth of the LSPR detection. **A)** Dose-response of a protein A surface for human IgG binding, ranging from ~ 7 ug/ml to 10 mg/ml. Notice the 3 repeats at 500 ug/ml IgG – the 4th highest curve. 60 ul of IgG is introduced at 30 ul/min to the LSPR surface, followed by a rinse of PBS. The green (25 sec) and blue (120 sec) arrows indicate reading time for generating the calibration curves in panel B. **B)** Shifts after 25 sec (green line) and 120 sec (blue line) are plotted against the IgG concentrations. The dashed line represents a fit to the logistic model and is used as a calibration curve. **C)** Illustration of the influence of the matrix on the performance of the LSPR sensor. The binding properties for a ProteinA/IgG binding are measured for IgG spiked in PBS and in cell culture media of non-expressing cells at an identical concentration (performed at a customer site). Notice the close relationship between the two media, indicating only a minimal perturbation of the media on the performance of the LSPR sensor. **D)** Illustration of the LSPR capability to compute kinetic parameters. The model used here has caboxybenzene sulfonamide on the surface and bovine carbonic anhydrase II, a 29kDa molecule, in solution at concentrations of 100, 33.3, 11.1, 3.33, 1.11 ug/ml. The gray line is the response of CAII on a reference surface lacking CBS on the surface. Fits with Scrubber 2 software yield a K_D of ~ 1.5 uM, consistent with results reported using SPR techniques. [9-10]

screening, epitope binning and mapping, or affinity and kinetic studies. LSPR technology clearly fits into these categories based upon recent published results. [4-6,8] Two applications of LSPR technology are more thoroughly discussed below: concentration monitoring and kinetic analyses.

The high degree of reproducibility of binding traces can be utilized for the evaluation of dose responses or titer of a particular analyte. In Figure 3A, the response of a protein A biosensor is measured when exposed to different concentrations of human IgG ranging from 7 ug/ml to 10 mg/ml. Notice the 3 overlapping repeats at 500 ug/ml. For quantitation, the LSPR sensor shift is read as a function of the analyte concentration at arbitrarily set check times. Figure 3B reports the reading after 25 sec and 120 sec for each concentration. The readings can be fit with a logistic model as shown by the dash lines through the data points. This approach establishes a calibration curve for the sensor, and/or batch of sensors, and is used for the determination of the test antibody concentration by comparing the shift produced by the test antibody with the calibration curve.

Fig. 3C compares the dose-response for human IgG measurements performed in PBS and crude media and indicates only a marginal difference. In fact, LSPR technology has been used to quantify the amount of IgG expressed by CHO cells, in media obtained from a fermenter, within a few percent of the value obtained by HPLC. More generally, LSPR technology is compatible with various matrices, including crude media, whole blood, cell lysates and other complex buffers containing up to 10% DMSO.

Similarly, LSPR technology is particularly useful for kinetic analyses because, upon injection and rinse steps, there is negligible bulk effect which minimizes the need for data manipulation, a potential major source of variance. Figure 3D represents the kinetic data for the binding and dissociation of Bovine Carbonic Anhydrase

