Biological applications of localised surface plasmonic phenomenae

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Abstract: Researchers and industrialists have taken advantage of the unusual optical, magnetic, electronic, catalytic, and mechanical properties of nanomaterials. Nanoparticles and nanoscale materials have proven to be useful for biological uses. Nanoscale materials hold a particular interest to those in the biological sciences because they are on the same size scale as biological macromolecules, proteins and nucleic acids. The interactions between biomolecules and nanomaterials have formed the basis for a number of applications including detection, biosensing, cellular and in situ hybridisation labelling, cell tagging and sorting, point-of-care diagnostics, kinetic and binding studies, imaging enhancers, and even as potential therapeutic agents. Noble metal nanoparticles are especially interesting because of their unusual optical properties which arise from their ability to support surface plasmons. In this review the authors focus on biological applications and technologies that utilise two types of related plasmonic phenomena: localised surface plasmon resonance (LSPR) spectroscopy and surface-enhanced Raman spectroscopy (SERS). The background necessary to understand the application of LSPR and SERS to biological problems is presented and illustrative examples of resonant Rayleigh scattering, refractive index sensing, and SERS-based detection and labelling are discussed.

1 Introduction

The intense scattering and absorption of light from noble metal nanoparticles is the source of some of the beautiful colours in stained glass windows and has attracted the interest of scientists for generations. These nanoparticles exhibit a strong UV-vis absorption band that is not present in the spectrum of the bulk metal [1–8]. Although scientists have learned that the characteristic hues of these noble metal nanoparticle suspensions arise from their strong interaction with light, the advent of the field of nanoparticle optics has allowed for a deeper understanding of the relationship between material properties such as composition, size, shape, and local dielectric environment and the observed colour of a metal suspension. An understanding of the optical properties of noble metal nanoparticles holds both fundamental and practical significance. Fundamentally, it is important to systematically explore the nanoscale structural and local environmental factors that cause optical property variation, as well as provide access to regimes of predictable behaviour. Practically, the tunable optical properties of nanostructures can be applied as materials for surface-enhanced spectroscopy [9–13], optical filters [14, 15], plasmonic devices [16–19] and sensors [20–34].

One of the most interesting properties of noble metal nanoparticles arises from their ability to support a localised surface plasmon resonance (LSPR). The LSPR results when the incident photon frequency is resonant with the collective oscillation of the conduction electrons of the nanoparticle. The simplest theoretical approach available for modelling the optical properties of nanoparticles is the Mie theory estimation of the extinction of a metallic sphere in the long wavelength, electrostatic dipole limit. In the following equation [35]:

\[
E(\lambda) = \frac{24\pi N A a^{3/2}}{\lambda \ln(10)} \left[ \frac{\varepsilon_i}{(\varepsilon_i + 2\varepsilon_m)^2 + \varepsilon_i^2} \right] \]

(1)

\(E(\lambda)\) is the extinction which is, in turn, equal to the sum of absorption and Rayleigh scattering, \(N A\) is the areal density of nanoparticles, \(a\) is the radius of the metallic nanosphere, \(\varepsilon_m\) is the dielectric constant of the medium surrounding the metallic nanosphere (assumed to be a positive, real number and wavelength independent), \(\lambda\) is the wavelength of the absorbing radiation, \(\varepsilon_i\) is the imaginary portion of the metallic nanosphere’s dielectric function, and \(\varepsilon_r\) is the real portion of the metallic nanosphere’s dielectric function. The LSPR condition is met when the resonance term in the denominator \((\varepsilon_r + 2\varepsilon_m)^2\) approaches zero. Even in this most primitive model, it is abundantly clear that the LSPR spectrum of an isolated metallic nanosphere embedded in an external dielectric medium will depend on the nanoparticle radius \(a\), the nanoparticle material \(\varepsilon_i\) and \(\varepsilon_r\), and the nanoenvironment’s dielectric constant \(\varepsilon_m\). Furthermore, when the nanoparticles are not spherical, as is always the case in real samples, the extinction spectrum will depend on the nanoparticle’s in-plane diameter, out-of-plane height, and shape. In this case the resonance term from the denominator of (1) is replaced with:

\[
(\varepsilon_r + \chi\varepsilon_m)^2
\]

(2)

where \(\chi\), a shape factor term [11] is a term that describes the nanoparticle’s aspect ratio. The values for \(\chi\) increase from two (for a sphere) up to, and beyond, values of 17 for a 5:1 aspect ratio nanoparticle. In addition, many of the samples
considered in this work contain an ensemble of nanoparticles that are supported on a substrate. Thus, the LSPR will also depend on interparticle spacing and the substrate dielectric constant.

LSPR excitation results in wavelength selective absorption with extremely large molar extinction coefficients \( \sim 3 \times 10^{11} \text{M}^{-1} \text{cm}^{-1} \) [36], resonant Rayleigh scattering [37, 38] with an efficiency equivalent to that of \( 10^6 \) fluorophors [39] and the enhanced local electromagnetic fields near the surface of the nanoparticle which are responsible for the intense signals observed in all surface-enhanced spectroscopies, e.g. surface-enhanced Raman scattering (SERS) and surface-enhanced fluorescence [11, 26, 27].

2 Synthetic methods to produce nanoparticles

One of the aims of any application, particularly for sensing research, is to be able to generate reproducible results. In nanobiological applications, this translates directly into a critical need to understand and control the synthetic methods used to generate novel nanoscale materials. At the nanoscale, there are many options available ranging from nanoparticles in solution, to surface-bound nanoparticles, to nanoparticles entrapped in a three-dimensional matrix. With this plethora of potentially useful nanomaterial classes, there exist a large number of fabrication methods to controllably and reproducibly produce these samples. In this Section, we will discuss the most pertinent synthetic methods used in biosensing application: chemical synthesis and lithographic techniques.

2.1 Nanoparticle synthesis by chemical reduction

The synthesis of inorganic nanocrystals with controllable shapes and sizes has been a driving force in much of materials research. This has been characterised by rapid progress in the past few years with synthesis of spheres [40, 41], rods [42–44], triangular prisms [45–47], disks [48–50], cubes [51] and branched nanocrystals [52]. These structures are compelling for their use in biolabelling and biosensing. In this review, we will concentrate on the synthesis and use of noble metal nanoparticles for biological sensing platforms. The most commonly prepared shapes are spheroids, triangular prisms, rods and cubes (Fig. 1). All of these shapes are based on the reduction of a metal salt to produce nanoparticles with varied shapes and sizes. A different but related synthetic technique is the electrochemical reduction of metal salts in the presence of a surfactant or template [53–57]. These methods are most effective at producing large aspect ratio particles. The effect of nanoparticle shape on the optical properties is clearly illustrated in Fig. 2, which shows the scattering spectra from four different morphologies [58].

2.2 Core-shell morphologies

Core-shell particles encompass a range of sample types from metal surrounding metal [59, 60] to dielectric surrounding metal. Most notably is the work of Ung et al. [61]. These particles are produced by first creating the metal cores by one of the standard reduction reactions described above. To stabilise the colloids and remove the citrate ions, the colloids are mixed and allowed to react with an aminoalkane or thioalkane. Once the citrate has been completely displaced, the colloidal solution is allowed to react with a solution of active silica to form the shell (Fig. 3a). After 24h, the particles can be used for further chemical modification or to increase the shell thickness [61–63]. Transmission electron microscopy (TEM) and dark-field optical micrographs of this type of core-shell structure are shown in Fig. 3b and c. Recently, Yin et al. [64] have developed a simpler and faster method to silica coat particles. Their method involves suspension of aqueous phase particles within an isopropanol/water mixture followed by the direct addition of a small fraction of tetraethyl orthosilicate (TEOS). The TEOS
quickly forms a silica shell around the nanoparticle indistinguishable from those created in the Ung et al. method. The Yin et al. method is rapid, but unsuitable for certain applications, notably the co-encapsulation of Raman-active species within the silica shell (see below), and the coating of particles formed in the presence of surfactants has met with limited success.

