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Silver nanoparticles: sensing and imaging applications

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1. Introduction

Recent advances in nanotechnology have allowed the development of robust, and highly sensitive and selective detection methods that are expected to address some deficiencies of conventional detection technologies. Within this context gold and silver nanoparticles have emerged as a powerful tool in sensing and imaging applications due to their surprising optical properties.

Although silver exhibits many advantages over gold, such as higher extinction coefficients, sharper extinction bands, higher ratio of scattering to extinction, and extremely high field enhancements, it has been employed far less in the development of sensors, with the exception of sensors based on surface enhanced spectroscopies. The reason for this is the lower chemical stability of silver nanoparticles when compared to gold. Nevertheless, recent developments include means of protecting efficiently silver nanoparticles that offer far improved chemical stabilities. As a consequence, silver nanoparticles are rapidly gaining in popularity and several research groups have begun to explore alternative strategies for the development of optical sensors and imaging labels based on the extraordinary optical properties of these metal nanoparticles.

In the present chapter, we will focus on recent developments regarding silver nanoparticles and their emerging sensing applications.

2. Surface plasmon

Noble metal nanoparticles display unique optical properties that differentiate them form their bulk counterparts. Probably the most fascinating finding, regarding this peculiar optical performance, is that they often exhibit strong extinction bands in the visible spectrum, and therefore bright and gaudy colours, that are not present in the spectrum of the bulk metal. Although these colours are reminiscent of molecular dyes, it is important to

emphasize that their fundamentals are different. While the spectra of molecular dyes can be understood only in terms of quantum mechanics, the extinction spectra of metal nanoparticles can be treated in terms of classical electromagnetism. Moreover, metal nanoparticles also scatter light with high efficiency and, unlike molecular dyes, their extinction spectra are really a combination of both absorption and scattering (Kelly et al., 2002). The interaction of the oscillating electromagnetic field of the light with metal nanoparticles, results in the collective coherent oscillation of the metal conduction electrons with respect to the nanoparticle positive lattice. At a particular frequency of the light this process is resonant, receiving the name of *Localized Surface Plasmon Resonance*, LSPR (Figure 1), and is the responsible of the strong extinction band exhibited by the nanoparticle. Additionally to the extremely high molar extinction coefficients and resonant Rayleigh scattering, LSPR also results in enhanced local electromagnetic fields near the surface of the nanoparticle (Novotny & Hecht, 2006).

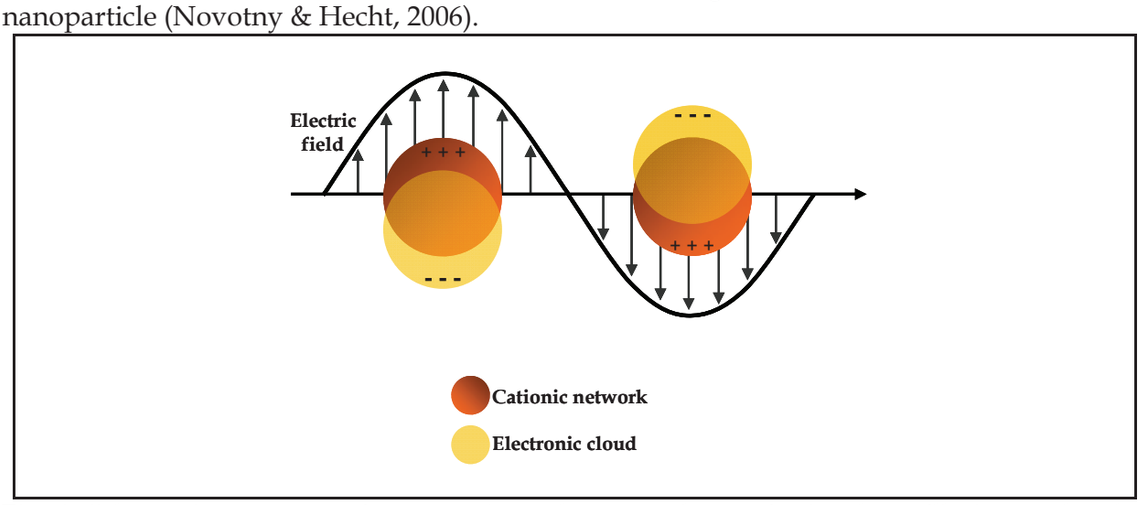


Fig. 1. Schematic representation of surface plasmon (electronic cloud) oscillation under the effect of an electromagnetic field

The first theoretical approach for modelling the optical properties of nanoparticles proposed by Mie, within classical electromagnetic formalisms, is still in common use today. According to the Mie theory, the resonance condition is achieved when the real part of the dielectric function of the metal equals the dielectric function of the surrounding medium (Mie, 1908). Therefore, the LSPR frequency depends both on the nanoparticle itself and on the medium where it is dispersed. Two important consequences arise from this dependency, on the one hand, the LSPR is tunable, i.e., its frequency can be modified through changes in the nanoparticle composition, size and shape (Kelly et al., 2003). On the other hand, metal nanoparticles are sensitive to their local environment, i.e., changes in the dielectric properties of their surroundings results in LSPR shifts that can be measured. Both tunability and sensitivity of LSPR convert metal nanoparticles in materials of choice for optical sensing and imaging applications. The most suitable metals are silver and gold, since the localized plasmon resonance condition mentioned above is satisfied at visible light frequencies. Additional advantages of these metal nanoparticles include simple preparation methods for a wide range of sizes and shapes and easy surface conjugation to a variety of ligands.