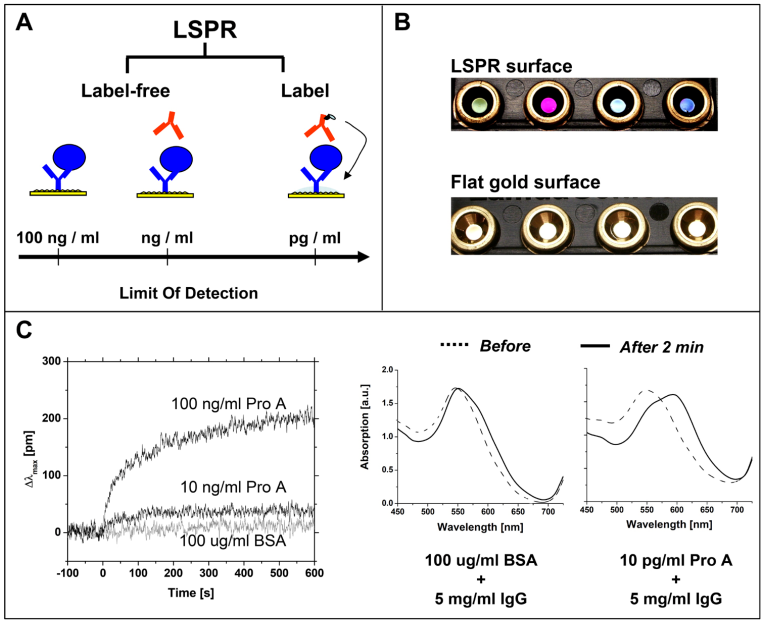

Figure 4

A) Illustration of the label-free and labeled assay implementation using a LSPR surface. In the labeled case, an enzyme is linked to a specific protein. Similar to an immunoassay, the enzyme here is used to convert a soluble substrate into an insoluble product. As soon as the product deposits on the surface, the LSPR color changes. After 2 min of reaction with an estimated 2 nm of product deposited on the surface, the color of the surface becomes blue and corresponds to a shift of 50-100 nm with respect to the original plasmon. **B)** The proof that color change is specific to a LSPR surface, and not due to the absorption of the material deposited on the surface, becomes evident based on the comparison of LSPR and regular gold sensors for the detection of various amounts of alkaline phosphatase. While LSPR sensors show a significant dose-response, the regular gold sensor shows minimal response. **C)** Use of label-free and labeled LSPR for the detection of residual protein A (Pro A), in a background of 5 mg/ml IgG. In the left panel, residual Pro A is spiked in a solution of 5 mg/ml IgG; Pro A is detected label-free in real-time to about 10 ng/ml or 2 ng/mg-IgG of Pro A; in the right panel, the sandwich antibody is labeled with alkaline phosphatase, and its plasmon is read. After addition of the substrate and two minutes of incubation, the LSPR array is read again to screen for a positive response. The graphs on the right represent the reading of a control surface exposed to BSA (lacking exposure to protein A) and a surface exposed to 10 pg/ml or 2 pg/mg-IgG of Pro A. Spectroscopically, the positive surface displays a 44 nm shift, while the control shifts by only 6 nm.

II, a 29 kDa protein, to immobilized sulfonamide ligands. Various repeats show the reproducibility of the responses and allow the computing of the kinetic parameters using Scrubber 2™, software designed for data analysis and in use for SPR. The rate constants (k_{on}, k_{off} and K_D) are compatible with values reported in the literature using SPR.[9-10]

From label-free to labeled detection for enhancing sensitivity

During bioprocessing, the detection of host-cell proteins, the amount of protein A leaching from purification columns, and the absence of live mycoplasma are critical for batch validation. These tests are currently performed with methods orthogonal to label-free techniques. Often, they rely on ELISA assays referred to a third-party lab. While results are being determined, production is in a stand-by mode.

While label-free LSPR does not require sample pretreatment, its sensitivity is restricted to the 10-200 ng/ml range of detection dependent on the protein being analyzed. In order to bridge the gap between label-free and labeled technology, LSPR detection can be enhanced with the use of an enzyme-labeled protein capable of selectively binding to the antigen of interest, such as the low level contaminants mentioned above. With a single reading platform, it becomes possible to expand levels of detection into the pico- to femto- gram range for an assay time of less than 30 minutes. The principle of LSPR-labeled detection is illustrated in Fig. 4A. An enzyme, such as alkaline phosphatase, is used to convert a substrate into an insoluble form. Upon deposition on the LSPR surface, the position of the plasmon peak red-shifts by an amount between 20 and 100 nm (Fig. 4B). The color shift is due to the deposition of the insoluble substrate onto the sensor surface, whereby the nanostructured surface acts as a transducer. It is important to note that the color of the precipitate is not measured, but rather its presence. For example, the reduction rate of nitro-blue tetrazolium (NBT) by alkaline phosphatase (0.002-0.007 OD/min [11]) corresponds to a deposit on the surface of 2-5 nm/min. On the other hand, the LSPR surface is capable of detecting deposition of NBT < 0.1 nm, because such amount would cause a measurable index of refraction change at the surface in the order of ~ 10^{-3}. [8] This is the reason why detection is much faster and sensitive than the traditional ELISA when the same type of experiment is performed on the LSPR surface

