

Fluorescence Responses of Single Organic Dye Molecules as (Bio-)Reporters in Complex Nano- Environments

Dissertation

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Abstract

Fluorescent organic molecules have found tremendous interest in modern photonics, sensing, and life sciences due to their sensitivity to the local chemical and photonic nano-environment. However, understanding the underlying photochemical and photophysical mechanisms remains challenging, as conventional ensemble spectroscopic methods often obscure critical details arising from inherent structural heterogeneity and dynamic processes. Single-molecule spectroscopy overcomes these limitations by directly probing the individual molecular behaviors in complex nano-environments.

In this thesis, a spectroscopic-computational framework is established, centered on a fundamental study of single-molecule tautomerization in the prototypical organic dye: hypericin. Tautomerization, driven by proton migration via quantum tunneling, leads to interconversion between distinct structural isomers (tautomers). By combining polarization-resolved single-molecule confocal microscopy with quantum-chemical calculations, this methodology allows the spatial determination of transition dipole moments (TDMs) and the unambiguous identification of individual tautomeric species. Furthermore, the tautomerization rates can be tailored both chemically, via different polymer hosts and isotope effects, and photonically, using a $\lambda/2$ Fabry-Pérot microcavity.

Beyond hypericin, this combined spectroscopic-computational methodology is successfully extended to two further applications: (i) investigating complex excited-state photokinetics in a stimulus-responsive donor-acceptor-donor (DADn) system, and (ii) elucidating surface-enhanced Raman scattering (SERS) in rhodamine 6G (R6G) confined within nanoparticle-on-mirror (NPoM) geometries. These case studies highlight the versatility and robustness of this approach, offering valuable insights into the design of functional molecular systems and paving the way for broader applications in nano-photonics, including but not limited to SERS and related optical platforms.

Zusammenfassung

Fluoreszierende organische Moleküle besitzen, aufgrund ihrer einzigartigen Sensitivität gegenüber lokalen chemischen und photonischen Umgebungsbedingungen, eine herausragende Bedeutung für moderne photonische Technologien, Sensoranwendungen und lebenswissenschaftliche Fragestellungen. Die detaillierte Aufklärung der zugrundeliegenden photophysikalischen und photochemischen Prozesse stellt jedoch nach wie vor eine zentrale wissenschaftliche Herausforderung dar, da konventionelle Ensemble-spektroskopische Methoden durch strukturelle Heterogenitäten und komplexe dynamische Prozesse bedingte Schlüsselphänomene häufig nicht auflösen können. Die Einzelmolekül-Spektroskopie überwindet diese Einschränkung, indem sie das Verhalten einzelner Moleküle in komplexen Nano-Umgebungen direkt zugänglich macht.

Im Rahmen dieser Doktorarbeit wird ein innovativer, methodenintegrativer Ansatz präsentiert, der spektroskopische Untersuchungen mit quantenchemischen Berechnungen kombiniert. Den konzeptionellen Kern bildet dabei eine fundamentale Studie zur Tautomerisierung des prototypischen organischen Farbstoffs Hypericin auf Einzelmolekülebene. Dieser durch protonengekoppelten Elektronentransfer via Quantentunneln getriebene Prozess führt zur reversiblen Umwandlung zwischen verschiedenen strukturellen Isomeren (Tautomeren). Durch die Entwicklung einer polarisationsaufgelösten konfokalen Einzelmolekül-Fluoreszenzmikroskopie in Kombination mit hochgenauen quantenchemischen Rechnungen gelingt erstmals sowohl die präzise räumliche Bestimmung der Übergangsdipolmomente (TDM) als auch die eindeutige spektroskopische Identifikation individueller Tautomere. Besonders bemerkenswert ist die demonstrierte Möglichkeit, die Tautomerisierungsraten sowohl durch chemische Modifikationen (Variation der Polymermatrix und Isotopeneffekte) als auch durch photonische Kontrolle mittels $\lambda/2$ -Fabry-Pérot-Mikroresonatoren gezielt zu steuern.

Das hier entwickelte Verfahren wurde anschließend erfolgreich auf zwei weitere Fragestellungen angewandt: (i) die Untersuchung komplexer angeregter

Photokinetiken in einem stimuli-responsiven Donor-Akzeptor-Donor-(DADn)-System und (ii) die Analyse der oberflächenverstärkten Raman-Streuung (SERS) von Rhodamin 6G (R6G) in einer Nanopartikel-auf-Spiegel-(NPoM)-Anordnung. Diese Fallstudien unterstreichen die Vielseitigkeit und Robustheit des entwickelten Ansatzes, der nicht nur wertvolle Einblicke in das Design funktionaler molekularer Systeme bietet, sondern auch ein großes Potenzial für zukünftige Anwendungen in der Nano-Photonik, insbesondere im Bereich SERS und verwandter optischer Plattformen, aufzeigt.

Abbreviations

A	Acceptor
Abs	Absorption
Ac	Acceptor, (2Z,2'Z)-3,3'-(2,5-bis(hexyloxy)-1,4-phenylene)bis(2-(3,5-bis(trifluoromethyl)phenyl)acrylonitrile)
AIMD	Ab Initio Molecular Dynamics
APD	Avalanche Photodiode
APDM	Azimuthally Polarized Doughnut Mode
BeT	Back Electron Transfer
BLA, Δ_{BL}	Bond Length Alternation, Change in Bond Length Alternation
CHCl ₃	Chloroform
cm	Centimeter
CS	Charge-Separated (CS)
CT	Charge Transfer
CW	Continuous Wave
D	Donor
d	Distance
DAD	Donor-Acceptor-Donor
DADn	Donor-Acceptor-Donor triads with different spacer lengths
DCM	Dichloromethane (solvent)
DCM	4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran (laser dye)
DFT	Density Functional Theory
DOS	Density of States
ET	Energy Transfer
eT	Electron Transfer
EtOH	Ethanol
eV	Electron Volt
<i>f</i>	Oscillator Strength
FGR	Fermi's Golden Rule
FL	Fluorescence
fs	Femtosecond
FWHM	Full Width at Half Maximum
GHz	Gigahertz
GS/S ₀	Ground State
HF	Hartree-Fock
HOMO	Highest Occupied Molecular Orbital
Hz	Hertz
IC	Internal Conversion
IRF	Instrument Response Function
ISC	Intersystem Crossing
LCAO	Linear Combination of Atomic Orbital
LDOS	Local Density of States
LT	Low Temperature
LUMO	Lowest Unoccupied Molecular Orbital
LW	Long Wavelength
m	Meter
MD	Molecular Dynamics

MeOH	Methanol
MHz	Megahertz
mm	Millimeter
MO	Molecular Orbital
ms	Millisecond
n	Refractive Index
n	Non-Bonding Lone-Pair Orbital
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NPoM	Nanoparticle-on-Mirror
ns	Nanosecond
OLED	Organic Light-Emitting Diode
PAH	Polycyclic Aromatic Hydrocarbon
PCM	Polarizable Continuum Model
PDT	Photodynamic Therapy
PES	Potential Energy Surface
PH	Phosphorescence
PMMA	Poly(methyl methacrylate)
PR	Photoreaction
ps	Picosecond
PVA	Polyvinyl Alcohol
Q	Quenching
QM	Quantum Mechanics
QM/MM	Quantum Mechanics/Molecular Mechanics
R6G	Rhodamine 6G
RISC	Reverse Intersystem Crossing
RPDM	Radially Polarized Doughnut Mode
RT	Room Temperature
s	Second
SCF	Self-Consistent Field
SERS	Surface-Enhanced Raman Scattering
S_n/T_n (S_1/T_1)	(First) Singlet/Triplet Excited State
SOC	Spin-Orbit Coupling
SW	Short Wavelength
TCSPC	Time-Correlated Single Photon Counting
TD	Time-Dependent
TD-DFT	Time-Dependent Density Functional Theory
TDM	Transition Dipole Moment
TERS	Tip-Enhanced Raman Spectroscopy
THz	Terahertz
TS	Transition State
TTL	Transistor-Transistor Logic
TTTR	Time-Tagged Time-Resolved
UV	Ultraviolet
VC	Vibronic Coupling
VIS	Visible
VR	Vibrational Relaxation
XC	Exchange-Correlation
XRD	X-Ray Diffraction
γ_G	Gaussian Width
μm	Micrometer
μs	Microsecond
τ_F , τ_{coh} , τ_{deph}	Fluorescence Lifetime, Coherence Lifetime, Dephasing Time

List of Publications

a) Accepted Publications

1. Monitoring Tautomerization of Single Hypericin Molecules in a Tunable Optical $\lambda/2$ Microcavity
Liangxuan Wang, Quan Liu, Frank Wackenhut, Marc Brecht, Pierre-Michel Adam, Johannes Gierschner, Alfred J. Meixner, *J. Chem. Phys.* **2022**, 156, 014203.
<https://doi.org/10.1063/5.0078117>
2. Deuterium Isotope Effect in Single Molecule Photophysics and Photochemistry of Hypericin
Liangxuan Wang, Quan Liu, Andrea Buchwald, Frank Wackenhut, Marc Brecht, Johannes Gierschner, Alfred J. Meixner, *ChemPhysChem* **2024**, 25, e202400374.
<https://doi.org/10.1002/cphc.202400374>
3. Donor-Acceptor-Donor Triads with Flexible Spacers: Deciphering Complex Photophysics for Targeted Materials Design
Siyang Feng, **Liangxuan Wang**, Begoña Milián-Medina, Alfred J. Meixner, Min Sang Kwon, Soo Young Park, Reinhold Wannemacher, Johannes Gierschner, *Adv. Mater.* **2023**, 35, e2306678. <https://doi.org/10.1002/adma.202306678>
4. Higher-Order Cavity Modes for SERS in a Gold Nanosphere-on-Mirror Configuration
Felix Schneider, Lukas Lang, **Liangxuan Wang**, Johannes Gierschner, Alfred J. Meixner, Monika Fleischer, Dai Zhang, *J. Phys. Chem. C* **2024**, 44, 18807.
<https://doi.org/10.1021/acs.jpcc.4c03962>
5. Angularly Resolved Tip-Enhanced Raman Spectroscopy
Felix Schneider,^{#,1} Tim Parker,[#] **Liangxuan Wang**, Michel Rebmann, Yang Zhao, Eric Juriatti, Heiko Peisert, Alfred J. Meixner, Johannes Gierschner, Lingyan Meng, Dai Zhang, *Angew. Chem. Int. Ed.* **2025**, e202506152.
<https://doi.org/10.1002/anie.202506152>

b) Manuscript submitted and ready for submission

1. Competitive Sorption of Glyphosate and Phosphorus Ligands at Al₂O₃ Surface: Experimental Insights and DFT Analysis
Mingshuai Wang, **Liangxuan Wang**, Philipp R. Martin, Daniel Buchner, Johannes Lützenkirchen, Johannes Gierschner, Alfred J. Meixner, Stefan B. Haderlein, to be submitted.

¹ [#] These authors contributed equally to this work.

c) Further publications to which I have contributed during the research period: 2021-2025

1. Synthesis and Photophysics of Color-Pure Blue Emitters Featuring Oxaboraphenanthrene as a Novel Structural Motif
Sunwu Song, Siyang Feng, **Liangxuan Wang**, Begoña Milián-Medina, Reinhold Wannemacher, Johannes Gierschner, Min Sang Kwon, *Tetrahedron*, **2025**, 175, 134522. <https://doi.org/10.1016/j.tet.2025.134522>
2. Paper and Pencil Design of Color-Pure Organic Emitters: The Curious Case of Xanthene Dyes
Morteza Eskandari,[#] **Liangxuan Wang**,[#] Begoña Milián-Medina, Johannes Gierschner, *J. Phys. Chem. A*, **2025**, 129, 1599.
<https://pubs.acs.org/doi/10.1021/acs.jpca.4c07313>
3. Fluorescence Modulation through the Inverted Energy Gap Law in Triply N–B←N-Containing Windmill-Shaped Triazines
Yi Feng, **Liangxuan Wang**, Hongcheng Gao, Jiadong Zhou, Matthias Stolte, Honglin Qiu, Linlin Liu, Victor Adebayo, Martial Boggio-Pasqua, Frank Würthner, Johannes Gierschner, Zengqi Xie, *Angew. Chem. Int. Ed.* **2025**, 64, e202416425.
<https://doi.org/10.1002/anie.202416425>
4. Rational Design of Color-Pure Blue Organic Emitters by Poly-Heteroaromatic Omni-Delocalization
Sunwu Song,[#] Siyang Feng,[#] **Liangxuan Wang**,[#] Jinwon Jun, Begoña Milián-Medina, Reinhold Wannemacher, Jaesang Lee, Min Sang Kwon, Johannes Gierschner, *Adv. Mater.* **2024**, 36, e2404388.
<https://doi.org/10.1002/adma.202404388>
5. Anomalous Deep-Red Luminescence of Perylene Black Analogues with Strong π - π Interactions
Ningning Tang, Jiadong Zhou, **Liangxuan Wang**, Matthias Stolte, Guojing Xie, Xinbo Wen, Linlin Liu, Frank Würthner, Johannes Gierschner, Zengqi Xie, *Nat. Commun.* **2023**, 14, 1922. <https://doi.org/10.1038/s41467-023-37171-y>
6. Theoretical and Experimental Evidence of Two-Step Tautomerization in Hypericin
Quan Liu,[#] **Liangxuan Wang**,[#] Juan Carlos Roldao, Pierre-Michel Adam, Marc Brecht, Johannes Gierschner, Frank Wackenhut, Alfred J. Meixner, *Adv. Photonics Res.* **2021**, 2, 2000170. <https://doi.org/10.1002/adpr.202000170>
7. 1,6-Diazapyrene: A Well-Defined Small-Size Prototype System for Nitrogen-Containing PAHs
Indranil Bhattacharjee,[#] **Liangxuan Wang**,[#] Nerea Gonzalez-Sanchez,[#] Begoña Milián-Medina, Reinhold Wannemacher, Rafael Ballesteros, Rafael Ballesteros-Garrido, Johannes Gierschner, *J. Phys. Chem. A* **2025**, 129, 4471.
<https://doi.org/10.1021/acs.jpca.5c01474>
8. Highly Emissive Single Benzene Fluorophores across Solid and Solution Phases: Elucidating the Molecular Origin of Bright Emission
Jung-Moo Heo, Jihyun Park, Maria F. Flórez-Angarita, **Liangxuan Wang**, Changhoon Yu, Jinho Choi, Hochul Woo, Begoña Milián-Medina, Adam Matzger, Min Sang Kwon, Johannes Gierschner and Jinsang Kim, *Nat. Commun.* **2025**, 16, 5560. <https://doi.org/10.1038/s41467-025-60316-0>

1. Personal Contribution

1.1 Publication – 1 –

Monitoring Tautomerization of Single Hypericin Molecules in a Tunable Optical $\lambda/2$ Microcavity

Liangxuan Wang, Quan Liu, Frank Wackenhut, Marc Brecht, Pierre-Michel Adam, Johannes Gierschner, Alfred J. Meixner, *J. Chem. Phys.* **2022**, *156*, 014203. <https://doi.org/10.1063/5.0078117>

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Contribution to this joint publication:

This is the first publication of this thesis. The work was conceptualized by Dr. Quan Liu and Dr. Frank Wackenhut. The specific experimental design and implementation, including the setup of measurement instruments and the execution of simulations, were performed by **Liangxuan Wang** and Dr. Quan Liu. The data processing and interpretation were performed by **Liangxuan Wang** and Dr. Quan Liu, with the self-written MATLAB codes developed by Dr. Quan Liu and Dr. Frank Wackenhut. **Liangxuan Wang** and Dr. Quan Liu wrote the original draft. Prof. Dr. Alfred J. Meixner and PD Dr. Johannes Gierschner supervised the work in the groups, and provided guidance on data interpretation, paper writing and revisions. All the authors discussed the results and commented on the paper.

1.2 Publication – 2 –

Deuterium Isotope Effect in Single Molecule Photophysics and Photochemistry of Hypericin

Liangxuan Wang, Quan Liu, Andrea Buchwald, Frank Wackenhut, Marc Brecht, Johannes Gierschner, Alfred J. Meixner, *ChemPhysChem* **2024**, *25*, e202400374. <https://doi.org/10.1002/cphc.202400374>

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Contribution to this joint publication:

This is the second publication of this thesis. The fundamental scientific concept underlying this publication was initially proposed by **Liangxuan Wang**, Dr. Quan Liu and Dr. Frank Wackenhut. **Liangxuan Wang** performed the experiments, conducted the quantum-chemical calculations and analyzed the experimental and calculated results. **Liangxuan Wang** and Dr. Quan Liu set up the measurement instruments. The self-written MATLAB codes for data processing were developed by Dr. Quan Liu and Dr. Frank Wackenhut. **Liangxuan Wang** wrote the original draft. Prof. Dr. Alfred J. Meixner and PD Dr. Johannes Gierschner supervised the work in the groups, paper writing and revisions. All the authors discussed the results and commented on the paper.

1.3 Publication – 3 –

Donor-Acceptor-Donor Triads with Flexible Spacers: Deciphering Complex Photophysics for Targeted Materials Design

Siyang Feng, Liangxuan Wang, Begoña Milián-Medina, Alfred J. Meixner, Min Sang Kwon, Soo Young Park, Reinhold Wannemacher, Johannes Gierschner, *Adv. Mater.* **2023**, 35, e2306678. <https://doi.org/10.1002/adma.202306678>

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Contribution to this joint publication:

This is the third publication of this thesis. The (TD-)DFT calculations were performed by Dr. Siyang Feng and **Liangxuan Wang**. **Liangxuan Wang** offered guidance to Dr. Siyang Feng on the quantum-chemical calculations, conducted molecular dynamics simulations, contributed to writing the manuscript, supervised by PD Dr. Johannes Gierschner and Prof. Dr. Alfred J. Meixner. Dr. Siyang Feng did the spectroscopic experiments and evaluation, supervised by Dr. Reinhold Wannemacher and PD Dr. Johannes Gierschner. Prof. Dr. Min Sang Kwon and Prof. Dr. Soo Young Park provided the compounds. The work was conceptualized by Prof. Dr. Min Sang Kwon (molecular design), Prof. Dr. Reinhold Wannemacher (spectroscopic analysis), Prof. Dr. Begoña Milián-Medina (quantum chemistry treatment) and PD Dr. Johannes

Gierschner (photophysics analysis); The paper was primarily written by PD Dr. Johannes Gierschner and revised by all authors.

1.4 Publication – 4 –

Higher-Order Cavity Modes for SERS in a Gold Nanosphere-on-Mirror Configuration

Felix Schneider, Lukas Lang, Liangxuan Wang, Johannes Gierschner, Alfred J. Meixner, Monika Fleischer, Dai Zhang, *J. Phys. Chem. C* **2024**, 44, 18807.

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Contribution to this joint publication:

This is the fourth publication of this thesis. Felix Schneider fabricated the rhodamine 6G (R6G) samples, performed the confocal Raman, surface-enhanced Raman scattering (SERS) and reference measurements, processed all experimental results and wrote the manuscript under the supervision of Prof. Dr. Dai Zhang. Lukas Lang and Felix Schneider performed the dark-field scattering measurements. Lukas Lang fabricated the gold cone reference sample, performed the boundary element method (BEM) simulations, and contributed to the manuscript. Lukas Lang and Prof. Dr. Monika Fleischer contributed significantly to discussions regarding the plasmonic configuration. **Liangxuan Wang** performed the (time-dependent) density functional theory ((TD-)DFT) calculations for the R6G system and contributed to the manuscript, supervised by Prof. Dr. Alfred J. Meixner and PD Dr. Johannes Gierschner. Prof. Dr. Alfred J. Meixner and PD Dr. Johannes Gierschner obtained funding, participated in writing the manuscript and contributed significantly to the scientific discussion. Prof. Dr. Dai Zhang conceived the project, obtained funding, participated in writing the manuscript, coordinated the scientific discussion and provided overall direction. All authors discussed the results and commented on the manuscript.

2. Objectives and Expected Outcomes

2.1 Motivation

The development of single-molecule microscopy and spectroscopy has been driven by the desire to observe and characterize the individual nanoscale behavior of single molecules in complex environments. Conventional ensemble measurements, which average over large populations of (presumed identical) molecules, have long limited the ability to resolve molecular heterogeneity. In contrast, a single molecule can serve as a local reporter of its “nano-environment”; and this allows the construction of the actual distribution of experimental parameters. Such distributions reveal deeper mechanistic insights into the underlying nano-environmental effects, especially when the system under study is sensitive to the local environment. This advantage is particularly critical for fluorescent organic dyes, which respond susceptibly to nano-environmental effects, such as the change in temperature, polarity^[5] or pH,^[6,7] as well as the presence of ions,^[8] aggregation,^[9] and other nano-environmental factors^[6,10]. Environmental effects directly influence the nature of the emitting state, and hence its fluorescence decay characteristics.

In this context, single-molecule spectroscopy becomes a powerful tool. It enables the optical excitation of the specific molecule of interest, and the observation of an individual molecule embedded in condensed matter.^[11] Single-molecule spectroscopy not only reveals static heterogeneity, which stems from distinct probe-environment interactions, but also uncovers dynamic heterogeneity arising from conformational and structural interconversions within individual molecules. The ability to detect such dynamics at the single-molecule level enables the direct observation of short-lived intermediates and rare kinetic events that are otherwise hidden in ensemble experiments.^[11]

However, a comprehensive investigation requires systems with pronounced nano-environmental sensitivity and well-defined structural motifs. Hypericin, a naturally occurring photoactive pigment with a complex landscape of conformational and structural (tautomeric) isomerization, represents an ideal model system. Its excited-state dynamics is inherently sensitive to proton migration and structural rearrangements, which can be further modulated by chemical or photonic nano-environments. This sensitivity makes hypericin an excellent prototype for systematically investigating how nano-environmental factors regulate single-molecule structural dynamics.

To address this challenge, this thesis develops and validates a spectroscopic-computational framework designed to directly access the structural dynamics of individual molecules, using hypericin as a model system. By integrating state-of-the-art single-molecule confocal microscope and spectroscopy with environment-embedded quantum-chemical modeling, we aim not only to resolve the distinct structural variations and their photokinetics but also to control such dynamics through rational nano-environmental engineering. The developed methodology is envisioned to be broadly applicable to a wider class of functional molecular systems beyond hypericin, including stimuli-responsive molecules and other nanoscale spectroscopic platforms, such as surface-enhanced Raman scattering (SERS).

2.2 Scope of This Thesis

This thesis is centered on the development, validation, and applications of a spectroscopic-computational approach to gain a comprehensive understanding of nano-environmental effects on the optical responses of environment-sensitive probes. This combined framework bridges high-resolution confocal microscope and spectroscopy with state-of-the-art quantum-chemical modeling to achieve single-molecule insights.

Chapter 3 starts with an overview of the principles of photophysics. Moreover, it covers fundamental concepts of single-molecule confocal microscope and spectroscopy, including antibunching, time-correlated single photon counting (TCSPC), higher-order laser modes, and microcavities. It also outlines the theoretical foundations of

computational chemistry, covering representative methods such as Hartree-Fock (HF), density functional theory (DFT), and molecular dynamics (MD).

Chapter 4 constitutes the core of this thesis, showcasing practical applications for probing single molecules (systems) under a wide range of conditions, from cryogenic to ambient temperatures, and from free-space environment to optical cavities.

Section 4.1 establishes a spectroscopic-computational approach using hypericin as a model system, focusing on the quantitative characterization of its stereochemistry, photophysics and photochemistry at the single-molecule level. This section demonstrates the direct observation of tautomerization, which involves proton migration along the molecular perimeter. By systematically tailoring the local chemical and photonic nano-environment, the tautomerization rate is effectively modulated. Computational studies complement the experimental findings by providing mechanistic insights into the associated photophysical and photochemical processes, including geometries and energies for different isomers, potential energy surfaces, transition states and the tautomerization pathways.

Section 4.2 applies this spectroscopic-computational methodology to a stimulus-responsive system of donor-acceptor-donor (DADn) triads with flexible σ spacer lengths, addressing its complex excited-state photokinetics. These molecules exhibit two distinct conformations, i.e. extended and stacked forms, whose interconversion responds dynamically to their local environment. Molecular dynamics (MD) simulations have been used to elucidate the competition between the radical ion pair and the exciplex formation. Furthermore, quantum-chemical calculations provide further insight into the geometries, electronic properties and optical responses. This quantitative insight is essential for the rational design of novel exciplex-based materials applicable to sensing, OLEDs, and lasing.

Section 4.3 further illustrates the versatility of this spectroscopic-computational framework by extending its application to surface-enhanced Raman scattering (SERS) systems. Specifically, a computational case study on rhodamine 6G (R6G) molecules confined in a nanoparticle-on-mirror (NPoM) geometry is presented. Raman spectroscopy is a powerful analytical technique that provides a molecular vibrational “fingerprint”. Its sensitivity is greatly enhanced through plasmonic nano-structures.

Objectives and Expected Outcomes

This study employs a cluster model to elucidate the origins of the Raman response, and polarization-dependent enhancements observed at individual NPoM “hot spots”. Theoretical modeling provides essential insights into local field effects, vibrational mode assignment, and SERS selectivity. This work not only validates the generalizability of this methodology but also showcases its potential for exploring structure-property relationships in a broader class of nano-photonic systems.

3. Introduction of Methods

3.1 Principles of Molecular Photophysics

Photoluminescence refers to any emission of light that occurs from electronically excited states.^[12] Depending on the nature of the involved excited state, photoluminescence is formally divided into two categories: *fluorescence* and *phosphorescence*. For molecules with a singlet ground state (GS or S_0), fluorescence refers to the radiative transitions from an excited singlet state (S_n , spin-allowed), whereas phosphorescence is observed in the deactivation of triplet states (T_n , spin-forbidden).

3.1.1 Organic Conjugated Systems

Fluorescence is commonly observed in conjugated polycyclic aromatic hydrocarbons (PAHs), where molecules are electronically excited by absorbing light at a certain wavelength (usually in the UV or visible range), followed by emission at a longer wavelength. This phenomenon was first observed by Sir J. F. W. Herschel, who reported the fluorescence in sunlight of quinine, a naturally occurring fluorophore found in tonic water.^[13] Many other natural or synthetic fluorophores are encountered in research and daily life. For instance, anthracene and perylene are widely used for environmental monitoring of oil pollution, while rhodamine 6G (R6G) and fluorescein are frequently used in dye lasers as the gain media to create the stimulated emission.

Conjugation arises from networks of sp^2 -hybridized carbon atoms featuring adjacent and overlapping p-orbitals, perpendicular to the molecular backbone. This π -electron delocalization over alternating single and double bonds forms an extended conjugation system, which is effectively described by the linear combination of atomic orbitals (LCAOs) framework.^[14] In a simple one-electron (de)excitation scheme, the transition generally occurs between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO).^[15] This HOMO-LUMO energy

gap typically ranges a few electron volts (eV), allowing the absorption and emission of light at visible and near-infrared wavelengths depending on the conjugation length.

The transition between two electronic states is governed by the transition dipole moment (TDM, $\vec{\mu}_{if}$), which is non-zero if the transition is allowed. The TDM is defined as the matrix element between the initial (i) and final (f) state wavefunctions Ψ_i and Ψ_f

$$\vec{\mu}_{if} = \underbrace{\langle \Psi_{i,el} | \hat{\mu} | \Psi_{f,el} \rangle}_{\text{electronic}} \underbrace{\langle \Psi_{i,sp} | \Psi_{f,sp} \rangle}_{\text{spin}} \underbrace{\langle \Psi_{i,vib} | \Psi_{f,vib} \rangle}_{\text{vibrational}} \quad (3-1)$$

where $\hat{\mu}$ is the dipole moment operator. This expression can be factorized into contributions from *electronic*, *spin*, and *vibrational* operators.

The corresponding oscillator strength (f), quantifying the transition probability, is given by

$$f = 3.408 \times 10^{56} [\text{eV}^{-1} \text{C}^{-2} \text{m}^{-2}] E_{A,\text{vert}} \cdot \vec{\mu}^2 \quad (3-2)$$

where $E_{A,\text{vert}}$ is the vertical transition energy expressed in electron volts (eV).

The **selection rules**, essential for differentiating between allowed and forbidden transitions, can be derived directly from the electronic, spin, and vibrational components in Equation (3-1).

The **symmetry selection rule** states that a transition is allowed if the irreducible representations Γ of the initial Γ_i , the final Γ_f and the dipole operator Γ_μ contain the totally symmetric element,

$$\Gamma = \Gamma_i \cdot \Gamma_\mu \cdot \Gamma_f \quad (3-3)$$

The symmetry selection rule may be violated by vibrations (Herzberg-Teller coupling).^[16]

The **spin selection rule** permits only transitions between states with identical spin multiplicities ($M=2s+1$), that is, only $S_m \leftrightarrow S_n$ and $T_m \leftrightarrow T_n$ are allowed, while singlet-triplet ($S_m \leftrightarrow T_n$) transitions are theoretically forbidden. However, such transitions may occur via spin-orbit coupling (SOC), particularly in systems (i) containing heavy atoms

such as bromine and iodine, or (ii) via an El-Sayed allowed process, where the wavefunctions of the initial and final states exhibit different (orthogonal) orbital characters. The latter is often observed in a twisted geometry with perpendicular molecular orbitals, making transitions

$$^1(n, \pi^*) \leftrightarrow ^3(\pi, \pi^*) \quad ^3(n, \pi^*) \leftrightarrow ^1(\pi, \pi^*) \quad (3-4)$$

allowed, while transitions

$$^1(n, \pi^*) \leftrightarrow ^3(n, \pi^*) \quad ^1(\pi, \pi^*) \leftrightarrow ^3(\pi, \pi^*) \quad (3-5)$$

are forbidden. Here, n is a non-bonding lone-pair orbital, and $\pi^{(*)}$ refers to a(n) (anti-)bonding π orbital.

Other less restrictive rules are **non-zero differential overlap** and **alternant pairing**. The former concerns the so-called charge-transfer (CT) transitions or $n \rightarrow \pi^*$ transitions, where weak intensities appear due to the limited spatial overlap between the participating molecular orbitals.

3.1.2 Jablonski Diagram

The photophysical (and photochemical) processes that may occur after photoexcitation are usually visualized in a Jablonski diagram (see Figure 3-1). This diagram illustrates the singlet ground (S_0), the first and higher excited singlet states (S_1 , S_n) and triplet states (T_1 , T_n). Each electronic state comprises a manifold of vibrational sublevels, labeled by their vibrational quantum numbers ($v = 0, 1, 2, \dots$). Absorption, fluorescence and phosphorescence (radiative processes) are represented by straight arrows, while non-radiative processes such as vibrational relaxation (VR), internal conversion (IC), intersystem crossing (ISC), and reverse intersystem crossing (RISC) are indicated by wavy arrows.

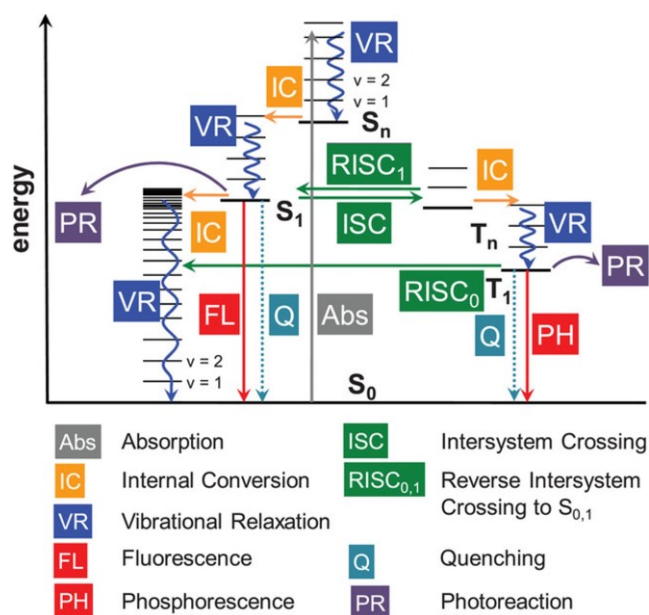


Figure 3-1: Jablonski diagram illustrating the key photophysical and photochemical processes in organic molecules. The diagram shows singlet ($S_0, S_1, \dots, S_n$) and triplet states ($T_1, \dots, T_n$), along with their vibrational sublevels ($v = 0, 1, 2, \dots$). Radiative processes (absorption (Abs), fluorescence (FL) and phosphorescence (PH)) are shown as by straight arrows, while non-radiative processes (internal conversion (IC), with subsequent vibrational relaxation (VR), (reverse) intersystem crossing (ISC, RISC) are indicated by wavy arrows. Quenching (Q) and photoreaction (PR) are also included to represent possible photochemical pathways. Reproduced with permission from [J. Gierschner et al., Adv. Optical Mater. 2021, 9, 2002251](#),^[9] with the permission of © Wiley-VCH.

Light absorption (Abs, ca. 10^{-15} s) occurs vertically (**Franck-Condon principle**) as the electronic excitation is much faster than the geometrical (or nuclear) reorganization (**Born-Oppenheimer approximation**^[17]). Upon photoexcitation to an excited state S_n , the molecule rapidly relaxes to the lowest vibrational quanta ($v=0$) of the same state by (thermal) vibrational relaxation (VR, ca. 10^{-13} s or less) through energy dissipation to the surroundings. Internal conversion (IC, ca. 10^{-8} - 10^{-13} s) for $S_n \rightarrow S_{n-1}$ is usually fast due to the small energy separations between the excited states until the S_1 is reached. As the energy gap between S_1 and S_0 is typically much larger, IC is much slowed down compared to that for higher excited states, making fluorescence (FL, ca. 10^{-4} - 10^{-9} s) a competitive pathway.

Under steady-state conditions, fluorescence from higher excited states is hardly observed, as explained by **Kasha's rule**, which states that *photon emission (fluorescence or phosphorescence) occurs in an appreciable yield only from the lowest excited state of a given multiplicity*.^[18] Notable exceptions exist, such as azulene and

its derivatives, which display observable S_2 fluorescence due to their unusual electronic structures.^[19-21] Specifically, the large S_2 - S_1 energy gap suppresses IC, while the relatively small S_1 - S_0 gap facilitates rapid IC, allowing S_2 emission to compete effectively.

Molecules in S_1 may also undergo intersystem crossing (ISC, ca. 10^{-3} - 10^{-12} s) to an acceptable triplet state T_{acc} , if the condition for an effective spin-orbit coupling (SOC) is fulfilled. Once in the triplet manifold, similar internal conversion (IC) and vibrational relaxation (VR) processes occur, and phosphorescence ($T_1 \rightarrow S_0$) may be observed. Phosphorescence is typically red-shifted and exhibits much longer lifetimes than fluorescence due to the spin-forbidden nature of the transition. Additionally, reverse intersystem crossing (RISC) can return the molecule from the triplet manifold back to S_1 , particularly in thermally activated delayed fluorescence (TADF) systems.^[22,23]

The excess energy can also be transferred to other molecules via photophysical and/or photochemical processes. Photophysical processes involve transitions where the molecular identity is preserved, while photochemical processes convert a molecule into another chemical species. The latter includes quenching (Q) by photoinduced energy or electron transfer in the presence of appropriate quenchers, and possible photoreactions (PR) such as photocyclization, tautomerization.