Over the last decade, several types of sensors have been developed on the basis of the plasmonic properties of noble metal nanoparticles i.e., extremely high molar extinction coefficients and resonant Rayleigh scattering on one side, and enhanced local electromagnetic fields near the surface of the nanoparticle on the other. These properties are inherent to a given detection mechanism and a given detection technique, enabling the classification of sensors in two main groups depending on the type of interaction involved, and therefore measured, between the metal nanoparticle and the analyte molecule. The first group encompasses sensors involving LSPR frequency shift, due to the interaction between nanoparticle and target molecule. Within this group, two different sensors may be distinguished, depending on the origin of LSPR changes: aggregation sensors and refractive index sensors. In aggregation sensors the LSPR shift is due to the plasmon coupling of nanoparticles in close proximity, in refractive index sensors the LSPR shift is due to changes in the local refractive index of the medium. The second main group of sensors is based on the enormous electromagnetic field enhancement in the vicinity of noble metal nanoparticles, which results in the so called surface enhanced spectroscopies, such as Surface Enhanced Raman Spectroscopy (SERS) and Metal Enhanced Fluorescence (MEF). This simple classification scheme is resumed in Table 1 that collects also the associated measurement techniques.

Sensor	Mechanism		Measurement/technique
Aggregation	LSPR-shift origin:	near-field electromagnetic coupling	Extinction/UV-Vis spectroscopy
Refractive Index		local refractive index changes	Extinction/UV-Vis spectroscopy Elastic Scattering/Dark-field microscopy
SERS	local electric field enhancement		Inelastic Scattering/Raman spectroscopy
MEF			Fluorescence

Table 1. Optical plasmonic sensors

In the following sections of this chapter, the principles of these sensors will be introduced and recent application examples for silver nanoparticles will be given.

3. Chemical sensing based on silver nanoparticles LSPR shift

3.1. Chemical sensing based on interparticle plasmon coupling: Aggregation sensors

The enhancement of electric fields on the nanoparticle surface, owing to plasmon resonance, decays over a distance on the order of the nanoparticle size. This means that when nanoparticles aggregate, i.e., approach each other within this distance, their fields interact leading to interparticle plasmon coupling and, consequently, to coupling-induced LSPR shift (Kreibig & Vollmer, 1995).

Generally, naked silver nanoparticles are stabilized against aggregation by means of anions (e.g., Cl^- or $citrate^{3-}$) or polymers (e.g., polyvinilpyrrolidone) absorbed on their surfaces. However, changes in dispersant media such as pH and ionic strength can cause nanoparticle destabilization and aggregation. When the nanoparticle aggregates, its LSPR is red-shifted and broadened (Figure 2). This effect has been exploited in the design of so-called

aggregation sensors that form the basis of simple, highly sensitive and low cost colorimetric assays, which have been applied to the detection of small molecules, DNA, proteins, toxic metal ions and pollutants.

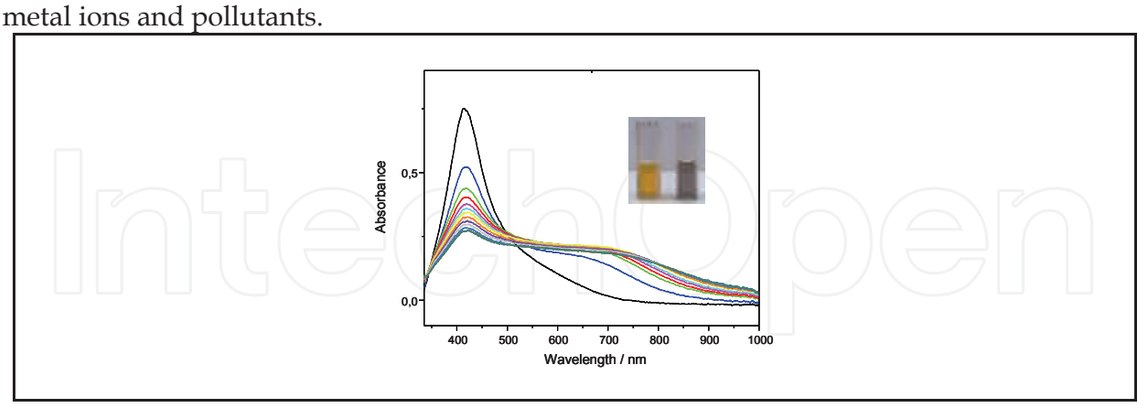


Fig. 2. Absorbance spectra of silver nanoparticles in aqueous solution before (black line) and after aggregation due to increasing salt concentrations (coloured lines). The suspension colour changes from yellow to blue.

In some cases, the proper analyte of interest is able to promote nanoparticle aggregation. An interesting example of this type of analyte-induced detection system has been recently described by Huang and co-workers. The authors have established a colorimetric analytical method for the detection of an anti-inflammatory drug, berberine hydrochloride, by means of citrate stabilized silver nanoparticles. Citrate stabilized silver nanoparticles exhibit a negatively charged surface rendering yellow stable dispersions in water due to the electrostatic repulsion between nanoparticles. In the presence of positively charged berberine hydrochloride, nanoparticles aggregate and suspension colour changes from yellow to green or blue, depending on the aggregation degree. By adjusting pH and ionic strength values of the medium, a good correlation between the absorbance change at the plasmon wavelength of the nanoparticle, and the berberine concentration was achieved, with detections limits of 1.3×10^{-8} M. Assay reliability was validated in berberine contained in commercially available drugs (Ling et al, 2008).

However, this method is restricted to cases where the analyte of interest is able to induce nanoparticle aggregation and no other substance present in the sample interferes by aggregating the nanoparticle. To overcome these limitations, it is necessary to endow aggregation sensors with selectivity. For this purpose, molecular recognition events have been employed to achieve selective colorimetric assays. The general strategy consists in functionalizing the silver nanoparticle surface with a monolayer of molecular recognition ligands. Aggregation is induced by specific recognition and binding to the target (Figure 3). The plasmon band is broadened and red-shifted as a function of the aggregation degree; therefore the change in absorbance is related to the target concentration.

