Biosensing Empowered by Molecular Identification: Advances in Surface Plasmon Resonance Techniques Coupled with Mass Spectrometry and Raman Spectroscopy

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Abstract

Surface plasmon resonance (SPR) has been utilized extensively in bioanalysis since the early 90s due to its unique combination of label-free and highly sensitive robust detection. In recent years, SPR has been coupled with and complimented by several other techniques that provide the qualitative and molecular information that SPR lacks. In this paper, advances in studies that present molecular identification to quantitative measurements by SPR are reviewed. In particular, coupling of SPR measurements and MALDI mass spectrometry (MALDI-MS) has been broadly explored where plasmon-assisted ionization upon gold substrates appears to demonstrate many added benefits. Imaging SPR analysis, upon coupling, has contributed to new analytical capability that proves to be powerful with elevated throughput. The combination of SPR and Raman spectroscopy has also found a wide range of applications in providing molecular information that promotes quantitative analysis. Similar to MALDI, the plasmonic thin-film metal substrates provide a desired surface structure and property for Raman enhancement, improving subsequent molecular recognition. Instrumentation advancements and improvements in coupling efficiency has facilitated a series of SPR sensor developments where the plasmonic properties of the surface contribute uniquely to the performance of the coupled technique.

Keywords

Surface Plasmon Resonance, SPR imaging, Matrix Assisted Laser Desorption Ionization (MALDI-MS), Surface Enhanced Raman Spectroscopy (SERS), Biosensors

1. Introduction

Surface Plasmon Resonance (SPR) is a powerful, label-free optical method that relies on the propagation of electromagnetic waves along a thin plasmonic metal surface (usually gold).[1] Aside from gold, other plasmonic materials including silver[2] and aluminum[3] have been utilized for SPR sensing applications, and alternative plasmonic materials are reviewed in depth by Naik et al.[4] These waves or surface plasmon polaritons (SPPs) are extremely sensitive to changes within the metal-dielectric boundary area. Therefore, adsorption of molecules within this boundary layer generates changes in the local refractive index, which alters the resonance condition for the photon-electron coupling. This effect produces a shift in the minimum reflectivity angle or wavelength, allowing for quantification of surface binding and molecular interactions[1] without the need of fluorescent labels (Figure 1, top). SPR is commonly used in pharmaceutical research[5] and has been extensively utilized to characterize protein/antibody affinities.[6-9] The quantitative detection of binding events via SPR is also the basis for various biosensors,[10-20] providing a valuable platform for applications ranging from disease detection[21, 22] to drug development.[23, 24] However, one limitation of the SPR technique is its inability to provide molecular identification information. Therefore, linkage of SPR with other analysis techniques, either as a secondary confirmation approach or through the newly added capability to obtain desired molecular recognition, appears to be an attractive solution. Mass spectrometry (MS) is the golden standard for molecular compound classification[25] and thus has been viewed as the top choice for coupling with SPR analyses. However, due to the nature of SPR measurement that relies on adhesion of analyte to the substrate surface, complimentary compound determination techniques generally require a surface-based ionization approach to provide effective analysis directly on the chip.

A number of platforms have been proposed to couple SPR and MS techniques for a more exhaustive analysis of protein-ligand binding interactions investigated with SPR. For example, analysis of SPR eluants by Liquid Chromatography Mass Spectrometry (LC-MS) was first utilized to identify target analytes without a surface ionization technique to identify target analytes.[26] More recently, a spray tip connected to the outlet of an SPR flow system was used for direct analysis in real time, resolved with the SPR signal.[27] These liquid-based MS techniques rely on sampling of the coupling of SPR liquid samples with MS. A review by Stigter et al. on coupling of SPR with MS techniques has been published with discussion of LC-ESI-MS

for quantification and identification of target ligands.[28] However, Matrix Assisted Laser Desorption Ionization (MALDI)-MS remains as the premier MS technique for joining with SPR due to ease of chip transfer, complimentary surface analysis, and potential for whole protein detection (Figure 1, middle). In recent years, advances have been made in SPR and MALDI-MS instrumentation, providing new avenues for realizing the coupling in different settings and with enhanced capability, which have contributed to acquisition of a greater breadth of information.

Another powerful molecular investigation technique that has been linked to SPR is Raman spectroscopy (Figure 1, bottom). Raman spectroscopy is broadly utilized for material recognition based on unique Raman signatures of the molecular constituents in a sample. The capabilities of Raman have been markedly increased due to recent instrumentation advancements, allowing analysis of biological materials in their native states in solution.[29] An added benefit of SPR-Raman coupling is that the plasmonic materials employed in SPR have been shown to provide a substantial amplification of Raman signal known as surface-enhanced Raman scattering (SERS).[30] The coupling of SERS has been reported for identifying molecular constituents post SPR analysis[31] and SERS detection has been integrated with SPR analysis into a single instrument.[32]

2. Coupling of SPR with MS Techniques of Different Ionization Methods

SPR analysis relies on capture of target analyte through biological interactions, similar to affinity chromatography[33], but it also provides further benefits of binding kinetics analysis and quantitative detection in various modes. When complemented by MS methods, the quantitative analysis is then uplifted with the capability of presenting additional molecular identification information. The combination has been realized on sensor surfaces with MALDI and through sample elution using ESI ionization methods. Though SPR gas sensors have been reported[34],[35, 36], the majority of publications still focus on the solid-liquid interface sensing. Furthermore, there is very little work concerning combined gas-phase SPR and MS instrumentation. Therefore, we will focus primarily on solid-liquid interface SPR coupled with mass spectrometry. Ionization of analyte from this interface is undoubtfully important and variance in ionization modes presents different limitations and sample preparation requirements.

Ionization techniques employed must meet two criteria: compatible with SPR surfaces and capable of effectively ionizing common types of analytes from SPR studies.

2.1. *Matrix Assisted Laser Desorption (MALDI)*

The most common ionization system for combined SPR-MS analysis is MALDI, which can be conducted with the transfer of SPR chips to a MALDI-MS instrument following a single step of matrix application. The ease in coupling of the two surface-based techniques has solidified them as a major methodology yielding complimentary data to support biomolecule detection. One of the limitations of MALDI analysis is in the low mass range, as coelution of matrix peaks below 500 m/z could complicate spectra evaluation.[37] As such, MALDI-MS is commonly utilized for soft ionization of larger molecular weight compounds with whole protein ionization capabilities.[38] As SPR is predominantly utilized to analyze large biomolecule targets the techniques match well and coelution with matrix peaks is generally not a concern. One important aspect in SPR's large biomolecular measurements is the use of small molecules such as thiols to form gold-thiol covalent linkages, allowing the formation of underlying monolayers that serve as the foundation for immobilization of capture molecules or anti-fouling function.[39] Interestingly, some of these small molecule thiols have been shown to eliminate the matrix requirement of MALDI, thus greatly simplifying the SPR-MALDI analysis.[40] Due to these factors, MALDI is an ideal method for ionizing captured macromolecules such as proteins on an SPR sensor chip with the combined benefits of high ionization efficiency and ease of transfer. Now capable of collecting both quantitative and molecular identification information, complementary usage of these two techniques effectively removes many of the limitations of each individual method.

2.2. *Desorption Electrospray Ionization (DESI)*

DESI utilizes electrically charged droplets directed at a solid surface to desorb and ionize molecules. This ionization method is effective for a variety of samples due to its high throughput sampling and operation at atmospheric conditions.[41] These advantages make DESI an attractive ionization method to join with SPR as it provides functions similar to MALDI, with the added potential for matrix-free, ambient ionization of captured molecules. However, protocols for transferring SPR chips to DESI set-ups need to be carefully evaluated. Neielen *et*

al. published the first report demonstrating coupling of the two techniques by utilizing a modified DESI source to detect mycotoxins, which are naturally occurring fungal toxins.[42] Mycotoxin deoxynivalenol, a secondary metabolite of fungi commonly found in various foods and beers, was ionized by positioning the SPR chip in front of the MS inlet, followed by introduction of spray solvent and application of voltage. They were able to observe characteristic m/z peaks for hydrogen, ammonium, sodium, and potassium adducts from antibody captured deoxynivalenol. From their study methanol was found to work as an ideal solvent due to its optimal electrospray performance and ability to disrupt antibody-antigen interactions. This disruption was considered a key component in this work as captured analyte molecules could not be desorbed and ionized without this step. DESI proves to a method of high potential for coupling with SPR from its unique ability of matrix-free, ambient ionization while still maintaining the throughput off MALDI analysis. Though, more research into this analysis combination is needed.

2.3 Liquid Chromatography Electrospray Ionization (LC-ESI)

Electrospray ionization (ESI), another broadly used MS ionization method, has also been linked to SPR[26, 27], primarily through liquid chromatography (LC). A major hurdle for the coupling between surface-based SPR and liquid-based MS, however, is the necessity of an elution step to collect the captured analyte for LC-MS instrumentation.[26] Recent work[43] has shown UPLC-QTOF/MS's feasibility to identify active ingredients from a traditional Chinese medicine known as *Radix Paeoniae Alba*. In this study, two compounds, paeoniflorin and paeonol, were identified as potential active ingredients based on a SPR affinity study with TNF-R1. Given that the combination of SPR with liquid-based MS techniques has been previously reviewed[28], we will focus instead on the progresses of SPR coupling with surface-based MS techniques – primarily MALDI.

