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Doctoral Dissertation

**Towards Stable Single-Molecule SERS
Measurements with High Temporal Resolution**

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List of Abbreviations

AA	L-ascorbic acid
AgFON	silver film over nanospheres
ALD	atomic layer deposition
APTES	(3-aminopropyl)triethoxysilane
AuBPs	gold nanobipyramids
AuNPs	gold nanoparticles (spherical)
AuNRs	gold nanorods
BPE	<i>trans</i> -1,2- <i>bis</i> (4-pyridyl)ethylene
CB[<i>n</i>]	cucurbit[<i>n</i>]uril
CoV	coefficient of variation
CRS	coherent Raman spectroscopy
CTAB	hexadecyltrimethylammonium bromide
CTAC	hexadecyltrimethylammonium chloride
DFT	density functional theory
DMSO	dimethyl sulfoxide
EBL	electron-beam lithography
EM-CCD	electron-multiplying charge-coupled device
ET	exposure time
FDTD	finite-difference time-domain
FIB	focused ion beam
FSRS	femtosecond stimulated Raman scattering
FWHM	full width at half maximum

List of Abbreviations

ISRS	impulsive stimulated Raman spectroscopy
KHD	Kramers–Heisenberg–Dirac
LSPP	localized surface plasmon polariton
LSPR	localized surface plasmon resonance
MG	malachite green
NA	numerical aperture
NIR	near-infrared
NPoM	nanoparticle on mirror
Pc	porphycene
PMMA	poly(methyl methacrylate)
PVP	poly(vinylpyrrolidone)
SAMs	self-assembled monolayers
SE-FSRS	surface-enhanced femtosecond stimulated Raman scattering
SEM	scanning electron microscopy
SERS	surface-enhanced Raman scattering
SE-SRS	surface-enhanced stimulated Raman scattering
SM-SERS	single-molecule surface-enhanced Raman scattering
S/N	signal-to-noise ratio
SPP	surface plasmon polariton
SPR	surface plasmon resonance
SRG	stimulated Raman gain
SRL	stimulated Raman loss
SRS	stimulated Raman scattering
TA	transient absorption
TEM	transmission electron microscope
TEOS	tetraethyl orthosilicate
Th	thionine
TMA	trimethylaluminum
TTM	two-temperature model
UV	ultraviolet
Vis	visible

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Introduction

Raman scattering provides vibrational spectra that define a unique chemical fingerprint for the material under study. However, Raman scattering is generally very weak, and to achieve single-molecule sensitivity, it is necessary to employ enhancement mechanisms, such as resonance or local-field enhancement. Local-field enhancement is usually associated with noble metal nanoparticles, which, due to their specific interactions with electromagnetic radiation, can produce a strong local electromagnetic field near their surfaces, thereby enhancing weak Raman scattering. In the described near-field scattering enhancement process, the nanoparticle behaves like an optical antenna — a nanodevice capable of efficiently converting freely propagating optical radiation into localized energy. [1, 2] Advances in nanofabrication now make it possible to produce optical antennas tailored for specific applications, for example, to enhance Raman scattering or fluorescence.

Raman scattering is a two-photon process in which inelastic scattering of photons occurs. The optical nanoantenna can enhance the efficiency of the Raman process by amplifying the local electromagnetic field at the excitation wavelength, thereby enhancing the interaction between light and the molecule. Simulations using methods of classical electrodynamics, providing information about the distribution of the electromagnetic field around optical nanoantennas and the enhancement factors calculated on their basis, indicate that the highest enhancement factors are achieved in ultra-small

cavities. The enhancement within the cavity reaches anomalous values that enable the detection of single molecules.

A characteristic feature of Raman spectra originating from single molecules located in cavities is the observation of temporal and spatial fluctuations in both signal amplitudes and the spectral positions of Raman bands. In order to investigate the origin of these fluctuations and the interrelationships between both types of fluctuations, temporal evolution measurements are performed, in which a sequence of spectra from a single molecule located in the cavity is recorded. However, single molecules that are not chemisorbed on the surface remain highly mobile, especially at room temperature. Therefore, lowering the temperature can help reduce their motion, thereby providing additional signal stabilization.

Molecules localized in cavities are dynamic systems that experience extremely intense and highly localized electromagnetic fields. Consequently, these systems undergo a range of kinetic processes, including adsorption and surface diffusion. In practice, this means that molecules diffuse into and out of the cavity, adsorb and desorb from the metal surface, and also have a certain degree of freedom to move across the metal surface. One solution to this problem is the chemical functionalization of the studied molecule, for example, by introducing a functional group with a strong chemical affinity for the metal. However, such a modification may significantly affect the optical properties of the investigated molecule, altering its absorption spectrum and making it more difficult to record Raman spectra at the single-molecule level. This implies the need to develop new methods to stabilize the Raman signal from single molecules to record reproducible signals and, consequently, expand the applicability of single-molecule detection using Raman techniques.

Temporal resolution remains a significant challenge in time-resolved studies based on spontaneous Raman scattering. In recent years, numerous reports have demonstrated temporal resolutions on the order of single microseconds in single-molecule measurements. [3–5] However, a major limitation of these studies is that the recorded Raman spectra exhibit poorly defined spectral features. In practice, this prevents many types of anal-

yses, such as tracking the relative temporal evolution of selected Raman bands. On the other hand, microsecond time resolution still represents a time-averaged result, since the intrinsic time scales of molecular motions occur on femtosecond or picosecond scales. Achieving such temporal resolution at the single-molecule level therefore requires the implementation of nonlinear vibrational spectroscopy, which employs at least two short laser pulses, commonly referred to as the pump and probe. When these pulses overlap in space and time, they generate a Raman signal in which the vibrational modes are prepared coherently. This coherent excitation, enhanced by an optical antenna, may enable single-molecule sensitivity while maintaining high temporal resolution synchronized with ultrafast dynamics.

In this doctoral dissertation, we investigated problems related to single-molecule spectroscopy, with particular emphasis on fluctuations in the amplitude and spectral position of Raman bands. Given the mobility of single molecules at room temperature, we also recorded sequences of spectra under cryogenic conditions to provide a more detailed description of the influence of temperature on spectral fluctuations and the photostability of single molecules. In these studies, we aimed to record Raman spectra with the highest possible temporal resolution while maintaining a high signal-to-noise ratio (S/N), enabling further analysis of Raman spectral sequences using correlation-based methods. In the next part of the work, we focused on controlling spectral fluctuations of single molecules by implementing supramolecular complexes that stabilize the molecules within the plasmonic cavity. Using quantum-chemical calculations, we explained the influence of supramolecular stabilization on several properties, including photostability and detection in the single-molecule regime. In the final part of the dissertation, we developed a methodology based on a nonlinear Raman scattering process enhanced by optical antennas. In this chapter, we present results obtained from colloidal suspensions of optical nanoantennas instead of single-molecule measurements, as our objective was to understand the origin of the unusual spectral shape and to describe the accompanying effects. Based on optimization studies and the preparation of highly monodisperse nanoantenna systems, we propose an explanation for

the rarely observed spectral shapes in vibrational spectroscopies.

The results presented in this dissertation deepen our understanding of single-molecule spectroscopy by providing new insight into the mechanisms underlying fluctuations of molecules localized within plasmonic cavities. Improving stability and detection by controlling fluctuations through supramolecular interactions enables extending single-molecule measurements to other Raman-based techniques. Finally, the development and optimization of measurement parameters based on antenna-enhanced nonlinear Raman scattering represent a key step toward transitioning from the macroscopic to the microscopic scale, including measurements of single nanoparticles and molecules in real time on the timescales relevant to the formation and breaking of chemical bonds.

Chapter 2

Theory

2.1 Plasmonic nanocavities

2.1.1 Properties of metals

The optical and conductive properties of metals are determined by their conduction electrons. Free electrons determine the strong response of metals to electromagnetic fields, being responsible for their high electrical conductivity and characteristic behavior in time-varying fields. In structures with nanometric dimensions, such as plasmonic nanocavities, the interaction between the electromagnetic field and conduction electrons leads to strong field localization and enhanced loss effects. Therefore, an accurate description of the dielectric response of metals, within the framework of Maxwell's equations, is a key element in the analysis and design of plasmonic nanosystems. [6]

The optical properties of metals can be described using the plasma model, in which a gas of free electrons with number density n moves relative to a static background of positively charged ion cores. The plasma model neglects the detailed lattice potential and electron–electron interactions. Instead, the influence of the electronic band structure is incorporated implicitly through an effective optical mass m assigned to each electron. When subjected to an external oscillating electromagnetic field, the elec-

trons undergo oscillatory motion, which is damped by scattering processes characterized by a collision frequency γ .

Illumination with electromagnetic radiation of appropriate frequency and momentum excites collective charge oscillations in the free-electron gas. Within the classical description, the equation of motion for an electron in the electron plasma subjected to an external electric field $\mathbf{E}$ can be written as:

$$m \frac{d^2 \mathbf{x}}{dt^2} + m\gamma \frac{d\mathbf{x}}{dt} = -e\mathbf{E} \quad (2.1)$$

where m is the effective optical mass of the electron, x is the electron displacement from its equilibrium position, γ is the collision (damping) frequency describing electron scattering processes, e is the elementary charge, E is the amplitude of the applied electric field, and t denotes time.

Assuming a harmonic time dependence of the external electric field $\mathbf{E}(t) = \mathbf{E}_0 e^{-i\omega t}$, the electron displacement is assumed to oscillate with the same frequency, $\mathbf{x}(t) = \mathbf{x}_0 e^{-i\omega t}$. The complex amplitude $\mathbf{x}_0$ accounts for any phase difference between the driving electromagnetic field and the resulting electron response through the following relationship:

$$\mathbf{x}(t) = \frac{e}{m} \frac{1}{\omega^2 + i\gamma\omega} \mathbf{E}(t) \quad (2.2)$$

The electrons that have been displaced contribute to the polarization $\mathbf{P}$ at the macroscopic level, which is defined by the formula:

$$\mathbf{P} = -ne\mathbf{x} = -\frac{ne^2}{m} \frac{1}{\omega^2 + i\gamma\omega} \mathbf{E} \quad (2.3)$$

Substituting the expression for the polarization $\mathbf{P}$ into the constitutive relation for the electric displacement field, $\mathbf{D} = \varepsilon_0 \mathbf{E} + \mathbf{P}$, we obtain:

$$\mathbf{D} = \varepsilon_0 \left(1 - \frac{\omega_p^2}{\omega^2 + i\gamma\omega} \right) \mathbf{E} \quad (2.4)$$

where $\omega_p = \sqrt{\frac{ne^2}{\varepsilon_0 m}}$ is defined as the plasma frequency of the free-electron gas. Consequently, the dielectric response of the free-electron gas can be

expressed as:

$$\varepsilon(\omega) = 1 - \frac{\omega_p^2}{\omega(\omega + i\gamma)} \quad (2.5)$$

The resulting expression is the classical form of the dielectric function in the Drude model, describing the response of a free electron gas with respect to the losses described by the collision frequency. In this model, the value of ω_p corresponds to the natural frequency of the collective oscillations of the free electron gas in the metal and is a quantity characteristic for a given metal. However, by taking into account the contribution of the dielectric background from bound electrons, it can be assumed to be approximately constant over the optical frequencies, leading to a generalized form of the dielectric function in the Drude model, which can be written as:

$$\varepsilon(\omega) = \varepsilon_\infty - \frac{\omega_p^2}{\omega(\omega + i\gamma)} \quad (2.6)$$

where ε_∞ is the high-frequency dielectric constant accounting for the contribution of bound electrons.

The dielectric function is a complex quantity. Its real part, $\text{Re}[\varepsilon(\omega)]$, describes dispersion, that is, how the electromagnetic field propagates through the material, whereas the imaginary part, $\text{Im}[\varepsilon(\omega)]$, accounts for energy losses and damping due to material response.

The Drude model provides a simple description of the optical plasma of free conduction electrons in a metal. However, this model is subject to fundamental limitations, leading to dielectric functions that deviate from experimental values. The Drude model treats electrons from the conduction band classically and ignores their quantum-mechanical nature. As a result, the experimental optical response data differ significantly from those calculated using the Drude model in certain photon energy ranges. This is due to photon absorption due to the excitation of interband transitions in the metal. Figure 2.1 compares the real and imaginary parts of the gold dielectric function calculated using the Drude model with the experimental data collected by Johnson and Christy. [7] For gold, the Drude model correctly describes only the lower energy range (<2 eV, which corresponds to a wave-

length >620 nm), while completely failing at higher photon energies (>2 eV, which corresponds to a wavelength <620 nm).

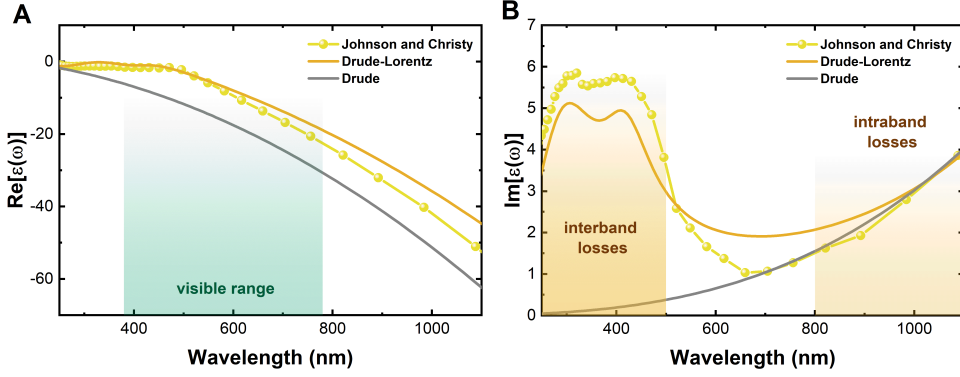


Figure 2.1: (A) Real and (B) imaginary parts of the dielectric function of gold, described by the Drude and Drude–Lorentz models and compared with experimental data measured by Johnson and Christy. [7]

To more accurately describe the dielectric function in the higher photon energy range, the Lorentz–Drude model was proposed (Fig. 2.1), which considers interband transitions as a set of harmonic oscillators, similar to the conduction band electrons in the Drude model. Consequently, the overall dielectric response is expressed as a superposition of the Drude term for free electrons and multiple Lorentzian oscillators representing interband transitions:

$$\varepsilon(\omega) = \varepsilon_{\infty} - \frac{\omega_p^2}{\omega(\omega + i\gamma)} + \sum_j \frac{f_j \omega_{p,j}^2}{\omega_{0,j}^2 - \omega^2 - i\gamma_j \omega} \quad (2.7)$$

where $\omega_{0,j}$, γ_j , and f_j are the resonance frequency, damping constant, and oscillator strength of the j -th Lorentz oscillator, respectively.

2.1.2 Surface plasmon-polariton

Surface plasmon polaritons (SPPs) are electromagnetic surface waves that propagate along the interface between a metal and a dielectric, arising from the coupling of photons with collective oscillations of conduction electrons.

Figure 2.2 schematically illustrates an SPP propagating at the metal–dielectric interface. In case of SPP, plasmons propagate along the metal–dielectric interface in the x–y plane over distances of tens to hundreds of micrometers, while exhibiting evanescent decay in the z-direction with 1/e decay lengths on the order of 200 nm. [8] Such propagation and decay characteristics give rise to sub-wavelength localization of the electromagnetic field at the surface, beyond the diffraction limit. SPPs exist only when the real part of the metal permittivity satisfies the relationship:

$$\varepsilon'_{\text{metal}} < -\varepsilon_{\text{dielectric}} \quad (2.8)$$

which ensures that the wavevector becomes larger than that of light in free space.

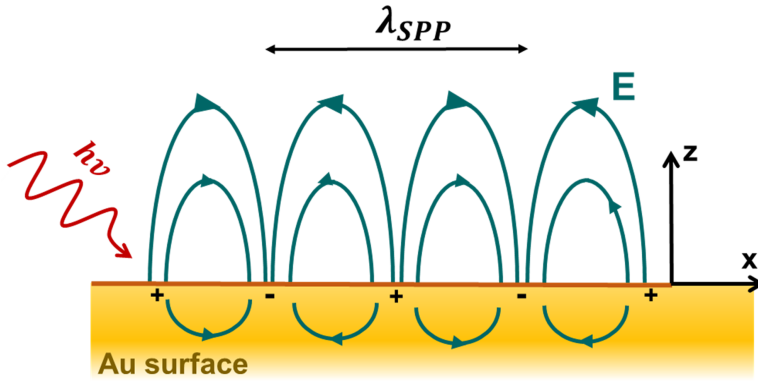


Figure 2.2: Schematic diagram illustrating a SPP propagating along a metal–dielectric interface, arising from the coupling of electromagnetic waves with collective charge oscillations in the metal.

The dispersion relation for an SPP at a flat interface is given by the following equation:

$$k_{\text{SPP}} = k_0 \sqrt{\frac{\varepsilon_{\text{metal}} \varepsilon_{\text{diel}}}{\varepsilon_{\text{metal}} + \varepsilon_{\text{diel}}}} \quad (2.9)$$

where k_0 is the free-space wavenumber. Because $k_{\text{SPP}} > k_0$, SPPs cannot be directly excited by freely propagating light due to the momentum mismatch unless special techniques for phase-matching are used. The most

common techniques for efficient SPPs excitation include prism coupling (Kretschmann [9] and Otto [10] configurations), grating coupling, highly focused beams, and charged particles. SPPs exhibit intense near-field enhancement and long-range propagation along the interface, with their propagation length limited by losses associated with the imaginary part of the metal permittivity $\varepsilon''(\omega)$. The distance over which an SPP propagates is strongly dependent on the excitation wavelength.

2.1.3 Localized surface plasmon polariton

Localized surface plasmon polaritons (LSPPs) are excited when the incident wavelength of light interacts with conduction electrons in metallic nanoparticles or nanostructures much smaller than the incident wavelength. The localized surface plasmon resonance (LSPR) is schematically pictured in Fig. 2.3.

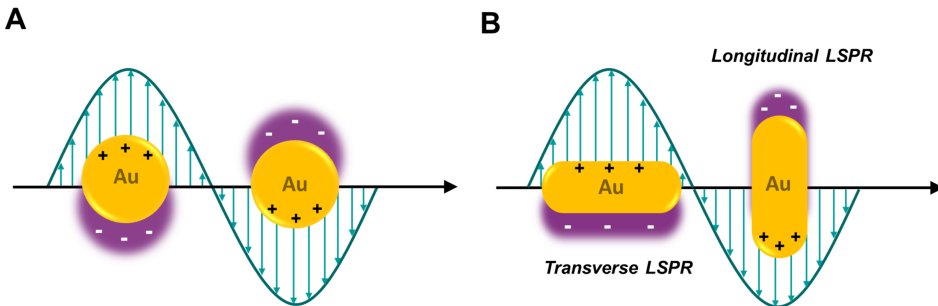


Figure 2.3: Schematic illustration of LSPRs in metallic nanostructures: (A) a spherical nanoparticle exhibiting a single, degenerate dipolar LSPR mode, and (B) a nanorod showing both transverse and longitudinal LSPR modes corresponding to electron oscillations along the short and long axes of the nanorod, respectively. In both cases, the purple cloud depicts the displacement of the conduction electron cloud relative to the positively charged atomic nuclei.

When the conduction electron cloud is displaced with respect to the nuclei, a restoring force due to Coulomb attraction between the electrons and the positive atomic core is generated. This force causes the electron cloud to oscillate around its equilibrium position. This collective oscilla-

tion is called the particle’s dipolar plasmon resonance. Besides the dipolar mode, higher-order plasmon excitations may occur, such as the quadrupolar mode. In this mode, different areas of the electron cloud oscillate in opposite directions compared to the applied electric field. These higher-order modes become significant when the nanoparticle size is comparable to the incident light’s wavelength, leading to spatially inhomogeneous electromagnetic fields across the particle that enable more complex charge oscillation patterns. [11] However, in this work, only nanoparticles with a size below 100 nm were used, so the quadrupole modes are not discussed here.

Unlike propagating SPPs, which travel along extended interfaces, LSPPs are spatially confined to nanoscale volumes, leading to enhanced and highly localized electromagnetic fields creating so-called “hot-spots” where electromagnetic fields can be amplified by orders of magnitude. The distribution of the electromagnetic field around the nanoparticle strongly depends on the geometry of the nanostructure.

The optical plasmonic resonances around different geometries of the nanostructures can be visualized based on finite-difference time-domain (FDTD) simulations. The FDTD method belongs to a group of numerical methods for simulating the interaction of electromagnetic waves with metallic nanoparticles. Typical FDTD simulation window with geometry used in this work is shown in Fig 2.4.

In FDTD, the simulation domain is a finite region discretized by a mesh containing an object (e.g., a nanoparticle with a specific geometry). Metallic nanoparticles are modeled using dispersive material models (such as Drude or Drude-Lorentz) or based on experimental data (such as Johnson and Christy [7]) to capture their frequency-dependent permittivity and plasmonic behavior. At the start of the FDTD simulation, the electromagnetic fields are calculated in each mesh cell by solving Maxwell’s equations. In the next step, the method updates the electric and magnetic field components in discrete time steps, allowing the temporal evolution of the electromagnetic field to be followed throughout the computational domain. FDTD enables detailed analysis of near-field enhancement, scattering, absorption,

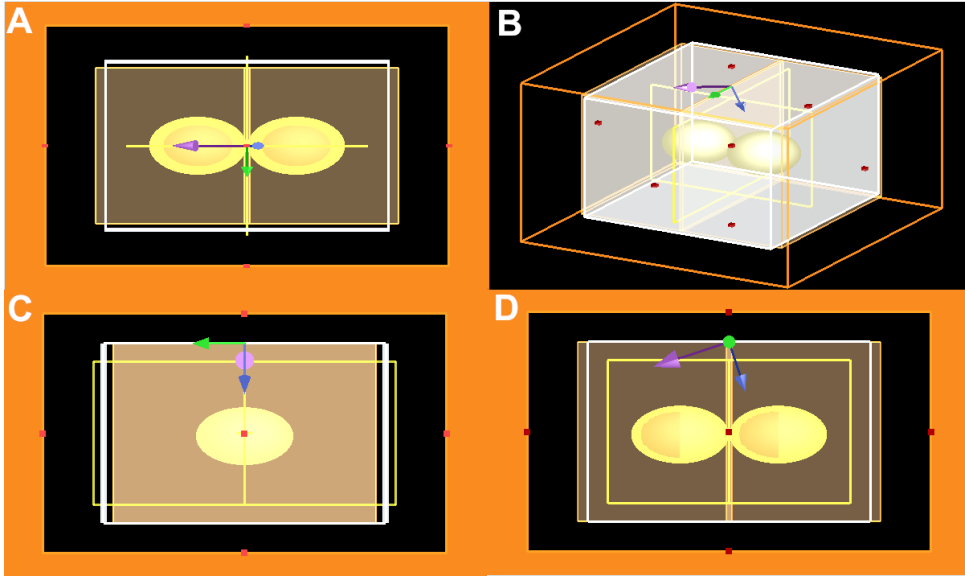


Figure 2.4: Typical FDTD simulation window with a dimer used as a model plasmonic nanocavity: (A) XY view, (B) perspective view, (C) YZ view, and (D) XZ view.

and resonance effects at the nanoscale.

The LSPR resonance condition depends on both material and geometry. For a small spherical nanoparticle in the quasistatic limit, the resonance occurs when [6]:

$$\text{Re} [\varepsilon_{\text{metal}}(\omega)] = -2 \varepsilon_{\text{diel}}, \quad (2.10)$$

a condition derived from solving the Laplace equation for electrostatics. The frequency of the dipolar LSPR mode depends on several parameters, including particle material, size, shape, environment, through its dielectric constant, as well as interparticle spacing. The above-mentioned factors ensure tunability of the optical response. Figure 2.5 shows the measured UV-Vis-NIR spectra of spherical nanoparticles and nanorods, demonstrating how increases in diameter (spherical nanoparticles) and length (nanorods) shift the LSPR.

Optical properties of metal nanoparticles under illumination are fundamentally governed by how they interact with the electromagnetic field in the far-field, where they exhibit LSPR. At resonance, these nanoparticles

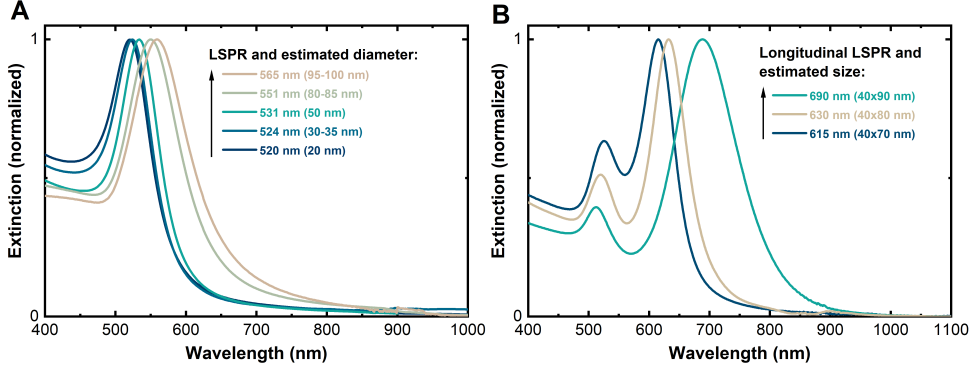


Figure 2.5: Extinction spectra of colloids of (A) spherical nanoparticles and (B) gold nanorods suspended in water. For spherical nanoparticles and nanorods, as the nanoparticle size increases, the LSPR position shifts toward longer wavelengths (red-shift).

show enhanced absorption and scattering relative to their geometric size, and these processes are quantified through cross-sections. For simple spherical nanoparticles, absorption, scattering, and extinction cross sections are given by [6]:

$$\sigma_{\text{abs}} = 4\pi k R^3 \text{Im} \left| \frac{\varepsilon(\omega) - \varepsilon_d}{\varepsilon(\omega) + 2\varepsilon_d} \right| \quad (2.11)$$

$$\sigma_{\text{sca}} = \frac{8\pi}{3} k^4 R^6 \left| \frac{\varepsilon(\omega) - \varepsilon_d}{\varepsilon(\omega) + 2\varepsilon_d} \right|^2 \quad (2.12)$$

$$\sigma_{\text{ext}} = \sigma_{\text{abs}} + \sigma_{\text{sca}} \quad (2.13)$$

where R is the nanoparticle radius, $k = 2\pi/\lambda$ is the optical wavevector, $\varepsilon(\omega)$ is the complex dielectric function of the metal, ε_d is the dielectric constant of the surrounding medium, and $\text{Im}(\cdot)$ denotes the imaginary part of a complex quantity.

For very small metal nanoparticles (typically $R < 20\text{--}30$ nm), the absorption cross-section is dominant because $\sigma_{\text{abs}} \propto R^3$ while $\sigma_{\text{sca}} \propto R^6$, meaning scattering grows much more rapidly and is negligible at small sizes. As the nanoparticle size increases (beyond $\sim 40\text{--}50$ nm), the scattering term increases rapidly and eventually exceeds absorption, making σ_{sca}

the dominant contribution to the total extinction; thus, large nanoparticles primarily scatter light while small ones mainly absorb it.

In the UV–Vis–NIR electromagnetic radiation range, noble metals such as gold, silver, and copper are capable of generating plasmonic resonances. Nevertheless, gold remains the most common choice for plasmonic research. Gold supports plasmonic resonances not only in the visible region of the electromagnetic spectrum but also in the near-infrared (NIR) range, where human tissues exhibit weak optical absorption, offering new opportunities for biomedical applications. Moreover, gold is biocompatible and only mildly cytotoxic. Gold surfaces can be easily modified, for example, by functionalization with thiol groups ($-\text{SH}$), forming stable covalent sulfur–gold ($\text{S}-\text{Au}$) bonds. Under ambient conditions, particularly in the presence of oxygen, gold is chemically stable and does not oxidize. [12]

2.1.4 Fabricating plasmonic nanocavities

Design and fabrication of plasmonic nanocavities with desired characteristics constitute a key aspect of plasmonics research. Over the years, numerous fabrication methods have been developed to produce nanocavities exhibiting unique optical properties. Nevertheless, achieving high reproducibility in nanocavity fabrication remains a practical challenge. Fabrication techniques for plasmonic nanocavities can be divided into two fundamental categories - "top-down" and "bottom-up" methods. [13, 14] Figure 2.6 shows a variety of plasmonic cavities obtained by the abovementioned techniques.

The "top-down" methods are analogous to micromachining, in which one begins with a bulk material or thin film and subsequently removes material in a selective manner to produce nanostructures. Within top-down strategies, a broad range of lithographic methods (electron-beam lithography (EBL), focused ion beam (FIB), or nanosphere lithography) [15–17] can be applied, as well as other techniques, including cryo-etching, electrochemical deposition, or reactive ion etching. [14, 18, 19] A key drawback of top-down fabrication methods is that they are often limited in achieving ul-

trasmall, atomically precise features — particularly sub-nanometer gaps — because they rely on physical patterning tools rather than molecular-level assembly. In addition, top-down processes like EBL or FIB can be expensive, slow, and not easily scalable.

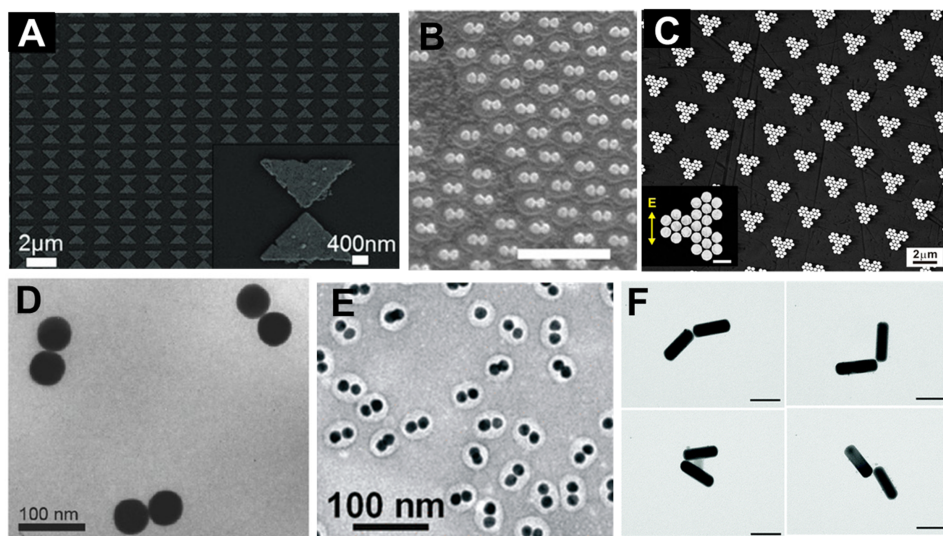


Figure 2.6: Plasmonic nanocavities with different geometries obtained using various methods. (A) SEM images of bow tie antennas on active silk films fabricated with nanoimprint lithography. Reproduced from [20]. (B) SEM image of a plane-view Ag-Ag dimer array on a Si substrate fabricated with nanoimprint lithography and shadow evaporation. Reproduced from [21]. (C) SEM image of plasmonic triphenylene structures fabricated by EBL. Reproduced from [22]. (D) SEM image of ideal dimers formed on a glass substrate synthesized by controlled assembly of monomeric nanospheres. Reproduced from [23]. (E) TEM image of dimers encapsulated in amphiphilic diblock copolymer separated through density gradient centrifugation. Reproduced from [24]. (F) TEM images of gold nanorod dimers synthesized by controlled assembly of monomeric nanorods. Reproduced from [25].

The "bottom-up" methods are based on wet-chemistry techniques. In this approach, molecules, colloids, or clusters act as building blocks that spontaneously assemble into the desired nanostructures. Wet-chemistry methods enable the formation of plasmonic nanocavities with precisely defined dimensions, often determined by the size of the organic linker employed, such as thiols [26], dithiols [27, 28], DNA origami structures [29],

or the macrocyclic cucurbit[n]uril family [30, 31]. A drawback of bottom-up techniques is the high heterogeneity of the resulting nanocavities, since aggregation processes occur spontaneously and progressively generate increasingly complex assemblies. [14, 32] Nevertheless, procedures exist that effectively increase colloidal monodispersity, achieving yields exceeding 90%. These methods utilize various chemical agents — such as linear polymers [33, 34], block copolymers [35, 36], or silica coatings — which form a protective shell around the cavity (or nanostructure), ensuring its stability under different conditions [37–39]. Subsequently, such heterogeneous mixtures with complex structural distributions can be separated using density-gradient centrifugation [40, 41] or gel electrophoresis, [42, 43] providing the desired systems in high yield.

2.2 Rayleigh and Raman scattering

Rayleigh scattering refers to the elastic scattering of light by particles much smaller than the wavelength of the incident radiation. In this process, the energy of the scattered photons remains identical to that of the incident photons, although their direction and polarization may change. This phenomenon is fundamental in explaining various optical effects, such as the blue color of the sky and the wavelength-dependent attenuation of light in transparent media.

Raman scattering is an inelastic light–matter interaction in which the scattered photons experience an energy shift corresponding to molecular vibrational or, in the case of gases, rotational transitions. This process was first predicted and described by Smekal in 1923, [44] and later experimentally demonstrated in 1928 by C. V. Raman and K. S. Krishnan. [45] Raman scattering is highly inefficient, and only a negligible fraction of the scattered photons (below 10^{-6}) undergo this process.

Raman scattering occurs when a molecule interacts with an incident photon and undergoes a transition from an initial stationary state to a virtual state, from which it subsequently transitions to a final stationary state.

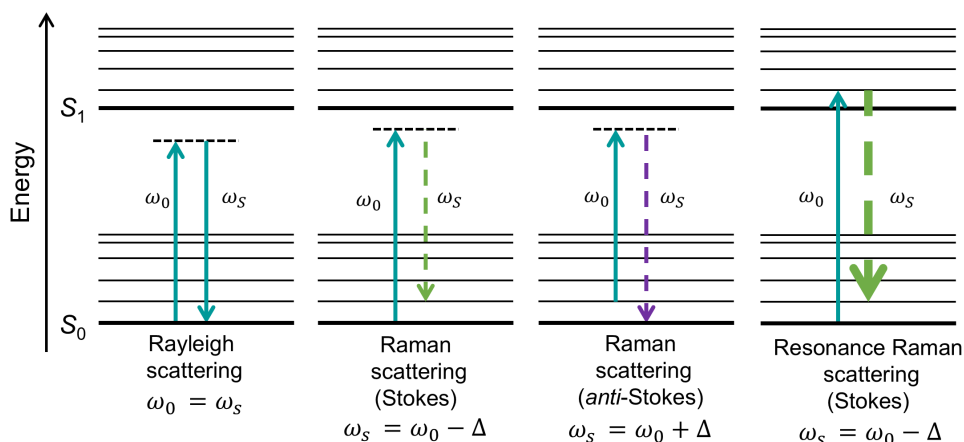


Figure 2.7: Energy-level diagram in the intensity representation illustrating Rayleigh scattering and non-resonant Raman scattering via virtual states (dashed lines), including Stokes and *anti*-Stokes processes, as well as resonance Raman scattering when the excitation energy matches a real electronic transition (S_1).

This virtual state, indicated by a dashed line in Fig. 2.7, is not a stationary state of the system and is not a solution of the time-independent Schrödinger equation. This virtual state does not correspond to a well-defined energy. [46] As a result, the energy of the scattered photon differs from that of the incident photon. When the molecule gains vibrational energy during the scattering process, the emitted photon has a lower energy (longer wavelength) than the incident one, a phenomenon known as the Stokes process. Conversely, when the molecule loses vibrational energy to the photon, the scattered photon has a higher energy (shorter wavelength), which corresponds to the *anti*-Stokes process. The Stokes and *anti*-Stokes lines appear symmetrically in the Raman spectrum relative to the excitation frequency, corresponding to the energy shifts of the scattered photons. However, when the excitation wavelength is tuned close to an electronic absorption band of the molecule the resonance Raman scattering occurs. Under these conditions, the virtual state involved in the scattering process lies energetically near a real electronic excited state, greatly enhancing the scattering efficiency. This enhancement selectively intensifies vibrational modes that are strongly coupled to the electronic transition, making resonance Ra-

man spectroscopy a powerful tool for probing chromophores and studying electronic–vibrational interactions in molecules.

The rate of photon emission in spontaneous Raman scattering, integrated over all directions, for a transparent sample of length z with an incident light intensity $I(\omega_0)$ is given by formula:

$$I(\omega_k) = N_d A z \sigma(\omega_k) I(\omega_0) \quad (2.14)$$

where N_d is the molecular number density, A is the illuminated area, and $\sigma(\omega_k)$ is the Raman cross section of the specific vibrational mode. The most significant material parameter is σ , which quantifies the responsiveness of the material. [47]

A theoretical description of Raman scattering can be formulated by treating both the molecule and the electromagnetic field within a classical framework. However, this approach exhibits inherent limitations. A refinement of the classical theory is provided by the semi-classical approach, in which the electromagnetic field is described classically, while the molecule is treated as a quantum system. [46]

According to the classical description, the origin of scattered radiation is considered to be the oscillating electric dipole induced in a molecule by the electromagnetic field of the incident light wave. This can be represented by the formula:

$$\mathbf{p} = \alpha \mathbf{E} \quad (2.15)$$

where $\mathbf{p}$ is the frequency-dependent linear induced electric dipole vector, α is the polarizability tensor of the molecule, and $\mathbf{E}$ is the electric field vector of the incident monochromatic radiation. The amplitude of the induced electric dipole at a given frequency allows the scattered intensity to be determined using the expression:

$$I = k'_\omega \omega_s^4 |p_0|^2 \sin^2 \theta \quad (2.16)$$

where the proportionality constant is defined as:

$$k'_\omega = \frac{1}{32\pi^2\varepsilon_0 c_0^3} \quad (2.17)$$

where I is the scattered intensity, ω_s is the scattered frequency, p_0 is the amplitude of the induced dipole moment, θ is the scattering angle, ε_0 is the permittivity of free space, and c_0 is the speed of light in vacuum.