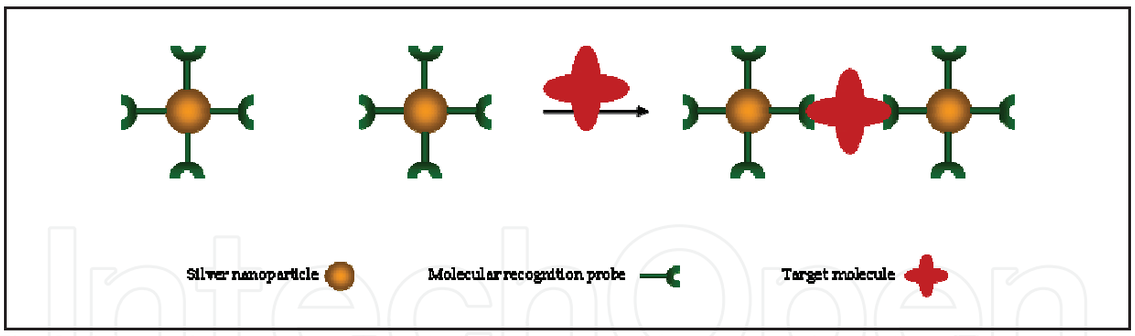


Fig. 3. Schematic representation of silver nanoparticles aggregation induced by molecular recognition events.

The hybridization properties of DNA make this biomolecule an especially suitable candidate in aggregation sensing applications. Silver nanoparticles conjugated to oligonucleotides have recently emerged as powerful tools for the detection of target DNA sequences, and have been used in the design of colorimetric assays based on aggregation induced by sequence-specific hybridization. Different conjugates nanoparticle-oligonucleotide have been proposed to this end (Chen et al., 2004; Liu et al., 2005; Lee et al., 2007).

Additionally, when gold (Jin et al., 2003) and silver (Thompson et al., 2008) nanoparticles are conjugated to oligonucleotides, sharper melting transitions are observed as compared to free oligonucleotides, allowing the differentiation between complementary strands and strands with one or more mismatches by both colorimetric and melting curve analysis. Taking advantage of this property, Mirkin and co-workers developed a "dual" detection method for single-nucleotide-polymorphisms. The authors employed two different types of probe systems, Au and Ag/Au core shell nanoparticles, which offer two different colour signatures for target DNA-directed colorimetric detection. The metal nanoparticles were conjugated to oligonucleotide probe strands, wild-type and single nucleotide mutant, and hybridized to the corresponding DNA targets. After hybridization, nanoparticles aggregated, and these aggregates could be dispersed again by heating them above the melting temperature of the duplex DNA interconnects. Mismatched hybridization, e.g. wild type strand functionalized nanoparticle-mutant DNA target, displayed lower melting points than their matching counterparts, and could be easily differentiated. As in this approach two different colour pairs are available, the method provides a cross-check, enhancing the reliability of the determination (Cao et al., 2005). Further improvement using silver nanoparticles offered higher sensitivity, due to the greater extinction coefficient of silver nanoparticles when compared to their gold analogues (Thompson et al., 2008). Anyway, with the achieved sensitivity, a PCR amplification step is still compulsory.

A completely different strategy, where the molecular recognition is performed away from the nanoparticle enabling the use of naked nanoparticles without loss of selectivity, has been very recently reported (Kanjanawarut & Su, 2009). In this strategy, charge neutral peptide nucleic acids (PNA) are used both as hybridization probe and to induce aggregation of citrate stabilized metal nanoparticles. In the absence of a complementary target DNA, PNA molecules, which remain free in solution, are able to coat nanoparticles inducing aggregation and, consequently, the colour changes in the colloidal solution. When a complementary DNA is present, PNA−DNA complexes are formed and nanoparticles do not aggregate, due to the stabilizing effect of the negative charges of the DNA strands in the

PNA−DNA complexes adsorbed on the nanoparticle surface. Nanoparticle behaviour could be easily followed by UV-Vis spectroscopy. This strategy enabled even single-base mismatch discrimination by means of adding NaCl to accelerate the aggregation process of the nanoparticles. Although both silver and gold citrate capped nanoparticles were employed to carry out the assay, silver rendered higher sensitivity.

Silver aggregation sensors are not restricted to DNA detection, and protein recognition has also been demonstrated. For example, carbohydrate binding proteins have been detected with carbohydrate stabilized nanoparticles. The tetrameric lectin concanavalin A (Con A) was detected by means of silver nanoparticles functionalized with mannose. Each Con A subunit contains a carbohydrate binding site that binds to a mannose ligand on the nanoparticle surface inducing nanoparticle aggregation (Schofield et al., 2006). Conversely, Con A can be utilized to detect a carbohydrate. Silver nanoparticles functionalized with dextran undergo aggregation in the presence of Con A, owing to the interaction of dextran with Con A. Competitive binding of glucose to Con A, is able to restore nanoparticles to solution, permitting the colorimetric detection of glucose. Although the detection limit obtained was below the physiological level of glucose, the specificity of this approach is not nearly sufficient for a diagnostic assay (Zhang et al., 2004a).

Aggregation based chemical sensing has also been used to monitor enzymatic reactions. Unmodified Ag nanoparticles have recently been used to develop a colorimetric assay to detect enzymatic reactions where adenosine triphosphate (ATP) is consumed, such as ATP dephosphorylation by calf intestine alkaline phosphatise and peptide phosphorylation by protein kinase. The assay relies on the capacity of ATP to protect the Ag nanoparticles against salt-induced aggregation. As ATP is consumed in the enzymatic process, nanoparticles begin to aggregate (Wei et al., 2008).