3. Optimization of SPR-MALDI towards Protein Analysis

MALDI was the first MS technique coupled with SPR, when it was used for identifying myotoxin *a* captured on an SPR sensor chip, based on characteristic m/z peaks.[44] The affinity-based capture of target analyte within a small area of the SPR chip was found to facilitate MALDI analysis despite the presence of only femtomole quantities of the analyte. A peak at m/z

4824 was identified as singly charged myotoxin *a,* which confirmed its capture in the sensing channel and was further verified by the control channel's lack of relevant peaks. Angiotension II and secretin were also added to separate channels that demonstrated the spatial separation of MALDI analysis between SPR sensing channels. This work presented the orthogonality of the SPR-MALDI-MS method through its ease of coupling, facilitation of highly sensitive analyte detection, and ability to provide both quantitative measurements of binding events with qualitative molecular identification of target molecules. This innovative work of coupling of SPR and MALDI set the stage to further apply MALDI analysis to a range of SPR sensing schemes. SPR-MALDI-MS has since been widely utilized to provide a number of interesting combinations of quantifiable measurements and qualitative results that will be discussed in the following sections.

3.1. *Optimization of SPR-MALDI Measurements*

Nelson and Nedelkov first reported optimization of the SPR-MALDI-MS technique[45], generating a new procedure to limit the sample lost for kinetically fast-off binding targets. They introduced the steps of reduced flow rates, shorter wash steps, carefully chosen matrix compounds, and a matrix aerosolization procedure to mitigate the sample loss issue and presented a more consistent SPR-MALDI-MS analysis. Furthermore, they developed a device to remove the gold capture substrate from the polystyrene support of Biacore sensor chips, alleviating the difficulties of fitting the whole chip into MALDI systems. Further work to optimize SPR-MALDI was done by Koenig and coworkers who developed a custom SPR fluid cell, which incorporated small pins into the side of the SPR flow cell that would not interfere with SPR measurements but could be transferred to MALDI.[46] These capture pins reduced contamination risks in transferring SPR captured biomolecules to MALDI systems as when the separation between the sensing areas is disturbed, cross contamination can occur. It allowed MS measurements and SPR binding kinetic analysis to be conducted without complex elution steps or destruction of the sensor chip. Additionally, it presents the potential for MS analysis of each capture step in a complex sensing process. Identification of bovine serum albumin (BSA) captured by surface immobilized antibodies was confirmed with MALDI-MS analysis, showing effectiveness of the method via specific capture of the protein through interaction with immobilized antibody.

3.2. Addressing Non-Specific Adsorption by SPR-MALDI

Aside from chip transfer and MALDI ionization issues, another limitation of the SPR-MALDI method is the non-specific binding of compounds in complex sample matrices. Biosensing in complex media is an important step for translating biosensors to clinical diagnosis with patients. However, complex media can complicate specific capture and thus compromise the detection of biomolecules with SPR[1], despite the development of various surface passivation schemes[47-51] providing ways to reduce non-specific interactions. This added complexity also makes MALDI analysis less effective as non-specifically captured protein can convolute MALDI spectra and mask target analyte peaks. Work by Aube et al. investigated nonspecific interactions and the resulting MALDI spectra.[52] Using five different pentapeptide self-assembled monolayers (SAMs) of varying polarity, they found significant differences in nonspecific adsorption of cell lysate. Chain length appeared to have minimal effect on nonspecific adsorption while hydrophobic peptides were more effective at preventing surface fouling. They then identified proteins and lipids from human cell lysate that non-specifically adsorbed to these SAMs using MALDI-MS (Figure 2). Here MALDI provided identification of the proteins that non-specifically adsorbed, augmenting SPR analyses that normally cannot determine protein identity. Collagen α-1(XX) chain, mitogen-activated protein (kinase)$_5$ 5, various ribosomal proteins, and 17 different lipids were found adsorbed to the sensor surface. Most of these peptide SAM surfaces yielded a significant reduction in non-specific interactions and this was confirmed by MALDI. Surfaces that did not prove to be antifouling can be further investigated using MALDI-MS to identify the molecular identity of fouling constituents. However, one needs to ensure that passivation schemes do not produce MALDI peaks that overlap with target analytes. One method that has been utilized to achieve this is the elution of bound analytes and subsequent spotting onto a MALDI plate to circumvent this problem.[53] The presence of non-specifically bound compounds in mass spectra was limited through a procedure of cleaning the flow system and targeted elution of the specifically bound molecules. An injection of elution solvent (2% formic acid) sandwiched between air bubbles was utilized to disrupt myoglobin binding and allow for its elution to a pipet that was removed upon observation of the second bubble. Transfer of the elution solution to the MALDI plate allowed detection of myoglobin peaks originally obscured by excess amounts of non-specifically adhering human serum albumin. These works provided the first examples in limiting the transfer of non-

specifically interacting proteins to MALDI-MS analysis. The developments of new capture schemes in SPR biosensing applications and the availability of various on-chip analysis methods have also stimulated advances in approaches capable of analyzing complex systems. For example, MS-MS has been applied to identify proteins with complex peptide fragments from "on-chip" trypsin digestion.[54] This group also reported the use of homemade chips by plasma-sputtering techniques, which reduced the cost of chips that are generally destroyed in MALDI analysis.[55] SPR capture and subsequent chip transfer to MALDI has continued to be the predominant form of the coupled SPR-MALDI technique.[56, 57]

Although tools have been developed to deal with complications of non-specific interactions and related problems of MALDI spectra complexity, whether SPR-MALDI coupling can really help understand the nature of non-specific interactions and their impact on bioanalysis remains to be verified. Breault-Turcot et al. investigated this issue through the adsorption of bovine serum proteins on four different SAMs by utilizing the SPR-MALDI platform[58], and identified BSA as the major non-specific adsorption culprit. Further analysis following a trypsin digestion of the adsorbed proteins confirmed the presence of apolipoprotein A-1, complement C3, SHC-transforming protein 1, and kininogen 2. Interestingly, each surface had distinct fouling proteomes, indicating that some affinity between specific proteins and the SAMs must exist. A digestion method was also used to determine the orientation of protein binding based on fragment ions, giving insight into the available cleavage sites that could be used to reveal the protein regions in contact with the surface. This work is important as it tackled the nature of non-specific binding interactions, which is key to optimizing SPR chips for biosensing and has demonstrated that chip digestion for MALDI analysis is an effective way to probe nonspecific interactions and protein orientation on an SPR surface.

3.3 *SPR-MALDI for Protein Analysis and BioSensing Applications*

MALDI-MS identification in conjunction with SPR has also been utilized to confirm and expand biosensor capabilities. Recently it has been demonstrated to confirm the capture and enrichment of ricin on an SPR sensor (Figure 3).[59] Monoclonal antibodies were oriented on the sensor surface using protein G, thereby enabling analysis of their binding to four different toxins (ricin, RCA120, abrin, and AAG). Using sandwich assays optimized through epitope mapping, these toxins could be reliably and sensitively detected. This optimized system was then

applied to analyze crude castor bean extract followed by MALDI-MS for confirmation of SPR results and identifying the captured proteins in the sandwich assay. The major protein captured from a crude extract of castor beans was found to be ricin, identified by using trypsin digestion of recovered sample from the SPR sensor surface. It is interesting to note that ricin from castor beans grown in different areas displayed different peptide fragment signatures. SPR affinity capture and quantification has also been combined with MALDI-MS to identify epitope regions of various proteins.[60-62] In their studies, SPR sensor chips were employed to determine protein binding constants with antibody or aptamer binding partners, and this was followed by proteolytic digestion and removal of unbound material, leaving epitope regions to be eluted and identified with MALDI-MS. This method was successful for the determination of epitopes of mono and polyclonal antibodies for myoglobin as well as epitopes for an Interleukin-8 antibody. It demonstrated the high capability of combining SPR quantification, K_D determination, and MALDI-MS recognition of point of interest (POI) regions such as epitopes in a protein.

4. Combining SPR Imaging with MALDI-MS Analyses

4.1 SPRi for MALDI

Surface plasmon resonance imaging (SPRi) is an advanced form of SPR that utilizes a CCD sensor to measure the intensity of light reflected from the whole sensor surface. By monitoring the reflection intensity (typically at a fixed angle), shifts in the resonant angle associated with the binding events can be identified and quantified based on the change in intensity values that occur from the reflectivity curve shift. This process allows for a video of the biosensor surface and the binding events occurring on the chip to be recorded that provides the same measurements as the angular scanning mode with added benefits of high throughput, multiplexed analysis through a microarray. Each spot on the microarray can be functionalized individually to allow for different binding interactions to be measured at once. SPRi provides a surface based quantitative analysis so the orthogonality of SPRi-MALDI remains as it does in SPR-MALDI. The first work of SPRi-MALDI was demonstrated by Grasso *et al*.[63] who utilized SPRi to capture and calculate protein surface coverage of anchored matrix metalloproteinases. This was accomplished by an on-spot trypsin digestion and transfer of resulting supernatant onto a MALDI plate for MS spectra acquisition. The results demonstrated

the feasibility of identifying different proteins with MALDI-MS that compliments the multiplex array functionality of SPRi. Different from the solution transfer strategy used in Grasso's work, an "on chip" SPRi-MALDI method was demonstrated by Nedelkov.[64] Antibody arrays were formed on a gold chip, transferred to an SPRi system where proteins were captured and quantified based on light intensity measurements. The chip was then washed, matrix was applied, and it was attached to a MALDI plate for MS analysis. Comparison against non-functionalized portions of the sensing chip was performed to account for possible nonspecific binding. This work further established the capability of coupled SPRi-MALDI method for array format quantification and identification of protein interactions and has since been applied to the analysis of many other biological molecular interactions.[65, 66]

The function of SPRi-MALDI for multi-target analysis has been additionally displayed with the analysis of anti-β-lactoglobulin and anti-ovalbumin on the same chip.[67] The respective antigens could be selectively detected when compared to the control spots that showed no sign of a binding event (Figure 4). The antibody chips were removed from the SPR instrument and applied to an adapted MALDI plate, allowing confirmation to be conducted by MS detection of whole proteins and associated peptide fragments following a trypsin digest. Protein peaks were found exclusively at locations arrayed with their antibody partner. Proteolytic ion peaks were further analyzed with MS/MS to identify obtained fragments as β-lactoglobulin and ovalbumin with mascot scores of 120 and 145, respectively. On-chip proteolysis of SPRi spots for subsequent MALDI analysis was also demonstrated by Ander *et al.* to identify RNA binding proteins.[68] Similar methodologies have been applied by Przybylski *et al*. to probe protein-GAG interactions that enabled the detection and quantification of complexes between heparin/heparin sulfate and cytokines.[69] An approach was established for optimizing on-chip digestion and MALDI peptide mass fingerprinting for confirming SPRi affinity analysis of mitochondrial intron Sc.ai5γ interaction with Mss116, a known cofactor. The authors screened mitochondrial lysate for other RNA interactive proteins and identified 4 distinct proteins aside from Mss116 that bound to the RNA using the SPRi affinity test. These works show the capability of SPRi-MALDI to simultaneously analyze different concentrations and targets within one chip, and affinity analysis of SPRi can be utilized to capture new unknown binding partners that can then be elucidated via MALDI confirmation, especially through the application of MS/MS methods.

