Frequency Shift Surface-enhanced Raman Spectroscopy Sensing: An Ultra-Sensitive Multiplex Assay for Biomarkers in Human Health

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Abstract

The sensitive and selective detection of biomarkers for human health remains one of the grand challenges of the analytical sciences. Compared to established methods (colorimetric, (chemi)-luminescent), Surface-Enhanced Raman Spectroscopy (SERS) is an emerging alternative with enormous potential for ultra-sensitive biological detection. Indeed even attomolar (10^{-18} M) detection limits are possible for SERS due to orders-of-magnitude boosting of Raman signals at the surface of metallic nanostructures by surface plasmons. However challenges remain for SERS assays of large biomolecules, as the largest enhancements require the biomarker to enter a "hot spot" nanogap in between metal nanostructures. The frequency shift *SERS* method has gained popularity in recent years as an alternative assay that overcomes this drawback. It measures frequency shifts in intense SERS peaks of a Raman reporter during binding events on biomolecules (protein coupling, DNA hybridization etc.) driven by mechanical transduction, charge transfer, or local electric field effects. As such, it retains the excellent multiplexing capability of SERS, with multiple analytes being identifiable by a spectral fingerprint in a single read-out. Meanwhile, like refractive index surface plasmon resonance methods, SERS measures a shift of an intense signal rather than resolving a peak above noise, easing spectroscopic resolution requirements. SERS frequency shift assays have proved particularly suitable for sensing large, highly-charged biomolecules that alter hydrogen bonding networks upon specific binding. Herein we discuss the frequency shift SERS method and promising applications in (multiplex) biomarker sensing, as well as extensions to ion and gas sensing, and much more.

Keywords: frequency shift sensing, surface plasmons, bioassay, surface-enhanced Raman scattering (SERS), biomarker detection, early diagnosis, multiplex assay

Assays for biomarkers that are characteristic indicators of disease, including

proteins, peptides, (micro)RNA, DNA, and small molecules, remain the focus of intense research and potential commercialization in the biomedical field. [1-3] Fast and accurate detection of (multiple) biomarkers is imperative for early diagnosis and prognosis, thus greatly improving patient outcomes. For example, early detection of lung cancer can improve prognoses with the 5-year survival rate of patients reaching 60-80%.[4] Just one example of the power of sensing multiple biomarker types, reported by Xu et al., showed that detecting the methylation level of specific sites on circulating tumor DNA (ctDNA) led to a >50% reduction in the rate of missed diagnoses of hepatocellular carcinoma (HCC) compared to the conventional method of assaying α-Fetoprotein (AFP) alone.[5] Commercialization accompanies advances in human health. For example a test kit developed by Hotgen Biotech company for early diagnosis of liver cancer has been approved by CFDA (China Food and Drug Administration) that is capable of high-throughput detection of three different biomarkers for liver cancer, greatly improving prognoses and the 5-year survival rate.[6]

Focusing on protein assays as a canonical example, conventional detection methods include various types of enzyme-linked immunosorbent assay (ELISA), colorimetric, chemiluminescent, Northern analysis, electrochemical, and fluorescence methods, and others.[7-9] Many of these are well-established clinically (for example chemiluminescent immunoassay (CLIA) for α-fetoprotein that is relatively abundant in human serum) and are currently utilized in the evaluation of COVID-19 serological assays.[10] However these methods have shortcomings including low sensitivity, high cost, and poor reproducibility, particularly when multiplex sensing of proteins present at low concentrations in serum is required.[11-15] Proteomics and metabonomics are emergent technologies for protein detection, however these technologies are rather complicated, time-consuming, and require sophisticated instrumentation and dedicated operaters.[16-18] New methods for sensing protein biomarkers with rapid, specific, sensitive, and especially multiplex features, is thus highly advantageous and commercially valuable.

Immunoassays based on surface-enhanced Raman spectroscopy (SERS) have the potential to overcome some of the drawbacks of conventional methods mentioned

above.[19, 20] Firstly, multiplex operation is facile due to the high resolution of Raman compared to UV/Vis absorption or luminescence spectra (thus multiple analytes can be identified via their spectral fingerprint in a single read-out). Secondly, the exceptionally high sensitivity of SERS that results from the amplification of Raman signals by surface plasmons can meet the clinical requirements for direct trace detection of biomarkers. Surface plasmons are generated by the strong coupling of light and collective electronic motions in metals, and can lead to sub-diffraction limit confinement of optical fields at metal/dielectric interfaces.[21] The SERS effect arises from the surface plasmon-induced amplification of both the excitation and Raman scattered optical fields for a molecule adsorbed on the surface of metallic (usually gold or silver) nanostructures. [22] This double effect means that SERS enhancement goes as the 4th power of the local optical field (E^4), and the total enhancement can reach 10^{11} compared with the ordinary (non-enhanced) Raman signals (NRS). This brings SERS to the limit of ultimate sensitivity, where single molecules can be detected directly, and where only fluorescence techniques can offer comparable sensitivity.[23-26]

Such enormous enhancements are only achievable however using 'sandwich' configurations, in which the molecule of interest can be inserted in a nanogap between two metallic nanoparticles, and where there is constructive interference of the surface plasmons on each nanostructure, known as the 'gap mode'. It is this gap mode that creates the 'hotspot' for SERS and methods for SERS biomolecule detection therefore often seek such sandwich motifs. This can be a particular challenge however when a large biomolecule of interest must be sandwiched between nanoparticles.[27]

If the biomolecule of interest does not itself have a large Raman scattering cross-section, indirect detection can be achieved by labeling with a Raman reporter molecule (a molecule with large Raman cross-section). A metal nanoparticle/analyte biomolecule/Raman reporter/metal nanoparticle conjugate is a common target for achieving sensitive protein detection in this way[28-31] as indicated in Figure 1a. However the complexity of the sandwich modality makes reproducibility an issue and usually involves long reaction times, leading to nonspecific adsorption on the Raman

reporter and decreasing sensitivity and specificity.

In 2012, Olivo and coworkers proposed an alternative SERS-based immunoassay which monitored frequency shifts in a Raman reporter's vibrational modes in response to the binding of a protein of interest, and associated modification of the environment of the Raman reporter.[32] 4-aminothiophenol (4-ATP) was employed as the Raman reporter as it could be bound to a SERS-active metal nanoparticle film via the thiol group, and could also be bound to an antigen via its amine. Thus bound, the 4-ATP Raman spectrum was found to be perturbed in quantitative correlation with the binding of an influenza-H1 (H1) protein to the antigen. Compared with the traditional SERS sandwich assays, Olivo's *SERS frequency shift* method required only one immunoreaction step, greatly decreasing the influence of nonspecific protein adsorption. Most importantly however, since the SERS spectrum central to the assay is that of a small-molecule Raman reporter, the more difficult task of inserting a large biomolecule in a hotspot between two nanoparticles was avoided. This type of sensing modality is shown schematically in Figure 1b.

Olivo attributed the frequency shift of the 4-ATP Raman bands to mechanical deformation upon binding of the protein. Since then, several other mechanisms for inducing SERS frequency shifts have been explored, including modified chemical and dielectric environments of the Raman reporter during binding events. In this way SERS frequency shift assays have proved particularly applicable to the sensing of large and highly-charged biomarkers, however they have also been developed for a wider range of targets, including small molecules, ions, and gases. Another attractive feature of the SERS frequency shift modality for ultra-sensitive detection is that a small shift in an intense signal must be measured, rather than the more difficult spectroscopic task of resolving a small peak out of a noisy background. In this way, a SERS frequency shift assay combines the multiplexing advantage of a typical SERS assay, with the ease of measurement of a refractive index shift assay (so-called 'SPR' method).