Other applications of protein functionalized silver nanoparticles that have been proposed include a colorimetric pH sensor based on the principle of pH dependent denaturation of proteins. Conformational changes in cytochrome c in response to pH changes could be measured over a wide range of pH values, as a result of plasmon shift in a system formed by silver nanoplates functionalized with the protein (Park et al., 2009).

A different approach to the molecular recognition events consists in functionalizing silver nanoparticles with complexing agents. Aggregation is achieved by formation of inclusion complexes between the functionalized nanoparticle and the guest molecule. Highly sensitive and selective colorimetric assays have thus been accomplished for the detection of histidine (Xiong et al., 2008; Li et al., 2009), pesticides (Xiong & Li, 2008) and Yb^{3+} ions (Han et al., 2009).

A very promising application of silver nanoparticles has been recently described. By means of the interdependency between the extent of nanoparticle coupling-induced LSPR shift and the distance between the interacting nanoparticles, Alivisatos and co-workers have developed a new class of molecular rulers based on plasmon coupling of single gold and silver nanoparticles (Sonnichsen et al., 2005). These “plasmon rulers” where used to study the dynamics of DNA hybridization on the single molecule level, with notorious advantages

when compared to conventional rulers based on Fluorescent Resonance Energy Transfer technique (FRET), e.g., scattering measurements offers better sensitivity and lower background signal than FRET, unlike reporter dyes used in FRET, the plasmon ruler neither blinks nor bleaches and affords upper distance limits of 70 nm as compared to approximately 10 nm for FRET.

3.2. Chemical sensing based on plasmon shifts with local refractive index

The silver nanoparticle LSPR is extremely sensitive to changes in local refractive index induced by analyte binding at or near the nanoparticle surface. The nanoparticle response to these changes can be measured as a LSPR shift in the UV-Vis extinction spectrum. This overall LSPR shift depends on surface coverage, i.e., on analyte concentration, allowing the development of refractive index sensors for quantitative determinations. Although other noble metals, such as gold, also exhibit high sensitivity to the local refractive index, the narrower plasmon bandwidth found in silver nanoparticles permits more accurate measurements of the LSPR shift (Lee et al., 2006).

As mentioned above, the extinction spectra of silver nanoparticles are really a combination of both absorption and scattering, and therefore the response of the LSPR to changes in local refractive index can also be measured by means of elastically scattered light. The Rayleigh scattering efficiency of a silver nanoparticle is 10^6 times higher than the fluorescence efficiency of a fluorescent dye molecule (Yguerabide et al., 1998), with the additional advantages that, unlike fluorescent probes, silver nanoparticles neither blink nor bleach. This extremely high scattering quantum yield of silver nanoparticles enables their use in highly sensitive imaging applications using dark-field optical microscopy, e.g. for imaging single living cells (Xu et al., 2004) as well as single receptor molecules on single living cells (Huang et al., 2007).

Apart from high sensitivity, selectivity for the analyte is also required to enable the use of silver nanoparticles in a detection system. For this purpose, the silver nanoparticle surface has to be tailored in such a way that only the target of interest binds to it. As in the previously described aggregation sensors, this can be achieved by functionalizing the nanoparticle surface with ligands capable of specifically binding the target molecule therefore eliminating non-specific surface adsorption (Haes & Van Duyne, 2003; Malinsky et al., 2001). Over the last decade, many functionalization protocols have been developed, nevertheless the most popular is the one based on using a thiolated intermediate linker or directly a thiolated derivative of the ligand. Such a popularity of thiolated compounds is mainly due to their capacity to form stable metal-sulphur bonds with the nanoparticle surface atoms under mild conditions. Moreover, silver nanoparticles modified with alkanethiol self-assembled monolayers exhibit extremely high short-range refractive index sensitivities (Malinsky et al., 2001; Sherry et al., 2006). The incorporation of different functionalities into the terminal position of the alkanethiol provides an easy way to tailor the chemical properties of the nanoparticle surface. These tailored silver nanoparticles have emerged as a powerful tool in the study of adsorption and binding events in biological systems (Ostuni et al., 1999), and have been extensively used in the design of refractive index sensors.

Basically, a refractive index sensor is any device that operates by transducing small changes in local refractive index in the silver nanoparticle surroundings into a measurable

wavelength shift response. A widely used configuration for refractive index sensors consists in a surface covered with silver nanoparticles that are coated with a recognition layer designed to bind a specific target molecule.

As first demonstrated by Van Duyne and co-worker, surface-confined triangular silver nanoparticles fabricated by nanosphere lithography on a glass substrate, render highly sensitive refractive index sensors. The recognition layer was constituted by a self-assembled mixed monolayer of alkanethiol molecules, comprising a thiolated compound with a terminal carboxylic acid group that could be used to attach biotin by common coupling chemistry. As a proof of concept, the biotinilated affinity sensor was assayed with streptavidin. Biotin-streptavidin binding induced a plasmon band shift, which was dependent on the streptavidin concentration. Exposure to 100 nM streptavidin, for example, rendered a LSPR red shift of 27 nm. After validation, its use was extended to the more realistic biotin-anti-biotin system with lower binding affinity typical of immunoassays. Limits of detection of 1 pM (streptavidin) and 100pM (anti-biotin) were obtained (Haes & Duyne, 2002). By using distinct ligand modified self-assembled monolayers to functionalize the triangular silver nanoparticles, this affinity sensor scheme has been extended to the optical detection of carbohydrate binding proteins (Yonzon et al., 2004), anti-amyloid-derived diffusible ligands focussed on the diagnosis of Alzheimer's disease (Haes et al., 2004) and small protein toxins (Zhu et al., 2009).