4.2 SPRi-MALDI Analysis in Complex Media

As previously mentioned, biosensing applications require the ability to perform detection in the complex media of patient or environmental samples. Therefore, investigations into the feasibility of identifying target biomolecules in these complex samples are important. To optimize the analysis requirements of SPRi-MALDI in complex biological fluids, interesting work has been performed that lead to detection of biomarkers in human saliva[70] and cell lysate.[71, 72] Musso *et al.*'s work to detect biomarkers in saliva[70] showed pure α-amylase could be selectively captured by antibodies immobilized on DCC/NHS activated self-assembled monolayer poly(ethylene oxide) biochips. This was followed by testing of diluted saliva for α-amylase and the capture and classification of another saliva biomarker, lysozyme. The use of SAMs to reduce nonspecific interactions played a key role in enabling detection in this complex sample, showing the coupled technique is effective for selective quantification and mass identification of proteins in a biological fluid.

Anders *et al*. reported a similar complex media analysis of ribosomal protein S6 kinase 2 (RPS6KA2) binding with designed ankyrin repeat proteins (DARPins) in SH-SY5Y cell lysate.[71] An array was utilized to determine kinetic constants for three selected DARPins. Their binding partners were then identified by "on-chip" MALDI of both intact proteins and digested peptides. Similar kinetics parameters and MALDI peaks were obtained for proteins in diluted cell lysate. Further analysis of biomarkers in bacteria lysate has been demonstrated by Lambert.[72] In this study, MALDI-MS analysis of cholera toxin spiked in bacterial lysates of *V. cholerae* and *E. coli* was achieved after an SPR quantification of cholera toxin using a GM1 functionalized interface. The complexity of the bacterial lysate samples led to significant surface fouling, but it did not compromise the SPR quantification through the use of appropriate controls. Furthermore, MALDI-MS demonstrated the ability to unambiguously identify cholera toxin specific peptides in a complex bacterial lysate after an on-chip digestion. A key step in this work is the suppression of non-specific binding and promotion of the signal of target analytes in biological fluids at the SPRi-MALDI interfaces, which contributes to the impressive results.

4.3. Advances in Coupling SPR imaging with MALDI imaging

MALDI imaging mass spectrometry (MALDI IMS) was first introduced in 1997 by Caprioli *et al*.[73] It allows for an ion image to be constructed through the combination of laser shots scanned across a thin slice of tissue sample, in which the laser shots are converted into

pixels that correlate to the intensity of selected peaks. This mapping function offers potential for combination with SPRi as array features align well with both techniques. The concept was first demonstrated for localization of target proteins to areas containing their complimentary antibodies and for revelation of non-specific interactions in other, unfunctionalized areas.[74] The study targeted the LAG3 protein, a marker for human breast cancer and tuberculosis, which was selectively detected in human plasma using SPRi. The capture of LAG3 was confirmed with MALDI-MS/MS, and an image of specific peptides from LAG3 and RSA was obtained to display the localization of LAG3 to the antibodies on the array spots. This example of the coupled SPRi-MALDI IMS technique clearly shows their potentials in complex bioanalysis. In addition to the features of a coupled technique that complement each other, it provides a visual identifier of peptides and their location across the sensing surface that is more attractive than traditional molecular identification.

Further coupling of SPRi with MALDI IMS was recently reported in a paper by Forest *et al*.[75] In this work, protein transfer from a mouse kidney tissue section to a SPRi sensor was accomplished through introduction of a 0.7mm spacer between the fixed tissue section and the SPRi sensing surface. Proteins were then allowed to equilibrate with PBS buffer and the binding over time was monitored via the SPRi reflectivity shift. The SPRi chip was then transferred and visualized with MALDI imaging. The transfer of protein across the spacer to the SPR surface was found to maintain spatial organization and allowed for quantitative measurement of proteins associated with specific locals of the tissue sample, as shown in Figure 5. Through coupling to MALDI imaging, the captured proteins could be identified and correlated with histological regions of the tissue sample. Based on m/z ratios, several proteins were determined to be localized to the cortex, medulla, or pelvic regions. These regions could also be differentiated by SPRi data that demonstrated differences in the amount of adsorbed materials. This work clearly shows the added functionality of the SPRi-MALDI IMS method, which could be a useful platform to quantify and identify proteins with the valuable addition of spatiality. However, the requirement of highly specific functionalization of the surface to the proteins of interest and limited understanding of non-specific binding could limit the applicability of work of this type and will require further study.

5. Coupling of SPR with Raman Spectroscopy

Raman spectroscopy utilizes inelastically scattered light to provide identification of specific functional groups through their unique vibrational, rotational, and other low-frequency modes. Recognition of these functional groups provides valuable fundamental information for determination of molecular structure as well as confirmation of the presence of target molecules.[76] Therefore, Raman spectroscopy has clear potential to be employed in conjunction with SPR to identify molecular constituents, especially when considering that surface enhanced Raman scattering (SERS) observed for molecules in close contact with plasmonic materials can enormously enhance weak Raman signals. As SPR analyses rely entirely on plasmonic surfaces, the added potential for significant Raman signal enhancement has motivated study into the coupling of SPR and SERS. Several authors have reported the use of surface plasmon polaritons in the Kretschmann configuration to improve the sensitivity of a variety of Raman approaches.[77, 78] One noted issue however, is that for optimal SERS analysis, rough surfaces are ideal[79], while as for SPR biosensing, smooth metal surfaces are preferred.[80] These conflicting requirements have prompted a broad investigation into using nanowire/nanorod hybrid structures.[81] Formation of a thiol monolayer on the nanostructure surfaces was examined with SPR-SERS, and by optimizing the nanostructure coverage and thickness, a maximized combination of SPR and SERS signal could be obtained. This analysis provided insight into the contradictory requirements of SPR and SERS sensing but demonstrated SERS' ability to identify molecules bound to an SPR sensing surface.

A recent example of hybrid structures for SPR/SERS measurement comes from Song *et al*.[82], who developed a dual-mode SPR/SERS nucleic acid assay utilizing a silver nanohole array covered with nanorods. Subwavelength nanohole arrays have been known for their extraordinary optical transmission (EOT) properties resulting from unique scattering orders caused by surface plasmons.[83] Local hot spots are generated across these surfaces, making them suitable for both LSPR and SERS measurements. However, upon further testing there was less Raman signal enhancement than expected.[84] In addition, LSPR lacks in both sensitivity and consistency when compared to a conventional propagating SPR assay. Song *et al*. resolved this limitation by using tilted nanorod deposition onto the nanoarray surface. High specificity for target nucleic acids, along with some degree of SPR signal enhancement, was achieved by immobilization of tetrahedral DNA probes to the nanorods. A 10-fold SPR signal enhancement and four times SERS enhancement was demonstrated. With a LOD of 0.51pM for SPR and

0.77fM for SERS, this work presents the feasibility of achieving high sensitivity for complementary SPR/SERS detection on the same surface. However, concerns over complex fabrication requirements, extensive preparation, and silver oxidation, leave the robustness, (and thus the appeal) of the system in question. Additionally, the procedure of running SPR and SERS measurements separately requires the substrate to be transferred between instruments, potentially further affecting the reliability of the system.