The molecular polarizability tensor depends on the positions of the nuclei and therefore varies with molecular vibrations. For a non-rotating molecule, this dependence can be expressed by expanding each component of the polarizability tensor in a Taylor series with respect to the normal vibrational coordinates:

$$\alpha_{ij} = \alpha_{ij}^{(0)} + \sum_k \left(\frac{\partial \alpha_{ij}}{\partial Q_k} \right)_0 Q_k + \frac{1}{2} \sum_{k,l} \left(\frac{\partial^2 \alpha_{ij}}{\partial Q_k \partial Q_l} \right)_0 Q_k Q_l + \dots \quad (2.18)$$

where $\alpha_{ij}^{(0)}$ is the value of the polarizability component at the equilibrium configuration, and $Q_k, Q_l, \dots$ are the normal vibrational coordinates corresponding to the molecular vibrational frequencies $\omega_k, \omega_l, \dots$. The summations extend over all normal modes, and the superscript “0” on the derivatives indicates that they are evaluated at the equilibrium configuration.

Under the harmonic approximation, all terms beyond the first order in the normal coordinate Q can be neglected. Considering a single normal vibrational mode Q_k , the polarizability component can therefore be written using only the linear term of its Taylor expansion:

$$\alpha_{ij} \approx \alpha_{ij}^{(0)} + \left(\frac{\partial \alpha_{ij}}{\partial Q_k} \right)_0 Q_k \quad (2.19)$$

The molecular polarizability tensor can be expressed in vector form as:

$$\alpha_k \approx \alpha_k^{(0)} + \alpha'_k Q_k \quad (2.20)$$

The vibrational motion of a molecule's nuclei can be accurately represented

as a harmonic oscillation of a normal coordinate Q_k , expressed as follows:

$$Q_k(t) = Q_k^{(0)} \cos(\omega_k t) \quad (2.21)$$

Substituting the expression for $Q_k(t)$ into Eq. 2.20, we obtain the following relation:

$$\alpha_k \approx \alpha_k^{(0)} + \alpha'_k Q_k^{(0)} \cos(\omega_k t) \quad (2.22)$$

Taking into account that the electric field of the incident electromagnetic wave can be described as $E = E_0 \cos(\omega_0 t)$, the time-dependent induced dipole moment is:

$$p = \left[\alpha^{(0)} + \alpha'_k Q_k^{(0)} \cos(\omega_k t) \right] E_0 \cos(\omega_0 t) \quad (2.23)$$

Using the cosine product identity, the expression can be rewritten as:

$$p = \alpha^{(0)} E_0 \cos(\omega_0 t) + \frac{1}{2} \alpha'_k Q_k^{(0)} E_0 \left[\cos((\omega_0 + \omega_k)t) + \cos((\omega_0 - \omega_k)t) \right] \quad (2.24)$$

The first term corresponds to Rayleigh scattering, while the latter term represents Raman scattering involving the Raman polarizability. All molecules exhibit Rayleigh scattering because they are polarizable. However, a vibrational mode is Raman active only if it produces a change in the molecular polarizability $\alpha'_k \neq 0$. Thus, the condition for Raman activity is that, for at least one component of the polarizability tensor, the plot of that component with respect to the normal coordinate has a non-zero gradient at the equilibrium position.

Although the classical model successfully predicts the characteristic vibrational frequencies of molecules, it cannot be extended to describe molecular rotations. Moreover, it offers no insight into how the quantity α'_k depends on intrinsic molecular properties, such as electronic energy levels, and therefore cannot account for the resonance Raman effect.

The semiclassical description of the Raman effect is based on the interaction between incident photons and the quantized vibrational and electronic energy levels of a molecule. [46] Within this framework, Raman scat-

tering processes, illustrated in Fig. 2.7, are described in terms of transition probabilities between an initial state $|i\rangle$ (with energy $\hbar\omega_i$) and a final state $|f\rangle$ (with energy $\hbar\omega_f$), typically evaluated using perturbation theory. [46] By employing the Schrödinger equation together with the interaction between the electric dipole-moment operator μ and an external electric field E , one can compute the transition polarizability tensor in the semiclassical approximation. Application of second-order time-dependent perturbation theory leads to the Kramers–Heisenberg–Dirac dispersion relation [46, 48], commonly referred to as the KHD formula:

$$\alpha_{\alpha\beta}^{(i\rightarrow f)}(\omega_0) = \frac{1}{\hbar} \sum_n \left[\frac{\langle f|\mu_\alpha|n\rangle\langle n|\mu_\beta|i\rangle}{\omega_{ni} - \omega_0 - i\Gamma_n} + \frac{\langle f|\mu_\beta|n\rangle\langle n|\mu_\alpha|i\rangle}{\omega_{nf} + \omega_0 + i\Gamma_n} \right] \quad (2.25)$$

where $\langle f|\mu_\alpha|n\rangle$ denotes the α component of the dipole transition moment between the states $|n\rangle$ and $|f\rangle$, $\langle n|\mu_\beta|i\rangle$ denotes the β component of the dipole transition moment between the states $|i\rangle$ and $|n\rangle$, μ_α and μ_β are the Cartesian components of the electric dipole moment operator, ω_{ni} is the transition frequency between states i and n , ω_0 is the angular frequency of the incident electromagnetic radiation (excitation field), and Γ_n represents the finite radiative lifetime (linewidth) of the intermediate state n .

Unlike classical theory, the semiclassical approach naturally incorporates molecular electronic structure and thus explains phenomena such as selection rules and resonance Raman enhancement. The intensity of resonance Raman scattering can be expected to be orders of magnitude greater than that of normal Raman scattering. This enhancement occurs because the denominator of the first term in Eq. 2.25 becomes very small when $\omega_0 \approx \omega_{ni}$, whereas it remains large when $\omega_0 \ll \omega_{ni}$. The second term in Eq. 2.25 for the Raman polarizability tensor is often negligible in the resonance case.

2.3 Surface-enhanced Raman scattering

Surface-enhanced Raman scattering (SERS) was first reported by Fleischmann *et al.* in 1974 for pyridine adsorbed on an electrochemically roughened silver electrode. [49] In 1977, two independent research groups proposed interpretations of the anomalously enhanced Raman signal, attributing the effect to the roughness of the electrode surface. Jeanmaire and Van Duyne identified the enhancement as resulting from an electromagnetic mechanism, [50] whereas Albrecht and Creighton suggested an alternative explanation based on a chemical mechanism, involving charge transfer between the molecule and the metallic substrate. [51] Later, Moskovits proposed that the strong Raman signal originates from the optical excitation of collective electron oscillations within the nanoscale metallic features on the surface. [52, 53] The details of the exact nature and magnitude of the enhancement factor remain subjects of ongoing discussion. However, it is widely accepted that Raman signal amplification arises from two contributing mechanisms — the electromagnetic and the chemical — of which the former provides the dominant contribution. [54]

The electromagnetic enhancement arises from the excitation of localized surface plasmon resonance in metallic nanostructures. When these plasmons are resonantly excited by light with an appropriate frequency, they produce highly intensified electromagnetic fields near the metal surface, particularly at sharp features or junctions known as hot-spots. Molecules located in these regions experience a much stronger local field, leading to a dramatic increase in the observed Raman scattering intensity. This enhancement can be summarized as:

$$G_{\text{EM}} = \beta^2(\omega_0) \beta^2(\omega_0 + \omega_v) \quad (2.26)$$

where $\beta(\omega_0)$ is the local field enhancement at the excitation frequency ω_0 , $\beta(\omega_0 + \omega_v)$ is the enhancement of the emitted (scattered) Raman field, and $\beta(\omega)$ represents the dimensionless enhancement factor indicating how much the local electric field is amplified by the plasmonic nanostructure. It is de-

defined as:

$$\beta(\omega) = \frac{|E_{\text{loc}}(\omega)|}{|E_0(\omega)|} \quad (2.27)$$

where $E_{\text{loc}}(\omega)$ is the local electric field at the position of the molecule, near the metal nanoparticle or surface, and $E_0(\omega)$ is the incident electric field, i.e., the laser field in free space without the nanostructure.

The plasmon resonance does not amplify the frequency of the incident radiation and the frequency of the Raman-scattered radiation to the same extent. However, for small vibrational frequencies, it can be assumed that $\beta(\omega_0) \approx \beta(\omega_0 + \omega_v)$. In this case, the electromagnetic enhancement scales as:

$$G_{\text{EM}} = \beta^4(\omega) \quad (2.28)$$

The electromagnetic enhancement does not require the analyte of interest to be in direct contact with the metallic surface, but does require it to be within a few nanometers. Masango *et al.* investigated the high-resolution distance dependence of SERS with a plasmonic substrate obtained by atomic layer deposition to control the thickness of the spacer. [55] Figure 2.8 presents the SEM image of this plasmonic substrate and high-resolution distance dependence of the SERS intensity measured on Ag film over nanospheres (AgFON). The results show that the SERS intensity decreases rapidly to approximately 20% of its maximal value at a distance of about 0.7 nm from the surface. [55] Beyond 1 nm, the intensity decays more gradually, reaching roughly 7% of the initial signal at a distance of 3 nm. [55] According to the electromagnetic mechanism, the magnitude of the enhancement is expected to be independent of the molecular species.

The chemical enhancement mechanism is a short-range effect that requires the molecule to be chemisorbed directly onto the metallic surface. This mechanism arises from interactions between the molecular orbitals of the adsorbed species and the conduction band of the metal. Such interactions induce charge-transfer processes that modify the molecular polarizability and enhance the Raman scattering cross-section of the vibrational

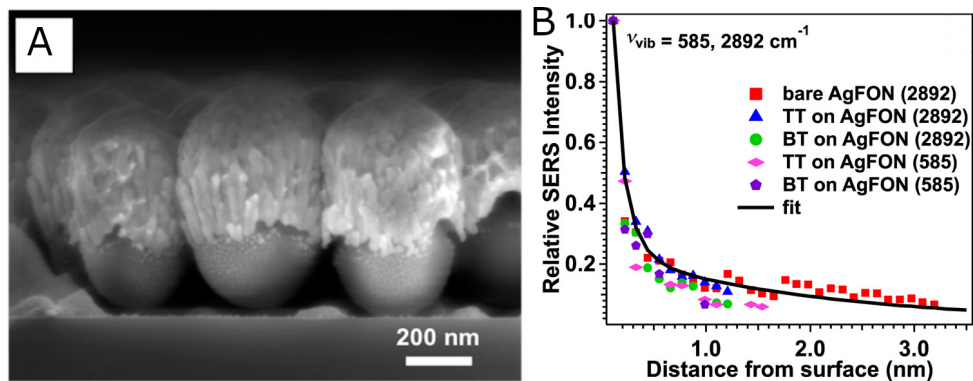


Figure 2.8: (A) SEM image of the AgFON substrate from side observation. (B) The high-resolution distance dependence of the SERS intensity measured on AgFON substrates coated with Al_2O_3 layers of varying thickness deposited by atomic layer deposition (ALD) at 55°C . The analysis is based on the symmetric C-H stretching mode at 2892 cm^{-1} and the symmetric Al- CH_3 stretching mode at 585 cm^{-1} originating from trimethylaluminum (TMA). For Al_2O_3 layers deposited on bare AgFONs, the SERS intensity decreases to approximately 20 % of its initial value when the TMA-(CH_3) $_x$ groups are located about 0.7 nm from the metal surface. Beyond a separation of 1 nm, the decay of the SERS signal becomes less pronounced. At a distance of approximately 3 nm from the surface, the SERS intensity is reduced to about 7 % of the initial value. Reproduced from [55].

modes. The chemical enhancement can be defined as follows:

$$G_{\text{CHEM}} = \frac{\sigma_k^{\text{ads}}}{\sigma_k^{\text{free}}} \quad (2.29)$$

where σ_k^{ads} and σ_k^{free} denote the Raman scattering cross-sections of the k -th vibrational mode of the adsorbed and free molecule, respectively.

The magnitude of the chemical enhancement strongly depends on the specific molecule being probed. Nevertheless, it is typically much smaller than the electromagnetic contribution.

The SERS enhancement factor can be defined as the ratio of the intensities of the bands observed in the SERS and Raman spectra, taking into account the number of molecules contributing to the recorded signal in each measurement. The magnitude of the enhancement factor depends on several parameters, including the material, shape, and size of the

plasmonic nanostructure, the characteristics of the excitation line (such as wavelength, polarization, or angle of incidence), as well as the intrinsic properties of the molecule and its surrounding environment. Experimentally determined enhancement factors typically range between 10^6 and 10^8 , enabling the detection and real-time observation of SERS spectra from single molecules. [56, 57] However, the enhancement factor is not uniformly distributed but rather confined to spatially localized regions (typically $< 100 \text{ nm}^2$). Feng *et al.* investigated the contribution of various enhancement sites to the overall SERS signal. [58] Their calculations demonstrated that only 63 out of one million molecules located at so-called “hot-spots” (with enhancement factors exceeding 10^9) contribute approximately 24% of the total SERS signal. In contrast, the coldest sites, which contain about 61% of the molecules, account for merely 4% of the overall SERS intensity.

SERS spectra may differ significantly from those obtained in spontaneous Raman scattering. This phenomenon arises from the interaction between the molecule and the metal surface, more specifically, from the orientation of the molecule relative to the vector of the electromagnetic field. Such an interaction can lead, among other effects, to perturbations in molecular symmetry, which in turn result in changes in the relative intensities of the bands observed in the SERS spectrum. Consequently, the SERS spectrum typically exhibits an enhancement of the Raman bands corresponding to those normal vibrations whose direction and Raman tensor are aligned with the vector of the electromagnetic field. In most cases, an enhancement of vibrational modes oriented perpendicular to the metal surface is observed, in contrast to those whose direction is parallel to the surface. In very rare cases, it is also possible to observe bands with zero intensity in spontaneous Raman spectra, or even bands that are symmetry-forbidden for the molecule. [59–61]

2.4 Femtosecond stimulated Raman scattering

Stimulated Raman scattering (SRS) is a third-order nonlinear coherent Raman process in which the simultaneous interaction of an intense pump photon and a Stokes photon with a molecular system selectively drives the vibrational transitions. In contrast to spontaneous Raman scattering, which is inherently weak and characterized by incoherent photon emission, SRS generates a coherent signal that is substantially stronger and propagates along the direction of the excitation photons. While spontaneous Raman scattering relies on random emission events, the presence of the Stokes field in SRS induces coherent stimulated emission, resulting in orders-of-magnitude enhancement in signal strength. [62]

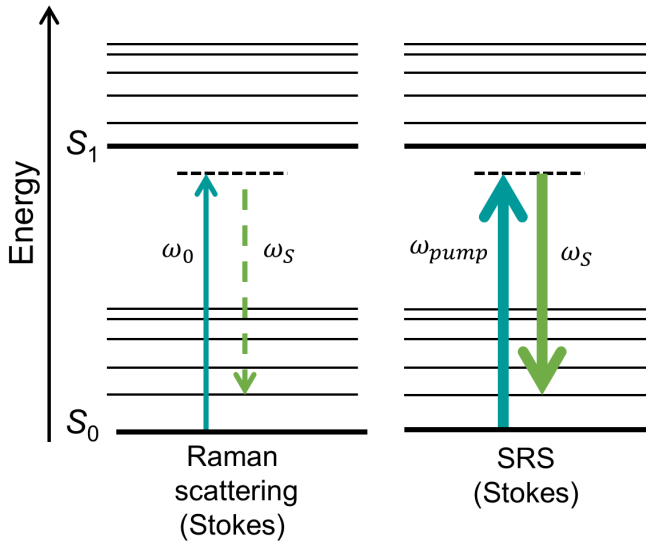


Figure 2.9: Energy-level diagram in the intensity representation illustrating Raman scattering and the SRS process.

The Jabłoński diagram of the SRS process is shown in Fig. 2.9. From a quantum-mechanical perspective, the mechanism is straightforward: upon interaction with the medium, a photon of frequency ω_{pump} is absorbed (annihilated) and a photon of frequency ω_S is emitted (created). The energy difference $\omega_{\text{pump}} - \omega_S$ is transferred to the material, exciting a vibrational

mode. The SRS process can therefore be quantified either by monitoring the depletion of photons in the pump beam or by measuring the amplification of photons in the Stokes beam. These detection approaches are referred to as stimulated Raman loss (SRL) and stimulated Raman gain (SRG), respectively. Importantly, both SRL and SRG provide equivalent Raman spectral information.

The magnitude of the SRS signal is proportional to the imaginary part of the third-order nonlinear susceptibility tensor, which provides direct information about absorptive processes and energy dissipation associated with a specific vibrational mode, as well as to the product of the pump and Stokes beam intensities, I_{pump} and I_{Stokes} , respectively. This relationship can be written as:

$$I_{\text{SRS}} \propto \text{Im}[\chi^{(3)}(\Omega)] I_{\text{pump}} I_{\text{Stokes}} \quad (2.30)$$

where $\chi^{(3)}(\Omega)$ denotes the third-order nonlinear susceptibility evaluated at the Raman frequency $\Omega = \omega_{\text{pump}} - \omega_{\text{Stokes}}$. The quantities I_{pump} and I_{Stokes} represent the intensities of the pump and Stokes optical fields, respectively.

Femtosecond stimulated Raman scattering (FSRS) extends the capabilities of conventional SRS by employing ultrashort laser pulses that allow direct probing of molecular vibrations on femtosecond timescales. In the scientific literature, the acronym FSRS is used to describe both the two-pulse steady-state technique and the three-pulse time-resolved method. In the two-pulse FSRS technique, only two laser pulses are used: a narrowband picosecond Raman pump pulse and a broadband femtosecond probe pulse. These two pulses interact simultaneously with the sample, generating SRG or SRL that reports on the vibrational spectrum under steady-state conditions without explicit time resolution of molecular dynamics. In contrast, the three-pulse FSRS technique employs three laser pulses: an additional femtosecond actinic pump pulse, followed by the Raman pump and broadband probe pulses. The actinic pump initiates a process, photochemical reaction or excite the system to a more energetic electronic state. After a time delay from the photoexcitation picosecond Raman pump pulse and femtosecond probe pulse interact with the system in order to create vibra-

tional coherence and generate the stimulated Raman signal that hold the time-resolved structural information about the investigated system. [63–66] However, in this dissertation, whenever FSRS is referenced, it exclusively refers to the two-pulse implementation and the measurement of stationary states.

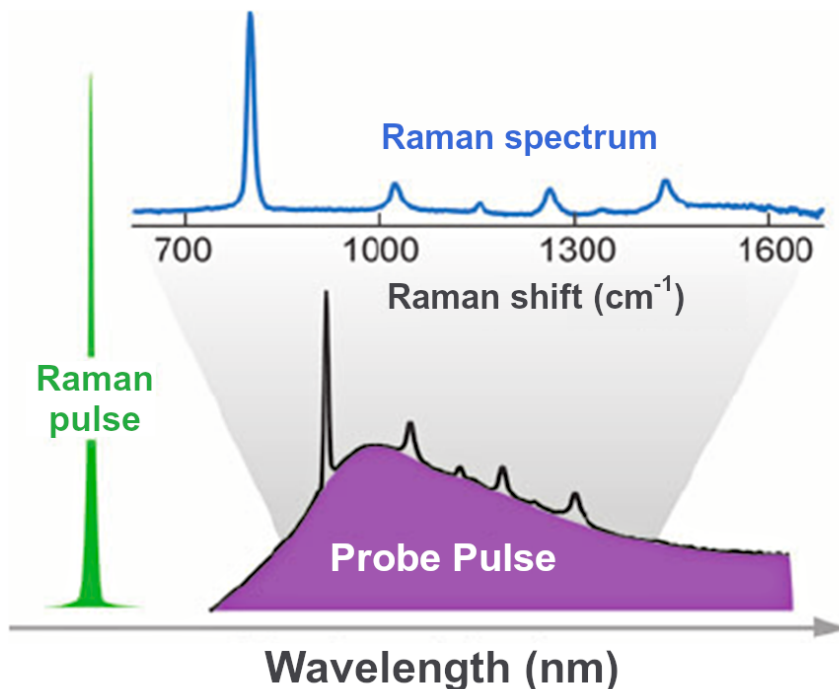


Figure 2.10: Illustration of the spectra used in broadband probing in FSRS spectroscopy. The Raman pulse is a narrowband picosecond pulse (green), while the probe pulse is a broadband femtosecond pulse (purple). Dividing the spectrum of the probe pulse in the presence of the Raman pulse (black) by the spectrum of the probe pulse in the absence of the Raman pulse (purple) yields the stimulated Raman gain spectrum (blue). Reproduced from [63].

In ground-state FSRS, the simultaneous interaction of a narrow-bandwidth picosecond Raman pump pulse with a broadband femtosecond continuum probe pulse results in stimulated photon transfer from the high-intensity Raman pump pulse to the weak probe pulse. As a consequence, sharp vibrational enhancement features appear in the probe spectrum at frequencies whose difference from the Raman pump frequency corresponds

to the vibrational resonances of the molecule. [63] A schematic illustration of the interaction between the Raman pump and probe pulses is shown in Fig. 2.10.

The spectrum of stimulated Raman gain $\text{Gain}_{\text{FSRS}}(\omega)$ is obtained by dividing the probe spectrum measured in the presence of the Raman pump pulse by the probe spectrum recorded with the Raman pump pulse blocked, according to the following relation:

$$\text{Gain}_{\text{FSRS}}(\omega) = \frac{I_{\text{probe+Raman}}(\omega)}{I_{\text{probe}}(\omega)} \quad (2.31)$$

where $I_{\text{probe}}(\omega)$ represents the measured spectral profile of the probe pulse recorded in the absence of the Raman pump pulse, and $I_{\text{probe+Raman}}(\omega)$ represents the measured spectral profile of the probe pulse in the presence of the Raman pump pulse.

The Raman pump defines the spectral resolution of the vibrational features, while the femtosecond probe ensures ultrafast temporal resolution, enabling a snapshot of the instantaneous molecular structure. This configuration allows the vibrational signatures to be extracted with high signal-to-noise ratio and minimal spectral congestion, even in complex molecular systems. Consequently, FSRS provides a powerful means of resolving subtle structural changes and mode couplings that are inaccessible with spontaneous or stationary Raman methods.

Chapter 3

Research Objective

The general objective of this doctoral dissertation was to improve understanding and control of the physical and chemical processes occurring in systems involving molecules confined within plasmonic nanocavities. In particular, the interactions of such systems with light were investigated using surface-enhanced Raman spectroscopy and surface-enhanced femtosecond stimulated Raman spectroscopy. The specific objectives included:

1. Elucidating the temporal evolution of single-molecule SERS spectra at both room and cryogenic temperatures with improved temporal resolution.
2. Controlling fluctuations in single-molecule SERS spectra through encapsulation of the investigated molecule within a macrocyclic supramolecular host.
3. Optimizing a methodology based on surface-enhanced femtosecond stimulated Raman scattering as a novel measurement tool for investigating process dynamics on ultrafast timescales.

Chapter 4

Temporal Evolution of Single-Molecule Surface-Enhanced Raman Spectra at Room and Cryogenic Temperatures

4.1 Introduction

4.1.1 Detecting single molecules by SERS

Single-molecule spectroscopy has opened new avenues for investigating molecular dynamics with a level of detail and resolution that was previously inaccessible. It offers significant advantages over conventional ensemble experiments performed in a cuvette, where the measured signals are averaged over a large number of molecules. By probing individual molecules one at a time, this approach reveals heterogeneity in structure, dynamics, and reactivity that is completely hidden in bulk measurements. It allows direct observation of rare events, transient states, and stochastic fluctuations that are washed out in ensemble averages. Single-molecule techniques also enable the study of molecular dynamics in real time without the need for synchronization across a population. In contrast to standard “in cuvette” experiments, which provide only mean values, single-molecule spectroscopy yields distributions and correlations that offer deeper mechanistic insight. As a result, it has become a crucial tool for understanding complex chemical and biological systems at the most fundamental level.

Over the past several decades, a broad range of analytical techniques has been developed to mitigate and overcome the limitations imposed by ensemble averaging, including single-molecule fluorescence spectroscopy, scanning tunneling microscopy, and atomic force microscopy. These single-molecule methods can provide detailed information on molecular electronic states, surface topography, and even the response of individual molecules to applied stretching or torsional mechanical forces. However, despite their strengths, many of these techniques remain limited by a lack of intrinsic chemical specificity. To address the limitations of the aforementioned techniques, Raman scattering spectroscopy is employed. As a complementary method to infrared spectroscopy, Raman spectroscopy provides detailed information on molecular structure through the observation of vibrational modes. Consequently, Raman spectroscopy constitutes a valuable addition to the set of techniques used for investigating molecules as individual enti-

ties.

The first experimental evidence of single-molecule detection using Raman spectroscopy was independently reported in 1997 by Nie and Emory [67] as well as by Kneipp and co-workers. [68] Both studies demonstrated that SERS can provide sufficiently large signal enhancements to overcome the intrinsically weak Raman scattering cross-section. Nie and Emory demonstrated single-molecule sensitivity by detecting the Raman spectra of individual rhodamine 6G molecules adsorbed onto Ag nanoparticles. [67] Kneipp *et al.* reported a statistical analysis of signal fluctuations of single-molecule SERS spectra of crystal violet using Ag colloids as a plasmonic substrate. [68] Together, these pioneering works established SERS as a viable technique for single-molecule vibrational spectroscopy. They marked a turning point in the field by demonstrating that Raman spectroscopy could achieve chemical specificity at the ultimate limit of single-molecule detection.

To confirm that a measured Raman spectrum originates from a single molecule, three main approaches are currently employed. These include (i) the use of ultra-low analyte concentrations, (ii) monitoring the fluctuations of the Raman signal until the occurrence of a sudden and irreversible signal drop of the signal, [69–71] and (iii) the bi-analyte approach, in which two spectroscopically distinguishable analytes are used simultaneously to statistically verify single-molecule detection. [72, 73] Together, these methods provide complementary and widely accepted criteria for validating single-molecule Raman measurements. Their combined application significantly increases the reliability and rigor of single-molecule spectroscopic analysis.

4.1.2 Origins of single-molecule SERS fluctuations

Fluctuations in SERS spectroscopy signals are recognized as a hallmark of single-molecule events. In many early studies, pronounced temporal variations in the SERS intensity were interpreted as evidence of single-molecule detection, serving as an alternative validation approach to experiments based on ultra-low analyte concentrations. Despite numerous experiments and proposed explanations, the origin of signal fluctuations in SM-SERS spectra

remains a highly debated topic within the SERS community. Nevertheless, it is generally accepted that SM-SERS fluctuations can be categorized into two types: temporal and spectral.

Temporal fluctuations are defined as absolute and/or relative changes in the intensities of Raman bands as a function of time. An interesting phenomenon accompanying temporal fluctuations is blinking, which is commonly observed in single-molecule fluorescence. It consists of the systematic appearance and sudden disappearance of the SERS signal (on-off behavior) over a broad time scale, ranging from microseconds to minutes. In recent years, this phenomenon has been the subject of intensive research, leading to an assessment of the influence of irradiation energy, electrolyte concentration, temperature, electromagnetic enhancement, as well as excitation wavelength on the specific blinking behavior. A qualitative analysis of blinking events in SERS conducted by Itoh *et al.* classifies it into (a) stable, (b) fluctuating, and (c) intermittent. [74] In subsequent studies, two interpretations were proposed. According to the first, blinking was attributed to unstable adsorption of the molecule on the nanoparticle surface, resulting in variations in the molecule-hot-spot distance and, consequently, changes in the enhancement factor. The second interpretation highlighted shifts in the plasmonic resonance induced by local photothermal effects, which also lead to variations in the enhancement factor.

Relative changes in Raman band intensities over time-dependent SM-SERS trajectories are typically attributed to the rotational motion of molecules within a plasmonic nanocavity. This is related to the fact that the molecular symmetry of a molecule adsorbed on a metal surface is reduced, and that the plasmonic cavity can selectively enhance those vibrational modes for which the molecular vibration vectors and the corresponding Raman tensors are aligned with the electromagnetic field vector of the plasmonic cavity. Relative changes in the intensities of Raman bands whose Raman tensors are mutually orthogonal indicate a change in molecular orientation, corresponding to rotational motion of the molecule.

Spectral fluctuations, often referred to as spectral diffusion, correspond to sudden and sharp changes in the positions of individual Raman bands

by approximately $\pm 5 \text{ cm}^{-1}$ relative to the spectrum averaged over a given time trace recorded for a single molecule. In contrast to temporal fluctuations, in which only changes in the intensities of Raman bands occur, spectral fluctuations involve modifications of the vibrational structure of the molecule. In 2016, Benz *et al.* introduced the concept of "picocavities" — sub-nanometer-scale regions whose mode volume is smaller than 1 nm^3 . [75] Picocavities are formed when a single atom from the surface of a nanoparticle, referred to as an adatom, establishes a "coordination bond" with a fragment of the molecule. Comprehensive DFT calculations indicate that, for each molecule, a well-defined stable adatom–molecule configuration exists, which in straightforward cases shows good agreement with experimental picocavity SERS observations. [76] However, experimental data are typically limited to thiol-ended molecules.

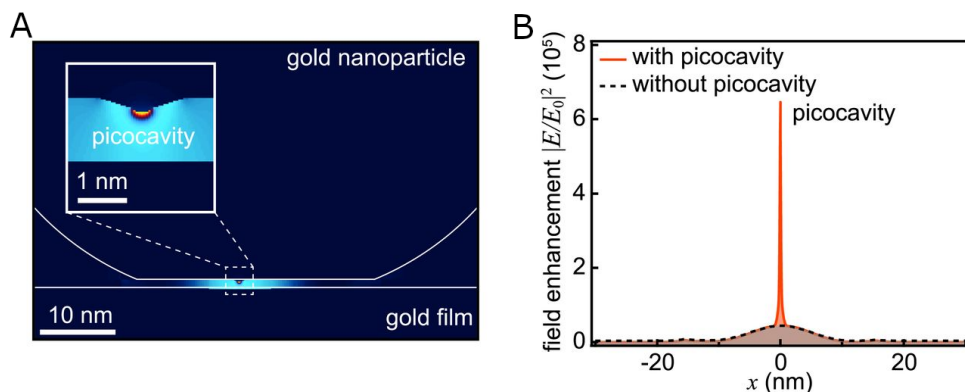


Figure 4.1: (A) Schematic illustration of a “picocavity” formed on the surface of a nanoparticle, showing sub-nanometer localization of the optical field (enlarged view). (B) Near-field intensity distribution. Reproduced from [75].

The appearance of Raman bands at new frequencies during the acquisition of time-resolved SM-SERS spectra may be associated with the occurrence of a chemical reaction. Tallarida *et al.* observed spectral diffusion of Raman bands that took place during a photoisomerization process of azobenzyl thiols adsorbed on Au(111). [77] The hallmark of single-molecule *cis-trans* isomerization manifests as anticorrelated flip-flop behavior between two distinct spectral states, characterized by discrete on and off lev-

els. [77] Choi *et al.* demonstrated that SERS can monitor single-molecule catalytic reactions in real-time. [78] The study was focused on the dimerization of 4-nitrobenzenethiols, which has previously been studied at an ensemble level. Li *et al.* probed in real-time by SERS a photo-induced carbon-carbon bond cleavage reaction between xanthene and phenyl groups of a single rhodamine B isothiocyanate molecule. [71] Recently, SERS was used to monitor a single-enzyme catalytic reaction by utilizing a DNA origami-based plasmonic antenna. [79]

In most cases, temporal and spectral fluctuations coexist during the acquisition of single-molecule SERS signals. However, to date, no clear and unambiguous relationship between these two types of fluctuations has been demonstrated. Consequently, the underlying mechanisms governing their mutual interplay remain an open question and constitute an important challenge in the interpretation of single-molecule SERS experiments.

4.2 Materials and methodology

4.2.1 Materials

Gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) ($\geq 99.9\%$ trace metals basis), hexadecyltrimethylammonium bromide (CTAB) ($> 99.5\%$), sodium borohydride (NaBH_4) (99%), silver nitrate (AgNO_3) ($\geq 99\%$), and L-ascorbic acid (AA) (99%) were obtained from Sigma-Aldrich. Acetone (HPLC, 99.9%), hydrogen peroxide (30%), nitric acid (63%), hydrochloric acid (38%), and sulfuric acid (98%) were purchased from CHEMPUR. Porphycene- d_0 and porphycene- d_{12} were provided by Prof. Jacek Waluk. All chemicals were used without further purification. Deionized water (Elix, Millipore, $> 15 \text{ M}\Omega \cdot \text{cm}^{-1}$) was used in all experiments. All glassware was thoroughly cleaned with aqua regia and subsequently rinsed with deionized water.

4.2.2 Plasmonic nanocavities preparation and characterization

We performed SM-SERS measurements using aggregated gold nanorods (AuNRs) as plasmonic nanocavities. Porphycene (Pc) was used as a Raman reporter molecule.

AuNRs were synthesized using the seed-growth method, as described by Nikoobakht and El-Sayed. [80] In the first step, the seed solution was prepared as follows: 5 mL of 0.05 mM HAuCl_4 was mixed with 5 mL of 20 mM CTAB solution in a 20 mL vial. Next, 0.6 mL of ice-cold 0.01 M NaBH_4 was injected into the HAuCl_4 -CTAB mixture under vigorous stirring (1 200 rpm). The solution changed color from yellow to brownish yellow. Vigorous stirring of the seed solution was continued for 2 min. The seed solution was aged at room temperature for 30 min before use. To prepare the growth solution, CTAB (5 mL, 0.20 M) was mixed with AgNO_3 (100 μL , 4 mM) solution at 25 °C. To this solution, 5.0 mL of 1 mM HAuCl_4 was added, and after gentle mixing, 70 μL of 0.0788 M AA was added. After adding AA, the growth solution changed color from dark yellow to colorless. The final step was the addition of 60 μL of the seed solution to the growth solution at 30 °C. The resultant mixture was stirred for 30 s and left undisturbed at 30 °C for 12 h for the growth of nanorods. The color of the solution gradually changed from colorless to a bluish hue.

AuNRs, prior to deposition, were centrifuged several times to remove excess of CTAB and residual chemicals remaining after synthesis. For this purpose, 1.5 mL of AuNRs (used as synthesized) were placed in an Eppendorf tube and centrifuged at 10 000 rpm for 10 min using a centrifuge (Eppendorf MiniSpin). The supernatant solution was subsequently removed by decantation, and 1 mL of deionized water was added. This procedure was repeated at least five times until no further foaming was observed. After the final centrifugation step, approximately 100–200 μL of water was added to the precipitate. The prepared AuNR solution (10–20 μL) was then deposited onto a cover glass (No. 1, Brand) that had been previously cleaned with piranha solution and allowed to dry under ambient conditions. After

drying, an ethanolic solution containing an equimolar mixture of Pc-d₀ and Pc-d₁₂ (total concentration 1×10^{-10} M) was applied to the prepared substrate (5 μ L) and left to dry again under ambient conditions.

4.2.3 SERS measurements

SERS and single-molecule SERS spectra were measured using a home-built multifunctional microspectroscopy system designed and built by Dr. Sylwester Gawinkowski. [81] Figure 4.2 shows a schematic of the system with minor modifications for measurements at cryogenic temperatures. The system consists of two components: an excitation part and a detection part.

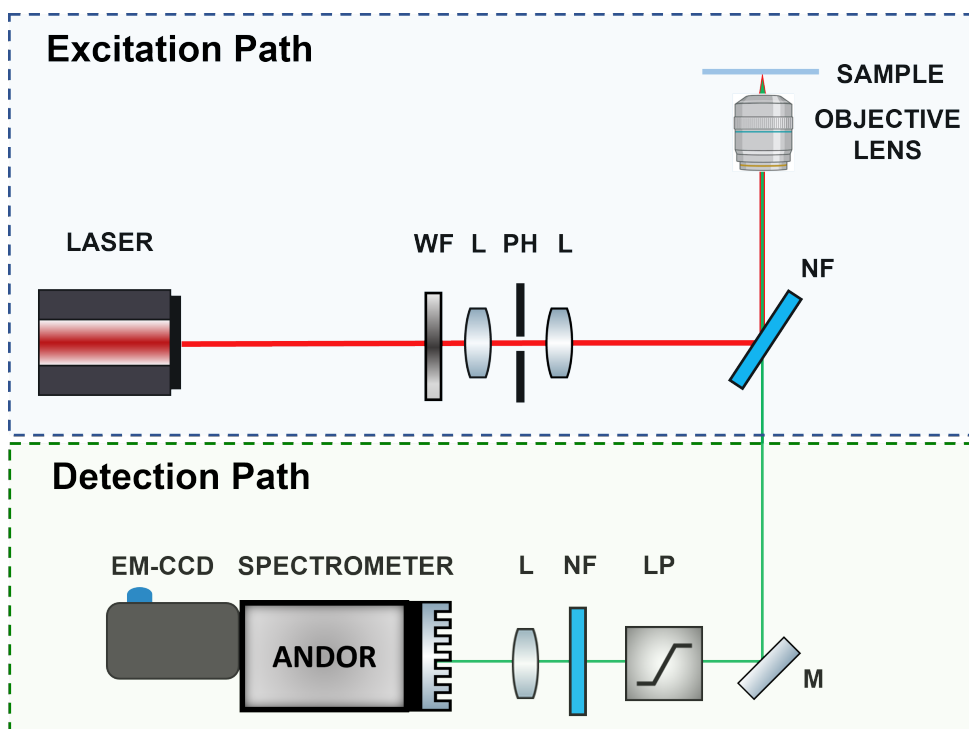


Figure 4.2: Schematic of the home-built multifunctional microspectroscopy system. Tunable neutral-density filter wheel (WF), lens (L), long-pass filter (LP), mirror (M), pinhole (PH), and notch filter (NF).

The laser beam emitted by a He–Ne laser (LASOS) had a wavelength of $\lambda = 632.8$ nm. The beam was subsequently directed through a tun-

able wheel optical density filter (FW1AND, Thorlabs), which was used to adjust the excitation intensity at the sample. The laser beam with the appropriate intensity was then directed to a notch filter (633 nm, OptiGrate) to clean the laser light, which functioned as a beamsplitter in the optical system. Next, the beam was focused onto a pinhole (P25D, Thorlabs) using a lens. After passing through the pinhole, another lens collimated the beam, which was then directed into the inverted microscope (ECLIPSE Ti2-U, Nikon) and focused onto the sample from below using a microscope objective. Three types of microscope objectives were used in this study: (i) C Epiplan-Apochromat 50 $\times$, NA = 0.95, Zeiss; (ii) CFI Apochromat TIRF 100XC, NA = 1.49, Nikon; and (iii) Semi-Apochromat LMPLFLN 50 $\times$, NA = 0.5, Olympus (used only for measurements at low temperatures). The sample was mounted on an XYZ translation stage (H117E1N4/G, Prior), which provided precise positioning and enabled scanning with step sizes of 100 nm.