Another form of core-shell particles has a metal shell surrounding a dielectric core. The dielectric core, usually silica, is produced by the Stöber method to produce monodispersed silica cores ranging in size from 80–500 nm [65, 66]. These particles are then functionalised with 3-aminopropyltrimethoxysilane. These functionalised particles are then reacted with small gold nanoparticles. The gold particles provide nucleation sites for the growth of the metallic shell from metal salts in solution. NH4OH is added to produce preferential deposition onto the surface, rather than initiate new particle formation in solution (Fig. 3d) [65–69]. The TEM and dark-field optical micrographs of this type of nanoshell are shown in Figs. 3e and 3f.

2.3 Lithographic techniques

All nanoparticle fabrication methods discussed thus far are used to create suspensions of nanoparticles in solution. Another class of techniques addresses substrate-bound nanostructure fabrication. The standard approach for making substrate-bound nanostructures is electron beam lithography (EBL). In EBL, the desired pattern is serially produced by exposing a thin layer of photo-resist to high energy electrons, followed by chemical development and deposition of the noble metal, Fig. 4a. Several research groups have focused on the optical properties of two-dimensional arrays in order to utilise these nanoparticle assemblies as surface-enhanced spectroscopy substrates [70–72]. While EBL provides exquisite control over nanoscale morphology, it is an expensive and time-consuming technique. An alternative method for the large-scale production of surface-bound nanoparticle arrays is nanosphere lithography (NSL).

NSL is a powerful fabrication technique to inexpensively produce nanoparticle arrays with controlled shape, size, and interparticle spacing [73]. Every NSL structure begins with the self-assembly of size-monodispersed nanospheres of diameter \( D \) to form a two-dimensional colloidal crystal deposition mask [8, 73–75]. As the solvent evaporates, capillary forces draw the nanospheres together, and the nanospheres crystallise into a hexagonally close-packed pattern on the substrate. As in all naturally occurring crystals, nanosphere masks include a variety of defects that arise as a result of nanosphere polydispersity, site randomness, point defects, line defects, and polycrystalline domains. Typical defect-free domain sizes are in the 10–100 \( \mu m \) range.

Following self-assembly of the nanosphere mask, a metal or other material is then deposited by thermal evaporation, electron beam deposition, or pulsed laser deposition, Fig. 4b. After metal deposition, the nanosphere mask is removed, leaving behind surface-confined nanoparticles with triangular footprints.

3 Sensing with noble metal nanostructures

It is apparent from (1) that the location of the extinction maximum of noble metal nanoparticles is highly dependent...
on the dielectric properties of the surrounding environment and that wavelength shifts in the extinction maximum of nanoparticles can be used to detect molecule-induced changes surrounding the nanoparticle. As a result, there are at least four different nanoparticle-based sensing mechanisms that enable the transduction of macromolecular or chemical-binding events into optical signals based on changes in the LSPR extinction or scattering intensity shifts in LSPR \( \lambda_{\text{max}} \), or both. These mechanisms are: (i) resonant Rayleigh scattering from nanoparticle labels in a manner analogous to fluorescent dye labels [38, 39, 76–82]; (ii) nanoparticle aggregation [83]; (iii) charge-transfer interactions at nanoparticle surfaces [35, 84–88]; and (iv) local refractive index changes [20, 23–25, 27, 28, 84, 89–93].

3.1 Solution-phase nanoparticle sensing

Solution-phase nanoparticle-based sensing is a simple, yet powerful detection modality. Because many molecules of interest, particularly biological molecules, are in the aqueous phase, it is desirable to have a sensitive and specific detection system that is homogenous with the phase of the target molecule, thereby decreasing the need for extended sample preparation. Aggregation-based detection has become a mainstay in the clinical community since the development of the latex agglutination test, (LAT) in 1956 [94]. In the LAT and similar tests, biomolecular-specific antibodies are conjugated to latex microspheres which, when mixed with a solution (e.g. blood or urine) containing the target antigen, cause the latex spheres to form visible aggregates. Whereas LATs are effective and quite rapid (15 min to 1 h), they are inherently insensitive, relying on high concentrations of analytes and on the human eye as a detector.

In contrast, for solution-phase LSPR-based sensing, signal transduction depends on the sensitivity of the surface plasmon to interparticle coupling. When multiple particles in solution that support a localised surface plasmon are in close proximity (i.e. interparticle spacings less than the nanoparticle diameter), they are able to interact electromagnetically through a dipole coupling mechanism. This broadens and red shifts the LSPR, and small clusters of particles possess LSPR properties similar to those of a larger single particle. Two methods of detection readily lend themselves to monitoring these changes in the position of the LSPR: (i) UV-visible (UV-vis) extinction (absorption plus scattering); and (ii) resonant Rayleigh scattering spectroscopy.

Several papers have been published on a gold nanoparticle-based UV-vis technique for the detection of DNA. This colourimetric detection method is based on the change in absorbance spectra (i.e. colour) as particles are brought together by the hybridisation of complementary DNA strands [95, 96]. The limits of detection (LOD) reported are in the range of tens of femtomoles of target oligonucleotide. These nanoparticle aggregation assays represent a 100-fold increase in sensitivity over conventional fluorescence-based assays [77]. Recently, gold nanoshells, that is, silica beads with a thin gold coating, have been used to detect antigens in whole blood [22]. In these studies, gold nanoshells were functionalised with a specific immunoglobulin. The nanoshells were designed so that they exhibit plasmon resonances in the near-infrared between the water absorption band and the absorption of hemoglobin. Upon addition of the nanoshells to whole blood that contains the appropriate antigen, the plasmon resonance broadens, the intensity decreases, and a slight red shift in the plasmon resonance occurs. This immunoassay, which occurs in less than 30 min, is capable of detecting picogram/millilitre quantities of antigens. Sensing based on resonant Rayleigh scattering detection is a potentially more powerful, yet less well published, method than the colourimetric technique mentioned above [38, 97, 98]. Figure 5 demonstrates a simple sensing experiment, wherein the observed scattering colour changes as gold nanoparticles are drawn together by protein-protein interactions [99]. The disadvantages of this type of experiment is that the aggregation of the particles, necessary to induce interparticle coupling and generate a signal, is often irreversible, difficult to quantitate, and can lead to sufficient aggregation of the nanoparticles that they settle out of solution.

3.2 Nanoparticle labelling

Whereas the application of noble metal nanostructures as optical data storage elements is still under development, companies have already been formed to exploit noble metal nanostructures as biological labels. Nanoscale cylinders with stripes of different metals have been used as immunoassay tags; readout mechanisms have been based on both the extinction [98] and reflectivity [100]. In another example, Simman and Burshteyn [101] have applied thin coatings of Ag or Au to latex microspheres that then act as flow cytometry labels. The noble metal island films make it easy to identify different subpopulations of white blood cells without significantly changing the bead’s flow characteristics. When the labelled strand of DNA is exposed to a complimentary single strand of DNA immobilised on a glass substrate, the Au nanoparticle is bound to the surface. A flatbed scanner can be used to identify DNA matches.