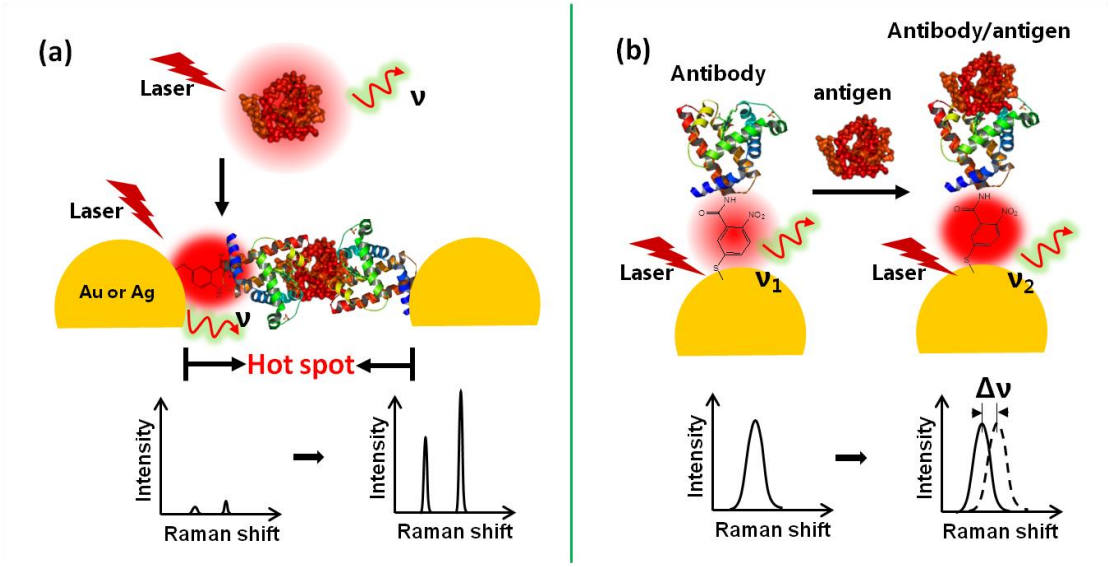

Figure **1** Schematic of SERS sensing modalities (a) sandwich, in which the biomolecule must be included in the 'hot-spot' of optical field localization between two nanoparticles (SERS from the biomolecule or a reporter is detected), and intensity of the SERS signal correlates with concentration; and (b) frequency shift sensing, in which the binding of a biomolecule to a Raman reporter molecule influences the mechanical, chemical, or local dielectric environment of the reporter, causing shifts in its intense Raman bands that correlate with biomolecule concentration.

We note here that monitoring frequency shifts in Raman spectra has been of great utility for decades. In the early 1970s for example, Raman frequency shift was used to investigate the effect of static uniaxial stress on silicon,[33] and to detect atmospheric pollutants (e.g. oil smoke and automobile exhaust gas).[34] Frequency shift is widely used for the measurement of stress in (111) silicon, for example to investigate residual stress in porous silicon films due to lattice mismatch between the film and substrates .[35,36]

In this Minireview however, we focus specifically on the use of SERS frequency shift assays for biomolecule and small molecule assaying, first discussing the various mechanisms that induce frequency shifts, and secondly discussing assay fabrication and the incorporation of metallic nanostructures. Thirdly we highlight the advantages of SERS frequency shift assays for multiplex protein detection, and more recent

assays for RNA/DNA, ions and small molecules. We conclude with a discussion of challenges and opportunities awaiting the adoption of SERS-based frequency shift methods for trace (bio) molecule detection.

The mechanisms of SERS frequency shift-based detection

The mechanism underlying SERS frequency shifts can be varied with more than one effect present, adding to complexity but at the same time offering opportunity for a wide range of assays to be developed. As mentioned, Olivo *et al.* proposed a mechanism based on mechanical deformation, or 'nano-stress' as shown in Figure 2a. They proposed that the sensor structure, the aforementioned binding of 4-ATP to a metallic nanoparticle substrate and further binding of antibodies to the 4-ATP, builds up significant steric strain in the substrate (particularly antibody-antibody repulsion). Upon specific recognition and binding of the H1 antigen, this steric strain is released, reducing the stiffness of the -C-S- bond of 4-ATP and shifting its frequency to lower energy in quantitative correlation with the presence of bound antigen.[32]

Assays in which SERS frequency shifts are induced by chemical rather than mechanical effects have also been developed. For example, Zhao *et al.* found that the intermolecular hydrogen bonding of aniline to p-mercaptobenzoic acid (MBA) leads to a shift in the frequency of some bands in the SERS spectra that are attributed to charge transfer resonance between MBA and Ag nanoparticles (NPs, Figure 2b).[37] In brief, any binding-induced interaction that modifies the environment of the Raman reporter has potential to be used as a basis for a SERS frequency shift assay. For example, Cronin *et al.* observed vibrational Stark shifts for thiolated-benzonitrile molecules bound to an electrode surface with applied local electric field, showing that modification in the dielectric environment around a Raman reporter is another effective way to induce frequency shift detection (Figure 2c).[38] The exceptional variety of possible mechanical, chemical, or local electric field effects on Raman spectra has led to the variety of SERS frequency shift assays that have been developed

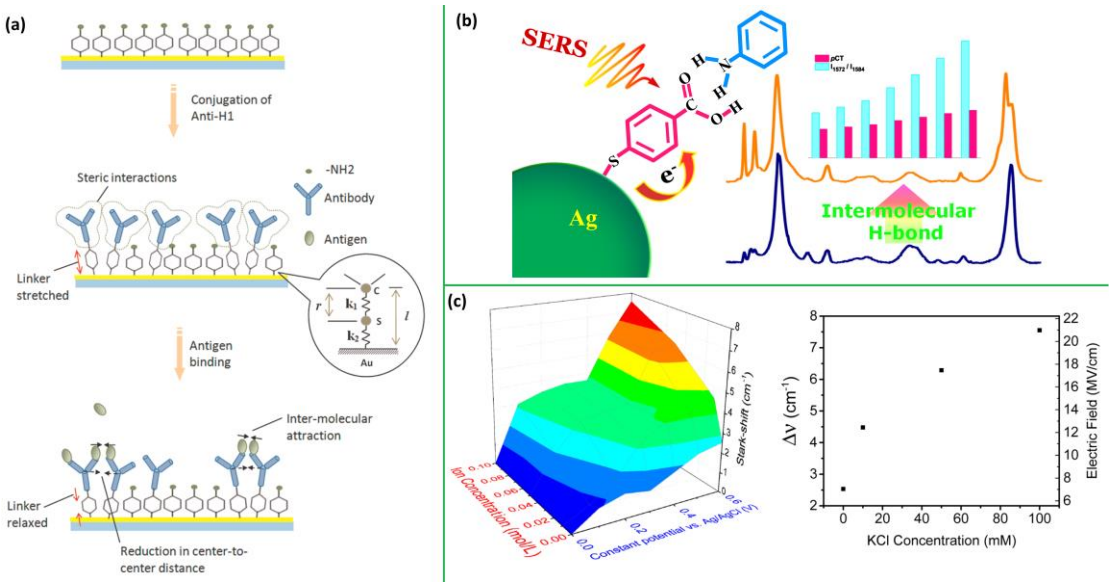

Figure 2 Examples of SERS frequency shifts arising from (a) mechanical deformation of the reporter; (b) intermolecular charge transfer (peak shape changes take place in concert with frequency shifts); (c) dielectric environment changes. Images reproduced from **a** ref. 32, **b** ref. 37 and **c** ref. 38 with permission from ACS.

in recent years. The various perturbations to the SERS spectra take the form not only of peak shifts but also peak shape change, further broadening the applicability of this form of assay.[28]

SERS Frequency shift detection systems

The ability to measure shifts in intense Raman bands depends mainly on the precision of the instrument and avoids the more onerous task of resolving a peak above noise at ultralow analyte concentrations. Importantly, the precision of standard Raman spectrometers for peak determination is very often sufficient for determining analyte-induced Raman shifts down to even femtomolar concentrations of analyte.