The scattered light beam passed through a beamsplitter before being directed to a long-pass filter (VersaChrome TLP01-704, Semrock). After passing through the long-pass filter, the beam was directed onto a second notch filter to remove residual Rayleigh scattering. Finally, the signal beam was focused by an achromatic lens ($f = 100$ mm) onto the entrance slit of a monochromator (Kymera 328i, Andor) and recorded using an EM-CCD camera (iXon Ultra DU-888U3-CSO-EXF, Andor). The monochromator was equipped with four diffraction gratings (150, 300, 600, and 1200 grooves/mm), enabling the selection of an appropriate spectral resolution for hyperspectral mapping. With this configuration, the system was capable of recording Raman spectra with a resolution of approximately 4–5 cm^{-1} , starting from Raman shifts of about 100 cm^{-1} , depending on the selected spectral range.

A CMOS camera (DCC1545M) was mounted at the exit port of the microscope. The camera was used for previewing the sample, adjusting the relative positions of the sample and objectives, and observing wide-field images through the microscope oculars. Visual inspection during positioning and alignment of the sample, laser spot, and objectives was enabled by the camera.

Measurements at cryogenic temperature were performed using a heating and freezing cryostat (Linkam TMS 600) with liquid nitrogen as the cooling medium. The temperature setting accuracy was 0.1 °C. The sample inside the cryostat was secured with cryogenic tape.

Home-written MATLAB software was developed to control the system and perform data acquisition. The software provided automated control of the translation stage for precise sample positioning, as well as control of the EM-CCD camera for hyperspectral raster mapping of Raman spectra and recording time-resolved traces. The acquired data were stored in a MATLAB-compatible format, enabling straightforward loading and subsequent numerical analysis and processing. Time-resolved SERS spectra with a temporal resolution below 0.5 s were recorded using commercial Solis software controlling the monochromator.

4.2.4 Quantum-chemical simulations

The initial geometries of the Pc molecule and Pc complexes containing Au adatoms or clusters were generated and pre-optimized using the Gabedit software. Subsequent geometry optimizations and vibrational frequency calculations were carried out with Gaussian 16 using the PBE1PBE hybrid functional in combination with the Def2TZVPP basis set. All structures were fully optimized prior to performing vibrational analyses. The nature of the stationary points was verified by confirming the absence of imaginary frequencies. Raman spectra were obtained by converting the calculated Raman activities from Gaussian output files into intensities, taking into account the temperature and vibrational frequencies, and were visualized using Chemcraft software. [82, 83]

4.3 Results and discussion

4.3.1 Plasmonic nanocavities characterization

AuNRs were synthesized using the seed-mediated growth method and characterized by SEM imaging and UV–Vis–NIR spectroscopy. Characterization of AuNRs is presented in Fig. 4.3. Based on the SEM images, a morphological analysis of individual AuNRs was performed, revealing that the average length of the AuNRs was 72 ± 6 nm, while the average width was 41 ± 5 nm. The extinction spectrum of AuNRs dispersed in water was measured over the spectral range of 400–900 nm and exhibited LSPR peaks at wavelengths of 524 nm and 624 nm, corresponding to the transverse and longitudinal LSPR bands, respectively.

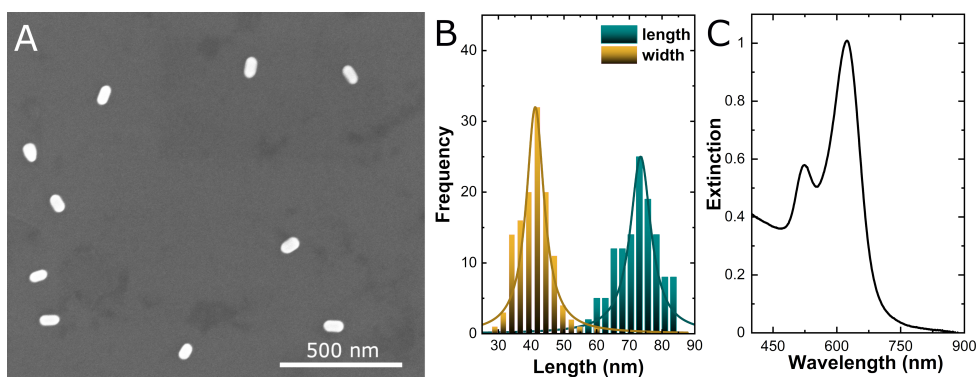


Figure 4.3: Characterization of AuNRs: (A) SEM image, (B) distribution of length and width of AuNRs, and (C) the extinction spectrum of AuNRs suspended in water.

4.3.2 Detection of single-molecule SERS spectra of porphycene (Pc)

Porphycene (Pc) was first observed at the single-molecule level using fluorescence spectroscopy [84–86] and scanning tunneling microscopy (STM) [87–90] Kumagai *et al.* investigated an individual Pc molecule adsorbed on a Cu(110) surface using STM. [88] Their results demonstrated that, under

these conditions, the molecule adopts a previously unobserved rare *cis* tautomer, which becomes more stable than the *trans* tautomer. Furthermore, the authors reported that the *cis*–*cis* tautomerization can be controlled through the atomic-scale placement of a single copper adatom in the vicinity of an individual Pc molecule.

Gawinkowski *et al.* demonstrated the detection of single-molecule spectra of Pc using Au and Ag nanoparticles as SERS substrates. [69] Single-molecule SERS (SM-SERS) detection of Pc was established using a bi-analyte approach, in which two isotopologues were employed. The single-molecule regime was reached when the total concentration of both isotopologues was reduced to 10^{-10} M. At this concentration, however, non-deuterated Pc was observed significantly more frequently than Pc-d₁₂, indicating that Pc-d₁₂ possesses a higher diffusion coefficient on the Au nanoparticle surface. In the same study, the authors found that Pc molecules exhibit photostability enhanced by approximately two orders of magnitude when adsorbed on Au or Ag surfaces compared with solution-phase fluorescence measurements. They attributed the increased stability of adsorbed Pc relative to the free molecule to a reduction in the lifetime of the lowest excited singlet state, which consequently decreases the probability of intersystem crossing to the triplet state. Temporal evolution measurements of SM-SERS spectra of Pc revealed the presence of a rare *cis* tautomeric form, which appeared only sporadically and spontaneously, while the *trans* form remained dominant. This *cis* form had previously been observed only on Cu(110) single-crystal surfaces using STM. [88] Nogala *et al.* also reported the detection of single-molecule spectra of Pc using Au nanostructures on indium tin oxide as a plasmonic SERS substrate. [91]

Time-resolved SERS spectra of single Pc molecules were recorded using aggregated AuNRs as the plasmonic substrate. In each case, the trajectory of the SM-SERS spectra was recorded until a sudden, drastic intensity decay occurred, as shown in Fig. 4.4. During the time course of the SM-SERS spectra of Pc, we observed strong fluctuations of the intensity of Raman bands, as well as spectral shifts, which are a hallmark of single-molecule SERS spectra.

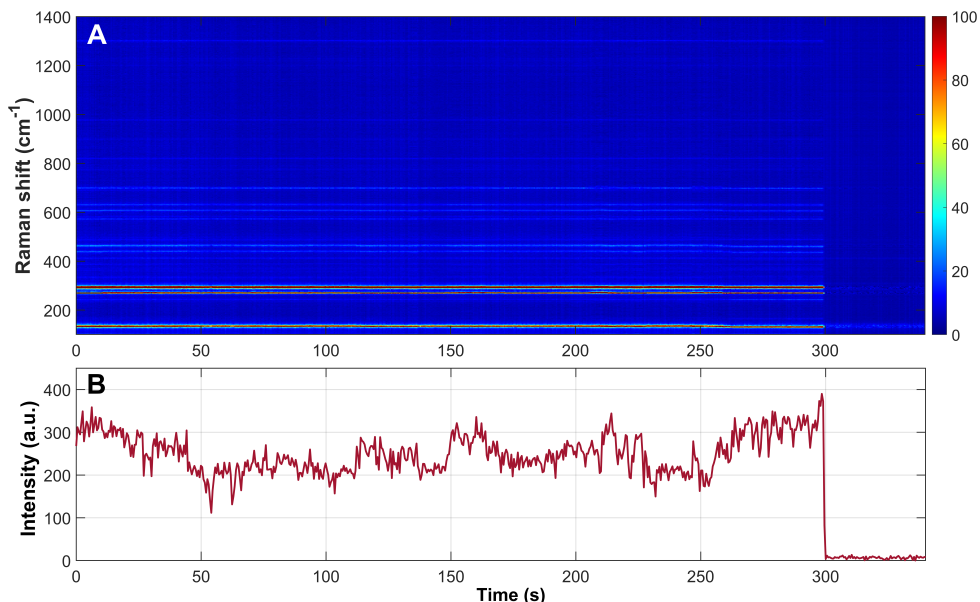


Figure 4.4: Example of the temporal evolution of SM-SERS spectra of Pc measured with a 0.5 s accumulation time and a 633 nm excitation wavelength at cryogenic temperature ($T = 83$ K). (A) Spectral time-dependent trajectory with (B) corresponding fluctuations of the SERS signal intensity for the Raman band at 343 cm^{-1} .

The sudden and abrupt disappearance of the SERS signal most often results from the decomposition of the molecule in the hot-spot, due to the fact that the large local enhancement of the electromagnetic field increases the cross-section for both scattering and absorption. The lack of observation of the Raman signal from the decay products of Pc molecules is likely due to the lack of resonance enhancement, as the decay products do not absorb electromagnetic radiation in the excitation region. SERS signal loss can also result from hot-spot destruction, for example, due to local melting or fragmentation of the metallic nanostructure, which drastically reduces the local enhancement factor. However, the risk of hot-spot destruction should not be significant if the molecule is excited with a low-power continuous-wave laser. This risk may increase if a pulsed laser is used, which can generate very high peak powers, leading to intense local heating and nonlinear light-nanostructure interactions.

The final factor contributing to the rapid decay of the SERS signal af-

ter a specific exposure time is the molecule's migration from the hot-spot. This model suggests that Pc has some freedom of movement within the plasmonic cavity, encompassing both translational and rotational motions, although these have not yet been clearly observed for Pc. The lack of direct experimental evidence for rotation may be due to the limited time resolution of the measurements.

The measurement of the temporal evolution of SM-SERS spectra of Pc was the fundamental measurement in this chapter, based on which the photostability of Pc at extreme temperatures — room and cryogenic — was investigated, and the amplitude and position fluctuations of selected vibrational Raman modes of Pc were analyzed.

4.3.3 The photostability of SM-SERS of Pc

In this section, we focus on the photostability of single molecules in SERS experiments at room and cryogenic temperatures. Molecule photostability is a key factor limiting the time and quality of SERS measurements, especially at high laser power densities and substantial local electromagnetic field enhancement. The temperature of the sample is of fundamental importance in this context because it determines the intensity of non-photon relaxation processes, diffusion, and photochemical reactions that lead to molecular degradation. Measurements conducted at room temperature are heavily burdened by thermal and dynamic effects, which lead to a rapid decrease in signal (known as photobleaching), whereas cryogenic conditions slow down these processes.

Figure 4.5 shows a comparison of the SERS spectra lifetimes of single Pc molecules at two extreme temperatures: (A) 298 K and (B) 83 K. At both temperatures, over 120 time-traces of SM-SERS spectra of Pc were recorded until a drastic and sudden drop in SERS spectral intensity was observed, which determined the observation of a single molecule. The recorded time traces of SM-SERS spectra of Pc were grouped into four categories: (a) 0–10 s, (b) 11–100 s, (c) 101–1000 s, and (d) > 1000 s. Figure 4.5 clearly indicates the increased photostability of Pc under SERS conditions at 83 K, which

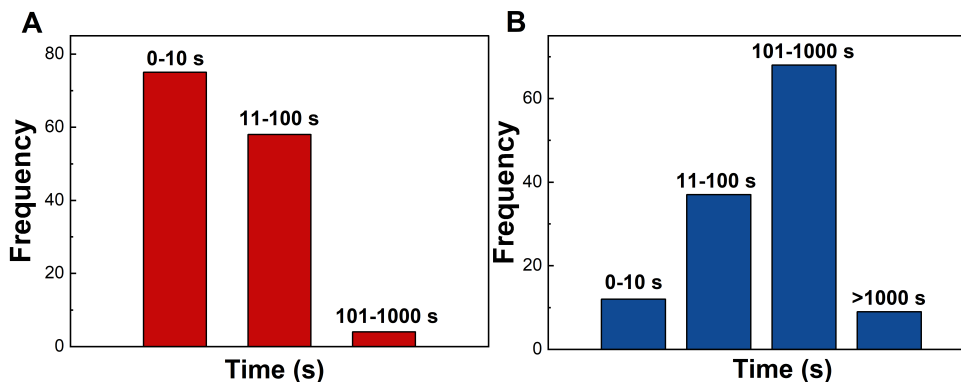


Figure 4.5: Histograms of the lifetimes of SM-SERS spectra of Pc measured at (A) 298 K and (B) 83 K. Temporal evolutions of SM-SERS spectra of Pc were recorded on AuNRs with a single-spectrum acquisition time of 0.5 s and a laser power of 50 μ W. At both temperatures, the sample was maintained under an N_2 atmosphere.

translates into an average 15-fold longer lifetime of a single Pc molecule. We suggest that the increased photostability at cryogenic temperature is a consequence of the strong suppression of photochemical processes and the slowing down of diffusion processes in the plasmonic cavity region, which leads to the stabilization of the molecule–metal system.

4.3.4 Temperature-dependent temporal and spectral fluctuations of SM-SERS spectra of Pc

In the previous section, we demonstrated that cryogenic temperature increases the lifetimes of SERS spectra of single Pc molecules. However, at both temperatures, we observed significant fluctuations in the SERS signal intensity as well as spectral fluctuations in the Pc Raman bands. In this section, we focus on analyzing the relationship between these two types of fluctuations in SM-SERS spectra with a single spectrum accumulation time of 0.5 s. To quantitatively describe these relationships, we used Spearman rank correlation coefficients, which — unlike the classical Pearson correlation — do not require the assumption of a linear relationship or normal distribution of the analyzed data and are more robust to the influence of outliers. The use of this correlation measure is particularly justified in the

case of SM-SERS signals, which are inherently characterized by strong fluctuations and potential nonlinearity of the studied dynamic processes due to the plasmonic environment.

Statistical analysis of Spearman rank correlation coefficients was performed based on the measured SERS time courses for single Pc molecules at both 298 K and 83 K. In this analysis, we focused on investigating the relationship between three Raman vibrational modes of Pc, namely $2A_g$, $3A_g$, and $4A_g$. These vibrational modes correspond to Raman bands of Pc-d₀ (183 cm⁻¹, 343 cm⁻¹, and 369 cm⁻¹) and Pc-d₁₂ (179 cm⁻¹, 327 cm⁻¹, and 349 cm⁻¹), respectively. [92] Before analysis, the experimental data were preprocessed by applying noise reduction, spectral smoothing via the Savitzky–Golay filter, and Lorentzian fitting to selected Raman bands.

Figure 4.6 presents a comparison of the distributions of Spearman correlation coefficients between the Raman band position and the amplitude of the SM-SERS signal for selected pairs of Pc Raman vibrational modes at two extreme temperatures: 298 K and 83 K. The distributions of the correlation coefficients were approximated by a Lorentzian function. The analysis reveals fundamentally different correlation behaviors between these two parameters at both temperatures.

At room temperature, the correlation coefficients for the Raman band amplitude indicate strong correlations (0.60–0.75), whereas those for the Raman band position reflect weak correlations (0.17–0.32). The Lorentzian-approximated distributions of the Spearman correlation coefficients for both the SM-SERS signal amplitude and the Raman band position are narrow, resulting in comparable full width at half maximum (FWHM). At cryogenic temperature, the correlation coefficients for the Raman band amplitude – similar to those observed at room temperature – indicate strong correlations (0.80–0.90), whereas the correlation coefficients for the band position reflect weaker correlations (0.27–0.42). However, the Lorentzian-approximated distributions of the Spearman correlation coefficients for the SM-SERS signal amplitude and the Raman band position differ markedly. The correlation coefficients for the SM-SERS amplitude are described by a narrow distribution, whereas those for the Raman band position are char-

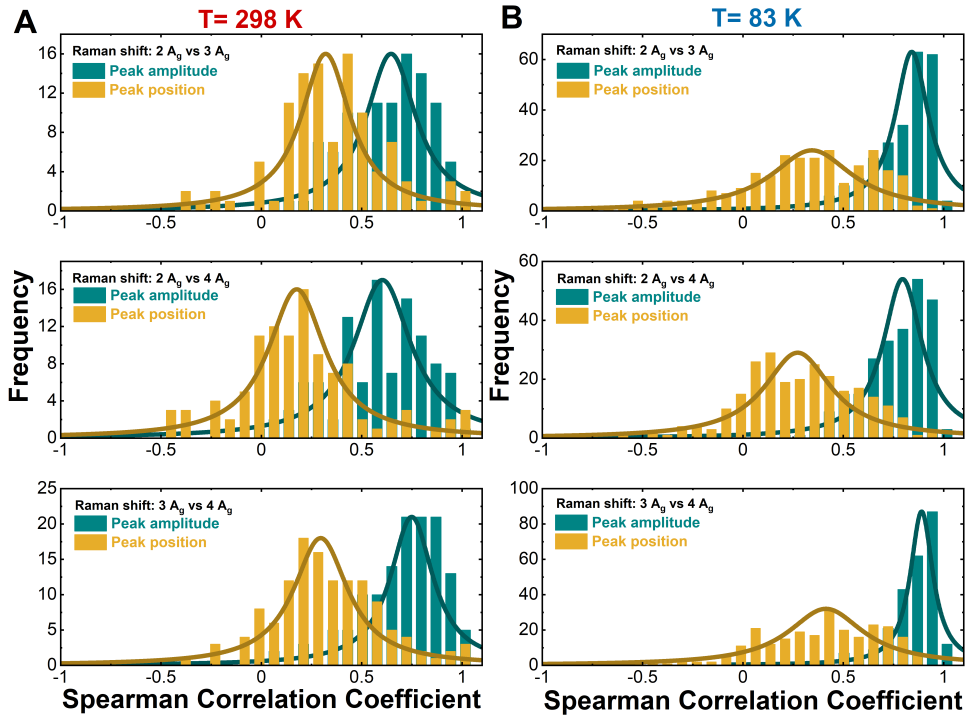


Figure 4.6: Distributions of Spearman correlation coefficients between Raman peak-position fluctuations and SERS-signal amplitude changes for single-molecule SERS spectra of Pc at room temperature (A) (298 K) and cryogenic temperature (B) (83 K) for selected pairs of Raman bands. Each distribution was fitted with a Lorentzian function. SM-SERS spectra were recorded with 0.5 s accumulation time and power of 50 μ W on the sample. Sample sizes corresponding to each pair of Raman modes — $2A_g$ vs. $3A_g$, $2A_g$ vs. $4A_g$, and $3A_g$ vs. $4A_g$ — were as follows: $n = [105, 105, \text{and } 130]$ for 298 K, and $n = [\text{in all cases } 248]$ for 83 K.

acterized by a broad distribution suggesting higher heterogeneity.

Regardless of the temperature, the pattern of the distribution of Spearman correlation coefficients remains similar, i.e., in the case of the Raman signal amplitude, it is higher relative to the Raman peak position. This distinct correlation behaviour suggests that SM-SERS fluctuations arise from at least two largely decoupled mechanisms. Comparison of the Spearman coefficient distributions indicates that the processes responsible for the fluctuations in the SM-SERS spectra are temperature-dependent to a varying degree. In the case of the correlation coefficient distribution for the SERS

signal amplitude, lowering the temperature from 298 K to 83 K shifted the distribution toward stronger correlations and decreased the mean FWHM. The distribution of Raman band position correlation coefficient values also shifted toward stronger correlations after lowering the temperature to 83 K, but these values are still low compared to the mean values of the SERS signal amplitude correlation coefficient. The distribution of correlation coefficient values changed significantly, from narrow at 298 K to broad at 83 K. This observation suggests that reducing temperature might slow down the process responsible for variation in Raman peak position. The same process at 298 K was dominated by fast thermal dynamics, while at 83 K, the distribution pattern of the Spearman correlation coefficient reveals static, site-specific heterogeneity in metal–molecule configurations.

The temporal resolution of the recorded SM-SERS spectral time traces, based on which the relationship between signal amplitude and Raman band position shown in Fig. 4.6 was examined, was 500 ms. This value is sufficient to investigate the influence of temperature on the processes responsible for SM-SERS spectral fluctuations. However, it remains too coarse to unambiguously identify the specific mechanism governing a given type of fluctuation. This is due to the fact that, particularly at room temperature, molecules are highly mobile, and despite probing a single molecule, a spectrum acquired with a 500 ms integration time represents an average over multiple orientations of the molecule adsorbed on the metal surface.

4.3.5 Temperature-dependent temporal and spectral fluctuations of SM-SERS spectra of Pc with improved time resolution

In the previous section, we demonstrated that fluctuations in the amplitude and position of Raman bands are characterized by distinct distributions of Spearman correlation coefficients, which are also temperature-dependent. In this section, we continue our investigation of the relationship between Raman band amplitude and position, based on measurements of the time

evolution of SM-SERS spectra with a time resolution of less than 500 ms. Reducing the accumulation time of a single spectrum significantly reduces the (S/N), which prevents correlation analysis due to the low intensity of the SERS spectra. To overcome this problem, we employed the following approach: while reducing the accumulation time of a single spectrum, we simultaneously increased the effective energy density focused on a single molecule. This approach proved effective, ultimately allowing us to record SM-SERS spectra with a time resolution of 2 ms at room temperature and 10 ms at cryogenic temperature, while maintaining a high (S/N).

Figure 4.7 shows how increasing the time resolution of a single SERS measurement affects the correlation distribution of the Raman band position (left column) and the SERS signal amplitude (right column) at 298 K. The results of the Spearman correlation coefficient analysis were grouped depending on the accumulation time of a single SERS spectrum, i.e., 500, 15-30, and below 9 ms, and then summarized in a common graph. By decreasing the time resolution of a single SM-SERS spectrum measurement, we observe a shift in the distribution of Spearman correlation coefficient values toward weaker correlations for both the Raman band position (A) and the signal amplitude (B). However, the change of this shift differs between the Raman band position and the signal amplitude.

Figure 4.8 shows how increasing the time resolution of a single SERS measurement affects the correlation distribution of the Raman band position (left column) and the SERS signal amplitude (right column) at 83 K. At 83 K, due to the use of a microscope objective with working distance (WD) = 10.6 mm and NA = 0.5, we did not achieve a time resolution for a single spectrum measurement below 10 ms. However, we managed to record SM-SERS spectra of Pc with a high (S/N). The results of the correlation analysis of the Spearman coefficients were grouped by accumulation time for a single SERS spectrum (500, 50-100, or 10-15 ms) and summarized in a single graph. At 83 K, we observed a similar trend to that at 298 K. Namely, increasing the time resolution of single-SERS-spectrum measurements shifted the distribution of Spearman correlation coefficient values towards weaker correlations.

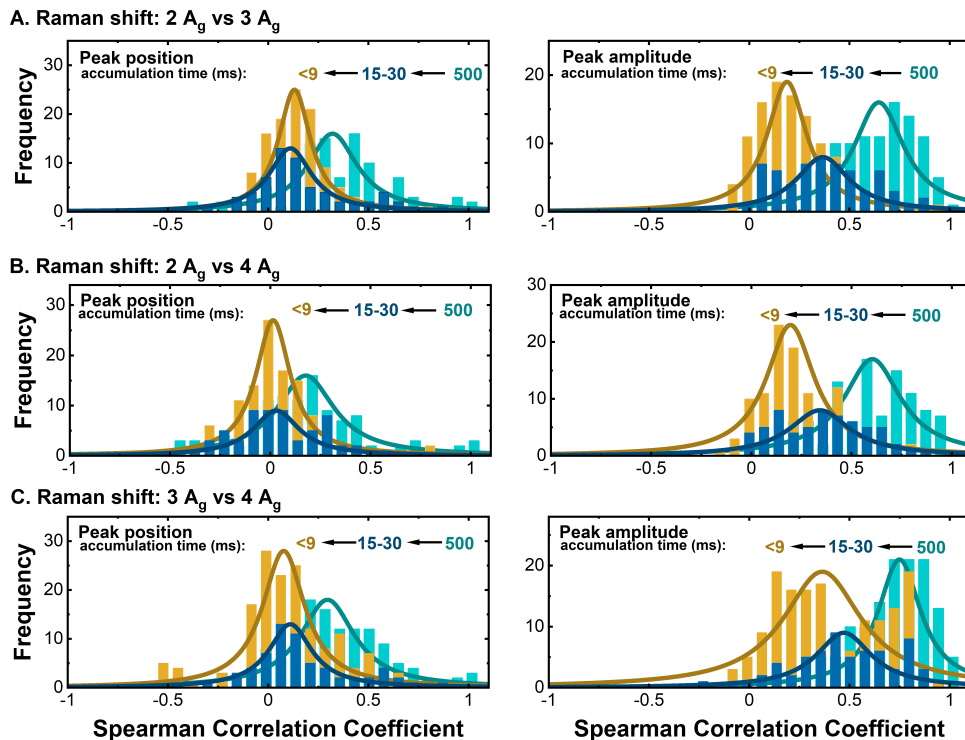


Figure 4.7: Distributions of Spearman correlation coefficients between Raman peak-position fluctuations and SERS-signal amplitude changes for single-molecule SERS spectra of Pc at room temperature (298 K) for selected pairs of Raman modes with improved temporal resolution. Each distribution was fitted with a Lorentzian function. SM-SERS spectra were recorded with accumulation times of 500, 15–30, and below 9 ms and corresponding powers of 50 μ W, 70 μ W, and around 0.3 mW on the sample. Sample sizes corresponding to each pair of Raman modes — $2A_g$ vs. $3A_g$, $2A_g$ vs. $4A_g$, and $3A_g$ vs. $4A_g$ — were as follows: $n = [105, 105, \text{and } 130]$ for 500 ms, $n = [56, 61, \text{and } 62]$ for 15–30 ms, and $n = [112, 112, \text{and } 156]$ for < 9 ms.

Figure 4.9 shows the dependence of the average Spearman correlation coefficient for Raman band position and signal amplitude as a function of the time resolution with which the SERS spectrum of a single molecule was recorded at two distinct temperatures (A) 298 K and (B) 83 K. At both temperatures, we observed a shift in the mean value of the distribution of Spearman correlation coefficients toward weaker correlations. Nevertheless, the mean values of the Spearman correlation coefficient at 83 K indicate greater variation, particularly for the Raman band amplitude, as evidenced by a

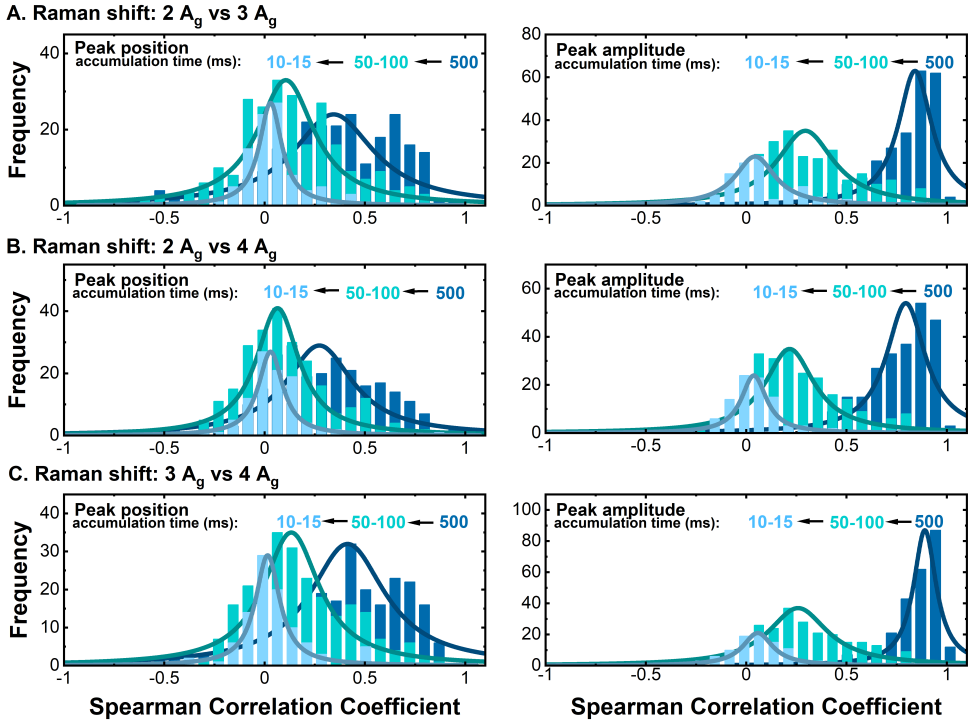


Figure 4.8: Distributions of Spearman correlation coefficients between Raman peak-position fluctuations and SERS-signal amplitude changes for single-molecule SERS spectra of Pc at cryogenic temperature (83 K) for selected pairs of Raman modes with improved temporal resolution. Each distribution was fitted with a Lorentzian function. SM-SERS spectra were recorded with accumulation times of 500, 50–100, and 10–15 ms and corresponding powers of 50 μ W (for 500 ms), and around 1 mW (for the rest) on the sample. Sample sizes corresponding to each pair of Raman modes – $2A_g$ vs. $3A_g$, $2A_g$ vs. $4A_g$, and $3A_g$ vs. $4A_g$ – were as follows: $n = [\text{in all cases } 248]$ for 500 ms, $n = [245, 245, \text{and } 247]$ for 50–100 ms, and $n = [95, 96, \text{and } 96]$ for 10–15 ms.

steeper distribution of data points. Moreover, at 83 K, the mean value of the Spearman correlation coefficient is close to zero, regardless of fluctuations in either the amplitude or the position of the Raman bands, indicating that these parameters are largely uncorrelated and evolve independently, suggesting the absence of a common mechanism governing their fluctuations.

In the final section of this sub-chapter, we summarize the fluctuations

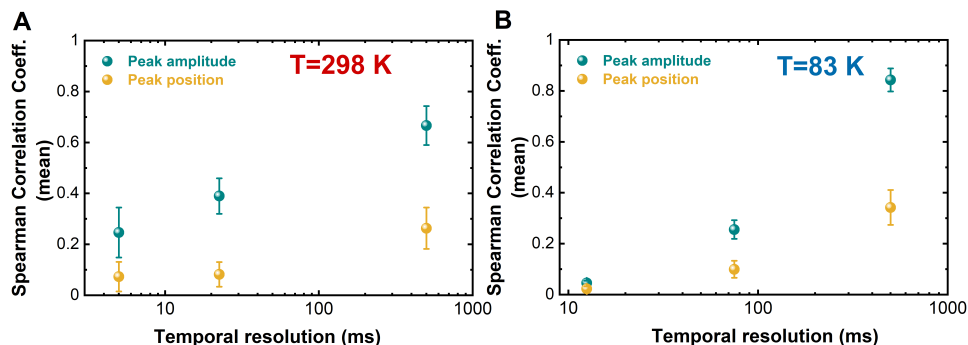


Figure 4.9: Effect of decreasing the temporal resolution (logarithmic scale) of a single SERS spectrum measurement on the mean value of the Spearman correlation coefficient for the Raman band position and the SERS signal amplitude as a function of temperature. (A) $T = 298$ K and (B) $T = 83$ K.

in the amplitude and position of the Raman band in SERS spectra recorded on single molecules. Rigorous measurements of SM-SERS spectra as a function of temperature, as well as with increased time resolution, provided evidence of the complex nature of the mechanisms responsible for fluctuations in SM-SERS spectra. For identical acquisition times within a single spectrum, we observed that lowering the temperature affects fluctuations in the Raman band position more than fluctuations in signal intensity over time. Simultaneously, time-resolved measurements of SM-SERS spectral evolution indicate that, regardless of environmental temperature, the amplitude fluctuations in SERS spectra are more readily captured as the temporal resolution increases, yielding observations undetectable with a 0.5 s integration time.

4.3.6 Interpretation of temporal and spectral fluctuations in SM-SERS spectra of Pc

In the preceding subchapters, we demonstrated that fluctuations in single-molecule SERS spectra arise from a complex mechanism driven by at least two distinct processes. Given that SERS spectral fluctuations depend on the nature of the molecule, this subchapter focuses on a qualitative inter-

pretation of the results obtained in the previous sections by proposing the specific processes responsible for fluctuations in the SM-SERS signal amplitude and for spectral fluctuations of the Raman bands.

Fluctuations in the amplitude of SM-SERS spectra of Pc were observed over a wide range of acquisition times for individual spectra. However, in most cases, the changes were synchronous, i.e., the intensities of all Raman bands increased or decreased simultaneously. Lowering the temperature to 83 K and/or improving the temporal resolution of single-spectrum acquisition revealed relative changes between Raman bands that, to the best of our knowledge, have not been previously reported in the literature. In most cases, these changes were observed between the $2A_g$ and $4A_g$ vibrational modes, whose displacement vectors were presented in Fig. 4.10. Figure 4.11 shows a representative time trace together with SM-SERS spectra in which relative changes between Raman bands are evident. Interestingly, the changes between the modes were not continuous but instead exhibited step-like behavior.

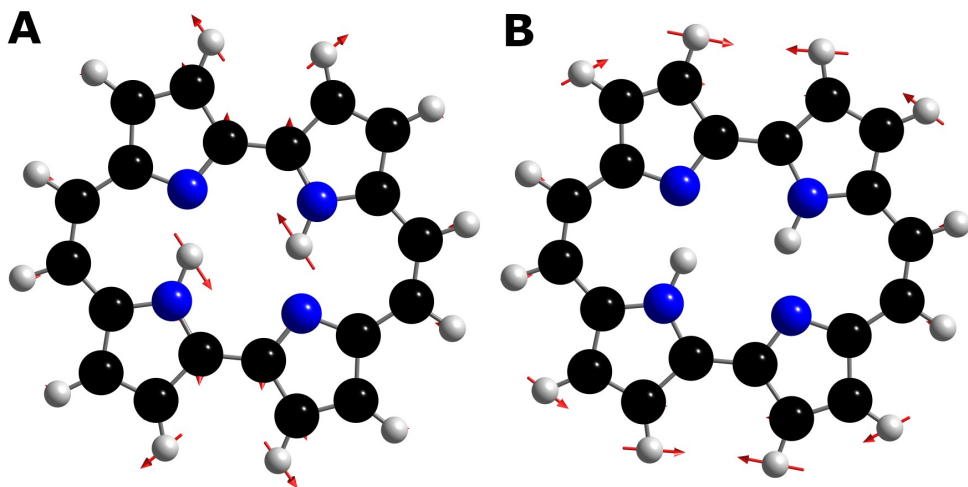


Figure 4.10: Visualization of displacement vectors for the (A) $2A_g$ and (B) $4A_g$ vibrational modes of Pc calculated using the PBE1PBE/Def2TZVPP method.

Relative changes in the amplitude of Raman bands corresponding to the vibrational modes $2A_g$ and $4A_g$ under resonance excitation conditions are contrary to the theory of resonance Raman scattering. In the general form

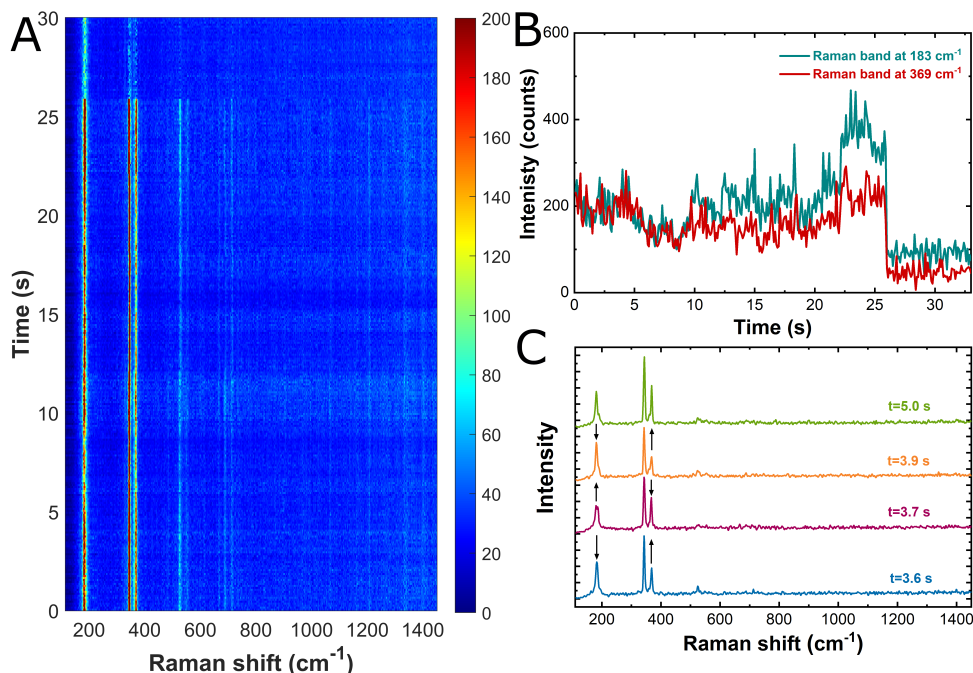


Figure 4.11: (A) Temporal evolution of SM-SERS spectra of Pc during which amplitude fluctuations were observed in the form of relative intensity changes of the 183 cm^{-1} and 369 cm^{-1} bands. (B) Intensity fluctuations of the 183 cm^{-1} and 369 cm^{-1} bands during the acquisition of the time trace. (C) SM-SERS spectra of Pc extracted from the time trace, highlighting the character of the amplitude changes of the selected Raman bands.

of the KHD formalism (Eq. 2.25) discussed in sub-chapter 2.2, the resonance Raman scattering amplitude involves a sum over all intermediate states of the molecule. In the case of resonance excitation, i.e., excitation close to the first electronic transition $S_0 \rightarrow S_1$, which in Pc overlaps spectrally with the excitation wavelength (633) (Fig. 4.12), the sum is dominated by the contribution from the excited state S_1 . Consequently, the Raman tensors of totally symmetric vibrational modes become approximately proportional to the product of the transition dipole moment components $\mu_i \mu_j$ and exhibit similar polarization properties.