Noble metal nanoparticles can go beyond acting as labels; recent advances show that changes in nanoparticle optical properties can act as the signal transduction mechanism in chemosensing and biosensing events. Haes et al. [26] and Malinsky et al. [84] have exploited the extreme LSPR sensitivity of NSL-fabricated nanoparticles to changes in local refractive index in order to sense small molecules, amino acids, proteins, and antibodies. The LSPR shifts systematically to lower energies as the local dielectric constant increases; accordingly, chemisorption of alkanethiols of increasing length cause a systematic red-shift.
in the LSPR (3 nm/methylene unit) [84]. These nanoparticle sensors take advantage of tunable noble metal nanostructure optical properties in order to sensitively and selectively detect trace levels of the target molecule. These experiments are further detailed below.

3.3 Sensing on surfaces
A possible method for circumventing the difficulty of irreversible aggregate formation is to synthesise nanoparticle clusters bound to substrates. Using this method, gold or silver nanoparticles can be chemically attached in a random fashion to a transparent substrate to detect proteins [32, 89]. In this format, signal transduction depends on changes in the nanoparticles’ dielectric environment induced by solvent or target molecules (not via nanoparticle coupling). Using this chip-based approach, a solvent refractive index sensitivity of 76.4 nm/RIU has been found and a detection of 16 nM streptavidin can be detected [32, 89]. This approach has many advantages including: (i) a simple fabrication technique that can be performed in most labs; (ii) real-time biomolecule detection using UV-vis spectroscopy; and (iii) a chip-based design that allows for multiplexed analysis.

3.4 Sensing with nanoparticle arrays
The development of biosensors for the diagnosis and monitoring of diseases, drug discovery, proteomics, and the environmental detection of biological agents is an extremely significant problem [102]. Fundamentally, a biosensor is derived from the coupling of a ligand-receptor binding reaction [103] to a signal transducer. Much biosensor research has been devoted to the evaluation of the relative merits of various signal transduction methods including optical [104, 105], radioactive [106, 107], electrochemical [108, 109], piezoelectric [110, 111] magnetic [112, 113], micromechanical [114, 115] and mass spectrometric [116, 117]. The development of large-scale biosensor arrays composed of highly miniaturised signal transducer elements that enable the real-time, parallel monitoring of multiple species is an important driving force in biosensor research. Recently, several research groups have begun to explore alternative strategies for the development of optical biosensors and chemosensors based on the extraordinary optical properties of noble metal nanoparticles. Recently, it was demonstrated that nanoscale chemosensors and biosensors can be realised through shifts in the LSPR $\lambda_{\text{max}}$ of triangular silver nanoparticles. These wavelength shifts are caused by adsorbate-induced local refractive index changes in competition with charge-transfer interactions at the nanoparticle surface. In this review, a detailed study is presented demonstrating that triangular silver nanoparticles fabricated by NSL function as extremely sensitive and selective nanoscale affinity chemosensors and biosensors. It will be shown that these nanoscale biosensors based on LSPR spectroscopy operate in a manner totally analogous to propagating surface plasmon resonance (SPR) sensors by transducing small changes in refractive index near the noble metal surface into a measurable wavelength shift response.

3.5 SPR sensors and their relationship to LSPR sensors
The potential of propagating SPR biosensors was recognised in early 1980s by Lidberg et al. [118], who were able to detect immunoglobulin antibodies by observing the change in critical angle when the antibodies bind selectively on a Au film. Propagating surface plasmons are evanescent electromagnetic waves that travel along the flat smooth metal-dielectric interfaces and arise from oscillations of the conduction electrons in the metal [119]. SPR sensors detect the local refractive index changes that occur when the target analyte binds to the metal film or nanoparticles. Surface refractive index sensors have an inherent advantage over optical biosensors that require a chromophoric group or other label to transduce the binding event, as in the labelling experiments described above.

It was realised that the sensor transduction mechanism of this LSPR-based biosensor is analogous to that of SPR sensors (Table 1). Since their original discovery, SPR changes have been used in refractive-index-based sensing to detect analyte binding at or near a metal surface and has been widely used to monitor a broad range of analyte-surface binding interactions including the adsorption of small molecules [120–122], ligand-receptor binding [123–126], protein adsorption on self-assembled monolayers [127–129], antibody-antigen binding [130], DNA and RNA hybridisation [131–134] and protein-DNA interactions [135].

Important differences to appreciate between the SPR and LSPR sensors are the comparative refractive index sensitivities and the characteristic electromagnetic field decay lengths. SPR sensors exhibit large refractive index sensitivities ($\sim 2 \times 10^6$ nm/RIU) [120]. For this reason, the SPR response is often reported as a change in refractive index units. The LSPR nanosensor, on the other hand, has a modest refractive index sensitivity ($\sim 2 \times 10^3$ nm/RIU) [84]. Given that this number is four orders of magnitude smaller for the LSPR nanosensor in comparison to the SPR sensor, initial assumptions were made that the LSPR nanosensor would be 10 000 times less sensitive than the SPR sensor. This, however, is not the case. In fact, the two sensors are very competitive in their sensitivities. The short (and tunable) characteristic electromagnetic field decay length $l_d$ provides the LSPR nanosensor with its enhanced sensitivity [26, 27]. These LSPR nanosensor results indicate that the decay length, $l_d$, is $5–15$ nm or $\sim 1–3\%$ of the light’s wavelength and depends on the size, shape, and composition of the nanoparticles. This differs greatly from the 200–300 nm decay length or $\sim 15–25\%$ of the light’s wavelength for the SPR sensor [120]. Also, the smallest footprint of the SPR and LSPR sensors differ. In practice, SPR sensors require at least a $10 \times 10 \mu$m area for sensing experiments. For LSPR sensing, this spot size can be minimised to a large number of individual sensing elements ($1 \times 10^{10}$ nanoparticles for a $2 \mu$m spot size, nanosphere diameter = 400 nm) down to a single nanoparticle (with an in-plane width of $\sim 20$ nm) using single nanoparticle measurement techniques [81]. The nanoparticle approach can deliver the same information as the SPR sensor, thereby minimising its effective pixel size to the sub-100 nm regime. Because of the lower refractive index sensitivity, the LSPR nanosensor requires no temperature control whereas the SPR sensor (with a large refractive index sensitivity) requires temperature control. The final and most dramatic difference between the LSPR and SPR sensors is cost. Commercialised SPR instruments can vary between $150 000–300 000$, whereas the prototype and portable LSPR system costs less than $5 000$.

There is, however, a unifying relationship between these two seemingly different sensors. Both sensors’ overall response can be described using the following equation [120]:

$$\Delta \lambda_{\text{max}} = m \Delta n (1 - \exp(-2d/l_d))$$

where $\Delta \lambda_{\text{max}}$ is the wavelength shift response, $m$ is the refractive index sensitivity, $\Delta n$ is the change in refractive index induced by an adsorbate, $d$ is the effective adsorbate...
layer thickness, and $l_d$ is the characteristic electromagnetic field decay length. It is important to note that for planar SPR sensors, this equation quantitatively predicts an adsorbate’s effect on the sensor. When applied to the LSPR nanosensor, this exponential equation approximates the response for adsorbate layers but does provide a fully quantitative explanation of its response [26, 27]. Similar to the SPR sensor, the LSPR nanosensor’s sensitivity was realised to arise from the distance dependence of the average induced square of the electric fields that extend from the nanoparticles’ surfaces.