5.1 Simultaneous SPR and Raman Analysis

When considering the coupling of SPR with other methods, chip transfer for subsequent analysis is of paramount importance as there might be sample loss and contamination during the process, risk of which exponentially increases with the transfer time. As a result, automation or instrument design for combination is the center of research aimed at clinical applications. Consequently, the consolidation of SPR-SERS into a singular instrument has been accomplished with minimal modification of existing systems (Figure 6)[32]. Using a Kretschman configuration, SPR shifts and SERS intensity could be observed with a single laser on a typical flat gold SPR chip. As previously discussed, a smooth surface is not necessarily ideal for SERS; however, this group managed to demonstrate a uniform and predictable Raman signal while also maintaining the sensitivity of SPR measurements by employing a Nile blue monolayer on the substrate surface. Further research into the simultaneous acquisition of SPR and SERS signals has been conducted by Kim *et al*.[31] They demonstrated the capability of SPR-SERS to differentiate between specific and nonspecific binding of streptavidin-functionalized nanoparticles (Figure 7). They formulated biotinylated Au-films and introduced streptavidin functionalized nanoparticles to monitor binding interactions over time using SPR and SERS. SPR binding shifts could be correlated with SERS spectra specific to streptavidin. SERS spectra could be further utilized to differentiate biotin bound and nonspecifically adsorbed streptavidin based on unique Raman signatures as demonstrated by Wang and Schultz.[85] These analyses clearly present the capacity of SPR-SERS coupling for molecular recognition and tracking of binding interactions. However, there are a few potential labeling issues that can arise from these simultaneous analyses. As tag molecules are commonly utilized in Raman applications[86], they may have the unintended effect of erasing the label-free benefits of the SPR sensors. As such, it will be important to properly formulate the combined sensors to avoid tag molecules or to

introduce the tags only in secondary amplifications steps, where labeling does not impact the interaction of interest, as has been done for SPR with plasmonic nanoparticles.[87, 88]

Development of new, stable, and easily fabricated substrates that fulfill the often-competing requirements for SPR measurement and Raman signal enhancement is a crucial step for dual-mode sensing to be practically and widely applied. Recently a Kretschmann-style sensing system for simultaneous SPR/SERS sensing was demonstrated using a nanoporous gold film (NPG).[89] Upon exposure to light NPGs showed both propagating SPR and LSPR effects, making them ideal candidates for dual-mode SPR/SERS systems.[90] While this phenomenon has been known for some time, traditional NPGs were prepared by chemical dealloying, which are ill-suited for optimization of key SPR and SERS properties such as film thickness and pore size. Hence, Chen *et al*.[89] developed a new method in which film thickness could be controlled during Ag-Au alloy sputtering and pore size reduced through low temperature dealloying. They demonstrated an optimized film prepared by depositing a 3nm chromium adhesion layer followed by a 10nm gold layer. Next, a 50nm layer of Ag-Au alloy was sputtered onto the film and dealloying was carried out with a HNO_3 solution for 24 hours at -18^{o}C, resulting in a relatively smooth (due to small pore size) gold surface appropriate for both SPR and SERS. Interestingly, Raman enhancement was observed using near-IR excitation of 785nm where LSPR effects should be muted, meaning that the same SPPs are likely responsible for both SPR and Raman enhancement. With the long-term stability and an NP-free detection scheme, this NPG film represents a significant leap in the practicality of dual-mode SPR/SERS in research and clinical applications.

5.2 Alternative SPR/SERS Platforms

Simultaneous SPR/SERS sensing is not limited to the Kretschmann configuration, and researchers are beginning to branch out to other configurations that may be more compatible. One example is the recent integration of dual-mode SPR/SERS in a fiber-optic based platform (Figure 8)[91]. Fiber optic integration as the coupling element for SPR has grown in popularity due to the benefits in weight, fabrication, sensitivity, response time, and remote sensing potential.[92] This work developed an SPR/SERS sensing platform with a Au/Ag bimetallic structure sandwiching a semiconductive MoS_2 layer. The SPR signal is detected through the fiber optic while the Raman signal is generated through direct exposure of the surface to a 532nm

laser (Figure 8). The result is a highly sensitive platform that allows for simultaneous SPR and SERS detection. In particular, the SPR sensitivity was high with a remarkable improvement over many previously developed fiber-optic sensors. SERS performance, however, was less impressive with a LOD for R6G in the nM range. SERS showed a linear correlation from 0.625%-5% when testing glucose solutions, but surface saturation negatively affected the results at higher concentrations.

A particularly unique new platform was reported by Song *et al*., in which they developed a dual-mode SPR/SERS method utilizing a catalytic hairpin assembly-induced gold nanoparticle network.[93] Signal is generated only upon the formation of a network of two differently functionalized AuNPs (probe 1 and probe 2) on ITO in the presence of miRNA-652 (Figure 9). Following a method previously reported by Li et al.[94, 95], dark-field microscope images were acquired and the SPR signal was extracted from the integral optical density (IOD) of the dark-field microscopic results. However, lack of uniformity in the distribution of plasmonic hotspots in AuNP networks resulted in significant SERS signal inconsistency. To account for this, the authors employed a ratiometric sensing scheme between 6-carboxyl-X-rhodamine (ROX) and an internal standard of 4-mercaptobenzoic acid (4-MBA). The result of this work is a highly sensitive (LOD of 42.5fM for SPR and 2.91fM for SERS) and orthogonal method for detection of nucleic acids.

6. Other SPR-Coupled Techniques

MALDI-MS and SERS represent the mainstream approaches in SPR-coupled chemical identification, but there are certainly other options that have been explored. One recent example is the amplification of specific nucleic acids and their detection in real-time by using both SPR and fluorescence spectroscopy.[96] Amplification was carried out using loop-mediated isothermal amplification (LAMP) in a cell wedged between an SPR module and a fluorescence detection device (Figure 10). As the LAMP process proceeds, the refractive index of the solution decreases, leading to a negative SPR response correlating to the amount of the target sample. This was performed simultaneously with fluorescence spectroscopy, a well-established method of detection widely used in quantitative or real-time PCR and LAMP. As demonstrated with other SPR-coupled methods, utilizing simultaneous detection provides a more robust system that

provides a much-improved understanding of the reaction dynamics and fewer chances for errors that may cause misdiagnosis.

Whereas SPR coupled techniques covered to this point have been utilized for detection of some chosen analyte and/or non-specifically adsorbed molecules, it should be mentioned that there are other coupled strategies that aim at chemical characterization of the sensing surface and their functionalization. Integration of surface characterization methods into SPR platforms offers the potential for rapid assurance of surface quality, thereby improving the robustness of the system and reproducibility of results. The most common of these methods is x-ray photoelectron spectroscopy (XPS), which provides information on chemical composition, relative abundances, and chemical states of surfaces.[97] Some examples of XPS integration with SPR substrates include quantification of carbohydrates and oligo(ethylene glycol) in glycoarray SAMs[98], functional group analysis of chitosan-graphene oxide films[99], and confirmation of S-Hg bond formation on a WS_2 film used for Hg^{2+} sensing.[100]

Conclusion and Future Outlook

SPR analyses can provide valuable information about molecular recognition, binding and dissociation kinetics, and function as a label-free quantitative detection scheme for biomarkers. However, SPR is limited by the inability to identify the molecular structure of bound analytes, and this has inspired a range of research efforts utilizing a coupling strategy to enable molecular identification on top of quantitative analysis. Presently, MS has proved to be an ideal technique to couple with SPR measurements. In particular, MALDI-MS has become the dominant ionization scheme for the coupling, due to its surface-based operation and compatibility with most SPR-appropriate analytes. SPR-MALDI coupling has been further expanded to include the imaging function and capability (i.e., SPRi-MALDI IMS), presenting a unique advantage for multiplex studies of protein interactions with high throughput capabilities. Given the high potential in biomedical research, we anticipate many other forms of couplings between SPR and MS will emerge with the enhanced performance needed to interrogate increasingly complex systems.

Raman spectroscopy has been used for coupling with SPR, and the integration of the two techniques into a single instrument indicates the desire and availability of compact devices for field work with greater efficiency. The imaging capability of SPRi will likely further increase the applications with benefits from Raman's surface mapping capacity as has been seen with MALDI techniques. The technical barrier to practically combining the two imaging techniques has been the acquisition time of traditional Raman imaging, which can take hours for a single image. The recent demonstration of ultrafast Raman imaging [101] could eliminate the blockade and enable SPR and Raman imaging to be achieved in tandem. Another issue is that the imaging area can differ significantly between SPR and Raman, which makes direct comparison difficult, though adopting microscopic optical settings in both methods could partially alleviate the problem. As technological advancements improve the speed and resolution compatibility of SPR and Raman imaging the potential for coupling will become an inevitability.

The couplings of SPR with recognition analyses discussed in this work represent significant advances in analytical technology and have generated new tools that promote our ability to detect, analyze, quantify, and identify molecular targets on an SPR chip. Altogether, these advancements represent potentially powerful improvements to our ability to analyze more complex systems and provide reliable clinical diagnoses.