Resonance effects, including changes in the symmetry of the Raman tensor, have been the subject of some theoretical studies. Meyer *et al.* dis-

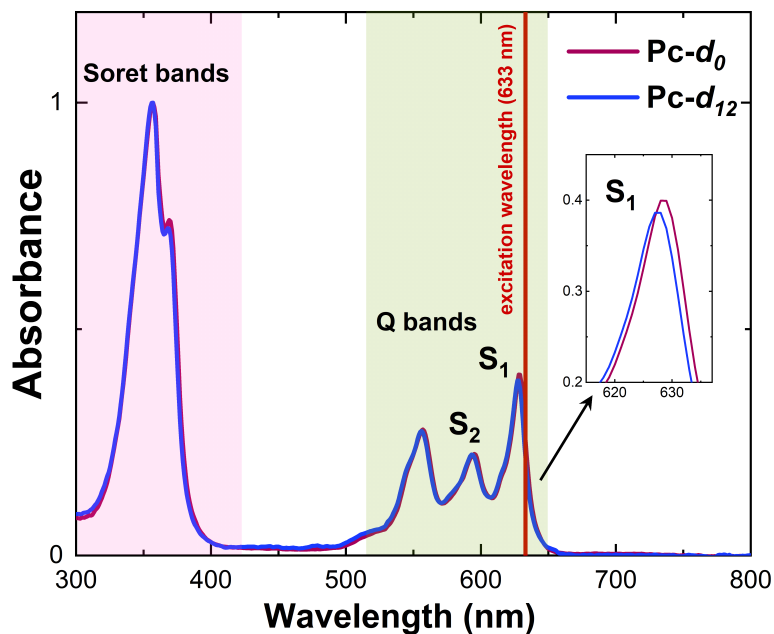


Figure 4.12: Electronic absorption spectra of Pc- d_0 and Pc- d_{12} in ethanol. The positions of the Q and Soret bands are indicated, together with the excitation line (633 nm), which overlaps with the first excited state (S_1).

cussed a case of benzenethiol for which Raman frequencies and tensors were calculated using time-dependent DFT. [93] Calculations were performed for several excitation wavelengths, starting from a wavelength away from the electronic transition and then shifting the excitation toward lower wavelengths until the molecular resonance overlapped. Tuning the excitation from off-resonance to near-resonance led to a gradual increase in Raman activity and a change in mode symmetry, altering the shape of the Raman tensors. When the excitation wavelength overlapped with the first electronic transition, the Raman activity increased significantly, and the orientation and symmetry of the Raman tensors for the selected modes remained consistent with the transition dipole moment, i.e., along the long axis of the molecule, despite the fact that in the case of excitation at a wavelength beyond the electronic transition, the orientation of the tensors in question was orthogonal. [93]

The presented consideration of the influence of resonance effects on

the symmetry of Raman tensors is crucial for the interpretation of microscopic measurements of SM-SERS spectra. In practice, it means that when a molecule is excited in its molecular resonance, the amplitude changes between the Raman bands in the SM-SERS spectra should be characterized by strong correlations, i.e., they should change in the same way. However, in the case of SM-SERS Pc, we observed relative changes in the intensity of selected Raman bands. These changes cannot be explained based on Raman tensor analysis, especially their orientation.

The relative intensity changes we observed were not accompanied by the appearance of new frequency bands, which allows us to rule out the formation of permanent interactions between Pc and Au adatoms from the nanoparticle's surface layer. Nevertheless, it is likely that a Pc molecule located near the sharp edge of the nanoparticle may experience an alternating electromagnetic field localized with atomic resolution. This localized field behaves analogously to the tip in tip-enhanced Raman scattering, selectively amplifying specific modes, which may lead to disappearance of some Raman bands and relative changes in Raman bands amplitudes. [94, 95] Chen *et al.* performed a high-resolution tip-enhanced Raman scattering simulation with resonance excitation and visualized the Raman polarizability density distributions of the individual modes of Pc. It was shown that confined near-field during Raman scattering may selectively enhance specific vibrational modes of Pc. [96]

We observed spectral fluctuations in the positions of Raman bands in SM-SERS spectra of Pc both at room temperature and under cryogenic conditions, as well as over a wide range of SM-SERS spectral accumulation times. As the temporal resolution of single-spectrum acquisition improved, the frequency of rapid and abrupt Raman band shifts increased. However, compared to the total number of recorded signals originating from individual Pc molecules, this number remained small (<10%). At room temperature, spectral fluctuations occurred significantly more frequently than at cryogenic temperatures. A characteristic feature of the spectral fluctuations is the appearance of a Raman band at a new frequency, along with the partial disappearance of bands previously present in the SERS spectrum. This case

is particularly significant, as it indicates that the nature of these changes is associated with specific interactions between the molecule and the nanoenvironment in which it resides.

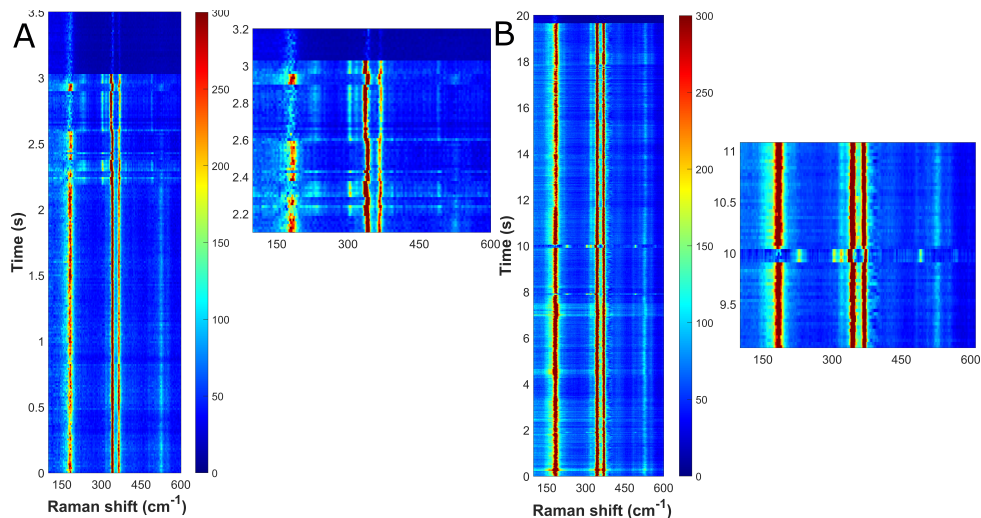


Figure 4.13: Temporal evolution of SM-SERS spectra of Pc, during which spectral fluctuations were observed, i.e., sudden and rapid shifts of Raman bands relative to the averaged spectrum. The selected figures show the time evolution of the SM-SERS spectra of Pc, along with a zoom-in on the observed spectral fluctuation (shown on the right relative to the time trace). (A) ET = 15 ms, T = 298 K; (B) ET = 32 ms, T = 298 K

Figure 4.13 shows representative temporal evolutions of SM-SERS spectra of Pc, in which fluctuations in the positions of Pc Raman bands became apparent during the measurements. In most cases, the time-dependent SM-SERS spectra of Pc exhibited Raman band position fluctuations characterized by similar behavior. Specifically, together with a sudden shift of the 343 cm^{-1} band, we observe the disappearance of the Raman band at 183 cm^{-1} . In most cases, the 369 cm^{-1} band remained unchanged. We also observed the emergence of new Raman bands at frequencies around 228, 305, 319, and 493 cm^{-1} . The observed spectral changes persisted for a relatively short time compared to the total duration of the recorded SM-SERS Pc spectral time trace.

The observed changes in the time traces most likely result from the

sudden, spontaneous formation of so-called picocavities, i.e., structures in which local interactions occur between Au atoms on the nanoparticle facets and a molecule located within the plasmonic cavity. However, to elucidate deeply and qualitatively the sudden and abrupt shifts of Raman bands observed during the acquisition of SM-SERS spectra of Pc, we performed DFT simulations in which the Pc molecule was placed in the vicinity of a single Au atom, two Au atoms, four Au atoms, or a cluster composed of 21 Au atoms, as well as two large clusters composed of 20 Au atoms located symmetrically along Pc molecule. Comparison of the experimental SM-SERS spectra recorded from different plasmonic nanocavities with spectra calculated using quantum chemical methods indicated that the most probable geometry responsible for the observed spectral shifts of the Raman bands is the Pc molecule-single Au atom configuration. Figure 4.14 shows the optimized geometry of this system, while Fig. 4.15 presents a comparison of selected experimental spectra extracted from the SM-SERS trajectory of Pc, with those calculated for the Pc-single Au atom model.

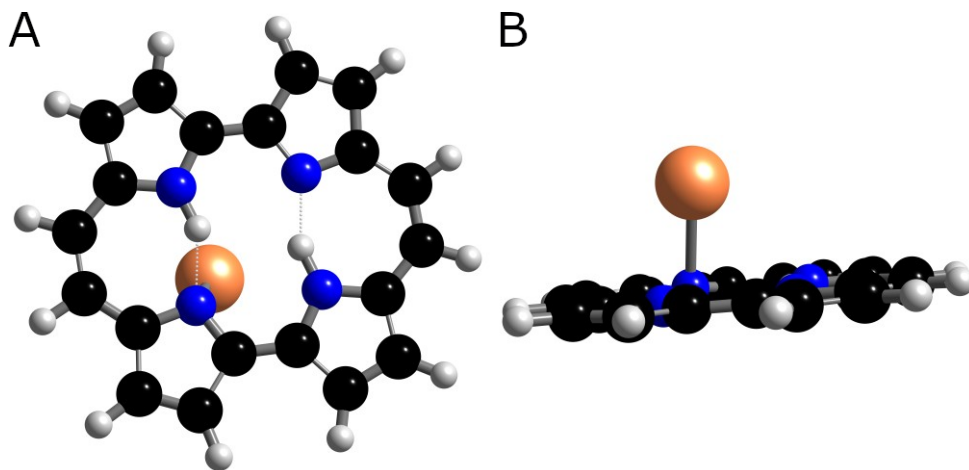


Figure 4.14: Optimized geometry (DFT) of Pc with a single Au atom from (A) top view and (B) side view.

When comparing spectral fluctuations with large Raman band shifts, we found that, in most cases, the character of the changes is similar. Specifically, a shift of the 343 cm^{-1} band toward lower frequencies is accompanied

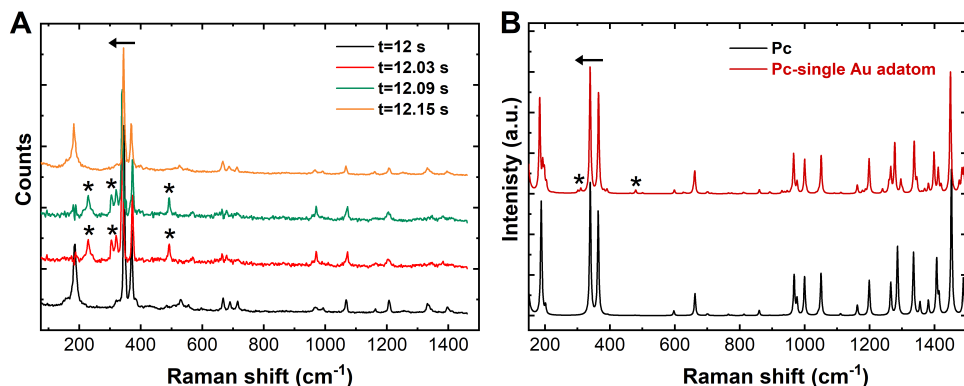


Figure 4.15: (A) Comparison of experimental SM-SERS spectra of Pc extracted from a time trace in which strong spectral fluctuations were observed, marked with an asterisk (*) (ET = 32 ms, Power ~ 0.3 mW, T = 298 K). (B) Theoretical (DFT) spectra of Pc and Pc with an optimized geometry, including a single Au adatom, where the bands appearing at new frequencies are marked with an asterisk (*).

by the disappearance and/or a reduction in the intensity of the 183 cm^{-1} band, as illustrated in Fig. 4.13. Similar spectral-shift behavior observed in SM-SERS spectra recorded from different plasmonic nanocavities may suggest the presence of a recurring geometry responsible for the observed changes. Baumberg suggested that, for each molecule, there exists a configuration involving an Au adatom that is characterized by a stable geometry. [76] This could explain the reproducibility of spectral fluctuations observed in the single-molecule SERS spectra of Pc. The previously mentioned observation that, at cryogenic temperatures, the frequency of occurrence of spectral fluctuations with large Raman shifts was lower compared to room temperature may result from the fact that adatom generation is significantly more difficult under cryogenic conditions. [76]

4.4 Conclusions

In this chapter, we presented results of studies regarding fluctuations in single-molecule SERS spectra at both room and cryogenic temperatures. We used aggregated gold nanorods (AuNRs) as the plasmonic nanocavi-

ties, while porphycene (Pc), in the form of an equimolar mixture of isotopologues, served as the Raman signal reporter.

In the first part of our study, we analyzed the effect of temperature on the lifetime of single molecules in SERS. We demonstrated that at a cryogenic temperature of 83 K, the lifetimes of single Pc molecules are statistically 15 times longer compared to those at room temperature (298 K).

In the main part of the chapter, we focused on the analysis of fluctuations in single-molecule SERS spectra, which included changes in both the amplitude and the position of Raman bands. The performed analysis of the Spearman correlation coefficient revealed that fluctuations in the amplitude and position of Raman bands exhibit different distribution profiles. Regardless of temperature, the distribution of Spearman correlation coefficients indicates that amplitude fluctuations are characterized by higher correlation values, which means that changes in the intensity of Raman bands are more strongly correlated in time than changes in their positions, indicating a greater uniformity of the mechanism responsible for amplitude fluctuations.

In subsequent studies, we placed particular emphasis on improving the temporal resolution during SM-SERS spectrum acquisition. The approach we employed reduced the acquisition time of a single spectrum while simultaneously increasing the energy flux to maintain a high (S/N). The applied methodology proved to be effective, enabling us to record SM-SERS spectra of Pc with an acquisition time of 2 ms, which represents the maximum temporal resolution of the measurement system due to the detection method used (EM-CCD). We demonstrated that as the temporal resolution of the SM-SERS spectral measurements increases, the distributions of the Spearman correlation coefficient, for both amplitude fluctuations and Raman band position fluctuations, shift toward weaker correlations. We also observed that the shift of the Spearman correlation coefficient distributions is temperature-dependent, suggesting that the dynamics of the processes responsible for the observed spectral fluctuations are strongly coupled to temperature.

Finally, we proposed a qualitative interpretation of the statistical anal-

ysis of the Spearman correlation coefficient by suggesting the processes responsible for the different types of fluctuations. In the case of amplitude fluctuations in the SM-SERS spectra of Pc, we observed rare relative intensity changes between Raman bands corresponding to the $2A_g$ and $4A_g$ vibrational modes of Pc, respectively. This observation contradicts the theory of resonant Raman scattering and cannot be explained based on Raman tensor analysis. We speculate that these changes may result from the selective amplification of selected Pc vibrational modes by the cavity atoms. The observed spectral fluctuations, during which rapid and abrupt shifts of Raman bands were detected, were explained — with the support of DFT calculations — as the formation of so-called picocavities, in which the Pc molecule interacts with an Au adatom originating from the surface of the nanoparticle.

Chapter 5

Stabilization of Single-Molecule SERS Signal via Supramolecular Encapsulation with Cucurbit[7]uril

The contents of this chapter are based on:

Patryk Pyrcz and Sylwester Gawinkowski, "Supramolecular Stabilization of Single-Molecule SERS: Cucurbit[7]uril Encapsulation of Thionine", ACS Phys. Chem Au, 6, 1, 57–68 (2026)

5.1 Introduction

The temporal evolution of single-molecule SERS spectra provides a unique set of information that, when supported by quantum-chemical calculations, allows us to decipher the actual motion of the molecule within the plasmonic nanocavity. However, monitoring the trajectory of single-molecule SERS spectra is susceptible to significant fluctuations, both spectral and temporal, the possible origins of which were discussed in the previous chapter. In parallel with research on the origins of SM-SERS signal fluctuations, new strategies are being developed to control the complexity of the fluctuations and stabilize the SERS signal. Strategies used so far, i.e., covalent attachment, formation of self-assembled monolayers, and encapsulation in polymer matrices, are often the cause of undesirable effects. For example, they may modify the analyte's intrinsic Raman signature, introduce unwanted background signals, or fail to sufficiently constrain the molecular motions that lead to signal fluctuations. [97] Given the considerable potential of SM-SERS applications, it remains essential to deepen our understanding of the mechanisms underlying fluctuations and to develop new methods that enable their effective control or substantial reduction.

Here, we present an approach that improves single-molecule detection while also reducing SM-SERS signal intensity fluctuations. The method is based on host-guest chemistry, in which the macrocyclic molecule cucurbit[7]uril (CB[7]) is used. By encapsulating the guest molecule, CB[7] does not introduce any chemical modifications to its structure. The architecture of CB[7] is distinctive among other supramolecular complexes, such as cyclodextrins, calixarenes, or pillararenes. Its rigid structure, combined with the presence of carbonyl groups, enables the formation of stable connections with Au nanostructures, while its precisely defined cavity size makes it ideal for SERS measurements. As the guest molecule, we employed thionine — a heterocyclic dye derived from phenothiazine (Fig. 5.1) Thionine is characterized by a well-defined SERS spectrum and is capable of forming complexes with CB[7]. [98] In this chapter, we address three previously underexplored aspects (i) the influence of CB[7] encapsulation of analyte

molecules on the SERS spectrum in both dry and aqueous environments, (ii) the photostability of the host–guest complex under plasmonic excitation, and (iii) the detection along with the temporal evolution of SM-SERS spectra for both the host–guest complex and free molecules at room temperature.

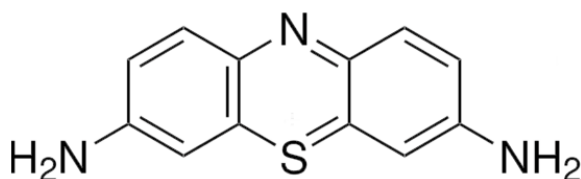


Figure 5.1: Chemical structure of thionine.

5.1.1 Guest-host supramolecular chemistry: cucurbit[n]uril (CB[n])

Cucurbit[n]urils (CB[n]) are pumpkin-shaped macrocyclic molecules composed of repeating units ($n = 5 - 8, 10$) of methylated glycoluril arranged in a ring (Fig. 5.2A). [30] The structure of CB[n] (Fig. 5.2B) is highly symmetric and has been characterized by various spectroscopic methods and X-ray crystallography. [99, 100]

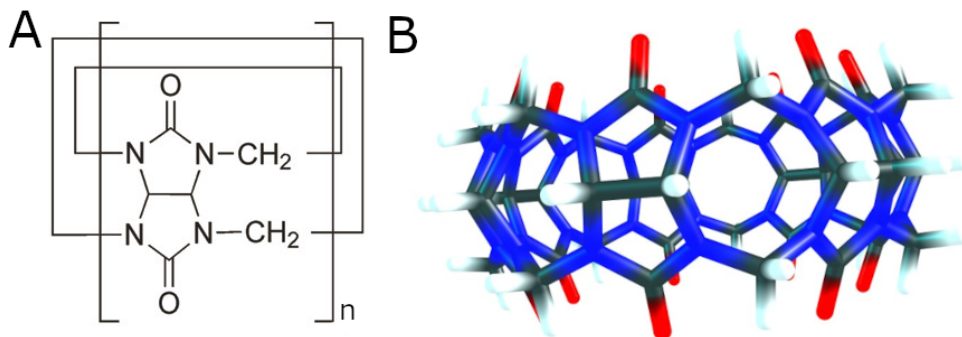


Figure 5.2: (A) Chemical structure of the monomer unit (methylated glycoluril) of CB[n] ($n = 5-8, 10$). (B) Chemical structure of CB[7].

The physical dimensions of different CB[n] are presented in Table 5.1. Crystallographic studies show that the only parameter that does not change with the increase in glycoluril units is the height of CB[n], which is 9.1 Å. The remaining parameters, i.e., portal diameter, cavity diameter, and the volume of the CB[n] cavity, increase systematically with the number of glycoluril units. Due to the precisely defined cavity size, specific CB[n] have the ability to encapsulate guest molecules of specific sizes. Therefore, CB[5] is best suited for encapsulating gases, whereas CB[6] can bind alkyl chains, CB[7] can host small aromatic molecules, and CB[8] is capable of simultaneously complexing with two guest molecules. These observations highlight the importance of guest size in the formation of CB[n]:guest complexes. This steric factor in molecular host–guest recognition was quantitatively described by Mecozzi *et al.*, who introduced the concept of the packing coefficient, defined as the ratio of the guest volume to the volume of the host cavity. [101]

Table 5.1: Physical dimensions of different uncomplexed CB[n].

	CB[5]	CB[6]	CB[7]	CB[8]	CB[10]
Portal diameter (Å)	2.4	3.9	5.4	6.9	9.5–10.6
Cavity diameter (Å)	4.4	5.8	7.3	8.8	11.3–12.4
Cavity volume (Å ³)	82	164	279	479	870
Height (Å)	9.1	9.1	9.1	9.1	9.1

The physicochemical properties of CB[n] vary depending on the number of glycoluril units of CB[n]. Water solubility is highest for CB[5] and CB[7], while CB[6] and CB[8] remain modestly soluble in water. However, the solubility increases dramatically in concentrated acid solutions. CB[n] are characterized by high thermal stability, with decomposition temperatures above 370 °C in all cases. [30]

The molecular structure of the CB[n] macrocyclic molecule is characterized by the presence of carbonyl groups located next to the inner and outer portals of CB[n]. The presence of electron-rich carbonyl groups is associated with a negative electrostatic potential at the CB[n] portals. Although

the portals of CB[n]s possess strong electronegativity, their internal cavities are surrounded by the fused rings of glycoluril subunits, which block any functional groups or lone electron pairs from being accessible inside. As a result, the inner cavity of CB[n]s is highly hydrophobic and exhibits a strong tendency to encapsulate hydrophobic molecules.

Apart from the physical dimensions and hydrophobic nature of the CB[n] cavity, a key driving force for the formation of CB[n]-guest complexes in aqueous solution is the so-called release of high-energy water. Water molecules confined within the inner cavity of CB[n] exhibit properties that differ markedly from those of bulk water. [102, 103] In particular, water-water interactions inside the cavity are considerably less favorable than in bulk, primarily due to weaker Coulombic interactions between the confined water molecules. During complex formation, these high-energy water molecules are displaced by guest molecules, leading to both enthalpic and entropic changes. This replacement results in an overall increase in Gibbs free energy, which drives the assembly of the host-guest complex. Among the CB[n] family, CB[7] shows the greatest energetic gain from water expulsion, as it contains the largest number of high-energy water molecules, whose energies are also significantly higher than those of bulk water. [104]

5.1.2 Assembly of cucurbit[n]uril on gold surface

CB[n] molecules can be deposited on surfaces in several ways. In case of metal surfaces, especially Au surface, direct immobilization occurs through electrostatic interactions between the electron-rich carbonyl portals of CB[n] and the Au surface. For CB[7] specifically, Fourier transform infrared reflection absorption spectroscopy and X-ray photoelectron spectroscopy (XPS) analyses revealed that CB[7] interacts with Au via its electronegative carbonyl portals, which are oriented perpendicular to the Au surface. [105] Additionally, CB[n] can be localized on the surface through interactions with immobilized guest molecules. Finally, CB[n] can be positioned on the surface after prior functionalization of its homologues, followed by covalent

attachment to the surface. While the electrostatic interactions of CB[n] primarily occur with metallic substrates, the other two binding strategies can be applied to a broader range of materials, including silica and quantum dots.

Electrostatic interactions between the carbonyl portals of CB[n] and the Au surface play a crucial role in SERS spectroscopy, notably in terms of plasmonic nanocavities fabrication. These interactions enable CB[n] to act as a "molecular glue" that links Au nanoparticles in solution. When a CB[n] or guest-CB[n] solution is added to a colloid of nanoparticles, such as spherical particles, gradual aggregation occurs, leading to the formation of dimers, trimers, tetramers, and increasingly longer chains, eventually resulting in larger nanoparticle aggregates. [32, 106, 107] These oligomers possess a well-defined cavity size, reflecting the precisely defined dimensions of CB[n]. The aggregation process can be monitored in real time by measuring extinction spectra, where the gradual disappearance of the single-mode plasmonic peak of Au spherical monomers is accompanied by the emergence of new plasmonic modes due to nanoparticle coupling, commonly named as chain modes. Similarly, CB[n] molecules or guest-CB[n] complexes can assemble on a flat Au surface to form self-assembled monolayers (SAMs). [108] The formation of such SAMs begins by immersing a planar Au substrate into a saturated aqueous solution of CB[n] or guest-CB[n], where CB[n] molecules physisorb onto the surface. In a subsequent step, Au nanoparticles can be drop-cast onto the substrate to create plasmonic nanocavities, known as nanoparticle-on-mirror (NPoM) geometry. [109, 110] Figure 5.3 shows a schematic of the NPoM cavity used in this work, along with a magnified view of the cavity containing the Th-CB[7] complex.

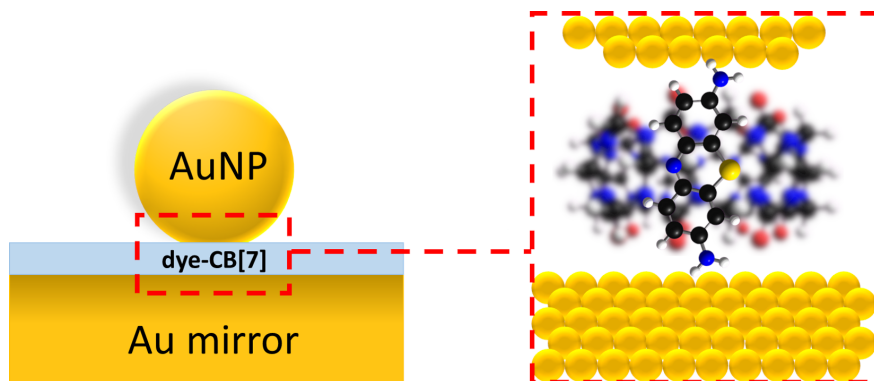


Figure 5.3: Schematic representation of the NPoM geometry used in our work: a spherical Au nanoparticle on the top separated from an Au mirror by a CB[7] monolayer partially loaded with dye (Th) molecules.

5.2 Materials and methodology

5.2.1 Materials

Gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) ($\geq 99.9\%$ trace metals basis), sodium citrate dihydrate (99%), thionine acetate (Th) ($\geq 85\%$), cucurbit[8]uril hydrate ($> 99.5\%$), and polyvinyl-pyrrolidone (PVP) (average $M_w \sim 55,000$) were obtained from Sigma-Aldrich. Cucurbit[7]uril hydrate ($> 99.5\%$) was sourced from Strem Chemicals, Inc. Acetone (HPLC, 99.9%), hydrogen peroxide (30%), nitric acid (63%), hydrochloric acid (38%), and sulfuric acid (98%) were purchased from CHEMPUR. A gold (99.99%) sputtering target (disc) was purchased from Mennica Polska S.A. All chemicals were used without further purification. Deionized water (Elix, Millipore, $> 15 \text{ M}\Omega \cdot \text{cm}^{-1}$) was used in all experiments. All glassware was thoroughly cleaned with aqua regia and subsequently rinsed with deionized water.

5.2.2 Gold nanoparticles preparation

Spherical Au nanoparticles (AuNPs) were synthesized via a seed-mediated growth method, following established literature protocols. [27, 111] The initial Au seed particles were produced using the citrate reduction method

originally introduced by Turkevich and co-workers. [112]

The initial synthesis was designed to produce AuNPs with an average diameter of approximately 50 nm. The procedure began with seed preparation. A sodium citrate solution (340 mM, 0.5 mL) was added to a vigorously stirred, boiling solution of HAuCl_4 (0.254 mM, 99.5 mL) under a reflux condenser. The reaction mixture was maintained at 90 °C for 30 minutes to form the seed particles. For the subsequent growth phase, freshly prepared stock solutions of HAuCl_4 (25 mM) and sodium citrate (60 mM) were utilized. In the first growth step, 30 mL of the seed solution was mixed with 20 mL of distilled water in a round-bottom flask, followed by the addition of sodium citrate solution (60 mM, 0.46 mL). The mixture was then heated to 90 °C and held at this temperature for 5 minutes. HAuCl_4 solution (0.23 mL) was added in two equal portions, with a 35-minute interval between each addition. After the second addition, distilled water (20 mL) was added to the mixture. The second growth phase began with the addition of sodium citrate solution (0.956 mL), and the mixture was again heated to 90 °C for 5 minutes. HAuCl_4 solution (0.956 mL in total) was subsequently added in two equal portions (0.478 mL each), spaced 35 minutes apart. Finally, 27.2 mL of distilled water was added to the reaction mixture to complete the synthesis.

The second synthesis was carried out to produce AuNPs with an average diameter of approximately 100 nm. Similar to the previous procedure, the synthesis began with seed preparation. In this case, HAuCl_4 (25 mM, 0.5 mL) was added to a boiling sodium citrate solution (2.2 mM, 75 mL). The reaction mixture was maintained at 90 °C for 30 minutes to generate seed particles. To facilitate seed growth, freshly prepared stock solutions of HAuCl_4 (25 mM) and sodium citrate (60 mM) were used. The growth process was carried out in three successive stages, all maintained at 90 °C. In the first growth stage, two aliquots of HAuCl_4 (0.1 mL each) were added to 15.1 mL of the seed solution, with a 35-minute interval between additions. Subsequently, 14.8 mL of distilled water was added. In the second stage, sodium citrate solution (0.556 mL) was added to the reaction mixture. After a 5-minute incubation, two portions of HAuCl_4 (0.278 mL each) were added

at 35-minute intervals. The reaction mixture was then diluted with 30 mL of distilled water. During the final growth stage, 1.133 mL of sodium citrate solution was added, followed by the addition of two equal portions of HAuCl_4 (0.567 mL each), separated by a 35-minute interval, after an initial 5-minute delay. Finally, the volume of the reaction mixture was adjusted by adding 36.5 mL of distilled water.

5.2.3 Plasmonic nanocavities preparation and characterization

Two types of plasmonic nanocavities differing in geometry and local electromagnetic field enhancement were fabricated. The first type is Au oligomers, composed of at least two spherical Au nanoparticles, connected into chains and larger aggregates. The second type is NPoM geometry, consisting of an individual spherical Au nanoparticle placed on a continuous Au surface (Au mirror).

Aggregation of the as-synthesized spherical AuNPs was induced by mixing with either an aqueous Th solution (0.05 mM, 50 μL) or a Th-CB[7] complex solution (aqueous mixture containing 0.1 mM CB[7] and 0.1 mM Th, 50 μL). A portion of the resulting Au oligomers solution (10 μL) was then drop-cast onto a piranha-cleaned microscopic cover glass (Brand, No. 1) and allowed to dry under ambient conditions. For measurements involving solution-phase SERS spectra, an aqueous PVP solution (100 μL , 13 wt.%) was added to the Au oligomers to stabilize the colloid and prevent further aggregation. [33]

Flat Au substrates were prepared according to the method described by Hegner. *et al.* [113] Briefly, a 70 nm-thick Au layer was thermally evaporated onto a clean silicon wafer using a sputter coater (Q150TES, Quorum). A cover glass (No. 1, Brand) was then glued to the Au-coated surface using Epo-Tek 377 epoxy glue, prepared by mixing both components in a 1:1 weight ratio. The assembly was cured at 150°C for 2 hours and allowed to cool slowly. After curing, the glass was detached, exposing a smooth Au surface. The resulting Au substrates were functionalized by immersion in

a Th–CB[7] solution — an aqueous mixture containing 1 mM CB[7] and 0.1 mM Th, or 0.01 mM Th for single-molecule detection — for a minimum of 24 hours. For control samples lacking CB[7], the Au substrates were immersed in aqueous Th solutions at concentrations of either 5 μ M or 0.05 μ M (for single-molecule detection). After incubation, all substrates were rinsed with deionized water and dried under a nitrogen stream. Subsequently, Au nanoparticles were drop-cast onto the functionalized Au substrates. After approximately 5 minutes, excess nanoparticles were removed by rinsing with water, and the substrates were dried with nitrogen.

The Au nanoparticles were analyzed using a 10 kV scanning electron microscope (SEM) (FEI Nova NanoSEM 450) to investigate the morphology of home-synthesized colloids. UV-Vis-NIR spectroscopy, performed using a Shimadzu 3100 spectrophotometer, was employed to characterize the optical properties of Au colloidal suspensions and aqueous solutions of Th, Th–CB[7], and Th–CB[8]. Extinction spectra of Au colloids were measured in PMMA cuvettes (Brand), whereas absorption spectra of Th, Th–CB[7], and Th–CB[8] were obtained using a quartz cuvette (110-1-40, Hellma).

5.2.4 Raman and SERS measurements

Raman and SERS measurements were conducted using an inVia Renishaw Raman microspectrometer, equipped with a Leica microscope and a thermoelectrically cooled CCD detector (1021×256 pixels). SERS spectra of dry substrates were acquired using a $50\times$ Zeiss objective (NA = 0.95, C Epiplan-Apochromat), whereas spectra of liquid samples were recorded in a 10 mm quartz cuvette (104-002-10-40, Hellma) using a 30 mm lens in a back-scattering geometry. The scattered light was dispersed using holographic gratings with 1200 or 1800 grooves/mm. Two laser lines were employed for excitation: 632.8 nm (HeNe, Renishaw) and 785 nm (HPNIR785). SERS mapping was performed by raster-scanning the sample in the horizontal (XY) plane, with Raman spectra collected at each point. Intensity distribution maps were generated using Renishaw Wire 5.6 software, which also facilitated the removal of cosmic ray artifacts.

A part of the SERS measurements, specifically those tracking single-molecule trajectories of the Th–CB[7] complex, was carried out using a custom-built SERS microscopy setup described in detail in subchapter 4.2.3. All experimental parameters — including excitation wavelength, exposure time, laser power at the sample, and objective — were kept consistent with those used in the inVia Renishaw spectrometer.

5.2.5 Numerical simulations

Simulations of the electric field distribution within plasmonic nanocavities were carried out using ANSYS LUMERICAL FDTD. The nanocavities were modeled either as a Au nanosphere positioned atop a thick Au film to replicate NPoM geometry, or as a dimer composed of two Au nanospheres to represent the geometry of Au oligomers observed experimentally. The interparticle or particle–mirror gap was set to 0.9 nm. Within the gap region, a uniform mesh size of 0.1 nm was applied in all directions to ensure high spatial resolution. Outside the gap region, the mesh size was set to 1 nm. The dielectric function of Au was taken from the dataset provided by Johnson and Christy. [7] To simulate experimental illumination conditions involving a high-numerical-aperture objective, FDTD calculations were performed using a p-polarized total-field scattered-field plane wave source at two angles of incidence: $\theta = 0^\circ$ and $\theta = 71.28^\circ$.

5.2.6 Quantum-chemical simulations

For molecular simulations, the initial geometry of CB[7] was obtained from the Cambridge Structural Database (refcode: FUYHIR). [114] The starting geometries of Th molecule and the Th–CB[7] complex were constructed and pre-optimized using Gabedit software. Further geometry optimizations and vibrational frequency calculations were performed with Gaussian 16, employing the PBE1PBE hybrid functional and the Def2TZV basis set. All geometries were fully optimized prior to vibrational analysis. The identification of true stationary points was confirmed by the absence of imaginary

frequencies. Raman intensity spectra were derived from the calculated Raman activities using output files from Gaussian and visualized with Chemcraft software. [82, 83]

Visualization of Raman tensors for selected vibrational modes — both before and after Th encapsulation within CB[7] — was carried out using a home-written Matlab code, developed based on the methodology described in Ref. [54].

5.3 Results and discussion

5.3.1 Optical properties of thionine (Th) and Th–CB[*n*]

Thionine (Th) is known to form a stable complex with CB[7] in aqueous solution, with a large binding constant of $1.35 \times 10^7 \text{ M}^{-1}$. [98] Figure 5.4 presents the DFT-optimized structure of Th encapsulated inside CB[7].

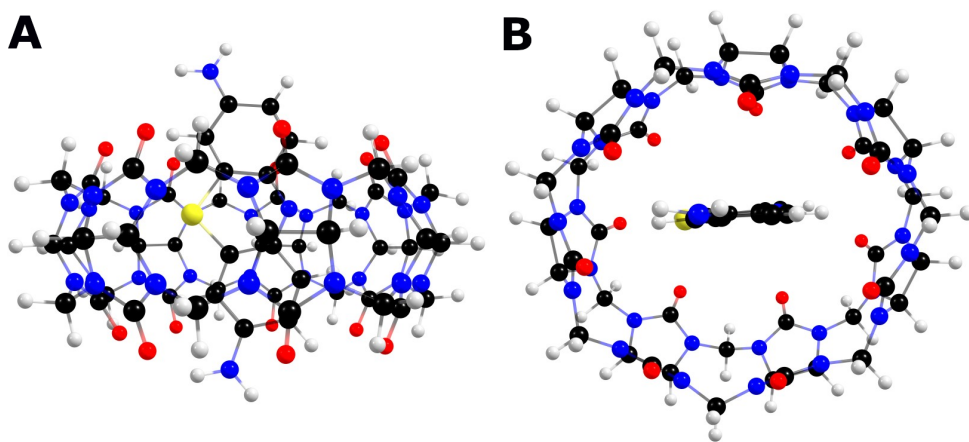


Figure 5.4: (A) Side and (B) top views of the DFT-optimized structure of Th–CB[7].