The particularities of the nanostructured LSPR surface are illustrated in Fig. 4B where antigen and alkaline phosphatase have been immobilized in parallel on both the LSPR and regular gold surfaces. After 2 minutes of substrate conversion, there is a minimal change on the regular gold chip even at the higher antigen dose, while clear and distinct coloration appears on the LSPR chip. A real-case scenario for the detection of residual protein A, in a background of 5 mg/ml IgG, was undertaken to demonstrate the feasibility of this approach. While the label-free detection is able to detect down to ~ 10 ng/ml of protein A in a solution with 5 mg/ml of IgG (fig. 4C left), the implementation of the labeled method was capable of detecting down to ~ 10 pg/ml of protein A. The labeled-approach is a semi-quantitative end-point assay at this time, but there are indications that it can be recast into a real-time quantitative assay as technology developments are advanced in the future.

Conclusions

Monitoring through LSPR only requires a source of white light and a spectrometer. Also, the platform is largely insensitive to temperature and ultra-precise alignment and therefore can be assembled at a fraction of the cost of conventional SPR. Because of its engineering simplicity, LSPR is amenable to miniaturization for certain point-of-use assays. Academic labs have already reported Palm-size prototypes based on LSPR. [12] The emergence of a commercial LSPR platform that reads the absorption of white light by a nanostructured thin film holds great promises for basic protein science and for applications such as QC/QA. It permits label-free quantitation of biomolecules at the ng/ml level, yet makes possible the detection of contaminants in a solution at femtogram to picogram levels, using the addition of labels which provides notable improvements over current ELISA testing by enhancing the sensitivity and speed of assay responses. LSPR technology benchmarks equivalently with current state-of-the art SPR instruments in terms of data quality, sensitivity and dynamic range, while also possessing the advantage of being scalable relative to throughput. In its current inception, the LSPR instrument can read 8 channels simultaneously with future modules enabling a greater number of channels.

References:

[1] Faraday M, Experimental Relations of Gold (and Other Metals) to Light. Philos. Trans. R. Soc. London, 1857; 147: 145.

[2] Mie G, Beiträge zur Optik truber Medien, speziell kolloidalet Metallösungen. Ann. Phys., 1908; 25: 376-445.

[3] Kreibig U, Vollmer M. Optical Properties of Metal Clusters, Springer Series in Materials Science, vol. 25, Springer, Berlin, 1995.

[4] Xia Y, Halas NJ editors. Synthesis and Plasmonic Properties of Nanostructures. MRS Bulletin, 2005 May; 30 (5): 329-408.

[5] For a review article, see: Anker JN, Hall WP, Lyandres O, Shah NC, Zhao J, van Duyne RP, Biosensing with Plasmonic Nanosensors. Nature Materials, 2008; 7: 442-453.

[6] Englebienne P, Use of colloidal gold surface plasmon resonance peak shift to infer affinity constants from the interactions between protein antigens and antibodies specific for single or multiple epitopes. Analyst, 1998; 123: 1599-1603.

[7] Knoll W, Interfaces and thin films as seen by bound electromagnetic waves. Ann. Rev. Phys. Chem, 1998; 49: 569-638.

[8] Willets KA, van Duyne RP, Localized Surface Plasmon Resonance Spectroscopy and Sensing. Ann. Rev. Phys. Chem., 2007; 58: 267-297.

[9] Day YSN, Baird CL, Rich RL, Myszka DG, Direct Comparison of Binding equilibrium, Thermodynamic, and rate constants determined by surface- and solution-based biophysical methods. Protein Science, 2002; 11: 1017-1025.

[10] Jecklin MC, Schauer S, Dumelin CE, Zenobi R, Label-free Determination of Protein-Ligand Constants using Mass Spectrometry and Validation using Surface Plasmon Resonance and Isothermal Titration Calorimetry. J. Mol. Recognit. 2009; 22: 319-329.

[11] Virella G, Thompson T, Haskill-Strowd R, A new quantitative nitroblue tetrazolium reduction assay based on kinetic colorimetry. Journal of Clinical Laboratory Analysis 1990; 4 : 86 – 89.

[12] Neuzil P, Reboud J, Palm-Size Biodetection System Based on Localized Surface Plasmon Resonance. Anal. Chem. 2008; 80: 6100:6103.