For example, the precision of peak position determination using the DXR Smart Raman spectrometer from Thermofisher is rated by the company at 0.022 cm^{-1}.[39] Our own work with this spectrometer (using 780 nm, 80 mW, 10 μm diameter focal spot laser excitation and 20s integration time detection of aromatic thiol SERS peaks), suggests that the standard deviation of repeated measurements of the peak Raman

shift on the same sample and same position was slightly greater at 0.03 cm^{-1}.[40] Even where data has been collected at 1 cm^{-1} intervals, the peak position can be determined to a precision of < 0.1 cm^{-1} either by fit to a b-spline function, or to voig profiles after background subtraction. [41]

Given a Raman peak determination precision of ca. 0.03 cm^{-1}, what is the reproducibility and limits to the measurements of SERS frequency shifts? We carefully addressed this issue for SERS frequency shifts observed upon binding microRNA to a complementary single strand DNA/mercaptobenzoic acid (MBA) monolayer chemisorbed to a silver nanoparticle film.[40] The frequency shift in the MBA Raman peak at ~1585 cm^{-1} was measured after incubating the substrate with the microRNA in concentrations ranging from 10^{-6} to 10^{-16} M, for measurements on 5 different substrates from one batch, for multiple batches, and for two different laser excitation wavelengths.[40] The average standard deviation in the measured frequency shifts across all these variables was 0.04 cm^{-1}.

This measured uncertainty in frequency shift is consistent with uncertainties of 0.3 cm^{-1} in the two peak position determinations required to calculate it adding in quadrature, as expected for random and uncorrelated errors. Defining the limit of detection (LOD) for an assay as 3 times this uncertainty gives a value of 0.12 cm^{-1} as the minimum significant frequency shift for assaying with a standard Raman spectrometer.[40]

In contrast, the shifts in Raman reporter bands upon binding with analytes can be of the order of several to tens of wavenumbers. Again, drawing on our previous work for typical examples, a Raman reporter bound to an antibody showed Raman peak shifts of 0.5 to 2.5 cm^{-1} in the presence of 10^{-13} to 10^{-10} M of the corresponding antigen. [41] Elsewhere, a Raman reporter bound to single strand DNA showed Raman peak shifts of 0.1 to 1.6 cm^{-1} in the presence of 10^{-16} to 10^{-6} M of an anti-sense microRNA. [42] Clearly, ultra-sensitive frequency shifts assays are very achievable without resort to specialist high-resolution Raman spectrometers.

Optimization of SERS frequency shift detection systems also must focus on the SERS substrate, the Raman reporter, and the probe for capturing the biomolecule of interest. Generally, a uniform response of the substrate is desired for the best sensitivity and reproducibility of the assays.

In terms of uniform SERS substrates, the major challenge is to produce nanostructured metallic films with appropriate hotspots for orders-of-magnitude SERS enhancement, at a high and uniform density such that point-to-point on a substrate, and substrate-to-substrate, uniform spectra of the Raman reporter can be obtained. One simple approach is to produce random films of nanoparticles where positional control of hotspots is sacrificed for speed and low cost of fabrication. More sophisticated technologies are also employed to improve positional reproducibility,

Table 1. Common Raman reporters used in SERS frequency shift sensing, including the vibrational bands that are commonly analyzed

Raman reporter	wavenumber (cm^{-1})	band assignments	References
4-mercaptobenzoic acid （MBA）	~1585	aromatic C-C stretches	[48]
5,5’-dithiobis(succinimidyl-2-nitro-Benzoate) (DSNB)	~1335	nitro stretch	[28]
2-Amino-6-purinethiol (6TG)	~1299	ring C-N stretches	[49]
4-mercapto-phenylboronic acid (4-MPBA)	~1586, ~1574	totally(8a) and nontotally (8b) symmetric vibrational modes of the benzene ring	[50]
2-nitro-5-mercaptobenzoic acids (TNBs)	~ 1342	symmetric stretching of $\nu(NO_2)$ mode	[51]
thiophenol (TP)	~1075	in-plane ring	[52]

	breathing mode coupled with ν(C-S)

including nanopatterned substrates, nanosphere lithography, and electrostatic assembly. [42-47]

In terms of the Raman reporter, again a uniform layer on the substrate surface is preferred such that the reporters lie in as uniform a SERS enhancement environment as possible. An appropriate choice is often small thiolated aromatics. These have a large Raman cross section for SERS sensing, thiolate groups for tight binding to Ag/Au substrates, and can be further functionalized to allow the conjugation of the capture probe for recognition of the targeted biomolecule (occasionally, some SERS-active molecules function as both Raman tags and capture probes, further simplifying the system). In the case of multiplex sensing particularly, Raman reporters which have binding-sensitive vibrational peaks that are isolated from the peaks of other Raman reporters in the combined spectrum are also necessary. Some of the Raman reporters that have shown great utility in SERS frequency shift assays are summarized in Table 1.

In terms of the capture probe, specificity of recognition of the target biomolecule in a complicated environment is the primary priority. They should also be robust to the conditions of sensor fabrication and assay testing. Finally they should have functional groups available for facile chemical bonding to the Raman reporter. Capture probes exhibiting antigen-antibody recognition, RNA/DNA hybridization, and ligand recognition have all been employed successfully. As mentioned above, some Raman reporters can also act as capture probes, 2-nitro-5-mercaptobenzoic acids (TNBs) (Figure 6a) for example can form multiple hydrogen bonds with melamine, and was selected both as reporter and as probe for the recognition and detection of melamine in milk samples by shift assay.[51]

Applications of SERS Frequency Shift Assays

Protein biomarker detection

As discussed above, mechanical stress, charge transfer, and dielectric environment changes (Stark shifts) can contribute to SERS frequency shifts for Raman reporters bound to plasmonic substrates. When they are conjugated with a capture probe, such shifts can occur in quantitative correlation with the concentration or molecular weight of the targeted biomolecule. As a result, biomolecules such as proteins and microRNAs that are generally macromolecular and feature multiple charges are ideal targets for SERS frequency shift-based biosensing. Furthermore, immunological recognition and DNA hybridization are some of the most specific and robust recognition reactions in nature. As a result a large array of SERS frequency shift assays have been designed for biomolecular targets. For example, Mascareñas's group designed a c-Fos peptide receptor frequency shift-based assay for the oncoprotein