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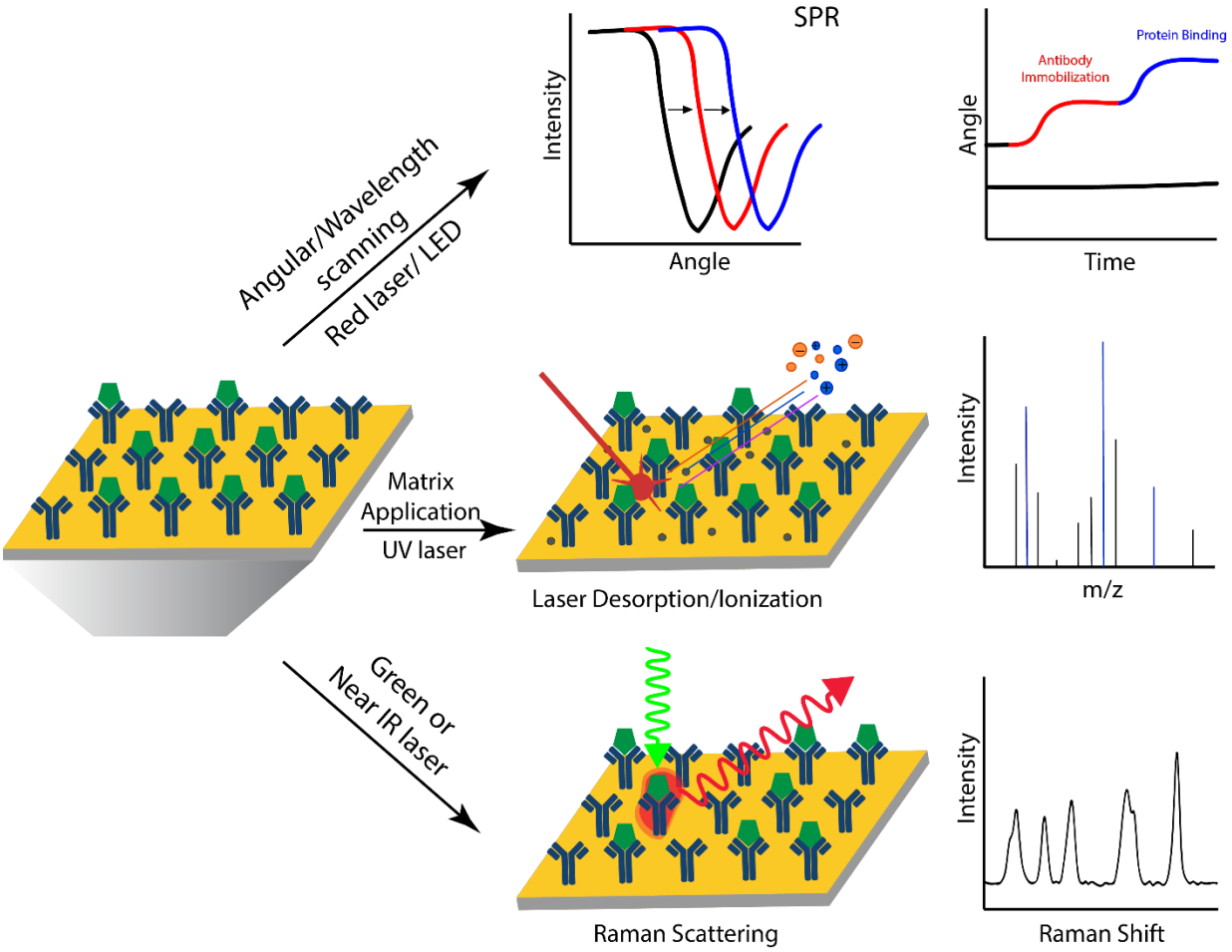

Figure 1. A schematic of the coupled SPR analyses. (Top) SPR sensor chips utilized to track resonance shifts through angular or wavelength scanning. (Middle) the same SPR chips transferred to the MALDI-MS platform for ionization with a UV laser. (Bottom) SPR chips subjected to inelastic scattering of green or near-IR light for Raman spectroscopic analysis.

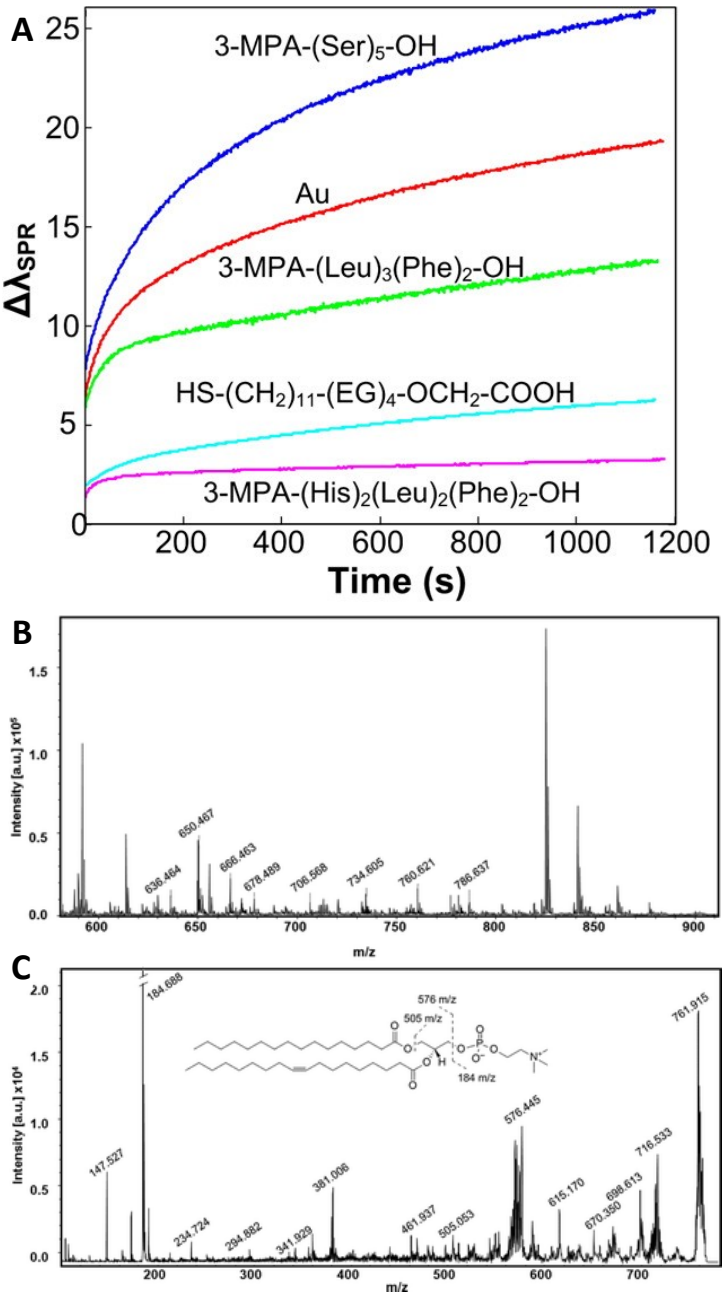

Figure 2. Resulting SPR sensorgrams and MALDI-MS spectra of nonspecific adsorption from whole cell lysate on SAMs of varying peptide polarity. These results show reduction in nonspecific adsorption for three peptide monolayers (pink, blue, and green) compared to the gold substrate. While one peptide surface, 3-MPA-(ser)5-OH, actually increased nonspecific adsorption. (a) Non-specific adsorption from cell lysate onto peptide SAMs. (b) MALDI-MS spectra of lipids adsorbed onto 3-MPA-(ser)5-OH SAM (blue in a). (c) MS/MS spectra obtained from the lipid peak at m/z 761.9. Adapted from [52].

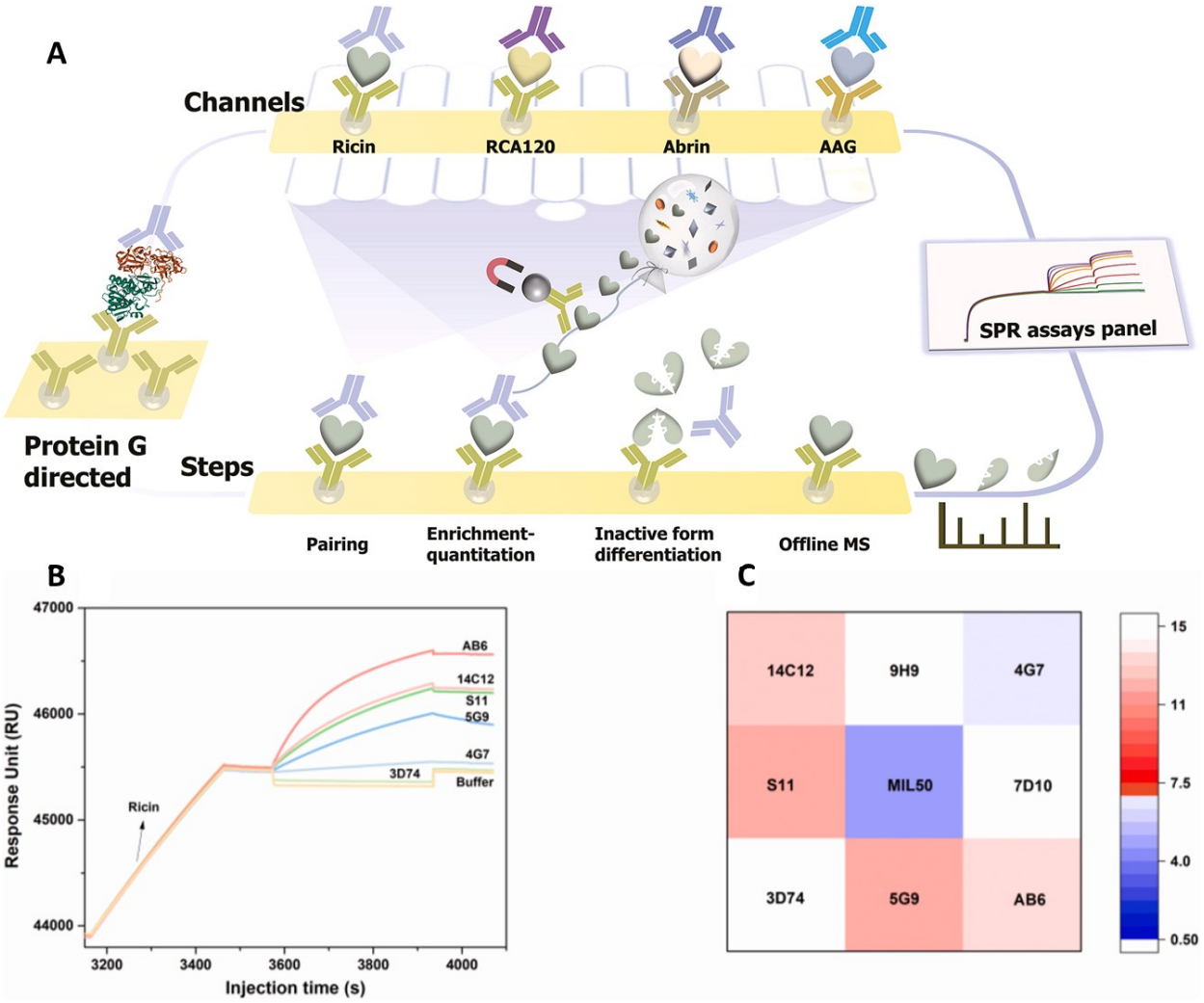

Figure 3. Demonstration of antibody-toxin binding methodologies, resulting SPR responses and epitope maps obtained from MALDI-MS analysis are shown in this figure. (a) Scheme demonstrating the combination of protein G SPR assays and MALDI-MS peptide identification. (b) Binding curves and (c) epitope mapping of mAbs to ricin on a protein G sensor chip. Adapted from [59].