3.1.3 Fluorescence Rate, Fluorescence Lifetime and Quantum Yield

Fluorescence emission is inherently stochastic, as each molecule independently emits photons. Among the key photophysical parameters, fluorescence lifetime and quantum yield are most commonly used to characterize the excited-state behavior of molecules.

The fluorescence lifetime describes the average residence time in the excited state, either for a single molecule monitored individually or for an ensemble of identical molecules, averaged collectively. For a fluorophore exhibiting a single-exponential decay, the time-dependent fluorescence intensity is given by

$$I(t) = I_0 e^{-t/\tau_F} \quad (3-6)$$

where I_0 is the initial intensity at $t=0$, and τ_F denotes the fluorescence lifetime.

Typical fluorescence lifetimes range from a few to several hundred nanoseconds (ns), while phosphorescence lifetimes range from microseconds (μs) up to milliseconds (ms), and some even up to seconds (s). The lifetime in the absence of non-radiative processes is called the **intrinsic or natural lifetime** τ_0 , and it is given by

$$\tau_0 = \frac{1}{k_r} \quad (3-7)$$

where k_r is the radiative rate. Both radiative (k_r) and non-radiative (k_{nr}) rates contribute to the deactivation of the excited state. The observable fluorescence lifetime τ_F is accordingly reduced to

$$\tau_F = \frac{1}{k_r + k_{nr}} \quad (3-8)$$

The fluorescence quantum yield Φ_F , which is defined as the ratio of radiative decay to total decay

$$\Phi_F = \frac{k_r}{k_r + k_{nr}} \quad (3-9)$$

Non-radiative processes (k_{nr}) include internal conversion (IC) with subsequent vibrational relaxation (VR), intersystem crossing (ISC) to the triplet manifold, quenching (Q) by appropriate quenchers, and/or possible photochemical reactions (PR).

$$k_{nr} = k_{IC} + k_{ISC} + k_Q \cdot c_Q + k_{PR} \quad (3-10)$$

where k_{IC} and k_{ISC} denote the rates of IC and ISC, respectively; and k_Q and c_Q refer to the quenching rate and the quencher concentration, respectively. For rigid molecular systems, k_{IC} can often be described reliably within **Fermi's golden rule** (FGR, vide infra). However, in flexible ("floppy") molecules, the non-radiative pathways may become highly sensitive to subtle geometric or electronic changes. In such cases, conical intersections (CI) must be explicitly considered, as they may dominate the

deactivation dynamics of the excited state by providing a barrierless and ultrafast route for internal conversion (for further details, see Ref.^[24-26] and literature cited therein).

3.1.4 Environmental Effects

The photophysical and photochemical properties of fluorescent molecules are highly sensitive to their nano-environment, where both radiative (k_r) and non-radiative (k_{nr}) rates are profoundly affected by various factors. These include temperature, pressure, viscosity, solvent polarity and relaxation dynamics, free volume of the polymer host, specific intra- and intermolecular interactions (e.g. hydrogen bonding), proton transfer, photochemical reactions (e.g. photoisomerization, quenching), oxygen permeability of the polymer host, isotope effects, and modifications of the local optical environment. These (nano-)environmental effects regulate key fluorescence characteristics such as (i) spectral position and bandwidth, (ii) fluorescence intensity, (iii) fluorescence lifetime τ_F , (iv) quantum yield Φ_F , (v) photostability and (vi) fluorescence intermittency (photoblinking).

Fluorescence Intensity and Quantum Yield

The fluorescence intensity and the fluorescence quantum yield Φ_F are highly sensitive to the competition between radiative (k_r) and non-radiative (k_{nr}) pathways. Different polymer hosts produce different chemical environments in terms of polarity, rigidity, free volume, and specific probe-host interactions. In particular, rigid matrices can suppress non-radiative channels, leading to enhanced fluorescence intensity and increased quantum yields.^[9,27-35] On the other hand, polarity alters the electronic state ordering and vibrational coupling, either promoting or hindering non-radiative pathways depending on the molecular structure.^[9,36] Furthermore, specific interactions such as hydrogen bonding have been recognized as an effective promoter of non-radiative decay via facilitating internal conversion (IC). Hydrogen-bond-assisted relaxation channels typically result in shortened fluorescence lifetimes and substantially reduced quantum yields.^[37-40]

Spectral Position and Band Shape

Unlike atomic spectra with sharp lines, molecular electronic absorption and emission spectra show vibronic progressions due to vibronic coupling, i.e. interactions between electronic and vibrational degrees of freedom. These features become particularly evident under cryogenic spectroscopy, where environmental fluctuations are minimized, allowing resolution of narrow zero-phonon lines and vibronic fine structure.^[14,41,42]

In solution or in the solid state at room temperature, both static and dynamic molecular fluctuations (e.g. conformational heterogeneity and solvent interactions) broaden the individual vibronic lines so that they overlap to form broad, asymmetric bands.^[43-47] This spectral broadening arises from two principal contributions: *inhomogeneous* and *homogeneous* broadening.^[42,44-46,48,49]

Inhomogeneous broadening stems from *static* heterogeneities in local molecular environments, causing a distribution of transition energies across the ensemble. For example, in disordered polymer hosts or liquid solutions, molecules experience diverse local electric fields, which shift the ground and excited states differentially, resulting in spectral heterogeneity.^[50,51] In contrast, crystalline hosts provide highly uniform local environments and conformations, significantly narrowing the inhomogeneous line widths.^[42] In polar environments, large fluctuations in local fields further amplify such inhomogeneous broadening.^[51,52]

On the other hand, homogeneous broadening originates from the finite coherence lifetime (τ_{coh}) of the excited state, which is fundamentally determined by the excited-state lifetime (τ_{ex}) and the dephasing time (τ_{deph}). The former τ_{ex} is given by Fermi's golden rule and depends on the square of the transition dipole moment (TDM) and the optical mode density (vide infra). The latter τ_{deph} quantifies the frequency of perturbations, i.e. *dynamic* environmental heterogeneities, such as solvent collisions, phonons, or local field fluctuations. The shorter the τ_{deph} is, the broader the homogeneous line width becomes. At cryogenic temperatures, environmental fluctuations are suppressed, τ_{deph} becomes long, and homogeneous broadening is primarily limited by τ_{ex} , leading to very sharp zero-phonon lines.^[48,49,53]

Beyond these broadening mechanisms, the vibronic spectral band shape is further governed by the strength of vibronic coupling, which includes both linear and quadratic contributions.^[9] Within the first-order perturbation approximation, linear vibronic coupling follows Fermi's golden rule

$$P_{if} = \frac{2\pi}{\hbar} \langle \Phi_i | H' | \Phi_f \rangle^2 \rho(E_f) \quad (3-11)$$

where P_{if} is the transition probability, H' the perturbation operator, and $\rho(E_f)$ the density of vibrational final state with the energy E_f .^[9] Vibronic coupling strength of a given mode is large if the geometrical changes upon photoexcitation coincide with the normal coordinates of that vibration.^[54-56]

To illustrate the interplay of vibronic coupling and environmental broadening, we consider stilbene and cyanine dyes, both of which possess identical resonance structures. Stilbene (a polyene-like molecule) exhibits significant change in bond length alternation (Δ_{BL}) between its ground and excited states, leading to a large vibronic coupling and broad vibronic structures. In contrast, cyanine dyes possess nearly identical ground- and excited-state geometries owing to equivalent resonance structures, resulting in narrow-band emission,^[57] *an example of minimized vibronic coupling and homogeneous broadening*. However, this non-alternating situation in cyanine can be violated by ion-pairing effects in nonpolar solvent.^[57-59] For example, the proximity of counterion $\text{Br}^\ominus$ induces asymmetric polarization in the polymethine backbone, significantly increasing the Δ_{BL} , and consequently broadening the spectrum.^[57] A similar yet milder broadening is observed in rhodamine 6G (R6G, a xanthene dye), due to its inherently localized structural relaxation upon photoexcitation (for further details, see Section 4.3.1).^[60,61]

At ambient temperatures, quadratic vibronic coupling and solvent relaxation dominate, collapsing fine vibronic features into overlapping Gaussian-shaped spectra. These bands typically exhibit a full width at half maximum Gaussian width (FWHM, γ_G) of approximately 0.07 eV, giving rise to characteristic asymmetric broad emission observed at room temperature.^[9,43-47,57,62,63] Simultaneously, extensive structural reorganization upon photoexcitation induces pronounced Stokes shifts,^[64,65] typically

observed around 1500 cm^{-1} in rhodamine dyes and up to 6000 cm^{-1} for laser dyes such as DCM².^[66]

Excimers and exciplexes: In concentrated solutions, molecules may form excimers or exciplexes. An excimer, as defined by J. B. Birks, is a molecular dimer which is *associated* in an excited electronic state but is *dissociative* (i.e. would dissociate in the absence of external restraints) in its electronic ground state.^[67] Pyrene and its derivatives are classical examples,^[68-70] showing a broad, structureless excimer band (D-band, $\sim 480\text{ nm}$) that progressively dominates over the well-resolved monomer fluorescence (M-band, $350\text{-}400\text{ nm}$) as the concentration increases.^[71-73] The unstructured and red-shifted nature of the excimer emission originates from strong inter-molecular vibrational coupling and geometry relaxation within the excimer state.^[74] On the other hand, an exciplex, classified as either intra- or inter-molecular, is an excited-state complex formed between two chemically distinct species. Such complexes are often referred to as exciplex complexes, and typically involve donor-acceptor interactions.^[67,74-76]

Photonic Modifications

Photonic nano-environments play a crucial role in modulating fluorescence properties via the Purcell effect. By embedding molecules into Fabry-Pérot microcavities (Section 4.1) or plasmonic nanocavities (Section 4.3), the local density of states (LDOS) is altered. This allows selective modification of the radiative rate k_r without affecting non-radiative channels k_{nr} , leading to tunable fluorescence lifetimes and quantum yields. The enhanced k_r suppresses intersystem crossing (ISC) to triplet states, thus improving photostability (the survival time) by reducing triplet-state-mediated photobleaching and other photoreactions originating from triplet states. Such photonic control has been applied to tailor excited-state dynamics without chemical modification.

² Laser dye DCM: 4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)- 4H-pyran.

Ensemble vs. Single-Molecule Observables

Ensemble-averaged spectroscopy reports statistical mean values of photophysical parameters such as fluorescence lifetime and quantum yield by integrating signals from a large number of molecules. This typically results in Gaussian distributions of observables that often obscure the underlying heterogeneity originating from diverse local nano-environments.^[77] Consequently, information about molecular sub-populations or rare events is lost, and the measured values only reflect the statistically averaged behavior.

In contrast, single-molecule spectroscopy overcomes this limitation by directly resolving the photophysical and photochemical behaviors of individual molecules. Distributions of fluorescence intensity, lifetime, quantum yield, and spectral features (spectral shifts, line widths, and vibronic structures) become experimentally accessible observables, offering valuable insight into nano-environmental heterogeneity.^[78-80] A characteristic feature of single-molecule fluorescence is photoblinking, originating from the transition between dark and bright states. Moreover, fluorescence intensity varies significantly among different emitters due to orientation-dependent excitation efficiency, determined by the angle between the molecular transition dipole moment (TDM) and the excitation field $\vec{E}$. Fluctuations in fluorescence properties (fluorescence intensity, spectral diffusion^[81]) reflect the dynamic changes of the local nano-environment and may reveal possible processes such as conformational isomerization, photochemical reactions (vide infra, see Section 4.1). All these factors contribute to broad, non-Gaussian and often skewed fluorescence intensity distributions. Notably, in weakly emissive or strongly quenched single-molecule systems, signal enhancements via microcavities or plasmonic nanostructures are frequently required to enhance the radiative rate (k_r) via the Purcell effect.^[82-85]

On the other hand, single-molecule quantum yields are generally lower than ensemble averages due to nano-environmental heterogeneity and statistical effects.^[83,86] Therefore, a major challenge in single-molecule studies arises in the determination of absolute quantum yields. Unlike ensemble measurements, where the fluorescence quantum yield can be directly determined from the ratio of emitted (N_{em}) to absorbed (N_{abs}) photons, single-molecule experiments suffer from uncertainties in local

excitation intensity and absorption cross-section. Therefore, single-molecule quantum yields are usually estimated indirectly from measured fluorescence lifetimes and relative brightness, often requiring reference systems or theoretical modeling to obtain semi-quantitative estimates.^[87]

3.2 Single-Molecule Imaging and Spectroscopy

The state-of-the-art single-molecule confocal microscope and spectroscopy offer outstanding sensitivity, stability, and spatial resolution, enabling direct observation of photophysical and photochemical processes at the single-molecule level. Compared to ensemble-averaged techniques, single-molecule measurements reveal not only sharper spectral features due to the absence of inhomogeneous broadening but also dynamic events such as spectral diffusion, photoblinking, or photobleaching, which originate from the stochastic nature of interactions between the probe and its nano-environment.

A major advantage of single-molecule confocal microscope and spectroscopy is its versatility across a wide range of conditions, from cryogenic to ambient temperatures, from free-space environment to optical cavities. In addition, the implementation of higher-order laser modes, specifically azimuthally (APDM) and radially (RPDM) polarized doughnut beams, enables polarization-resolved imaging and spectroscopy. These techniques provide not only direct access to the three-dimensional (3D) orientation of transition dipole moments (TDMs),^[88-92] but also to the polarization-dependent spectral responses.^[4,93,94]

In this thesis, the experimental setup has been specifically designed and optimized for the stable and sensitive detection of single-molecule signals. The system allows the simultaneous acquisition of confocal fluorescence images, fluorescence intensity time traces, fluorescence lifetimes, and polarization-resolved Raman spectra. This methodological framework forms the basis for systematically investigating nano-environmental effects, which will be discussed in the case studies in Chapter 4.

3.2.1 Single-Molecule Confocal Microscope and Spectroscopy

The optical measurements in Section 4.1 were performed by a home-built inverted confocal microscope and spectroscopy, equipped with higher-order laser modes. A schematic of the experimental setup is shown in Figure 3-2.

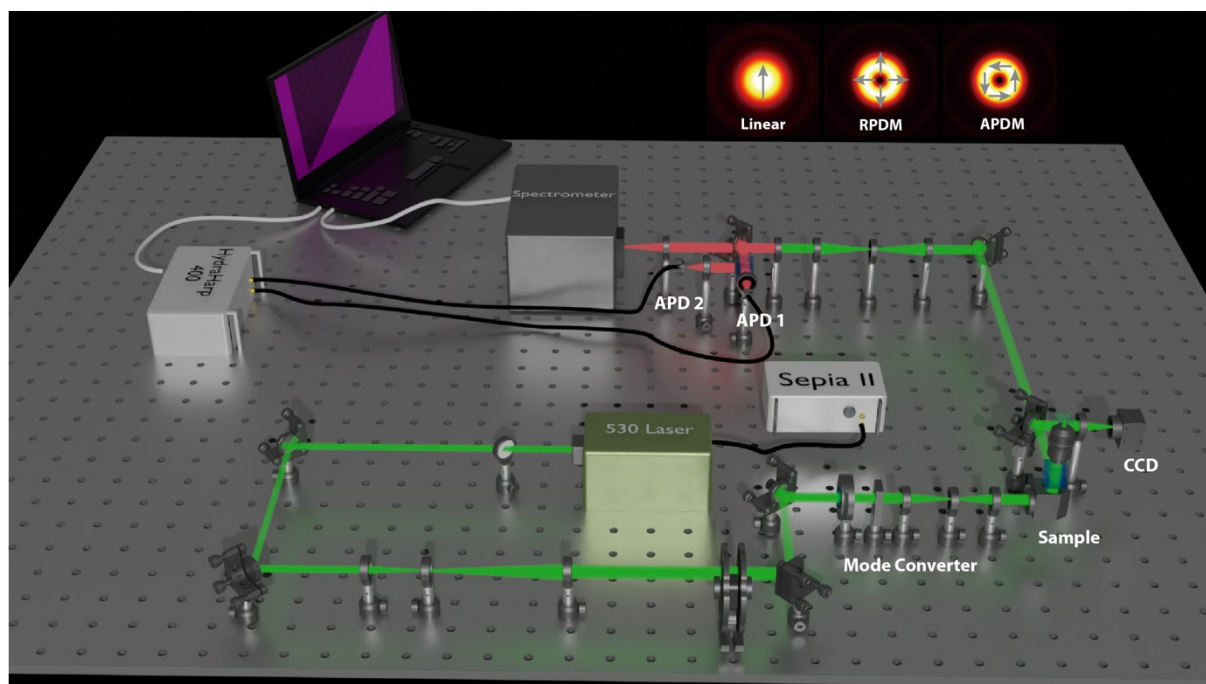


Figure 3-2: Schematic of the home-built confocal microscope and spectroscopy, equipped with a mode converter for modifying the beam profile and polarization. The inset (top right) shows the intensity cross section of a linearly polarized Gaussian beam, an azimuthally (APDM) polarized doughnut mode, and a radially (RPDM) polarized doughnut mode. Reproduced from [L. Wang et al., J. Chem. Phys. 7 January 2022; 156 \(1\): 014203](#),^[1] with the permission of AIP Publishing.

The sample was excited by a pulsed laser (530 nm, pulse duration < 100 ps, 20 MHz, LDH-P-FA-530 L, PicoQuant). The excitation beam was directed into a high numerical aperture oil-immersion (NA=1.46, 64 $\times$, Carl Zeiss), which simultaneously focused the excitation beam onto the sample and collected the fluorescence signal. Furthermore, the laser mode profile could be switched between a linearly polarized Gaussian beam and higher-order doughnut modes. Specifically, a commercial mode converter (Polarization Converter, ARCoptix) was used to generate azimuthally (APDM) and radially (RPDM) polarized doughnut modes (vide infra).

The samples were mounted onto a scanning piezo-stage (P-527.3CL, Physik Instrumente) for raster-scanned image acquisition. A 532 nm long-pass filter (532LP Edge Basic, Semrock) was placed in the detection path to remove the residual excitation light. The confocal pinhole ensured the spatial filtering of out-of-focus fluorescence, leading to high signal-to-background ratios suitable for single-molecule detection. The collected fluorescence signal was subsequently directed either (i) to two avalanche photodiodes (APDs, SPCM-AQR-14, PerkinElmer) for time-resolved measurements, including fluorescence intensity time traces, fluorescence lifetime, photon correlation, or (ii) to a grating spectrometer (SP-2500i, Princeton Instruments) for spectra analysis.

Time-correlated single photon counting (TCSPC) measurements were performed using a HydraHarp 400 (PicoQuant) module, synchronized with the pulsed laser. Each detected photon was registered as a time-tagged time-resolved (TTTR) file.^[95] In standard fluorescence lifetime measurements, only one APD was used for optimal signal-to-noise ratio with a timing resolution of 64 ps. For antibunching measurements (vide infra), two APDs and a 50/50 beam splitter were employed with a timing resolution of 1 ps. Additional short-pass filters were placed in front of the APDs to suppress the after-pulsing effect.^[95] Collected TCSPC-related data were analyzed using SymPhoTime 64 (PicoQuant). Spectra and scanning confocal fluorescence images were processed using custom MATLAB scripts.

In single-molecule experiments, it is essential to ensure that only one molecule is present within the focal volume, which is typically achieved by working with extremely low sample concentrations ($\sim 10^{-10}$ M). The presence of single molecules can be further verified by antibunching measurements (see Section 3.2.2). Moreover, a sufficient signal-to-noise ratio is required, which is achieved by optimizing the combination of a tightly focused excitation volume, minimizing background impurities, maximizing absorption cross-section and quantum yield, and ensuring good photostability and minimal population of dark states such as triplet states.

3.2.2 Antibunching

The presence of single molecules can be confirmed by antibunching measurements. Antibunching represents a characteristic signature of single-photon emitters, manifested by the sub-Poissonian photon statistics. It provides unambiguous evidence for the identification of individual quantum emitters, including single molecules, quantum dots, carbon nanotubes, and defect centers such as nitrogen vacancy (NV) centers in diamond, as well as applications based on single-photon sources.^[96,97]

The physical origin of antibunching lies in the fundamental limitation that a single-photon emitter can emit only one photon per excitation-relaxation circle. This process can easily be described using a simplified two-level energy diagram, where upon photoexcitation, an emitter in an excited state requires a finite time before it relaxes back to the ground state by emitting a photon. Consequently, the temporal separation between consecutive photon emission events is predominantly governed by the excited-state lifetime. This effect is known as antibunching, representing the sub-Poissonian nature of the statistics.

The statistical properties of single-photon emission are often quantitatively characterized by the second-order correlation function $g^2(\tau)$ ^[48]

$$g^2(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} \quad (3-12)$$

where $I(t)$ is the photon detection intensity at time t , and τ is the time delay between two photon detection events. This can be understood as the probability to detect a photon at time $(t+\tau)$ after one was detected at the time point t .

For an ideal single-photon emitter, the presence of an antibunching dip at $\tau=0$ with $g^2(\tau=0) \rightarrow 0$ directly evidences the absence of coincident photon emission (see Figure 3-3). This behavior is independent of a continuous-wave (CW) or a pulsed excitation. The typical result of an antibunching experiment in CW excitation is a flat line with a notable “intensity dip” at zero time delay, while in a pulsed excitation, $g^2(\tau)$ shows periodic peaks at intervals corresponding to the laser repetition period, with a suppressed or absent peak at $\tau=0$.

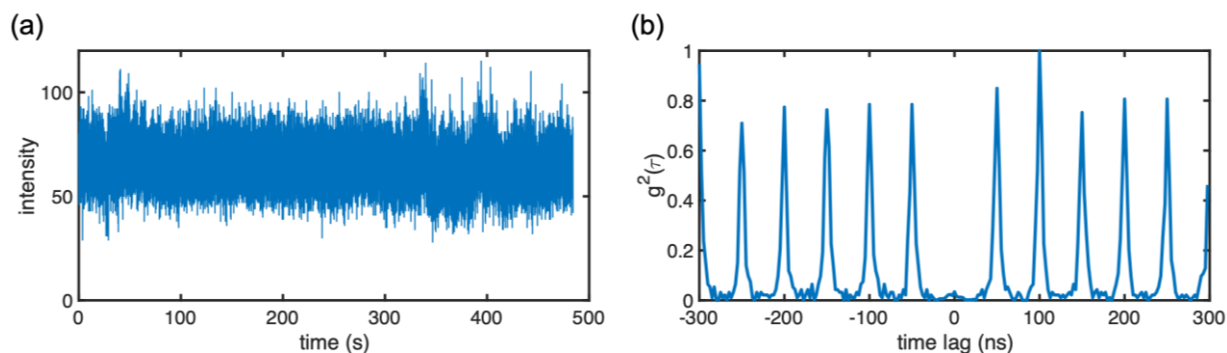


Figure 3-3: (a) Representative fluorescence intensity time trace of a single hypericin molecule embedded in a PMMA polymer host (concentration: 10^{-10} M), and (b) the corresponding second-order photon correlation function (antibunching). The pronounced antibunching dip at $g^2(\tau=0) \rightarrow 0$ provides direct evidence for single molecule detection.

Experimentally, photon antibunching is measured using a Hanbury Brown-Twiss (HBT) interferometer setup,^[98] in which the emitted photons are equally split by a 50/50 beam splitter, and directed onto two independent single-photon sensitive detectors, typically avalanche photodiodes (APDs). The time intervals between successive detection events are histogrammed by a time-correlated single-photon counting (TCSPC) module, yielding quantitative assessment of the antibunching effect.

3.2.3 Time-Correlated Single Photon Counting (TCSPC)

Time-correlated single-photon counting (TCSPC) is a highly sensitive and widely used method for quantifying fluorescence lifetimes, and analyzing photon emission dynamics.^[95,99] This technique is particularly advantageous for experiments suffering from (i) insufficient temporal resolution, and/or (ii) extremely low photon flux. Typical fluorescence lifetime in organic conjugated systems ranges from only a few hundred picoseconds (ps) to several tens of nanoseconds (ns).^[95] In single-molecule measurements, the detected fluorescence signal often consists of only a few photons per excitation-relaxation cycle, which imposes demanding requirements on both the sensitivity and time resolution of the detection system.^[100]

TCSPC addresses these challenges by statistically reconstructing fluorescence decay profiles from photon arrival times accumulated over multiple excitation-relaxation cycles.^[100] Instead of directly recording complete transient waveforms, TCSPC registers individual photon arrival times relative to the excitation pulse, accumulating

them into a histogram. This histogram represents the probability distribution of the photon emission times, and directly reflects the fluorescence decay curve.^[100]

The key assumption of TCSPC is the low probability of multi-photon detection within a single excitation-relaxation cycle.^[95] To preserve proper single-photon statistics, the detection probability per excitation pulse is typically kept below 5%.^[100] This avoids the so-called photon “pile-up” effect, where the system registers the first photon but misses subsequent ones due to the detector and electronics’ inherent dead time, leading to systematic distortion of the decay curve.^[100] As a practical guideline, only one in 20 to 100 excitation pulses should generate a detected photon. For example, when using an 80 MHz pulsed laser system, the detection rate should not exceed 4 MHz to preserve statistical validity.^[100]

In a simple case, the time-dependent fluorescence intensity generally follows a single-exponential decay $I(t) = I_0 e^{-t/\tau_F}$ (Equation 3-6). However, the experimentally measured histogram $S(t)$ does not directly represent the fluorescence intensity, but rather the convolution of this decay $I(t)$ with the instrument response function (IRF) $R(t)$

$$S(t) = \int_0^{\infty} R(t') I(t - t') dt' \quad (3-13)$$

where $R(t)$ accounts for the temporal broadening introduced by (i) the finite response time of the detector, (ii) the width of the excitation laser pulses, and (iii) the timing jitter of the electronics.^[12] The IRF width typically ranges from a few tens to hundreds of picoseconds, depending on the specific detector and timing system. To accurately determine the intrinsic fluorescence lifetime, $I(t)$ is extracted via iterative deconvolution or re-convolution fitting procedures based on the measured IRF.^[95,100]

3.2.4 Higher-Order Laser Modes

Upon photoexcitation, the transition dipole moment (TDM) orientation of a molecule is directly related to its molecular orientation. In a single-molecule confocal microscope, the absolute three-dimensional (3D) TDM orientation can be determined by employing higher-order laser modes, specifically azimuthally (APDM) and radially (RPDM) polarized doughnut beams.

As shown in Figure 3-4, higher-order laser modes are generated by passing a linearly polarized Gaussian beam through a mode converter^[101] (polarization converter, ARCOptix), consisting of a twisted nematic liquid crystal cell. The converter induces a spatially varying polarization distribution by rotating the local polarization vector through the angularly dependent twist of the liquid crystal molecules. Depending on the applied voltages, the generated polarization pattern is determined by the relative orientation of the incident beam to the converter's optical axis, allowing the device to produce either azimuthal or radial polarization. A spatial filter (15 μm pinhole) is used to remove undesired higher-order spatial frequency components, and to improve the purity of the doughnut modes.

The incident linearly polarized Gaussian beam has a uniform intensity profile at any cross-section perpendicular to the optical axis. After mode conversion, the resulting higher-order laser beams (APDM and RPDM) exhibit distinct polarization-resolved electric field distributions within the focal plane. The RPDM provides both transverse (in-plane) and longitudinal (out-of-plane) electric field components, with the longitudinal component being dominant. In contrast, the APDM exclusively generates in-plane electric field. The intensity profiles and polarization distributions are shown in Figure 3-4(c, d).

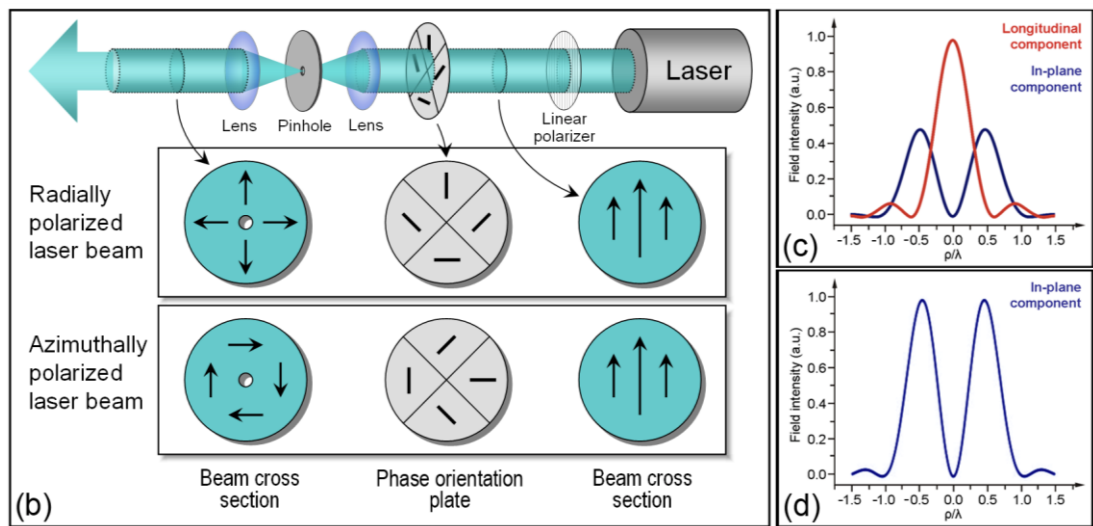


Figure 3-4: Scheme of the mode converter that generates azimuthally (APDM) and radially (RPDM) polarized doughnut modes from the linearly polarized Gaussian beam. Reproduced from Figure 3(b-d) in [A. M. Chizhik et al., Nanophotonic Materials VI. SPIE, 2009, 7393: 31-41](#),^[102] with the permission of Proceedings of SPIE, Regular series.

The fluorescence intensity pattern observed under higher-order excitation directly reflects the transition dipole moment (TDM) orientation. The excitation efficiency at each spatial position is determined by the projection of the local excitation field onto the TDM. For example, molecules with TDMs aligned perpendicular to the sample surface (vertical dipoles) are primarily excited by the longitudinal component of the RPDM, producing a circularly symmetric pattern with a single bright spot. Conversely, molecules with TDMs parallel to the surface (horizontal dipoles) generate two symmetric lobes due to the interaction with the in-plane electric fields of both APDM and RPDM. In the more general case, where the TDM is tilted relative to the surface, the fluorescence pattern becomes asymmetric, and its shape provides direct information on the tilt angle and the azimuthal orientation. The combined analysis of the RPDM and APDM pattern thus allows for the precise determination of the absolute three-dimensional (3D) orientation of the TDM.^[89,92,103]

This polarization-resolved approach is particularly valuable, as it provides direct access to molecular orientation and its dynamic fluctuations under varying nano-environmental conditions. In particular, it enables the precise determination of the absolute 3D orientation of individual TDMs, which is essential for identifying specific isomeric (tautomeric) species (Section 4.1); and facilitates a detailed investigation of polarization-dependent spectral properties (Section 4.3).

3.2.5 Microcavity

A microcavity is an optical resonator designed to confine electromagnetic fields into a volume comparable to or smaller than the wavelength λ of light, thereby significantly modifying light-matter interactions.^[104] This confinement enhances the local density of optical states (LDOS) and alters the spontaneous emission properties of embedded emitters. One of the most widely used configuration is the Fabry-Pérot microcavity (see Figure 3-5) due to its straightforward design and efficient mode confinement.

A Fabry-Pérot microcavity consists of two parallel mirrors (M1 and M2) with reflectivities of R_1 and R_2 , separated by an adjustable distance d , and filled with a medium of refractive index n .

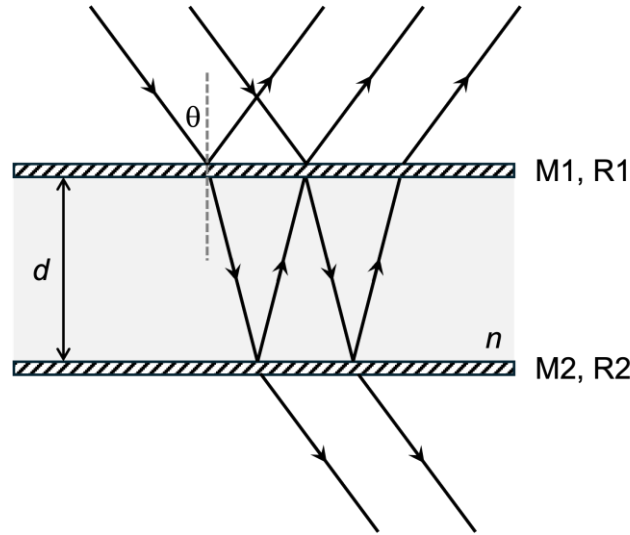


Figure 3-5: Schematic of a planar Fabry-Pérot microcavity consisting of two parallel mirrors (M1 and M2) with reflectivities R1 and R2, separated by a distance d . The medium inside has a refractive index n . Light entering the cavity undergoes multiple reflections, leading to an optical path difference of $\delta = 2d\sqrt{n^2 - \sin^2\theta}$ between successive beams.

Light undergoes multiple reflections between the mirrors, resulting in constructive or destructive interference depending on the optical path difference^[48]

$$\delta = 2d\sqrt{n^2 - \sin^2\theta} \quad (3-14)$$

where θ is the angle of incidence. For normal incidence ($\theta=0$), this reduces to $\delta=2nd$. The on-resonance condition is achieved when the accumulated round-trip phase shift φ equals an integer multiple of 2π , i.e. $m=1, 2, 3, \dots$,^[48]

$$\varphi = \frac{4\pi nd}{\lambda} = 2\pi m \quad (m \in \mathbb{Z}) \quad (3-15)$$

The on-resonance condition occurs at discrete cavity distance d with a linear relationship of the half wavelength $\lambda/2$, i.e. $m=1, 2, 3, \dots$, described by^[48]

$$d = \frac{m\lambda}{2n} \quad (3-16)$$

The resulting properties can be analyzed by considering the effect of introducing light of wavelength λ , from one side and calculating how much get transmitted through the other side. Assuming negligible absorption or scattering losses within the cavity, that transmission T is expressed by^[48]

$$T = \frac{1}{1 + (4\mathcal{F}^2/\pi^2)\sin^2(\varphi/2)} \quad (3-17)$$

where $\mathcal{F}$ is the finesse of the cavity^[48]

$$\mathcal{F} = \frac{\pi(R_1 R_2)^{1/4}}{1 - \sqrt{R_1 R_2}} \quad (3-18)$$

A higher finesse $\mathcal{F}$ leads to sharper resonances, with phase line width (full width at half maximum, FWHM) inversely proportional to finesse $\mathcal{F}$ ^[48]

$$\Delta\varphi_{\text{FWHM}} = 2\pi/\mathcal{F} \quad (3-19)$$

The resonance line width $\Delta\omega$ inversely proportional to the finesse of the cavity^[48]

$$\Delta\omega = \frac{\pi c}{nd\mathcal{F}} \quad (3-20)$$

The quality factor Q characterizes the spectral selectivity^[48]

$$Q = \frac{\omega}{\Delta\omega} \quad (3-21)$$

Higher quality factor Q corresponds to narrower line widths and longer photon lifetimes. The cavity supports discrete resonant frequencies, where the molecular fluorescence emission can couple into resonant photonic modes, the angular resonance frequencies of the latter condition is^[48]

$$\omega_m = m \frac{\pi c}{nd} \quad (3-22)$$

Molecular fluorescence emission can couple to resonant photonic modes if the transition frequency of the molecule matches a cavity mode, modifying the spontaneous emission rate. The light-matter interaction strength depends fundamentally on three parameters:^[48] (i) the photon decay rate of the cavity κ ; (ii) the molecular non-resonant decay rate γ ; (iii) the molecule-cavity coupling parameter g_0 .