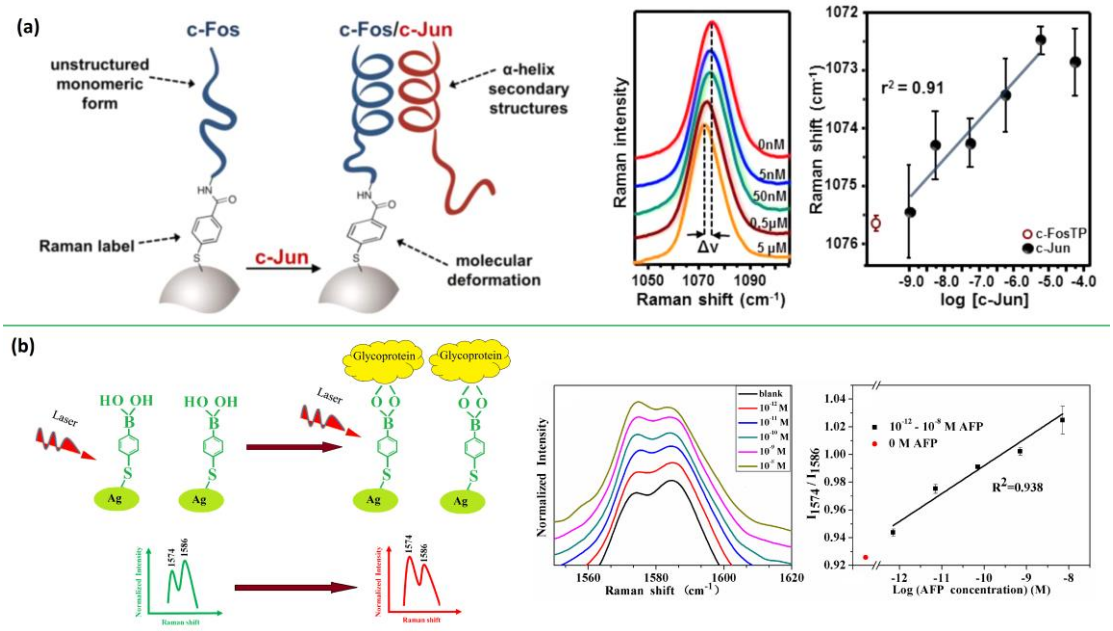

Figure 3 (a) Schematic of c-Fos/c-Jun dimerization on a metal surface and the resulting deformation of the Raman label (reporter) structure (left), and the Raman label SERS peak shift spectra and plot as a function of protein concentration (right). (b) Schematic representation of simultaneous capture and detection of glycoproteins by a ratiometric SERS shift assay (left), the SERS peaks around 1580 cm^{-1} of the

reporter 4-mercapto-phenylboronic acid (4-MPBA) upon binding with glycoprotein AFP in the range 10^{-12} to 10^{-8} M (centre), and ratio of the shifting peaks with glycoprotein concentration (right). Images reproduced from **a** ref. 43 with permission from ACS, **b** ref. 53 with permission from RSC..

c-Jun with nanomolar sensitivity (Figure 3a).[43] Our group employed 4-mercapto-phenylboronic acid (4-MPBA) as both a glycoprotein-specific recognition biosensor and as a Raman reporter for the antibody-free assay of glycoproteins (Figure 3b).[52] Olivo *et al.* established the 'nanostress' SERS sensor for quantitative detection of H1 influenza protein based on frequency shift already discussed in detail above.[32, 54] The peak frequency of the 1580 cm^{-1} benzene ring stretching mode of 4-ATP shifted following recognition of H1 influenza protein. Zhao's group systematically analyzed the transformation of the Raman probe MBA in SERS-based protein detection.[55] These single-target SERS frequency shift assays showed a performance superior to conventional ELISA.

Frequency shift-based multiplex assay in protein/nucleic acid detection

In practical applications and particularly for clinical diagnosis, assays that can detect multiple biomolecular targets are highly valuable as they can reduce the chance of false positives being recorded. Therefore, multicomponent detection is an issue of major significance. Multicomponent detection is implementable for SERS frequency shifts assays by preparing substrates with multiple reporter/capture probe conjugates, with distinguishable Raman peaks. This can be achieved by random chemisorption of the reporters, however we have shown that improvements can be gained by micro-contact printing patterned regions of a single reporter, for up to three different reporters, on a single substrate.[41] The individual binding events/frequency shifts occurring in each region can be detected simultaneously by exciting each region with a single, larger laser excitation spot. We attribute the improvement in assay performance to a reduction in cross-talk between binding events for the different biomolecules targeted.

To achieve this goal, our group developed a high temperature modification of the Tollen's method for preparing dense films of highly faceted ~ 45 nm diameter silver particles (Figure 4). [41] We then used micro-contact printing to print uniformly distributed micro-domains of individual Raman reporters on the same Ag NP substrate. As shown in Figure 4, square-patterned and stripe-patterned PDMS stamps were used for stamping the substrate for simultaneous detection of two and three biomarkers, respectively.[40, 41]

These substrates were employed for SERS frequency shift immunoassay of hepatocellular carcinoma (HCC) protein biomarkers, α-Fetoprotein (AFP) and Glypican-3 (GPC3).[41] AFP is an established biomarker for early diagnosis of HCC, the most common form of liver cancer. Serum GPC3 has good sensitivity (40−60%) and excellent specificity (>90%) for HCC, and when detected in combination with AFP can increase the sensitivity of HCC diagnosis to 80%.[56] A PDMS stamp covered

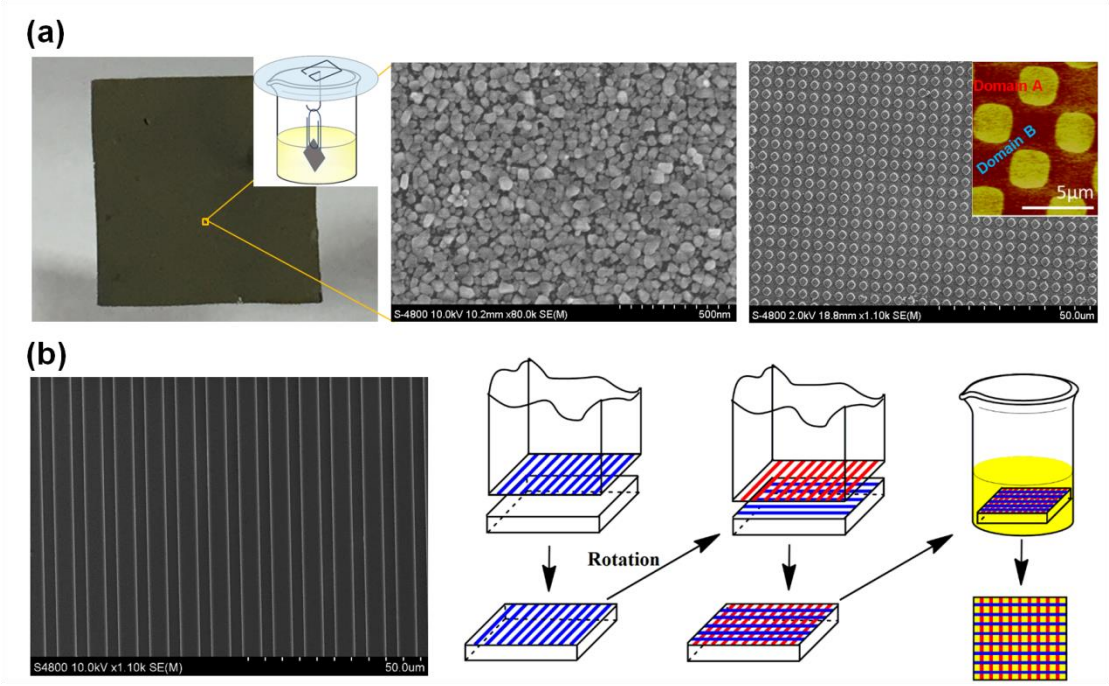

Figure 4 (a) The preparation of SERS frequency shift substrate consisting of silver nanoparticles, via a high temperature modification of the Tollen's method (left), SEM image of the silver nanoparticle film (centre), and SEM image of a PDMS stamp used

for microcontact printing of Raman reporter MBA in 2×2 μm squares on the substrate (inset: Lateral Force Microscopy image showing the integrity of domains of 11-aminoundecanethiol chemisorbed to atomically flat gold using the PDMS stamp); (b) SEM image of a ridged/striped PDMS substrate and the scheme for stamping of three Raman reporters for simultaneous detection of multiple analytes. Images reproduced from **a** ref. 41 and **b** ref. 40 with permission from ACS.

with MBA (reporter 1) was used to contact the Ag NP film surface, leaving 2×2 μm square-shaped regions of MBA self-assembled monolayer (SAM) on the surface. The rest of the Ag NP film was chemisorbed with a second thiolated Raman reporter 2, DSNB (5,5'-dithiobis(succinimidyl-2-nitro-benzoate). By tuning the reaction conditions, capture probes for the two protein biomarkers were linked to each reporter separately, resulting in uniformly distributed and non-interfering regions of reporter/capture probe conjugates which could be photoexcited by a single laser spot and a SERS spectrum collected. Simultaneous AFP and GPC3 detection from PBS solutions exhibited sensitivity down to sub-picomolar concentrations, at least 2 orders of magnitude higher sensitivity than possible for conventional ELISA. The sensing capability of this platform in human sera was demonstrated by detecting the amount of AFP and GPC3 in liver cancer patient serum, where again, the protein biomarkers were recognized by the capture probes in each micro-domain and the induced frequency shifts measured. These results corresponded accurately with the values measured by ELISA when considering the 100× more dilute samples.