Here, we verified the formation of the Th–CB[7] complex using optical absorption spectroscopy (Fig. 5.5). Th exhibits two distinct absorption peaks whose relative intensities vary with concentration, indicating dimer formation. The peak at 600 nm corresponds to monomeric Th in solution, while the peak around 560 nm, which becomes more prominent at

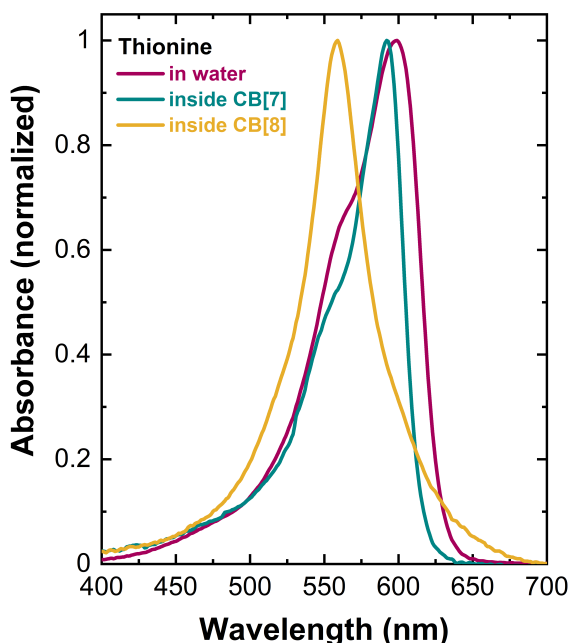


Figure 5.5: Electronic absorption spectra of Th (0.1 mM), Th-CB[7], and Th-CB[8] in aqueous solution.

higher concentrations, indicates the presence of Th dimers (face-to-face aggregation) [115, 116]. When Th is encapsulated by an excess of CB[7], the monomer peak shifts slightly from 600 nm to 593 nm, and the absorption band becomes narrower. Additionally, the intensity of the dimer-related peak is greatly reduced. This suggests that CB[7] effectively suppresses dimerization by encapsulating individual Th molecules within its cavity, thereby inhibiting dye aggregation. In contrast, a control experiment with the larger cucurbit[8]uril (CB[8]), which can encapsulate two Th molecules, showed only the 560 nm dimer peak and no signal at 600 nm, confirming that Th primarily exists in dimeric form in this case (Fig. 5.5). [98, 116]

5.3.2 Plasmonic nanocavities characterization

Au nanoparticles were synthesized using the seed growth method and characterized through SEM imaging and optical spectroscopy. SEM-based morphological analysis of individual spherical particles showed average diame-

ters of 48 ± 10 nm (Fig. 5.6B) and 97 ± 2 nm (Fig. 5.6D). Based on extinction measurements, the position of surface plasmon resonances (SPR) was determined to be 531 nm for nanoparticles with a diameter of 48 nm and 565 nm for nanoparticles with a diameter of 97 nm, respectively. Both SPR values correspond to the dipolar mode of an isolated Au nanoparticle.

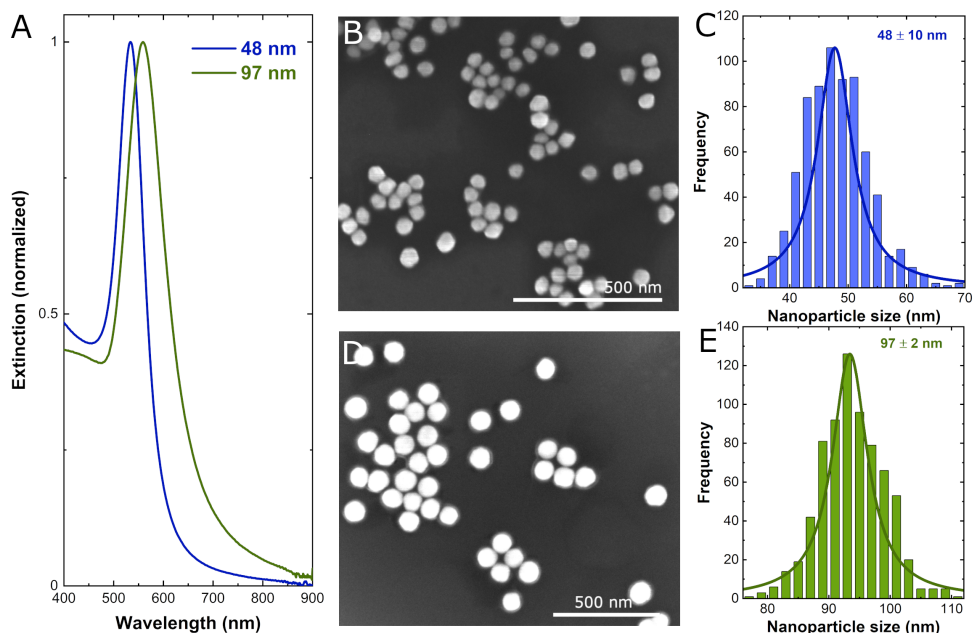


Figure 5.6: Spectral and morphological characterization of home-synthesized spherical Au nanoparticles, including (A) extinction spectra, (B and D) representative SEM images, and (C and E) diameter distribution.

5.3.3 Effect of Th encapsulation in CB[7] on its SERS spectrum

The influence of Th encapsulation by CB[7] on the SERS spectrum was examined using a plasmonic cavity composed of at least two spherical Au nanoparticles, each with a diameter of 48 nm - Au oligomers. SERS spectra of both uncomplexed Th and the Th-CB[7] complex were acquired under non-resonant excitation at 785 nm in order to avoid pre-resonance effects and to monitor spectral changes upon CB[7] inclusion. Measure-

ments were performed in both anhydrous (Fig. 5.7A) and aqueous environments (Fig. 5.7B). The use of non-resonant excitation eliminates resonance-induced intensity variations that could result from CB[7] encapsulation, shifting the electronic transition energy of Th. The SERS spectra recorded under pre-resonance excitation at 633 nm exhibited nearly identical intensities for both wet and dry samples.

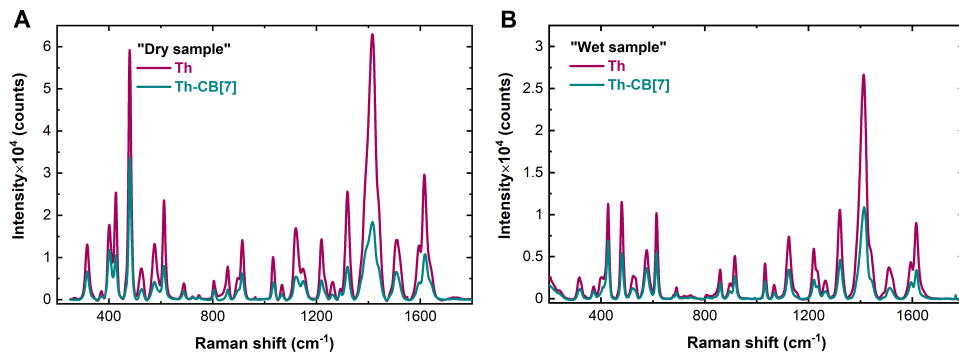


Figure 5.7: Baseline-corrected average SERS spectra of Th and Th-CB[7] mixed with spherical Au oligomers (A) as deposited on glass and dried under ambient conditions, and (B) in colloidal solution. SERS spectra were recorded with 785 nm excitation.

The Th concentration in all samples was maintained at 2 μM to ensure a uniform molecular distribution on the nanoparticle surface. Fang *et al.* investigated the relationship between the fraction of molecules at each Raman enhancement factor and the contributions of the different site enhancement factors to the overall SERS signal. [58] Based on their analysis, it can be reasonably assumed that the probed Raman signal originates from the same number of molecules.

A comparative analysis of the bulk SERS spectra of Th and Th-CB[7] reveals that encapsulation does not influence the Raman shift values of the vibrational modes of the guest molecule, which is consistent with results obtained in the absence of a plasmonic substrate. Chen *et al.* reported changes in the Raman shift values of specific vibrational modes of ferrocene after encapsulation in CB[7] in solid-state samples. [117] However, the magnitude of these Raman shift changes was small (approximately 10 cm^{-1} or less).

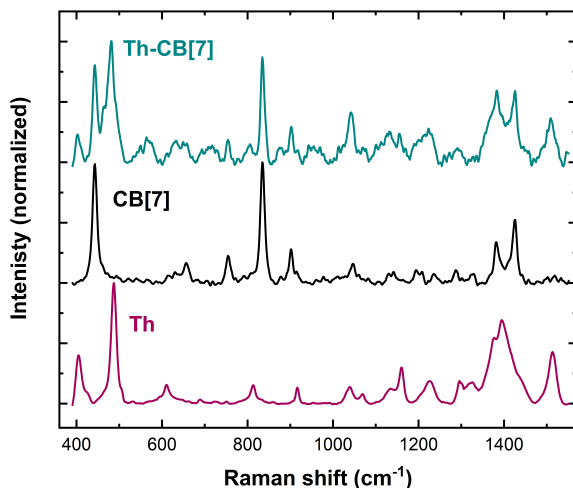


Figure 5.8: Room-temperature spontaneous Raman spectra of an aqueous solution of Th (~ 0.9 mM), CB[7] (~ 25 mM), and Th-CB[7] measured with an excitation wavelength of 785 nm (90 s, 30 acc, 28.6 mW on sample). Spectra are baseline-corrected and normalized for better visibility.

Regardless of the environment, a reduction in intensity was observed for nearly all the molecular vibrational modes for the encapsulated molecule compared to its freely bound and oriented counterpart within the nanocavity. This suggests a restricted ability of the molecule to undergo oscillatory motion inside CB[7].

For the “dry” sample, the relative intensity changes between Th and the Th-CB[7] complex ranged from approximately 33% (for the 401 cm^{-1} band) to as much as 73% (for the 1262 cm^{-1} band). In the “wet” environment, these changes varied from 38% (for the 427 cm^{-1} band) to 67.8% (for the 1509 cm^{-1} band). Figure 5.9A presents the variation in Th vibrational mode intensities between wet and dry environments. The circular points with error bars indicate the environments where a greater difference in intensity is observed.

The average percentage change in Th intensity before and after encapsulation in CB[7] suggests that, in the aqueous environment, the changes in Raman band intensities are slightly smaller (Fig. 5.9B). The high polarity of water may be responsible for such changes, as it leads to interactions be-

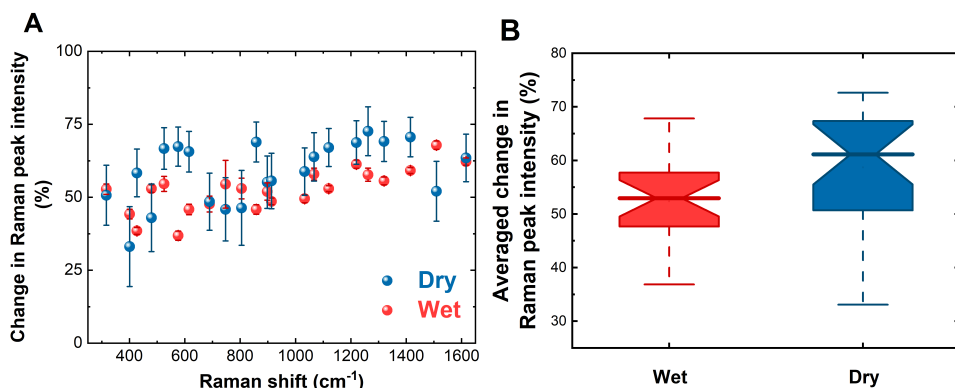


Figure 5.9: (A) Change in the intensity of Th Raman bands under wet and dry conditions. (B) Averaged percentage change in the Th signal intensity (median $\pm$ distribution).

tween water and the Th–CB[7] complex mediated by hydrogen bonds and solvation effects. [104, 118] Such interactions may stabilize the Th–CB[7] complex by restricting the intrinsic deformational degrees of freedom associated with the inclusion of the guest molecule into CB[7]. Consequently, this leads to smaller changes in SERS signal intensity after dye encapsulation in CB[7] compared to an anhydrous environment.

Encapsulation of Th by CB[7], followed by the introduction of the Th–CB[7] complex into the plasmonic cavity, may affect the strong distance dependence of the near-field enhancement, as shown in Fig. 2.8. Moreover, steric effects as well as cavity-size effects are known to significantly influence SERS intensities. [70] Encapsulation of Th by CB[7], followed by the introduction of the Th–CB[7] complex into the plasmonic cavity, may affect the strong distance dependence of the near-field enhancement, as shown in Fig. 2.8. Moreover, steric effects as well as cavity-size effects are known to significantly influence SERS intensities. [70] However, Th (≈ 1.5 nm in length) is longer than the CB[7] cavity (≈ 0.9 nm) and therefore adopts an orientation nearly perpendicular to the metal surface upon inclusion, as illustrated in Fig. 5.10C. The presence of terminal --NH_2 groups may cause the molecular orientation to be perpendicular to the metal surface, regardless of whether Th is present on the Au surface as a free molecule or encapsu-

lated within CB[7], due to the strong chemical affinity of the -NH_2 groups for the Au surface. As a result, the size of the plasmonic cavity remains unchanged, suggesting that the reduction in SERS intensity (on the order of several tens of percent) is not caused by a change in the plasmonic cavity size. Apart from the overall decrease in intensity resulting from the encapsulation of Th by CB[7], no significant qualitative differences are observed in the SERS spectra.

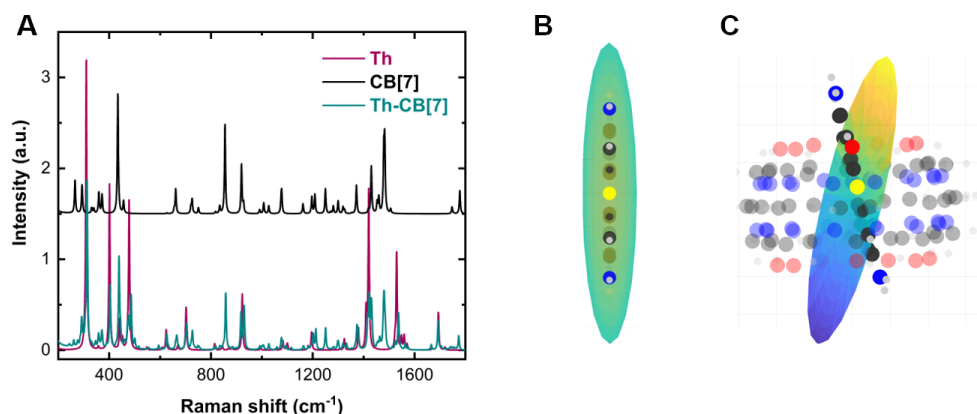


Figure 5.10: (A) Simulated Raman spectra of Th, CB[7], and the Th–CB[7] complex. Graphical visualization of the Raman polarizability tensor corresponding to the 316 cm^{-1} mode of (B) free Th and (C) Raman tensor representation of Th encapsulated within CB[7].

The experimentally observed changes in Raman peak intensities are largely consistent with the simulated spectra (Fig. 5.10A). Variations in Raman band intensities resulting from CB[7] encapsulation may be attributed to modifications in the Raman tensor, arising from structural changes induced by spatial constraints within the CB[7] cavity and by interactions between the Th molecule and CB[7], whereas the distance- and steric effects are negligible. In the case of Th, the most Raman-active vibrational modes have tensors elongated along the long molecular axis, which, in combination with the perpendicular electromagnetic field vector generated by the plasmonic cavity, provides the most efficient enhancement and thus the most intense SERS signal. Encapsulation of Th within CB[7] disrupts this symmetry, leading to a decrease in SERS intensity because the Raman

tensor is no longer ideally aligned with the plasmonic cavity. Such effects are illustrated for the Raman tensor of 316 cm^{-1} mode in Fig. 5.10B and Fig. 5.10C. Density functional theory (DFT) simulations indicate that the Th molecule inside CB[7] is slightly tilted relative to the longest axis of the CB[7] skeleton, in agreement with previous reports [98]. However, the observed change in the tensor tilt direction is in the opposite direction to that of Th.

5.3.4 The photostability of Th–CB[7] complex in plasmonic environment

The photostability of uncomplexed Th and the Th–CB[7] complex was compared using two types of plasmonic nanocavities: nanoparticle-on-mirror (NPoM) structures and spherical Au oligomers, both of which provide strong electromagnetic field confinement, enabling SM-SERS detection. [119, 120] Two sizes of spherical Au nanoparticles were employed, 48 nm and 97 nm in diameter, producing cavities with plasmonic resonances matched to 633 nm and 785 nm excitation, respectively. For 633 nm excitation, a partial spectral overlap occurs between the molecular resonance and the plasmonic cavity mode. In contrast, 785 nm excitation allows SERS signal acquisition without contributions from the resonance Raman effect.

For each sample set, SERS raster maps were collected over consecutive measurement cycles. The spatially averaged SERS intensity exhibited a systematic decline with successive acquisitions. Figure 5.11 and Fig. 5.12 illustrates the decay profile of normalized and background-corrected SERS intensities along with corresponding exponential decay fits. The observed signal loss is likely caused by photodecomposition of analyte molecules within electromagnetic hot-spots or by their diffusion away from these regions under strong field gradients. Sigle *et al.* suggested the possibility of occasionally removing the guest molecule from the CB[7] cavity under incident laser illumination, which led to changes in the temporal average SERS signal. [121] The decay kinetics revealed distinct behaviours between the two nanocavity types. NPoM samples displayed monoexponential decay,

whereas Au oligomers data were best described by a biexponential fit. This difference reflects variations in structural uniformity: NPoM architectures generate hot-spots with similar energy densities, leading to homogeneous photodegradation rates and thus single-exponential decay. In contrast, the morphological diversity of Au oligomers produces a broad distribution of hot-spot field strengths, resulting in multiple decay components that correspond to different photodecomposition rates.

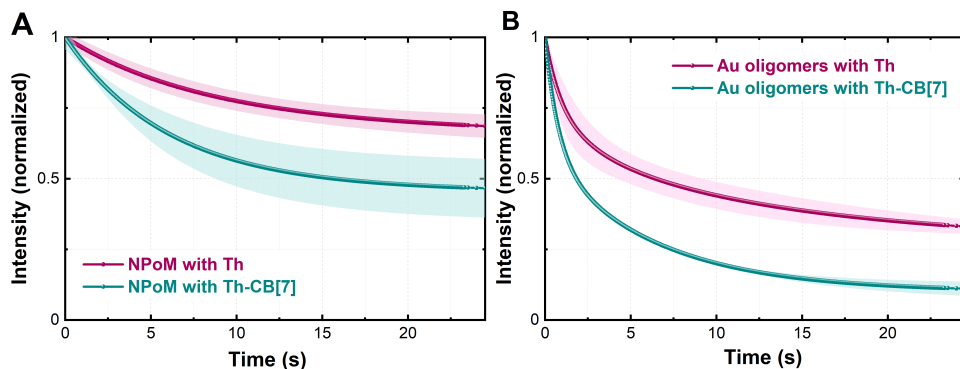


Figure 5.11: SERS signal decay profiles for Th and Th-CB[7] registered with excitation at 633 nm from (A) NPoM geometry and (B) Au oligomers. Each curve represents the baseline-corrected signal averaged over a ($20 \times 20 \mu\text{m}$) SERS map ($1 \mu\text{m}$ step size) and fitted with a (A) monoexponential decay or (B) a biexponential decay. Measurements were performed with an integration time of 0.5 s per spectrum and a laser power of $17 \mu\text{W}$ on the sample. Shaded regions around each curve represent standard deviations; replicates were obtained from distinct regions ($n = 5$ NPoM, $n = 3$ Au oligomers), with 50 maps collected per region.

In both NPoM and Au oligomers configurations, the Th-CB[7] complex exhibited a faster SERS signal decay than uncomplexed Th, independent of the excitation wavelength. To quantify the degradation rate, SERS decay profiles were compared after 11.5 s and 23.5 s of continuous illumination. For the NPoM samples, illumination at 633 nm for 11.5 s resulted in signal decreases of 25% for Th and 46% for Th-CB[7]. Under 785 nm excitation, the corresponding reductions were 28% and 50%, respectively. After 23.5 s of illumination, signal losses were more pronounced under both resonant and non-resonant conditions. However, the decay rate slowed compared to the initial 11.5 s period. In Au oligomers cavities, the largest SERS signal

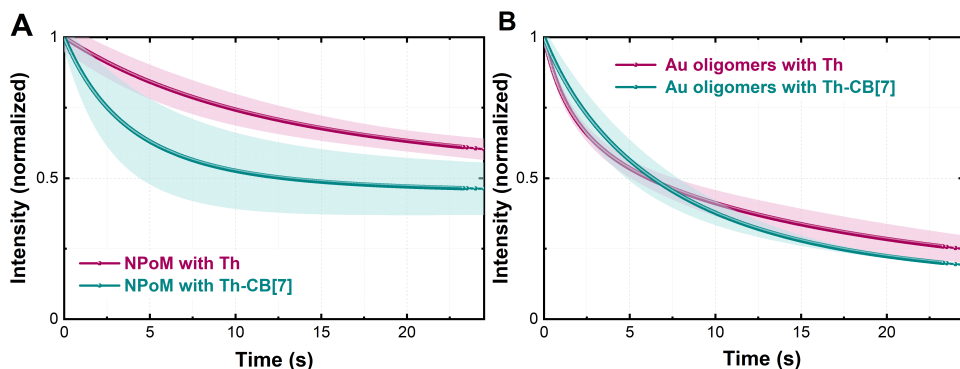


Figure 5.12: SERS signal decay profiles for Th and Th-CB[7] registered with excitation at 785 nm from (A) NPoM geometry and (B) Au oligomers. Each curve represents the baseline-corrected signal averaged over a ($20 \times 20 \mu\text{m}$) SERS map ($1 \mu\text{m}$ step size) and fitted with a (A) monoexponential decay or (B) a biexponential decay (B). Measurements were performed with an integration time of 0.5 s per spectrum and a laser power of $60 \mu\text{W}$ on the sample. Shaded regions around each curve represent standard deviations; replicates were obtained from distinct regions ($n = 5$ NPoM, $n = 3$ Au oligomers), with 50 maps collected per region.

decrease under resonant conditions was observed after 11.5 s, amounting to 58% for Th and 82% for Th-CB[7]. Under non-resonant excitation, the signal decay values at this time point were comparable for both systems and within the experimental margin of error. After 23.5 s of illumination, further signal reduction was observed. Nevertheless, as in the NPoM case, the rate of SERS signal degradation was less pronounced than during the first 11.5 s of exposure.

The significantly faster SERS signal decay observed upon the inclusion of the molecule within CB[7] in the different types of plasmonic nanocavities may be attributed to statistically higher enhancement factors within the hot-spot populations on the samples. Macrocyclic host CB[7], due to the presence of carbonyl groups in its structure, is capable of forming stable interactions through electrostatic forces between the Au surface and the carbonyl portals. This nano-junction, characterized by a well-defined size of 0.9 nm, ensures a strong and reproducible SERS signal. To visualize the plasmonic field enhancement as a function of the nanocavity geometry, FDTD simulations were conducted for two models: spherical Au

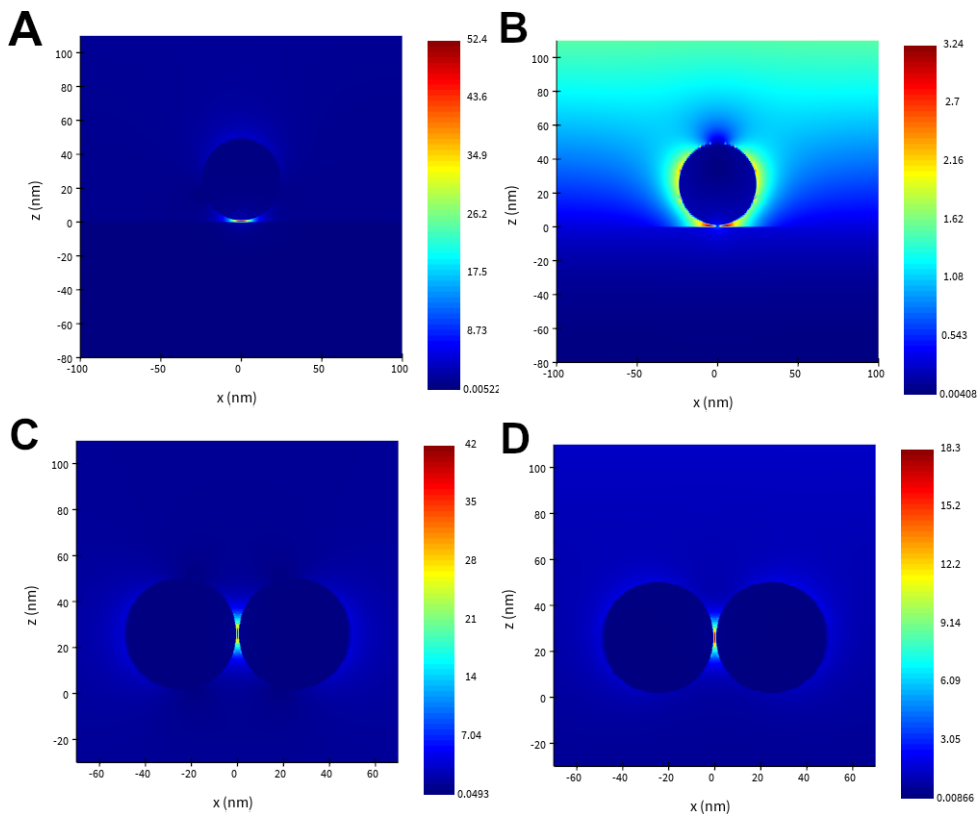


Figure 5.13: Distribution of the electric field enhancement (FDTD) in nanocavities composed of nanoparticles with a diameter of 48 nm. NPoM illuminated at (A) 71.28° and (B) 0° . Au nanosphere dimer illuminated at (C) 0° and (D) 71.28° .

nanoparticle on a thick Au layer (NPoM) and a dimer consisting of two Au nanospheres. These models replicate the plasmonic cavity geometries employed in the experiments. The calculated enhancement factor within the cavity is higher for a cavity constructed from at least two spherical Au nanoparticles compared to an NPoM system. This indicates that oligomeric cavities should exhibit a faster decay of the Raman signal due to the higher enhancement of the electromagnetic field in such plasmonic systems. Moreover, the real enhancement factors may be significantly higher due to the nanoparticle's facet, as the nanoparticles used are not ideal nanospheres. AuNPs' surface facetting and other deviations from perfect sphericity can generate even stronger localized fields at sharp edges and vertices. [109]

Molecules in the vicinity of these “sub-hot-spots” potentially experienced a much higher SERS enhancement factor.

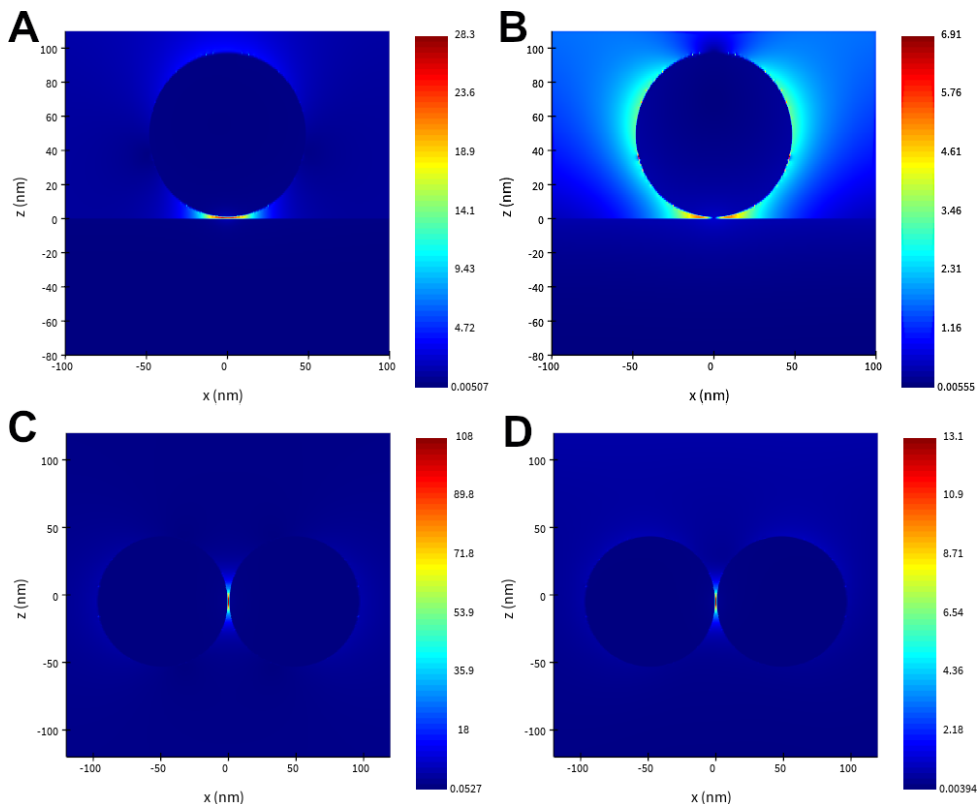


Figure 5.14: Distribution of the electric field enhancement (FDTD) in nanocavities composed of nanoparticles with a diameter of 97 nm. NPoM illuminated at (A) 71.28° and (B) 0°. Au nanosphere dimer illuminated at (C) 0° and (D) 71.28°.

The SERS signal decay profiles over time for Th and Th-CB[7] differ significantly for the same type of nanocavity under the same excitation wavelength. For both nanocavity types, the Th-CB[7] decay profile shows a more dynamic trend. This behavior may be attributed to the substantial signal enhancement caused by the strong interaction between the plasmon gap mode and the emitter, which boosts the resonance Raman effect. Encapsulation of Th within CB[7] aligns the transition dipole moment of Th with the electric field vector generated by the nanocavity upon excitation. Since the transition dipole moment of Th lies along its long axis, the complexed

Th inside CB[7] undergoes more efficient electronic excitation. In contrast, free Th molecules exhibit a much broader range of attachment configurations with the nanocavity, often without optimal positioning at the gap center. Moreover, unlike Th complexed with CB[7], free Th molecules can adopt multiple orientations relative to the electromagnetic field direction inside the cavity, leading to greater heterogeneity. It is worth mentioning that without CB[7], a guest molecule may experience lower stability due to variations in local temperature [122, 123] or electromagnetic field gradients. [124, 125] The constrained geometry imposed by CB[7] encapsulation therefore promotes more uniform and intense SERS signals, albeit at the cost of increased photodegradation rates due to sustained exposure to high field intensities.

5.3.5 Detection and temporal evolution of SM-SERS spectra of Th and Th–CB[7]

Single-molecule SERS detection of Th and Th–CB[7] was performed using an NPoM nanocavity at room temperature under molecular pre-resonance conditions ($\lambda = 633$ nm). Figure 5.15 presents the results of SERS mapping acquired with a spatial resolution of $0.5\ \mu\text{m}$ for NPoM cavities containing Th (Fig. 5.15A) and Th–CB[7] (Fig. 5.15B).

The results indicate that the frequency of detectable signals originating from single molecules is approximately several times higher for the Th–CB[7] compared to the free Th molecule. This higher detectability of Th–CB[7] complex may be related to the molecular orientation within the hot-spot. Single-molecule detection of Th encapsulated in CB[7] is more probable due to the alignment of the transition dipole moment of Th with the electric field vector of the nanocavity, resulting in enhancing the resonance Raman intensity to levels sufficient for single-molecule detection. In contrast, the number of hot-spots detectable for single Th molecules is significantly lower than for Th–CB[7] under identical measurement conditions. This may be attributed to the fact that most Th molecules lie flat on the Au surface. [126] DFT calculations suggest that the majority of the

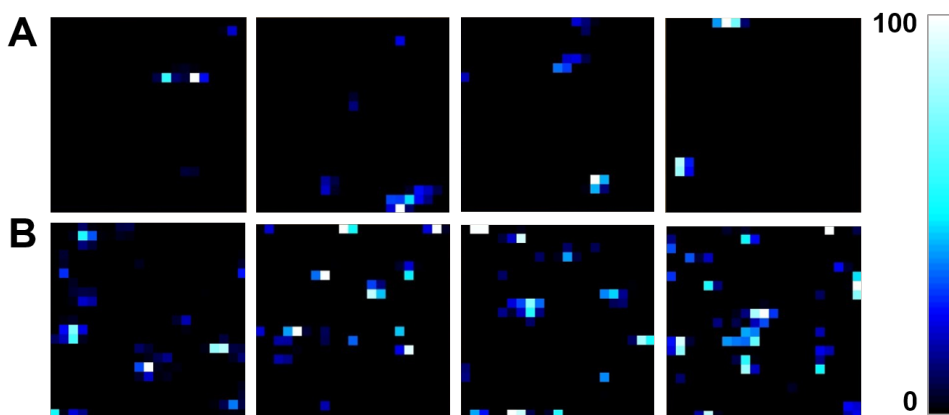


Figure 5.15: SERS maps ($10 \times 10 \mu\text{m}$) of an NPoM for (A) Th and (B) Th-CB[7], obtained using 633 nm excitation with 17 μW laser power, 0.5 s pixel integration time, and a step size of $0.5 \mu\text{m}$.

Raman tensors associated with Th's most intense vibrational modes are ellipsoidally oriented along the molecule's long axis. Such a geometry is inefficient for Raman scattering, as the tensor does not align with the nanocavity's electric field vector, leading to the absence of a detectable SERS signal. Nevertheless, a vertical orientation of Th is also plausible due to the presence of two amino groups at its termini that may interact with the Au surfaces and promote a perpendicular alignment of the molecule within the nanocavity. However, the number of such favorable geometries appears to be limited, as evidenced by the results of the SM-SERS mapping. Both amino groups can also bind to the same Au surface, with this interaction being further strengthened by the sulfur atom's high affinity for Au. [126] Comparison with the Th-CB[7] complex indicates that, while this particular orientation of free Th is not highly favored, it is still observed with measurable frequency in the SM-SERS maps.

The single-molecule SERS spectra taken from NPoM nanocavity were observed through the time-dependent evolution of the SERS signal in order to confirm that the recorded spectra originated from a single molecule. Figure 5.16 presents a temporal evolution of SERS spectra registered with pre-resonance excitation (633 nm) from single Th-CB[7] (Fig. 5.16A), and

registered on single Th (Fig. 5.16B).

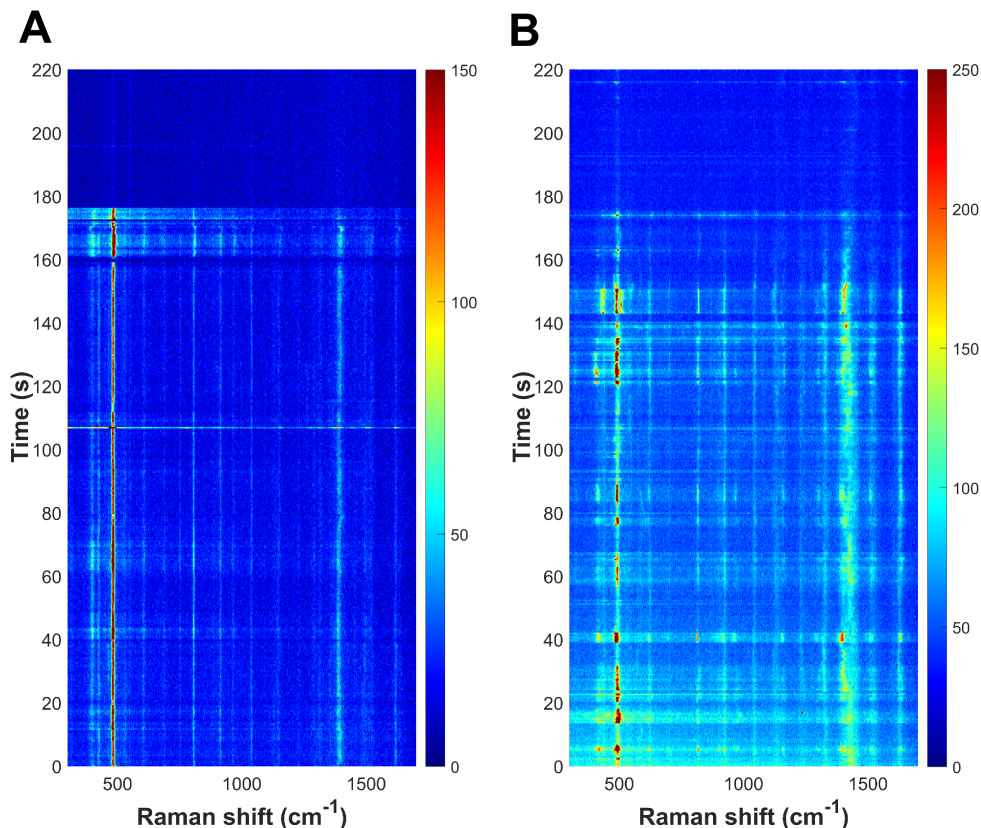


Figure 5.16: Time-resolved SERS spectra of (A) a single Th-CB[7] complex and (B) a single free Th molecule acquired with a 0.5 s integration time using 633 nm excitation.

In case of both samples we can observe appearing of intensity fluctuations of the Raman peaks, which is a hallmark of SERS measurements at the single-molecule and a few-molecule level. However, in the case of a single Th molecule, we observed significant and dramatic jumps in the intensity of selected Raman bands. The duration of these sudden and anomalously intense jumps in the amplitude was less than 1 second. At room temperature, molecules can exhibit high mobility, particularly when subjected to a strong electromagnetic field. The temporal resolution in our experiment, limited by the CCD camera, was insufficient to thoroughly investigate changes occurring over such short timescales. Therefore, we

hypothesize that the anomalously intense SM-SERS spectra represent an averaged signal composed of multiple molecular orientations within the hot-spot. Compared to Th the intensity fluctuations of a single Th-CB[7] exhibited a more stable fashion with reduced fluctuation amplitudes. Both time-dependent traces exhibit characteristic features of SERS spectra measured in the single-molecule regime, namely bleaching, which is often associated with a sudden and drastic decrease in SERS signal intensity (Fig. 5.17).