In general, the interaction of a quantum system with its optical field in a cavity can be divided into two regimes: the strong coupling regime ($g_0 \gg \kappa, \gamma$) and the weak coupling

regime ($g_0 \ll \kappa, \gamma$).^[48] In the strong coupling regime, the emission is considered to be reversible due to the fact that the photon is reabsorbed by the molecule before its loss from the cavity. In contrast, the emission in the weak coupling regime is an irreversible process, where the radiative rate k_r is altered compared with that in free space.^[48] The latter phenomenon was first considered by Purcell in 1946,^[85] the radiative rate k_r is enhanced by the **Purcell factor** P , defined by

$$P = \frac{k_{r,c}}{k_r} = \frac{3Q(\lambda/n)^3}{4\pi^2 V_0} \quad (3-23)$$

where $k_{r,c}$ is the radiative rate in the (micro-)cavity, k_r is the radiative rate in free space, and V_0 is the modal volume of the (micro-)cavity.

Given that the cavity mode has an angular frequency of ω_c with a FWHM of $\Delta\omega_c$. The density of states (DOS) $\rho(\omega)$ ^[48]

$$\int \rho(\omega) d\omega = 1 \quad (3-24)$$

results in a Lorentzian line-shape function^[48]

$$\rho(\omega) = \frac{2}{\pi\Delta\omega_c} \frac{\Delta\omega_c^2}{4(\omega - \omega_c)^2 + \Delta\omega_c^2} \quad (3-25)$$

A perfect on-resonance condition is satisfied when $\omega_0 = \omega_c$, $\rho(\omega)$ then can be rewritten as^[48]

$$\rho(\omega) = \frac{2}{\pi\Delta\omega_c} = \frac{2Q}{\pi\omega_0} \quad (3-26)$$

In Section 4.1.3, a two-layer Fabry-Pérot microcavity is implemented to control the photophysics and photochemistry of single hypericin molecules. Transfer matrix method (TMM)^[105] simulations are employed to model the resonance spectrum, considering the multilayer structures.

3.3 Computational Treatment

In this thesis, computational chemistry is employed as an essential complement to interpret and rationalize experimental findings, including both spectroscopic and microscopic data. The combination of experiments and simulations permits a comprehensive understanding of the structural, electronic, photophysical and photochemical properties of the studied systems. To address the specific challenges arising from nano-environmental effects, particular attention is given to methods capable of accurately describing ground- and excited-state electronic structures, their potential energy surfaces, and the associated molecular conformational and structural dynamics. The selection of computational approaches is carefully evaluated to meet the requirements of each system investigated in this work. Wavefunction-based methods, represented by Hartree-Fock (HF), provide the foundation for constructing molecular orbitals and establishing electronic structure models. Density functional theory (DFT) and its time-dependent extension (TD-DFT) offer an efficient and widely accepted framework for describing ground and excited states of medium to large molecular systems. Classical molecular dynamics (MD) simulations provide valuable insights into conformational dynamics and explicit solute-solvent interactions, which are essential for interpreting experimentally observed solvent effects on structure and dynamics. The following sections outline the theoretical foundations and practical considerations of these methods, with a particular emphasis on their application to the case studies in Chapter 4.

3.3.1 Quantum Chemical Foundations

The time-independent, non-relativistic **Schrödinger equation** has been regarded as the cornerstone of quantum mechanics for describing molecular systems

$$\hat{H}|\Psi\rangle = E|\Psi\rangle \quad (3-27)$$

The wavefunction Ψ represents one of the states of the system (state function), and depends on all the particles' coordinates. According to the probabilistic interpretation, its modulus squared corresponds to a **probability density**, indicating the likelihood of finding a particle at a certain position. Here, $\hat{H}$ is the Hamiltonian operator, and E is

the energy of the system in that state. The Hamiltonian $\hat{H}$ in atomic units (a.u.) is expressed as

$$\hat{H} = \underbrace{-\sum_{i=1}^e \frac{1}{2} \nabla_i^2}_{\hat{T}_e} - \underbrace{\sum_{A=1}^N \frac{1}{2M_A} \nabla_A^2}_{\hat{T}_N} - \underbrace{\sum_{i=1}^e \sum_{A=1}^N \frac{Z_A}{r_{iA}}}_{\hat{V}_{eN}} + \underbrace{\sum_{i=1}^e \sum_{i<j}^e \frac{1}{r_{ij}}}_{\hat{V}_{ee}} + \underbrace{\sum_{A=1}^N \sum_{A<B}^N \frac{Z_A Z_B}{R_{AB}}}_{\hat{V}_{NN}} \quad (3-28)$$

where the Hamiltonian operator is decomposed into the kinetic energy operator, which in turn contains nuclear and electronic terms, $\hat{T}_N$, $\hat{T}_e$; as well as into the potential energy operator, which contains electron-nucleus attraction $\hat{V}_{eN}$, electron-electron repulsion $\hat{V}_{ee}$, and nucleus-nucleus repulsion $\hat{V}_{NN}$ terms. M_A , $Z_{A/B}$, r_{iA} , R_{AB} and r_{ij} are the atomic mass of nucleus A, atomic number of nuclei A/B, distance between electron i and nucleus A, distance between nuclei A and B, and distance between electrons i and j, respectively.

However, the Schrödinger equation of a many-body system is challenging and cannot be solved for more than two particles. The Born-Oppenheimer approximation^[17] (vide supra) addresses this by separating electronic and nuclear motions, leading to an electronic Schrödinger equation at fixed nuclear positions. Within this framework, nuclei and electrons are treated as point particles, and relativistic effects, including spin-orbit coupling, are neglected. These assumptions form the basis for most electronic structure methods, including the Hartree-Fock approach introduced below.

3.3.2 Hartree-Fock Method

The **Hartree-Fock (HF)** method represents a foundational ab initio approach in electronic structure theory and marked a breakthrough in the development of modern computational chemistry. It is a wavefunction-based approximation, in which each electron is treated as an independent particle moving in an average field generated by all other electrons and the nuclei. This approximation transforms the many-electron Schrödinger equation into a set of coupled one-electron equations that are solvable through the self-consistent field (SCF) procedure (vide infra).

Molecular and Spin Orbitals in Hartree-Fock Theory

To illustrate the origin of **molecular orbitals (MOs)** in this framework, it is instructive to start with the simplest system: the hydrogen atom (H). Consisting of one electron and one proton, its electronic state is *exactly* described by an **atomic orbital (AO)**, obtained from the analytical solution of the Schrödinger equation. A natural extension is the hydrogen molecular cation (H_2^+), consisting of one electron and two protons. In this picture, the electron occupies a molecular orbital (MO), which is delocalized over both nuclei.

In polyatomic molecules, molecular orbitals are approximated as **linear combinations of atomic orbitals (LCAOs)**, allowing the description of delocalized electrons and chemical bonding. Within the HF method, each electron is assigned to a spin-orbital, which is the product of a spatial molecular orbital and a spin function (either α or β). These spin-orbitals form the basis for many-electron wavefunctions, which are used to construct a single Slater determinant (vide infra) to ensure the anti-symmetry required by the Pauli exclusion principle. The spatial orbitals are optimized variationally, and their expansion coefficients are obtained through an iterative self-consistent field (SCF) procedure, in which the orbitals and the mean-field potential are updated until convergence.

To determine the optimal MOs, one must first define the electronic Hamiltonian that governs the system (in atomic units, a.u.)

$$\hat{H}_{\text{elec}} = - \sum_{i=1}^{\text{electrons}} \frac{1}{2} \nabla_i^2 - \sum_{i=1}^{\text{electrons}} \sum_{A=1}^{\text{nuclei}} \frac{Z_A}{r_{iA}} + \sum_{i=1}^{\text{electrons}} \sum_{i < j}^{\text{electrons}} \frac{1}{r_{ij}} \quad (3-29)$$

$$\sum_{i=1}^{\text{electrons}} \hat{h}_i = \text{"separable"} \quad \underline{\underline{\text{"non-separable"}}}$$

where an infinitely heavy point nucleus and many non-interacting electrons are assumed. The first two terms are “separable” and can be written in the context of the one-electron Hamiltonian, whereas the final term represents an interaction potential with all of the other electrons and renders the problem inherently many-body and non-separable. To address this “many-body” problem numerically, Hartree (1928)^[106]

proposed an iterative “**self-consistent field**” (SCF) procedure that averages over the “mean field” of all the other electrons.

Hartree-Fock Equations and the Variational Principle

Although the mean-field approximation is severe, the true wavefunction should explicitly include spin and must be anti-symmetric with respect to the interchange of electrons due to the Pauli exclusion principle. Hence, one must take an anti-symmetric linear combination of products of spin-orbitals. This principle was first pointed out by Fock^[107,108] and by Slater^[109] in 1930. A self-consistent field (SCF) calculation that uses anti-symmetrized spin-orbitals is called a Hartree-Fock (HF) calculation. The total electronic wavefunction is written as a single **Slater determinant** of spin-orbitals^[110]

$$\Psi(1,2, \dots N) = \frac{1}{\sqrt{N!}} \det [\chi_i(j)] \quad (3-30)$$

where $(N!)^{-1/2}$ is a normalization factor. By applying the variational principle to this form, one obtains the **Hartree-Fock (HF) equations**

$$\hat{f}_i u_i = \varepsilon_i u_i, \quad i = 1, 2, \dots, n \quad (3-31)$$

where u_i is the i^{th} spin-orbital; $\hat{f}_i$ is the Fock operator, which represents an effective HF Hamiltonian. The eigenvalue ε_i is the corresponding spin orbital energy.^[111]

In a closed-shell system, i.e., all the electrons are spin-paired (two per occupied orbital), the one-electron **Fock operator** is defined for each electron as,

$$\begin{aligned} \hat{f}_i &= -\frac{1}{2} \nabla_i^2 - \sum_{A=1}^N \frac{Z_A}{r_{iA}} + V_i^{\text{HF}}\{j\} \\ \hat{f}_i &= -\frac{1}{2} \nabla_i^2 - \sum_{A=1}^N \frac{Z_A}{r_{iA}} + \sum_{j=1}^{N/2} (2\hat{J}_{ij} - \hat{K}_{ij}) \end{aligned} \quad (3-32)$$

where the final term (the HF potential V_i^{HF}) is $\sum_{j=1}^{N/2} (2\hat{J}_{ij} - \hat{K}_{ij})$. The $\hat{J}_{ij}$ and $\hat{K}_{ij}$ are the Coulomb and the exchange operators, respectively. The Coulomb operator $\hat{J}_{ij}$ represents the electrostatic electron-electron repulsion energy; the exchange operator

$\hat{K}_{ij}$ has no simple physical interpretation but arises from the anti-symmetry of the wave function with respect to electron exchange.^[111]

Self-Consistent Field Procedure

The solution to the Hartree-Fock (HF) equations is obtained through the self-consistent field (SCF) procedure. This iterative algorithm starts with an initial guess for molecular orbitals (MOs) and constructs the corresponding Fock operator; the resulting eigenvalue problem is solved to yield updated orbitals, which are used to reconstruct the Fock operator. This cycle is repeated until convergence is achieved, meaning that the input and output orbitals agree within a pre-defined threshold. The final result corresponds to a stationary point of the total HF energy, representing the best single-determinant approximation under the given basis and functional form.

Basis Set Expansion

Originally, the Hartree-Fock (HF) equations were solved numerically,^[112,113] but this is only practical for small, highly symmetric systems, such as single atoms and diatomic molecules. Roothaan (1955)^[114] reformulated the HF method using a basis set expansion, making the calculation of accurate molecular self-consistent field (SCF) wavefunctions feasible. The spatial orbitals ϕ_i are expressed as linear combinations of a set of one-electron basis functions χ_s

$$\phi_i = \sum_{s=1}^b c_{si} \chi_s \quad (3-33)$$

In theory, a complete and orthonormal basis set is required for an exact representation of the molecular orbitals. In practice, if b is sufficiently large and the basis functions χ_s are well chosen, one can represent the molecular orbitals (MOs) with negligible error.

Electron Correlation and Post-Hartree-Fock Methods

The Hartree-Fock (HF) method is based on the mean-field approximation and suffers from the one-electron nature of the Fock operators. It provides an exact description of electron exchange interactions, but neglects dynamic electron correlation, i.e. instantaneous Coulomb interactions between electrons of opposite spin. As a result,

HF energy (E_{HF}) systematically overestimates the total energy. The difference between the HF energy and the exact non-relativistic energy (E_{exact}) defines the **correlation energy**^[110,111,115]

$$E_{\text{corr}} = E_{\text{exact}} - E_{\text{HF}} \quad (3-34)$$

Electron correlation energy (E_{corr}) is always negative. To recover this missing correlation, **post-Hartree-Fock** (post-HF) **methods** have been developed. There are three main methods for including electron correlation: configuration interaction (CI),^[116,117] many-body perturbation theory (MBPT, e.g. Møller-Plesset (MP) perturbation theory^[118,119]), and coupled cluster (CC)^[120], which systematically build upon the HF reference wavefunction to account for electron correlation at increasing computational cost.

The primary advantage of the Hartree-Fock (HF) method is its ability to reduce an unsolvable many-body problem into a tractable set of one-electron equations. It provides a physically intuitive molecular orbital (MO) picture and a reliable starting point for more advanced correlated methods. However, HF neglects dynamic electron correlation, leading to systematic overestimation of total energies and inaccurate descriptions of effects such as dispersion and charge transfer.

3.3.3 (Time-Dependent) Density Functional Theory

While the Hartree-Fock (HF) method is a fully ab initio wavefunction-based approach, density functional theory (DFT) introduces a conceptually distinct framework that shifts the focus from solving the many-electron Schrödinger equation to determining the molecular **electron density** $\rho(\vec{r})$, and then computing the total electronic energy as a functional of this density.

In quantum mechanics, the probability of finding an electron at a point $\vec{r}$ is determined by integrating the square of the many-electron wavefunction over the coordinates of the remaining electrons.

$$\rho(\vec{r}) = N \sum \int d^3r_2 \dots \int d^3r_N |\psi(\vec{r}_1, \vec{r}_2, \dots, \vec{r}_N)|^2 \quad (3-35)$$

This definition highlights that electron density $\rho(\vec{r})$ is a function of only three spatial coordinates, independent of the total number of electrons N . This significant reduction in dimensionality allows DFT to efficiently simulate medium- to large-size systems, in contrast to the exponential scaling of wavefunction-based methods, thereby enabling significantly reduced computational cost compared to wavefunction-based methods.

Hohenberg-Kohn Theorems

The entire field of the density functional theory (DFT) is built upon two fundamental mathematical theorems, proven by Hohenberg and Kohn and later used in the derivation of a set of equations by Kohn and Sham in 1964.^[121] The first theorem, which states that *the ground-state energy from Schrödinger's equation is a unique functional of the electron density*, relates the ground-state wavefunction with the ground-state electron density. This theorem is an existence theorem, asserting that the non-degenerate ground-state electronic energy E_0 is a functional of ρ_0 and is given by,

$$E_0 = E_0[\rho_0] = \bar{T}_s[\rho_0] + \int \rho(\vec{r})v(\vec{r})d\vec{r} + \bar{V}_{ee}[\rho_0] \quad (3-36)$$

where $\bar{T}_s[\rho_0]$ is the kinetic energy of a non-interacting reference system, and $\bar{V}_{ee}[\rho_0]$ represents the electron-electron energy. Both functionals are universal, independent of the external potential $v(\vec{r})$. However, there has been so far no practical way to calculate E_0 from ρ_0 because neither $\bar{T}_s[\rho_0]$ nor $\bar{V}_{ee}[\rho_0]$ is known.

The second theorem provides a variational principle, stating that *the true ground-state electron density minimizes the energy functional $E_v[\rho_{tr}]$* , where E_v is the energy functional for every trial density function ρ_{tr} . For any trial density ρ_{tr} , the variational principle gives

$$E_v[\rho_{tr}] \geq E_v[\rho_0] \quad (3-37)$$

This provides a practical approach for finding the ground-state density by minimizing $E_v[\rho]$.^[111] These theorems establish the theoretical justification for DFT and were later extended to degenerate systems by Levy.^[122]

Kohn-Sham Formalism

To make DFT practically applicable, Kohn and Sham proposed an auxiliary system of N non-interacting electrons that reproduces the same ground-state density as the real N interacting system, i.e. $\rho_s(\vec{r}) = \rho_0(\vec{r})$.^[123] The Hamiltonian for the auxiliary system is

$$\hat{H}_s = -\sum_{i=1}^N \left[-\frac{1}{2} \nabla_i^2 + v_s(\vec{r}) \right] \equiv \sum_{i=1}^N \hat{h}_i^{KS} \quad (3-38)$$

where $\hat{h}_i^{KS}$ is the Kohn-Sham one-electron Hamiltonian, and $v_s(\vec{r})$ is an effective potential consisting of three terms: the external potential $V_{\text{ext}}(\vec{r})$, the classical Hartree Coulomb potential $V_H[\rho](\vec{r})$, and the exchange-correlation potential $V_{\text{XC}}[\rho](\vec{r})$.

The resulting Kohn-Sham equations are

$$\left[-\frac{1}{2} \nabla^2 + V_{\text{ext}}(\vec{r}) + V_H[\rho](\vec{r}) + V_{\text{XC}}[\rho](\vec{r}) \right] \varphi_i(\vec{r}) = \varepsilon_i \varphi_i(\vec{r}) \quad (3-39)$$

where $\varphi_i(\vec{r})$ represents the spatial molecular orbitals, and ε_i is the corresponding orbital energy. In this way, the ground-state electron density can be written as

$$\rho(\vec{r}) = \sum_{i=1}^{\text{occ}} |\varphi_i(\vec{r})|^2 \quad (3-40)$$

The formulation makes Kohn-Sham equations resemble those of Hartree-Fock theory, but include an additional **exchange-correlation (XC) energy** $E_{\text{XC}}[\rho]$

$$E[\rho] = E_0[\rho] + E_{\text{XC}}[\rho] \quad (3-41)$$

The XC energy $E_{\text{XC}}[\rho]$ is defined by two unknown functionals

$$E_{\text{XC}}[\rho] = \Delta \bar{T}[\rho] + \Delta \bar{V}_{ee}[\rho] \quad (3-42)$$

where $\Delta \bar{T}[\rho]$ is the kinetic correlation energy, i.e. the difference between the true and non-interacting kinetic energies; and $\Delta \bar{V}_{ee}[\rho]$ includes non-classical electron-electron interactions. The exchange-correlation potential V_{XC} is found as the functional derivative

$$V_{\text{XC}}(\vec{r}) \equiv \frac{\delta E_{\text{XC}}[\rho(\vec{r})]}{\delta \rho(\vec{r})} \quad (3-43)$$

Exchange-Correlation Functionals

The accuracy of a density functional theory (DFT) calculation depends critically on the choice of the exchange-correlation (XC) functional. This exchange-correlation (XC) energy E_{XC} contains the following components: (i) the kinetic correlation energy $\Delta\bar{T}$; (ii) the exchange energy (arising from the anti-symmetry of the wavefunction); (iii) the Coulomb correlation energy (reflecting electron-electron repulsion) and (iv) a correction for self-interaction errors.^[111]

Local-Density Approximation (LDA)

The local density approximation (LDA) assumes that the electron density at each point in space can be treated as that of a uniform electron gas. In this approximation, the exchange-correlation (XC) energy depends solely on the local value of the electron density

$$E_{XC}^{LDA}[\rho] = \int \rho(\vec{r}) \varepsilon_{XC}[\rho(\vec{r})] d\vec{r} \quad (3-44)$$

where ε_{XC} is the exchange-correlation (XC) energy per electron in this uniform electron gas with the electron density ρ .

This quantity can be decomposed into exchange (ε_X) and correlation (ε_C) contributions

$$\varepsilon_{XC}(\rho) = \varepsilon_X(\rho) + \varepsilon_C(\rho) \quad (3-45)$$

The exchange part (ε_X) is analytically known and given by the Dirac formula^[124,125]

$$\varepsilon_X(\rho) = -\frac{3}{4} \left(\frac{3}{\pi} \right)^{1/3} (\rho(\vec{r}))^{1/3} \quad (3-46)$$

and the correlation part (ε_C) has been constructed in various approaches, such as Vosko-Wilk-Nusair (VWN),^[126] and Perdew-Wang^[127]. While LDA often yields reasonable bond lengths and vibrational frequencies, it typically overestimates dissociation energies.^[128]

Generalized Gradient Approximation (GGA)

The generalized gradient approximation (GGA) improves upon the local-density approximation (LDA) by considering an inhomogeneous electron gas. The physical idea behind GGA is to make the exchange and correlation (XC) energies dependent not only on the electron density but also of its spatial gradient^[129-131]

$$E_{XC}^{GGA}(\rho^\alpha, \rho^\beta) = \int f(\rho^\alpha(\vec{r}), \rho^\beta(\vec{r}), \nabla\rho^\alpha(\vec{r}), \nabla\rho^\beta(\vec{r})) d\vec{r} \quad (3-47)$$

where f is a model function of the α - and β -spin densities and their gradients. Similar to the LDA approach, the total exchange-correlation energy E_{XC}^{GGA} is typically decomposed into separate exchange and correlation contributions, which are treated independently through distinct functional forms.

To further improve accuracy, **meta-GGA (mGGA)** functionals incorporate second derivatives of the electron density ρ and/or the Kohn-Sham kinetic energy density $\tau_{\alpha/\beta}$

$$E_{XC}^{mGGA}(\rho^\alpha, \rho^\beta) = \int f(\rho^\alpha(\vec{r}), \rho^\beta(\vec{r}), \nabla\rho^\alpha(\vec{r}), \nabla\rho^\beta(\vec{r}), \nabla^2\rho^\alpha(\vec{r}), \nabla^2\rho^\beta(\vec{r}), \tau_\alpha, \tau_\beta) d\vec{r} \quad (3-48)$$

where the Kohn-Sham kinetic-energy density for spin- α electrons is defined as

$$\tau_\alpha = \frac{1}{2} \sum_{\text{occ}} |\nabla\theta_{i\alpha}^{\text{KS}}|^2 \quad (3-49)$$

where $\theta_{i\alpha}^{\text{KS}}$ is the occupied Kohn-Sham orbitals for spin- α electrons.^[111]

Hybrid Functionals

Hybrid functionals approximate exchange-correlation (XC) functionals by incorporating a portion of exact exchange from Hartree-Fock (HF) theory; the remaining XC energy is taken from other sources, such as ab initio or empirical methods, to improve performance.

A widely used global-hybrid GGA functional is B3LYP (the Becke, 3-parameter, Lee-Yang-Parr), with 20% HF exchange^[132,133]

$$E_{\text{XC}}^{\text{B3LYP}} = E_{\text{X}}^{\text{LDA}} + a_0(E_{\text{X}}^{\text{HF}} - E_{\text{X}}^{\text{LDA}}) + a_{\text{X}}(E_{\text{X}}^{\text{GGA}} - E_{\text{X}}^{\text{LDA}}) + E_{\text{C}}^{\text{LDA}} + a_{\text{C}}(E_{\text{C}}^{\text{GGA}} - E_{\text{C}}^{\text{LDA}}) \quad (3-50)$$

with the coefficients $a_0=0.20$, $a_{\text{X}}=0.72$, and $a_{\text{C}}=0.81$. $E_{\text{X}}^{\text{GGA}}$ (Becke^[130]) and $E_{\text{C}}^{\text{GGA}}$ (Lee et al.^[134]) are incorporated alongside with the LDA for the correlation functional $E_{\text{C}}^{\text{LDA}}$ (Vosko et al.^[126]). The B3LYP functional has been shown to perform well and remains a competitive choice for various computational chemistry applications.

The introduction of GGA and hybrid functionals in the early 1990s revolutionized computational chemistry and contributed to the awarding of the 1998 Nobel Prize in Chemistry to Walter Kohn and John A. Pople.

Dispersion and Long-Range Corrections

A major deficiency of most exchange–correlation (XC) functionals, as well as wavefunction-based methods, is their inadequate treatment of intra- and inter-molecular London dispersion interactions^[135,136] between non-bonded atoms.^[137] Such weak, non-covalent forces between non-bonded atoms play a vital role in biomolecular systems, π - π stacking in conjugated organic molecules, and van der Waals complexes such as dimers and clusters.^[137-141] To address this issue, S. Grimme and his co-workers introduced empirical dispersion corrections into DFT by adding an additional energy term

$$E_{\text{DFT-D}} = E_{\text{DFT}} + E_{\text{disp}} \quad (3-51)$$

This approach is called **DFT-D** method. Among its variants, DFT-D3 has become one of the most widely adopted versions, in which the "3" denotes its third version.^[142] This model incorporates dynamic polarizabilities obtained from first-principles time-dependent density functional theory (TD-DFT) calculations, which allow for accurate dispersion modeling in different chemical environments. Additionally, it employs fractional atomic coordination numbers for interpolation.^[143] A more recent variant, DFT-D4,^[143] further improves upon this by explicitly incorporating the actual electronic structure into the dispersion correction, offering greater accuracy. However, this improvement comes at the cost of increased computational cost and limited software support at present.

In parallel, pure exchange-correlation (XC) functionals (LDA, GGA) tend to overestimate local (short-range) contributions while underestimating non-local (long-range) contributions, due to the limitations of treating long-range Coulomb interactions explicitly. To address this, range-separated hybrid functionals partition the electron-electron Coulomb term into short-range and long-range components^[144]

$$\frac{1}{r_{12}} = \underbrace{\frac{1 - \text{erf}(\omega r_{12})}{r_{12}}}_{\text{short-range (SR)}} + \underbrace{\frac{\text{erf}(\omega r_{12})}{r_{12}}}_{\text{long-range (LR)}} \quad (3-52)$$

where ω is a range-separation parameter that controls the transition between short- and long-range contributions; and erf denotes the error function.

One prominent example is the Coulomb-attenuating method (CAM), implemented in hybrid functionals such as CAM-B3LYP. In this scheme, the Coulomb interaction is expressed as a weighted sum of HF and DFT exchange^[145]

$$\frac{1}{r_{12}} = \underbrace{\frac{1 - [\alpha + \beta \text{erf}(\omega r_{12})]}{r_{12}}}_{\text{short-range (SR)}} + \underbrace{\frac{\alpha + \beta \text{erf}(\omega r_{12})}{r_{12}}}_{\text{long-range (LR)}} \quad (3-53)$$

In this formulation, the Hartree-Fock (HF) exchange fraction is α at short range and $(\alpha + \beta)$ at long range. For reference, a global hybrid functional corresponds to $\beta = 0$ while a pure range-separated (long-range corrected) functional corresponds to $\alpha = 0$ and $\beta = 1$.

Modern long-range corrected functionals, such as ω B97X-D, incorporate CAM and empirical dispersion to provide robust performance across a wide range of molecular systems.

Time-Dependent Density Functional Theory

Density functional theory (DFT) was originally developed and limited to time-independent systems, i.e., the ground state. However, several DFT-based theories have been extended to describe excited electronic states. Among the available extensions, the most widely adopted is linear-response time-dependent DFT (LR-TD-DFT), commonly referred to as TD-DFT.

Analogous to the Hohenberg–Kohn theorem,^[121] TD-DFT is based on time-dependent perturbation theory, where a system initially in its ground state is perturbed by a weak, oscillating, and spatially uniform electric field. This theory foundation is formulated and proven by the **Runge-Gross theorem**,^[146] which states that *the time-dependent wavefunction is equivalent to the time-dependent electronic density, and the excited time-dependent electron density $\rho(\vec{r}, t)$ determines the time-dependent external potential $V_{\text{ext}}(\vec{r}, t)$ of a system.*

TD-DFT has proven reliable for calculating vertical excitation energies,^[147] and it provides good predictions for excited-state equilibrium geometries and vibrational frequencies in many systems. However, TD-DFT is limited to single excitations from the ground state to low-lying excited states, fails for molecules with long-chain conjugated π -electron systems, and struggles with excited states involving charge transfer.^[148,149] Furthermore, TD-DFT results are highly sensitive to the choice of the exchange-correlation (XC) functional. Therefore, the reliability of TD-DFT calculations must be critically evaluated against the nature of the system and benchmarked, when possible, with experimental or higher-level theoretical data.

Despite these limitations, TD-DFT offers significant advantages in calculating excited states for medium- to large-sized molecular systems, where more sophisticated methods are computationally prohibitive. However, the search for a universally suitable exchange-correlation (XC) functional remains a significant challenge.

Polarizable Continuum Model

The **polarizable continuum model (PCM)** is a widely used shape-adapted, implicit solvation model in computational chemistry for modeling solvation effects in molecular systems. Rather than representing individual solvent molecules explicitly, PCM treats the solvent as a homogeneous polarizable dielectric continuum that surrounds the solute.

This model calculates the solvation free energy (G_{sol}) as the sum over three terms

$$G_{\text{sol}} = G_{\text{es}} + G_{\text{dr}} + G_{\text{cav}} \quad (3-54)$$

where G_{es} is the electrostatic contribution, G_{dr} is the dispersion and repulsion interactions, and G_{cav} is the cavitation energy required to create a cavity in the solvent.

The cavity is constructed from interlocking van der Waals spheres centered on the solute atoms. The reaction field generated by the polarizable continuum is modeled by apparent surface charges, which interact with the electronic distribution of the solute. When coupled with quantum mechanical (QM) methods, PCM provides a computationally efficient way to incorporate major solvent effects on molecular properties and reactivity, leading to improved accuracy in spectroscopic predictions and reaction energetics in solution.^[150,151] However, PCM has limitations in precisely describing specific solute-solvent interactions such as hydrogen bonding. In such cases, more realistic explicit solvent models or mixed quantum mechanics/molecular mechanics (QM/MM) approaches may be more appropriate.

3.3.4 Molecular Dynamics

Molecular dynamics (MD) simulations provide valuable insights into the dynamic behavior of systems by allowing atoms and molecules to interact over a fixed period of time. In classical molecular dynamics simulations, the trajectories of atoms and molecules are determined by numerically solving Newton's equations of motion for a system of interacting particles. The forces between the particles and their potential energies are typically determined using inter-atomic potentials or molecular mechanics force fields. Molecular dynamics enables the computation of both equilibrium thermodynamic and dynamical properties at finite temperatures, with the accuracy of results largely dependent on the chosen force field.

The fundamental concept behind molecular dynamics is the ability to study the average behavior of a many-particle system. Central to this approach is the **ergodicity hypothesis**, which asserts that *time averages* obtained from molecular dynamics simulations are equivalent to the *ensemble averages* predicted by statistical mechanics. This allows molecular dynamics to approximate thermodynamic equilibrium without explicitly sampling every possible micro-state (or dynamical state) in the ensemble. Furthermore, ergodicity ensures that, given sufficient simulation time, molecular dynamics explores all relevant configurations in the system's phase space.

Classical molecular dynamics simulations are computationally efficient, and have been successfully applied across diverse fields, including chemical physics, materials science, and biophysics. It is widely used to study molecular diffusion, conformational changes, and interactions in large-scale systems such as membranes, nucleic acids, and proteins.^[152]

On the other hand, **ab initio molecular dynamics (AIMD)** offers an alternative approach by calculating inter-atomic forces on-the-fly using quantum mechanical (QM) methods instead of empirical force fields.^[152-154] This method allows for more accurate simulations of chemical phenomena, such as bond breaking, bond forming, and electronic polarization effects.^[152-154] However, its significantly higher computational cost limits its application to relatively smaller systems and short simulation timescales.

To address the trade-off between accuracy and computational cost, hybrid **quantum mechanics/molecular mechanics (QM/MM)** approaches have been developed.^[155] In this framework, the chemically active region of interest is treated using quantum mechanical methods such as AIMD or DFT, while the surrounding environment is described with classical force fields.^[156] This method has been successfully applied to biomolecular simulations such as studies of enzymatic mechanisms and metal-protein interactions.^[157]

Ongoing advances in algorithms and computational resources are continuously expanding the applicability and predictive power of MD-based simulations. These methods now play a pivotal role not only in academic research but also in industrial applications, such as electrolyte optimization for solid-state batteries,^[158-160] heterogeneous catalysis,^[161,162] and molecular-level insights into drug delivery.^[163,164]

4. Results and Discussion

4.1 Monitoring and Controlling Tautomerization in Single Hypericin Molecules

4.1.1 Photophysics and Photochemistry of Hypericin

Hypericin (Figure 4-1) is a naturally occurring photoactive pigment, and has been identified as an effective photoreceptor in *Hypericum perforatum* plants,^[165,166] commonly known as St. John's wort.^[167,168] Due to its anti-viral and anti-retroviral properties, hypericin has been intensively investigated in biochemical and biomedical studies. In particular, hypericin has been recognized as one of the most promising photosensitizers for photodynamic therapy (PDT).^[169,170] Upon photoexcitation, the singlet excited state (S_1) of hypericin undergoes highly efficient intersystem crossing (ISC) to the triplet manifold; subsequently enabling the generation of singlet oxygen (1O_2) via energy transfer to molecular oxygen (3O_2); this active oxygen species is primarily responsible for the therapeutic action.^[171,172] In light of its considerable pharmaceutical potential, hypericin has been a model system for fundamental investigations of its photophysics and photochemistry. In this work, a spectroscopic-computational approach is established to quantitatively explore the photophysical and photochemical mechanisms of hypericin at the single-molecule level.

The foundational research of hypericin has been systematically examined and reviewed by H. Falk and his colleagues.^[167,173-178] Earlier conformational and tautomeric analyses were carried out at the force-field theory level, identifying four conformers and ten tautomers, along with a qualitative potential energy surface and the corresponding energy barriers, similar as illustrated in Figure 4-2(a). However, such force-field-based descriptions suffer from limited accuracy, particularly for transition states. In the following, a state-of-the-art theoretical study at the (time-dependent) density functional theory ((TD-)DFT) level will be employed to quantitatively map the conformational and tautomerization processes. Furthermore,

the conformational cycle will be expanded into a three-dimensional (3D) landscape, by introducing a new coordinate for hydrogen(H)-flipping at the hydroxyl (-OH) ends. Last but not least, transition states governing the tautomerization process will be characterized and analyzed.



Figure 4-1: (a) *Hypericum perforatum*, commonly known as St John's wort,^[179] the natural source of hypericin. (b) Molecular structure of Hypericin (^PM-Q_{7,14}) featuring an extended π -conjugated system. The methyl substituents at the *bay* region induce a distinct distortion of the aromatic parent backbone.