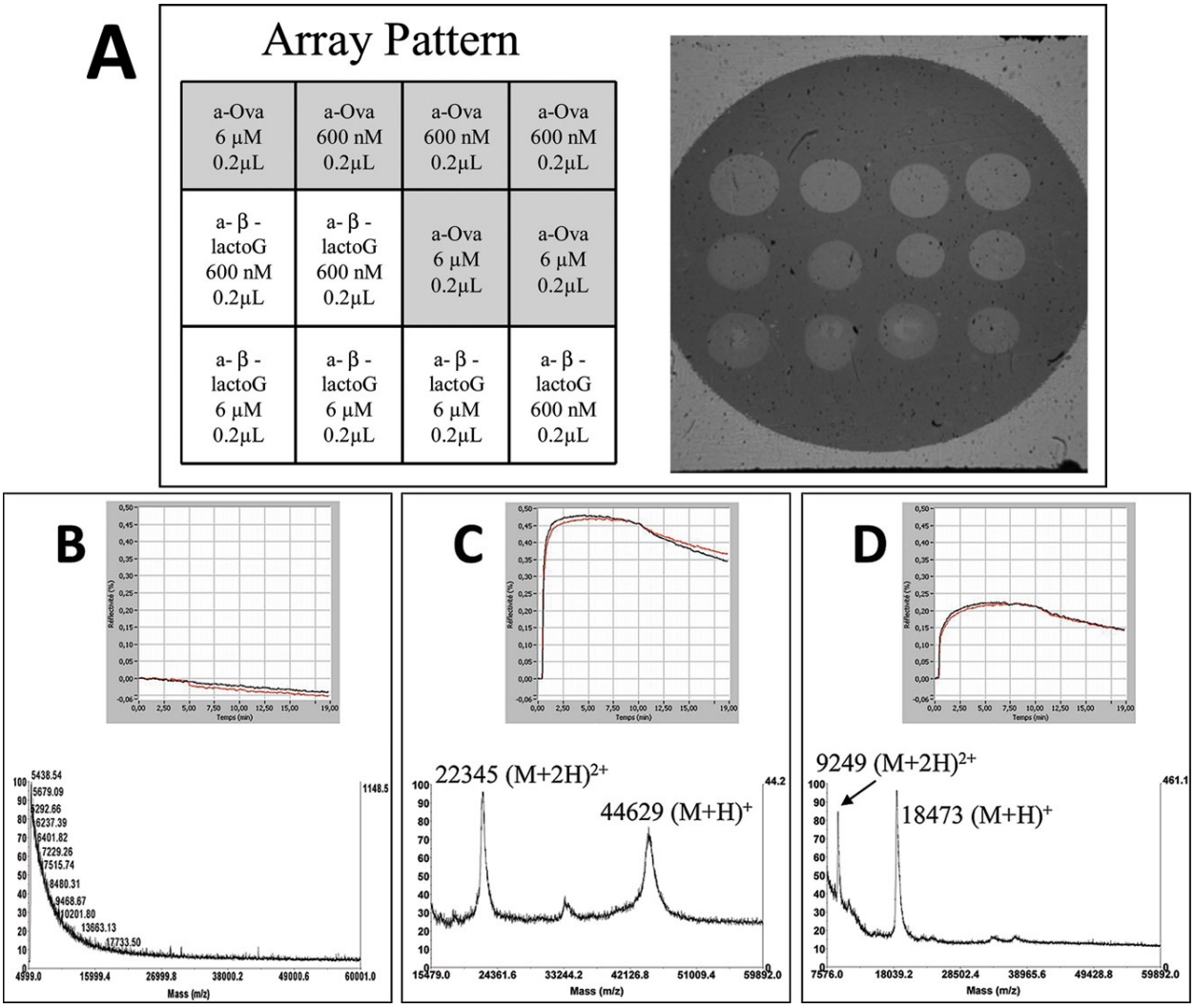

Figure 4. SPRi and MALDI-MS analysis of sensor arrays for sensitive detection of proteins ovalbumin and lactoglobulin and molecular confirmation of its antibody-based capture. (a) SPRi array pattern concentrations and array image. (b-d) SPRi sensorgrams and MALDI-MS spectra of an unarrayed section of the chip (b), anti-ovalbumin-arrayed spot (c), and anti-β-lactoglobulin-arrayed spot (d), respectively. Adapted from [67].

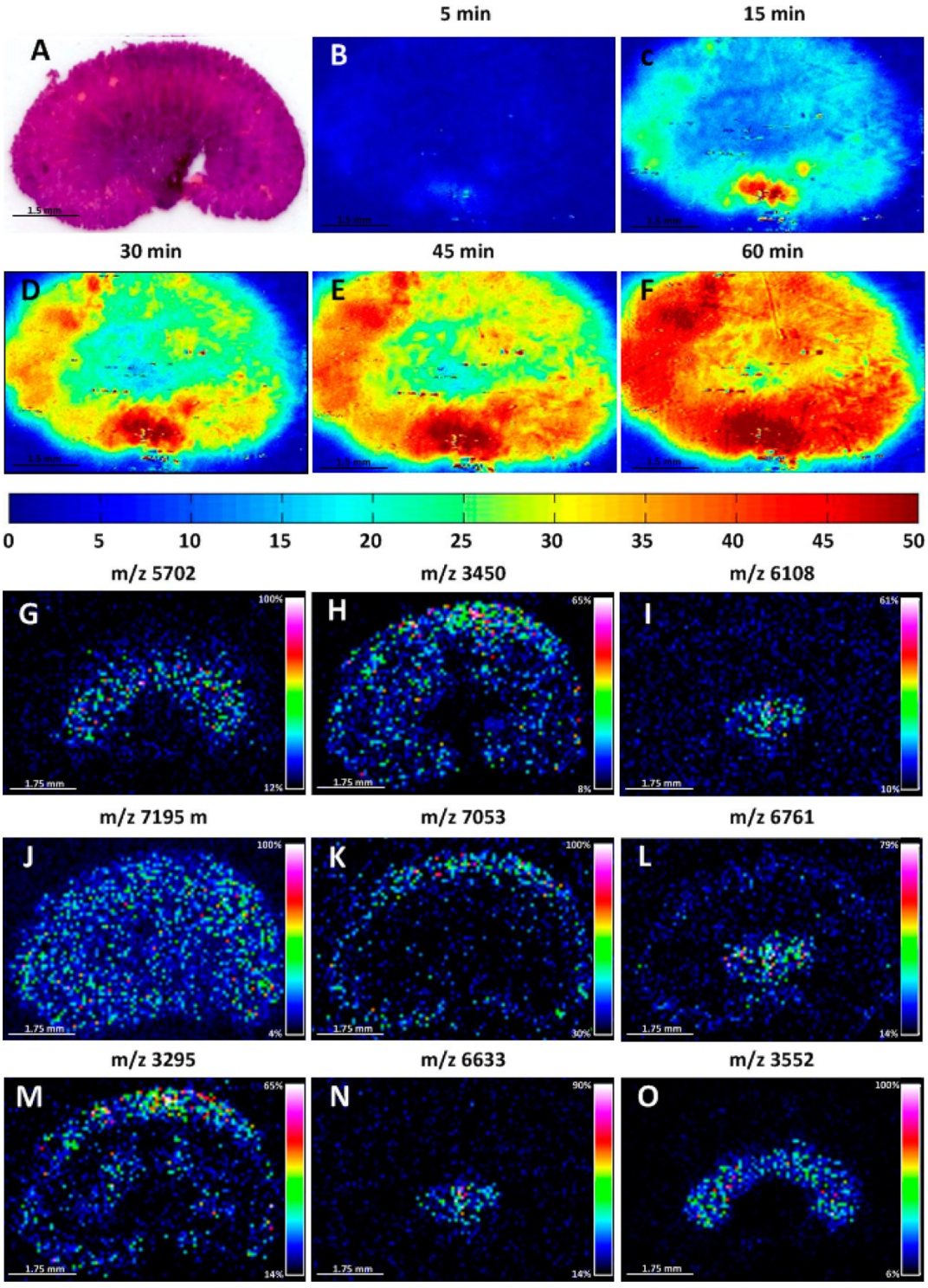

Figure 5. Images of a 20 µm thick rat kidney slice obtained via optical, SPR, and MALDI imaging methodologies. (A) H&E stained kidney slice after 60 min of protein transfer. (B-F) SPRi images of adsorbed proteins over a 60 min time interval. (G-O) MALDI IMS images of selected proteins after 60 minutes of protein transfer to the SPRi analysis chip. Adapted from [75].