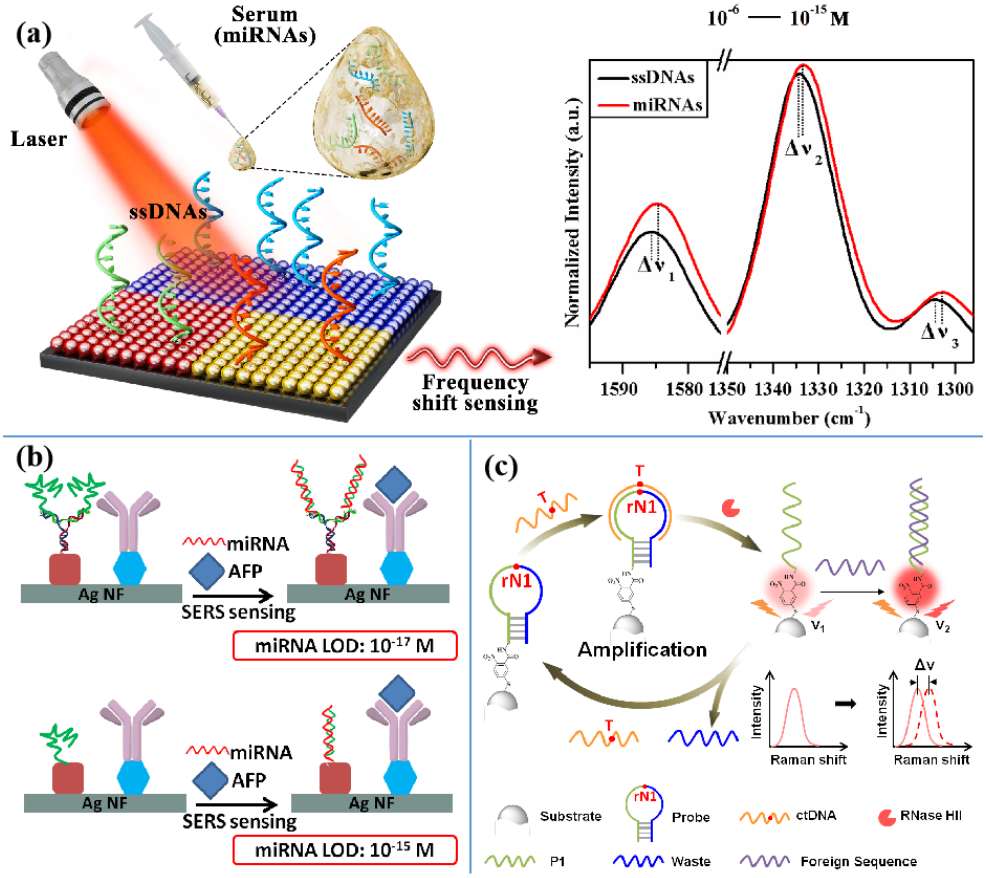

Figure 5 (a) Simultaneous microRNA and protein sensing by SERS frequency shift combined with micro-contact printing method. (b) Branched DNA for multiplex assaying of serum miRNAs biomarkers with an improved detection limit. (c) Frequency shift detection of ctDNA with specially designed hairpin DNA. Images reproduced from **a** ref. 40, **b** ref. 57 and **c** ref. 58 with permission from ACS.

Later our group further improved the assay, achieving simultaneous detection of both proteinacous and nucleic acid biomarkers. MicroRNAs (miRNAs) are small, endogenous, noncoding RNAs with approximately 22 nucleotides that are emerging as an important class of biomarkers for many human diseases. The direct and comparatively fast and cheap nature of SERS frequency shift-based assays can complement the current gold-standard nucleic biomarker detection technique, polymerase chain reaction (PCR), for quantifying circulating miRNAs.

Our group developed assays for simultaneous detection of three miRNAs, or

α-fetoprotein and two miRNAs, for the early diagnosis of liver cancer with higher accuracy, as shown in Figure 5.[40] In these works, DNA that was anti-sense to the target miRNAs was attached to the Raman reporter to induce a specific binding event and the reporter SERS frequency shift. Multiplex miRNA detection is especially useful in clinical diagnosis applications since dysregulation of a single biomarker is not necessarily predictive of a certain disease. Measuring a suite of biomarkers affords more reliable information, reducing false positives. The limit of detection (LOD) of the frequency-shift sensing platform for three miRNAs, miR-26a-5p, miR-223 and miR-27a-3p, was shown to be at sub-femtomolar concentrations. This exceptional sensitivity was attributed to the miRNA binding event disturbing the anti-sense DNA monolayer on the SERS substrate, effecting multiple Raman reporters per single binding event.

Since miRNA-223 is known to be down-regulated for hepatocellular carcinoma (HCC) and up-regulated for intrahepatic cholangiocarcinoma (ICC) from quantitative real time (qRT) PCR studies,[59] patterned substrates with just two domains were utilized to simultaneously detect AFP and miRNA-223 for the discrimination between HCC and ICC (normally this discrimination can only be achieved via biopsy in early stages of disease). The frequency shift SERS method combined with micro-contact printing as applied to direct, multiplex detection of serum microRNA and protein biomarkers was shown to be a highly flexible and effective tool for the early detection and discrimination of primary liver cancers.

By proper probe design, the detection limit of the SERS frequency shift sensing method can be further reduced. To achieve this goal, we subsequently employed a branched DNA, together with microcontact printing, to realize multiplex sensing of different kinds of liver cancer biomarkers simultaneously. The branched DNA included multiple complementary ‘sticky ends’ which act as capture probes for target microRNA, but also features a rigid, branched core of double-stranded DNA that increases the distance between sticky ends, exposing more capture probes for higher sensitivity. The branched DNA enabled 2 orders of magnitude increase in sensitivity for microRNA detection over single-stranded DNA (Figure 5b). The limit of detection

reached as low as 10 attomolar (S/N = 3) for miR-223.[57]

For accurate and sensitive detection of the typically pico-to-femtomolar serum concentration of ctDNA of lung cancer, our group designed an RNA binding site, embedding a DNA-rN1-DNA hairpin probe for specific ctDNA recognition. This frequency shift SERS sensing platform had a detection limit for ctDNA of 1.2×10^{-16} M and functioned well in human physiological media. Furthermore, this sensor could recognize one single base pair mutation (KARS G12D mutation) from 10,000 normal analogues (KARS G12D normal), indicating the high specificity of the sensor for ctDNA detection (Figure 5c).[58]