Conformational Isomerization of Hypericin

Hypericin is a dye molecule with an extended π -conjugated system, whereby two methyl substituents at the *bay* region induce a distortion of the aromatic parent backbone (Figure 4-1(b)). Moreover, the structural diversity arises from six hydroxyl (-OH) and two carbonyl (C=O) groups, along with two methyl (-CH₃) groups at the *bay* region of the naphthodianthrone core, giving rise to multiple conformational and constitutional isomers.^[167] Following the established nomenclature,^[176] the most stable conformer is ^PM-Q_{7,14}, denoting carbonyl (C=O) groups positioned at sites 7 and 14 (Figure 4-1(b)).

In this work, a quantitative description of conformational isomerization, arising from torsions within the molecular scaffold and its substituents, was revealed.^[103] They are classified as the “propeller” (^PM-Q, ^PP-Q) and the “butterfly” (^BM-Q, ^BP-Q) types (see Figure 4-2). As discussed in the pioneering work of H. Falk and his co-workers,^[167,176,177] the conformer pairs ^PM-Q/^PP-Q and ^BM-Q/^BP-Q are isoenergetic enantiomers related by reflection symmetry, while the conformer pairs ^PM-Q/^BM-Q and ^PP-Q/^BP-Q are diastereomers. The “propeller” forms are energetically favored, with a

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lower Gibbs free energy of 0.09 eV. The conformational interconversion proceeds via two transition states: ¹T (hydroxyl-plane distortion) and ²T (methyl-plane distortion), with computed Gibbs free energy barriers of $\Delta E_{\text{Gibbs}}(^1\text{T})=2.55$ eV and $\Delta E_{\text{Gibbs}}(^2\text{T})=3.45$ eV, respectively. The larger barrier for ²T originates from the pronounced steric hindrance between the adjacent methyl substituents at the *bay* region (see Figure 4-2(b)).

In addition to the skeletal torsions, hypericin exhibits a new conformational coordinate, corresponding to the hydrogen(H)-flipping at the hydroxyl groups, see Figure 4-2(b). In this process, two hydroxyl protons alternately engage in forming an intra-molecular hydrogen (H) bond with neighboring hydroxyl oxygen atoms, modulating the directions of the H-bond (upwards vs. downwards). Owing to the C₂ molecular symmetry of hypericin, proton transfer in this bond-shifting process leads to a structurally equivalent species. The energy barriers in this coordinate are estimated to be 1.50 eV for “propeller” and 2.48 eV for “butterfly” conformers, respectively.

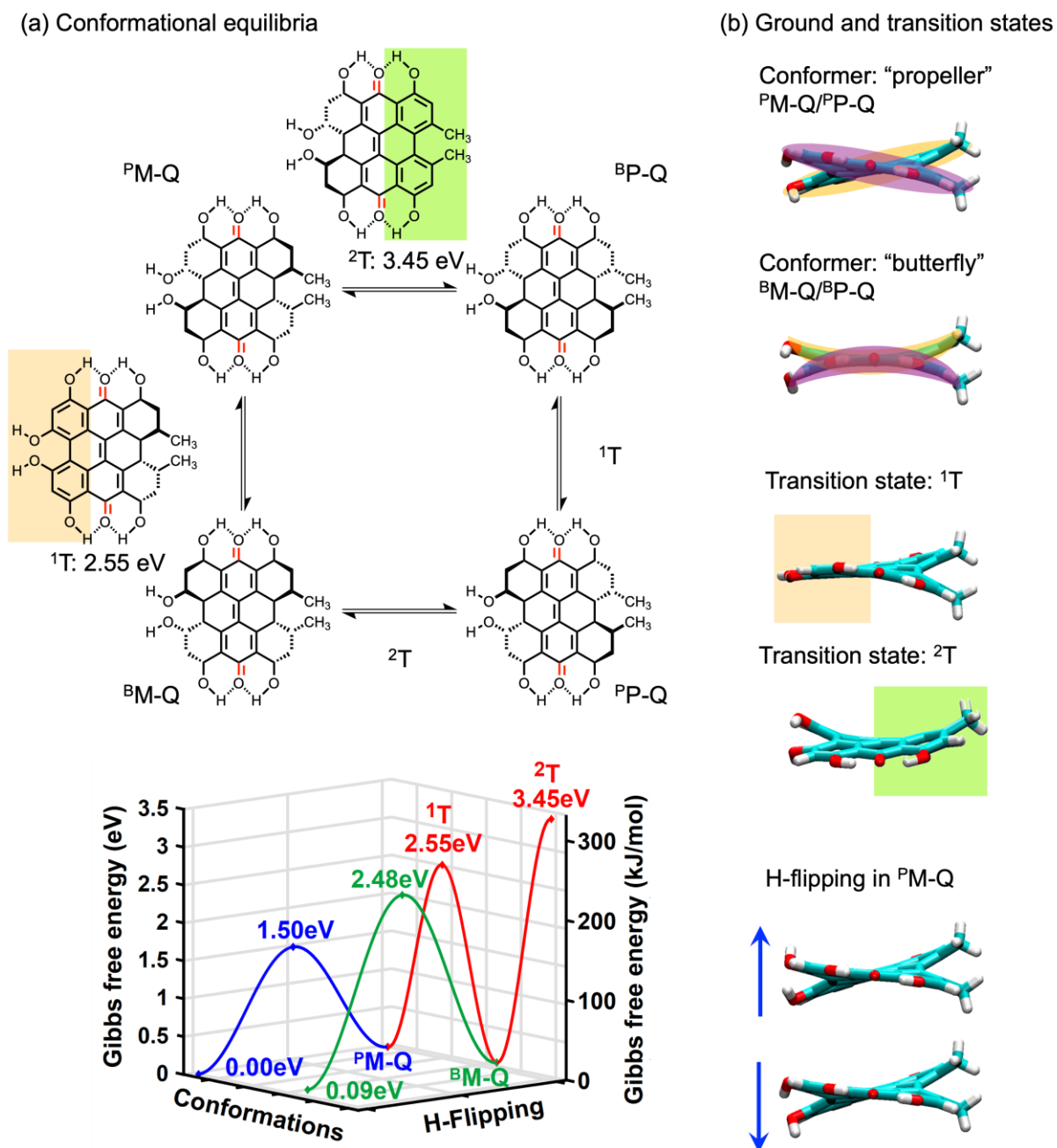


Figure 4-2: (a) Conformational equilibria of the most stable neutral conformers of hypericin with carbonyl (C=O) groups at positions 7 and 14 (highlighted in red). The upper panel illustrates the conformational isomerization cycle, including transition states (1T and 2T). The lower panel introduces a newly identified coordinate of hydrogen(H)-flipping at the hydroxyl ends at the bay region. Reproduced from [Q. Liu et al., Adv. Photonics Res 2021](#),^[103] with the permission of © Wiley-VCH. (b) Optimized ground- and transition-state geometries of the relevant conformers. The "propeller" and "butterfly" forms arise from torsional distortions of the molecular backbone and its substituents. The H-flipping occurs at the bay region, yielding a structurally equivalent species due to C_2 symmetry. Calculations were performed at the D3-B3LYP/6-311G(d) level.

Tautomerization of Hypericin

The peripheral protons of hypericin can undergo structural interconversion, commonly referred to as tautomerization, resulting in the formation of multiple constitutional isomers (tautomers). Previous computational studies, as well as our own work, identified ten energetically accessible tautomers, interconnected via single-proton transfer pathways (see Figure 4-3).^[92,103,173,176,177] Due to the low energy barriers and the fast interconversion rates, experimental evidence for tautomerization has historically remained elusive; neither direct observation nor indirect identification through conventional characterization techniques has been able to resolve distinct tautomers or capture their dynamic interconversions. However, quantum-chemical calculations can provide mechanistic insights by resolving the potential energy surface (PES) that governs such interconversions.

Conceptually, tautomerization can be classified into two categories depending on the origin of the proton donor: intra- and inter-molecular tautomerization. Figure 4-3 illustrates the ten possible tautomeric structures. Starting from the thermodynamically most stable tautomer ^PM-Q_{7,14}, the migration of a *peri*-positioned proton to an adjacent carbonyl oxygen results in the formation of a new hydroxyl group, leading to five intra-molecular tautomers (blue structures in Figure 4-3). In contrast, inter-molecular tautomerization is mediated by an external proton donor, such as a proton-rich host; herein, proton migration occurs not only at the *peri* but also at the *bay* region (green structures in Figure 4-3).

Theoretical studies provide a clear picture of the geometrical and electronic description of these tautomers (for details, see Table 4-1). Two key torsional angles, θ_{OH} and θ_{Me} , are defined to describe the degree of structural distortion in the parent backbone (see Figure 4-3). This distortion is induced by steric hindrance between hydroxyl and methyl substituents at the *bay* region. The Gibbs free energies E_{Gibbs} and the relative stabilities ΔE_{Gibbs} for all tautomers were systematically investigated, as summarized in Table 4-1. Among these accessible species, ^PM-Q_{1,7}, ^PM-Q_{7,13}, and ^PM-Q_{3,7} exhibit the lowest relative Gibbs free energy ($\Delta E_{Gibbs} \approx 0.4\text{--}1.3$ eV) and are therefore expected to actively participate in the dynamic tautomerization network.

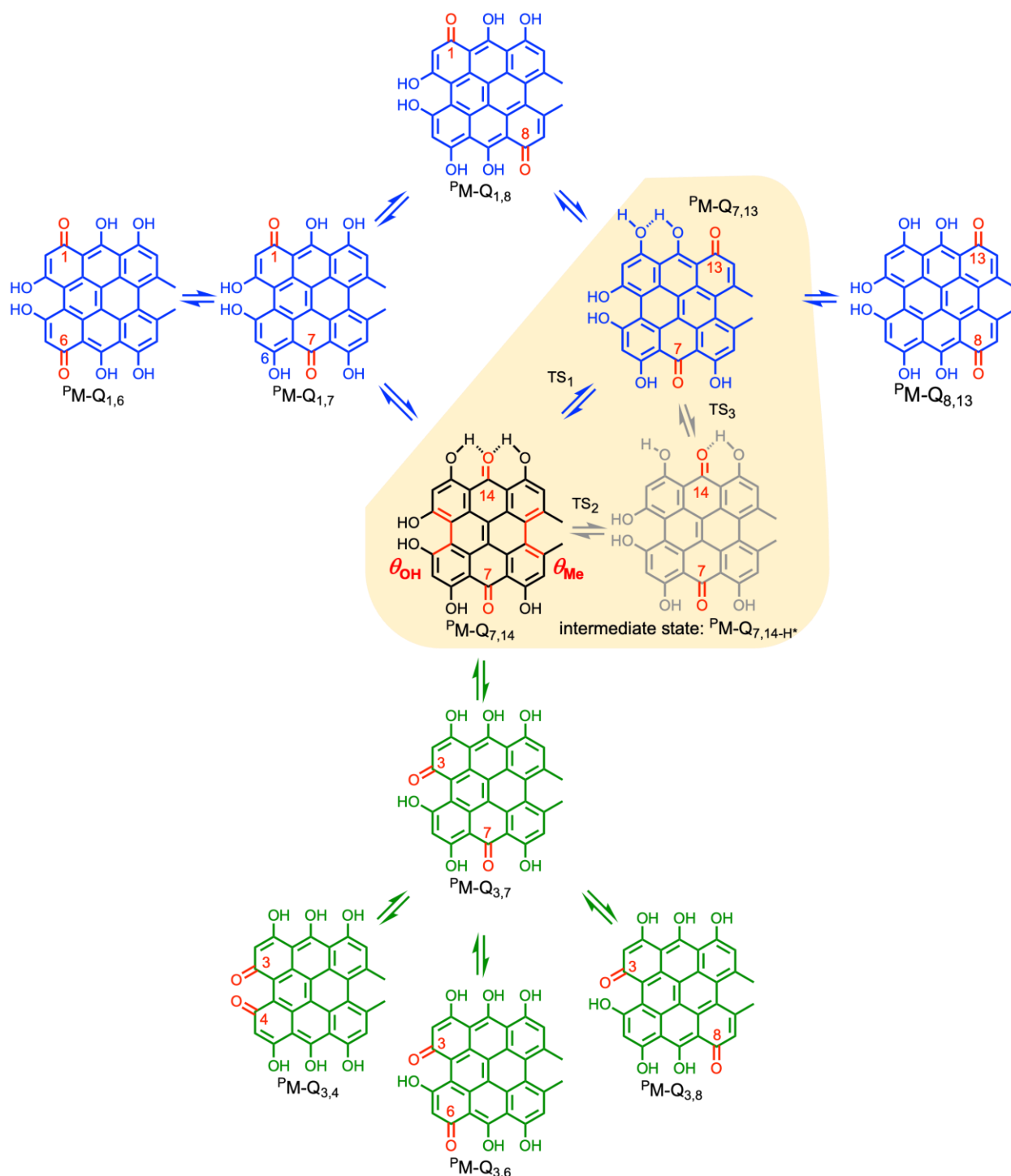


Figure 4-3: Intra-molecular [blue] and inter-molecular [green] tautomerization network of hypericin. The thermodynamically most stable tautomer, ${}^{\text{PM}}\text{-Q}_{7,14}$, defined as the carbonyl (C=O) groups at positions 7 and 14, serves as the central species. Intra-molecular proton transfer (blue arrows) yields five tautomers, while additional tautomers (green structures) require inter-molecular proton donation, typically facilitated by the local environment. The orange panel highlights the tautomerization between ${}^{\text{PM}}\text{-Q}_{7,14}$ and ${}^{\text{PM}}\text{-Q}_{7,13}$, accessible via (i) a single-proton transfer pathway with a higher energy barrier of 1.58 eV; and (ii) a stepwise two-proton transfer mechanism involving an intermediate state ${}^{\text{PM}}\text{-Q}_{7,14}\text{-H}^*$ (gray) and two lower energy barriers of 0.79 eV and 1.10 eV. Two key torsional angles (θ_{OH} , θ_{Me} , defined in Scheme 4-1) that reflect the parent backbone distortion are defined in ${}^{\text{PM}}\text{-Q}_{7,14}$. Calculations were performed at the D3-B3LYP/6-311G(d) level.

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Table 4-1: Optimized geometries and relative electronic, Gibbs free energies of five intra-molecular [blue] and four inter-molecular [green] tautomers of $^{\text{P}}\text{M-Q}_{7,14}$. An intermediate state $^{\text{P}}\text{M-Q}_{7,14-\text{H}^*}$ is given to elucidate the possible intra-molecular tautomerization pathways between $^{\text{P}}\text{M-Q}_{7,14}$ and $^{\text{P}}\text{M-Q}_{7,13}$. The dihedral angles θ_{OH} and θ_{Me} represent torsional distortions induced by hydroxyl and methyl groups, respectively, at the *bay* region of the hypericin backbone. These angles reflect deviations from planarity and are key indicators of local structural deformation. The definitions and positions of these angles are illustrated in Scheme 4-1. Calculations were performed in vacuum and in methanol (MeOH, PCM model) at the D3-B3LYP/6-311G(d) level of theory.

Tautomer $^{\text{P}}\text{M-Q}$	ΔE_{elec} (eV)	ΔE_{elec} (kJ/mol)	ΔE_{Gibbs} (eV)	ΔE_{Gibbs} (kJ/mol)	θ_{OH} (°)	θ_{Me} (°)
vacuum						
1,6	2.70	259.43	2.56	245.79	27	36
1,7	1.37	131.50	1.30	124.79	27	34
1,8	2.83	271.23	2.67	256.76	27	33
7,13	1.43	137.25	1.34	128.47	29	31
7,14 (ref.)	0	0	0	0	28	33
8,13	2.69	257.98	2.52	242.34	30	30
3,4	1.74	167.46	1.68	161.59	29	34
3,6	1.90	182.22	1.79	171.43	24	36
3,7	0.44	42.23	0.39	37.06	22	34
3,8	1.81	173.62	1.68	160.86	23	33
7,14-H* (intermediate state)	0.64	61.16	0.61	58.48	29	33
MeOH						
1,6	1.97	189.09	1.88	180.61	27	36
1,7	1.01	97.12	0.96	92.15	27	34
1,8	2.19	210.65	2.08	199.60	29	33
7,13	1.07	102.97	1.00	95.71	28	32
7,14 (ref.)	0	0	0	0	27	33
8,13	2.01	193.06	1.89	181.49	29	31
3,4	1.29	123.94	1.25	119.73	30	34
3,6	1.30	124.35	1.21	116.30	25	36
3,7	0.25	24.28	0.20	19.35	22	34
3,8	1.32	127.10	1.20	115.14	23	34
7,14-H* (intermediate state)	0.47	45.17	0.46	43.81	28	34

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Table 4-2: Optimized geometries and relative electronic, Gibbs free energies of transition states, involved in the intra-molecular tautomerization between $^P\text{M-Q}_{7,14}$ and $^P\text{M-Q}_{7,13}$. Two mechanistic pathways are considered: (i) a single-proton pathway via TS_1 ; and (ii) a stepwise two-step pathway via an intermediate state $^P\text{M-Q}_{7,14-\text{H}^*}$ with two transition states: TS_2 and TS_3 . The dihedral angles θ_{OH} and θ_{Me} represent torsional distortions induced by hydroxyl and methyl groups, respectively, at the *bay* region of the hypericin backbone. The definitions and positions of these angles are illustrated in Scheme 4-1. Calculations were performed in vacuum and in methanol (MeOH, PCM model) at the D3-B3LYP/6-311G(d) level of theory.

Tautomer $^P\text{M-Q}$	ΔE_{elec} (eV)	ΔE_{elec} (kJ/mol)	ΔE_{Gibbs} (eV)	ΔE_{Gibbs} (kJ/mol)	θ_{OH} (°)	θ_{Me} (°)
vacuum						
TS_1	1.67	159.87	1.58	151.74	26	30
TS_2	0.86	82.81	0.79	75.98	28	33
TS_3	1.19	113.91	1.10	105.47	28	31
MeOH						
TS_1	1.45	138.75	1.38	132.19	25	30
TS_2	0.74	70.88	0.67	64.60	29	33
TS_3	1.08	103.25	0.98	94.53	27	31

In a second step, an investigation was conducted on the transition states (TS) associated with the representative intra-molecular tautomeric pair, $^P\text{M-Q}_{7,14} \rightleftharpoons ^P\text{M-Q}_{7,13}$. As illustrated in the beige panel of Figure 4-3, two distinct pathways become possible. The *single-proton pathway* proceeds via the transition state TS_1 , and encounters a substantial barrier of 1.58 eV due to the pronounced geometrical distortion observed at position 14. Alternatively, this process can proceed via a *two-step mechanism* involving an intermediate state ($^P\text{M-Q}_{7,14-\text{H}^*}$), where the first proton transfer occurs at position 1 with an energy barrier (TS_2) of 0.79 eV, followed by a second proton transfer at position 14 with an energy barrier (TS_3) of 1.10 eV (Table 4-1 and Table 4-2). This *two-step* pathway, characterized by minimal geometrical distortions ($\Delta\theta_{\text{OH}}, \Delta\theta_{\text{Me}} \approx 1^\circ$) at the *bay* region, is energetically preferred and is more likely to prevail in experiments. These angles quantify deviations from planarity and are defined in Scheme 4-1.

In summary, quantum-chemical calculations provide a detailed understanding of the stereochemistry of hypericin, including conformational isomerization, tautomerization, as well as the corresponding potential energy landscape and transition states. However, the direct experimental verification of tautomerization dynamics and the identification of individual tautomers remain challenging due to their subtle structural variations and fast interconversion rates. This limitation calls for advanced experimental strategies, as addressed in the following section.

4.1.2 Monitoring Tautomerization in Single Hypericin Molecules³

This section is centered on a spectroscopic-computational approach to monitor tautomerization dynamics of hypericin at the single-molecule level. Tautomerization, driven by the rearrangement of the carboxyl (C=O) and hydroxyl (-OH) groups, results in a distinct reorientation of the S_0 - S_1 electronic transition dipole moment (TDM). By direct observation of TDMs by our home-built confocal microscope with higher-order laser modes, in combination with quantum-chemical calculations for accurate orientation information, individual tautomeric species can be unambiguously identified.

Our home-built single-molecule imaging and spectroscopy experiments were performed at ambient conditions using our home-built setup, for instrumentation details, see Figure 3-2. Figure 4-4(a) illustrates a typical fluorescence image of single hypericin molecules at a $10 \times 10 \mu\text{m}^2$ scale. This fluorescence image presents isolated diffraction-limited bright spots with Gaussian intensity profiles; the close-up views on the right further reveal the single-molecule behaviors, including photobleaching and fluorescence intensity fluctuations. The corresponding spectroscopic data (Figure 4-4(b)) exhibit the characteristic photoblinking and the subsequent photobleaching. Such variations in emissivity (or dynamic intensity fluctuations) can be attributed to the (temporal) dark states, primarily involving triplet-state transitions or other less- or non-emissive states.^[180] Photon antibunching measurements confirm the presence of single molecules within a minimum spatial distance of $1 \mu\text{m}$.

³ The experimental work in this section was performed by Dr. Quan Liu and Dr. Frank Wackenhut.

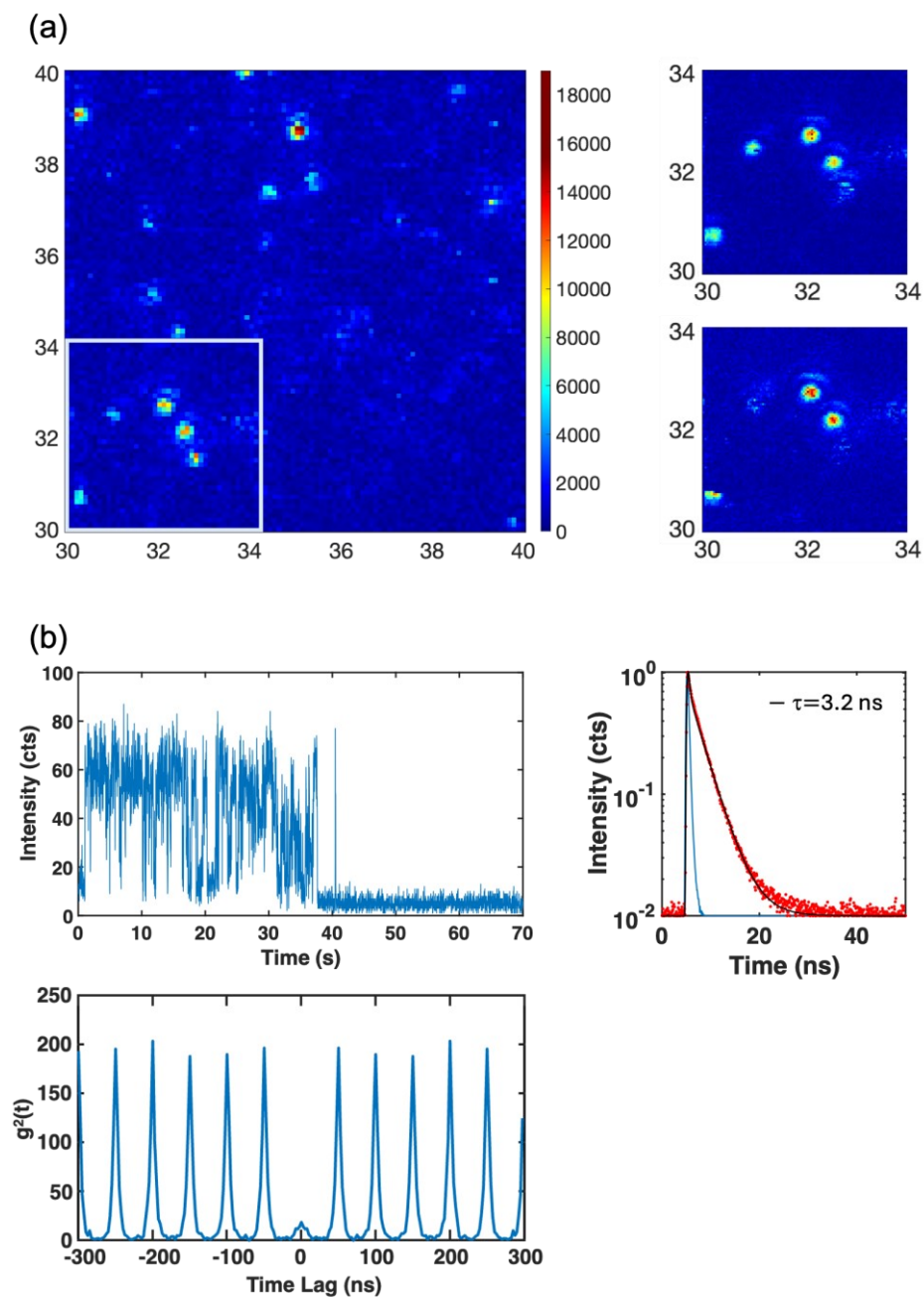


Figure 4-4: (a) Typical fluorescence images of single hypericin molecules embedded in PVA acquired by our home-built single-molecule confocal microscope ($10 \times 10 \mu\text{m}^2$ scan area, 10 ms per pixel). The isolated diffraction-limit spots exhibit Gaussian intensity profiles. The close-up images (right) highlights the characteristic single-molecule features, including long-lived dark state and (stepwise) fluorescence intensity fluctuations. Reproduced from [L. Wang et al., ChemPhysChem 2024](#)^[2], with the permission of © Wiley-VCH. (b) Representative fluorescence intensity time trace showing characteristic photoblinking behavior under continuous excitation, with an abrupt transition to a dark state (the corresponding fluorescence lifetime is $\tau_F = 3.2 \text{ ns}$). Photo antibunching confirm the presence of single hypericin molecules embedded in PVA matrix under ambient conditions.

When embedded in a rigid polymer matrix, hypericin exhibits considerably slowed tautomerization compared to its rapid dynamics (up to MHz) in solution, allowing individual tautomers to persist for seconds or minutes.^[1,89,91,92,103,181-184] Stepwise fluorescence intensity variations (Figure 4-4(b)) serve as signatures of tautomerization events. The fixed molecular orientation within the polymer leads to TDM reorientations during tautomerization, resulting in apparent fluorescence fluctuations due to their varying alignment with the excitation field.

In order to determine the absolute TDM orientation, azimuthally (APDM) and radially (RPDM) polarized doughnut modes were employed.^[92] As shown in Figure 4-5, an abrupt image pattern flip accompanied by fluorescence intensity changes, independent of lifetime changes, indicates tautomerization events. The APDM exclusively probes the in-plane TDM component, while RPDM reveals both in-plane and out-of-plane components. The shape and size of the asymmetric two-lobe pattern observed in APDM and RPDM allow for a full three-dimensional (3D) reconstruction of the TDM orientation.

Figure 4-5 shows a sequence of images for three representative molecules (arranged in pairs: a1-a2, b1-b2, c1-c2, d1-d2). Molecule 1 exhibits stable patterns, indicating the absence of tautomerization. Molecule 2 displays ambiguous ring shapes in a1 and c1, suggesting that tautomerization occurs faster than the image acquisition time. For molecule 3, four distinct excitation patterns are clearly distinguished, confirming the coexistence of the four stable tautomers of hypericin. Correlating experimental observations with quantum-chemical calculations and applying a rotation matrix to the ensemble of the TDMs of all potential tautomeric species, the tautomerization trajectory was determined in accordance with the imaging sequence: ${}^{\text{P}}\text{M-Q}_{3,7} \rightarrow {}^{\text{P}}\text{M-Q}_{1,7} \rightarrow {}^{\text{P}}\text{M-Q}_{7,14} \rightarrow {}^{\text{P}}\text{M-Q}_{7,13}$.

This spectroscopic-computational approach marks the first successful direct observation of single-molecule tautomerization. It establishes a solid foundation for further advancing this methodology to control tautomerization rates and systematically investigate nano-environmental effects, as elaborated in the following sections.

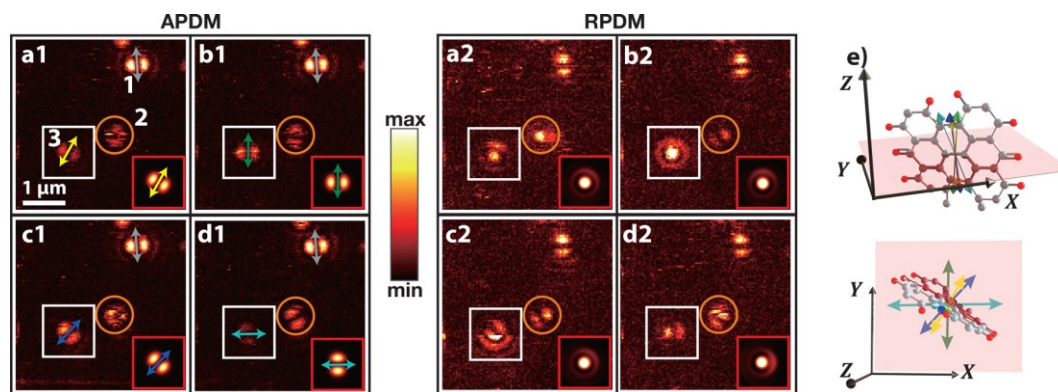


Figure 4-5: Representative excitation patterns of three single hypericin molecules embedded in PVA, recorded using azimuthally (APDM) and radially (RPDM) polarized doughnut modes ($10 \times 10 \mu\text{m}^2$ scan area, 10 ms per pixel). Panels a1-d1 are excited with APDM, selectively probing the in-plane TDM component, while panels a2-d2 display RPDM excitation, revealing both in-plane and out-of-plane TDM components. Molecule 1 exhibits stable patterns, indicating the absence of tautomerization. Molecule 2 shows ambiguous ring-shaped patterns, suggesting that tautomerization occurs faster than the acquisition time. Molecule 3 displays four distinct excitation patterns, corresponding to the four different tautomers. Inset images in red squares ($1.2 \times 1.2 \mu\text{m}^2$) present simulation results. Colored arrows correspond to the transition dipole moments (TDMs) of the tautomers. (e) The TDM orientation of Molecule 3 and its in-plane projection, which reveal the reorientation associated with tautomerization. Reprinted with permission from [Q. Liu et al., J. Phys. Chem. Lett. 2021, 12, 3, 1025](#).^[92] © 2021 American Chemical Society.

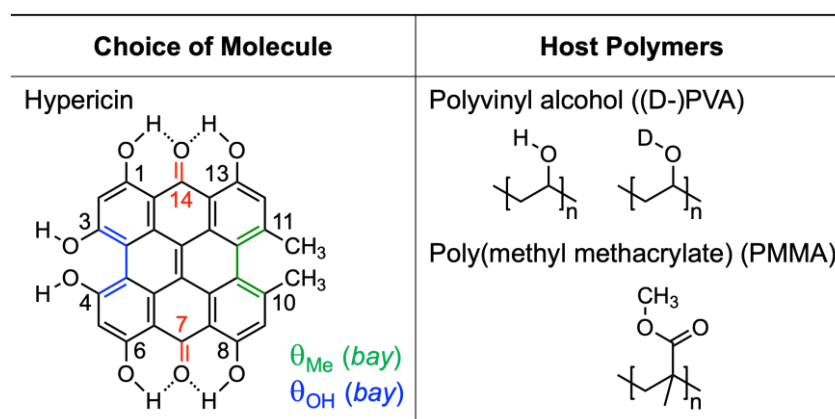
4.1.3 Controlling Tautomerization in Single Hypericin Molecules

Proton migration around the perimeter (the “outer” part) renders hypericin highly sensitive to interactions with its local (nano-)environment. Since tautomerization proceeds via quantum tunneling, the available residence time in the excited state directly governs the probability of proton transfer. Thus, controlling the excited-state fluorescence lifetime provides an effective strategy to regulate tautomerization rates. This section demonstrates two complementary approaches: (i) chemical modification via different polymer hosts and isotope effects, and (ii) photonic modification via the embedding hypericin inside a $\lambda/2$ Fabry-Pérot microcavity.

Chemical Modification via Polymer Hosts and Isotope Effects

The local chemical environment, as reflected by macroscopic parameters such as the dielectric constant, oxygen permeability and solvent and/or water content of the polymer, can play a significant role in influencing the excited-state lifetime, dark state dynamics and probe survival time (defined as the duration before a long-lived dark

state or a total photobleaching).^[2,182] From a computational perspective, these observations point to the geometrical and electronic changes that occur in the excited states. This section is dedicated to the investigation of specific probe-host interactions. For this purpose, PVA and PMMA were selected due to their transparency and high light transmittance (Scheme 4-1).



Scheme 4-1: Chemical structures of hypericin and the host polymers: PVA, deuterated PVA (D-PVA), and PMMA. Two key dihedral angles at the bay region of hypericin, θ_{Me} (methyl, green) and θ_{OH} (hydroxyl, blue) groups, are highlighted.

The ensemble fluorescence lifetime of hypericin embedded in a PVA polymer host was reported by us earlier,^[182] giving an average lifetime $\tau_F = 4.2 \pm 0.9$ ns and a quantum yield of $\Phi_F = 12\% \pm 1\%$. Such ensemble values have been utilized as benchmarks for subsequent single-molecule studies. In stark contrast, single-molecule spectroscopic data have revealed substantial heterogeneity, arising from specific probe-host (hypericin-polymer) interactions and variations in molecular orientation.

To systematically modulate the local chemical nano-environment, hypericin was embedded into two representative polymer hosts: polyvinyl alcohol (PVA) and poly(methyl methacrylate) (PMMA). The most notable feature of PVA is the prevailing hydroxyl group (see Scheme 4-1), which can form hydrogen (H/D) bonds with hypericin; thus facilitating a substantial non-covalent interaction between the probe and the polymer host. Notably, stepwise fluorescence intensity fluctuations observed prior to the long-lived dark state, as shown in Figure 4-6(a), indicate tautomerization events. These intensity changes result from the reorientation of the transition dipole moment (TDM) due to proton migration, directly reflecting the tautomerization process at the single-molecule level. In contrast, PMMA can be considered as an “innocent” polymer host, as it lacks functionalities that cause specific strong probe-host

interactions; however, its low oxygen permeability limits the photobleaching, while enhancing its photostability (see Figure 4-6).^[185] PVA is higher in polarity. Hosts with a higher dielectric constant generally lead to a higher reaction field in response to the static electric dipole moment of an embedded molecule (for further details, see Ref.^[186-188] and literature cited therein). For simplification, we denote the systems as H- or D-hypericin in H- or D-PVA, H- or D-hypericin in PMMA.

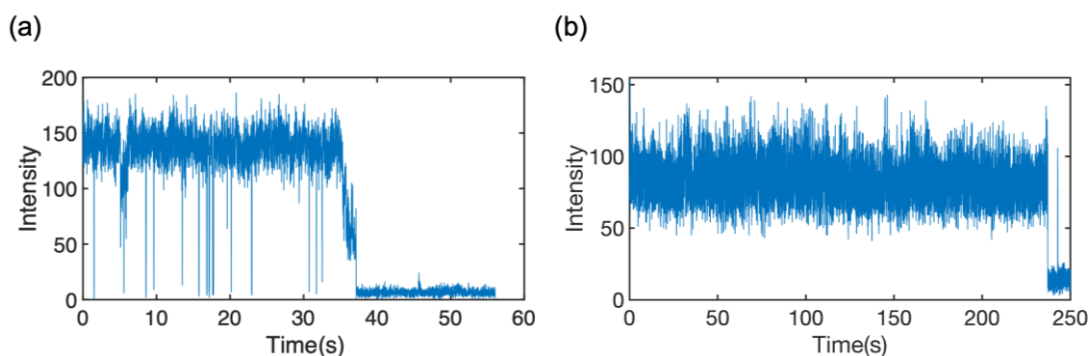


Figure 4-6: Representative fluorescence intensity time traces of single D-hypericin molecules embedded in (a) D-PVA and (b) PMMA under ambient conditions. The traces show characteristic single-molecule photoblinking behavior, with D-PVA exhibiting stronger photoblinking compared to PMMA. In (a), stepwise fluorescence intensity fluctuations observed prior to the long-lived dark state indicate tautomerization dynamics.