Interestingly, it has been reported that when chromophores are used as ligands, amplified spectral response to target binding can be achieved. For this to happen, the LSPR frequency of the silver nanoparticles has to be close to the molecular resonance of the chromophore, so that they couple (Haes et al., 2006; Zhao et al., 2007). This enhanced sensitivity has been recently applied to detect the binding of low molecular weight substrates and inhibitors (Zhao et al., 2008) as well as different drugs (Das et al., 2009) to human cytochrome P450.

Although the previous examples in this section were based on triangular nanoparticles, many other nanoparticle shapes are usable in refractive index sensors, such as rhombic silver nanoparticles (Zhu et al., 2008), and spherical silver nanoparticles (Gish et al., 2007). Groves and co-workers have recently developed a label-free LSPR sensor constituted by silver nanocubes covered with a self-assembled alkanethiol monolayer and interfaced with a glass-supported model membrane. This sensor was used to monitor and quantify static and dynamic protein binding to the membrane (Galush et al., 2009).

Refractive index sensors are also highly interesting for pollutant detection, for example, very recently a sensor based on silver colloidal nanoparticles has been reported for the detection of volatile organic compounds (Chen & Lu, 2009).

Further exploration of the potential of silver nanoparticles in refractive index sensors showed that a single silver nanoparticle monitored by dark-field microscopy can be used to sense local refractive index changes (McFarland & Van Duyne, 2003). Moreover, single silver nanoparticles functionalized with a single ligand molecule have demonstrated their capacity to constitute an optical sensor by themselves. These "single nanoparticle sensors" permit the target detection at the single molecule resolution and opens the door to studying single

molecular interactions of chemical functional groups on the surface of nanoparticles. Sensing and imaging of a single human cytokine molecule, tumor necrosis factor-α (TNFα), have been recently achieved by employing small silver nanoparticles (2.6 nm) functionalized with a single antibody molecule. Such as small nanoparticle size provides intrinsic single molecule detection volumes per nanoparticle, affording high sensitivity and a notably wide dynamic range (0-200 ng/mL) suitable for diagnostic purposes in diseases inducing increased TNFα levels (Huang et al., 2008).

Other examples involving silver nanoparticles in sensing and imaging applications associated to dark field microscopy include the development of a microarray-based DNA hybridization assay to screen for a given polymorphic site in the breast cancer gene BRCA1 (Oldenburg et al., 2002), the study of the action of antibiotics upon the cell membrane of living bacterial cells (Kyriacou et al., 2004; Xu et al., 2004) and imaging nanoparticle binding to fungi (Weinkauf & Brehm-Stecher, 2009).

4. Chemical sensing based on inelastic light scattering: SERS

Raman scattering is an extremely inefficient process. When a molecule interacts with visible light, most of the light is absorbed and only a small fraction is inelastically scattered to render the Raman spectrum of the molecule. As a consequence, Raman cross sections are extremely small, less than $10^{-29} cm^2 sr^{-1}$, i.e., about 10 orders of magnitude smaller than that of infrared absorption, and 14 orders of magnitude smaller than that of fluorescent dye molecules. This means that the detection sensitivity of Raman spectroscopy is intrinsically low and, in order to achieve the high sensitivity required in biological or pollutant samples, the scattering intensity should be greatly increased.

Nevertheless, when the Raman spectrum is registered for a molecule located in the vicinity of a metal nanoparticle, or a metallic surface with nanometric roughness, the Raman cross section can be amplified dramatically. This enhancement effect is known as *Surface Enhanced Raman Scattering* or Surface Enhanced Raman Spectroscopy (SERS). SERS is probably one of the most powerful techniques currently available for sensing applications since, additionally to an extremely high sensitivity, it provides valuable structural information on the analyzed molecule.

Although SERS effect has not been completely elucidated, two enhancement mechanisms are generally recognized to contribute to SERS effect, the electromagnetic mechanism and the chemical mechanism. In the electromagnetic mechanism, local electric fields in the surroundings of the metal nanoparticle are enhanced due to the surface plasmon excitation, leading to more intense electronic transitions in molecules located near the nanoparticle, and enhanced Raman scattering (Schatz et al, 2006). The chemical mechanism consists in changes in the polarizability of the molecule, owing to charge transfer interaction between electronic states of the molecule and the metal nanoparticle surface, which results in increased Raman signals (Otto & Futamata, 2006). Even though these two mechanisms contribute to the enhancement, the electromagnetic one plays a major role when compared to the chemical one which is much smaller. Moreover, whereas the chemical mechanism depends on the properties of the analyte molecule, the electromagnetic mechanism only depends on tunable

optical properties of the metal nanoparticles that can be optimized in order to obtain higher SERS enhancements. In this sense, electromagnetic mechanism plays a key role in the development of improved SERS substrates, and current efforts directed to optimize nanostructures will be further discussed within this section.
The most active metal in SERS is silver, followed by gold and copper, in that order. SERS enhancement factors as high as 10^{14}-10^{15} have been described for silver nanoparticles (Kneipp et al., 1997; Xu et al., 1999; Futamata el al., 2002), enabling detection limits as low as a single molecule (Nie & Emory, 1997; Kneipp et al., 1997; Le Ru et al., 2006). The enormous sensitivity achieved in SERS permits the ultrasensitive and ultraselective detection of biomolecules (Ji et al., 2005; Fabris et al., 2008) as well as pollutants (Guerrini et al., 2009a; Abalde-Cela et al., 2009).

The IR signals of molecules nearby silver nanoparticles are also enhanced, giving rise to *Surface Enhanced Infrared Absorption Spectroscopy* (SEIRAS). However, SERS presents the considerable advantage over SEIRAS that aqueous samples may be examined, due to the very weak Raman scattering of water as compared to strong IR absorption. This is extremely significant for biological samples.