SERS frequency shift assays of small molecules, ions, gases, and aggregates

(1) Small molecules

When designing SERS frequency shift assays for small molecules it is often possible for the reporter to directly bind the target of interest without a capture probe intermediate. For example, our group developed a SERS frequency shift assay for the detection of melamine in milk with a LOD as low as 10^{-12} M, in which 2-nitro-5-mercaptobenzoicacids (TNBs) was used as both Raman reporter and capture probe (Figure 6).[51] The method was further applied to the monitoring of melamine in both raw milk and milk powder. We also developed a sensitive and single-step reaction assay of glucose, with 4-mercapto-phenylboronic acid (4-MPBA) functioning as both Raman reporter and capture probe as presented in Figure 3b.[53] The frequency shift varied linearly in the range of 10^{-5}–10^{-2} M glucose and the detection limit reached 10^{-5} M. A similar system was also reported by Zhao *et al.* for SERS frequency shift-based aniline detection.[37] Lu *et al.* achieved SERS frequency shift sensing of theophylline by identifying conformational changes of an aptamer binding to theophylline.[60] Frequency shift assaying can also be applied in chemical reaction tracing for small (or bio-)molecules. Balakrishnan *et al.* employed Raman vibration spectroscopy in combination with FTIR spectroscopy for ^{18}O isotopic shift discrimination, to detect and quantify Met oxidation in various therapeutic proteins, including mAbs, fusion proteins, and antibody-drug conjugates (Figure 6b).[61] Zhao

and Ozaki reported a frequency shift-based SERS study for the enantioselective discrimination of alcohols which opens new avenues for fast and novel enantio-sensing.[62]

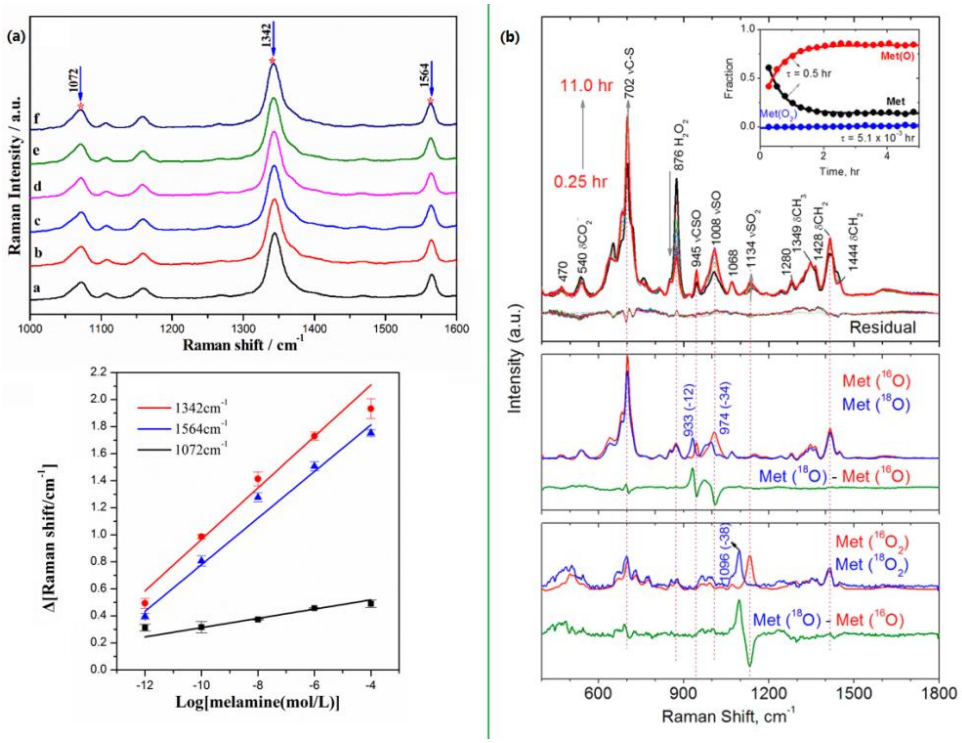

Figure 6 (a) Frequency shift of 2-nitro-5-mercaptobenzoic acids (TNB) for the detection of melamine in milk powder. (b) Oxidation of free Met by ^{16}O or ^{18}O isotopic hydrogen peroxide monitored by *in situ* Raman spectroscopy. Images reproduced from **a** ref. 63 with permission from Elsevier and **b** ref. 61 with permission from ACS.

(2) Ions

Specific and quantitative detection of ions via SERS is a challenge due in part to the low Raman cross-sections of ions, and in part due to the general non-specificity of electrostatic binding.[26] SERS-pH sensing and SERS hydrogen bond (H-bond) sensing has been an active field in recent years.[62, 64] Olivo *et al.* developed a SERS-based pH sensing system using an arene chromium tricarbonyl-linked aminothiophenol ($Cr(CO)_3$–ATP) as a novel Raman reporter. The frequency of the CO stretching vibration at 1820 cm^{-1} is shifted strongly, depending on the pH value (Figure 7a).[64]

Our group developed a highly sensitive and selective frequency shift-based detection of aqueous Zn(II) ions with a detection limit of 10^{-14} M, which was applied to drinking water and also to measurements of cellular uptake of Zn(II). (Figure 7b,c).[63]

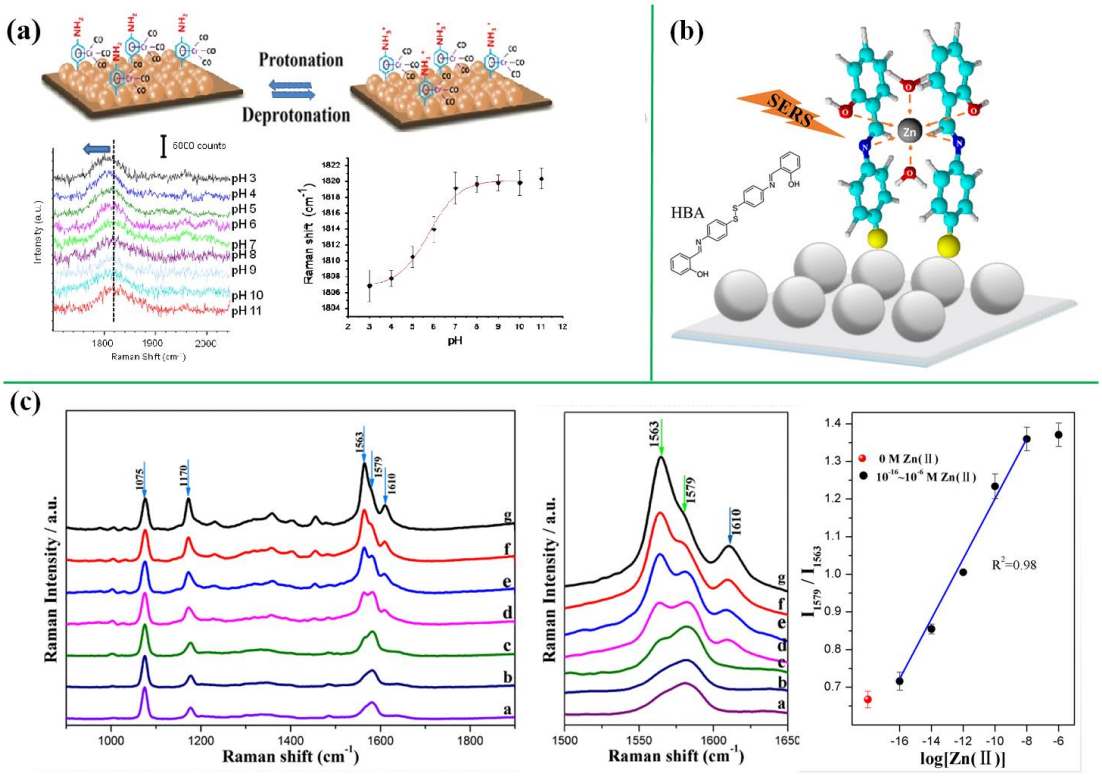

Figure 7 (a) $Cr(CO)_3$–ATP immobilized bimetallic SERS substrate for pH sensing. (b) Scheme of frequency shift detection of Zn(II) by N,N-bis(2-hydroxybenzylidene)-4-aminophenyl disulfide (HBA); (c) Zn(II) detection based on frequency shift of the Raman reporter HBA. Images reproduced from **a** ref. 64 with permission from Elsevier, **b** and **c** ref. 63 and with permission from RSC.