To quantitatively evaluate the single-molecule nano-environmental and isotope effects on hypericin, statistical analysis of survival time and photoblinking dynamics was performed (Figure 4-7). Here, the survival time is defined as the period until the long-lived dark state (and/or irreversible photobleaching) occurs, while the photoblinking behavior is characterized by the on-off (bright-dark) ratio, expressed as the proportion of the “on” period (bright state) relative to the total survival time. A reduced on-off ratio indicates more pronounced photoblinking. As illustrated in Figure 4-7, hypericin in PMMA exhibits significantly longer survival times and higher on-off ratios compared to PVA, for both H- and D-hypericin species. Deuteration further reduces the survival time and enhances photoblinking, indicative of its impact on non-radiative decay pathways (*vide infra*).

Figure 4-8 complements these findings by providing fluorescence lifetime distributions, which clearly show longer lifetimes for hypericin in PMMA relative to PVA, with a further moderate increase upon deuteration. These observations collectively highlight the critical role of both polymer hosts and isotope effects in modulating the excited-state dynamics of hypericin.

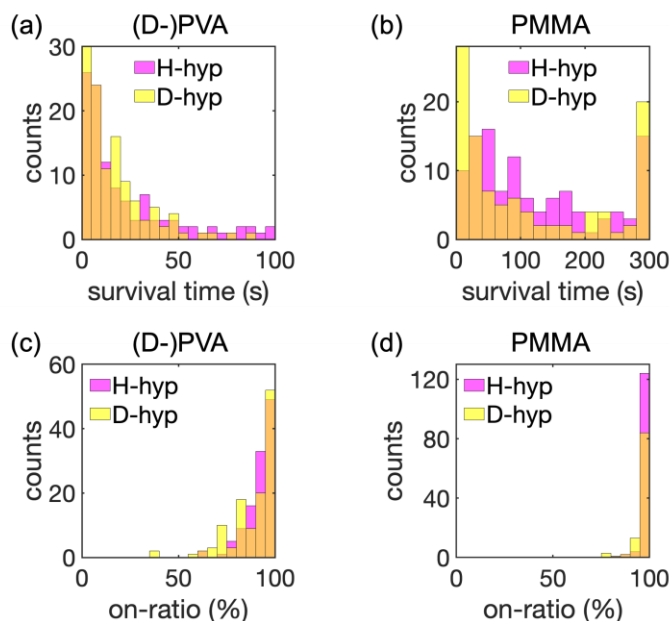


Figure 4-7: Statistical analysis of photoblinking and survival times of single H-(D-)hypericin embedded in different polymer hosts. (a,b) Histograms of the on-off (bright-dark) ratios, quantifying the fraction of the total survival time spent in the on (bright) state; (c,d) Survival time distributions. Hypericin in PMMA exhibits significantly longer survival times and higher on-off ratios than in PVA, indicating suppressed photoblinking and enhanced photostability. Deuteration further shortens survival times and enhances photoblinking dynamics. Sample size: $n=115$ (H-Hyp/H-PVA), $n=121$ (D-Hyp/D-PVA), $n=131$ (H-Hyp/PMMA), $n=104$ (D-Hyp/PMMA). Orange bars represent overlapping values. Reproduced from [L. Wang et al., ChemPhysChem 2024](#),^[2] with the permission of © Wiley-VCH.

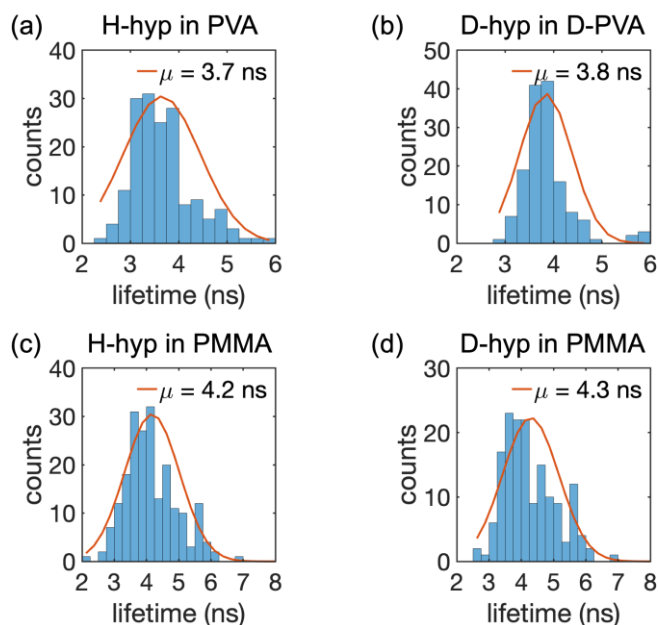


Figure 4-8: Histograms of derived single-molecule fluorescence lifetimes τ_F for single H/D-hypericin molecules in (a) PVA ($n=172$), (b) D-PVA ($n=147$), (c) PMMA ($n=175$) and (d) PMMA ($n=159$). The red curves correspond to fitted normal distributions associated with the mean values (μ). Deuteration leads to a modest increase in lifetime ($\Delta\tau_F \approx 0.1$ ns) due to reduced internal conversion (IC), in line with the calculated zero-point energy reduction of 0.37 eV. This subtle isotope effect is particularly evident in PVA due to additional probe-host H-(D-) bonds. Reproduced from [L. Wang et al., ChemPhysChem 2024](#),^[2] with the permission of © Wiley-VCH.

The observed host-dependence and isotopic effects can be rationalized by the interplay of inter- and intra-molecular effects. The inter-molecular contributions primarily originate from specific interactions between hypericin and the polymer host. To elucidate such interactions, ground- and transition-state geometries of hypericin were optimized using both implicit methanol (MeOH, PCM model) and explicit polymer cluster models (PVA, PMMA). The results reveal that H-(D-)hypericin embedded in H-(D-)PVA experiences pronounced structural distortion, particularly at the *bay* region. The formation of the inter-molecular H-(D-)bonds between H-(D-)hypericin and the hydroxyl groups of H-(D-)PVA restricts the conformational freedom of hypericin, leading to a more distorted geometry compared to that in PMMA. These geometrical differences are particularly evident in the calculated dihedral angles (defined at the *bay* and *peri* regions, see Scheme 4-1), which directly characterize the deformation of the aromatic scaffold of hypericin (see Table 4-3).

Table 4-3: Calculated hypericin geometries at the ground (GS) and the transition (TS) state in methanol (MeOH, PCM), and explicit polymer cluster models (PVA, PMMA), using D3-B3LYP/6-31G(d) level of theory. Dihedral angle definitions are provided in Scheme 4-1.

Dihedral Angle (°)	MeOH (PCM)		PVA		PMMA	
	GS	TS	GS	TS	GS	TS
θ_{Me} (<i>bay</i>)	33	34	40	45	36	37
θ_{OH} (<i>bay</i>)	27	27	24	25	29	30
θ_{OH} (<i>peri</i>)	1	102	8	120	3	113

The intra-molecular contribution arises from the deuterium isotope effect. Although deuteration causes only minor structural changes, it significantly alters the non-radiative decay pathways.^[189-191] The non-radiative decay component (k_{nr}) comprises internal conversion (IC), vibrational relaxation (VR), intersystem crossing (ISC) towards the triplet manifold, and other photochemical channels such as tautomerization. Deuteration reduces the zero-point vibrational energy of 0.37 eV, and consequently affects vibrationally mediated processes such as internal conversion (IC),^[192] while leaving the intersystem crossing (ISC) channel essentially unaffected.^[36] The rate k_{IC} can be estimated from Fermi's golden rule.^[9] Here, k_{IC} decreases with decreasing vibrational frequency, so that k_{IC} decreases upon deuteration because of smaller energy spacing between vibrational quanta.^[14] In contrast, deuteration shows

a negligible effect on the radiative rate (k_r) as it is related to the oscillator strength (f) and energy (E) of the emitting state via the Strickler-Berg relation,^[193] which remain largely unchanged upon deuteration.

Despite the suppression of IC, the observed isotope effect on the fluorescence lifetime $\tau_F = 1/(k_r + k_{nr})$ of hypericin and related aromatic hydrocarbons^[194] is modest. This can be rationalized by the dominant role of ISC ($k_{ISC} \gg k_{IC}$) in hypericin, consistent with its efficient population of triplet states.^[195]

Photonic Modification via a $\lambda/2$ Fabry-Pérot microcavity⁴

In addition to chemical control, tautomerization of hypericin can be photonically modulated by tailoring the radiative decay rate k_r via the Purcell effect. This is achieved by enclosing molecules inside a $\lambda/2$ Fabry-Pérot microcavity, which selectively alters the local density of optical states (LDOS). The microcavity consists of two parallel mirrors (see Figure 4-9(a)), with an adjustable spacing d that can be finely tuned to fulfill the resonance condition between the cavity mode and the hypericin emission. The cavity mirror spacing d can be determined from the transmission spectra.

To construct the microcavity (Figure 4-9(a)), the bottom mirror was prepared on a cleaned cover slip by sequentially evaporating 1 nm Cr, 30 nm Ag, and 50 nm SiO₂, while the top mirror was a convex lens with a 25.8 mm curvature radius covered with 1 nm Cr, 50 nm Ag, and 50 nm SiO₂ layers, respectively. The Cr layer serves as an adhesion layer, while the SiO₂ layer acts as a spacer to protect the two mirrors from mechanical damage and to avoid quenching of the molecule on top of the silver film.

Figure 4-9(c) shows single-molecule fluorescence lifetime distributions of single hypericin molecules measured under different cavity spacings d . The distribution recorded under free-space conditions shown in Figure 4-9(b) serves as a reference for quantifying cavity-induced lifetime modulation. When the cavity is tuned to be on resonance with the emission spectrum of hypericin, a significant reduction in the

⁴ The transfer matrix method (TMM) simulation in this section was performed by Dr. Quan Liu and Dr. Frank Wackenhut.

excited-state lifetime is observed compared to free-space conditions, consistent with the Purcell effect.

Figure 4-9(d) further compares experimental results with simulations as a function of cavity spacing d . The Purcell effect can either reduce or increase the fluorescence lifetime by up to threefold, depending on whether the emitted photons are on or off resonance within the microcavity. In this simulation, the non-radiative rate (k_{nr}) is assumed to remain constant, and only the radiative rate (k_r) is modulated by the Purcell effect. The observed agreement between experiment and simulation confirms that the lifetime reduction originates exclusively from the Purcell-enhanced radiative decay. Two lifetime minima, located at cavity spacings of 170 nm and 390 nm, correspond to the Purcell factor maxima. The reduced fluorescence lifetime effectively limits the residence time available for proton transfer, thereby suppressing the tautomerization.

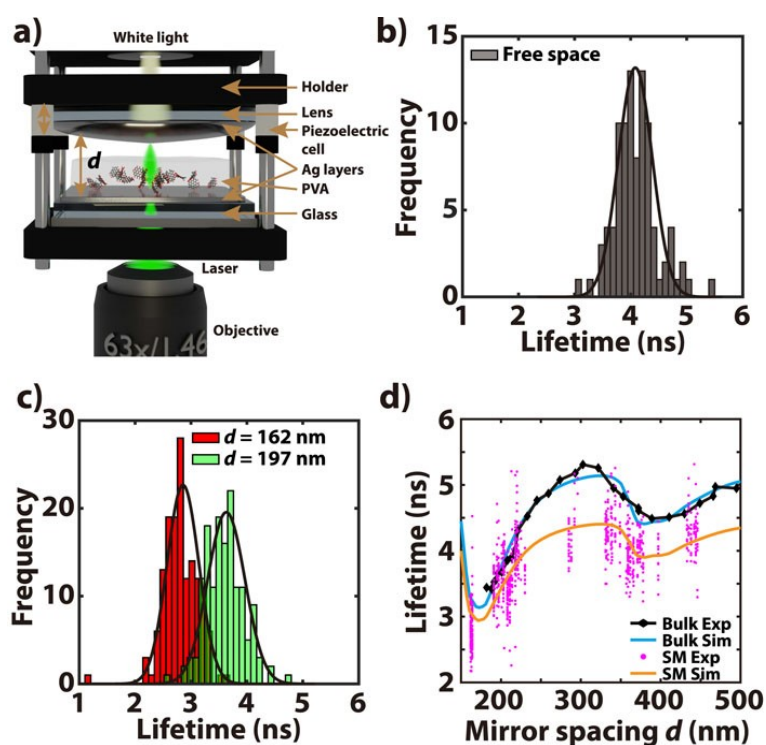


Figure 4-9: (a) Schematic drawing of a $\lambda/2$ microcavity embedding single hypericin molecules in a PVA matrix between two silver mirrors. Fluorescence lifetime histograms of single hypericin molecules in (b) free space, as reference; and (c) the microcavity at two representative mirror spacings: 162 nm [red] and 197 nm [green], both demonstrating a fluorescence lifetime reduction under resonance conditions. (d) Fluorescence lifetime of the ensemble (10^{-5} M, black curve) and single hypericin molecules (10^{-9} M, magenta dots) as a function of mirror spacing d . The solid curves are the best fit to the respective experimental data of the ensemble (blue curve) and single molecules (orange curve). Reproduced from [L. Wang et al., J. Chem. Phys. 7 January 2022; 156 \(1\): 014203](#),^[1] with the permission of AIP Publishing.

Beyond its impact on fluorescence lifetimes, the anisotropic local density of optical states (LDOS) within the microcavity strongly affects transition dipole moments (TDMs, $\vec{\mu}$) depending on their orientation. As predicted by Fermi's golden rule,^[53] the radiative rate (k_r) of a molecule, represented by the TDM $\vec{\mu}$, can be calculated as

$$k_{r,c}(d, z) \propto S(\omega) |\vec{\mu} \cdot \vec{\epsilon}_c|^2 \rho(\omega, d, z) \quad (4-1)$$

where the fluorescence spectrum $S(\omega)$ of hypericin is obtained experimentally;^[182] the cavity mode field $\vec{\epsilon}_c(\theta, \phi)$ is a vector property; the local density of optical states (LDOS) in the cavity $\rho(\omega, d, z)$ depends on the frequency ω , the mirror spacing d and the position z of the molecule in the microcavity. Both $\vec{\epsilon}_c(\theta, \phi)$ and $\rho(\omega, d, z)$ can be derived numerically using the transfer matrix method (TMM).^[105] The spectral-averaged Purcell factor $P(d, z)$ is defined as the ratio of the radiative rate in the microcavity $k_{r,c}$ to the free-space radiative rate k_r

$$P(d, z) = \frac{k_{r,c}}{k_r} = \frac{\int S(\omega) |\vec{\mu} \cdot \vec{\epsilon}_c|^2 \rho(\omega, d, z) \sin \theta \, d\theta d\phi d\omega}{\int S(\omega) |\vec{\mu} \cdot \vec{\epsilon}|^2 \rho(\omega) \sin \theta \, d\theta d\phi d\omega} \quad (4-2)$$

and the fluorescence lifetime inside the microcavity $\tau_{F,c}$ can be expressed as

$$\tau_{F,c} = \frac{1}{Pk_r + k_{nr}} \quad (4-3)$$

Notably, for a specific single molecule, the orientation of its TDM critically determines the strength of the Purcell effect, with the most significant enhancement occurring when the TDM lies parallel to the mirror surface and coincides with a LDOS maximum.

Hence, for molecules with fixed orientation embedded in an optical microcavity, changes in TDM orientation are typically correlate by variations in fluorescence properties, such as lifetime and intensity. As shown in Figure 4-10, single-molecule fluorescence trajectories exhibit stepwise fluorescence lifetime jumps, accompanied by intensity changes. Such variations serve as “fingerprints” of tautomerization-induced TDM reorientation, illustrating that photonic control not only modulates tautomerization but also directly reveals real-time observation of molecular dynamics.

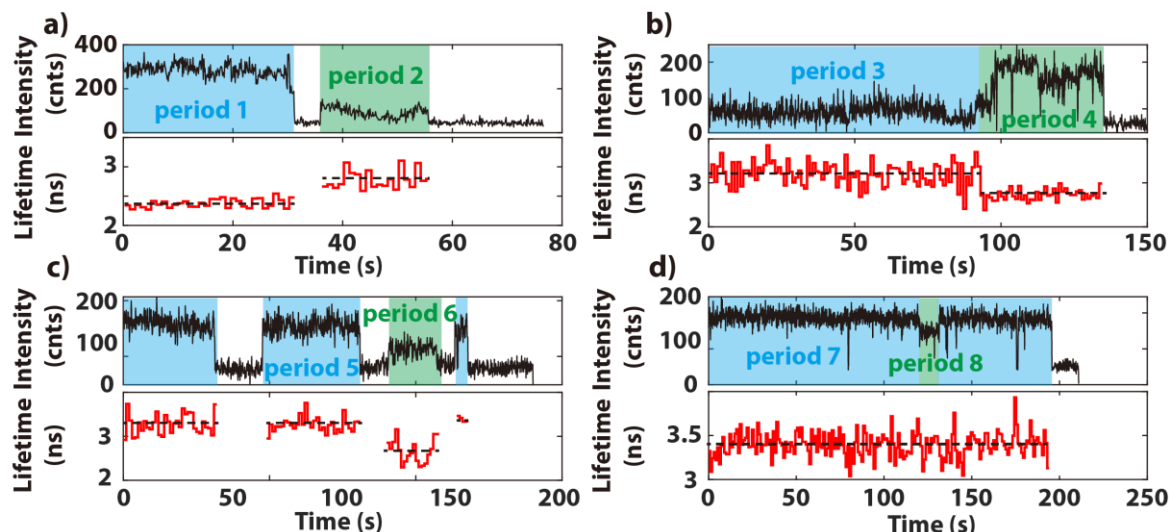


Figure 4-10: Fluorescence intensity time traces [top] and the respective fluorescence lifetime trajectories [bottom] are shown for four individual molecules in a microcavity at the following cavity mirror spacing: (a) 162 nm, (b) 197 nm, (c) 212 nm, and (d) 197 nm. The binning time is 10 ms for time traces and 1 s for lifetime trajectories. Reproduced from [L. Wang et al., J. Chem. Phys. 7 January 2022; 156 \(1\): 014203](#),^[1] with the permission of AIP Publishing.

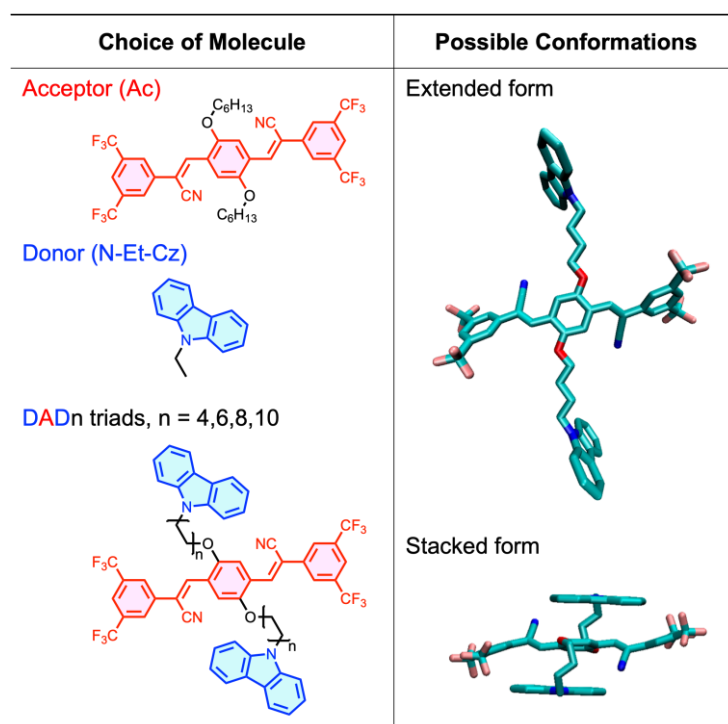
4.1.4 Summary and Perspectives

In this work, we established a comprehensive spectroscopic-computational framework for an in-depth investigation of hypericin at the single-molecule level. We systematically analyzed its stereochemistry, photophysics and photochemistry, and its pronounced sensitivity to the nano-environment. Combining quantum-chemical calculations with our home-built single-molecule imaging and spectroscopy, we successfully identified individual tautomeric species and directly observed tautomerization processes.

Furthermore, tautomerization rates can be effectively modulated by tailoring the fluorescence lifetime via (i) chemical modification through different polymer hosts and isotope effects, and (ii) photonic modification (Purcell effect) via embedding hypericin inside a $\lambda/2$ Fabry-Pérot microcavity. This spectroscopic-computational framework not only provides fundamental insights into the photophysics and photochemistry of hypericin, but also serves as a cornerstone for the next chapters, where we will extend this approach to case-specific systems and explore its general applicability in other optical platforms, i.e. surface-enhanced Raman scattering (SERS) systems, showcasing its potential as a universal strategy for probing optical responses modulated by nano-environments effects.

4.2 Photophysics and Origins of Single Donor-Acceptor-Donor Triads

In a recent study, a series of donor-acceptor-donor (DAD) triads was reported to exhibit fluorescence on-off switching with high-contrast ratios.^[196] An external stimulus allows the long-spacer candidates ($n \geq 6$) to switch between two distinct conformations.^[196] However, the underlying switching mechanism remains unclear, and the photokinetics in solution have not been systematically elucidated. Therefore, an in-depth investigation of the complex photokinetics of DADn triads with varying flexible spacer lengths was conducted.



Scheme 4-2: [Left] chemical structures of the acceptor (Ac), donor (N-Et-Cz) and donor-acceptor-donor (DADn) triads. [Right] Representative extended and stacked forms of an exemplary triad: DAD4.

4.2.1 Principal Photophysics of DADn Triads

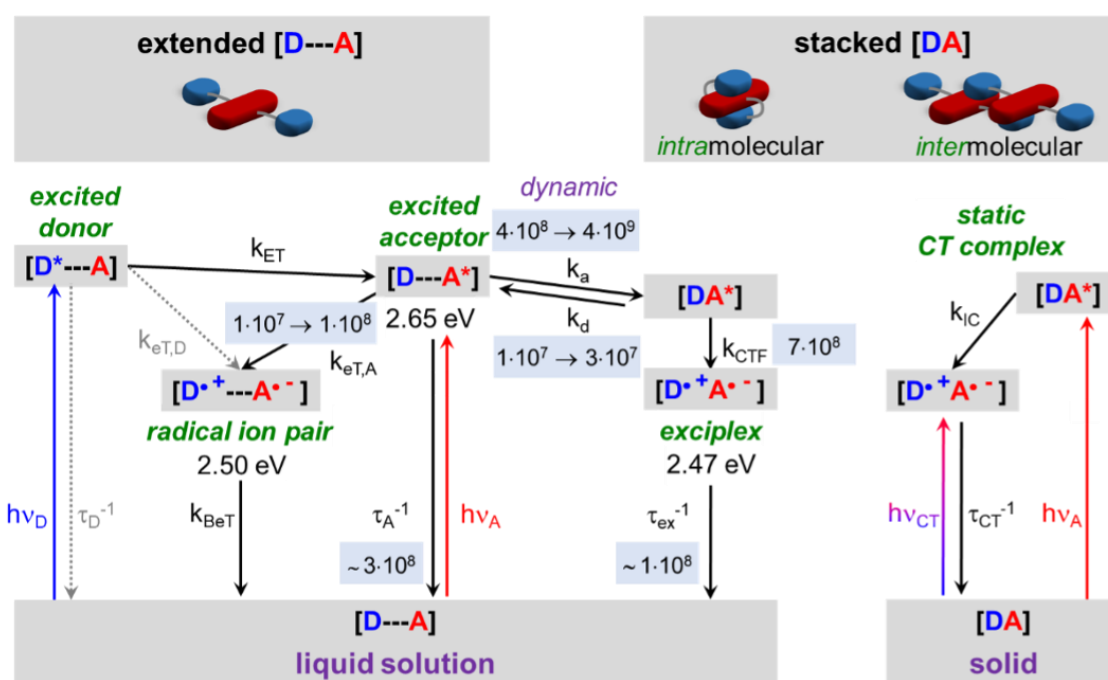
As shown in Scheme 4-2, the DADn triads consist of a highly fluorescent *p*-distyrylbenzene-type (DCS) unit as the acceptor (A), linked to two carbazole donors (D). The D and A moieties are covalently connected by a flexible non-conjugated spacer of varying lengths, namely an alkoxy bridge, $O(CH_2)_n$, where $n=4, 6, 8, 10$. High-contrast fluorescence on-off switching in the solid state can be induced by

external stimuli such as mechanical force, temperature etc.^[196] This has been attributed to the conformational isomerization between extended and stacked forms, corresponding to two distinct local minima along the potential energy surface (PES). The reference acceptor molecule (Ac) has the same structure as the acceptor moiety of the triads, but carries hexyloxy-groups (OC_6H_{13}) at the central ring. In addition, the compound N-ethylcarbazole (N-Et-Cz) serves as the reference donor molecule.

All-organic π -conjugated materials have attracted significant attention due to their versatile applications in organic solar cells (OSCs), organic light-emitting diodes (OLEDs), and organic field-effect transistors (OFETs). A key challenge in designing such materials lies in understanding the complex photophysical deactivation pathways. Among these, photoinduced charge-transfer (CT) states are critical for device performance. From a mechanistic perspective, effective population of the low-lying CT state (first excited state, S_1) upon direct or indirect photoexcitation requires a short spatial distance between donor (D) and acceptor (A) units. This can be achieved by (i) intra-molecular D–A systems connected directly^[197-199] or via short spacers^[199-203], or (ii) inter-molecular D:A complex. The generated CT state can be emissive, if the radiative rate k_r can successfully compete with the non-radiative rate k_{nr} . On the other hand, systems with large D–A separations generally prevent efficient population of such CT states upon direct photoexcitation. Nevertheless, CT states can still be dynamically generated in liquid solution in their excited states, commonly termed an exciplex;^[67,74,204] these typically occur in systems of mixed D and A species, in which high concentrations promote the formation of encounter complexes such as $[\text{D}-\text{A}^*]$ or $[\text{D}^*-\text{A}]$ during the S_1 lifetimes τ_F .

A summary of the basic photophysics in the current work is illustrated in Scheme 4-3. Upon photoexcitation, an excited donor (D^*) transfers its energy entirely to the acceptor (A) via resonant energy transfer (ET), generating an excited acceptor (A^*); the latter A^* can also be generated via direct photoexcitation of A. From here, A^* can be deactivated by (i) direct return to the ground state S_0 via emission or non-radiative decay (the latter typically includes internal conversion (IC), with subsequent vibrational relaxation (VR)), (ii) the photoinduced electron transfer (eT), or (iii) a dynamic exciplex formation.

The electron-transfer (eT) process creates a radical ion pair $[D^{\bullet+} \dots A^{\bullet-}]$, i.e. commonly termed a “charge-separated” (CS) state within extended forms,^[205] which is subsequently deactivated via back electron transfer (BeT) to the ground state S_0 .^[205,206] Alternatively, an exciplex $[D^{\bullet+}A^{\bullet-}]$ is formed via a pre-associated complex $[DA^*]$; this is a reversible dynamic process with an association constant k_a , and a dissociation constant k_d . The specific signature of the exciplex is a red-shifted, structureless fluorescence with a prolonged lifetime.



Scheme 4-3: Deactivation scheme of donor-acceptor-donor (DADn, $n=4,6,8,10$) triads within the singlet manifold under direct photoexcitation of D or A, generating excited donor (D^*) or excited acceptor (A^*). Apart from (i) direct deactivation from the excited acceptor $[D-A^*]$, (ii) electron transfer (eT) forms a free radical ion pair $[D^{\bullet+} \dots A^{\bullet-}]$; this process competes with (iii) the dynamic exciplex formation $[D^{\bullet+}A^{\bullet-}]$. The rate constants (s^{-1}) for DAD10 and DAD4 are obtained from a photokinetic analysis (see text). Reproduced from [S. Feng et al., Adv. Mater. 2023](#),^[3] with the permission of © Wiley-VCH.

4.2.2 Photokinetics of DADn Triads: Exciplex Formation⁵

As shown in Figure 4-11, the absorption spectrum of the reference acceptor (Ac) shows a structureless “double-hump” feature with maxima at 347 nm and 445 nm. The

⁵ The ensemble spectroscopic work in this section was performed by Dr. Siyang Feng under the supervision of Dr. Reinhold Wannemacher and PD Dr. Johannes Gierschner.

ensemble absorption spectrum of DADn in Figure 4-11 indicates a superimposed feature of those of the reference acceptor (Ac) and the reference donor (N-Et-Cz) molecules alone. Besides, there is no evidence to suggest the involvement of charge-transfer (CT) states, indicating that DADn triads tend to exhibit the extended form in solution. In contrast to absorption, the ensemble fluorescence properties vary significantly with the spacer length n . The fluorescence spectra of DAD10 and DAD8 are almost identical, closely resemble that of Ac. However, there is a slight broadening on the longer wavelengths. On the contrary, DAD4 indicates only a shoulder at this short wavelength (SW) at 535 nm but a significant portion at the long wavelength (LW) at 623 nm. Further ensemble spectroscopic studies confirm the existence of the prompt lifetime τ_{PF} ; this is observed to decrease strongly and monotonically with increasing spacer length n versus Ac, particularly in polar solvents, like in chloroform (CHCl_3). Moreover, no concentration dependence was found upon dilution. All evidence suggests that the LW is a result of an intra-molecular exciplex between the donor (D) and acceptor (A) moieties, i.e., the interconversion from the extended to stacked form, in which a CT state is formed dynamically by the folding of the spacer during the excited-state lifetime. This is consistent with the observed broad features of the LW emission, as typically found as a “fingerprint” of an exciplex.^[207-214] Spectral decomposition (as indicated as dashed lines in Figure 4-11) for all DADn triads indicates the exciplex (the stacked form) is responsible for the LW tails.

The rate constants for exciplex formation were determined via photokinetic analysis of available spectroscopic data, based on a three-state model: the ground state $[\text{D}-\text{A}]$, the excited-state acceptor $[\text{D}-\text{A}^*]$, and the pre-associated exciplex $[\text{DA}^*]$. The excited-state lifetimes of the excited acceptor $[\text{D}-\text{A}^*]$ and the exciplex $[\text{D}^{\cdot+}\text{A}^{\cdot-}]$ remain relatively constant, with $\tau_{\text{A}}^{-1} \approx 3 \times 10^8 \text{ s}^{-1}$ and $\tau_{\text{ex}}^{-1} \approx 1 \times 10^8 \text{ s}^{-1}$, respectively, although the radiative k_{r} and non-radiative rate k_{nr} show somewhat variations. The rate constant of electron-transfer (eT) quenching increases from $k_{\text{eT,A}} \approx 1 \times 10^7 \text{ s}^{-1}$ for DAD10 to $\approx 1 \times 10^8 \text{ s}^{-1}$ for DAD4 due to the shortened D–A separation. Meanwhile, the energy of the radical ion pair $[\text{D}^{\cdot+} \dots \text{A}^{\cdot-}]$ remains fairly constant according to the TD-DFT calculations. In contrast, the association rate for the pre-associated species $[\text{DA}^*]$ increases significantly, from $k_{\text{a}} \approx 4 \times 10^8 \text{ s}^{-1}$ for DAD10 (corresponding to an association time of

$t_a=2.5$ ns) to $k_a\approx 4\times 10^9$ s⁻¹ for DAD4 ($t_a=0.25$ ns). This difference indicates that the radical ion pair formation is a minor pathway in comparison with the exciplex formation.

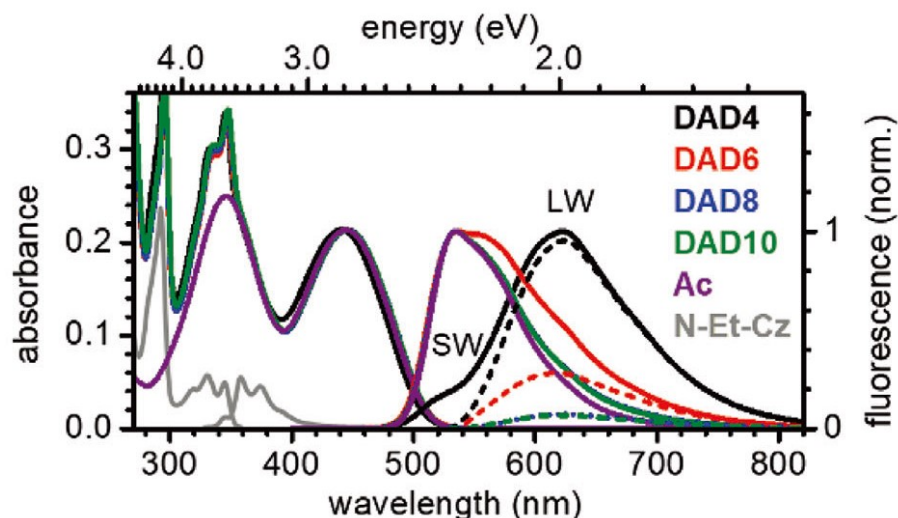


Figure 4-11: Absorption (left) and fluorescence (right) spectra of the compounds in 10 μ M chloroform (CHCl_3) at room temperature. The fluorescence spectrum of DAD8 [blue] is nearly identical to that of DAD10 [black], and is therefore shown as superimposed. Dashed lines indicate the long-wavelength (LW) components, obtained by spectral decomposition. For reference, the absorption and fluorescence spectra of the donor N-ethyl-carbazole (N-Et-Cz) are shown. Reproduced from [S. Feng et al., Adv. Mater. 2023,](#)^[3] with the permission of © Wiley-VCH.

Table 4-4: Spectroscopic data of the compounds in chloroform (CHCl_3) at room temperature, including short (SW) and long (LW) wavelength emissions with their corresponding emission maxima λ_{max} . Individual lifetimes (amplitudes) τ_i (A_i) are obtained from multi-exponential fits, obtained at $\lambda_{\text{det}}=536$ nm, 680 nm for SW and LW, respectively; τ_{PF} is the mean (intensity-weighted average) prompt lifetime (i.e. of the short components $\tau_{1,2}$). $\Phi_{\text{F,tot}}$ is the total fluorescence quantum yield, and $\Phi_{\text{F,i}}$ are the quantum yields of the SW and LW components, determined by spectral decomposition of the steady-state data in Figure 4-11 via the fractional intensities $x_{\text{SW,LW}}$.