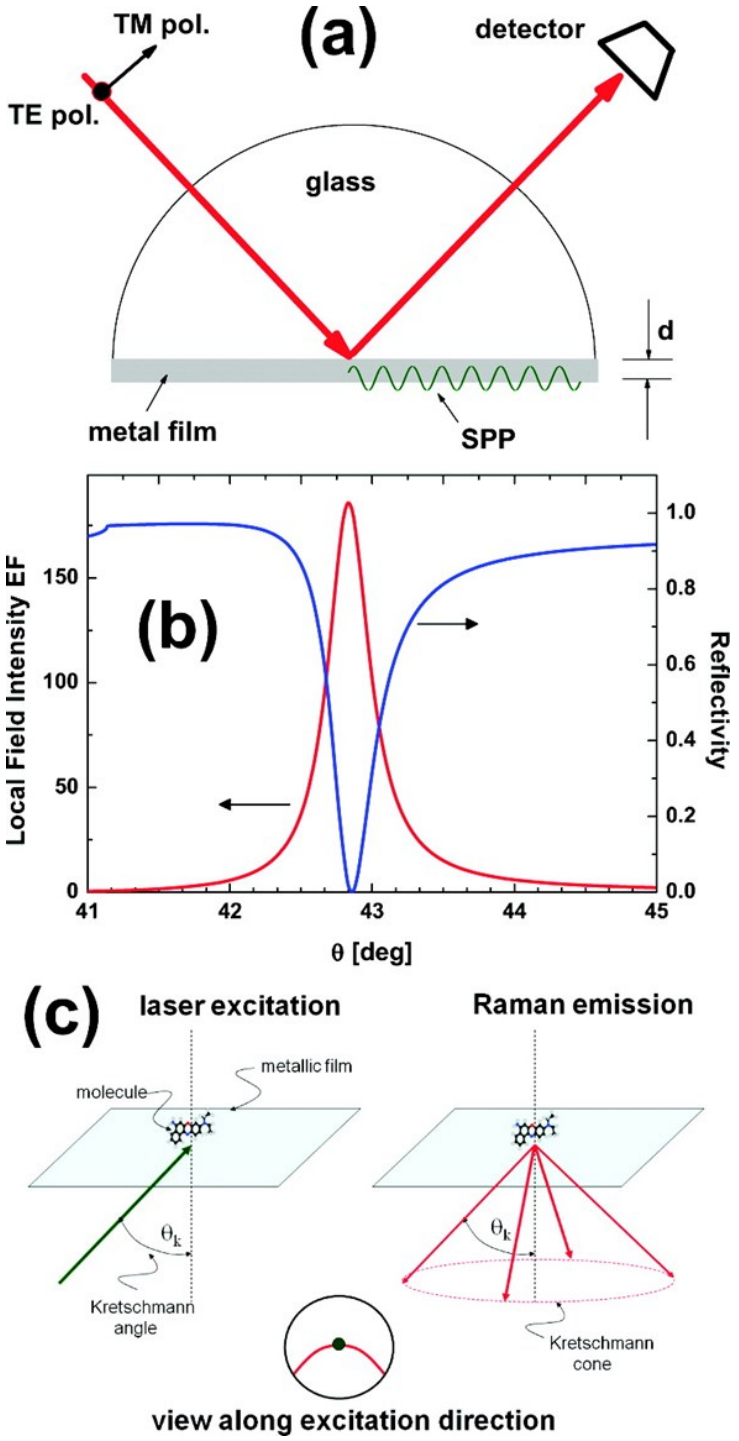

Figure 6. (a) Kretschmann configuration schematic of surface plasmon polariton excitation. (b) SPR reflectivity (blue) and associated local field intensity enhancement factor (red). (c) Indication of SPP excitation and Raman emission in a Kretschmann cone where backscattered light was collected to obtain both Raman and SPR signals from a single laser source. Adapted from [32].

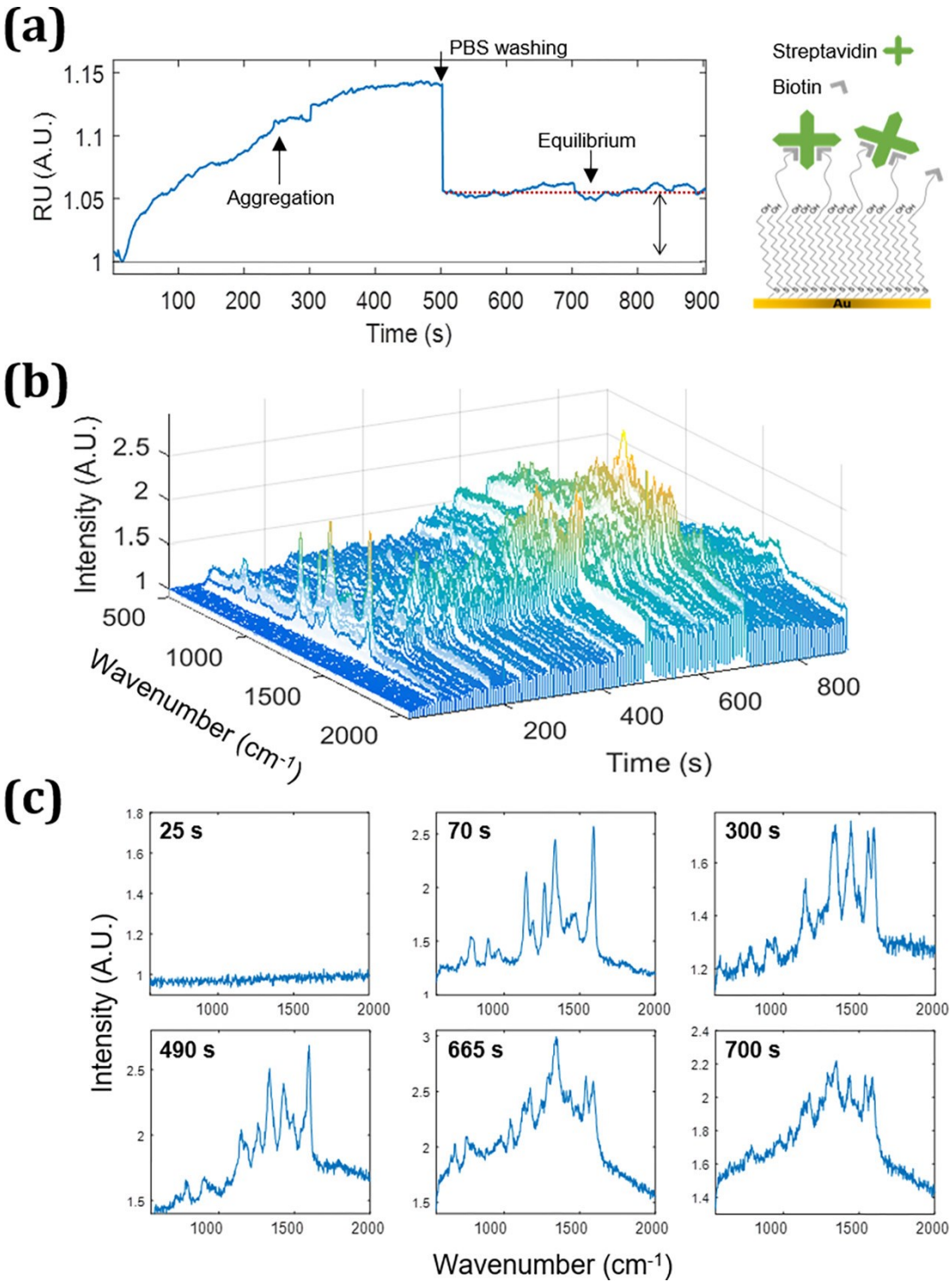

Figure 7. Simultaneous detection of SPR and SERS from streptavidin functionalized-AuNPs on a biotin/MUOH monolayer. The combination of which can be used to track aggregation and differentiate between biotin bound or nonspecifically adsorbed streptavidin. (a) SPR sensorgram; (b) Combination of SERS spectra obtained over the SPR analysis. (c) Individual SERS spectra at time points (25, 70, 300, 490, 665, 700s). Adapted from [31].