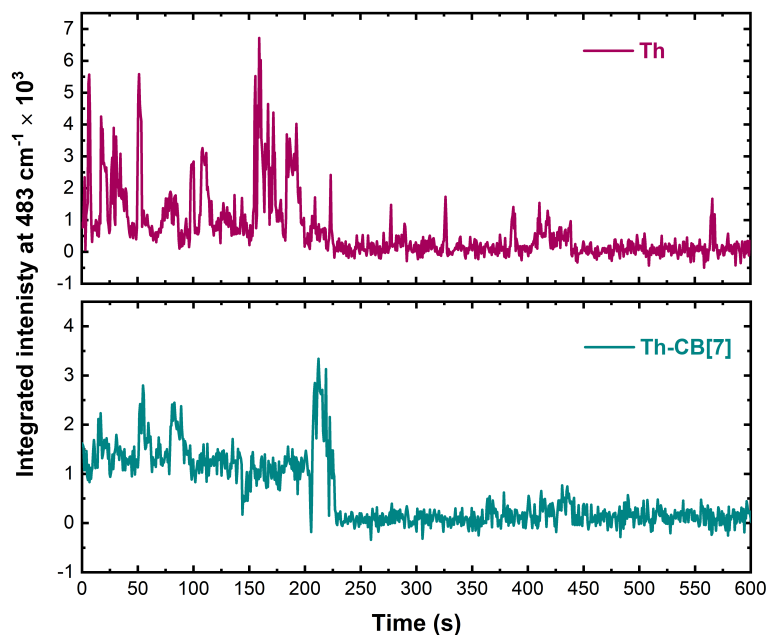


Figure 5.17: Extracted from Fig. 5.16, the temporal evolutions of the 483 cm^{-1} band as peak area integrals for Th and Th-CB[7].

The increased intensity fluctuations of individual Th molecules are reflected in the analysis of the coefficient of variation (CoV), defined as the ratio of the standard deviation to the mean signal intensity. Figure 5.18 presents a histogram of the CoV for a population of single Th and Th-CB[7] molecules taken from over 100 single-molecule events for each sample and fitted with a Lorentzian distribution. The average CoV for Th is 0.54, whereas for Th-CB[7] it is 0.41, corresponding to a 24% reduction in relative intensity variability upon encapsulation. Additionally, the distribu-

tion for Th exhibits a slightly broader spread, suggesting a higher degree of variability in fluctuation behavior among the molecules. These experimental results provide valuable insights into the dynamics of single molecules within the hot-spot.

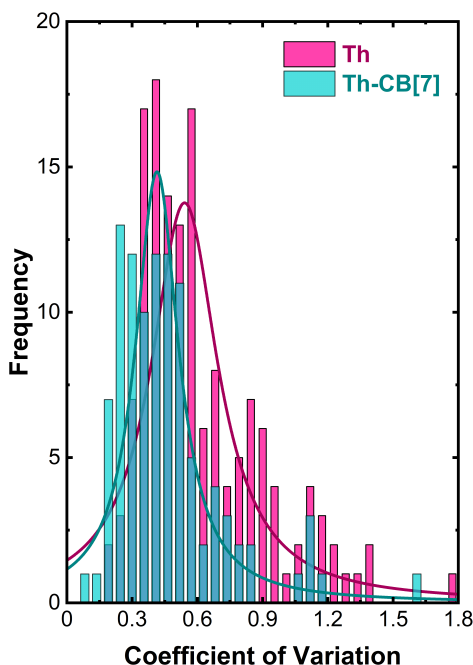


Figure 5.18: Histograms of the coefficient of variation (CoV) for the free Th and Th-CB[7] samples. The distribution of CoV values was fitted by the Lorentzian function.

The observed increase in CoV for Th can be attributed to the greater mobility of the molecule resulting from the possibility of performing rotational and translational movements in the hot-spot. The free molecule can adopt various orientations with respect to the local field polarization and occupy different spatial locations within the nanoscopic hot-spot volume, with each configuration contributing a unique enhancement factor to the time-averaged signal. In contrast, the mobility of Th encapsulated within CB[7] is restricted by the local nanoenvironment imposed by the CB[7] cavity. For instance, reorientation from a perpendicular to a parallel alignment relative to the nanocavity is forbidden, assuming CB[7] do not change its

orientation on the surface. Additionally, the presence of carbonyl portals in the CB[7] structure ensures the binding of CB[7] to the Au surface via electrostatic interactions, which eliminates the translational motion of Th–CB[7].

These results align with previous investigations by Lindquist *et al.*, who reported an analysis of the CoV as a function of the reaction environment (wet vs. dry) and the molecular binding within the hot-spot. [127] For a dry environment, it was found that a covalently bound molecule exhibited a mean CoV of 0.56, whereas a freely adsorbed and bound molecule had a mean CoV of 0.65. [127] Comparing the results of this study, it can be concluded that SM-SERS spectral fluctuations depend on the type of molecule due to different physicochemical affinities for the Au surface. Nevertheless, the encapsulation of the molecule in CB[7] can significantly reduce the degree of intensity fluctuations in SERS.

5.3.6 Investigation of correlations between different vibrational modes in SM-SERS spectra of Th and Th–CB[7]

Fluctuations in the SM-SERS Raman peak amplitude intensity do not occur independently but are accompanied by concurrent spectral fluctuations of Raman band positions, called spectral diffusion. An example of a single-molecule SERS trajectory with clearly visible spectral diffusion is presented in Fig. 5.19. During time-resolved measurements of SM-SERS spectra, spectral diffusion reveals itself in sudden and abrupt frequency shifts in Raman bands that persist for a certain period before spontaneously returning to their original positions or shifting to new Raman frequencies. These spectral jumps are a defining feature of SM-SERS, since ensemble measurements would smooth out such individual molecular events.

Encapsulation of Th inside CB[7] significantly restricts its molecular translational and rotational motion compared to a freely oriented and adsorbed molecule on an Au surface. Th adequately fills the CB[7] cavity, however, due to a packing coefficient of less than 1, a small degree of space remains, allowing conformational flexibility and small-amplitude molecu-

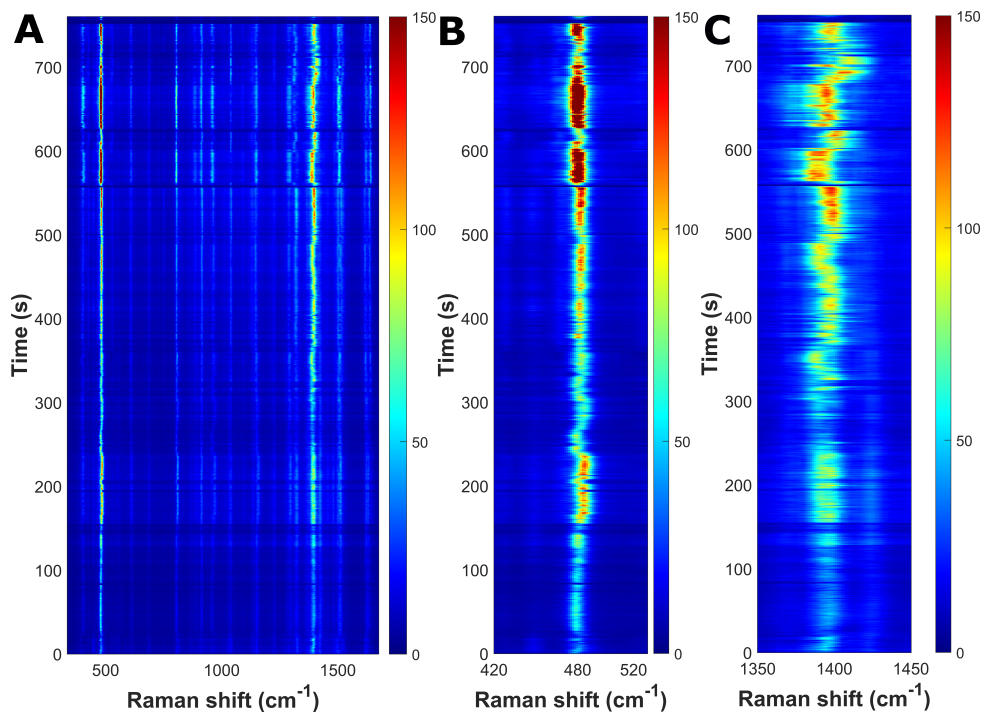


Figure 5.19: Temporal evolution of SM-SERS spectra of Th-CB[7] recorded with a 0.5 s integration time and a 633 nm excitation wavelength. Panel (A) shows the full spectral time trace, while panels (B) and (C) highlight spectral diffusion of Raman bands at approximately 483 and 1405 cm^{-1} , respectively.

lar motion in the CB[7] inner cavity.

To elucidate the complex fluctuation mechanism, we investigated the mutual relationship between peak position and peak intensity variations. For this purpose, we performed a statistical analysis of Spearman correlation coefficient for the following pairs of Th Raman bands: (1) 404 cm^{-1} vs. 483 cm^{-1} , (2) 483 cm^{-1} vs. 610 cm^{-1} , (3) 483 cm^{-1} vs. 805 cm^{-1} , and (4) 610 cm^{-1} vs. 805 cm^{-1} . The statistical population comprised over 100 SERS spectra recorded from single molecules. Analysis of the SM-SERS spectral data required preliminary processing of the experimental results, including noise reduction, spectral smoothing using the Savitzky–Golay filter, and fitting of the selected Raman bands with a Lorentzian function. Figure 5.20 presents a comparison of the distribution profiles of Spearman correlation coefficients for both the position and amplitude of each pair of Th Raman

bands, calculated for Th–CB[7] (Fig. 5.20A) and free Th (Fig. 5.20B).

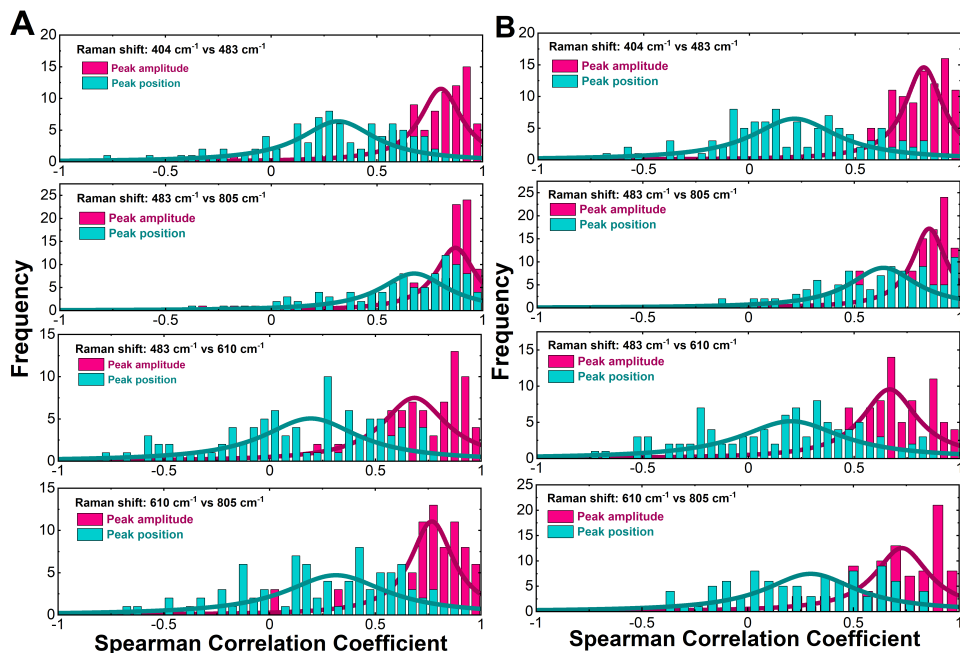


Figure 5.20: Distributions of Spearman correlation coefficients between variations of Raman peak position and SERS-signal amplitude changes for single-molecule SERS spectra of free (A) Th and (B) Th–CB[7] for selected pairs of Raman bands. Each distribution was fitted with a Lorentzian function. Sample sizes for each pair of Raman modes – 404 cm^{-1} vs. 483 cm^{-1} , 483 cm^{-1} vs. 610 cm^{-1} , 483 cm^{-1} vs. 805 cm^{-1} , and 610 cm^{-1} vs. 805 cm^{-1} – were $n = [87, 87, 99, 87]$ for Th and $n = [100, 95, 107, 92]$ for Th–CB[7].

The overall trend emerging from this analysis indicates a lack of correlation between band position fluctuations and SM-SERS signal amplitude variations. This suggests a complex mechanism underlying SM-SERS spectral fluctuations, likely involving at least two distinct processes. Furthermore, the distribution of Raman peak position correlation coefficients was notably broader than that of the amplitude correlation coefficients. This broad distribution suggests that the process responsible for variations in peak position is more dynamic or heterogeneous than that responsible for variations in peak amplitude. In contrast, the distribution of correlation coefficients for peak amplitude variations exhibited a narrow spread, indicat-

ing greater reproducibility of the observed changes. The mean correlation values based on Raman band amplitude ranged from 0.67 to 0.87, indicating a strong correlation. In contrast, correlation values based on Raman peak position were significantly lower, ranging from 0.19 to 0.67, corresponding to weak correlation.

Characteristic correlation pattern implies that SM-SERS fluctuations originate from at least two independent mechanisms. The strong peak amplitude correlations are likely driven by electromagnetic enhancement variations that affect all Raman bands simultaneously, whereas the weak position correlations suggest that spectral diffusion is governed by mode-specific chemical interactions. No influence of Th encapsulation in CB[7] on the correlation analysis of relative spectral and amplitude variations of Raman bands was observed, since mean correlation coefficients were comparable for both free and encapsulated molecules. This suggests that the fundamental fluctuation mechanisms remain unchanged upon complexation with CB[7].

Correlation analysis clearly indicates that encapsulation of Th in CB[7] does not result in inhibition of spectral and intensity fluctuations. However, in case of intensity fluctuations, we observed a reduction of the CoV values (Fig. 5.18), suggesting a reduced mobility of the molecule in CB[7].

Crystallographic studies have demonstrated that CB[*n*] undergoes ellipsoidal deformations upon guest molecule inclusion. [128] El-Barghouthi *et al.* conducted molecular dynamics simulations of methyl viologen inclusion in CB[*n*], revealing that while the rotation of the guest molecule within the CB[7] cavity is possible, it induces structural deformations in the diameter of CB[7]. [129] Ai *et al.* revealed the rotational motion of the confined guest molecule (aminoferrocene) inside the CB[7] cavity, based on analysis of the position shift and intensity change of the cyclopentadienyl ring of aminoferrocene. [130] Measurements were performed in electrolyte on plasmonic molecular junctions containing CB[7] or its host–guest complexes in a nanoparticle on a nanoelectrode geometry.

Taking into account the results presented in this thesis, along with literature reports — particularly molecular dynamics simulations — it can be

assumed that fluctuations in Raman band intensity arise from variations in the molecular orientation within CB[7]. However, in order to fully observe such motion, it would be necessary to significantly reduce the accumulation time of a single spectrum while simultaneously increasing the excitation energy flux, so as to maintain a high (S/N).

The variability in Raman peak positions reveals additional aspects of the underlying processes in the context of SM-SERS fluctuations. We speculate that dynamic shifts in Raman peak position may result from local and transient interactions between Au adatoms within the plasmonic cavity and the analyte molecule, as proposed by Benz *et al.* [75]. The formation and dissociation of such adatom–molecule complexes alter the local chemical environment, producing both minor and major vibrational frequency shifts. This model accounts for the broad distribution of correlation coefficients, suggesting that the process is largely stochastic, non-reproducible, and highly sensitive to local environmental conditions.

5.4 Conclusions

In this chapter, we have provided a comprehensive investigation of single-molecule surface-enhanced Raman scattering (SM-SERS), with particular emphasis on modulating molecular dynamics through supramolecular encapsulation. Using thionine (Th) as a model system, we demonstrated that encapsulation within cucurbit[7]uril (CB[7]) offers an effective strategy for enhancing the stability of SM-SERS signals while maintaining the defining features of single-molecule sensitivity and suppressing molecular dimerization.

In the first part of our study, we investigated the effect of encapsulation within CB[7] on the SERS spectrum of thionine (Th), using spherical Au oligomers as a model plasmonic nanocavity. Encapsulation within CB[7] leads to a pronounced reduction in nonresonant bulk SERS intensity without inducing shifts in Raman band positions, consistent with conformational constraints and modifications of molecular polarizability tensors,

as supported by density functional theory (DFT) calculations. We also observed that the intensity reduction in the SERS spectrum upon encapsulation in CB[7] depends on the surrounding environment. In an aqueous environment, the changes were less pronounced compared to dry samples, suggesting that water molecules may provide additional stabilization of the Th–CB[7] complexes.

In the subsequent part of this study, we investigated the photostability of Th–CB[7] complexes as a function of the plasmonic nanocavity geometry — namely, a nanoparticle-on-mirror (NPoM) configuration or spherical nanoparticle oligomers — as well as the excitation wavelength, chosen either to be resonant with or detuned from the electronic transition of the molecule. The SERS signal decay profiles exhibited more pronounced and dynamic behavior for nanocavities based on spherical oligomer geometries compared to those with an NPoM configuration. Finite-difference FDTD simulations of the electromagnetic field distribution indicate that oligomeric nanocavities are expected to exhibit higher enhancement factors, leading to faster SERS signal decay, in agreement with our experimental observations. Regardless of the plasmonic cavity geometry, encapsulation within CB[7] resulted in a more rapid decay of the SERS signal compared to molecules freely adsorbed on the metal surface. This accelerated SERS signal decay is most likely associated with changes in molecular orientation induced by encapsulation, as CB[7] promotes an alignment of the Raman tensor that is more optimally oriented with respect to the local electromagnetic field vector of the plasmonic nanocavity. As a consequence, an increased Raman scattering efficiency is achieved.

The final part of this work was devoted to investigating the effect of supramolecular encapsulation on the detection and fluctuation behavior of single-molecule SERS (SM-SERS) signals. The enforced molecular orientation within the CB[7] cavity, described in the previous section, has several important implications for single-molecule detection. We observed that the probability of detecting individual molecules increases several-fold, thereby rendering SM-SERS measurements more reliable and experimentally accessible. At the same time, this stabilized molecular orientation reduces un-

desirable signal fluctuations while preserving hallmark SM-SERS phenomena, such as blinking and photobleaching, thus confirming the retention of the fundamental single-molecule character. The constrained geometry also leads to more predictable and reproducible SERS responses. Time-resolved single-molecule SERS (SM-SERS) measurements revealed distinctly different fluctuation patterns for free molecules compared to those encapsulated within CB[7]. Free thionine (Th) exhibited rapid intensity “jumps” with high variability, whereas the Th–CB[7] complex displayed significantly more stable temporal behavior, characterized by a lower coefficient of variation across individual single-molecule trajectories. Analysis of the temporal evolution of single molecules revealed strong correlations in amplitude fluctuations but weaker correlations in peak positions, suggesting distinct underlying mechanisms governing these fluctuations.

The results of our study indicate that restricting molecular mobility via encapsulation in CB[7] reduces amplitude fluctuations, whereas correlation analysis reveals that spectral diffusion remains unaffected. In this context, the reduction in mobility is likely associated with the suppression of translational motion, as CB[7] effectively prevents the molecule from “escaping” the plasmonic hot-spot. Consequently, the statistical analysis enabled us to distinguish between two possible mechanisms underlying the observed fluctuations: molecular motion that is suppressed by CB[7] encapsulation, and substrate or adatom dynamics that remain unaffected by the encapsulation process.

Chapter 6

Surface-Enhanced Femtosecond Stimulated Raman Spectroscopy – Optimization of a Novel Measurement Tool for Single-Molecule Detection

The contents of this chapter are based on:

Patryk Pyrcz and Sylwester Gawinkowski, "Advancements in surface-enhanced femtosecond stimulated Raman spectroscopy: Exploring factors influencing detectability and shapes of spectra", *Nanophotonics*, 14, 1, 1–12 (2025)

6.1 Introduction

6.1.1 Temporal limits of SERS

The temporal resolution of routine single-molecule SERS spectral measurements is typically on the millisecond timescale, which results from the time-resolution limitations of the detectors used, namely CCD cameras. [131] However, the dynamics of many chemical and biochemical reactions occur on femtosecond and picosecond timescales. [132–136] Achieving such high temporal resolution using conventional SERS is currently unattainable, particularly at the single-molecule level. A viable solution to this limitation is the integration of SERS with the family of coherent Raman spectroscopies (CRS).

CRS represent a group of nonlinear optical techniques that probe molecular vibrational modes through the coherent interaction of multiple laser fields with molecules, resulting in the generation of phase-matched signals. [137] In contrast to spontaneous Raman scattering, which arises from incoherent, randomly phased molecular vibrations and yields weak emitted signals, coherent Raman processes drive a macroscopic vibrational coherence across the molecular ensemble. This collective excitation leads to strongly enhanced signal intensities, well-defined emission directions, and improved signal-to-noise ratio (S/N). Moreover, the coherent nature of these techniques enables selective excitation and readout of specific vibrational modes with high temporal resolution, allowing direct access to vibrational dynamics on ultrafast timescales. Nevertheless, one of the main drawbacks of coherent Raman spectroscopies is related to the physical nature of the sample, since these techniques typically require highly concentrated samples with relatively large Raman cross sections.

The concept of using a plasmonic nanocavity as an optical antenna has enabled SERS detection limits to be reduced to the single-molecule level. A similar approach has been explored for CRS. However, in contrast to SERS, the number of literature reports on plasmonic enhancement in CRS remains limited, mainly due to the experimentally demanding conditions required

to perform such measurements. Nevertheless, coupling plasmonic nanocavities with CRS is highly attractive, as it would enable real-time observation of the behavior and reactions of individual molecules, while also enabling the investigation of unique dynamical processes occurring near plasmonic surfaces, with relevance to plasmonically enhanced photovoltaics, catalysis, and plasmon-mediated chemistry. [138]

In 2011, the successful coupling of ultrafast nonlinear Raman spectroscopy in the form as FSRS with SERS resulted in the first experimental realization of surface-enhanced femtosecond stimulated Raman spectroscopy (SE-FSRS). [139]

6.1.2 Development of SE-FSRS

The first demonstration of SE-FSRS was conducted in the Van Duyne group. Frontiera *et al.* demonstrated ground-state SE-FSRS spectra on aggregated Au oligomers with adsorbed *trans*-1,2-bis(4-pyridyl)ethylene (BPE) molecules and capped in a silica shell. [139] The SE-FSRS experiment was performed using a 100 kHz ultrafast amplifier with a narrow bandwidth Raman pump pulse and a broadband femtosecond probe pulse. The recorded SE-FSRS spectrum was characterized by a strong negative background and a dispersive line shape of spectral lines. By changing the diameter of Au nanoparticles, Frontiera *et al.* tuned the energy of the plasmon resonance of the Au oligomers relative to the wavelengths of the Raman pump and probe pulses. [140] The change in plasmon resonance energy led to a change in the phase of the dispersion of the observed SE-FSRS peaks, which were modeled using a Fano function. The authors proposed that the Fano-like line shapes result from the interaction between the narrow molecular vibrational coherence and the broad plasmon resonance of the colloidal nanoparticles. For comparison, the spectral lines of the SERS spectrum were described by the Lorentzian function and were background-free.

In the following years, Van Duyne's group continued their research, providing original work aimed at improving the SE-FSRS technique. Gruenke *et al.* investigated the Au concentration and optical path length depen-

dences of SE-FSRS signal quality and signal-to-noise ratio. [141] Buchanan *et al.* reported the first ground-state SE-FSRS spectrum registered at a 1 MHz repetition rate, which was ten times greater than previously published SE-FSRS experiments. [142] With the 1 MHz system, the SE-FSRS measurements offer significantly improved signal-to-noise ratio with comparable gain to the 100 kHz system. Finally, Buchanan *et al.* studied the SE-FSRS spectra by varying the excitation wavelength of the picosecond Raman pump pulse. [143] The spectral line shapes differed dramatically with excitation wavelengths and showed a strong dependence on the frequencies of the incident fields relative to the surface plasmon resonance.

The presence of a dispersive and asymmetric background in SE-FSRS spectra makes it difficult to perform a quantitative analysis, allowing for an accurate estimation of the enhancement factor. Kumar *et al.* report time-domain Raman measurements of BPE-coated Au oligomers capped in silica shell using impulsive stimulated Raman spectroscopy (ISRS). [144] Unlike frequency-domain FSRS, ISRS operates in the time domain. In this technique, ultrashort pump pulses coherently excite Raman-active molecular vibrations, which are then directly observed as oscillations in the time-resolved absorption signal. The analysis using the Fourier transform uncovered the enhancement factor as high as $10^5 - 10^6$ with nanoJ energy at the pump pulse. The same group implemented an actinic pulse to photoexcite the LSPR band of BPE-coated Au oligomers and track the evolution of photoexcited plasmon-driven ultrafast dynamics of the adsorbate on metal nanoparticles. [145] Kumar *et al.* showed that LSPR excitation drives BPE through two transient states linked to hot-electron dynamics and weakened adsorption before returning to the initial steady state. [145]

Zong *et al.* have performed frequency-domain SE-SRS with chirped laser pulses at 80 MHz repetition rate coupled with a microscope. [146] The SE-SRS measurements was conducted on two isotopologues of adenine deposited on Au nanostructures. With a special denoising algorithm, they proved that the sensitivity of SE-SRS reached a single-molecule level. However, even at the single-molecule level with the frequency domain technique, the stimulated Raman signal is distorted by a strong and broad back-

ground.

Recently, Sun *et al.* have demonstrated the resonance Raman effect in SE-FSRS using malachite green (MG) as a Raman probe adsorbed on shell-isolated nanoparticles of Au or Ag for the first time. [147] Maximum enhancement factor $\sim 10^6$ has been obtained when the excitation wavelength matches with the plasmonic band of shell-isolated nanoparticles. Through combining shell-isolated nanoparticles with FSRS, Sun *et al.* investigated the ultrafast dynamics of photoinduced processes in a monolayer of MG adsorbed on Au surfaces. They found that MG undergoes intramolecular twisting within approximately 100 fs, and that the strong localized plasmonic field accelerates the structural evolution. [147]

In order to describe the dispersive shape of the SE-FSRS spectral lines and to explain the origin of the background, several theoretical models have been proposed. Mandal *et al.* explained the dispersive line shapes of reported SE-FSRS experiments by describing a theoretical model, according to which interference between the plasmon-enhanced third-order nonlinear signals originating from the molecular Raman response and the resonant plasmon emission or scattering results in a dispersive profile of SE-FSRS spectra. [148] Simulated SE-FSRS as a function of increasing enhancement factor showed increased dispersion. [148] Concurrently, McAnally *et al.* presented a coupled wave semiclassical theory to describe enhancement in SE-FSRS. [149] A central finding was that the plasmon-enhanced fields, which drive the vibrational dynamics of each normal mode, lead to dispersive line shapes in the SE-FSRS spectrum. Zong *et al.* [150] reported a theoretical model together with a phasor diagram to elucidate the observed dispersive nature of SE-FSRS spectra. According to this model, dispersive line shapes in SE-FSRS spectra should be observable at high enhancement factors, whereas at low enhancement factors the spectral band shapes are expected to approximate a Lorentzian profile. Moreover, the model predicts an increase in the dispersive character of the SE-FSRS line shape when the excitation wavelength approaches the spectral maximum of the plasmon resonance. [150]

The origin of the background was discussed by two independent groups.

Zong *et al.* suggest the photothermal effect of Au nanoparticle [150] performing SE-SRS measurements with 60 nm Au nanoparticles, while Reynkens *et al.* proposed the combination of photothermal and thermo-optic effects based on SE-SRS experiment with nanoplasmonic waveguide. [151] Photothermal effect is related to the modulated heating of Au nanoparticles as a consequence of absorption of the modulated Raman pump, which is associated with the change of the absorption and refractive index of the Au. The heat generated by linear absorption within the Au nanostructure influences the Stokes beam, thereby masking the Raman response of the investigated molecule.

Previous SE-FSRS measurements were mainly performed on aqueous colloids of aggregated Au nanoparticles as plasmonic substrates. [33, 139–144] These plasmonic nanoantennas were characterized by a broad spectrum of LSPR, due to the fact that the sample consists of different types of oligomers. As a consequence, theoretical models were not consistent with frequency-dependent SE-FSRS measurements. [143] Up to now, it has not been possible to perform SE-FSRS measurements with a plasmonic substrate that would be characterized by a high degree of monodispersity and thus a well-defined spectrum of LSPR.

6.2 Materials and methodology

6.2.1 Materials

Gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) ($\geq 99.9\%$ trace metals basis), sodium citrate dihydrate (99%), (3-aminopropyl)triethoxysilane (APTES) (97%), sodium silicate solution ($\text{NaOH} \geq 10\%$, $\text{SiO}_2 \geq 27\%$), tetraethyl orthosilicate (TEOS) (99.9%), *trans*-1,2-*bis*(4-pyridyl) ethylene (BPE) (97%), hexadecyltrimethylammonium bromide (CTAB) ($\geq 98\%$), hexadecyltrimethylammonium chloride (CTAC) (25 wt.% in H_2O), silver nitrate (AgNO_3) ($\geq 99\%$), sodium borohydride (NaBH_4) (99%), L-ascorbic acid (AA) (99%), sodium hydroxide (NaOH) ($\geq 97\%$), and dimethyl sulfoxide (DMSO) (99.9%) were obtained from Sigma-Aldrich. Ammonia solution ($\text{NH}_3 \cdot \text{H}_2\text{O}$) (28%), hydrochloric

acid (HCl) (35–38%), nitric acid (HNO₃) (65%), and ethanol (EtOH) (HPLC grade, 99.9%) were sourced from CHEMPUR. All chemicals were used without further purification. Deionized water (Elix system, Millipore, > 15 M Ω ·cm⁻¹) was used throughout the experiments. All glassware was thoroughly cleaned with aqua regia solution and rinsed with deionized water.

6.2.2 Plasmonic nanoantenna preparation and characterization

We performed SE-FSRS measurements using two types of plasmonic nanoantennas. These included spherical Au nanoparticles, each containing multiple cores (e.g., dimer, trimer, tetramer) functionalized with adsorbed BPE and embedded in a silica shell (BPE-AuNPs), as well as monodisperse silica-coated Au bipyramids (AuBPs) without a Raman reporter.

AuNPs were synthesized using a seed-mediated growth method based on previously reported protocols. [27, 111] The seed nanoparticles were prepared using the sodium citrate reduction approach originally developed by Turkevich and coworkers. [112] In brief, 0.5 mL of sodium citrate solution (340 mM) was added to 99.5 mL of boiling HAuCl₄ solution (0.254 mM) under vigorous stirring, and the mixture was maintained at 90 °C for 30 minutes. To grow the seed particles, stock solutions of HAuCl₄ (25 mM) and sodium citrate (60 mM) were first prepared. Then, 30 mL of the seed solution was mixed with 20 mL of water, and 0.46 mL of 60 mM sodium citrate was added to a round-bottom flask. The mixture was heated to 90 °C for 5 minutes. Following this, two portions of 0.23 mL HAuCl₄ were added to the solution, each separated by a 35-minute interval. After the additions, 20 mL of water was added. In a subsequent growth step, 0.956 mL of sodium citrate was introduced, and the mixture was again heated for 5 minutes. Two further additions of HAuCl₄ (0.478 mL each) were made, also at 35-minute intervals. During each of these additions, 27.2 mL of water was added. By repeating this growth cycle, larger Au nanoparticles were successfully obtained.

BPE-AuNPs were prepared following previously reported methods. [37,

38, 152] In a typical procedure, 25 mL of AuNP solution was used, with the concentration adjusted to ensure a maximum extinction of one at the plasmon resonance peak. A 1 mM ethanolic solution of BPE was added to the AuNP colloid to induce the formation of nanoparticle oligomers. The aggregation process of the monodisperse AuNPs was monitored in real time by recording extinction spectra. Once the desired level of oligomerization was reached, 40 μL of a 1 μM aqueous solution of APTES was added to the mixture under continuous stirring. After 10 minutes, 400 μL of a 1.2% aqueous sodium silicate solution was introduced. The reaction was stirred continuously for 48 hours, resulting in the formation of a thin silica shell around the AuNP oligomers. To increase the silica shell thickness, the Stöber method was employed, which involves the hydrolysis of TEOS as the silica source. [153]. The reaction mixture was first concentrated to approximately 4 mL, followed by the addition of 27 mL of EtOH. Next, 2.32 mL of $\text{NH}_3\cdot\text{H}_2\text{O}$ and 17.2 μL of TEOS were added while stirring. The reaction was allowed to proceed for another 48 hours. Afterward, the product was subjected to multiple centrifugation cycles (15 minutes at 5 500 rpm), with each cycle followed by washing the optical Au nanoantennas with water.

AuBPs were synthesized following the procedure outlined by Sánchez-Iglesias *et al.* [154], with sodium citrate dihydrate used in place of citric acid. The synthesis consisted of two main steps: seed preparation and nanoparticle growth. In the seed preparation step, an aqueous solution of HAuCl_4 (10 mL, 0.25 mM) was rapidly reduced by the addition of freshly prepared, ice-cold NaBH_4 (0.25 mL, 25 mM) in a 50 mM CTAC solution containing 5 mM sodium citrate dihydrate, under vigorous stirring at room temperature. A color change from pale yellow to brown signaled the formation of seed nanoparticles. After approximately 2 minutes, the reaction vial was sealed and placed in an oil bath at 80 °C for 90 minutes. During this period, the solution gradually changed color from brown to red. After heating, the vial was removed from the bath, allowed to cool, and stored at room temperature. For the growth phase, a solution was prepared at 30 °C by mixing CTAB (50 mL, 100 mM), HAuCl_4 (2.5 mL, 10 mM), AgNO_3 (0.5 mL, 10 mM), HCl (1 mL, 1 M), and AA (0.4 mL, 100 mM). To this growth solution, 1.25 mL

of the prepared seed solution was added under vigorous stirring. The reaction mixture was then left undisturbed with gentle stirring for at least 2 hours to allow the formation of AuBPs.

Silica-AuBPs were prepared following procedures reported in the literature. [155] A 12 mL suspension of AuBPs was purified by two rounds of centrifugation at 7 000 rpm for 10 minutes. The resulting precipitate was re-dispersed in 12 mL of water. Subsequently, 200 μ L of CTAB (0.1 M), 400 μ L of TEOS (1.75 wt.% in EtOH), and 120 μ L of NaOH (0.1 M) were added to the dispersion with gentle shaking. The reaction mixture was kept at room temperature under gentle shaking for 12 hours to allow the deposition of a silica shell onto the AuBPs. After the coating process, the samples were centrifuged at 5 000 rpm–6 500 rpm for 10 minutes. The final product was redispersed in 6 mL of water for further use.

The Au nanoparticles were analyzed using a 10 kV scanning electron microscope (SEM) (FEI Nova NanoSEM 450) to investigate the morphology of home-synthesized colloids and silica-coated optical nanoantenna. UV-Vis-NIR spectroscopy, performed using a Shimadzu 3100 spectrophotometer, was employed to characterize the spectral properties, mainly to estimate the position of LSPR of nanoantennas. Extinction spectra of all colloids were measured in PMMA cuvettes (Brand).

6.2.3 Raman and SERS measurements

Raman and SERS spectra were recorded on a home-built microspectroscopy system described in detail in subchapter 4.2.3. Minor changes were introduced only in the excitation path of the microscopic setup, while the detection path remained unchanged. The laser beam was emitted from a laser diode (HL6545MG or HL6748MG, Thorlabs) mounted in a thermoelectric head (LDM56/M, Thorlabs). The diode was controlled by adjusting the temperature and current using the following controllers: a temperature controller (TED200C, Thorlabs) and a current controller (LDC205C, Thorlabs). The laser beam was directed onto a tunable wavelength bandpass filter (VersaChrome TBP01-704, Semrock) and a tunable optical density filter (NDC-

50C-4M, Thorlabs). After passing through the filters, the laser beam was directed to the microscope and focused onto the sample using a 30 mm focal length objective (Renishaw). SERS spectra of the colloids were accumulated using a PMMA cuvette (Brand), while Raman spectra were collected in a quartz cuvette (111-10-40, Hellma). After interaction with the sample, the laser beam was guided toward the detection path of the microscope setup.

6.2.4 FSRS and SE-FSRS measurements

The FSRS and SE-FSRS measurements were carried out using the home-built setup constructed by Dr. Michał Nejbauer. [156] Figure 6.1 presents a schematic representation of the FSRS setup.

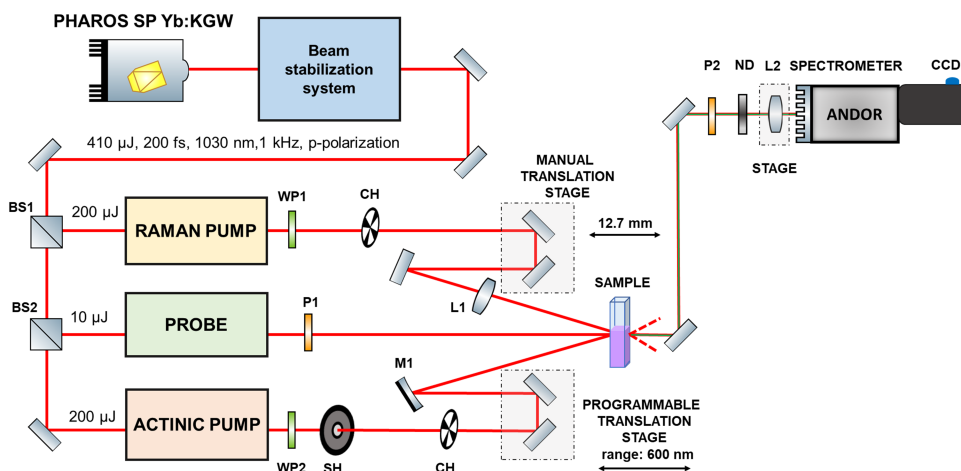


Figure 6.1: Schematic of the home-built FSRS system. Beamsplitters (BS), half-wave plate (WP), lenses (L), concave mirror (M1), polarizers (P), optical chopper (CH), electronic shutter (SH), and neutral-density absorptive filters (ND).

The optical setup is driven by a commercial femtosecond Yb:KGW laser system (Pharos, Light Conversion) which produces 200 fs pulses centered at 1030 nm with a 1 kHz repetition rate. The pulses pass sequentially through a beam direction stabilization system using two quadrant diodes, two kinematic holders with piezo actuators, and an autonomous electronic system for correcting the beam directional drift. After passing through the beam

stabilizer, the beam is directed onto the BS1 beamsplitter, which divides the beam in a ratio of approximately 50:50. Another beamsplitter, BS2, reflects a small part of the beam ($<5\%$), which is used to generate broadband probe pulses. The femtosecond probe (supercontinuum) pulses were generated by focusing a small portion of the fundamental beam onto a sapphire plate. The systems for generating Raman and actinic pulses have pulses with an energy of $200\ \mu\text{J}$. In this thesis, only ground-state measurements were carried out, for which narrowband Raman pump (ps) and broadband probe (fs) pulses were used. Therefore, the description of the system for generating ultrashort actinic pulses is not discussed here.