### 3.6 Real-time binding studies of SPR and LSPR sensors

To experimentally evaluate the relative performance of the two sensors, side by side comparisons were conducted. The exact procedures followed the standard experimental protocols established for the two sensing modalities [145].

For the SPR sensor, Au (50 nm) was evaporated onto glass coverslips with a thin Ti underlayer (10 nm). A BIAcore 1000 (Neuchâtel, Switzerland) was used for all propagating SPR measurements reported. The mannose-functionalised substrate was incorporated into BIAcore cassettes by gluing the chip into the cassettes, and measurements were reported as changes in resonance angle ($\Delta \theta$), where $1 \sim 10,000$ RU. The LSPR sensors were fabricated using NSL to create Ag nanoparticle arrays (50 nm). LSPR extinction measurements were taken using a fibre optically-coupled spectrometer. All spectra in this study were from macroscopic measurements obtained in transmission mode using unpolarised white light. A home-built flow cell was used to control the surrounding environment of the Ag nanoparticles and the introduction of analytes [84].

The self-assembled monolayer (SAM) was prepared by immersing the coverslips in an ethanolic solution containing maleimide-terminated disulphide (1 mM) and 11-mercaptoundecyl tri(ethylene glycol) disulphide (EG3, 1 mM). After 12 h, the coverslips were washed with ethanol and dried under a stream of nitrogen. The substrates presenting maleimide-functional groups were immersed in methanolic solutions of 5 mM mannose thiol for 40 min, providing $\sim 5\%$ sugar-immobilised surfaces. The mannose-functionalised sensors were then exposed to 19 $\mu$M concanavalin A (Con A) in PBS buffer for 20 min (Fig. 6).

### 3.7 Real-time comparison of LSPR and SPR detection of Con A

To directly compare the sensing capabilities of SPR and LSPR sensors, the real-time response of Con A binding to a mannose-functionalised flat surface SPR sensor (Fig. 6a) and the LSPR Ag nanosensor (Fig. 6b) were investigated. Con A is a 104 kDa mannose-specific plant lectin comprised of a tetramer with dimensions of 6.32, 8.69, and 8.93 nm and has four binding sites [146]. During the association phase, both the SPR and LSPR response of Con A binding to the mannose-functionalised Ag nanosensor was also probed. After the LSPR $\lambda_{\max}$ of the mannose-functionalised surface was recorded, 19 $\mu$M Con A in buffer was injected. The sensor was then flushed with buffer to remove both non-specifically bound and Con A bound as the 1:1 mannose complex. Figure 6a illustrates the real-time monitoring of 19 $\mu$M Con A by the SPR $\Delta \theta$ shift. Similarly, the real-time LSPR response of Con A binding to the mannose-functionalised Ag nanosensor was also probed. After the LSPR $\lambda_{\max}$ of the mannose-functionalised surface was recorded, 19 $\mu$M Con A was injected into the flow cell, then the sample was flushed with PBS buffer, and during this process the LSPR $\lambda_{\max}$ was measured in 5 s intervals for 20 min (Fig. 6b).

During the association phase, both the SPR and LSPR sensor showed a rapid response when Con A was exposed to the surface, which indicates strong Con A binding using two binding sites on the surface followed by weak 1:1 binding Con A binding as well as non-specific binding.
However, during the dissociation phase, when the Con A-bound SPR sensor surface was flushed with PBS buffer, the response decreased by 60% whereas the response decreased only by 14% for the LSPR sensor. It is hypothesised that this difference is the long-range decay length of the SPR sensor electromagnetic field ($l_d \approx 200 \text{ nm}$) [120], compared to that of the LSPR sensor ($l_d \approx 5-6 \text{ nm}$) [26, 27].

### 3.8 LSPR streptavidin and immunoglobulin sensing on nanoparticle arrays

As described above, nanoscale chemosensing and biosensing could be realised through shifts in the LSPR extinction maximum ($\lambda_{\text{max}}$) of these triangular silver nanoparticles [20, 23, 28, 84]. Signal transduction is an effect caused by shifts in wavelength produced by adsorbate-induced local refractive index changes in competition with charge-transfer interactions at the surfaces of nanoparticles. For these experiments, NSL was used to fabricate 25 and 50 nm Ag nanoparticles on glass or mica. The peak-to-peak wavelength shift noise of the baseline in repetitive experiments from this spectrometer is $\sim 0.5 \text{ nm}$. Taking the limit of detection as three times this value, one can conservatively estimate the limit of detection of an assay will arise from a wavelength shift of 1.5 nm.

### 3.9 Streptavidin sensing using LSPR spectroscopy

The well-studied biotin-streptavidin system with its extremely high binding affinity ($K_d \sim 10^{15} \text{ 1/M}$) is chosen to illustrate the ultra-sensitive attributes of these LSPR-based nanoscale affinity biosensors. The biotin-streptavidin system has been studied in great detail by SPR spectroscopy and serves as an excellent model system for the LSPR nanosensor [20]. Streptavidin, a tetrameric protein, can bind up to four biotinylated molecules (i.e. antibodies, inhibitors, nucleic acids, etc.) with minimal impact on its biological activity and therefore will provide a ready pathway for extending the analyte accessibility of the LSPR nanobiosensor.

In the streptavidin detection scheme, the $\lambda_{\text{max}}$ of the Ag nanoparticles were monitored during each surface functionalisation step (Fig. 7) [20]. First, the LSPR $\lambda_{\text{max}}$ of the bare Ag nanoparticles was measured to be 561.4 nm (step A in Fig. 7). To ensure a well-ordered SAM on the Ag nanoparticles, the sample was incubated in a 3:1 Octanethiol : mercaptoundecanoic acid (OT/MUA) for 24 h. The LSPR $\lambda_{\text{max}}$ (step B in Fig. 7) was measured to be 598.6 nm, a 38 nm red shift. Next, biotin was covalently attached via amide bond formation to carboxylated surface sites (step C in Fig. 7). The LSPR $\lambda_{\text{max}}$ after biotin attachment (step C in Fig. 7) was measured to be 609.6 nm, corresponding to an additional +11 nm shift. Exposure to 100 nM streptavidin, resulted in LSPR $\lambda_{\text{max}} = 636.6 \text{ nm}$ (step D in Fig. 7) corresponding to an additional +27 nm shift.

### 3.10 Anti-biotin sensing using LSPR spectroscopy

A field of particular interest is the study of the interaction between antigens and antibodies. For these reasons we have chosen to focus the present LSPR nanobiosensor study on the prototypical immunosassay involving biotin and antibiotin, an IgG antibody. Ag nanotriangles were synthesised using NSL to develop a LSPR biosensor that monitors the interaction between a biotinylated surface and free antibiotin in solution [28]. The importance of this study is that it demonstrates the feasibility of LSPR biosensing with a biological couple whose binding affinity is significantly...
lower ($1.9 \times 10^{-6} - 4.98 \times 10^{-8} \text{ M}$) than in the biotin/streptavidin model [20].

Each step of the functionalisation of the samples was monitored using UV-vis spectroscopy, as shown in Fig. 8 [28]. After a 24 h incubation in SAM, the LSPR extinction wavelength of the Ag nanoparticles was measured to be 670.3 nm (step A in Fig. 8). The LSPR wavelength shift due to biotin binding was measured to be $+12.7 \text{ nm}$, resulting in a LSPR extinction wavelength of 683.0 nm (step B in Fig. 8). At this stage, the nanosensor was ready to detect the specific binding of anti-biotin. Incubation in 700 nM anti-biotin resulted in a LSPR wavelength shift of $+42.6 \text{ nm}$, giving a $\lambda_{\text{max}}$ of 725.6 nm (step C in Fig. 8).