The largest Raman scattering enhancements have been described for molecules residing in gaps of a few nanometers between aggregated colloidal silver nanoparticles, the so called "Hot Spots" (Jiang et al., 2003; Michaels et al., 2000). This is attributed to plasmon-coupling between nanoparticles in close proximity (Su et al., 2003; Atay et al., 2004; Fromm et al., 2004; Jana & Pal, 2007). Other factors concerning the nanostructure have also been claimed to influence the enhancement effect, such as the presence of sharp edges and the LSPR coupling to Raman excitation source. On the basis of these considerations, a great deal of the current research effort in SERS focuses on the development of improved SERS active substrates for analytical purposes, by means of controlling composition, size, shape, and interparticle spacing of nanoparticles and their assemblies (Lin et al., 2009).
From the numerous methods actually available to fabricate SERS substrates, aggregation of colloidal nanoparticles is still preferred in applications requiring extremely high sensitivity. Indeed, silver nanoparticle aggregates have been found to be responsible for single-molecule detection in SERS, due to their high content in hot spots (Nie & Emory, 1997; Kneipp et al., 1997).
Aggregation can be achieved by increasing the ionic strength of the medium, (Jana & Pal, 2007). In this salt-induced aggregation strategy, attention has to be paid to salt election, as far as some ions have been claimed to enhance SERS effect, by means of surface activation, while others can even quench it (Doering & Nie, 2002). In fact, the SERS spectra of DNA and RNA mononucleotides may be obtained with high sensitivity when the silver colloid is aggregated with $MgSO_4$ instead of the more commonly used halide ions. (Bell & Sirimuthu, 2006)
Despite being widely utilized, owing to its simplicity and sensitivity, the aggregation method has several drawbacks such as low reproducibility and the lack of stability of the substrates. Very recently Mejías and co-workers have developed a new method for the obtention of highly reproducible films of silver nanoparticle aggregates that are strongly attached to the substrate and exhibit a high hot spots content (Figure 4). Due to the high stability of this SERS substrate, it can even be reused. Moreover, this substrate has shown its potential in quantitative SERS applications (Caro et al., 2008).

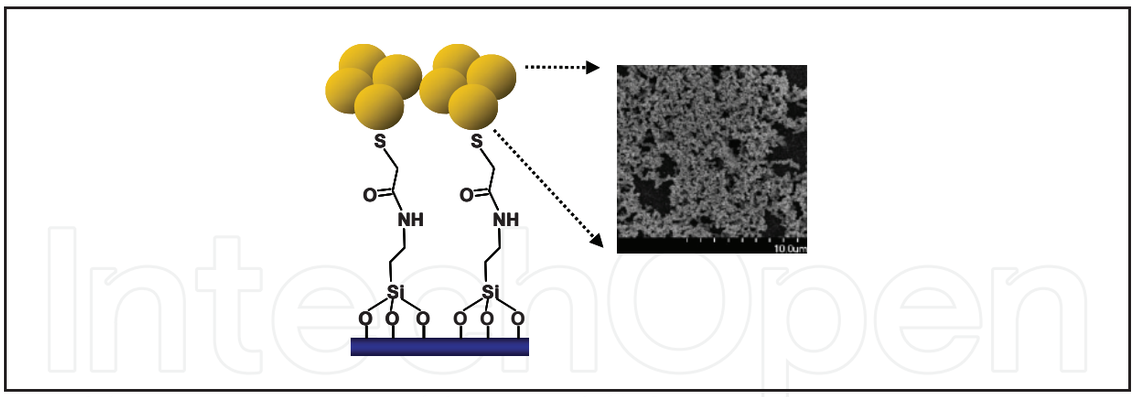


Fig. 4. Schematic representation of the sensor (left). Scanning electron microscopy image of silver nanoparticle aggregates on the sensor surface (right).

Although SERS is a selective technique, given that it provides a vibrational fingerprint of the analyte, distinguishing between closely-related species in complex samples, for example peptides that differ only slightly in sequence, is beyond the Raman discrimination capacity. Furthermore, a necessary condition for obtaining SERS activity is that the analyte molecule possesses affinity for the metal surface to ensure that it will approach the SERS substrate surface. If, to the contrary, the analyte of interest has no affinity for the surface at all, it does not matter how active the substrate is, it will not display SERS effect. These drawbacks have been overcome by coating the nanoparticle surface with a substance that promotes the adsorption or binds specifically to the target molecule. Such surface coated SERS sensors have made possible, for example, the detection of molecules with no affinity for uncoated surfaces, such as polycyclic aromatic hydrocarbons (Guerrini et al., 2009b), and the quantitative *in vivo* glucose measurement (Stuart et al., 2006).

Another interesting example of these modified SERS substrates, consist in a novel and fast method for the qualitative detection of protein kinase activity. To constitute the sensor, silver nanoparticles were deposited on a glass substrate and covalently linked to a target kinase-recognition peptide through a thiol group in the amine terminus of the peptide. After kinase reaction, the covalent attachment of the phosphate group to the peptide tyrosine residue resulted in a prominent change in the Raman spectra, exhibiting a collapse of the 848/828 cm^{-1} doublet to a single peak around 830 cm^{-1}. The reported method proved to be selective even in crude cell lysates (Yue et al., 2009).

In addition to the SERS active substrates, another attractive sensor arrangement has been described, the fiber-optic SERS sensor. In this type of sensor, the substrate is simultaneously part of the excitation and detection mechanism. For example, glass fiber tips coated by silver nanoparticles enabled the recording of spectra of biological samples, such as plant tissues or microbiological cells, with a high spatial resolution and avoiding sample photoinduced destruction due to the considerably reduced laser power required with this sensor (Gessner et al., 2002). In another example, crystal violet molecules, a contaminant in aquaculture, were detected in water far below the ppb detection limit (Lucotti & Zerbi, 2007).