Broadly tunable, narrow-bandwidth Raman pump pulses ($\sim 10\text{--}20\ \text{cm}^{-1}$) were generated using the method outlined by Nejbauer *et al.* [157] This method involves the direct parametric amplification of a highly chirped white light continuum by a narrow-bandwidth pump pulse, which is produced through sum-frequency mixing of two oppositely chirped femtosecond pulses. In the path of the Raman pulse beam, a half-wave plate (WP1) is placed to allow free rotation of the polarization, as well as an optical chopper (MC2000, Thorlabs) synchronized with the pump laser. The optical chopper physically blocks every other pulse in the sequence, enabling rapid comparison between the Raman signal and the reference signal. Subsequently, the Raman pulse beam is directed onto a pair of mirrors mounted on a translation stage, which allows temporal overlap between the Raman and probe pulses. The Raman pulse beam is then focused onto the sample by lens L1 to a spot size of approximately $50\ \mu\text{m}$. After generating Raman and probe pulses with appropriate parameters and alignment of optical paths, the beams are directed to the location where the sample is placed.

The sample in the form of an aqueous colloid was placed in a flow quartz cuvette (137-1-40, Hellma), in which the movement of the liquid was forced by pressing the tube using six rolling pump cylinders (Ismatec Reglo Digital ISM831C). This method of delivering the sample for measurements always ensured a fresh portion of the colloid at the place where the laser beams crossed. Thanks to this, the risk of photodegradation of the sample was significantly reduced, and the high quality of the measured signal was en-

sured.

During the measurement, the spectrum of the probe pulse is recorded. After passing through the sample, the probe pulse beam is directed onto neutral density filters (ND), which allow adjustment of the signal level reaching the detector. In the next step, lens L2 focuses the probe pulse beam onto the entrance slit of the spectrograph (Andor Shamrock SR 500i), to which a CCD camera (Andor Newton U971N) is connected.

6.2.5 Numerical simulations

FDTD simulations were carried out using the commercial software ANSYS LUMERICAL (2023). The AuBP was modeled as two five-sided frustums of pyramids stacked back-to-back. The silica shell was represented with an ellipsoidal geometry. In both models, dimensions were defined based on the average diameter and length obtained from SEM measurements. During the simulations, the electromagnetic excitation was represented as circularly polarized light using the total-field scattered-field method. A mesh size of 0.5 nm was applied in all directions of the simulated area. The refractive index of the surrounding medium was set to that of water 1.33, while the refractive index of silica was assumed to be 1.4. The dielectric function of Au was determined by fitting experimental data reported by Johnson and Christy. [7]

Here, FDTD simulations were used to calculate cross-sections for absorption, scattering, and extinction for AuBP.

6.2.6 Two-temperature model

The two-temperature model (TTM) [158–162] describes the evolution of the electron (T_e) and lattice (T_l) temperatures with time (t) as a pair of coupled differential equations (Eqs. 6.1 and 6.2) connected by the electron–phonon coupling constant g , which models the rate of energy exchange between

the electrons and phonons:

$$C_e(T_e) \frac{dT_e}{dt} = -g(T_e - T_l) \quad (6.1)$$

$$C_l \frac{dT_l}{dt} = g(T_e - T_l) \quad (6.2)$$

where C_l is the lattice heat capacity, $C_e(T_e) \approx \gamma T_e$ is the temperature-dependent electronic heat capacity, γ is the electron heat capacity constant, and g is the electron-phonon coupling constant. For bulk Au, $\gamma = 66 \text{ J m}^{-3} \text{ K}^{-2}$ and $g = (2.5 \pm 0.5) \times 10^{16} \text{ W m}^{-3} \text{ K}^{-1}$. [160, 163, 164] The temperature dependence of C_e means that the time scale for electron-phonon coupling depends on the initial electronic temperature. Change in the electron temperature (ΔT_e) was calculated (Eq. 6.3) as a function of Raman pump wavelength from the experimental extinction spectra and simulated optical cross sections:

$$\Delta T_e = \sqrt{T_0^2 + \frac{2 \times \%_{\text{abs}} \times E}{N \times V \times \gamma}} - T_0 \quad (6.3)$$

where $\%_{\text{abs}}$ represents the percentage of light absorbed, determined from the extinction spectrum of the measured sample and corrected based on the relative contribution of absorption versus scattering to the total extinction, as determined through simulations, T_0 is the ambient temperature [K], N is the number of AuBPs illuminated by the pump beam, V is the nanoparticle volume [m^3], and E is the average energy of the pump beam [J]. [160, 165, 166]

Disregarding the fact that the Raman pump pulse is a picosecond pulse, we use TTM to describe and interpret the experimental data. Based on TTM, we estimate the initial electron temperature of AuBPs after excitation with the Raman pump pulse. However, we want to emphasize that the presented analysis is only an approximation due to the model's limitations regarding temperature. [167, 168] Specifically, the temperatures we obtained exceed the range for which the model is applicable.

6.3 Results and discussion

6.3.1 Plasmonic nanoantenna characterization

The SERS plasmonic nanoantennas were analyzed using scanning electron microscopy (SEM) and UV-Vis-NIR spectroscopy. Representative SEM images of both sample types are presented in Fig. 6.2.

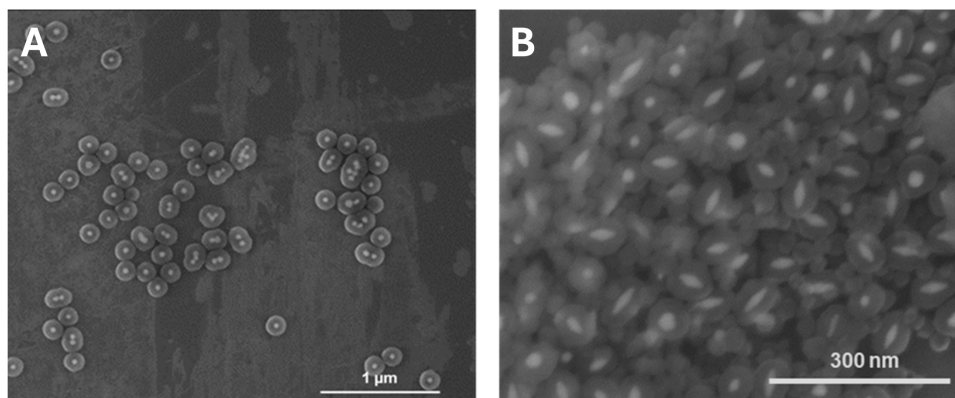


Figure 6.2: SEM images of silica-coated plasmonic nanoantenna's containing (A) spherical oligomer core with adsorbed BPE molecule and (B) single nanobipyramid.

The spherical Au nanoparticles observed in the SEM images had an approximate diameter of 50 nm and were embedded in a (60 ± 11) nm silica shell. Spherical Au monomers form oligomers with sub-nanometer plasmonic cavities between the nanoparticles, called electromagnetic hot-spots, making them an ideal substrate to investigate Raman scattering. The distribution of individual oligomers in the silica-coated BPE-AuNPs samples was determined from SEM images (Fig. 6.2) and was consistent across all prepared colloidal solutions. The distribution of oligomeric forms was approximately 59% monomers, 25% dimers, 8.5% trimers, 3% tetramers, 1.5% pentamers, and 3% larger clusters (Fig. 6.3). Despite the fact that monomers represent more than half of the sample population, no attempt was made to remove them from the sample by ultracentrifugation [40] due to their non-SERS-active character. [169]

The AuBPs measured about (49 ± 6) nm in length and (18 ± 2) nm

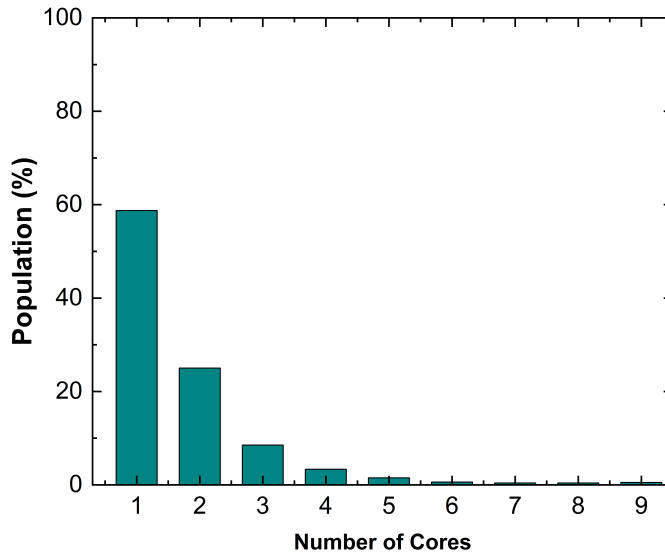


Figure 6.3: Histogram of the percent population as a function of the number of cores made for silica-coated BPE optical nanoantennas.

in width, with a silica shell thickness of around 70 nm. For silica-coated AuBPs, the colloid exhibited high monodispersity, as it contained only a single type of nanoparticle, with an LSPR FWHM of (53 ± 1) nm. Simulated values of absorption, scattering, and extinction cross sections for AuBPs obtained with FDTD simulations were as follows: $\sigma_{\text{abs}} = 5.78 \times 10^{-15} \text{ m}^2$, $\sigma_{\text{scat}} = 4.29 \times 10^{-16} \text{ m}^2$, and $\sigma_{\text{ext}} = 6.21 \times 10^{-15} \text{ m}^2$, respectively.

Table 6.1: Spectral properties of optical nanoantennas derived from UV-Vis-NIR extinction spectroscopy measurements.

Optical nanoantenna	Single AuNP mode	Oligomers mode	OD at single AuNP mode
silica-coated BPE AuNPs	537 nm	640–850 nm	0.80
silica-coated BPE AuNPs (suspended in DMSO)	538 nm	630–870 nm	0.80
Optical nanoantenna	SPR mode	LSPR mode	OD at single LSPR mode
silica-coated AuBPs	526 nm	625–790 nm	0.56

The extinction spectra of all types of optical nanoantennas were recorded in the range of 400 nm to 1100 nm. Figure 6.4 presents the extinction spectra of the obtained optical nanoantenna colloids, while Table 6.1 summa-

rizes the spectral properties of the nanoantennas, including the positions of surface plasmon resonances and the optical densities (OD) relevant to both SERS and SE-FSRS measurements. The resonance peak of single AuNPs corresponds to the plasmon mode of individual core nanoparticles, while the extinction maxima for oligomers are red-shifted to longer wavelengths. This red shift, along with a significantly broader extinction peak in oligomers compared to monomers, is attributed to the collective surface plasmon resonances of the multi-core nanoparticle assemblies.

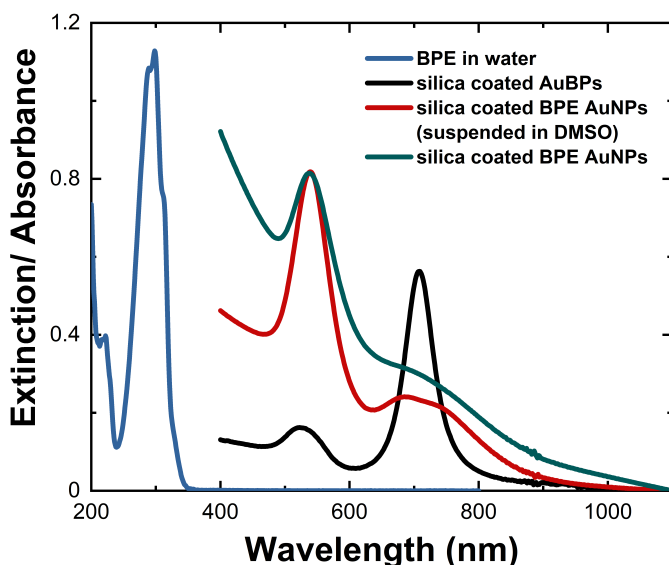


Figure 6.4: UV-Vis absorption spectrum of BPE in water and extinction spectra of home-synthesized optical nanoantenna colloids.

6.3.2 SE-FSRS at 1 kHz repetition rate

The comparison of SE-FSRS and SERS spectra of silica-coated BPE-functionalized Au nanoantennas is displayed in Fig. 6.5.

The SE-FSRS spectrum was obtained using a Raman pump pulse at 665 nm with an energy of 8 nJ per pulse and was averaged over 5 minutes. This represents the first successfully recorded SE-FSRS spectrum in the frequency domain at a 1 kHz repetition rate. Previously, the only research pre-

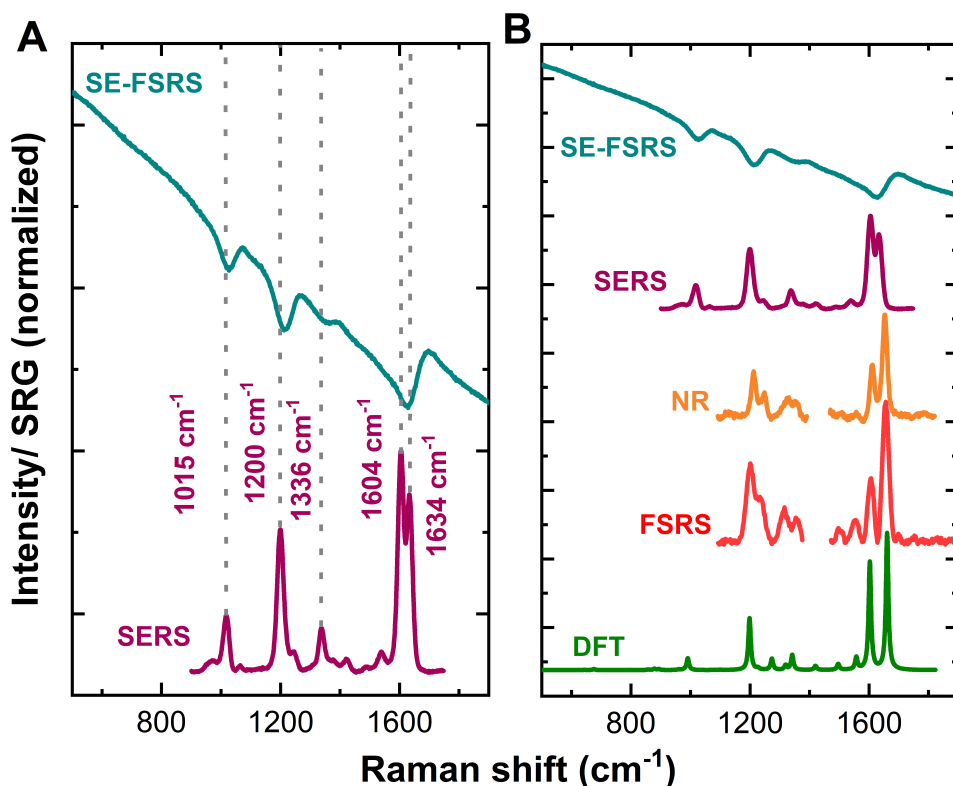


Figure 6.5: (A) Comparison of SERS (671 nm) and SE-FSRS (665 nm) spectra with the most intense BPE bands marked. (B) Comparison of surface-enhanced spectra of BPE with spontaneous Raman (NR) (661 nm), FSRS (665 nm), as well as simulated (DFT) spectrum. Surface-enhanced spectra were measured on silica-coated BPE-nanoantenna. Spontaneous Raman and FSRS spectra were registered on 50 mM BPE in DMSO (Raman peaks of solvent have been removed from spectra). In brackets wavelength of excitation is given. Theoretical spectrum was calculated using (B3LYP/ 6-31G(d,p)).

sented by Kumar *et al.* [144] used a Raman pump excitation at 1 kHz, but the spectrum was recorded in the time domain using a different setup configuration (SE-ISRS). The presented SE-FSRS spectrum and the SERS spectrum of BPE-functionalized nanoantennas show that the Raman peaks are in good agreement. However, in SE-FSRS, the spectral features exhibit a dispersive character, rather than the Lorentzian or Gaussian line shapes observed in the SERS spectrum. Notably, the Raman peaks at 1604 cm^{-1} and 1634 cm^{-1}

do not correspond quantitatively to those in the SERS spectrum, due to their close spacing in frequency, which causes overlapping dispersive features in the SE-FSRS measurement. Moreover, the SE-FSRS spectrum is distorted by the presence of a broad negative background.

Having obtained a frequency-domain SE-FSRS spectrum at 1 kHz with a high signal-to-noise ratio and well-characterized Raman resonances of BPE, we proceeded to perform optimization studies of SE-FSRS. The measurements on silica-coated BPE nanoantennas included investigations of (i) the effect of ps-Raman pump pulse energy, (ii) the time delay between the Raman pump and probe pulses, and (iii) the excitation wavelength of the Raman pump pulse on the amplitude and shape of the spectral lines observed in the SE-FSRS spectra. The final part of the study focused on investigating the broadband background using silica-coated AuBPs as plasmonic nanoantennas, which were characterized by high monodispersity.

6.3.3 Energy of ps-Raman pump pulse

We performed measurements of SE-FSRS spectra as a function of Raman pump pulse energy. Figure 6.6 shows the SE-FSRS spectra collected with constant acquisition time (5 min) but with varying energy in a single Raman pump pulse. The SE-FSRS spectra display a broad background signal, characterized by a gain on the Stokes side and a corresponding negative amplitude on the *anti*-Stokes side. With the increase of the Raman pulse energy, we observe an increase in the background intensity, which in the pulse energy range up to 8 nJ is linear. Above the pulse energy of 8 nJ, we observe saturation of the background signal. The amplitude of the dispersive Raman peaks of BPE increases linearly with the Raman pulse energy, reaching a maximum at 8 nJ, however, the largest SE-FSRS signal was achieved at 14 nJ. Above the Raman pulse energy of 14 nJ, we observe a gradual and linear decrease in the SE-FSRS signal intensity. With the increase of the Raman pulse energy, we also observe a broadening of the dispersive spectral lines.

In previous studies focused on the optimization of SE-FSRS, the depen-

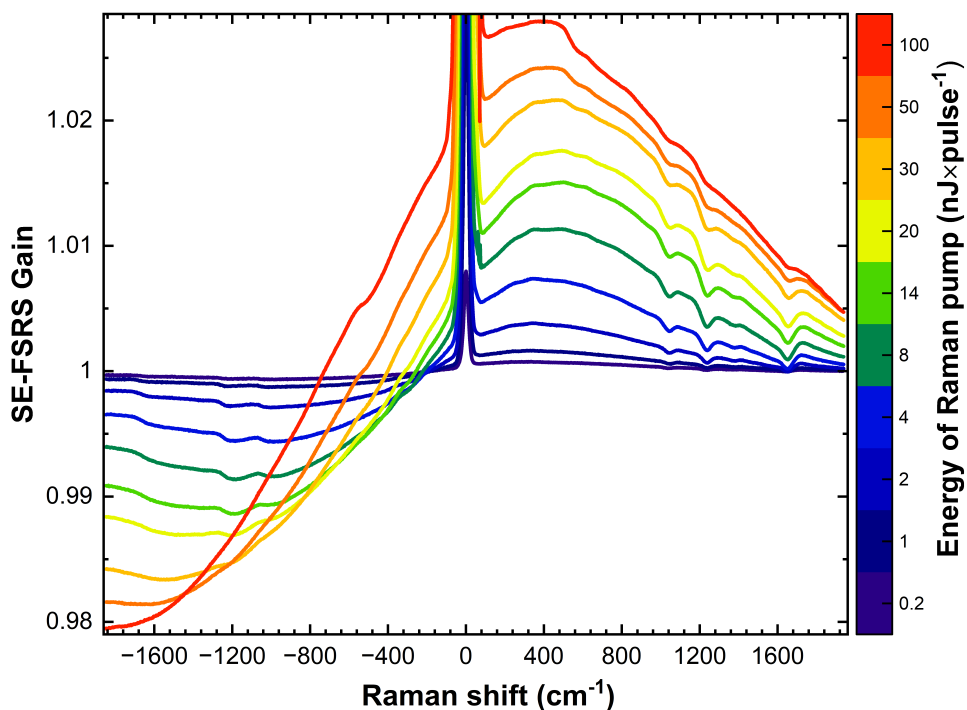


Figure 6.6: SE-FSRS spectra registered with varying the Raman pump pulse energies (from 0.2 nJ to 100 nJ) and constant acquisition time (5 min). The Raman pump pulse excitation wavelength was set to 665 nm to overlap the LSPR of BPE-nanoantenna.

dence of signal amplitude on Raman pulse energy was reported twice. However, the range of Raman pulse energies used in those studies was very limited. Frontiera *et al.* recorded SE-FSRS amplitudes using a maximum Raman pulse energy of 11 nJ [139], while Kumar *et al.* employed a maximum energy of 20 nJ. [144] In both studies, signal saturation with increasing Raman pulse energy was observed. However, due to the low energy range applied, no decrease in signal intensity was reported.

The loss in Raman signal amplitude at Raman pulse energies exceeding 14 nJ may result from photodamage to BPE molecules within the hot-spot. In parallel, photodamage of BPE molecules may be accompanied by photodegradation of hot-spots, or in general, plasmonic nanostructures. It is known that under picosecond or femtosecond irradiation, fragmentation,

melting, or fusion of nanoparticles may occur. [170–173] At elevated pulse energies, the electric field at the beam's focal point reaches approximately 10^8 V/m, however, in the hot spot, this value may be even higher due to localized energy. In extreme cases, the plasmonic enhancement of the electric field can increase the field by approximately three orders of magnitude, potentially causing Raman reporter ionization or dissociation. Consequently, when the Raman pump pulse energy surpasses 14 nJ, the degradation rate becomes so rapid that it cannot be offset by the relatively slow replacement of the sample at the focal point, even under forced colloid flow.

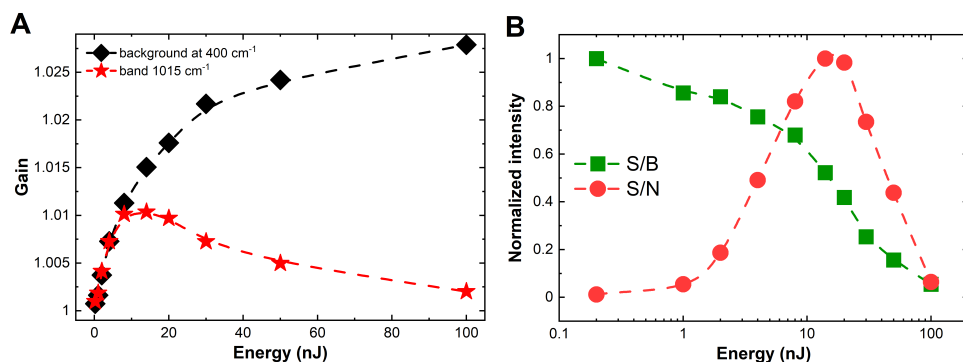


Figure 6.7: (A) Variation of the 1015 cm^{-1} BPE peak amplitude and the broadband background amplitude at a 400 cm^{-1} Stokes shift, measured across different Raman pump pulse energies. (B) Corresponding changes in the signal-to-noise (S/N) ratio of the 1015 cm^{-1} peak and the signal-to-background (S/B) ratio were also evaluated at varying pump pulse energies.

As shown in Fig. 6.7, increasing the Raman pump pulse energy results in a maximum signal-to-noise ratio (S/N) at approximately 14 nJ. However, concurrently, the signal-to-background ratio, defined as the ratio of the Raman band amplitude to the background intensity, declines rapidly (Fig. 6.7B). Furthermore, when the Raman pump pulse energy exceeds 8 nJ, noticeable broadening of the Raman bands and distortions in the background emerge (Fig. 6.8A and Fig. 6.8B). Taking into account the elevated risk of sample photodecomposition at higher pulse energies, the optimal Raman pump energy appears to be around 8 nJ. The reduction in amplitude and broadening of SE-FSRS features observed at higher pump energies were

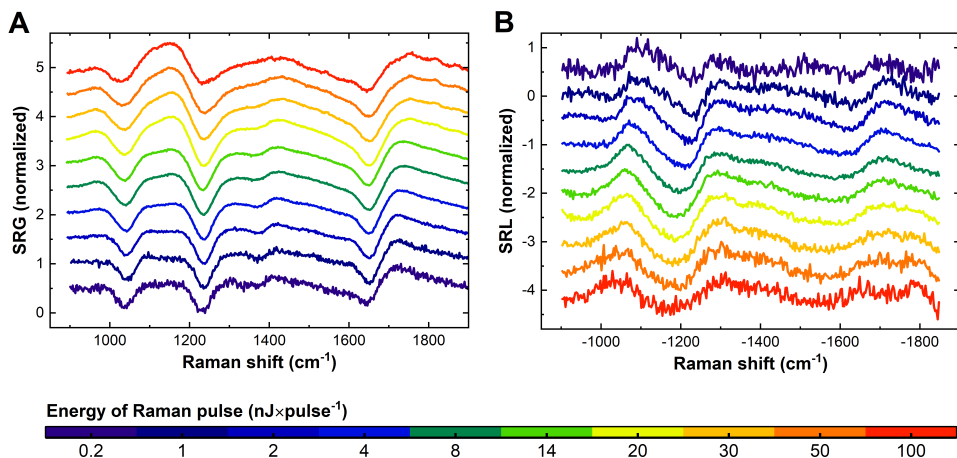


Figure 6.8: The baseline corrected and normalized SE-FSRS spectra as a function of Raman pump pulse energy for (A) Stokes and (B) *anti*-Stokes parts. For better visibility, the spectra are shifted by arbitrary amounts.

largely reversible upon lowering the pulse fluence. The only exception was a slight overall decrease in signal amplitude following prior exposure to Raman pump energies of 100 nJ or more. This residual reduction is attributed to irreversible photodecomposition of the sample, which occurred despite the use of a flow cuvette to maintain continuous circulation of the colloidal solution through the excitation region.

6.3.4 Temporal overlap of Raman pump and probe pulses

To investigate the nature of the vibrational coherence coupled to a plasmonic system profoundly, we explored the effect of pump–probe time-delay on the SE-FSRS spectra. The ultrafast response of the silica-coated BPE nanoantennas was compared with a standard sample - a solution of the BPE dissolved in DMSO with a concentration of 50 mM. The Raman pump beam was tuned to 665 nm to match the spectral region of the plasmon resonance band of nanoantennas, with the pulse energy set to approximately 8 nJ per pulse. The zero time-delay ($\Delta t = 0$ fs) was arbitrarily defined as the moment when the Raman band intensities reached their maximum. By convention, a negative time delay was defined as one in which the probe

precedes the temporal maximum of the Raman pump pulse.

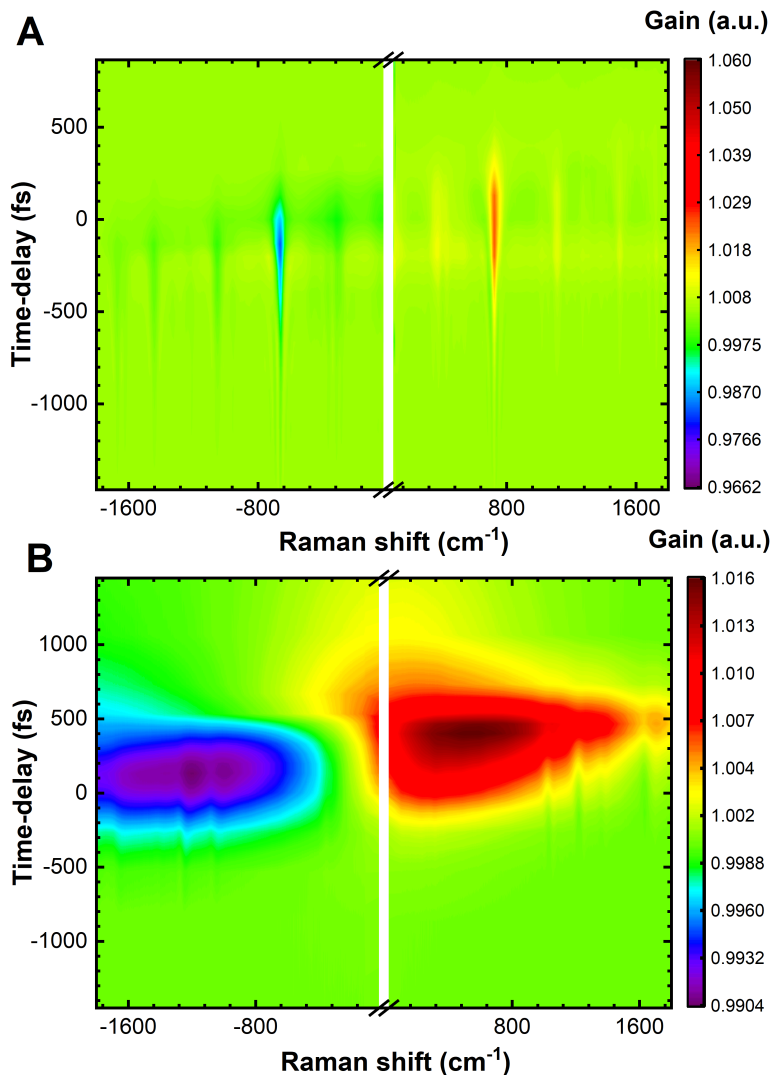


Figure 6.9: Time-delay dependence between the Raman pump and probe pulses, for both FSRs spectra of the (A) BPE dissolved in DMSO as standard and (B) SE-FSRs spectra of silica-coated BPE-nanoantennas sample.

Figure 6.9 presents FSRs data, plotted as a 2D map, examining time-delay dependence between the Raman pump and probe pulses, for both the (A) reference sample and (B) silica-coated BPE nanoantennas. The Raman spectra for both the BPE reference and the BPE-nanoantennas show

characteristic peaks that persist for a similar time duration. Vibrational features of the BPE dissolved in DMSO are characterized by the blue (negative slope) and red (positive slope) vertical features in the spectra, and are most intense when the pump and probe temporal maxima are overlapped, at ($\Delta t = 0$ fs). In the case of the BPE-nanoantenna sample, we may observe a broad transient absorption-like background from the nanoantenna plasmon resonance, where the *anti*-Stokes side of the SE-FSRS spectrum is dominated by a negative broadband background, equal in magnitude and width to the Stokes side features.

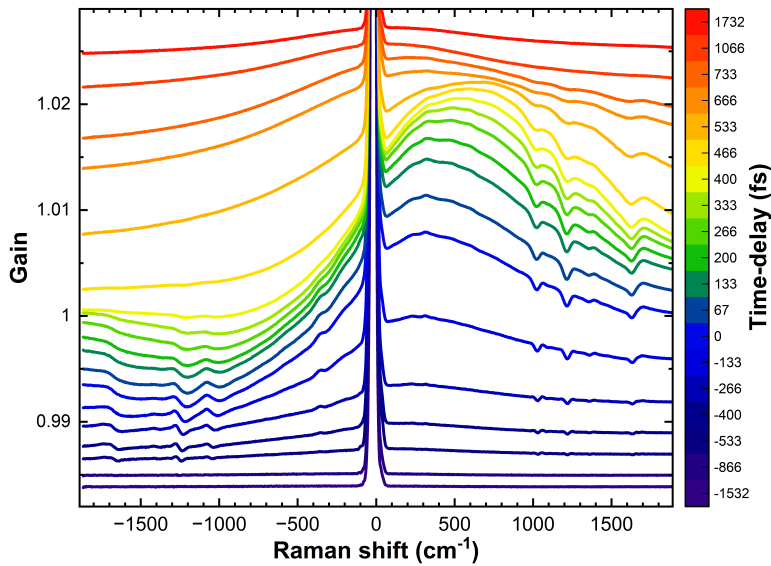


Figure 6.10: The SE-FSRS data examining time-delay dependence between the Raman pump and probe pulses for silica-coated BPE-nanoantennas sample.

The SE-FSRS time-delay spectra for the BPE-nanoantenna sample are presented in Fig. 6.10. Changing the time delay between the Raman pulse and the probe pulse leads to changes in the amplitude and shape of both the broad background and the Raman bands. With the temporal delay between pulses, the peak position of the broadband background appeared to shift at the Stokes part of the spectrum. At first, the peak was close to the laser line, then it gradually shifted toward the red side until the band reached its maximum amplitude, after which it moved back toward the laser line.

We observed notable changes in the line shapes of the nanoantenna-enhanced signals as a function of the pump–probe time delay. Variations in the time delay led to an initial increase and subsequent decrease in the amplitudes of these bands, accompanied by alterations in their shapes (Fig. 6.11). At early delays, the dispersive bands exhibited widths of approximately $25\text{--}30\text{ cm}^{-1}$, comparable to those seen in non-surface-enhanced FSRS spectra of BPE solution, and constrained by the temporal and spectral resolution of the instrumentation. As the time delay increased, further broadening occurred, leading to a reduction in spectral resolution. These modifications in SE-FSRS spectral line shapes qualitatively resembled those reported in resonance-FSRS studies, where the Raman pump pulse overlaps with an electronic transition of the analyte. [174, 175]

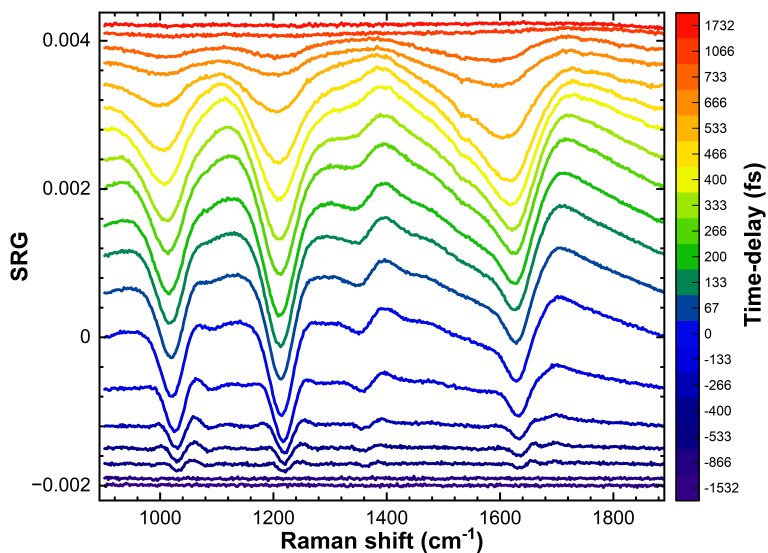


Figure 6.11: Baseline-corrected Stokes-shifted part of SE-FSRS spectra as a function of pump-probe time delay. Spectra are offset for visual clarity.

The observed behavior can be interpreted in terms of vibrational dephasing dynamics, similar to explanations provided in previous FSRS studies proposed by Frontiera *et al.* [140] Specifically, when the femtosecond probe pulse arrives after the picosecond Raman pump pulse has reached its temporal peak, the vibrational coherence is prematurely truncated, result-

ing in broader spectral features. In contrast, when the probe pulse coincides with the leading edge of the pump pulse, the full vibrational coherence is sampled, producing narrower peaks. Maximum peak intensity is observed when the two pulses overlap at their temporal maxima, where vibrational coherence generation is most efficient. These findings differ from those reported by Frontiera *et al.* [140], who observed no significant changes in nanoantenna line shapes with varying pump–probe time delays.

It was observed that the position of the broadband background varied with the pump–probe time delay (Fig. 6.10). The onset and temporal evolution of the background were delayed relative to the appearance of the BPE Raman bands (Fig. 6.12). The background reached its maximum intensity approximately 400 fs after the peak of the 1634 cm^{-1} BPE Raman band. While the temporal width of the Raman bands was roughly consistent with the pump pulse duration, the background exhibited an additional, slower component that extended beyond the pump pulse and did not decay to zero within the same time frame.

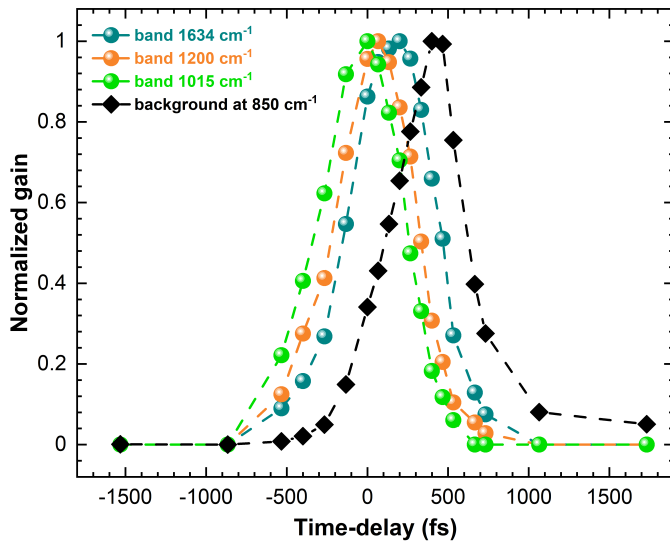


Figure 6.12: Gain dependence of Raman bands at Stokes shifts of 1015, 1200, and 1634 cm^{-1} , along with the background at 850 cm^{-1} , as a function of pump-probe time delay.

Our findings at this moment suggest that the beginning segment of the

pump pulse fluence is sufficient to initiate the process responsible for the observed broadband background. Since the duration of the probe pulse does not exceed 200 fs, the mechanism underlying background generation must occur on an even shorter timescale. In this context, even the early portion of the Raman probe pulse interacts with the sample, both triggering and probing the process that leads to background formation. As the probe pulse spans a broad spectral range, functioning as a supercontinuum (quasi-white light), it can effectively excite a wide array of plasmonic resonances. However, broadband background amplitude reaches a maximum approximately 400 fs after the maximum of the Raman peak, suggesting a significant time scale of the Raman process and formation of the background.

The most plausible mechanism contributing to the background involves plasmon excitation followed by relaxation of the electrons via electron-electron scattering, their thermalization with the crystal lattice, and eventual energy dissipation into the nanoantenna surrounding environment. [162]

6.3.5 Excitation wavelength of Raman pump pulse

We examined the SE-FSRS line shape and amplitude, as well as broadband background, as a dependence of Raman pump pulse excitation wavelength for the BPE-nanoantenna colloid. Due to the fact that the amplitude of the FSRS signal depends on the spatial and temporal overlap of the pump and probe pulses, the time-delay was separately tuned for each excitation wavelength. Minor variations in the overlap of these pulses can influence the intensities of the recorded spectra and lead to greater inaccuracies in estimating the gain across different excitation wavelengths. For that reason, silica-coated BPE-functionalized Au nanoantennas were measured in two different environments, aqueous and DMSO, respectively. In contrast to water, DMSO displays clearly detectable FSRS spectral bands. Since the excitations are electronically non-resonant with DMSO, the amplitudes of its FSRS bands were utilized as an internal reference for intensity calibration. Multiwavelength SE-FSRS spectra compared with the extinction of optical nanoantennas are shown in Fig. 6.13.