### 3.11 Concentration-dependent response

It was revealed that if the analyte solution was decreased, the LSPR wavelength shift response also decreased. Subsequently, the full response of the sensor was determined over a wide concentration range. For this reason, variable analyte concentrations were exposed to a biotinylated LSPR chip to test the sensitivity of the system to different molecules. Specifically, the LSPR $\lambda_{\text{max}}$ shift, $\Delta R$, against analyte concentration response curve was measured over the concentration range $1 \times 10^{-15} \text{ M} < [\text{streptavidin}] < 1 \times 10^{-10} \text{ M}$ and $7 \times 10^{-10} \text{ M} < [\text{anti-biotin}] < 7 \times 10^{-6} \text{ M}$ (Fig. 9) [20, 28]. The lines seen in Fig. 9 are not a fit to the data. Instead, the line was computed from a response model (a complete analysis of this model is described in [28]). It was found that this response could be interpreted quantitatively in terms of a model involving: (i) 1:1 binding of a ligand to a multivalent receptor with different sites but invariant affinities; and (ii) the assumption that only adsorbate-induced local refractive index changes were responsible for the operation of the LSPR nanosensor.

The binding curve provides three important characteristics regarding the system being studied. First, the mass and dimensions of the molecules affect the magnitude of the LSPR shift response. Comparison of the data with theoretical expectations yielded a saturation response, $\Delta R_{\text{max}} = 26.5 \text{ nm}$ for streptavidin, a 60 kDa molecule, and 38.0 nm for anti-biotin antibody, a 150 kDa molecule. Clearly, a larger mass density at the surface of the nanoparticle results in a larger LSPR response. Next, the surface-confined thermodynamic binding constant $K_a,\text{surf}$ can be calculated from the binding curve and is estimated to be $1 \times 10^{11} \text{ M}^{-1}$ for streptavidin and $4.5 \times 10^{7} \text{ M}^{-1}$ for anti-biotin antibody. These numbers are directly correlated to the third important characteristic of the system, the LOD. The LOD is less than 1 pM for streptavidin and 100 pM for anti-biotin antibody. As predicted, the LOD of the nanosensor studied is lower for systems with higher binding affinities such as for the well-studied biotin-streptavidin couple and higher for systems with lower binding affinities as seen in the anti-biotin antibody system. Given this information and analysis, a similar treatment can be made for virtually any ligand receptor system. It should be noted that the LOD of the system corresponds to the smallest reliable wavelength shift response induced by a given solution concentration. These ‘real’ LODs are often translated to the commonly reported surface coverage in terms of molecules. For the LSPR nanosensor, the surface coverage detection corresponds to $\sim 25$ streptavidin molecules per nanoparticle. In the previously described experiments, $\sim 10^7$ nanoparticles were probed (nanoparticle density $\sim 1 \times 10^{10}$ nanoparticles per square centimetre with a spot size with a 1 mm radius), and this corresponds to the detection of a $75 \times 10^7$ streptavidin molecules. A clear method to decrease the number of molecules detected would be to decrease the number of nanoparticles probed.

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**Fig. 8** LSPR spectra for each step of the preparation of the Ag nanobiosensor, and the specific binding of anti-biotin to biotin. Step A: Ag nanoparticles after modification with 1 mM 3-11MTA, $\lambda_{\text{max}} = 670.3$ nm; Step B: Ag nanoparticles after modification with 1 mM biotin, $\lambda_{\text{max}} = 683.0$ nm; and Step C: Ag nanoparticles after modification with 700 nM anti-biotin, $\lambda_{\text{max}} = 725.6$ nm. All spectra were collected in a N₂ environment. Reproduced with permission from [28]. Copyright 2003, The American Chemical Society.

**Fig. 9** The specific binding of streptavidin (left) and anti-biotin (right) to a biotinylated Ag nanobiosensor is shown in the response curves. All measurements were collected in a N₂ environment. The solid line is the calculated value of the nanosensor’s response. Reproduced with permission from [20]. Copyright 2002, The American Chemical Society. Reproduced with permission from [28]. Copyright 2003, The American Chemical Society.
This has been recently demonstrated as described in greater detail in a later Section.

3.12 Detection of disease markers using the LSPR sensor chip

Alzheimer’s disease is the leading cause of dementia in people over age 65 and affects an estimated 4000000 Americans [148]. Although first characterised almost 100 years ago by Alois Alzheimer, who found brain lesions now called plaques and tangles in the brain of a middle-aged woman who died with dementia in her early fifties [149], the molecular cause of the disease is not understood; and an accurate diagnostic test has yet to be developed. However, two inter-related theories for Alzheimer’s disease have emerged that focus on the putative involvement of neurotoxic assemblies of a small 42-amino acid peptide known as amyloid beta (Aβ) [150, 151]. The widely investigated ‘amyloid cascade’ hypothesis suggests that the amyloid plaques cause neuronal degeneration and, consequently, memory loss and further progressive dementia. In this theory, the Aβ protein monomers, present in all normal individuals, do not exhibit toxicity until they assemble into amyloid fibrils [152]. The other toxins are known as Aβ-derived diffusible ligands (‘ADDLs’). ADDLs are small, globular, and readily soluble, 3-24mers of the Aβ monomer [153] and are potent and selective central nervous system neurotoxins which possibly inhibit mechanisms of synaptic information storage with great rapidity [153]. ADDLs now have been confirmed to be greatly elevated in autopsied brains of Alzheimer’s disease subjects [154]. An ultrasensitive method for ADDLs/anti-ADDLs antibody detection potentially could emerge from LSPR nanosensor technology, providing an opportunity to develop the first clinical lab diagnostic for Alzheimer’s disease. Preliminary results indicate that the LSPR nanosensor can be used to aid in the diagnosis of Alzheimer’s disease [155, 156].

3.13 Sensing with single nanoparticles

The extension of the LSPR sensing technique to the single nanoparticle limit provides several improvements over existing array- or cluster-based techniques [157, 158]. First, absolute detection limits are dramatically reduced. The surface area of chemically prepared Ag nanoparticles is typically less than 20000 nm², which requires that a complete monolayer of adsorbate must constitute fewer than approximately 100 zeptomol. The formation of alkanethiol monolayers on Ag nanoparticles can result in a LSPR $\lambda_{\text{max}}$ shift of greater than 40 nm, a change that is over 100 times larger than the resolution of conventional UV-visible spectrometers [159]. This suggests that the limit of detection for single nanoparticle-based LSPR sensing will be well below 1000 molecules for small molecule adsorbates. For larger molecules such as antibodies and proteins that result a greater change in the local dielectric environment upon surface adsorption, the single molecule detection limit may be achievable [28]. Second, the extreme sensitivity of single nanoparticle sensors dictates that only very small sample volumes (i.e. attolitres) are necessary to induce a measurable response. This characteristic could eliminate the need for analyte amplification techniques (e.g., the polymerase chain reaction) required by other analytical methods. Third, single nanoparticle sensing platforms are readily applicable to multiplexed detection schemes. By controlling the size, shape, and chemical modification of individual nanoparticles, multiple sensing platforms can be generated in which each unique nanoparticle can be distinguished from the others based on the spectral location of its LSPR [81, 157, 158]. Several of these unique nanoparticles may then be incorporated into a one device, allowing for the rapid, simultaneous detection of thousands of different chemical or biological species.