Over the last years a new SERS sensor architecture has emerged as a powerful tool in biological assays, the SERS tags. Briefly, a SERS tag is a chemically encoded labeling agent. The basic structure of the SERS tag is formed by a SERS reporter molecule as labeling agent, i.e., a molecule with a high Raman cross-section, adsorbed on the surface of a silver nanoparticle that can be optionally further encapsulated in a protective shell. Once the basic structure is assembled, it may be conjugated to a recognition element, such as an antibody. In a detection process, the Raman signature of the SERS reporter molecule will reveal whether the labeling agent is present or not. SERS tags represent an excellent alternative to fluorescence-based encoding methods due to the advantages that they present when compared with traditional fluorescence labels, such as narrower emission peaks, increased number of optical signatures, high-level multiplexing, single laser excitation for detection of multiple labels and higher stability.

As mentioned above, a SERS tag can be obtained by simply adsorbing a SERS reporter molecule on the nanoparticle surface, this simple yet effective approach has been employed, for example, to develop a sandwich type immunosensor to detect hepatitis B surface antigen (Ji et al., 2005).

A wide variety of SERS tags have been reported, the most popular being the encapsulated ones, given that encapsulation prevents desorption of the SERS reporter molecule and increases the chemical stability of metal nanoparticles. As encapsulating agent, polymers (Stokes et al., 2006) and silica (Jun et al., 2007) are generally employed. Indeed, silica encapsulated SERS tags are being commercialized.

Exploiting the fact that the SERS effect is stronger in nanoparticle aggregates, Su and co-workers have developed an induced-aggregation method to obtain SERS tags. In this approximation, silver seed nanoparticles are grown, in the presence of appropriate SERS reporters, which are able to induce aggregation. Once the silver clusters reach the desired size, they are stabilized by the addition of bovine serum albumin (BSA). These SERS BSA-coated tags can be easily conjugated to antibodies by direct adsorption, for their use in immunoassays (Su et al., 2005). Covalent binding to antibodies is also possible by employing a cross-linked organic encapsulation with BSA and glutaraldehyde. These SERS tags have been used to map prostate specific antigen in tissue samples (Sun et al., 2007).

A different aggregation strategy has been recently reported by Fabris and co-workers who prepared silver nanoparticle dimers and small aggregates via a dithiol linker, which acts both as a “bridge” between the nanoparticles and as SERS reporter molecule. Further functionalization of these SERS tags with aptamers allowed highly sensitive protein detection (Fabris et al., 2008).

In a complex variation of the SERS tag scheme, Lee and co-workers have conjugated the optical properties of silver nanoparticles with the magnetic properties of iron oxide nanoparticles to obtain SERS encoded multiplex beads that afford both protein separation and identification (Jun et al., 2009). For this purpose sulfonated polystyrene-divinylbenzene beads were successively functionalized with magnetic iron oxide nanoparticles, silver nanoparticles, SERS reporter molecules and, finally, encapsulated with a silica shell. These

multiplex beads were tested as separation vehicles on the model system biotin–streptavidin. Multiplex beads retain both magnetic and SERS properties after silica coating and biotin functionalization, offering a wide range of applications in drug screening and combinatorial chemical synthesis. This combination of SERS detection and magnetic separation capacities by means of silver and magnetic nanoparticles, have also been used in a sandwich type immunoassay. In this assay, monoclonal antibody modified silica-coated magnetic nanoparticles, as separation tool, and polyclonal antibody Ag/SiO_2 core-shell nanoparticles embedded with a SERS tag, were employed to bind to target antigen, affording separation and detection. Using this strategy, concentration of human tumor marker α-fetoprotein up to 0.12 μg/ml was detected with a detection limit of 11.5 pg/ml (Gong et al., 2007).

Silver nanoparticles functionalized with SERS reporter molecules exceed the simple molecule detection frontier, and applications such as SERS cellular imaging (Kim et al., 2006; Hu et al., 2007), or intracellular pH sensing (Talley et al., 2004; Wang et al., 2008) are also feasible.

5. Chemical sensing based on metal enhanced fluorescence: MEF

Fluorescence detection still plays a major role in most of the biological assays currently used. Nevertheless, fluorescence detection at the single molecule level has several limitations arising from the use of organic fluorophores, such as low signal to background ratio, poor photostability, and strong photoblinking.

Metal nanoparticles are known to interact with nearby fluorophores affecting their emission intensity. When this interaction results in a fluorescence enhancement, the effect is known as *Metal Enhanced Fluorescence* (MEF). This effect can be understood as arising from two contributions. On the one hand the enhanced emission intensity from the fluorophore can be attributed to the enhanced local fields associated with the metal nanoparticle, as in the case of SERS. On the other hand, the interaction with the nanoparticle LSPR results in increased radiative and nonradiative decay rates and, consequently, increased quantum yield and decreased lifetime (Lakowicz et al., 2002a; Lakowicz, 2005; Cade et al., 2009). Additionally, and as a consequence of the decreased lifetimes, the photostability of the fluorophore increases (Lakowicz et al., 2002b). Considering the potential of MEF, it could therefore improve current fluorescence based techniques.

In contrast to other metals, which typically quench nearby fluorophores (Huang & Murray, 2002), silver nanoparticles enhance the luminescence of fluorophores when they are localized at a distance of 4-10 nm from the silver nanoparticle surface. For example, silver nanoparticles deposited on a glass substrate increase the emission intensity of the common biological label fluorescein by an average of at least three fold (Pugh et al., 2003) and, what is more, fluorescein self-quenching decreases even in highly labeled proteins, permitting the use of ultrabright over-labeled protein as labels (Lakowicz et al., 2003; Lukomska et al., 2004).