(3) Gas molecules

In principle, small, gaseous molecules do not influence Raman frequencies of reporter molecules in a dramatic way due to their low adsorption efficiencies. However, the accumulated effect of small gaseous molecules can be evidenced using frequency shift assays. For example, the surface potential of Au nanoparticles is subject to change by exposure to volatile organic compounds (VOCs). The SERS spectra of surface-bound Raman reporters such as 2,6-dimethylphenylisocyanide (2,6-DMPI) are also very susceptible to changes in surface potential of Au, and can

thus be used to assay the presence of VOCs. Using this methodology, a blue- or red-shift of 1 cm^{-1} in the SERS spectrum of 2,6-DMPI was reproducibly measured for the detection of volatile organic compounds (CCl_4 methanol, ammonia, and butylamine vapor) (Figure 8a,b).[65] Similarly, the Raman spectral characteristics of CO_2 can be affected by the environment, thus frequency shift assay was applied for the identification and quantitative analysis of CO_2. Zhang *et al.* analyzed the effects of temperature and pressure on the Raman spectral characteristics of CO_2 in different phases (Figure 8c). Temperature and pressure have significant effects on both the Raman peak frequencies and their full width at half-maximum (FWHM), potentially allowing accurate assaying of CO_2 phases in deep sea extreme environments.[66, 67]

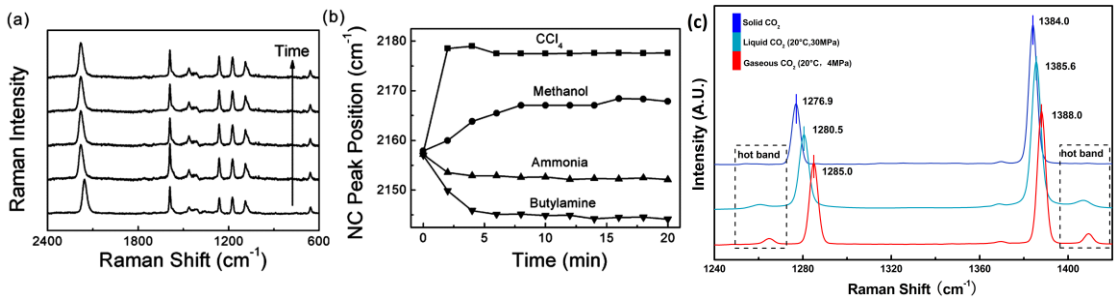

Figure 8 (a) Time-dependent SERS spectra of 2,6-dimethylphenylisocyanide (2,6-DMPI) on a gold nanoparticle film measured under a flow of CCl_4; (b) 2,6-DMPI SERS peak position variation under flows of CCl_4, methanol, ammonia, and butylamine; (c) Raman spectra of solid CO_2, liquid CO_2 (20°C, 30 MPa), and gaseous CO_2 (20 °C, 4 MPa). Images reproduced from **a** and **b** ref. 65 with permission from RSC, **c** ref. 66 with permission from OSA

(4) Peptide aggregation

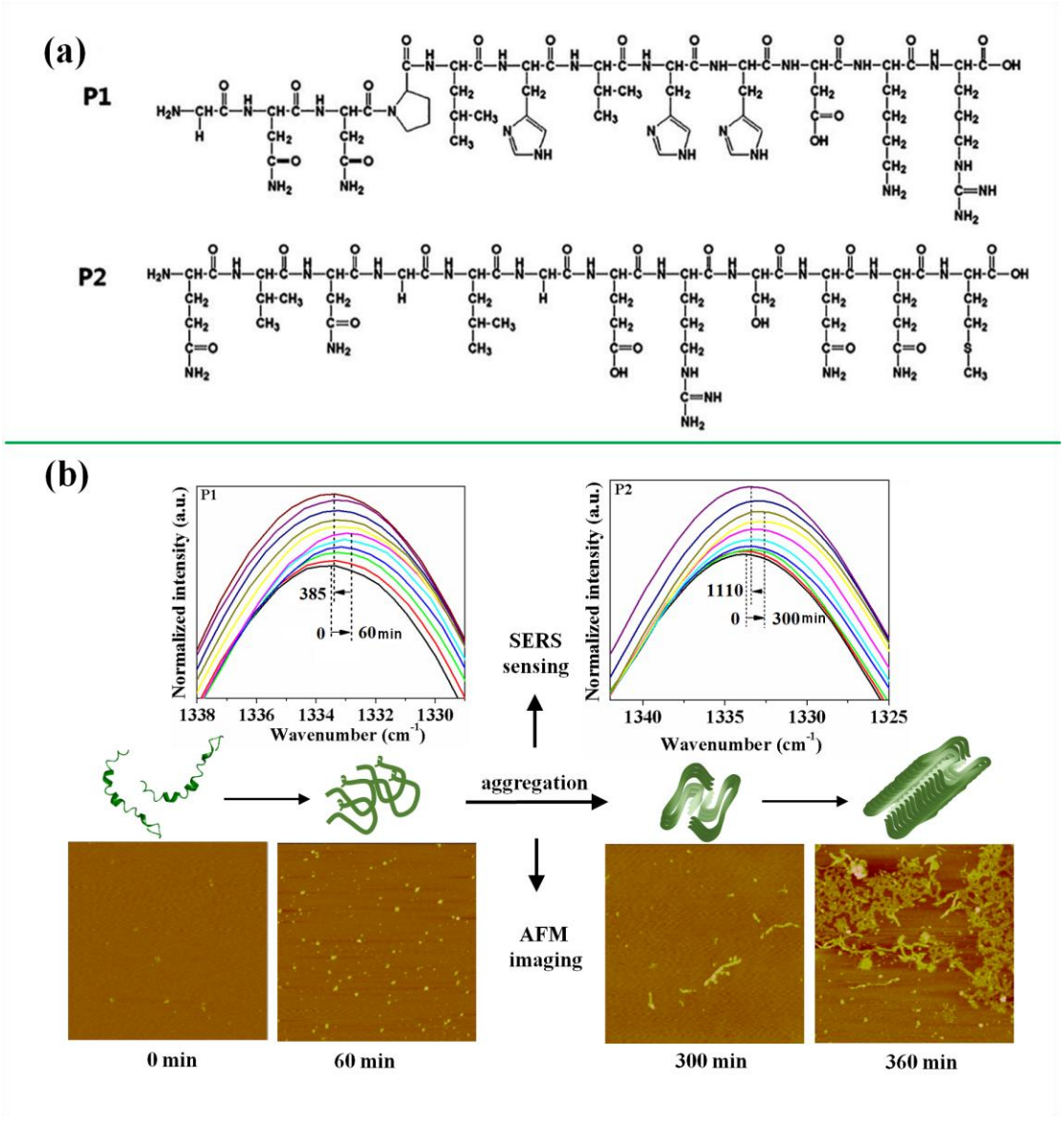

Figure 9 (a) Probes P1 and P2 for monitoring the aggregation dynamics of amyloid‑β protein (targeting monomers and fibers respectively). (b) The possible aggregation processes of amyloid‑β protein based on the frequency shift of Raman reporters, and associated AFM images. Images reproduced from **a** and **b** ref. 68 with permission ACS.