The key to exploiting single nanoparticles as sensing platforms is developing a technique to monitor the LSPR of individual nanoparticles with a reasonable signal-to-noise ratio [81]. UV-visible absorption spectroscopy does not provide a practical means of accomplishing this task. Even under the most favourable experimental conditions, the absorbance of a single nanoparticle is very close to the shot noise-governed limit of detection. Instead, resonant Rayleigh scattering spectroscopy is the most straightforward means of characterising the optical properties of individual metallic nanoparticles.

3.14 Experimental procedure for single nanoparticles

Colloidal Ag nanoparticles were prepared by reducing silver nitrate with sodium citrate in aqueous solution according to the procedure referenced above [40]. Immobilised particles on cover slips were inserted into a flow cell and were exposed to various dielectric environments or molecular adsorbates [81]. Prior to all experiments, the nanoparticles in the flow cell were repeatedly rinsed with methanol and dried under nitrogen. All optical measurements were performed using an inverted dark-field microscope equipped with an imaging spectrograph [81, 82]. The apparatus used in these experiments is shown in Fig. 10.

![Diagram of the instrumentation used for single nanoparticle spectroscopy.](image)

Fig. 10

a) Diagram of the instrumentation used for single nanoparticle spectroscopy.
b) Close-up of the flow cell to show illumination and collection geometry.

3.15 Single nanoparticle refractive index sensitivity

The local refractive index sensitivity of the LSPR of a single Ag nanoparticle was measured by recording the resonant Rayleigh scattering spectrum of the nanoparticle as it was exposed to various solvent environments inside the flow cell. As illustrated in Fig. 11, the LSPR $\lambda_{\text{max}}$ systematically shifts to longer wavelength as the solvent RIU is increased. Linear regression analysis for this nanoparticle yielded a refractive index sensitivity of 203.1 nm RIU⁻¹. The refractive index sensitivity of several individual Ag nanoparticles was measured and typical values were determined to be 170–235 nm RIU⁻¹ [81]. These are similar to the values obtained from experiments utilising arrays of NSL fabricated triangular nanoparticles [26, 84].
Streptavidin sensing with single nanoparticles

In nanoparticle array sensing, it has been demonstrated that it is possible to detect $< 100$ streptavidin molecules per nanoparticle (in a solution concentration of 1 pM). The biology community would like to reduce the amount of biological sample needed for an assay without amplification. For this reason, experiments have been conducted to detect streptavidin on single Ag nanoparticles [82]. After functionalisation with a capture biomolecule, the LSPR of an individual nanoparticle was measured to be 508.0 nm (curve (i) in Fig. 12). Next, 10 nM streptavidin was injected into the flow cell, and the $\lambda_{\text{max}}$ of the nanoparticle was measured at 520.7 nm, curve (ii) in Fig. 12. This +12.7 nm shift is estimated to arise from the detection of less than 700 streptavidin molecules. It is hypothesised that just as in the array format, as the streptavidin concentration decreases, a fewer number of streptavidin molecules will bind to the surface thereby causing smaller wavelength shifts.

Theoretical explanation of LSPR sensing

To model the experiment results presented above, the discrete dipole approximation (DDA) method [160, 161] a finite element-based approach to solving Maxwell’s equations for light interacting with an arbitrary shape/composition nanoparticle was used to calculate the plasmon wavelength in the presence or absence of an adsorbate with a wavelength-dependent refractive index layer thickness. Bare silver nanoparticles with a truncated tetrahedral shape were first constructed from cubic elements and one to two layers of the adsorbate were added to the exposed surfaces of the nanoparticle to define the presence of the adsorbate. All calculations refer to silver nanoparticles with a dielectric constant taken from Lynch and Hunter [162].

In this treatment the dielectric constant is taken to be a local function, as there is no capability for a non-local description within the DDA approach. There have been several earlier studies in which the DDA method has been calibrated by comparison with experiment for truncated tetrahedral particles, including studies of external dielectric effects and substrate effects [11, 71, 163] and based on this it is expected that DDA analysis will provide a useful qualitative description of the results. In particular, it has been demonstrated that the plasmon resonance shift close to the nanoparticle surface is dominated by hot spots while that farther away arises from colder regions around the nanoparticle surface. By comparing the overall maximum LSPR shifts of the nanoparticles, it was shown that increasing the aspect ratio of Ag nanotriangles produces larger plasmon resonances shifts and shorter-ranged interactions.

This behaviour can be easily compared to lightning rods (invented by Ben Franklin) which are placed at the top of a building, and are designed to provide a location where lightning will strike and from which the energy is carried harmlessly to the ground. A needle-like metal tip is used in a lightning rod, since this leads to high electric fields just above the tip when the building is charged relative to the atmosphere above it, providing a more likely location for lightning to strike. A similar description can be used to describe light’s interaction with tips of nanoscale silver and gold nanotriangles [26, 27]. This property is best displayed in Fig. 12.
in Fig. 13. This Figure was generated using the DDA method. This leads to fields around the nanoparticles whose magnitudes relative to the incident light are plotted in the Figure. The results show that the electromagnetic fields are greatly amplified (up to 65 000 times) in the region near the nanoparticle tips, leading to enhanced sensitivity to molecules that might be located there. This information can be used as a basis for constructing nanoparticle structures and understanding the sensing mechanism for surface-enhanced spectroscopies.

4 Biological applications of surface-enhanced Raman scattering

Inelastic photon scattering was first witnessed by Raman and Krishnan in 1928 [164] a process later to bear the name Raman scattering and to garner the authors a Nobel Prize in 1930. In the original Nature paper [164] the inelastic scattering effect was recognised by ‘its feebleness in comparison with ordinary (Rayleigh) scattering, and by its polarisation.’ Raman was able to observe this phenomenon by focusing a beam of sunlight through a telescope and a matic light, enabled the more practical detection of the one light scattered from chloroform. The development of the polarisation. Raman was able to observe this phenomenon comparison with ordinary (Rayleigh) scattering, and by its scattering effect was recognised by ‘its feebleness in

Fig. 13 Local E-field (plotted as contours of log(E²)) for a Ag nanoparticle (in-plane width = 100 nm, out-of-plane height = 30 nm). Reproduced with permission from [26]. Copyright 2004, The American Chemical Society

The electromagnetic effect gives rise to an enhanced Raman yield for a given molecular polarisability, whereas the chemical effect refers to a change in the polarisability itself. The theory of chemical enhancement (a) was invoked to account for a discrepancy between the enhancement predicted the electrical model (E) (10⁶) and the empirically determined enhancement (10⁸). It is thought that ‘active sites’, either crystal defects or adatoms on the surface of the metal surface undergo a charge-transfer process enabling the CHEM (a) enhancement [170]. The dependence of optimal SERS excitation frequency on the electrode potential suggests that new electronic states may arise upon adsorption of the analyte, which leads to resonant intermediate states for Raman scattering. The CHEM enhancement has been investigated, particularly the effect of halide ions on increasing SERS [173–176]. The recent discovery of single molecule SERS (SMSERS) by Nie and Emory [177] and Kneipp et al. [178] in 1997, has necessitated further refinements to the EM mechanism.