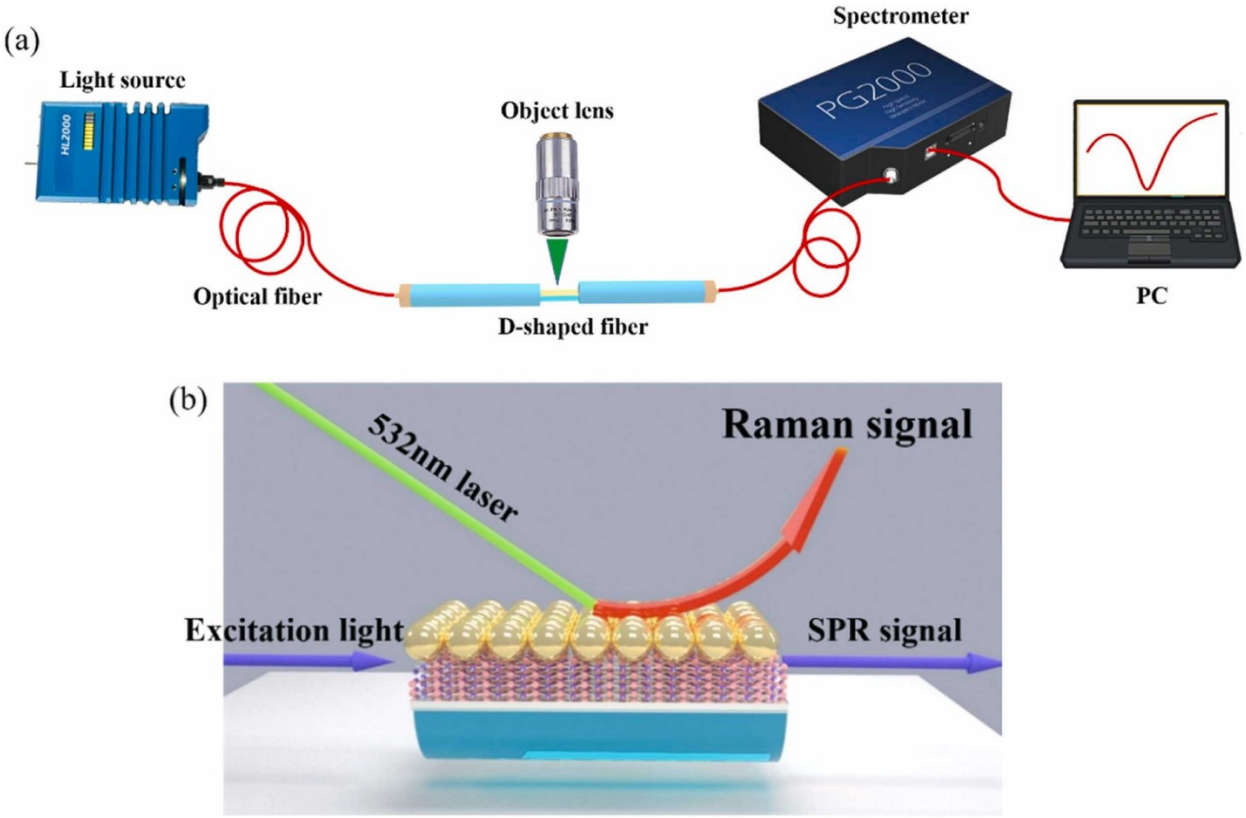

Figure 8. Diagram of fiber optic integrated SPR/SERS sensing platform. SPR excitation laser and generated SPR signal travel through the optical fiber and information is extracted by resonant wavelength change. Raman signal is generated through direct exposure of immobilized AuNPs on sensing surface. Adapted from [91].

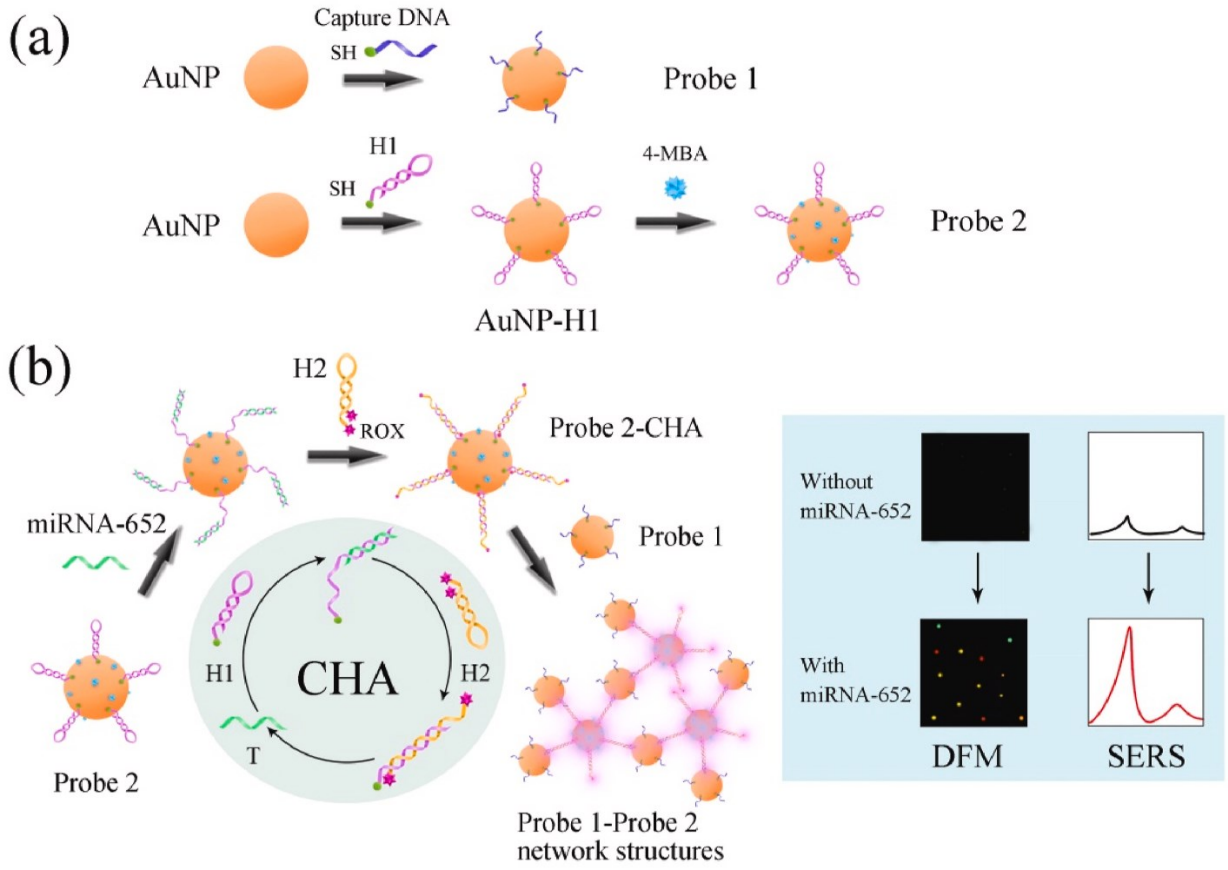

Figure 9. Schematic diagram of catalytic hairpin assembly-induced AuNP network biosensor for miRNA-652. (a) Method for the functionalization of probe 1 and probe 2. (b) Process/strategy for AuNP network formation and subsequent miRNA-652 detection. Adapted from [93].

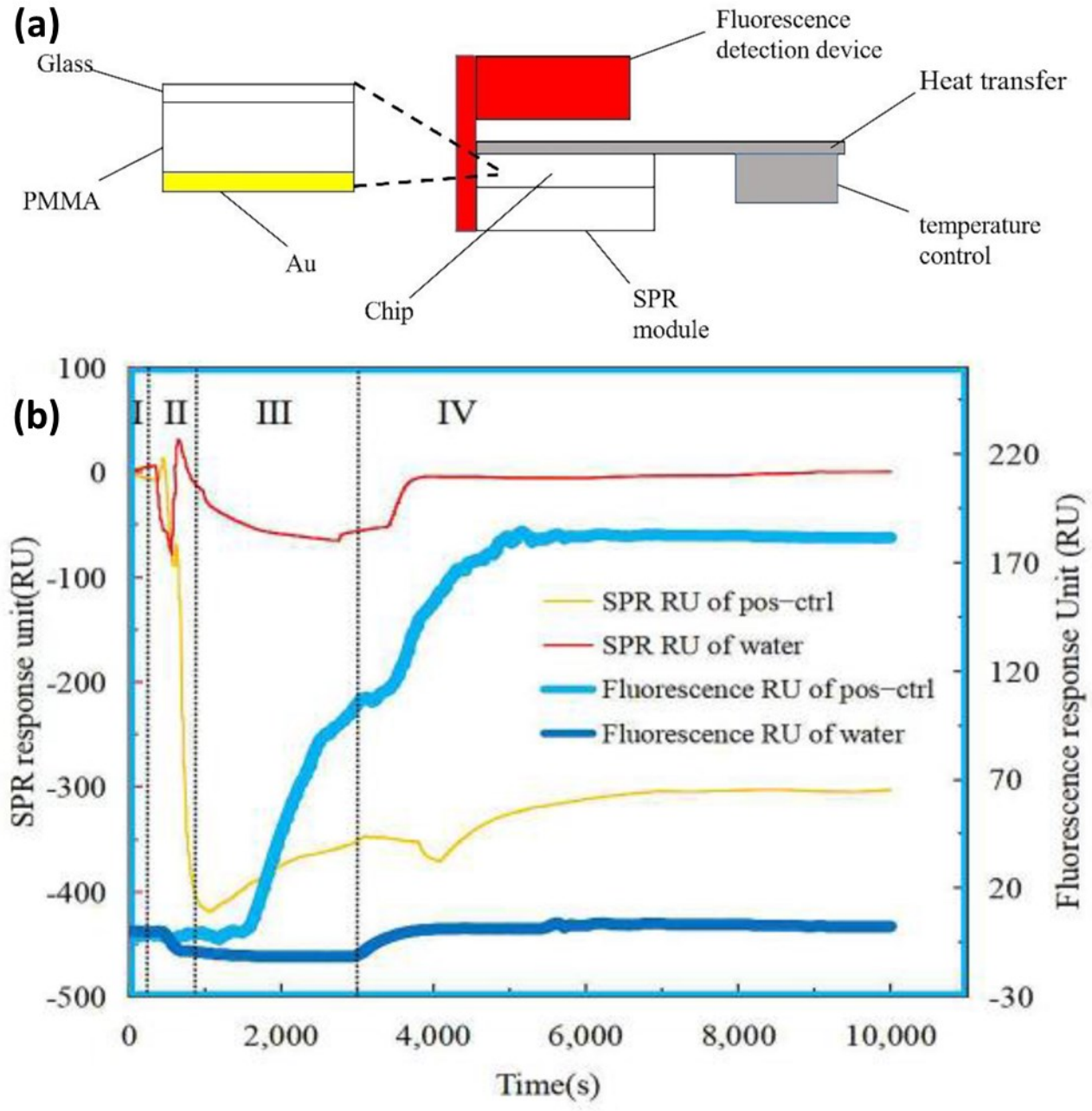

Figure 10. (a) Schematic of integrated SPR, fluorescence, and temperature control instrument for detection of LAMP products. (b) Results of combined SPR and fluorescence measurements with response for water and positive control. With the left axis indicating SPR response and the right axis displaying fluorescence response. Adapted from [96].

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