Probe		τ_1 (ns) ^{a)}	A_1	τ_2 (ns) ^{a)}	A_2	τ_{PF} (ns) ^{b)}	$\Phi_{\text{F,tot}}$	$\Phi_{\text{F,i}}$	λ_{max} (nm)
DAD4	SW ^{c)}	0.07	0.99	1.17	0.01	0.21	0.16	0.02	535
	LW							0.14	620
DAD6	SW ^{d)}	0.17	0.54	0.58	0.46	0.47	0.11	0.08	535
	LW	0.38	1.00	–	–	0.38		0.03	620
DAD8	SW	0.46	0.21	1.07	0.79	1.01	0.23	0.21	535
	LW	0.19	0.25	0.98	0.75	0.93		0.02	615
DAD10	SW	0.64	0.20	1.47	0.80	1.39	0.30	0.27	535
	LW	0.33	0.13	1.30	0.87	1.27		0.03	623
Ac	SW	0.87	0.12	3.47	0.88	3.39	0.41		535

^{a)} the accuracy is limited by the IRF (ca. 50 ps);

^{b)} intensity-weighted averaged lifetime; $\tau_{\text{PF}}=(A_1\cdot\tau_1^2+A_2\cdot\tau_2^2)/(A_1\cdot\tau_1+A_2\cdot\tau_2)$. Differences in τ_{PF} for SW and LW detection are within the experimental error of the setup;

^{c)} $\lambda_{\text{det}}=524$ nm; ^{d)} $\lambda_{\text{det}}=505$ nm.

Diffusion Model

To rationalize the association rate k_a extracted from the spectroscopic data, an initial estimation was made in a simple diffusion model. To further understand the conformational dynamics involved in exciplex formation, particularly the transition from the extended to the stacked form, molecular dynamics (MD) simulations were conducted to visualize the folding process.

As the exciplex association/dissociation (k_a/k_d) is a reversible process, the competition between electron transfer (eT_A) and exciplex formation cannot be described in a purely stochastic model due to the guided trajectory imposed by the molecular σ spacers. Nevertheless, as a first approximation, we assume that the extended and stacked forms can be bridged by a free diffusing donor (carbazole, Cz) unit in a simplified three-dimensional (3D) diffusion model. The diffusion constant D is then approximated by the 3D Stokes-Einstein equation

$$D = \frac{k_B T}{6\pi\eta R} \quad (4-4)$$

where R is the radius of the uncharged (spherical) particle. The corresponding diffusion length L_D in three dimensions (3D) is then

$$L_{D,3D} = \sqrt{6Dt} \quad (4-5)$$

while for a one-dimensional (1D) case, it is given by

$$L_{D,1D} = \sqrt{2Dt} \quad (4-6)$$

MD simulations of free Cz yielded a diffusion constant $D=1.1\pm0.3\times10^{-5}$ cm²/s in chloroform (CHCl₃), in good agreement with the literature value $D=1.3\pm0.3\times10^{-5}$ cm²/s in water^[215] and acetonitrile^[216]. The effective diffusion lengths L_D were derived from the optimized geometries of the extended and stacked forms, corresponding to Cz displacement distances of 1.2 nm for DAD4 and 1.9 nm for DAD10. Diffusion constants extracted from MD simulations are presented in Figure 4-12 and Table 4-5.

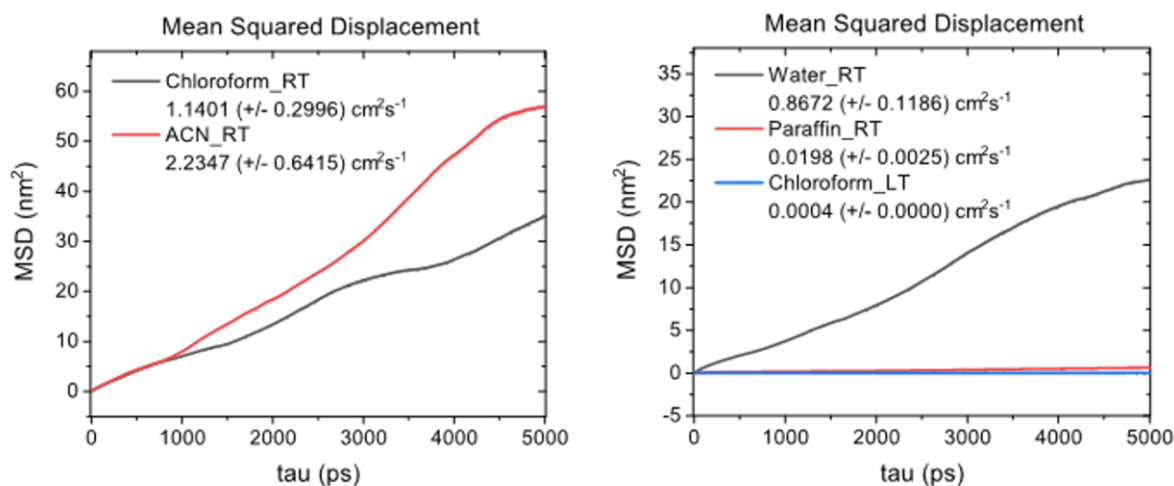


Figure 4-12: Mean squared displacement (MSD) analysis of free carbazole (Cz) in three dimensions (3D) across different explicit solvents at 298.15 K (room temperature, RT) and 77 K (low temperature, LT). Accurate error estimates are shown in the parentheses.

Table 4-5: Calculated diffusion constants of carbazole in three dimensions (3D) for different solvents, extracted from the mean squared displacements (MSD) analysis shown in Figure 4-12.

Carbazole	D [10^{-5} cm ² /s]
ACN RT	2.2347 ± 0.6415
Chloroform RT	1.1401 ± 0.2996
Chloroform LT	0.0004 ± 0.0000
Water RT	0.8672 ± 0.1186
Paraffin RT	0.0198 ± 0.0025

Table 4-6: Exciplex association times (t_a) for **DAD4** and **DAD10** in chloroform (CHCl₃) as determined from experiment (exp), a free three-dimensional (3D) diffusion model, a quasi-one-dimensional (1D) diffusion model, and molecular dynamics (MD) simulations. Reproduced from [S. Feng et al., Adv. Mater. 2023](#),^[3] with the permission of © Wiley-VCH.

	DAD4	DAD10
$t_{a, \text{exp}}$	0.25 ns	2.5 ns
$t_{a, 3D}$	0.18 ns	0.46 ns
$t_{a, 1D}$	0.55 ns	1.4 ns
$t_{a, MD}$	~0.2 ns	~1 ns

The experimental and calculated exciplex association times t_a for DAD4 and DAD10 in chloroform (CHCl_3) are summarized in Table 4-6. The free diffusion model in 3D (1D) predicts the reference association times $t_{a,3D}$ ($t_{a,1D}$) of 0.18 ns (0.55 ns) for DAD4, and 0.46 ns (1.4 ns) for DAD10, respectively. The experimental value for DAD4 ($t_{a,\text{exp}}=0.25$ ns) falls between the calculated 3D and 1D diffusion limits, which appears reasonable, consistent with a partially guided folding trajectory. The experimental value for DAD10 shows a larger deviation, suggesting greater structural constraints.

To further investigate the conformational dynamics, MD simulations were carried out for both DAD4 and DAD10, starting from their extended geometries. Full structural relaxation in explicit solvent at different temperature (298.15 K and 77 K) was performed to capture intrinsic molecular flexibility and spacer-guided folding behavior. Gyrate analysis, implemented in Gromacs, was employed to monitor the folding process by computing the time-resolved radius of gyration R_g and its components ($R_{g,x}$, $R_{g,y}$, $R_{g,z}$) along the x-, y-, and z-axes over time. As shown in Figure 4-13, the simulations reveal a distinct collapse in molecular size upon folding, indicated by a sharp decrease in the radii of gyration R_g , corresponding to the transition to the stacked state.

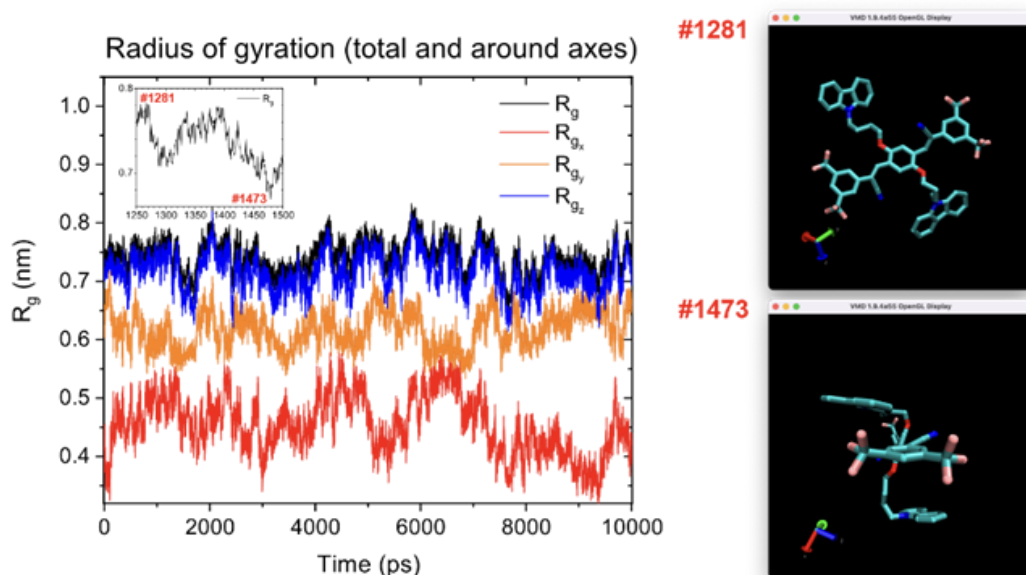


Figure 4-13: Representative gyration analysis of DAD4 in dichloromethane (DCM) at 298.15 K, showing the total and the axial components of the radius of gyration. The inset figure indicates one complete folding event, [right] from an extended (#1281) to a stacked (#1473) conformation. Reproduced from [S. Feng et al., Adv. Mater. 2023,^{\[3\]}](#) with the permission of © Wiley-VCH.

Quantum-Chemical Treatment⁶

In order to gain further insight into the underlying photokinetics from a thermodynamic perspective, quantum-chemical calculations were conducted to elucidate the geometrical and electronic features, as well as optical responses upon photoexcitation.

In this case study, the functional ω B97XD was applied to optimize the ground state (S_0) geometries, due to the inclusion of long-range correlation and dispersion correction. Subsequently, the functional PBE0 was employed for the time-dependent (TD) treatment, given its good agreement with the experimental observations. Despite ongoing challenges in accurately treating excited states with (partial) charge-transfer (CT) character, it has been shown that standard functionals like PBE0 and B3LYP can still perform reliably for states with very pronounced CT character; that is, the HOMO is predominantly localized on the donor (D) and the LUMO on the acceptor (A) with a vanishing oscillator strength f_{CT} ,^[197,199,217,218] as in this case study.

The optimized extended (C_2 symmetry) and stacked (C_i symmetry) conformers correspond to two local minima on the potential energy surface (PES), as confirmed by the absence of imaginary frequencies. The stacked conformation exhibits a lower electronic energy, by 1.54 eV in the case of DAD4; this is due to the “sandwich-like” Coulomb interaction between donor (D) and acceptor (A) moieties. However, the exact D–A positioning depends sensitively on the spacer length due to the nature of the σ bonds. In DAD4, the D units are situated on top and bottom of the central β -cyano-styryl moiety of A, while in those with longer spacers, the donors tend to position themselves on the outer α -cyano-styryl moieties of A. Furthermore, the *p*-distyrylbenzene-type (DCS) acceptor backbone demonstrates pronounced “twist elasticity”, and responds sensitively to the D positioning. This is reflected in two key torsional angles, as shown in Table 4-7. The stacked forms show greater geometric distortion in comparison with the reference acceptor Ac. As the spacer length

⁶ The quantum chemistry work in this section was performed by Dr. Siyang Feng and Liangxuan Wang.

increases, the change in torsional angles becomes more significant, leading to spectral drift, as discussed later in Section 4.2.3.

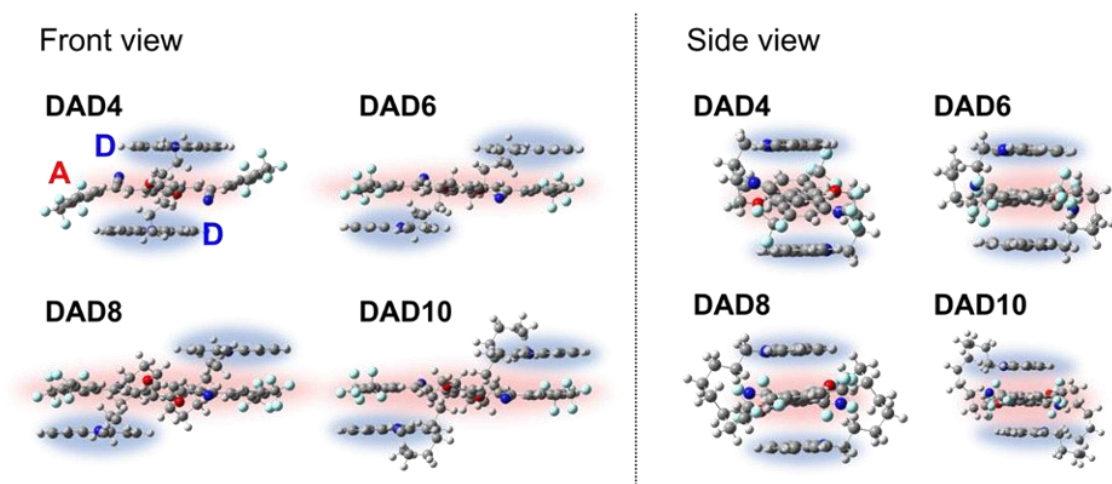
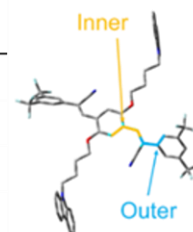


Figure 4-14: Optimized stacked geometries of all DADn triads calculated at ω B97XD/6-311G(d) level in chloroform (CHCl_3 , PCM model) (front view: left; side view: right).

Table 4-7: Calculated differences in electronic and Gibbs free energies, along with inner and outer torsional angles for all DADn triads at the ground state (S_0). All calculations were performed at the ω B97XD/6-311G(d) level in chloroform (CHCl_3 , PCM model).

		configuration	DAD4	DAD6	DAD8	DAD10	Ac
ΔE_{Elec} (eV)	extended		0	0	0	0	
	stacked		-1.54	-1.24	-1.59	-1.62	
ΔE_{Gibbs} (eV)	extended		0	0	0	0	
	stacked		-1.06	-0.75	-1.14	-0.61	
torsional angles (°)	extended	inner	31	29	29	29	30
		outer	34	33	33	34	35
	stacked	inner	29	27	30	27	
		outer	29	22	16	13	



On the basis of the optimized ground-state geometries, the minimum energy reaction pathway connecting the extended and the stacked forms was estimated by locating the energy barrier along the potential energy surface. The calculated energy barriers are 0.10 eV for DAD4 and 0.22 eV for DAD10. In the absence of external stimuli, this conformational interconversion can thus be considered thermodynamically barrierless, and is expected to be predominantly governed by kinetic factors.

4.2.3 Monitoring Exciplex Formation in Single DAD4 Triads⁷

So far, we have established a basic understanding of the complex photophysical deactivation pathways of DADn triads with varying spacer lengths. However, the driving force for this dynamic intra-molecular exciplex formation remains unclear. Investigating this single-molecule behavior in response to its nano-environment may offer deeper mechanistic insights into the competition between exciplex formation and photoinduced electron transfer. In addition, the dynamic nature of exciplex formation manifests in a strong dependence on local environmental parameters, including solvent polarity and viscosity, temperature variation, and the free volume of the polymer host.

In this preliminary experiment, DAD4 was selected as a model compound for single-molecule investigation under both ambient and cryogenic conditions. At low temperature (82 K), the absence of oxygen significantly enhances the photostability of the system, resulting in an extended survival time upon photoexcitation. The characteristic blinking behavior observed in Figure 4-15 confirms the detection of individual DAD4 molecules.

Furthermore, two distinct molecular conformations were identified in Figure 4-15: the extended DAD4 (ex-DAD4) gives a single short-wavelength (SW) emission band at around 535 nm, whereas the stacked form (st-DAD4) exhibits dual emission at 550 nm (SW) and 600 nm (long-wavelength, LW). Notably, a spectral shift is observed at the 30th frame in Figure 4-15(b), which is attributed to structural fluctuations involving the torsional angles within the acceptor backbone. As shown in Table 4-8, an increased torsional angle leads to a higher electronic energy at the ground state, and a corresponding blue shifted in emission.

These findings lay the groundwork for future experiments aimed at statistically analyzing the photophysics of single DADn triads under different environmental

⁷ The cryogenic work in this section was led by Jakob Keck.

conditions. Future efforts will focus on elucidating the mechanism of the exciplex formation and probing nano-environmental effects in this system. Ultimately, such understanding may allow for fine control over the competition pathways, including the acceptor emission, exciplex formation, and quenching by photoinduced electron transfer (eT), through precise control of the environmental factors.

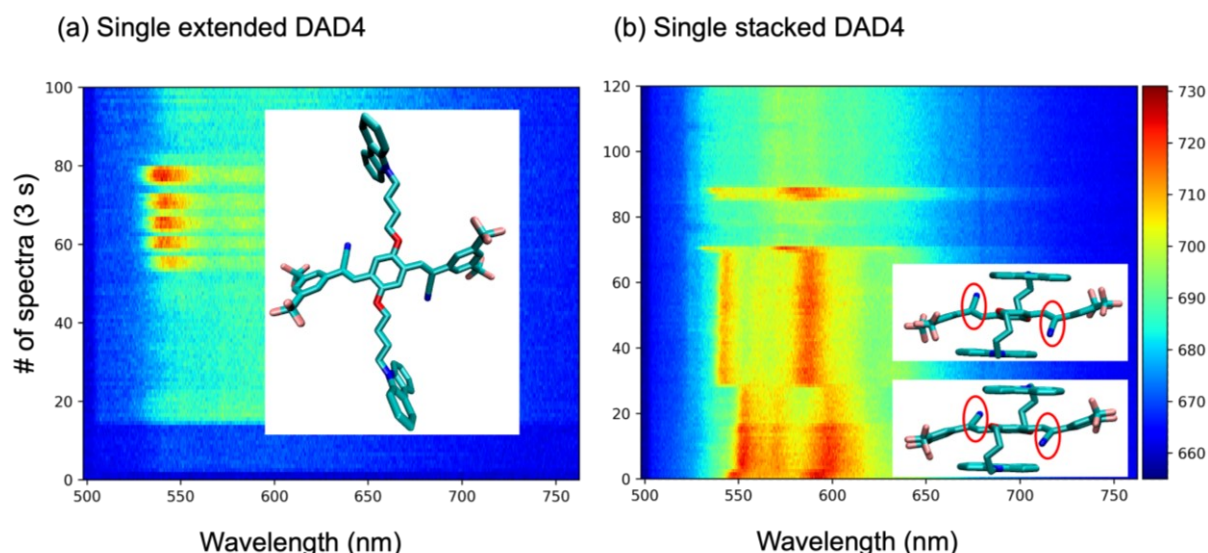
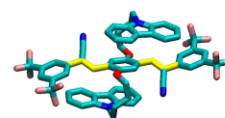


Figure 4-15: Time-resolved single-molecule fluorescence spectra for DAD4, recorded at 82 K with 3 s exposure time per frame (100 frames for (a) and 120 frames for (b)). DAD4 molecules were immobilized in PMMA matrix at a concentration of 10^{-10} M. Two conformations were observed, (a) the extended-DAD4 (ex-DAD4) exhibits a single short-wavelength (SW) emission at ~ 535 nm; and (b) the stacked-DAD4 (st-DAD4) shows dual emission bands at 550 nm (SW) and 600 nm (long-wavelength, LW). A sudden spectral shift was observed around the 30th frame in (b), attributed to structural fluctuations in the acceptor core. An increased torsional angle (marked in red circles) contributes to a blue-shifted emission spectrum.

Table 4-8: [Left] calculated differences in electronic and Gibbs free energies for DAD4 with different torsional angles at the ground state (S_0). [Middle] Corresponding short-wavelength (SW) and long-wavelength (LW) emission peaks, along with the corresponding oscillator strengths (f) from TD-DFT. [Right] The key torsional angle is highlighted in yellow. Ground-state geometry optimizations were performed at the ω B97XD/6-311G(d) level, and the time-dependent (TD) part was done using the PBE0 functional. All calculations employed the implicit PCM solvent model in chloroform (CHCl_3).

DAD4 with diff. torsional angles	ΔE_{elec} (eV)	ΔE_{Gibbs} (eV) at 298.15 K	Compounds	SW		LW	
				nm	f	nm	f
			Ac	463	0.6692		
			ex-DAD4	467	0.7315		
st-DAD4: 19°	Ref.	Ref.	st-DAD4: 19°	479	0.3919	523	0.0060
st-DAD4: 29°	0.00	0.61	st-DAD4: 29°	464	0.3630	501	0.0033
st-DAD4: 39°	0.00	0.06	st-DAD4: 39°	448	0.3088	482	0.0119
st-DAD4: 49°	0.05	0.15	st-DAD4: 49°	431	0.2526	460	0.0284



4.2.4 Summary and Perspectives

In this work, we have successfully extended the spectroscopic-computational methodology to a stimulus-responsive system of donor-acceptor-donor (DADn) triads with varying spacer lengths, addressing its complex excited-state photokinetics. By combining ensemble spectroscopy, molecular dynamics simulations, quantum-chemical calculations and single-molecule spectroscopy, we revealed the dynamic interplay between conformational interconversion, charge-transfer interactions, and nano-environmental modulation.

Our results show that the intra-molecular exciplex formation [D^+A^-] proceeds via a dynamic folding mechanism of the flexible spacer, which kinetically outcompetes the formation of the radical ion pair [$D^+ \dots A^-$] generated via photoinduced electron transfer (eT). The efficiency of exciplex formation is strongly influenced by spacer length, conformational freedom, and the local nano-environment, as evident by both ensemble and single-molecule measurements. The photokinetic analysis, supported by the diffusion model, highlights the crucial role of the spacer-guided dynamics in controlling the exciplex association rate. Molecular dynamics simulations provide direct visualization of the folding trajectories, while quantum-chemical calculations offer detailed mechanistic insights into conformational preferences and the correlation with the spectral variations. Importantly, single-molecule experiments proved particularly valuable for capturing in situ conformational fluctuations and exciplex dynamics, which are inaccessible via ensemble measurements.

This work marks a significant step towards validating our spectroscopic-computational approach for investigating single-molecule exciplex photophysics under controlled nano-environmental conditions. Future work will aim to elucidate how the nano-environment governs exciplex dynamics and to modulate competing relaxation pathways. Such mechanistic understanding is anticipated to guide the rational design of stimuli-responsive materials for advanced applications in optoelectronics, sensing, and other molecular devices with tailored excited-state properties.

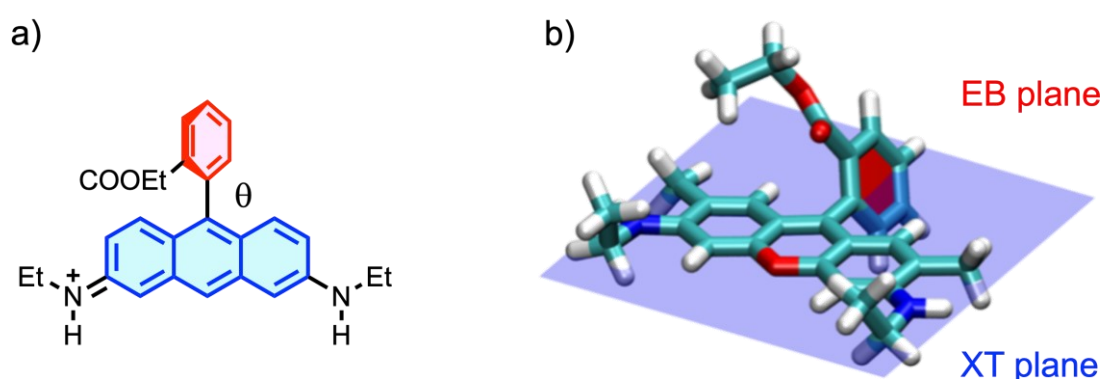
4.3 Photophysics and Surface-Enhanced Raman Scattering of Rhodamine 6G: A Computational Perspective

The confinement of optical fields within a cavity strongly modifies the light-matter interactions, leading to significant photonic enhancements. In addition to the $\lambda/2$ Fabry-Pérot microcavity discussed in Section 4.1.3, metal nanostructures offer even greater field enhancements by confining light at the nanoscale, forming plasmonic nanocavities. This characteristic feature of metals serves as the fundamental mechanism for a host of radiative and scattering processes associated with nanophotonic systems, particularly in the context of surface-enhanced Raman scattering (SERS).^[219-221] As one of the most sensitive analytical techniques, SERS greatly amplifies inelastic light scattering by factors up to 10^8 or even larger, enabling single-molecule detection in some cases.^[221]

The principal mechanism driving SERS is the excitation of localized surface plasmon resonances (LSPRs) in noble metal nanostructures such as gold (Au) and silver (Ag).^[222-228] Upon photoexcitation, collective oscillations of free electrons are induced, generating intense local electromagnetic fields at plasmonic “hot spots”, particularly at sharp edges or within nano/picometer gaps. These enhanced fields dramatically boost the Raman signals of nearby molecular adsorbates.^[4]

In this work, a nanoparticle-on-mirror (NPoM) geometry^[229,230] has demonstrated enormous Raman enhancements due to a highly confined and amplified electromagnetic field within the nanocavity. This NPoM configuration consists of rhodamine 6G (R6G, chemical structure shown in Scheme 4-4) located within the sub-nanometer gap between a gold nanoparticle and an ultrasmooth gold mirror. While the dipolar plasmonic gap mode (D-mode) has been extensively investigated,^[231,232] recent evidence highlights the importance of higher-order resonances, such as quadrupolar (Q-mode) contributions, especially under resonant excitation at the wavelength around 636 nm.

In order to systematically examine the polarization-dependent optical responses of single NPoM configurations, a linearly polarized beam as well as azimuthally (APDM) and radially (RPDM) polarized doughnut modes were employed.⁸ Complementing the experimental work, a generalized boundary element method (BEM)⁹ was used to obtain their local field distributions within the nanocavity. In addition, (TD-)DFT calculations were performed to assign characteristic vibrational modes and evaluate peak-specific SERS enhancements.



Scheme 4-4: (a) Structural formula and (b) DFT-optimized ground-state geometry of rhodamine 6G (R6G) in ethanol (EtOH). R6G consists of a planar xanthene chromophore ([right], blue, XT plane) with two ethylamine groups and one ethyl benzoate group ([right], red, EB-plane), giving it a net positive charge. (b) In the optimized geometry, the ethylamine group adopts the shown orientation with the torsional angle $\theta \approx 66^\circ$. Upon adsorption on a gold surface (see Figure 4-18(b)), the C-N bonds align with the XT-plane, while the benzoate group forms an EB-plane tilted relative to the XT-plane $\theta \approx 63^\circ$. Notably, the torsional angle θ responds sensitively to environmental conditions. Geometry optimization was carried out at the D3-B3LYP/6-311G(d) level using an implicit solvent model (PCM, EtOH). Reprinted with permission from [F. Schneider et al., J. Phys. Chem. C, 44, 18807 \(2024\)](#),^[4] © 2024 American Chemical Society.

⁸ The Raman spectroscopic work in this section was performed by Felix Schneider under the supervision of Prof. Dr. Dai Zhang.

⁹ The BEM simulation in this work was performed by Lukas Lang under the supervision of Prof. Dr. Monika Fleischer.

4.3.1 Principal Photophysics of Rhodamine 6G (R6G)

Rhodamine 6G (R6G) is a highly fluorescent xanthene dye (chemical structure shown in Scheme 4-4), consisting of a planar xanthene chromophore (XT plane), two ethylamine groups and one ethyl benzoate (EB plane) group. Due to its excellent photostability and photophysical properties, R6G has been widely used as a laser dye,^[233,234] a fluorescence standard (for quantum yield^[235] and polarization^[236]), as well as a molecular probe in bioimaging and sensing applications.^[237-244]

R6G exhibits a strong absorption maximum around 530 nm, accompanied by fluorescence emission in the 540-570 nm range, characterized by a small Stokes shift. R6G possesses a high fluorescence quantum yield of approximately 95%.^[245] As shown in Figure 4-16, the narrow emission profile originates from a minimal change in bond length alternation (Δ_{BL}) between the ground and excited states, which can be rationalized by its resonance structures and frontier molecular orbital characteristics.^[60]

To explore solvent effects, the ground- and first excited-state geometries of R6G were optimized both in the presence and absence of the Cl^- counterion, corresponding to the fully associated and dissociated cationic forms expected in less polar and polar solvents, respectively (i.e., chloroform and ethanol). In ethanol (Figure 4-16(b)), the emission spectrum exhibits a full width at half maximum (FWHM) of 0.20 eV (39 nm), while in chloroform (Figure 4-16(a)), ion pairing leads to a broadened FWHM of 0.23 eV (51 nm), in good agreement with the simulated spectra.^[61] Notably, this ion-pairing effect in R6G is considerably less pronounced than in cyanine dyes.^[57-59] This is ascribed to the high sensitivity of cyanines to the electric field induced by the counterion;^[57] while R6G undergoes only localized C–C bond shortening or elongation, yielding much smaller Δ_{BL} variations. Therefore, xanthene dyes are expected to be more resilient against solvent effects in comparison with cyanines.

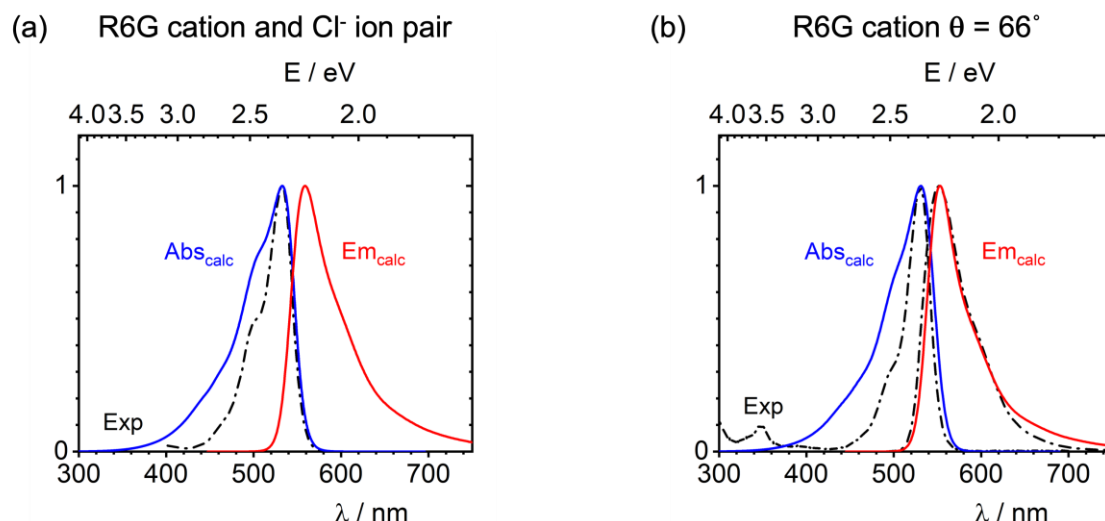


Figure 4-16: Experimental (black) and calculated absorption (blue, Abs_{calc}) and emission (red, Em_{calc}) spectra of rhodamine 6G (R6G) in two model configurations, both featuring a torsional angle of $\theta=66^\circ$. (a) R6G cation paired with Cl^- ion (simulating the associated form in chloroform); (b) isolated R6G cation in methanol (MeOH, simulating the dissociated form in ethanol). Fully converged convoluted spectra at the same temperature were obtained with the time-dependent method at 298.15 K, including a Gaussian broadening γ_G of 0.07 eV (full width at half maximum, FWHM). Energy offsets of 0.06 eV (absorption) and 0.09 eV (emission) were applied to align with experimental data. Experimental spectra were measured in (a) chloroform ($CHCl_3$)^[61] and (b) ethanol^[246] for comparison. Geometry optimizations were carried out at the D3-B3LYP/6-311G(d) level using an implicit solvent model (PCM, MeOH). All the emission spectra were further corrected by the λ^{-2} -factor when converted into the wavelength scale.

4.3.2 Surface-Enhanced Raman Scattering: A Computational Study

Rhodamine 6G (R6G) is extensively employed as a prototypical analyte in surface-enhanced Raman scattering (SERS) studies due to its large Raman scattering cross-section, high sensitivity, well-defined spectral “fingerprint”, and excellent adsorption onto metal nanostructures.^[247,248]

Polarization-resolved experimental SERS spectra (Figure 4-17(c)) reveal distinct enhancement patterns depending on the molecular vibrational modes and the polarization. Raman peaks at 610 cm^{-1} (aromatic C–C–C in-plane bending), 771 cm^{-1} (C–C–C aromatic in-plane bending and C–C–H out-of-plane bending) and 1642 cm^{-1} (aromatic C–C stretching) experience the strongest enhancement under linear and radial polarizations, with the latter giving the highest overall intensities. In contrast, the bands at 1311 cm^{-1} (aromatic C–C stretching, C–N–H in-plane bending), 1358 cm^{-1} (aromatic C–C stretching, C–N–H bending), and 1504 cm^{-1} (aromatic C–C stretching)

show significantly stronger enhancement under radial polarization, while 1189 cm^{-1} (aromatic C–C in-plane stretching) is preferentially enhanced under azimuthal polarization.

To rationalize the polarization-dependent enhancement factors for individual Raman peaks, vibrational mode analysis was conducted for both solvated and surface-adsorbed R6G (Figure 4-18). Particular attention was given to the adsorption geometry, specifically the molecular orientation with respect to the gold surface and the torsional angle (θ , defined in Scheme 4-4(a)) between the XT and EB planes.^[249,250] In this study, a cluster model was applied to provide a realistic electronic structure of the system. We adopted the suggested adsorption geometry, in which the XT core lies flat on a bilayer gold surface, with the nitrogen atoms of the ethylamine groups oriented toward the surface, and the ethyl and EB groups bent out of plane (see Figure 4-18(b)).^[249,251] This geometry corresponds to the thermodynamically most stable conformation. To account for local conformational dynamics within the SERS "hot spot", rotational averaging in the x-y plane was applied, allowing the ensemble-averaged experimental spectra to be reliably simulated using this thermodynamically favored adsorption geometry. For comparison, a complete vibrational analysis of R6G in ethanol was also performed.

Time-dependent DFT calculations predict an $S_0 \rightarrow S_1$ vertical transition energy of 2.66 eV ($\lambda=466\text{ nm}$). As the experimental excitation wavelength ($\lambda=636\text{ nm}$, 1.95 eV) lies close to the first absorption maximum $\lambda_{\text{max}}=530\text{ nm}$ (2.34 eV),^[246] pre-resonance conditions were considered with an energy offset of 0.32 eV. Figure 4-18(a) shows both non-resonance and pre-resonance Raman spectra of free R6G in ethanol, and non-resonance Raman spectrum of R6G adsorbed on a gold surface in the range from 250 cm^{-1} to 2000 cm^{-1} . Due to computational limitations, calculations with a larger basis set were restricted to free R6G in ethanol. For consistency, the starting geometry in solution was taken from the surface-adsorbed R6G structure and subsequently re-optimized in the solvated environment. Interestingly, the Raman spectrum of solvated R6G shows better agreement with the experiment compared to the cluster model, which may be attributed to the use of the larger basis set with diffuse functions. Therefore, the solution-phase calculation was used as a reference for vibrational mode assignment.