It is well established that the EM mechanism arises from optical excitation of surface plasmon resonances in small metal particles, which leads to a significant increase in the electromagnetic field strength at the particle surface. Initially, Moskovits [179] developed a resonant fractal theory to explain surface Raman enhancement in extensively aggregated colloids. For single particles and small aggregates, however, the case can be dramatically different than for an ensemble average of particles. Electromagnetic enhancement is attributable to the formation a LSPR, as described above. This polarises the particle with an electromagnetic field significantly higher than the applied field. Recent theoretical modelling, has greatly extended the nature of the plasmonic material (e.g. particle morphology, spacing, and coupling) beyond the simple spherical model developed by Mie, and is more useful in describing actual experimental conditions. The intensified field at rough or highly-curved surfaces enhances both the incident laser light
cation of small molecule drugs, and so forth. Reviews of these applications have recently been written by Cotton et al. [166], Nabiev et al. [167], and Petry et al. [168]. However, the low signal intensities and complications with spectral identification caused by the generation of a spectral response all species in a complex mixture and the concomitant confusion generated by closely spaced or overlapping Raman bands unfortunately hinder application of NRS. SERS can increase the scattering cross-section of a molecule in proximity to a nanoscale roughened surface by 10⁶ to 10¹⁴ times, potentially mitigating the two aforementioned disadvantages of NRS [169, 170]. SERS is a plasmonic phenomenon whereby specific molecules at or near a nanoscale roughened metal surface undergo a dramatic increase in the intensity of the observed inelastically scattered light. Since the discovery of SERS by Jeanmaire and Van Duyne [171] the exact mechanism of enhancement has remained controversial [172, 173]. However, it is generally agreed that two mechanisms contribute to the overall enhancement, a chemical effect (CHEM, a) and an electromagnetic (EM, E) enhancement. The intensity of Raman scattering is proportional to the square of P, the electric field-induced dipole moment. The P term is related to the molecular polarisability (a) and the electric field (E) experienced by the molecule. These relationships are mathematically expressed thus:

\[ G \propto P^2 \quad (4) \]

and

\[ P = aE \quad (5) \]

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and the scattered Raman light. Due to the dependence of the SERS signal intensity on the fourth-power of the EM field, the signal drops by a factor of ten for each nanometre separation between the molecule and the surface [180–182]. This electromagnetic enhancement can be qualitatively conceptualised as an ‘antenna’ effect, gathering and strengthening local electric fields.

4.1 Intrinsic SERS of target molecules
SERS provides not only enhanced signal intensities but also species specific spectra, so it is therefore unsurprising that many SERS experiments have been conducted that attempt to directly address molecules of biological interest and their chemical interactions. The first such experiment was conducted in 1980 by Cotton et al. on cytochrome c and hemoglobin, molecules with strong electronic absorbances in the visible. These intense absorbances were matched with the laser excitation wavelength, $\lambda_{ex}$, to eke out a few orders of magnitude extra enhancement by exploiting surface-enhanced resonance Raman spectroscopy (SERRS). The resonance effect has continued to make hemoglobin and cytochrome c popular proteins for study, particularly since the advent of SMSERS [183, 184]. In the same year as Cotton’s pioneering experiments, Billman and Otto [185] also demonstrated the successful detection of cyanide on roughened silver electrodes [188]. Cyanide, while not a biomolecule as such, is nonetheless of great interest in both biological, environmental, and defence circles in the detection of cyanides and similar molecules which have a direct impact on biological systems. As another example, SERS has been used on the bio-terror front for the detection of anthrax [186]. SERS has been demonstrated to successfully detect dipicolinic acid, the principle chemical component of $bacillus anthracis$ spores (15% wt) [187]. Since the 1980s, the number of biological applications of SERS (and NRS) has skyrocketed [166–168, 188–194]. Indeed significant strides have been made in the Raman spectroscopy of proteins and peptides using NRS, and progress continues as the use of SERS expands. SERS techniques have been applied to an impressive variety of systems [193, 195–207]. Initially, most SERS experiments were conducted on electrochemically roughened surfaces. However, since the discovery of SMSERS, researchers have increasingly tended to use colloidal systems as they are easy to prepare chemically and to model theoretically. Exceptions are common, however, Maxwell et al. [208] employed size-selected nanoparticle films to study amoxicillin and adenine, and the metal film-over-nanosphere (MFON) surfaces remain popular for their mechanical stability [209]. Remarkably, Kneipp et al. [210] have been able to obtain SERS spectra from gold nanoparticles sited within single living cells. Recently, Jarvis and Goodacre [211] have used SERS on aggregated silver colloids in combination with chemometric techniques to classify a collection of clinical bacterial isolates. SERS can also be used to study molecular interactions and dynamics, such a protein interactions [212–214] and pharmacokinetics [162, 215, 216]. Naturally, both SERS and NRS lend themselves to sensitive detection modalities for separation science [216–219].

4.2 SMSERS
Despite its tremendous power and potential the capricious nature of SMSERS has resulted in only a few publications on its biological application. In 1998, Kneipp et al. [220] were the first to successfully obtain SERS spectra from single nucleoside bases building on their experience with not only SMSRS [178] but also SERS detection of DNA [221, 222]. Shortley afterwards, Keller et al. [223] and Emory and Keller [224] brought SMSERS to bear on nucleosides. Recently, Xu et al. [225] have returned to the use of hemoglobin as the SERS analyte, but at the single molecule level. This 1999 study was augmented a year later by an excellent manuscript covering the contribution of the EM mechanism of SMSERS [226]. Since then, these researchers have combined SMSERS with fluorescence correlation spectroscopy to study HRP: a highly useful component in the bioanalytical toolkit [227]. SMSERS on myoglobin has reported by Bizzarri and Cannistraro [228] while Habuchi et al. [229] obtained SERRS spectra from the green fluorescent protein.

4.3 Limitations to intrinsic SERS
There are two considerable difficulties in applying SERS directly to biological problems. First, without resonance enhancement (as in the case of cytochrome c) it is still often difficult to obtain quality Raman spectra of biologically interesting molecules (e.g. drugs, protein, nucleic acids, etc.), particularly at the relatively low concentrations often necessitated by the expense or insolubility of the analyte. Even although SERS is potentially sensitive for single molecule spectroscopy, not all molecules, such as water, are efficient SERS scatterers. Also many molecules neither adsorb nor bind to the SERS active surface, making them difficult to detect, given the consequent lack of chemical (CHEM) enhancement and greatly diminished electromagnetic enhancement (EM). Approximately one-order of magnitude in SERS intensity is lost per nanometre distance the target molecule is from the enhancing substrate, making the detection of certain molecules, such as some ionic species, carbohydrates, etc., particularly problematic. Second, large macromolecules and numerous small molecules of biological importance have complex Raman spectra, and often have a number of overlapping peaks due to structural similarities in the molecule. For example all biomacromolecules are built from a limited set of building blocks; the approximately 20 amino acids for proteins, and eight nucleic acids for DNA and RNA. Teasing out specific information about a given biomacromolecule and its composition, conformation, or interactions is a particularly daunting, but not impossible task. The problem becomes particularly difficult in complex mixtures, which are the norm for biologically interesting systems such as environmental samples from contaminated waste sites, and the protein jungle of the cytosol.