Generally, MEF leads to 10-1000 fold intensity enhancements. Some factors affecting the enhancement degree have been described, such as the distance between the silver nanoparticle and the dye (Kühn et al, 2006; Anger et al., 2006), silver nanoparticle

aggregation (Zhang et al, 2004b; Zhang et al, 2007a) and the spectral overlap between the LSPR band of the silver nanoparticle and the emission band of the attached fluorophore (Chen et al., 2007).

The detection of DNA hybridization is of interest in numerous biological applications such as microarrays and PCR. To this end, Lakowicz and co-workers have studied in depth the MEF effect applied to DNA. They have found that silver nanoparticles enhance the intrinsic fluorescence of DNA (Lakowicz et al., 2001). Further research demonstrated that when DNA was labeled with a fluorescent probe, silver nanoparticles improved the fluorophore photostability (Malicka et al., 2002). The increased brightness of the fluorophore near silver islands enabled the measurement of the DNA hybridization kinetics as well as the development of the first MEF-based DNA hybridization assay (Malicka et al., 2003). Taking into account that silver nanoparticle LSPR also permits the detection of hybridization events, a further refinement was made and a dual detection method was developed. In this new approach, DNA hybridization was monitored through both nanoparticle absorbance and fluorophore emission spectral changes (Zhang et al., 2006). In these studies, the authors have made use of tiopronin monolayer-protected silver nanoparticles with the additional advantage that these nanoparticles are not cytotoxic (Castillo et al., 2008).

As fluorescent labeling is widely employed in immunoassays, efforts have been made to implement this technique by means of MEF, for example to detect insulin (Lochner et al., 2003) and myoglobin (Barnett et al., 2007).

Biological recognition events, such as antigen-antibody interaction or DNA hybridization, are often kinetically slow, requiring long incubation times. Recently, low-power microwave heating has been employed to accelerate these processes in MEF based assays. The thermal gradient created between the bulk aqueous medium and the metal nanoparticles during microwave heating has been claimed to play a major role in the observed faster biorecognition kinetics in such a microwave-accelerated MEF-based bioassay. This principle has been applied to immunoassays and DNA hybridization assays that could be completed within a few seconds with the benefits of increased sensitivity associated to MEF (Aslan & Geddes, 2008).

Silver nanoparticles have also demonstrated their potential in applications such as the development of solid substrate-based RNA capture assays (Aslan et al., 2006), label-free bioassays by means of enhancement of tryptophan fluorescence in proteins (Szmacinski et al., 2009), the detection of glucose (Aslan et al., 2004) and the development of a MEF ratiometric pH sensor, using a pH-sensitive fluorophore (Aslan et al., 2005).

The development of molecular imaging agents for fluorescent imaging of cells is of great interest in disease diagnosis and elucidation of signalling pathways. Silver nanoparticles, conjugated to target molecules and fluorophores, have emerged as promising tools for cell imaging applications (Zhang et al., 2007b). Advantages of their use as cell imaging agents owing to their increased brightness and decreased lifetimes have been recently reported. Single molecule detection in cells by means of fluorescence requires the utilization of intense laser beams, which cause cell damage. This undesirable effect can be reduced by means of

MEF, which permits the use of weaker laser beams. Furthermore, owing to shorter lifetimes afforded by MEF, which reduces the time that a fluorophore spends in the excited state when it is vulnerable to oxygen attack, photobleaching is also decreased (Borejdo et al., 2008). Moreover, lifetime images generally lack interest given that the lifetimes of most organic fluorophores are close to the value of cell autofluorescence resulting in background interference. However, useful lifetime images can be obtained thanks to the decreased lifetimes obtained in MEF (Zhang et al., 2008).

6. Conclusions

Silver nanoparticles possess many valuable optical properties that have opened the door to new approaches in sensing and imaging applications, offering a wide range of detection modes such as colorimetric, scattering, SERS, and MEF techniques, at extremely low detection limits. Moreover, nanoparticles have made possible the use of scattering imaging techniques and have brought valuable improvements to standard imaging techniques. Silver nanoparticles may be introduced explicitly as labels, or may already be required in the application design to perform other functionalities, such as drug delivery, and simply provide this extremely useful added benefit.

Although LSPR sensing offers many promising features, detection thresholds must be improved for most practical applications. A great deal of current research effort is directed towards the optimization of nanoparticle tunable properties to improve their response in LSPR sensors.

One of the most promising contributions of silver nanoparticles to sensing and imaging applications is SERS. In this highly active research field, hundreds of papers are published each year on both fundamental aspects and applications. SERS has risen to its potential in the ultrasensitive detection of a wide variety of compounds, ranging from chemical pollutants to biomolecules, with detection thresholds as low as a single molecule, and the added advantage to provide structural information on the target molecule. It is to be expected that future improvements in this technique will be directed towards the development of more robust and reproducible sensors with a maximum coverage of hot spots. Additionally to SERS sensors, encapsulated SERS tags have emerged as an alternative to traditional fluorescent labeling offering advantages such as improved stabilities and multiplexing capability. On the other hand, MEF has also shown a great potential to address the deficiencies of traditional fluorescent labeling.

Summarizing, although further research is required, future perspectives of silver nanoparticles include applications in multiplexing assays, massively parallel bioassays for high throughput screening of drugs, diagnostic level sensing for clinical assays, the study of biological interactions at the molecular level and the measurement of intermolecular distances of tens of nm with plasmon rulers.

7. References

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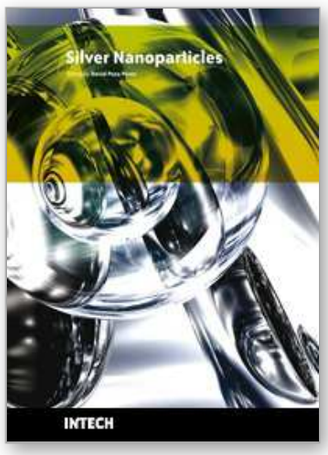
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