Comparison of the vibrational profiles highlights the strong dependence of Raman frequencies and intensities on the chemical and photonic environment, particularly for modes involving C–N–H and C–C–H bending in the amino groups. Furthermore, spectral clustering and vibrational mode mixing often result in overlapping bands, complicating spectral assignments. Therefore, we focused on three well-isolated vibrational modes (Figure 4-18): $\nu_1=394\text{ cm}^{-1}$, $\nu_2=610\text{ cm}^{-1}$ (both with a significant component perpendicular to the XT-plane, i.e. in-plane and out-of-plane modes), and $\nu_3=1189\text{ cm}^{-1}$ (with exclusive nuclear displacements within the XT-plane, i.e. in-plane mode).

Strikingly, while ν_3 shows maximal Raman intensity under azimuthal polarization, ν_1 and ν_2 are preferentially enhanced under radial polarization (Figure 4-17(c,d)). These observations are consistent with the proposed adsorption geometry, in which the XT plane of R6G lies parallel to the gold substrate, enabling anisotropic coupling between specific vibrational modes and the spatially structured optical fields in the nanoparticle-on-mirror (NPM) cavity.

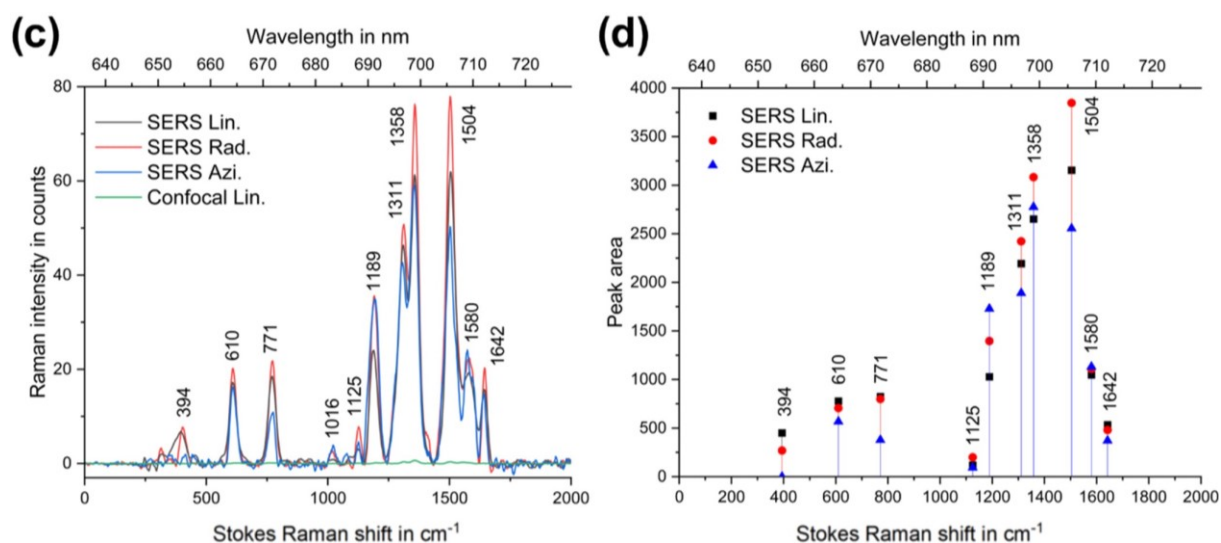


Figure 4-17: Polarization-resolved surface-enhanced Raman spectra of rhodamine 6G (R6G) under excitation at $\lambda_{\text{ex}}=636\text{ nm}$. Acquisition time $t_{\text{aq}}=30\text{ s}$ per spectrum, averaged over 10 measurements; laser power: $36\text{ }\mu\text{W}$. (c) Averaged background-subtracted Raman spectra of R6G in nanoparticle-on-mirror (NPM) configuration under linear (black), radial (red) and azimuthal (blue) polarization, compared to a confocal measurement without nanoparticles (green). (d) Comparison of averaged peak-integrated Raman intensities under different polarizations. While radial polarization leads to the strongest enhancement, distinct vibrational modes show pronounced polarization-dependent responses. Reprinted with permission from [F. Schneider et al., J. Phys. Chem. C, 44, 18807 \(2024\)](#),^[4] © 2024 American Chemical Society.

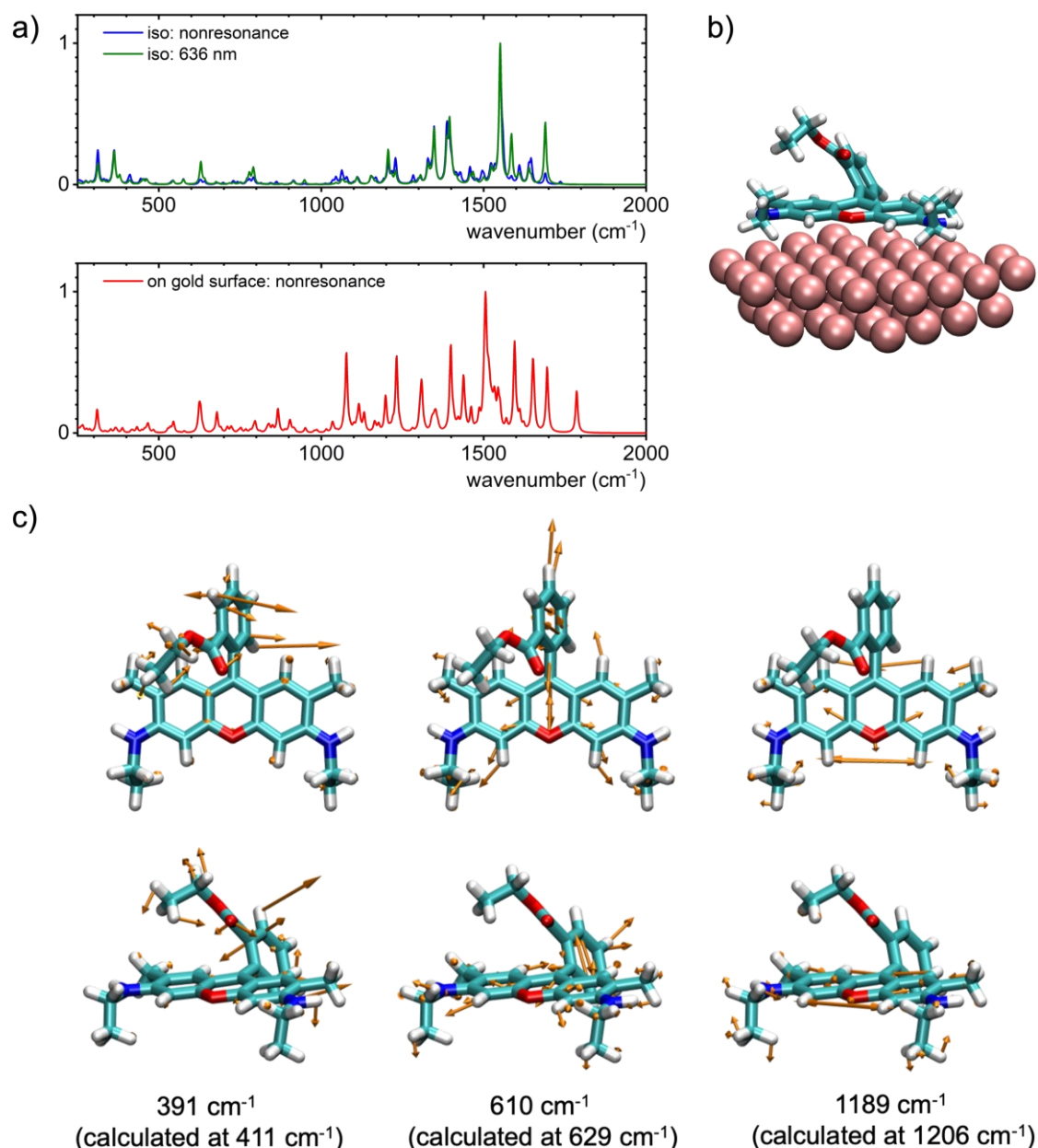


Figure 4-18: (a) Calculated Raman spectra of R6G in ethanol (EtOH, PCM model; top) and adsorbed onto a flat gold surface (bottom), obtained at D3-B3LYP/6-311+G(d) level. (b) Optimized adsorption geometry: the XT chromophore lies parallel to a bilayer gold surface; the ethylamine nitrogen atoms are oriented toward the surface, while the ethyl and EB groups bend out of plane. Atom color code: carbon (cyan), oxygen (red), nitrogen (blue), hydrogen (white), gold (pink). (c) Displacement vectors of three representative vibrational modes of free R6G in ethanol: $\nu_1 = 394 \text{ cm}^{-1}$ (calculated at 411 cm^{-1}) and $\nu_2 = 610 \text{ cm}^{-1}$ (calculated at 629 cm^{-1}), both with strong perpendicular components; $\nu_3 = 1189 \text{ cm}^{-1}$ (calculated at 1206 cm^{-1}), with displacements confined within the XT plane.

4.3.3 Summary and Perspectives

This work further extends the applicability of our spectroscopic-computational framework to plasmonic nano-environments. By integrating experimental observations with quantum-chemical modeling, this holistic and comprehensive methodology demonstrates its versatility and robustness in deciphering polarization-dependent surface-enhanced Raman scattering (SERS) responses. It provides a unified platform for connecting electronic structures, vibrational dynamics, and nano-environmental interactions across complex nano-photonic systems.

A key innovation in this work lies in the use of a cluster model to explicitly define the molecular geometry of surface-adsorbed R6G. This approach enables a realistic description of the adsorption conformation, which is critical for the accurate prediction of vertical excitation energies and for assigning vibrational frequencies of the molecule. This combined approach enabled us to correlate vibrational mode-specific enhancement and different laser polarizations. These findings highlight the critical role of the molecular orientation and higher-order plasmonic resonances in modulating the Raman responses, offering experimentally tunable parameters for optimizing SERS performance.

Despite these advancements, accurately modeling the local plasmonic field remains challenging, especially for more complex architectures such as tip-enhanced Raman scattering (TERS). Further developments in modeling such complex systems will be crucial for deepening our understanding of nano-optics, and for further establishing SERS as a powerful and reliable analytical technique across various scientific domains.

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Appendix

1. Monitoring Tautomerization of Single Hypericin Molecules in a Tunable Optical $\lambda/2$ Microcavity

Liangxuan Wang, Quan Liu, Frank Wackenhut, Marc Brecht, Pierre-Michel Adam, Johannes Gierschner, Alfred J. Meixner, *J. Chem. Phys.* **2022**, 156, 014203.

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2. Deuterium Isotope Effect in Single Molecule Photophysics and Photochemistry of Hypericin

Liangxuan Wang, Quan Liu, Andrea Buchwald, Frank Wackenhut, Marc Brecht, Johannes Gierschner, Alfred J. Meixner, *ChemPhysChem* **2024**, 25, e202400374.

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3. Donor-Acceptor-Donor Triads with Flexible Spacers: Deciphering Complex Photophysics for Targeted Materials Design

Siyang Feng, **Liangxuan Wang**, Begoña Milián-Medina, Alfred J. Meixner, Min Sang Kwon, Soo Young Park, Reinhold Wannemacher, Johannes Gierschner, *Adv. Mater.* **2023**, 35, e2306678.

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4. Higher-Order Cavity Modes for SERS in a Gold Nanosphere-on-Mirror Configuration

Felix Schneider, Lukas Lang, **Liangxuan Wang**, Johannes Gierschner, Alfred J. Meixner, Monika Fleischer, Dai Zhang, *J. Phys. Chem. C.* **2024**, 44, 18807.

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Monitoring tautomerization of single hypericin molecules in a tunable optical $\lambda/2$ microcavity

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ABSTRACT

Hypericin tautomerization that involves the migration of the labile protons is believed to be the primary photophysical process relevant to its light-activated antiviral activity. Despite the difficulty in isolating individual tautomers, it can be directly observed in single-molecule experiments. We show that the tautomerization of single hypericin molecules in free space is observed as an abrupt flipping of the image pattern accompanied with fluorescence intensity fluctuations, which are not correlated with lifetime changes. Moreover, the study can be extended to a $\lambda/2$ Fabry–Pérot microcavity. The modification of the local photonic environment by a microcavity is well simulated with a theoretical model that shows good agreement with the experimental data. Inside a microcavity, the excited state lifetime and fluorescence intensity of single hypericin molecules are correlated, and a distinct jump of the lifetime and fluorescence intensity reveals the temporal behavior of the tautomerization with high sensitivity and high temporal resolution. The observed changes are also consistent with time-dependent density functional theory calculations. Our approach paves the way to monitor and even control reactions for a wider range of molecules at the single molecule level.

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INTRODUCTION

A vibrant development of single-molecule microscopy and spectroscopy has been witnessed in recent years.^{1–9} One of the most fascinating aspects is that it allows us to study fundamental photophysical and chemical processes of individual quantum emitters or nanoparticles,^{10–12} thus revealing rare events and giving the distribution of certain characteristics without ensemble averaging, such as spectral fluctuations and on/off blinking,¹³ different emitting species in single proteins,^{14,15} inhomogeneous spectral behavior,^{16–18} spectral diffusion,^{19,20} time-varying triplet state lifetimes,²¹ individual isomers,^{22,23} and allowing us to explore the energy landscape of proteins.^{24,25} The technique becomes even more appealing if the photonic environment can be further tailored by placing the quantum emitters into an optical microcavity.^{26–28} Consequently, a variation of the radiative rate due to the Purcell effect is expected.^{29–31} In

recent years, we have conducted extensive studies in a Fabry–Pérot type microcavity at the single-molecule level and stressed the ability of the microcavities to tailor the optical and photophysical properties of single quantum emitters.^{26,32,33} A Fabry–Pérot microcavity consists of two parallel mirrors allowing multiple beam interference. By varying the mirror separation, the cavity resonance and the local mode structure of the electromagnetic field are changed together with the radiative coupling of the emitters to the field.^{34,35} The reduced transmission of a microcavity makes it crucial to achieve a high signal to noise ratio at the single-molecule level. Therefore, general candidates are, for instance, quantum dots and specially selected dye molecules with a high fluorescence quantum yield and good photostability. Indeed, it is important to extend the methodology to a broader range of objectives. Here, we present a first comprehensive study of single hypericin molecules inside a $\lambda/2$ Fabry–Pérot microcavity and show that the excited state lifetime trajectories of

single hypericin inside the microcavity can be monitored to reveal the temporal behavior of the tautomerization.

Hypericin is a natural drug widely found in more than 300 species of the genus *Hypericum* of the plant family Guttiferae.^{36–40} It has been intensively investigated in biochemical and biomedical studies due to its antiviral and antiretroviral properties,³⁶ and it is also reported to be one of the most promising photosensitizers used in photodynamic therapy (PDT).^{41–44} Hypericin tautomerization is a reversible chemical reaction, closely related to its antiviral activity, in which the position of labile proton changes.^{45,46} Time-dependent density functional theory calculations show that the transition dipole moments (TDMs) of its tautomers differ by a significant amount in orientation ($\sim 24^\circ$) and strength ($\sim 26\%$).⁴⁷ The absolute 3D spatial TDM orientation of a single hypericin molecule can be observed using confocal microscopy combined with higher order laser modes, namely, azimuthally polarized doughnut mode (APDM) and radially polarized doughnut mode (RPDM).^{48–51} The tautomerization transition can be directly witnessed by the reorientation of the TDM via the flipping of the fluorescence excitation image pattern. In order to obtain a better knowledge of the photodynamic mechanism of hypericin, it is crucial to understand the influence of the photonic environment on the tautomerization. In contrast to the usually used high quantum yield fluorescence dyes, hypericin is suffering from its high triplet yield and the rather weak fluorescence intensity. Hence, recording fluorescence excitation images by confocal scanning usually used in free space is not suitable for single hypericin molecule experiments in a microcavity.

The time a single hypericin molecule resides in a specific tautomeric form is referred to as the residence time. To examine the residence time on a sub-second time scale, we show that the excited state lifetime of single hypericin molecules inside a microcavity can be monitored to reveal the tautomerization. Time-correlated single photon counting (TCSPC) is the method of choice since it offers highly sensitive detection with a good temporal resolution (up to several picoseconds), while a confocal scan is limited by the scanning rate, normally requiring several seconds for image recording. The lifetime of the same single molecule is stable over time, as long as it resides in a rigid, homogeneous environment [e.g., embedded in poly(vinyl alcohol) (PVA) matrix]. Small fluctuation of the lifetime in free space can be explained by the variations of the local density of states (LDOS) caused by the local environment fluctuations.⁵² In comparison to free space, the anisotropic LDOS in the microcavity exposes a reorientation of the TDM associated with the tautomerization as a sudden distinct change in the fluorescence lifetime that is correlated in time with a respective change in the fluorescence intensity.

In this paper, we first investigate the tautomerization of single hypericin molecules in free space, which is observed as an abrupt flipping of the image pattern accompanied with large fluorescence intensity fluctuations, while the lifetime only changes slightly. These transient intensity fluctuations of a single hypericin molecule can be used to analyze the temporal tautomerization dynamics.⁴⁷ Next, we measure the fluorescence lifetime of hypericin in a tunable Fabry–Pérot microcavity at the single molecule level. Tautomerization induced reorientation of the TDM is further confirmed by the discrete lifetime changes of single hypericin molecules in the cavity. Recognizing hypericin tautomerization as a jump of the lifetime in a microcavity, the tautomerization residence time is obtained between

the transient changes in fluorescence lifetime. It is particularly interesting to explore the temporal behavior of the tautomerization and how it is altered by the photonic environment. This approach also allows us to study a wider range of molecules at the single molecule level.

EXPERIMENTAL SECTION

Confocal microscope setup

All measurements were performed with a home-built confocal microscope. The scheme of the setup is depicted in Fig. 1. The sample was excited by a pulsed laser (530 nm, 1.2 μ W in front of the objective) focused on the sample via a high numerical aperture (NA = 1.46, Carl Zeiss) oil objective lens. The laser mode profile can be switched between the linearly polarized doughnut mode, azimuthally polarized (APDM) doughnut mode, and radially polarized doughnut mode (RPDM). APDM and RPDM are generated by a commercial mode converter (Polarization Converter, ARCOptix). Scanning images were obtained by fixing the sample with magnets on a piezo-stage (P-527.3CL, Physik Instrumente) and raster scanning it through the fixed excitation focus. The fluorescence signal was collected by the same objective lens and sent to two avalanche photodiodes (APDs, SPCM-AQR-14, PerkinElmer). A time-correlated single photon counting module (TCSPC, HydraHarp 400, PicoQuant) was used for fluorescence lifetime and photon antibunching measurements along with linearly polarized excitation.

Materials and sample preparation

Hypericin (Burg-Apotheke, Königstein) was first dissolved in ethanol (Uvasol, Merck, Germany). Poly(vinyl alcohol) (PVA, Sigma-Aldrich) was dissolved in triply distilled water, and 2 μ l of hypericin solution was added to a 2 wt. % PVA solution to obtain the required concentration. Hypericin concentrations of 10^{-5} and 10^{-9} M were used for ensemble and single molecule experiments, respectively. All solutions were stored in the dark at 4 $^\circ$ C to minimize photobleaching. 20 μ l of a respective solution was spin-coated (6k rpm, 30 s) on a clean cover slip or on the lower mirror of the microcavity. The PVA polymer matrix forms a rigid environment that locks the position and rotation of molecules and protects them from oxygen.

Optical microcavity preparation

The microcavity used in this work is based on the tunable microcavity structure originally designed by Chizhik and co-workers.^{33,53} It consists of two mirrors: The bottom mirror was prepared on a cleaned cover slip by sequentially evaporating (Edwards EB3, 10^{-6} mbar, 0.1 nm/s) 1 nm Cr, 30 nm Ag, and 50 nm SiO₂, while the top mirror was a convex lens with a 25.8 mm curvature radius covered with 1 nm Cr, 50 nm Ag, and 50 nm SiO₂ layers, consecutively. The Cr layer was used as an adhesion layer, while the SiO₂ layer serves as a spacer to protect the two mirrors from possible mechanical damage and to avoid quenching of the molecule on top of the silver film. These two mirrors were assembled in a home-built mirror holder, and a piezo-actuator (KCI-PZ/M, Thorlabs) was used to approach the top mirror

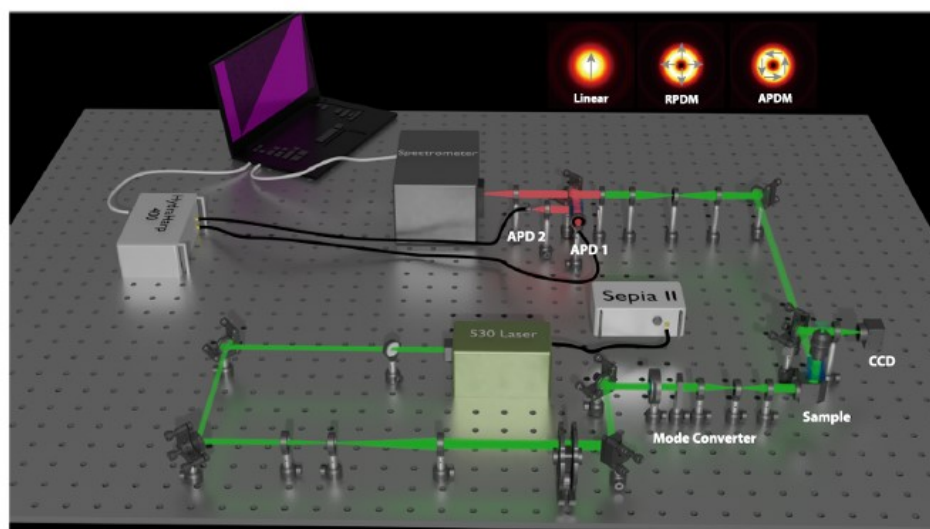


FIG. 1. Scheme of the confocal microscope equipped with a mode converter to alter the beam profile and its polarization. The inset (top right) shows the intensity cross section of a linearly polarized Gaussian beam, an azimuthally polarized doughnut mode (APDM), and a radially polarized doughnut mode (RPDM).

toward the fixed bottom mirror, where the fluorescence signal was collected.

RESULTS AND DISCUSSION

Hypericin offers a rich structural variety of conformational and constitutional or structural isomers due to the presence of six hydroxyl and two keto groups, as well as methyl groups in the bay region of the naphthodianthrone core.⁴⁰ The conformation and tautomerization pathways have been investigated in the ensemble^{54–56} and very recently at the single-molecule level.^{47,57} In a previous study, we showed that the chemical structure of a single hypericin molecule can be determined *in situ* by determining single molecule tautomeric states. Furthermore, from a theoretical point of view, we presented a clear classification of tautomerism process and the most probable tautomerization pathway.⁴⁷ We exemplify here in Fig. 2(a) the four most stable tautomers with the S_0 – S_1 TDM calculated by TD-DFT plotted as double red arrows indicating the relative strength of the TDMs. The largest in-plane angle difference between TDMs from $Q_{1,7}$ and $Q_{7,13}$ is 24° , and the out-of-plane angles differ by less than 2° .⁵⁷ Our experimental-computational approach confirmed the coexistence of these four tautomers and presented the single-molecule tautomerization dynamics via consecutive fluorescence excitation pattern scans. Here, $\Gamma_{exc} \propto |D \cdot E_{exc}|^2$ is the excitation rate depending on the local excitation field E_{exc} . The orientation of the TDM D can be directly determined from the excitation image pattern using higher order laser modes, i.e., APDM and RPDM.⁴⁸ The focal field intensity of the APDM, which forms a ring-shaped pattern, is strictly azimuthally polarized perpendicular to the optical axis (in-plane); hence, the excitation patterns of a single molecule always have a double lobe shape and directly display the in-plane orientation of the TDM. On the contrary, the focal field of a RPDM has a strong longitudinal field component in the center

along the optical axis (out-of-plane), surrounded by a toroidal weaker field component, which is radially polarized. This results in excitation patterns ranging from dot-like to asymmetric double lobe and finally to symmetric double lobe shapes. These different pattern shapes can be used to determine the out-of-plane orientation of the TDM. Therefore, two molecules that are fixed in space, i.e., no translation and rotation, and have differently oriented TDM appear with differently shaped excitation patterns. Consequently, tautomerization, which changes the orientation of the TDM with respect to the molecular structure, should appear as an abrupt change in the excitation pattern. Such a change can appear as a sudden change in an image scan or between consecutive images of the same molecule. Furthermore, if the transition between two tautomeric forms of a molecule is much faster than the time required for image acquisition, the observed excitation pattern is a superposition of the multiple patterns. In order to verify the tautomerization, we acquired consecutive fluorescence excitation images of the same spatial region until photobleaching of molecules occurred.

Four consecutive images, acquired at the same spatial region, are shown in Fig. 2(b). More than nine well separated single molecules can be observed. The second order correlation function has been measured on the same sample; clear antibunching (see Fig. S1 of the supplementary material) can be easily achieved for randomly selected bright spots, proving that single molecules are observed. Since all the molecules are embedded in a rigid PVA matrix, they cannot move or rotate and their local environment in the focus is supposed to be relatively homogeneous. Distinct intensity differences among different molecules can be due to various orientation of the molecule with respect to the excitation field, and local heterogeneities in the amorphous polymer matrix causing an intrinsic variation of the radiative and nonradiative decay rate. Alternating dark and bright pixels, e.g., molecules 1 and 2, indicate the typical photo blinking behavior of single molecules at the millisecond range. The TDMs of molecules 1–3 have a fixed orientation

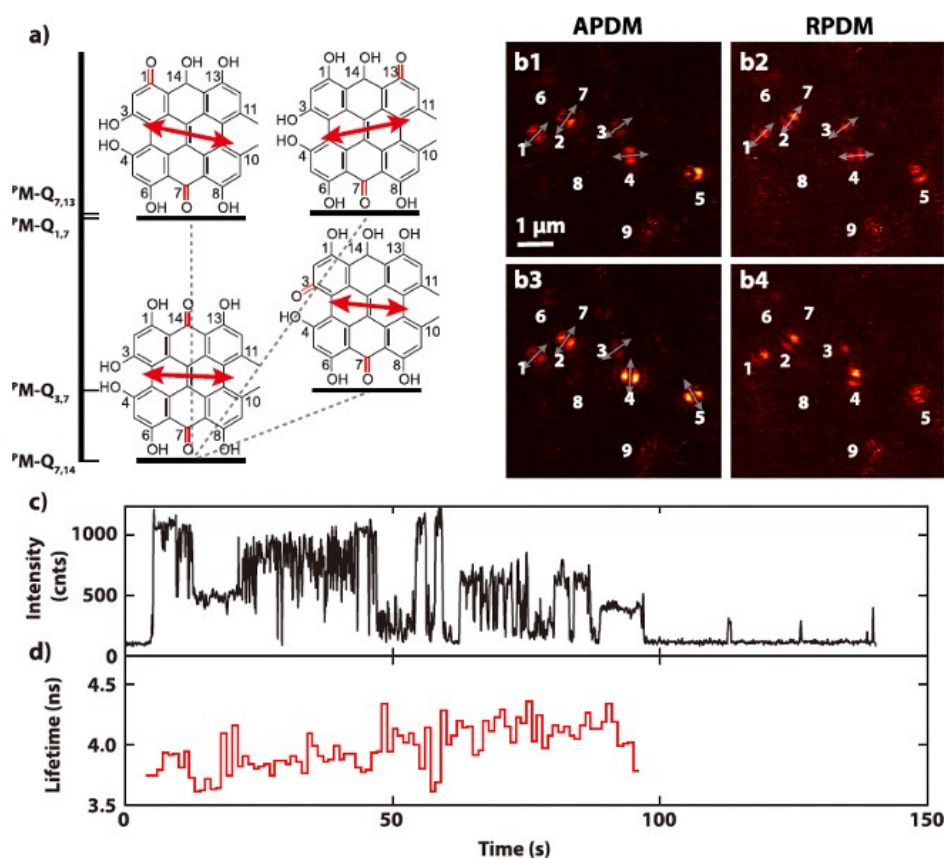


FIG. 2. (a) Gibbs free energy (calculated by DFT) diagram of the tautomers of hypericin overlaid with the tautomerization transition path. The four most stable tautomeric forms are displayed, and the red arrows indicate the orientation of the respective $\text{S}_0 \rightarrow \text{S}_1$ electronic transition dipole moments (TDMs). (b) Excitation fluorescence patterns of single hypericin molecules excited with the azimuthally polarized doughnut mode (APDM) and radially polarized doughnut mode (RPDM). The arrows indicate the in-plane orientation of the TDMs. The scanning range is $6 \times 6 \mu\text{m}^2$ ($150 \times 150 \text{ pixel}^2$, 5 ms/pixel). (c) Fluorescence time trace of a single hypericin molecule recorded with a linearly polarized Gaussian beam profile with a binning time of 10 ms and (d) the corresponding fluorescence lifetime trajectory with a binning time of 1 s.

during the scan series, while the TDMs of molecule 4 and molecule 5 reorient approximately by 90° from Figs. 2(b1)–2(b3) accompanied with obvious fluorescence intensity fluctuation. Especially in Fig. 2(b1), molecule 5 manifests the tautomerization transition as a sudden flip of the excitation pattern; this results in an overlap of two different pattern orientations. However, image pattern acquisition is limited by the scanning rate up to several minutes.

On the contrary, TCSPC allows us to record intensity fluctuations with a much higher temporal resolution. One representative example is presented in Fig. 2(c), which shows significant intensity fluctuation. We excite the same molecule continuously with a linear polarized laser to obtain a continuous fluorescence time trace and the steps between different levels in the intensity trajectory can be assigned to the tautomerization transition of a single hypericin molecule.^{47,57} Unlike the multi-stair behavior observed from multiple molecules, tautomerization transitions associated with a sudden orientation change in the transition dipole moment manifest themselves as stepwise intensity fluctuations with

a high chance to decrease to the background level in a single step due to photobleaching or triplet quenching via intersystem crossing.⁴⁰

Benefitting from the high sensitivity and high temporal resolution of time-correlated single photon counting (TCSPC), the temporal behavior of the tautomerization transition can be easily captured even at low intensity levels. This allows exploring the tautomerization transition in a microcavity, which is an effective approach to alter the spontaneous emission rate of a single molecule by the alteration of the LDOS, without changing its chemical structure or local environment. Before focusing on the impact of the cavity-induced changes in the photonic environment, it is important to exclude the influence of local environment fluctuations in free space. Figure 2(d) shows the fluorescence lifetime trajectory of the time trace in Fig. 2(c) with a binning time of 1 s. Local environment fluctuations cause random lifetime changes, while the intensity changes in free space are caused by tautomerization transitions and are not correlated with lifetime changes.

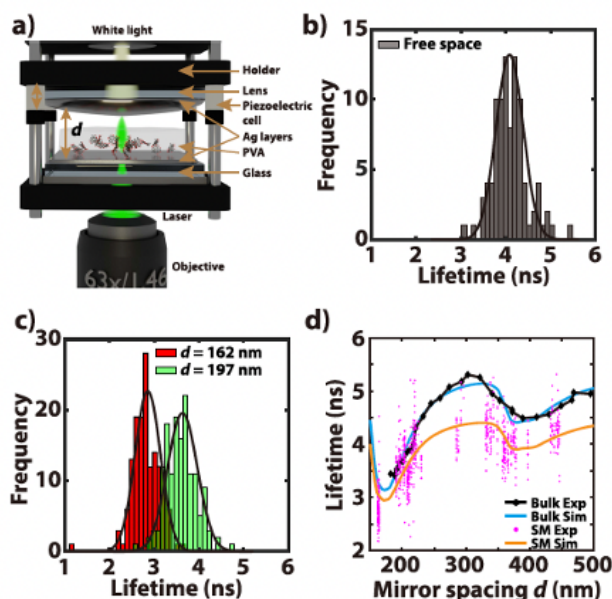


FIG. 3. (a) Schematic drawing of a $\lambda/2$ microcavity where single hypericin molecules in a PVA layer are positioned between two silver mirrors. Fluorescence lifetime histograms of single hypericin molecules (b) in free space and (c) in the cavity for two mirror spacings of 162 nm (red) and 197 nm (green). (d) Fluorescence lifetime of the ensemble (10^{-5} M, black curve) and single hypericin molecules (10^{-9} M, magenta dots) as a function of mirror spacing d . The solid curves are the best fit to the respective experimental data of the ensemble (blue curve) and single molecules (orange curve).

Figure 3(a) shows a schematic drawing of the microcavity. Fabrication details are given in the section titled “Experimental section.” As explained above, fluorescence lifetimes obtained from single molecule measurements present a distribution due to the distinct decay rates. Figures 3(b) and 3(c) show the distribution of the lifetime in free space and inside a cavity with fixed mirror spacing. A Gaussian function is used for fitting. The full width at half maximum (FWHM) of the fitting results is 1.01 ± 0.16 ns for free space, 0.97 ± 0.12 ns when the cavity mirror spacing is 162 nm, and 1.08 ± 0.14 ns when the cavity mirror spacing is 197 nm. Similar distributions with similar FWHMs are obtained for other cavity mirror spacings. The similar widths of the distributions suggest that the molecular structure and local environment have not significantly changed except for the photonic environment in the cavity leading to a shift of the whole distribution.

To elucidate the modification of the photonic environment by a cavity, fluorescence lifetimes of hypericin inside the microcavity were measured both at the ensemble and at the single molecule level as a function of mirror spacing d . The cavity mirror spacing is tuned from 160 to 500 nm as determined from the transmission spectra (for details, see Fig. S2 of the supplementary material). Figure 3(d) displays the results of the lifetime measurements with the cavity resonance tuned twice across the emission maximum of the molecular fluorescence. Two local minima are observed for a mirror spacing of 170 and 390 nm, respectively, which correspond to two maxima of the Purcell factor when the cavity is on resonance with the hypericin

emission maximum [Fig. S3(b)]. According to Fermi’s golden rule,⁵⁸ the spectral spontaneous emission rate of a molecule represented by the TDM D can be calculated as $k_r(\omega) \propto |D \cdot E|^2 \rho(\omega)$, where E represents the mode field and $\rho(\omega)$ is the spectral mode density. Taking into account the influence of the line shape of the fluorescence spectrum (ω), the spectral emission rate $k_{r,c}(\omega)$ of a molecule in the cavity is

$$k_{r,c}(\omega) \propto S(\omega) |D \cdot E_c|^2 \rho(\omega, d, z), \quad (1)$$

where the cavity mode field $E_c(\theta, \varphi)$ is a vector property, which is normalized to unity at the antinode. The local optical mode density in the cavity $\rho(\omega, d, z)$ depends on the emission frequency ω , the mirror spacing d of the cavity, and the position z of the molecule in the cavity. The spectral averaged Purcell factor $P(d, z)$, which is defined as the ratio of the spontaneous emission rate in the cavity $k_{r,c}$ to the free space rate k_r , is obtained as³⁴

$$P(d, z) = \frac{k_{r,c}}{k_r} = \frac{\int S(\omega) |D \cdot E_c|^2 \rho(\omega, d, z) \sin \theta d\theta d\varphi d\omega}{\int S(\omega) |D \cdot E|^2 \rho(\omega) \sin \theta d\theta d\varphi d\omega}. \quad (2)$$

The fluorescence spectrum $S(\omega)$ of hypericin is obtained experimentally.⁵⁹ $E_c(\theta, \varphi)$ and $\rho(\omega, d, z)$ can be derived numerically using the transfer matrix method (TMM).⁶⁰ The molecules are placed about half way between the mirrors inside the cavity, referring to a distance of $z = 90$ nm from the lower mirror separated by a thin silica layer and a PVA film. In an ensemble, the molecules and their TDMs D are assumed to be randomly orientated in the cavity. However, the TDM of a specific single molecule exhibits a particular orientation. Calculated Purcell factors for specific orientations of D are shown in Fig. S3(b).