4.4 Extrinsic SERS labelling
Several approaches have been reported to address the difficulties with direct SERS-based detection of biologically relevant targets. An attractive option is to indirectly detect the presence of interaction of a complex and/or weakly scattering molecule by labelling it with a small molecule with high intensity, well defined, and characteristic spectral features [230, 231]. The extrinsic labelling schemes described below are all variations on the ELISA (enzyme-linked immunosorbent assay). A graphical summary of the various extrinsic SERS methods is shown in Fig. 14. In a standard ELISA, molecular binding events are detected using antibodies (Abs) possessing a high affinity for the molecule of interest, and which are covalently bound to an enzyme (e.g. HRP). Once the target molecule is immobilised on a surface, the target is exposed to the detection AB/enzyme complex. The substrate for the enzyme is then introduced, and the enzymatic reaction allowed to occur. Typically the product of the enzymatic reaction is then detected by a change in a physical property, such a colour for HRP/OPD (ortho-phenylenediamine). The amount of target is
but more optimally enhancing 63 nm gold nanoparticles, improvements were realised for this method by using larger, molecules and Ab could be used for immunoassay readout. showed that colloidal gold with co-adsorbed organic multiplexed SERS detection of labelled polymerase chain reaction products, and Ni et al. [238] and Pham et al. [239] showed that colloidal gold with co-adsorbed organic molecules and Ab could be used for immunoassay readout. Improvements were realised for this method by using larger, but more optimally enhancing 63 nm gold nanoparticles, which had stronger spectral intensities and were intense enough to permit single particle imaging [240]. Innovative results have also been reported by Mulvaney et al. [241] and Doering and Nie [175] who prepared silica encapsulated nanoparticles for use in multiplexed bioassays. These encapsulated particles are stable in the presence of high salt (common in most biological buffers), and the silica shell protects not only the particle from the environment, but also the environment from the potentially toxic Raman reporter. In a significant departure from traditional SERS substrates, Cao et al. [242] prepared SERS-active nanostructures by seeded growth of silver on gold and demonstrated that these structures are useful for multiplexed nucleic acid detection. The seeded growth method was extended to protein detection and labelling in [243] and cell and tissue labelling in lab [99]. Both methods use small gold nanoparticles associated with both a reporter molecule for signal specificity and protein to confer biological activity [244]. After binding of the bioconjugates to the target, electroless deposition is employed to develop a shell of silver metal around the nanoparticle seed, in a process confusingly referred to as ‘silver enhancement’. The silver layer forms a complex fractal surface, providing the nanoscale roughness necessary for efficient SERS. Whereas Cao et al. [242] synthesised reporter-labelled DNA/protein conjugates, the method developed by Stuart et al. [99] functionalises amine reactive dye molecules directly onto existing protein gold bioconjugates. This later method not only is simpler and substantially less expensive, but also leads to higher reporter and protein levels which increases both the conjugate’s binding capacity and observed signal intensity.

4.5 Spectroscopic labelling of biological samples with enhanced probes

Since Raman scattering can be semi-quantitative, and does not photobleach, this method may prove useful to many biomedical researchers, for example, to those studying cancer. As a test bed, the technology was applied to tissue sections in a microarray format. Colon cancer/normal tissue microarrays were purchased from Zymed Laboratories Inc., and subsequently deparaffinised and re-hydrated according to the manufacturer’s protocol. Primary antibody (mouse anti-carcinoembryonic antigen, CEA) conjugation was achieved by following the manufacturer’s protocol, which could readily be automated. Secondary labelling with malachite green/anti-mouse gold particles and subsequent silver enhancement was performed as per standard immunogold and silver staining practice [245]. The limiting factor is not the spectroscopy, which is capable of single molecule sensitivity and large number of strongly SERS-active reporters for multiplexing, but the availability of suitably specific biomolecules. Fortunately, the CEA antibody binds to a wide range of colon cancer types, but has minimal interaction with normal tissue. Figure 15 shows the ability of the technology to discriminate between the cancerous tissues (red line) and tissues taken near (adjacent mucosa, blue line) and far from the tumor site (remote mucosa, black line). By recording the spectrum of the marking tissue, the ‘hump’ was eliminated by background subtraction. Also, the silver staining itself provides a rapid method of determining if particles were successfully bound, and serves as a secondary method of staining tissues [246]. Figure 15 shows, however, that the level of staining required to generate a SERS signal is quite low. To the unaided eye, there is no difference between the normal (Fig. 15b) and the cancer tissue sections (Fig. 15c). Even under microscopic examination, correctly identifying the silver-enhanced sections of the tissue can require skill and training. Spectroscopic labelling provides a rapid and unambiguous
differentiation, and the operator familiarisation time for the instrument would be minimal.

4.6 SERS using functionalised substrates
A secondary method of overcoming the difficulty in the direct SERS detection of problematic analytes is to employ a functionalised SERS surface. By coating the SERS active substrate with a SAM [247], hydro-gel [248] or polymer [249] substantial gains in signal can be realised from analytes whose SERS spectra are otherwise difficult or impossible to obtain. Whereas the specifics of the interactions between the functionalised surface and the complex mixture are inherently unique to each particular combination of analyte and surface, two general mechanisms operate simultaneously to provide the improved signal.

First, the material coating the SERS substrate can act as a barrier to undesired analytes. In this sense, the coating layer acts as a chemical and physical filter. For example, large species may be effectively excluded by controlling the pore size on a polymer substrate coating, or cations may be repelled by using a charged SAM bound to a SERS active surface. Stokes et al. [249] recognised the potential of combining polymers with SERS. The technique of SAM functionalisation has been largely pioneered by [203, 218, 250–252] who have used the selectivity of the SAM in the analysis of bilirubins and salicylate from whole blood, and drug compounds amongst other targets. From a biomedical and device perspective, perhaps the most interesting application of this selective SAM approach has been the detection glucose by the collaborative efforts at Northwestern University [253, 254].

4.7 SERS detection of glucose
The NIH estimates on the number of diabetics in the US is staggering. An estimated 17 000 000 people, or about 7% of the US population suffer from diabetes. Current treatment of diabetes consists of self-regulation of blood glucose levels through frequent ‘finger-stick’ monitoring. A faster, easier, automated, and less painful method for frequently measuring glucose levels would be of great individual, clinical, and societal benefit.

AgFONs with a monolayer of EG3 (Fig. 16a) were incubated for 1 h in glucose solutions (0–25 mM) of clinically relevant concentrations. SERS spectra were then measured from each sample. The chemometric method of partial least squares (PLS) is used when the spectrum of an analyte of interest is embedded within a complex background spectrum [255]. The result of PLS analysis is a plot of PLS predicted physiologically relevant glucose concentrations vs against actual glucose concentrations using leave-one-out cross-validation projected on a Clark Error grid. AgFON samples were made (sphere diameter = 390 nm, metal thickness = 200 nm), incubated for 19 hs in 1 mM EG3, and dosed in glucose solution (range: 0–25 mM) for 10 min. Each SERS measurement was made while samples were in an environmental control cell filled with glucose solution, using $l_{ex} = 632.8$ nm (3.25 mW, 30 s). Dashed line is not a fit, but rather represents a perfect prediction.
of predicted analyte concentration against actual analyte concentration. As shown in Fig. 16b, the SERS transduction mechanism successfully predicts glucose concentration quantitatively, with the vast majority of the data points falling within the A or B areas of the Clark-error grid [256].

5 Conclusions

We have highlighted representative research accomplishments in the area of the tunable LSPR of silver and gold nanoparticles in solution and on surfaces and the applications of two unique plasmonic phenomena (i.e. the LSPR and SERS) to biological applications and systems. We have provided the reader with a sufficiently deep, yet accessible, coverage of the underlying principles and theory behind surface plasmonics and its application to nanobiology. We have highlighted work in the field that is not only relevant and innovative, but also shows immediate promise for near-term implementation in real-world applications such as point-of-care diagnostics, environmental monitoring, proteomics, drug discovery, and fundamental biological research. Future commercialisation of nanoparticle devices relies on a better understanding of the interaction between nanoparticles and their immediate environment, as well as the development of techniques that will preserve nanoparticle optical activity under challenging biological conditions.

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