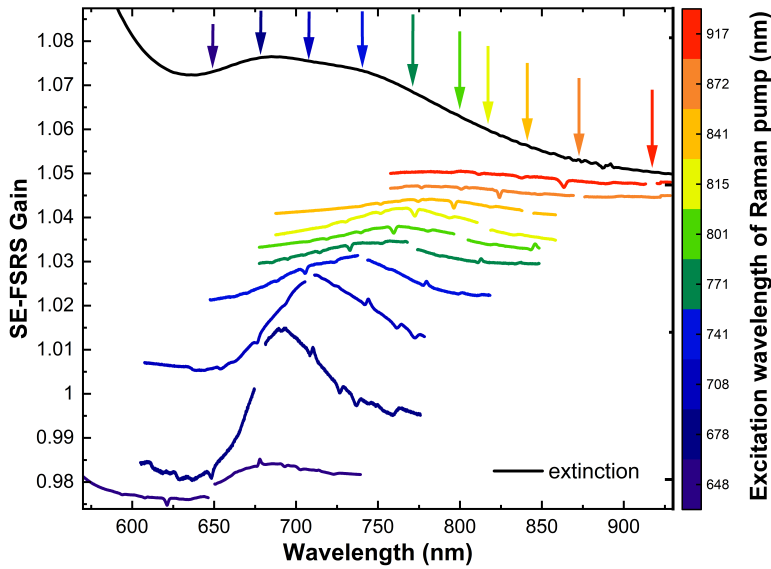


Figure 6.13: Dependence of SE-FSRS spectra on the excitation wavelength of the Raman pump pulse, schematically indicated by an arrow on the plasmon resonance of a silica-coated BPE nanoantenna suspended in DMSO.

We start by comparing Stokes and *anti*-Stokes signals of SE-FSRS spectra measured with a variable excitation wavelength of the Raman pump pulse. When the Raman pump is tuned to 648 nm, it lies between the single-mode and oligomers modes of the LSPR of the BPE-nanoantennas. With this excitation, the SE-FSRS spectrum, including the Stokes and *anti*-Stokes parts, was characterized by the highest S/N ratio (Fig. 6.14). However, the BPE signal from the optical nanoantennas was barely visible. Changing the excitation wavelength of the Raman pump to 678 nm resulted in the appearance of BPE signals on both the Stokes and *anti*-Stokes parts, which reached the maximum at this wavelength. As the excitation wavelength of the Raman pump pulse was shifted toward the infrared, a gradual decrease in the amplitude of the BPE signals was observed. Interestingly, the change in the amplitude of the BPE Raman bands as a function of the Raman pulse excitation revealed that the different Raman bands are characterized by varying decay times. The observed relationship is shown in Fig. 6.15 for the Raman bands at 1200 cm^{-1} and 1634 cm^{-1} , respectively, relative to the extinction

of the silica-coated BPE nanoantennas.

For Raman pump wavelengths of 648, 678, and 708 nm, we observed a higher amplitude of the Raman band at 1634 cm^{-1} . These Raman pump wavelengths spectrally overlapped with the extinction of the silica-coated optical nanoantenna arrays in the region where the colloid exhibited the highest energy. For Raman pump wavelengths above 708 nm, which spectrally corresponded to the range of nanoantennas with lower energy, we observed the opposite trend in terms of Raman band amplitude — the Raman band at 1200 cm^{-1} exhibited a higher amplitude. The dispersive shape of the SE-FSRS spectral lines changed significantly only when the Raman pump excitation wavelength overlapped with the maximum LSPR region of the optical nanoantenna arrays. Below the LSPR maximum, no significant changes in the shape of the BPE bands were observed upon changing the excitation wavelength within this spectral range. Wavelength-scanned SE-FSRS along the steep slope of the extinction band of BPE-nanoantenna indicates that the excitation wavelength has a strong effect on the enhancement factor. However, it affects only the amplitude of the dispersive bands, not their shape, which is in contradiction to findings reported by Zong *et al.* [150]

Next, we examine the broadband background pumped at several different Raman pump wavelengths. As shown in Fig. 6.13, the intensity and spectral profile of the broad background are dependent on the selected excitation wavelength of the Raman pump pulse. The spectral position of the maximum shifted toward the infrared as the Raman pump wavelength was tuned in that direction. Nevertheless, the rate of this shift did not show a consistent correlation with the wavelength change. For excitation wavelengths 648 and 678 nm, the maximum appeared on the longer-wavelength part of the laser line, whereas for excitations above 741 nm, it was located on the opposite part. At the Raman pump of 708 nm, the maximum of background overlaps with the laser line. The amplitude of the gain at the peak of the positive part of the broadband showed a partial correlation with the spectral position of the Raman pump excitation relative to the extinction spectrum, as illustrated in Fig. 6.15A. This correlation, however, was weak,

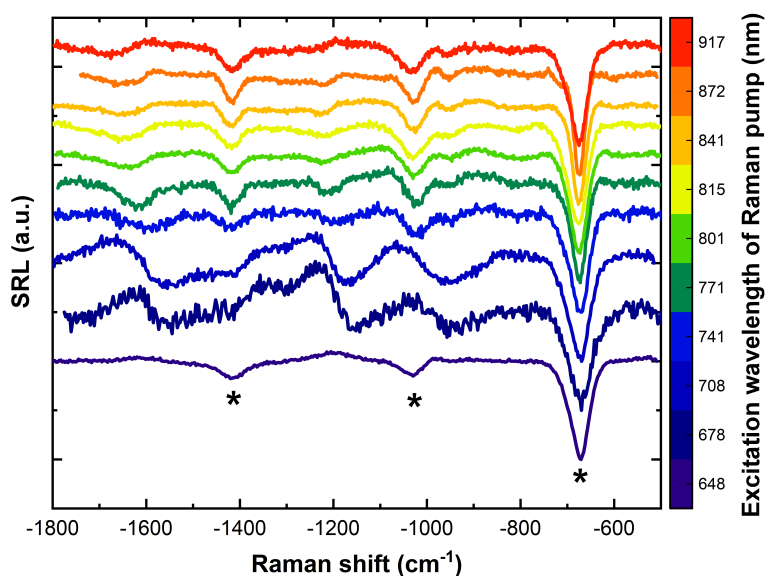


Figure 6.14: Background-corrected and normalized *anti-Stokes* part of SE-FSRS spectra from Fig. 6.13 and adjusted relative to the intensity of the internal standard 670 cm^{-1} band of solvent DMSO. Stars indicate the positions of the DMSO bands.

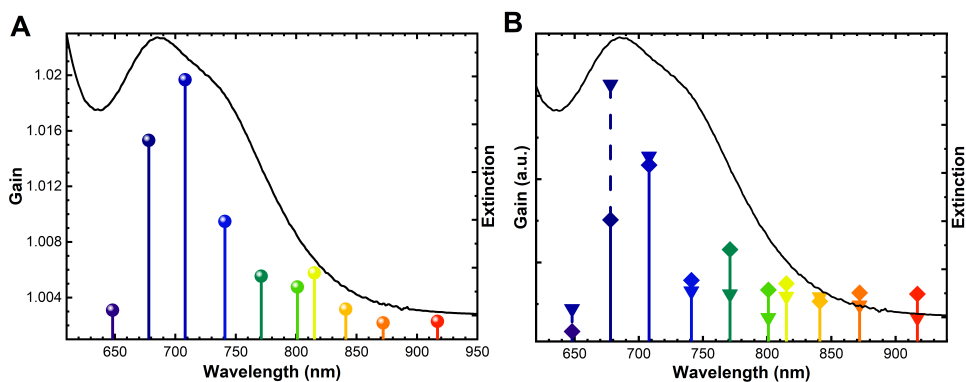


Figure 6.15: Comparison of the extinction spectrum of the silica-coated BPE nanoantenna (black line) and (A) the broadband background at its maximum, as well as (B) the amplitudes of the SE-FSRS bands of BPE at 1200 cm^{-1} (diamonds) and 1634 cm^{-1} (triangles), as a function of the excitation wavelength of the Raman pump pulse.

with the most intense backgrounds observed for the Raman pumps near the maximum peak of the extinction spectrum.

The analysis presented, supported by in-depth experimental studies of the dependence of amplitude and line shape — both of the Raman bands and of the broad background — on the excitation wavelength of the Raman pump pulse, provides a preliminary explanation of the observed relationships between the variables. It appears that the origin of the variability in SE-FSRS spectra as a function of Raman pulse excitation may lie in the intrinsic nature of the sample itself. Silica-coated assemblies of Au oligomers functionalized with BPE were found to comprise systems of varying degrees of structural complexity, ranging from monomers, through dimers and trimers, up to higher multimers, as illustrated previously in Fig. 6.3. The extinction spectrum of such a colloid represents the averaged extinction of the individual fractions present in the mixture, each of which, when considered as an isolated system, is characterized by a variable extinction maximum and a narrower extinction band compared to the averaged one. [41, 43]

During macroscopic SE-FSRS spectral measurements, the entire population of oligomers located within the region of pulses overlap is excited simultaneously. However, the most efficiently excited systems are those whose plasmonic resonance is tuned to the Raman pump wavelength. By adjusting the Raman pump wavelength, one can selectively excite specific oligomeric fractions within the whole population, which accounts for the variability of the broad background observed in SE-FSRS spectra. On the other hand, compared to monomeric systems, oligomeric assemblies exhibit significantly higher SERS enhancement factors, due to the strong concentration of the electromagnetic field in the nanocavity, which plays a decisive role in SE-FSRS measurements. [169]

The above considerations explain the significantly higher background signal amplitudes observed when the excitation is tuned to wavelengths of 678 nm and 708 nm, indicating that systems whose extinction maximum is close to the laser wavelength contribute most strongly to the generation of the background signal. Furthermore, the compositional complexity of the nanoantenna sample manifested itself in the shift of the maximum position of the broadband background peak in the SE-FSRS spectra.

6.3.6 Source of the broadband background

To gain deeper insight into the interaction between plasmon resonance and the SRS process, we performed an LSPR-dependent experiment using a silica-coated plasmonic nanoantenna consisting of a single AuBP as the core (Fig. 6.2B). This approach makes it possible to eliminate artifacts arising from ensemble measurements. A single AuBP serves as a high-quality periodic substrate with a narrow LSPR distribution.

We start by comparing the time-resolved SE-FSRS spectra of silica-coated AuBPs excited with three distant Raman pump wavelengths to cover the entire longitudinal plasmon mode range. Figure 6.16 illustrates the temporal evolution of a monodisperse sample of silica-coated AuBPs excited with Raman pump at 641, 707, and 764 nm. The time-resolved SE-FSRS spectra in all cases are dominated by bleaching of the broadband signal, whose spectrum strongly aligns with the extinction spectrum of the AuBPs nanoantenna presented in Fig. 6.17B. Regardless of the chosen Raman pump wavelength, the peak position remains unchanged, while the half-widths are narrower compared to the extinction spectrum of the nanoantennas. Similarly to the peak position, the band shape does not change with the chosen wavelength, but its amplitude does, reaching a maximum when the excitation wavelength coincides with the extinction maximum.

Time-resolved SE-FSRS spectra of silica-coated AuBPs are similar to ultrafast transient absorption (TA) spectra in the context of spectral shape. TA spectroscopy is a pump-probe technique where both pulses, pump and probe, are femtosecond pulses (typically 30-150 fs). The observed spectral signature of silica-coated AuBPs on SE-FSRS spectra (Fig. 6.16(A-C)) has a positive amplitude. However, a broad band with negative amplitude also appears on the right side of the positive band. The amplitude of this negative band also depends on Raman pump excitation, being the highest when Raman pump excitation aligns with the maximum extinction of the nanoantenna. Comparable regions of negative gain were detected in the SE-FSRS spectra of silica-coated BPE-nanoantenna samples, where the spectral location of this negative gain band partially overlapped with the extinction band

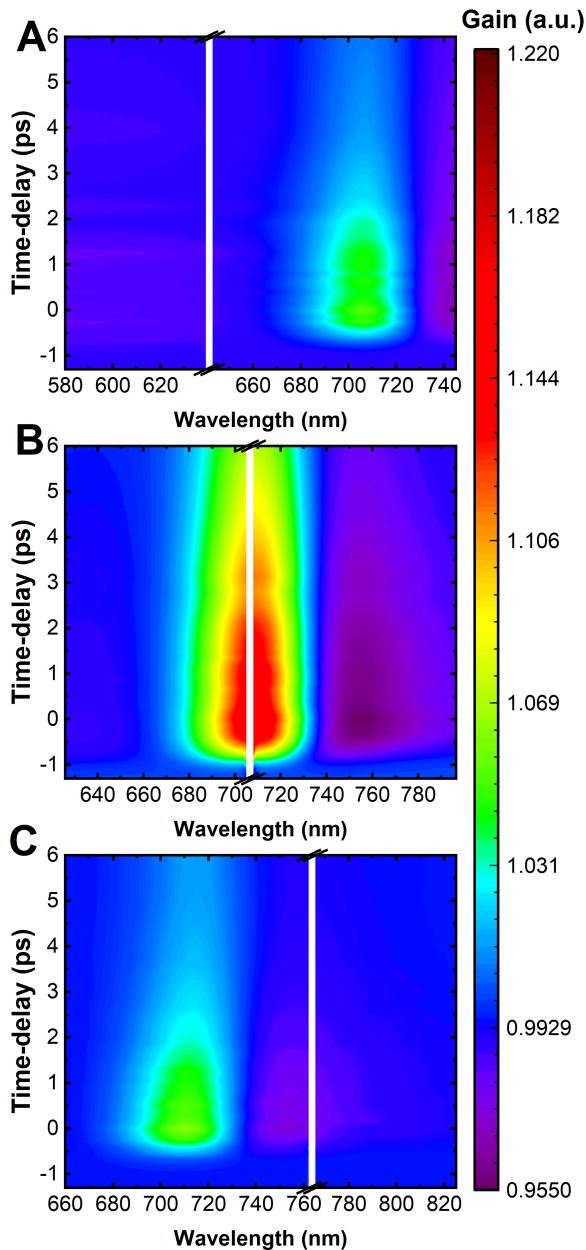


Figure 6.16: Time-resolved SE-FSRS spectra of silica-coated AuBP nanoantennas with Raman pump excitation at wavelengths of (A) 641 nm, (B) 707 nm, and (C) 764 nm. The spectra were recorded with a single Raman pump pulse energy of 5 nJ with an acquisition time of 10 s.

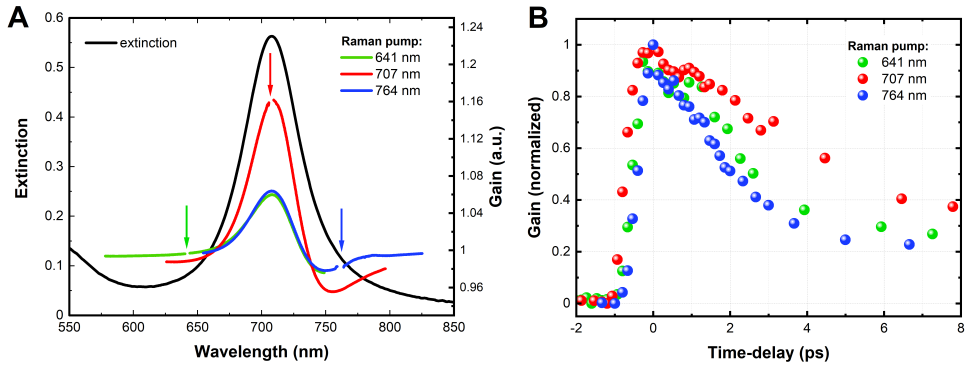


Figure 6.17: (A) Extinction spectrum of silica-coated AuBP nanoantennas overlapped with SE-FSRS spectra at 641 nm, 707 nm, and 764 nm, extracted from Fig. 6.16 at zero time delay. Arrows indicate the spectral positions of the Raman pump excitation wavelengths. (B) Temporal evolution of the maximum intensity of the amplified band as a function of excitation wavelength.

and shifted together with the positive gain band during the wavelength-scanned experiment. In contrast, for monodisperse silica-coated AuBPs, the spectral positions of both the negative and positive gain features remained unchanged under different excitation conditions. This effect, associated with spectral regions of both negative and positive gain, is most likely driven by changes in the sample's extinction induced by Raman pump pulse excitation.

Considering the analogy between FSRS and TA, we can conclude that the broad background observed in the SE-FSRS spectra has a fundamental origin arising from TA changes of Au nanoparticles upon their excitation by the Raman pump pulse. Based on studies of the TA spectra of Au nanoparticles, it can be inferred that the broadband background observed in the SE-FSRS spectra arises from plasmonic excitation, with the absorbed energy subsequently dissipated as heat. The precise time-scales of the individual stages of energy redistribution following plasmonic excitation may vary depending on the nanoparticles' size, shape, and material composition. [162, 176, 177]

Metal nanoparticles can exhibit very large absorption and scattering cross sections since many electrons contribute to the LSPR. [178, 179] Ab-

sorption of photons by a metal nanoparticle creates a non-equilibrium distribution of electrons and holes above and below the Fermi level with energies up to the incident photon energy. In chronological order, immediately after pump excitation, plasmon resonance begins to dephase on a very fast time scale (~ 10 fs time scale). The change in electron population induced by the pump pulse, called athermal distribution, converts by electron–electron scattering on a timescale of ~ 100 fs into a thermalized distribution governed by a Fermi–Dirac statistics that may be assigned an effective electron temperature. The effective electron temperature depends on pump laser energy, therefore values of effective electron temperature can reach up to several thousands of Kelvin. Next, on a 1–5 ps time scale, the metal nanoparticle starts to equilibrate electron and lattice temperatures via electron–phonon coupling. Electron–phonon coupling reduces the effective electron temperature due to the mismatch between the heat capacities of electrons and the lattice. Cascade of photophysical processes ends with energy dissipation in the surrounding environment on cooling the temperature on much longer time scale (10–100 ps). [162, 180–182]

Each stage of the energy dispersion process can influence the extinction properties of the nanoparticles, as observed in the temporal evolution of their spectra. However, due to the temporal resolution of the FSRS setup — particularly the Raman pump pulse (1 ps), which must remain in the picosecond range to preserve Raman resolution — the broadband signal we detect mainly corresponds to the final stage of the energy dissipation process. At this stage, heating of the nanoparticles and their immediate surroundings leads to noticeable modifications in the TA spectra.

Time-resolved SE-FSRS experiments with variable Raman pump excitations show a broadband signal decay up to approximately 6 ps (Fig. 6.16). For Raman pump excitations that do not coincide with the maximum extinction of silica-coated AuBPs, this timescale was sufficient to capture the main decay. However, when the Raman pump excitation overlaps with the maximum extinction of the nanoantenna colloid, a significant signal amplitude is still observed after 6 ps. For this reason, we recorded time-resolved SE-FSRS spectra with extended decay times, up to several tens of picoseconds,

using two distinct Raman pump excitations: 641 nm and 707 nm (Fig. 6.18). Similar to the temporal evolution observed on shorter timescales (Fig. 6.16), the long-scan measurements reveal a much slower decay of the broadband signal when the Raman pump excites the plasmonic substrate at its LSPR maximum.

To describe the behaviour of a plasmonic nanoantenna in the early phase of the dynamic process of dissipation energy, we employed the two-temperature model (TTM), which proved highly effective in interpreting the results of absorbance measurements obtained via TA. For the data presented in Fig. 6.16, the maximum electron temperatures calculated for different Raman pump pulse excitations were as follows: 4492.4 K at 641 nm, 7733.2 K at 707 nm, and 4195.1 K at 764 nm. Based on these electron temperature values, we determined the corresponding electron heat capacities, since the electronic heat capacity depends on the temperature of the electron distribution. The calculated values were $3.0 \times 10^5 \text{ J}\cdot\text{K}^{-1}\text{m}^{-3}$ at 641 nm, $5.2 \times 10^5 \text{ J}\cdot\text{K}^{-1}\text{m}^{-3}$ at 707 nm, and $2.8 \times 10^5 \text{ J}\cdot\text{K}^{-1}\text{m}^{-3}$ at 764 nm. It should be emphasized that these values correspond to the initial electron temperature immediately after pump laser excitation. Over time, however, the colloid approaches equilibrium with its surroundings, resulting in energy transfer to the metal lattice and a subsequent increase in its temperature. [183]

The obtained values of electronic temperatures are in good agreement with time-resolved SE-FSRS spectra of silica-coated AuBPs presented in Fig. 6.16. The highest value of electronic temperature aligns with the longest decay of SE-FSRS amplitude signal, which corresponds to the Raman pump excitation at the spectral maximum of extinction of silica-coated AuBPs. In contrast, values of electronic temperatures obtained for Raman pump excitation wavelengths for the side spectral positions of colloid extinction are very similar. In the context of dynamic dissipation of energy, it means a much longer time for the plasmonic system to reach the surrounding temperature after excitation at the highest energy of the plasmonic antenna.

The decay profiles presented in Fig. 6.17B reveal an exponential decay of the background signal amplitude, corresponding to a temperature decrease caused by energy transfer to the metal lattice. Simulations indi-

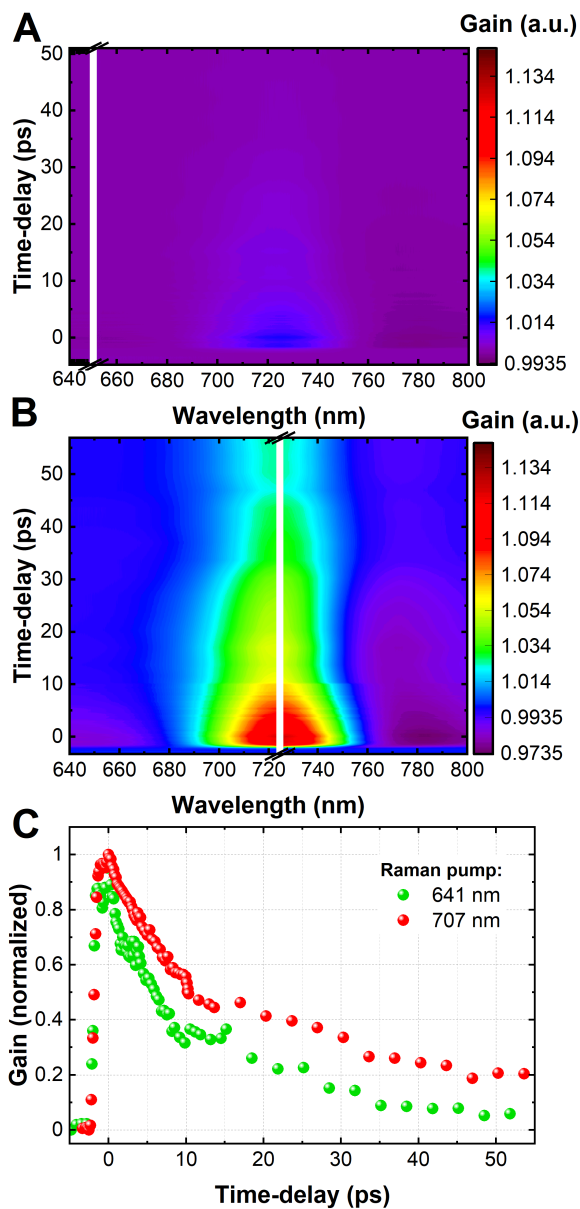


Figure 6.18: Time-resolved SE-FSRS spectra of silica-coated AuBP nanoantennas with Raman pump excitation at wavelengths of (A) 650 nm and (B) 725 nm, exposed to long illumination. The spectra were recorded with a single Raman pump pulse energy of 5 nJ with an acquisition time of 10 s. (C) Temporal evolution of the maximum intensity of the amplified band as a function of excitation wavelength.

cate that the lattice temperature is approximately 6–7 times lower than the initial electron temperature. [165] When comparing the specific electronic heat capacity with that of the lattice (for bulk Au, $2.4 \times 10^6 \text{ J} \cdot \text{K}^{-1} \text{ m}^{-3}$) [165], we observe comparable values, suggesting a potential expansion of the nanoparticle without leading to its destruction. Frontiera *et al.* reported a decrease in the extinction of optical nanoantennas in the near-infrared plasmon resonance region after several weeks of exposure [139]. However, they did not observe abnormally fused or melted nanoparticles in TEM images, but rather a lighter contrast ring surrounding the silica shells. This observation is consistent with our predictions and is supported by the TTM-based approximation.

Finally, we compare time-resolved SE-FSRS spectra between silica-coated BPE Au oligomers and monodisperse silica-coated AuBPs. Both types of plasmonic nanoantenna display significant differences in the context of the signal's decay dynamics. This variation appeared to be linked to the considerably smaller size of the spherical nanoparticles. Although some aggregation occurred within the colloid, the sample mainly consisted of very small monomers and dimers (Fig.6.2). Consequently, the decay proceeded much faster, with the peak intensity dropping on a timescale comparable to the pump pulse duration. Only a weak residual tail of the decay signal extended beyond 1 ps. In contrast, for AuBPs, the initial decay stage lasted several picoseconds, and the overall relaxation extended for at least several tens of picoseconds. These observations are consistent with the reports of Hu *et al.* [184] and Chiang *et al.* [166], who highlighted strong dependencies of TA decay on the size and shape of Au nanoparticles, with markedly shorter decay times observed for smaller particles.

6.4 Conclusions

In this chapter, we demonstrate for the first time surface-enhanced femtosecond stimulated Raman spectroscopy (SE-FSRS) spectra over a broad spectral range at a repetition rate of 1 kHz. SE-FSRS spectra, along with op-

timization studies of the key parameters influencing background intensity and the spectral profiles of Raman bands, were acquired using silica-coated spherical Au nanoparticle oligomers.

In the first step, we investigated the effect of the Raman pulse energy on the shape and intensity of both the background signal and the Raman bands. We observed that, with increasing single-pulse Raman energy, the amplitude of the SE-FSRS signal initially increases, reaches a maximum, and then decreases sharply. In contrast to the SE-FSRS signal amplitude, the background intensity increased monotonically with increasing single-pulse energy. In the next step, we examined the influence of the temporal delay between the Raman pump pulse and the probe pulse, which revealed variations in both the broadband background and the SE-FSRS spectral features. For the first time, we observed an asymmetric broadband background profile on which the SE-FSRS signal is superimposed.

In the final stage, we studied the effect of the Raman excitation wavelength. We found that the amplitudes of both the background and the SE-FSRS signal are strongly correlated with the extinction spectra of the optical nanoantenna systems. To elucidate the origin of the background signal, we employed optical nanoantennas with a high degree of monodispersity for the first time in SE-FSRS experiments. Measurements performed on single Au nanobipyramids at different Raman excitation wavelengths showed that, regardless of the excitation wavelength, the spectral position of the background remains unchanged and its shape closely follows the extinction spectrum of the nanobipyramids. The temporal evolution of the background amplitude as a function of pump-probe delay further indicates that the background originates from plasmonic excitation and the subsequent thermal energy redistribution within the plasmonic nanoparticles.

Conclusions and Future Outlook

7.1 Conclusions

In this doctoral dissertation, the overall objective was to improve understanding and control of physical and chemical processes involving molecules in plasmonic nanocavities. A plasmonic nanocavity is the central component of an optical nanoantenna, which enhances molecular signals and improves detection. Using light, we investigated the interactions of molecules with the environment in plasmonic nanocavities by recording surface-enhanced Raman scattering (SERS) and surface-enhanced femtosecond stimulated Raman scattering spectra (SE-FSRS).

In the first part of the dissertation, we focused on the issue of the temporal evolution of surface-enhanced Raman scattering spectra of single molecules (SM-SERS). In particular, we devoted attention to the problem of fluctuations in the SM-SERS signal, as well as attempts to control it through appropriate modifications of the molecular environment. Based on the performed SERS and SM-SERS spectral measurements, as well as on comparisons between the experimental spectra and spectra obtained from quantum-chemical calculations (DFT), we formulated the following main conclusions:

1. We demonstrated that cryogenic temperature leads to an extension

of the lifetimes of SM-SERS spectra.

2. We decoupled amplitude fluctuations from spectral fluctuations of Raman bands by analyzing correlations using Spearman correlation coefficients, demonstrating that amplitude fluctuations are always characterized by higher Spearman correlation coefficients than those associated with fluctuations in band positions. We established that both amplitude fluctuations and spectral fluctuations in Raman band positions depend on temperature and on the acquisition time of a single SERS spectrum. As the temporal resolution of single SM-SERS spectrum measurements increased, we observed the same trend for both amplitude and Raman band position fluctuations — namely, a shift in the distribution of Spearman correlation coefficients toward negative correlations.
3. We proposed that, in the case of Pc, the mechanism responsible for amplitude fluctuations is the selective enhancement of vibrational modes by atomically confined near-field cavities. In contrast, spectral fluctuations may arise from the formation of so-called picocavities — spontaneously forming local interactions between Pc and individual Au adatoms.
4. We demonstrated that encapsulation of the molecule in CB[7] reduces SM-SERS amplitude fluctuations while simultaneously increasing the probability of single-molecule detection. We proposed a mechanism in which enhanced detectability results from a change in molecular orientation induced by encapsulation in CB[7], leading to a favorable orientation of the Raman tensor with respect to the electromagnetic field vector in the plasmonic nanocavity. This favorable molecular arrangement results in higher Raman process efficiency, which, in practice, manifests as faster SERS signal decay profiles for molecules confined in CB[7] than for molecules freely oriented on the metal surface.
5. We established that, in the case of Th, the distributions of Spearman

correlation coefficients are generally similar to those observed for Pc; that is, amplitude fluctuations are characterized by higher Spearman correlation coefficients than fluctuations in Raman band positions. This suggests that distinct mechanisms responsible for SM-SERS spectral fluctuations may be a characteristic feature of SM-SERS spectra and are independent of the type of molecule.

In the second part of the doctoral dissertation, we focused on the development of a methodology based on surface-enhanced femtosecond stimulated Raman scattering as a new tool in the field of vibrational spectroscopy, with great potential for studying single-molecule dynamics in real time. Based on the SE-FSRS spectral measurements, we conducted optimization studies that enabled us to formulate the observations and main conclusions presented below.

1. We demonstrated that excessively high energy in a single Raman pump pulse degrades the SE-FSRS signal, leading to a decrease in SE-FSRS amplitude.
2. We observed, across the entire spectral range, a globally asymmetric background profile in the SE-FSRS spectra, as well as a lack of correlation between the SE-FSRS signal amplitude and the background amplitude.
3. We showed that the background observed in SE-FSRS spectra reflects the spectrum of plasmonic resonances (the extinction spectrum) of optical nanoantenna systems. Moreover, using nanoparticle systems with high monodispersity, we demonstrated that the spectral position of the background remains constant and is independent of the excitation wavelength. We propose that the background originates from a photothermal effect associated with local temperature changes in the nanoparticles.

7.2 Future Outlook

The results presented in this dissertation have allowed us to answer some important questions about measurements of single-molecule SERS spectra. Although the findings do not exhaust the topic, they provide more precise directions for future research. Below, we present selected issues that may constitute a continuation of the studies described in this doctoral dissertation.

1. **Lock-in Detection:** In the SM-SERS spectral detection technique used in this doctoral dissertation, employing an EM-CCD camera, the temporal resolution of full spectral acquisition was typically on the order of milliseconds. Although modern EM-CCD and CCD cameras can achieve much higher frame rates by reducing the pixel region, shortening the acquisition time proportionally decreases the number of detected photons. This, in turn, lowers the S/N. As a result, the real temporal resolution of spectral measurements is often limited by photon statistics rather than by the detector readout speed alone. An interesting approach to partially overcome this limitation is phase-sensitive signal detection using a lock-in amplifier. This technique enables the extraction of weak modulated signals from noise and can improve the effective S/N, provided that the detected photon flux remains above the detector sensitivity threshold. Coupling such phase-sensitive detection with a Raman microscope may therefore enable the observation of SM-SERS signal fluctuations on microsecond time scales. To our knowledge, this method has not been used for single-molecule SERS detection.
2. **Plasmonic nanocavities with 2D materials:** Plasmonic enhancement is a phenomenon that strongly depends on the separation distance, as illustrated in Fig. 2.8. Therefore, there is a need to explore new spacer materials that would enable the fabrication of ultra-small plasmonic nanocavities. We propose NPoM cavities incorporating a spacer consisting of a monolayer of graphene or hexagonal boron nitride (h-BN).

The thickness of such a monolayer is only 0.33–0.34 nm, making it more than 2.5 times thinner than CB[7]. In addition, these cavities are expected to exhibit greater stability than metallic spacers, which are susceptible to photothermal damage and oxidation. Despite reports in the literature demonstrating SERS coupling with 2D materials and promising preliminary results [185, 186], reliable single-molecule detection remains a significant challenge.

3. **Coupling SE-FSRS with a microscope:** microscopic studies of the SE-FSRS phenomenon constitute a relatively small part of the development of this technique. Although the single-molecule limit has been achieved [146], the acquisition of a complete frequency-domain spectrum within a single measurement remains a significant experimental challenge. Experimental studies should be complemented by theoretical investigations, particularly those related to spectral modeling, because it is currently known that the SE-FSRS signal — at least for molecules excited off molecular resonance — is distorted by the plasmonic resonance. The NPoM nanocavities used in this dissertation may provide a good plasmonic substrate due to their high reproducibility and ease of fabrication. Coupling SE-FSRS with a microscope would enable the creation of a unique tool capable of recording “molecular movies” of molecules near metal surfaces, similar to the research reported by Apkarian and Halas groups. [187, 188]

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Curriculum Vitae

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List of Publications

- **Patryk Pyrcz** and Sylwester Gawinkowski. Supramolecular Stabilization of Single-Molecule SERS: Cucurbit[7]uril Encapsulation of Thionine. *ACS Physical Chemistry Au*, 6(1): 57-68, 2026.
- **Patryk Pyrcz** and Sylwester Gawinkowski. Advancements in surface-enhanced femtosecond stimulated Raman spectroscopy: exploring factors influencing detectability and shapes of spectra. *Nanophotonics*, 14(1): 1-12, 2025.
- Karolina Gronkiewicz, Lars Richter, Fabian Knechtel, **Patryk Pyrcz**, Paul Leidinger, Sebastian Günther, Evelyn Ploetz, Philip Tinnefeld, and Izabela Kamińska. Expanding the range of graphene energy transfer with multilayer graphene. *Nanoscale* 16(28): 13464-13470, 2024.
- Agata Kołodziejczyk, Aleksandra Wróblewska, Mariusz Pietrzak, **Patryk Pyrcz**, Kacper Błaziak, and Rafał Szmigielski. Dissociation constants of relevant secondary organic aerosol components in the atmosphere. *Chemosphere* 351: 141166, 2024.
- Agata Kołodziejczyk, **Patryk Pyrcz**, Kacper Błaziak, Aneta Pobudkowska, Kumar Sarang, and Rafał Szmigielski. Physicochemical properties of terebic acid, MBTCA, diaterpenylic acid acetate, and pinane-diol as relevant α -pinene oxidation products. *ACS Omega* 5(14): 7919-7927, 2020.

- Agata Kołodziejczyk, **Patryk Pyrcz**, Aneta Pobudkowska, Kacper Błaziak, and Rafał Szmigielski. Physicochemical properties of pinic, pinonic, norpinic, and norpinonic acids as relevant α -pinene oxidation products. *The Journal of Physical Chemistry B* 123(39): 8261-8267, 2019.

List of Conferences Contributions

- Micro symposium IChF PAN 2026. Oral presentation: *Advancements in surface-enhanced femtosecond stimulated Raman spectroscopy: exploring factors influencing detectability and shapes of spectra.*
- Molecules and Light, Poznań, Poland, 2025. Oral presentation: *Impact of spatial confinement on probing single-molecule surface-enhanced Raman scattering in plasmonic nanocavity.*
- Micro symposium IChF PAN 2025. Poster: *Influence of local nanoenvironment on single-molecule SERS and SERS spectra and their temporal evolution.*
- 28th International Conference on Raman Spectroscopy (ICORS), Rome, Italy, 2025. Poster: *Temporal evolution of single-molecule SERS spectra in plasmonic nanocavity.*
- Doctoral Seminar organized by Leibniz Institute of Photonic Technology (IPHT), Dornburg, Germany, 2024. Poster + short oral presentation: *Surface-enhanced femtosecond stimulated Raman spectroscopy.*
- Micro symposium IChF PAN 2024. Poster: *Plasmon resonances responsible for the dispersive line shape of surface-enhanced femtosecond stimulated Raman signal.* (Award for the best poster)

- 12th International Conference on Advanced Vibrational Spectroscopy (ICAVS), Kraków, Poland, 2023. Poster + short oral presentation: *Plasmon resonances responsible for the dispersive line shape of surface-enhanced femtosecond stimulated Raman signal.*
- 27th International Conference on Raman Spectroscopy (ICORS), Long Beach, USA, 2022. Poster: *Surface-enhanced femtosecond stimulated Raman spectroscopy: effects of the energy of plasmon resonances on dispersive line shapes in spectra.*
- Micro symposium IChF PAN 2022. Poster: *Towards ultrafast plasmonics: Surface-enhanced femtosecond stimulated Raman spectroscopy.*
- 11th International Conference on Advanced Vibrational Spectroscopy (ICAVS), Kraków, Poland, 2021. Poster flash presentation: *Wavelength-scanned surface-enhanced Raman excitation spectroscopy – from macroscopic measurements to microscopic research of single gold oligomers.*

Research Projects

- National Science Center Grant No. 2021/43/D/ST5/01929: *Structurally tuned and nanostructured polymers conducting in both the positive and negative potential range as electrode materials for supercapacitors and molecular interconnections* Dr. Eng. Kamila Łepicka, 07.2025–09.2026.
- National Science Center Grant No. 2020/39/B/ST4/01523: *Fast molecular dynamics in plasmonic nanocavities* Dr. Sylwester Gawinkowski, 01.2025–06.2025.
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- National Science Center Grant No. 2017/27/B/ST4/02822: *Development of ultrafast stimulated Raman scattering spectroscopy enhanced by optical nanoantennas* Dr. Sylwester Gawinkowski, 02.2019–09.2020.

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