Applications of SERS frequency shift sensing beyond isolated molecular/ionic sensing have also been uncovered. For example, the SERS spectral shifts of a Raman reporter (DSNB) upon Amyloid-β (Aβ) protein binding were recorded for both *in situ* Aβ42 aggregation monitoring, and for Aβ42 monomer and fiber detection.[68] To do this, two affinity peptides were selected by biopanning and used for specific binding

of Aβ42 monomers and fibers respectively (Figure 9a). At the beginning of the process, Aβ42 monomers were the majority component in solution, recognized and captured by the P1 affinity peptide. A second probe P2 with affinity for fibers was also present to track Aβ42 monomer folding and aggregation into fibers with increasing incubation time (Figure 9b). The sensing of aggregation kinetics in such systems may be useful as they have been linked to clinical manifestation of Alzheimer's disease.

Conclusion and Outlook

SERS offer several advantages over colorimetric and luminescence detection methods due to its ultra-high detection sensitivity and multiplexing capability. Traditionally, SERS assays have been based on SERS intensity variations in 'sandwich' motifs, but in recent years variations such as frequency shift methods have become prominent. Through surveying the literature of frequency shift assays developed over recent years, we hope we have brought into focus some important advantages of this exciting and growing subset of SERS-based assays. Firstly, they are exceptionally suited for assaying large and highly polar molecules that during binding induce large Raman reporter frequency shifts, either by mechanical or charge effects. Biomolecule analytes like proteins and DNA/RNA are thus exemplary targets and frequency shift assays discussed herein have been shown to be equal to or superior to current gold-standard clinical methods such as chemiluminescence and PCR for their detection in human biofluids. In particular, the SERS frequency shift method is expected to be well suited to predicting mutation number and position in ctDNA, which could give important insights into the origin of cancers and thus assist clinical diagnoses. Secondly, these ultra-sensitive SERS-based assays can be performed with standard Raman spectrometers and in spectroscopic terms are easier to perform than their intensity-based cousins, relying on precision of peak position rather than the resolution of small peaks on a noisy background. Thirdly, the variety of Raman reporters with distinct SERS spectra available makes multiplex detection of biomarkers much easier to achieve for SERS frequency shift assays than it is for

refractive index-based peak shift methods (e.g. SPR), which is incredible important for accurate and early disease diagnosis and prognosis. Finally, SERS frequency shift-based techniques of enormous variety are starting to emerge, focusing on diverse analyte types including ions, gases, and even protein aggregates. Uniting this variety of frequency shift assays with large data set analysis may realize online intelligent clinical diagnosis.

Despite the success of all these SERS frequency shift assays, many critical challenges remain. Improvements to SERS frequency shift assays will come on the one hand by addressing challenges that affect all SERS-based assays. These include developing cheap and large area (and potentially roll-to-roll fabrication of) SERS substrates with reproducible and strong scattering response. For example, most frequency shift-based detection methods have been designed to work on macro-scale flat substrates, which limit their possible application *in vitro and in vivo*. Could the substrates be miniaturized, even down to the single nanoparticle level, to enable SERS frequency shift assays to function in micro/nanoscale cellular environments?

Another common challenge for SERS assays is the range of available Raman reporters in terms of scattering cross-section, functionality and controlled surface-assembly. A limited choice of capture probes also restricts applications. One promising solution discussed herein is the screening for capture probes via the bio-panning technique, which enabled the assay for amyloid-β protein aggregation dynamics to be developed.

Especially importantly for frequency shift assays is that the quantifiable effect of the binding interaction be collective in nature, that is, the binding of a single target biomarker should affect more than a single Raman reporter/capture probe to amplify the sensitivity of the assay. This is thought to occur in several SERS Frequency shift assays where binding of a biomarker disrupts an otherwise ordered self-assembled monolayer of Raman reporter/capture probes conjugates on a SERS-active substrate. This includes protein binding inducing steric strain in a monolayer of Raman reporter-bound antibodies,[32] and microRNA binding disrupting Raman reporter-bound ssDNA monolayers.[40]

In this review, we have presented a systematic description of SERS frequency shift-based assays and their application in quantitative detection of biomolecules and other analytes in a range of contexts. With the wide range of applications already demonstrated, its high adaptability to the detection environment, and most importantly, its multiplex quantitative detection ability, we believe SERS frequency shift assays will continue to advance and increase their impact in the near future.

Declaration of competing interest

The authors declare no financial interests.

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Vocabulary

Surface plasmons: collective oscillations (waves) of light coupled with free electrons at a metal surface. Raman scattering: Inelastic scattering of light from, for example, molecules for which the Raman scattered light differs in energy from the excitation light by an energy equivalent to a vibrational energy of the molecule. SERS: Surface-enhanced Raman scattering referring to the phenomenon, or Surface-enhanced Raman spectroscopy referring to its spectroscopic detection, an enhancement of Raman scattering due to a surface plasmon-induced increase in the intensity of excitation and scattered light fields. Frequency shift sensing: Detection of a molecule of interest not by the appearance/intensity of a spectroscopic peak due to that molecule, but by the detection of a shift in an already existing spectroscopic peak within the system, the shift in the peak being quantifiably related to interaction with the molecule of interest. Multiplex assays: A sensing platform in which multiple molecules of interest can be quantified simultaneously. MicroRNA: Small,

endogenous, noncoding RNAs with approximately 22 nucleotides that have multiple functions in the human body and emerging diagnostic relevance for human health. Amyloid-β (Aβ) 42 protein: A dominant component of fibrillar aggregates (plaques) prevalent in the human brain and suspected of a link to, and of having diagnostic potential of, Alzheimers disease.

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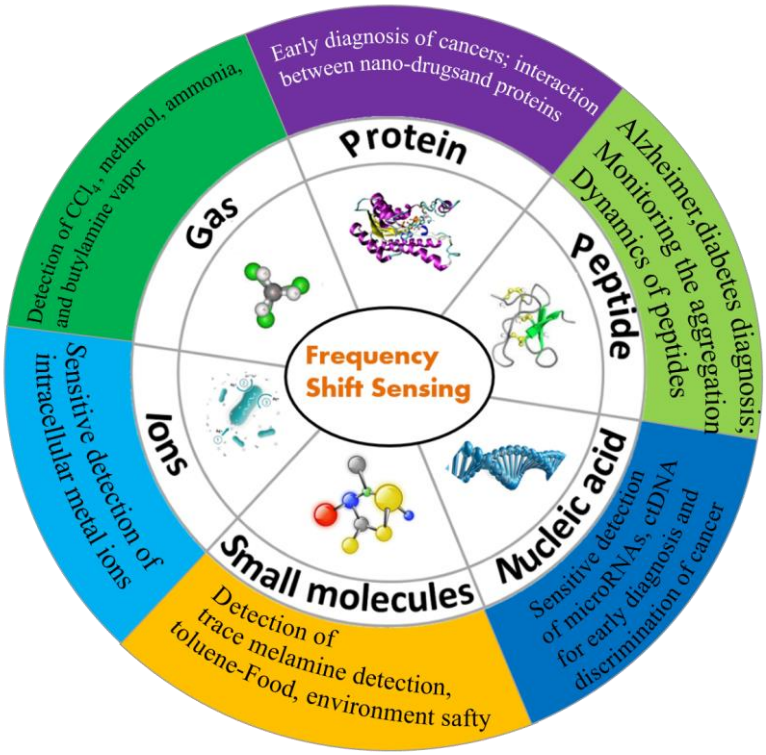

SERS frequency shift assay combines the multiplexing advantage of a typical SERS assay and has gained popularity in recent years as an alternative sensing method. This Minireview summarizes the particular applications of this assay to the sensing of large and highly-charged biomarkers as well as a wider range of targets, including small molecules, ions, and gases.