Once the Purcell factor is available, the excited state lifetime inside the cavity, τ_c , can be determined by the following equation:⁶¹

$$\frac{1}{\tau_c} = k_{r,c} + k_{nr} = Pk_r + k_{nr} = \frac{1}{\tau_0} (1 - \Phi_F + P\Phi_F), \quad (3)$$

where $k_{r,c}$ is the radiative rate in the cavity and k_{nr} is the nonradiative rate, only depends on the local surrounding host, and is specific for the PVA matrix. $\Phi_F = \frac{k_r}{k_r + k_{nr}}$ is the quantum yield. Experimental data in Fig. 3(d) are fitted using Eq. (3). The results are plotted as solid blue and orange lines. We note that the free space excited state lifetime τ_0 and the quantum yield Φ_F are the only free parameters for the curve fitting^{61,62} and are $\tau_0 = 4.78 \pm 0.06$ ns, $\Phi_F = 12.2\% \pm 1.2\%$ for the ensemble and average values of $\tau_0 = 4.16 \pm 0.09$ ns, $\Phi_F = 9.6\% \pm 1.8\%$ for the single molecule measurements, respectively. While the single molecule free space lifetime is notably smaller than the lifetime in the ensemble, both values are in good agreement with experimental data from literature.⁵⁹ Shorter fluorescence lifetimes are typical for single molecule experiments since the person performing the experiments tends to select the brighter single molecule fluorescence spots, hence preferentially selecting those molecules with a large radiative rate and a short lifetime, whereas darker molecules have smaller radiative rates and longer lifetimes are missed. This is even more the case for single hypericin inside the cavity. The quantum yield values found here are smaller than the value measured in pure dimethyl sulfoxide ($\Phi_F \approx 20\% \pm 3\%$),^{40,63} which suggests that hypericin is very sensitive to the local environment. A decrease in the quantum yield of hypericin

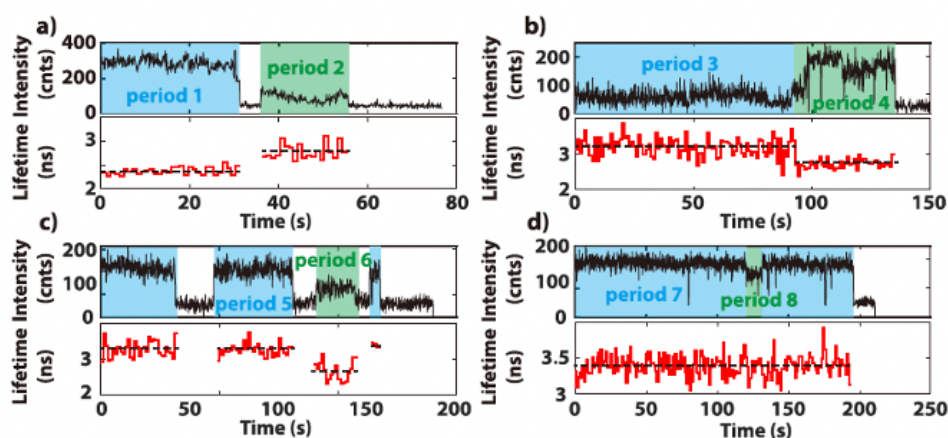


FIG. 4. Fluorescence intensity time traces together with the respective fluorescence lifetime trajectories are shown for four individual molecules in a microcavity at the following cavity mirror spacing: (a) 162 nm, (b) 197 nm, (c) 212 nm, and (d) 197 nm. The binning time is 10 ms for time traces and 1 s for lifetime trajectories.

is also reported with a continuous increase in the water content in dimethyl sulfoxide–water mixtures.⁶³

Finally, we focus on single molecules inside the cavity. According to Eq. (2), the Purcell factor is different for molecules with different orientation of the TDM D with respect to the optical axis (i.e., the cavity mode field) even if all other parameters are kept constant. The fluorescence intensity time traces and the lifetime trajectories of four individual molecules in a microcavity for different mirror spacings are presented in Fig. 4. The mirror spacings are in the $\lambda/2$ range in order to achieve larger Purcell factors. In the time traces, the background level is around 50 counts with 10 ms binning time. Compared to the single molecule in free space [Fig. 2(c)], the detected fluorescence intensity is five to ten times weaker inside the cavity. This is due to the reduced transmittance of the cavity [the transmittance is typically around 10%–20% in experiments (see Fig. S2 of the supplementary material)] as compared to the samples without mirrors. All examples exhibit typical, aforementioned intensity fluctuations and single step bleaching dynamics. Inside the cavity, the molecular structure and the local environment have not changed. The discrete lifetime changes in single molecules correlate directly with the intensity fluctuations and hence very likely reflect the tautomerization induced reorientations of the TDMs. The fluorescence intensity of the single molecule in Fig. 4(a) decreases from 236 counts in period 1–41 counts in period 2, which correlates with an increase in the lifetime from 2.4 to 2.8 ns. The average Purcell factor can be calculated accordingly and is 8.0 during period 1 and 5.6 during period 2. For this calculation, we have used the average values for the fluorescence lifetime and quantum yield in free space, $\tau_0 = 4.16$ ns and $\Phi_F = 9.6\%$, as obtained from fitting the experimental single molecule data. Comparing with calculated Purcell factor for different TDM orientations [see Fig. S3(b) of the supplementary material, as indicated by the red dashed line], we can conclude that the TDM is oriented almost parallel to the mirror planes in period 1 (close to $D_1 = [0^\circ, 80^\circ, 1]$, $P_{162\text{nm}} = 8.1$), while it has an out-of-plane orientation in period 2 (close to $D_2 = [45^\circ, 55^\circ, 1]$, $P_{162\text{nm}} = 5.6$). The difference in angle θ [see Fig. S3(a) of the supplementary material] between D_1 and D_2 is in the range of the achievable maximum

angle difference between different tautomers, which is 24° according to TD-DFT calculations.^{47,57} Accordingly, the fluorescence intensity in period 1 should be 1.2 times of the one in period 2 if only the Purcell effect ($\Phi_F = \frac{P_{T_F}}{P_{T_F} + P_{T_M}}$, $\Phi_{F,\text{period1}}/\Phi_{F,\text{period2}} = 1.2$) is considered. However, the experimental data show 5.8 times intensity difference, which is most likely caused by a difference in the excitation rate and the collection efficiency. Accounting for the angle dependence of the dipole radiation,⁶⁴ the collection efficiency in period 1 is larger than in period 2. Additionally, the relative orientation of TDMs of the molecule with respect to the linear polarization of the excitation field leads to different excitation rates at different periods. In the end, considering the joint contribution of the Purcell effect, the excitation rate, and the collection efficiency, it is very well possible to have a sixfold intensity difference ($I_{\text{period1}}/I_{\text{period2}} = 5.8$) between period 1 and period 2. Figure 4(b) shows a similar time trace of another molecule, and there are also two distinct periods from 0 to 80 s (period 3) and from 100 to 135 s (period 4). A Purcell effect induced intensity enhancement $\Phi_{F,\text{period4}}/\Phi_{F,\text{period3}} = 1.4$ is found, and the experimental fluorescence intensity ratio $I_{\text{period4}}/I_{\text{period3}}$ is 2.8.

In contrast to Figs. 4(a) and 4(b) where higher fluorescence intensity corresponds to smaller lifetime, the single molecule in Fig. 4(c) has a higher fluorescence intensity for larger lifetimes. This clearly shows the complicated interplay of the Purcell effect, the excitation rate, and the collection efficiency. Again, two periods are defined in Figs. 4(c), i.e., period 5 (0–32, 50–81, and 115–118 s) and period 6 (91–108 s). In this case, the Purcell effect induced intensity enhancement $\Phi_{F,\text{period6}}/\Phi_{F,\text{period5}}$ is 1.5, while the experimental intensity in period 6 is weaker compared to period 5 ($I_{\text{period6}}/I_{\text{period5}} = 0.6$). This implies that the excitation rate or the collection efficiency decreases, even though the Purcell factor has increased in period 5 compared to period 6. Figure 4(d) shows another example with intensity fluctuations between 130 and 135 s and no lifetime variation. Here, tautomerization most likely occurs as an in-plane reorientation of the TDM, i.e., only the excitation rate will change, while the LDOS is constant; hence, the lifetime will stay constant, too.

CONCLUSIONS AND OUTLOOK

Using confocal microscopy combined with higher order laser modes, we successfully observe the tautomerization of hypericin at the single molecule level. Furthermore, we extend the studies from free space to a microcavity. By varying the mirror spacing, the local mode structure of the electromagnetic field is altered in a controlled way. As a result, the spontaneous emission rate is modified by the Purcell effect and gives rise to a decrease or an increase in the fluorescence lifetime. A theoretical model is introduced to discuss the modification of the photonic environment by a cavity and shows good agreement with the experimental data. Moreover, the excited state lifetime trajectories of single hypericin molecules inside a microcavity are monitored to reveal the temporal behavior of the tautomerization. These transient intensity fluctuations can be further analyzed using the fluorescence correlation function.⁴⁷ The investigation of the fluorescence time trace illustrates a much more detailed tautomerization process. For a molecule that is embedded in a solid host matrix such that it cannot translate or reorient, we attribute a distinct jump of the lifetime and fluorescence intensity to a reorientation of the TDM associated with the tautomerization inside a microcavity. In combination with a microcavity, the temporal behavior of hypericin tautomerization is monitored with high sensitivity and high temporal resolution. We further prove that the observed changes are consistent with TD-DFT calculations. Our approach allows us to study a wider range of molecules at the single molecule level, especially when the molecule is in a microcavity where less fluorescence signal can be collected. This may pave the way to monitor and control reactions within an optical microcavity.

SUPPLEMENTARY MATERIAL

As the [supplementary material](#), we present confocal scan images of single hypericin molecules and second order fluorescence intensity correlation traces that show antibunching, and measured and simulated white light transmission spectra of a $\lambda/2$ Fabry-Pérot resonator as a function of cavity mirror spacing, and a diagram illustrating the orientation of the TDM in the microcavity with respect to the optical axis that is used as a basis for calculating the Purcell factor as a function of cavity mirror spacing.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Deuterium Isotope Effect in Single Molecule Photophysics and Photochemistry of Hypericin

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The peripheral protons of the dye molecule hypericin can undergo structural interconversion (tautomerization) between different isomers separated by a low energy barrier with rates that depends sensitively on the interaction with local chemical environment defined by the nature of host material. We investigate the deuterium (D) isotope effect of hypericin tautomerism at the single-molecule level to avoid ensemble averaging in different polymer matrices by a combined

spectroscopic and computational approach. In the 'innocent' PMMA matrix only *intramolecular* isotope effects on the internal conversion channel and tautomerization are observed; while PVA specifically interacts with the probe via H- and D-bonding. This establishes a single molecular picture on *intra*- and *intermolecular* nano-environment effects to control chromophore photophysics and -chemistry.

Introduction

Optical detection of single molecules in condensed matter has expanded far beyond the early low-temperature studies in crystals, to investigate single molecules in cells, in solution and in polymers. Detecting individual molecules optically gains the major advantage from avoiding ensemble averaging, providing direct access to inhomogeneous static or quasi-static distribution in nano-environments. As a consequence, it hence allows to observe kinetics, for instance, diffusion^[1] and binding processes of nucleic acid^[2] and proteins.^[3] Especially, conformational dynamics are of interest, since they are difficult to observe or are hidden in a classical ensemble measurement.^[4] Moreover, it allows access to spatial^[5] as well as structural^[6] information.^[7]

Tautomerization is an *intramolecular* chemical reaction defined as structural interconversion of a molecule between two isomers separated by a low energy barrier, in which the position of a labile proton changes.^[8] Particularly interesting is that the tautomerization rate in liquid solutions at room temperature is often on the order of MHz, but it can range over orders of magnitude depending on the temperature and the local environment. Embedding tautomerizable single molecules at low concentration in various polymer matrices at room temperature has unequivocally revealed that the rate in these molecules can vary dramatically reduced over time, such that tautomerization can be frozen for seconds and longer under ambient conditions.^[4c]

Hypericin (Scheme 1), as a natural active ingredient in *Hypericum perforatum* (St. John's wort), has found tremendous interest in bio- and biomedical applications as photosensitizer, multifunctional agent in drugs due to its anti-depressive, anti-neoplastic, anti-tumor and anti-viral activity.^[9] Furthermore, hypericin is not only of medical interest, but also a fascinating molecule for the investigation of fundamental physical and

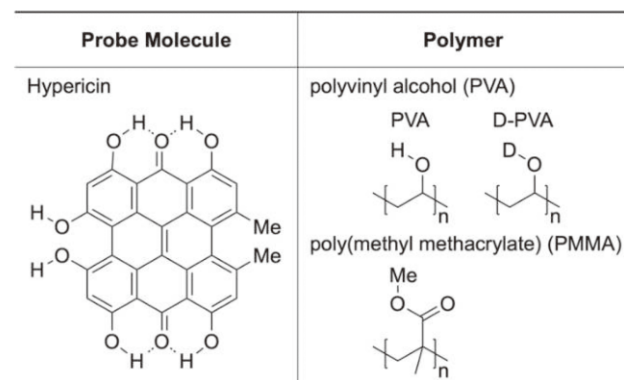
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Scheme 1. Chemical structure of hypericin and the host polymers: PVA and PMMA.

chemical processes, as it can undergo de-/protonation, conformational transitions and tautomerization.^[10] Proton migration around the perimeter of hypericin gives rise to ten tautomeric isomers.^[10c,11] Direct observation of the tautomerization of hypericin in the polymer matrix was achieved in preliminary studies using confocal microscopy combined with an azimuthally polarized or radially polarized doughnut mode (APDM/RPDM) laser beam.^[11] The tautomerization in hypericin occurs at the 'outer' part of the molecule and is hence very sensitive to the interaction of the chromophore with the host molecules. By altering the local optical and chemical nano-environment, one can modulate the radiative as well as non-radiative rates.^[11–12] The modification of the local photonic environment can be achieved independently by enclosing the molecule between the two mirrors of a $\lambda/2$ Fabry-Pérot microcavity and varying their distance.^[12b] As a result, the spontaneous emission rate is modified by the Purcell effect and leads to a variation in fluorescence lifetime.^[12b]

In this work, we focus on the local chemical environment as reflected by the dielectric constant, oxygen permeability and solvent and/or water content of the polymer; all these factors can strongly influence the excited-state lifetime, dark state dynamics and survival time (photo-bleaching) of the probes. Furthermore, the investigation is extended to deuterated environments; deuteration causes only minor structural change, but has a considerable impact on the non-radiative decay path.^[13] Due to the low pK_a (1.8) at the bay hydroxyl groups,^[10b,14] H/D exchange can be easily achieved in solution. In addition, fluorescence correlation spectroscopy (FCS)^[15] is used to analyze temporal dynamics of hypericin tautomerization; this allows to study how the tautomerization rate is influenced by the matrix in which the molecule is embedded or by exchanging the tunneling proton with deuterium. Last but not least, the experiments are complemented by (time-dependent) density functional theory (TD)DFT calculations, in which hypericin was embedded in an explicit solid matrix through a cluster model; this allows to study the *intermolecular* interactions, that largely influence the curvature of potential energy surface, and may give indications for the tunneling effect.

Experimental Section

Confocal Microscope

All measurements were performed with a home-built confocal scanning microscope. The sample was excited by a pulsed laser ($\lambda=530$ nm, pulse duration < 100 ps, 20 MHz, LDH-P-FA-530 L, PicoQuant, 3 μ W in front of the objective), focused on the sample via a high numerical aperture (NA=1.46, 63 $\times$, Carl Zeiss) oil objective lens. Scanning images were obtained by fixing the sample with magnets on a piezo-stage (P-527.3CL, Physik Instrumente). The fluorescence signal from the excitation spot was collected by the same objective lens and sent to two avalanche photodiodes (APDs, SPCM-AQR-14, PerkinElmer) via a 50/50 beam splitter. The APDs were connected to a time-correlated single photon counting (TCSPC) module (HydraHarp 400, PicoQuant), synchronized with the transistor transistor logic (TTL) signal, generated by the pulsed laser; a TTL from the APD is recorded for each detected photon and

saved as a time-tagged time-resolved (TTTR) file.^[16] The presence of single hypericin molecules was confirmed by antibunching measurements. (see Figure S1 in SI)

Materials and Sample Preparation

Hypericin (Burg-Apotheke, Königstein) was dissolved in ethanol (Uvasol, Merck) or in methanol- d^4 (99% atom D, Sigma-Aldrich), respectively, for H/D exchange; in the following denoted as D-hypericin. Poly (vinyl alcohol) (PVA, Mowiol 40–88, Sigma-Aldrich) was either dissolved in triply distilled water or in deuterium oxide (99.9% atom D, Sigma-Aldrich) (2 wt. %) for a complete H/D exchange of the hydroxyl group; in the following denoted as D-PVA, respectively. Poly (methyl methacrylate), PMMA (in anisole, AR–P 631-679 e-beam resists for nanometer lithography)^[17] is commercially available. H- and D-hypericin concentrations of 10^{-9} M were used for single-molecule experiments. To prevent photobleaching as far as possible, the sample solutions were stored in a dark refrigerator. The preparation of thin films was carried out by spin-coating (20 μ L solution approx. 6000 rpm over a period of 20 seconds). All the measurements were conducted under a laminar nitrogen flow to minimize the impact of oxygen quenching.

Computational Methods

The electronic ground, excited and transition state optimizations on the full structure of the system were carried out by (TD)DFT calculations. In all cases, the B3LYP functional with the standard 6–31G(d) basis set with dispersion correction (D3) was applied, as defined in the Gaussian16 program package.^[18] As the work focuses on the nano-environmental effect on single hypericin molecules, we propose a realistic model of the single hypericin molecule surrounded by an explicit polymer matrix, describing specific solvent-solute interactions at the electronic level. A full geometry optimization of hypericin in PVA/PMMA was carried out. (see Figure 1(e)) In detail, one hypericin molecule was surrounded by either seven PMMA or 14 PVA oligomers (with $n=5$ repeating units) within the Packmol package,^[19] followed by 10 ns molecular dynamics (MD) simulations with Gromacs^[20] adopting the GAFF force field, restraining the positions of the hypericin structure. All energy minima were confirmed by the absence of imaginary frequencies, whereas the transition states were characterized by one imaginary frequency with a proper mode, referring to a local maximum (a saddle point along the potential energy surface) and reflecting the dynamic pathway between two local maxima.^[21] For the details of all calculations, see SI.

Results and Discussion

Hypericin is a dye molecule with a large π -electron system, whereby the methyl-substitutions at the *bay* region contribute to a distorted aromatic parent backbone (see Figure 1(d)). Besides, hypericin offers a rich structural variety of conformational and constitutional or structural isomers due to the presence of six hydroxyl and two keto groups, as well as naphthodianthrone core.^[10b] Ensemble fluorescence lifetime measurements of H-hypericin in a PVA polymer matrix were described by us earlier,^[22] giving an average lifetime of $\tau_f=4.2\pm0.9$ ns and a quantum yield of $\Phi_f=12.2\%\pm1.2\%$. Such polymer matrices are commonly used in single molecule measurements to immobilize and photo-stabilize the

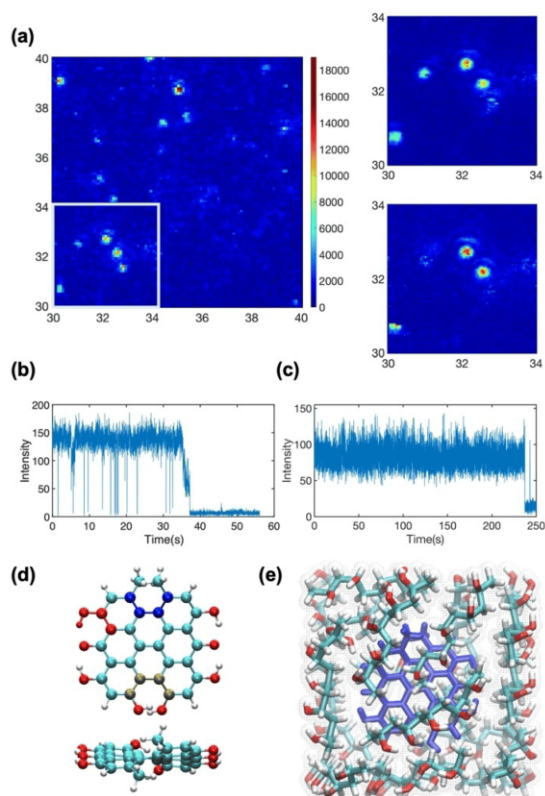


Figure 1. Fluorescence properties of single hypericin molecules. (a) Fluorescence images of single D-hypericin molecules embedded in D-PVA matrix. The inset is a close-up scan and shows a characteristic photo-bleaching event during the scanning process, scan unit in μm ; (b) and (c) two exemplary intensity time traces of single D-hypericin molecules in D-PVA and PMMA with a binning time of 10 ms; (d) chemical structure of hypericin with three dominating dihedral angles, blue: θ_{Me} (bay); green θ_{OH} (bay); and red: θ_{OH} (TS), for details see Table 2; (e) the cluster model of hypericin embedded in polymer matrix.

fluorophore.^[23] In the current work, however, specific polymer-probe interactions are investigated. For this, we used PVA and PMMA, as both polymers are transparent with high light transmittance. The most pronounced feature of the PVA structure is the prevailing hydroxyl group (Scheme 1), which can form hydrogen (H/D) bonds with H- or D-hypericin, thus providing significant non-covalent interaction between the probe and the matrix. The deuterium isotope effect is expected to be observed in PVA as the D-bond is stronger compared to the H-bond due to the lower zero-point vibrational energy of the D-bond (*vide infra*).^[24] PMMA, on the other hand, can be considered as an ‘innocent’ polymer host, as it does not provide functionalities which cause specific strong interactions; however it benefits from its high oxygen permeability.^[25] Due to PVA’s higher polarity, PVA exhibits a dielectric constant of 2.6,^[26] while that of PMMA is 1.8.^[17] Generally, matrices with a higher dielectric constant lead to a higher reaction field in response to the static electric dipole moment of an embedded molecule (for details, see Ref.^[27] and literature cited therein).

Typical fluorescence images of single hypericin molecules at a $10 \times 10 \mu\text{m}^2$ scale, acquired by our home-built setup, are

illustrated in Figure 1(a) on the left. The fluorescence images show isolated bright circular spots with a diffraction-limited diameter Gaussian-shaped intensity distribution; further details can be observed in the close-up views on the right side of Figure 1(a). Antibunching confirmed that single molecules with a minimal spatial distance of $1 \mu\text{m}$, which is much larger than the diffraction limited focal spot of the confocal microscope, are observed; this is sufficient to detect only one molecule within the focal volume at a time. As discussed in former works,^[11–12,22] variations in emissivity (or dynamical intensity fluctuation) can be attributed to (temporal) dark states of hypericin; the latter show populating kinetics resembling those of the triplet state or in other less- or non-emissive states.^[28] In order to investigate the blinking dynamics of hypericin, single molecule intensity time traces were recorded. Two examples, namely D-hypericin in D-PVA (Figure 1(b)) and PMMA (Figure 1(c)) with a binning time of 10 ms were recorded until the dark state was reached and no further emission signal could be recorded after a duration of at least 20 s. The two exemplary intensity time traces show the characteristic ‘blinking’ phenomenon consisting of discrete on-off (or bright-dark) transitions which agrees well with Figure 1(a). D-hypericin in D-PVA shows a higher intensity with stronger fluctuations during the ‘on’ periods. The fluctuations are magnitudes greater than the statistical noise, or instabilities of the excitation intensity as the off-state 40–55 s in Figure 1(b). This indicates on-off transitions on a much shorter timescale than seconds and are likely to be caused by intersystem crossing to a triplet state. In addition, shortly before the occurrence of persistent bleaching, there is often a sudden drop in intensities, indicating the tautomerization process. In stark contrast, D-hypericin in PMMA is rather stable not only in terms of fluctuation dynamics but also in terms of photostability. The decrease in on-off transition and longer survival time correspond qualitatively to the partial quenching due to the low oxygen exposure.

The influence of the local environments (PVA, PMMA) on the survival time and blinking dynamics (the on-off ratio) were analyzed statistically. The survival time is defined as the time before total photobleaching. Figure 2(a) and (b) illustrate the survival time for a single (D-)hypericin molecule in (D-)PVA and PMMA, respectively. At the first glance, we find the survival time in PMMA is much longer than that in PVA due to the low oxygen exposure. Applying an exponential fitting function on the data in Figure 2(a) and (b), we determine an average survival time of 28 s (22 s) for (D-)hypericin in (D-)PVA and 164 s (117 s) in PMMA. Deuteration contributes to a shorter survival time in the same polymer matrix.

The same fluorescence intensity time traces were used to study the blinking behavior by determining the on-off ratio, that is, the proportion of the ‘on’ period (bright state) to the total survival time (‘on’ and ‘off’ period). The ‘on’ period is defined as the fluorescence above the threshold (10% above the background intensity). An increased blinking corresponds to a decreased on-off ratio. Figure 2(c) and (d) presents the results of ‘on’ ratio for (D-)hypericin in PVA and PMMA. It can be clearly seen that (D-)hypericin in PMMA exhibits a significant amount of ‘on’ period with k_{on} close to 100%, reflecting a rather

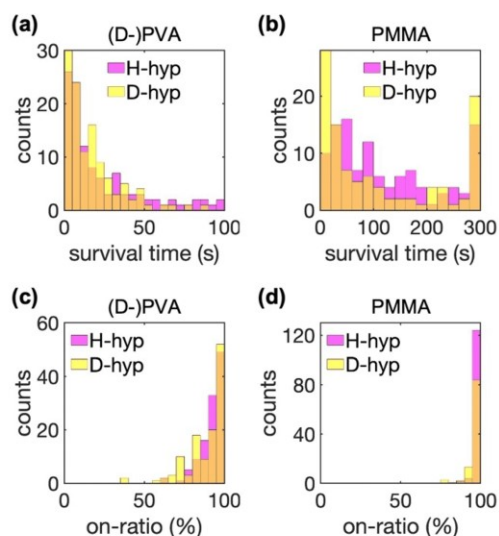


Figure 2. Statistics of single hypericin molecules' blinking behaviour. (a) and (b) shows the histogram of the survival times of single hypericin in different polymer matrices ($n=115$ for H-hypericin in PVA, $n=121$ for D-hypericin in D-PVA, $n=131$ for H-hypericin in PMMA, $n=104$ for D-hypericin in PMMA). Orange bars represent the overlap.

suppressed blinking dynamics, whereas the on-times of the molecules in PVA are competitive to their off-times with a significantly smaller k_{on} . Obviously, oxygen shortens the on-times of single molecules in PVA. This could be partially explained by the assumption that oxygen quenches the fluorescence by static quenching through an intermediate state, which then reversibly changes into another non- or less fluorescence state. Apart from the effect of oxygen on the blinking phenomena, we notice that deuterium plays an important role. Deuteration not only shortens the survival time, but also enhances the blinking on a much longer timescale. The fluctuations of the fluorescence intensity of D-hypericin seem to be much more pronounced than those H-hypericin. The origin of these observations will be discussed in the following.

Changes in the blinking dynamics are often accompanied by changes in the fluorescence lifetime. Therefore, we performed single-molecule TCSPC for (D-)hypericin in (D-)PVA and PMMA. As shown in Table 1, the average fluorescence lifetime τ_F is much longer in PMMA than in PVA, with no significant difference in standard deviation between H- and D-hypericin. A reduction of the fluorescence lifetime in PVA is observed, which

Table 1. Hydrogen/Deuterium isotope effects on fluorescence lifetimes τ_F of (H-/D-)hypericin (in ns). Empirical mean μ and standard deviation σ for the normal distribution and the median of fluorescence lifetimes of single hypericin molecules in different polymer matrices.				
Matrix	Probe	μ [ns]	σ [ns]	Median [ns]
PVA	H-hypericin	3.7	0.8	3.7
D-PVA	D-hypericin	3.8	0.5	3.8
PMMA	H-hypericin	4.2	0.9	4.0
PMMA	D-hypericin	4.3	0.9	4.0

can be ascribed to H-bonding, as discussed in ensemble measurements.^[29] Moreover, the fluorescence lifetime τ_F increases somewhat by deuteration in PVA (see Table 1).^[29] The small deuterium isotope effect can be understood by analyzing τ_F , given by

$$1/\tau_F = k_r + k_{nr} \quad (1)$$

with k_r and k_{nr} as the radiative and non-radiative rates. Deuteration shows a negligible effect on k_r as the latter is related to the oscillator strength (f) and energy (E) of the emitting state (via the Strickler-Berg relation^[30]), which are hardly changed upon deuteration (see SI for details). The component k_{nr} incorporates all other terms, which contribute to the overall decay rate, i.e. internal conversion (IC, with subsequent vibration relaxation, VR), intersystem crossing (ISC) towards the triplet manifold, and photochemical reactions like tautomerization, etc.^[31] Deuteration has a pronounced effect on k_{IC} , but a negligible effect on k_{ISC} .^[32] Therefore, the small isotope effect in hypericin, as also observed in related aromatic hydrocarbons,^[33] suggests that $k_{ISC} \gg k_{IC}$, which agrees with the high triplet population of hypericin.^[34] For IC, the rate k_{IC} can be estimated from Fermi's golden rule.^[30b] Here, k_{IC} decreases with decreasing vibrational frequency (see SI for details), so that k_{IC} decreases upon deuteration because of smaller energy spacing between vibrational quanta (see SI for details).^[35] This is in fact reflected in the much smaller total zero-point energy (0.37 eV) in the DFT calculations, while there is no significant difference in electronic and Gibbs free energies. This in all rationalizes the small, but notable increase of τ_F of D-hypericin in both polymer matrices in comparison with H-hypericin; see Figure 3. In any case, besides this *intramolecular* effect, we note a much

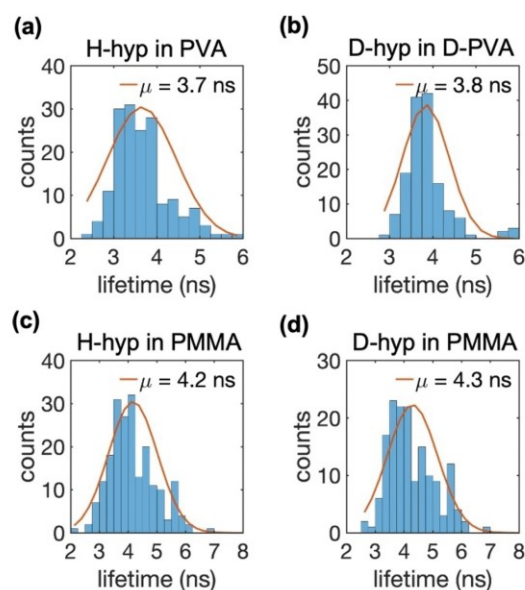


Figure 3. Histograms of derived fluorescence lifetime of single (H-/D-)hypericin molecules in (a) PVA ($n=172$), (b) D-PVA ($n=147$), (c) PMMA ($n=175$) and (d) PMMA ($n=159$). The red curves correspond to fitted Normal distributions associated with the mean values.

stronger effect for PVA than in PMMA; this points to a significant *intermolecular* contribution, which is evidently related to the possibility of H(D) bonding in the (D-)PVA matrix. This is expected to restrict the conformational freedom of hypericin, which in turn limits IC_{F} ,^[30b,36] this is indeed in line with the experimentally observed significant increase of τ_{F} and a narrower distribution for D-hypericin in D-PVA, as shown in Figure 3(b) and Table 1.

It is well established that tautomerization occurs via tunneling,^[37] in organic molecules this is governed by the vibrational overlap. We thus expect that deuteration decreases the tautomerization rate, again due to the decreased energy spacing of the vibrational quanta; this was in fact observed in (ensemble) studies on various systems with largely different time constants.^[38] In hypericin, tautomerization takes place in the second time range,^[11] at a single molecule level, this can be monitored by fluorescence correlation spectroscopy (FCS),^[39] which accumulates statistics from this complex many-event dynamics.^[7b,12a,40] The intensity autocorrelation function (ACF), which reflects fluorescence intensity fluctuation dynamics,^[41] can be calculated directly from the TCSPC data (with temporal resolution of 64 ps), as shown in Figure 4. The characteristic time of the fluctuations can be then extracted from a fluctuation time trace record with a model function. Here, we take into account two dynamic components, that is the triplet state lifetime (i.e. the short-time scale τ_1 ; i.e. in milliseconds) and the tautomerization process (the long-time scale τ_2 ; i.e. in seconds).

$$g^2(\tau) = G(\tau) - 1 \cong Ae^{-(\frac{\tau}{\tau_1})^{\beta_1}} + Be^{-(\frac{\tau}{\tau_2})^{\beta_2}} \quad (2)$$

It is noted that, if the multiple events by accident occur within the same temporal integration time, the evaluated

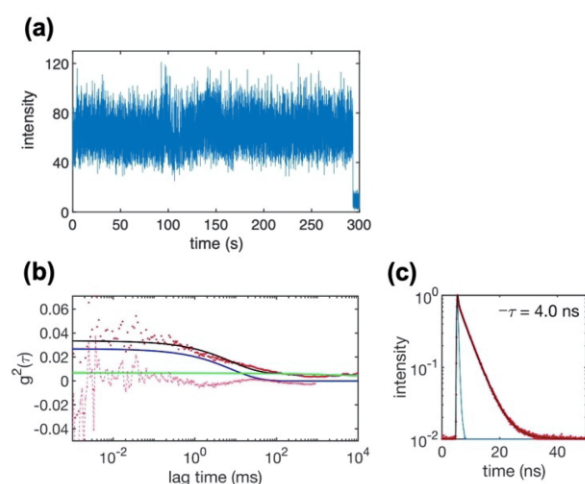


Figure 4. (a) The intensity time trace of a single hypericin molecule in PMMA and the corresponding (b) intensity autocorrelation functions $g^2(\tau)$ (the black curve) as well as (c) the fluorescence lifetime. The molecule shows a short-time blinking ($\tau_1 = 7.8$ ms, the blue curve) and a long-time fluctuation ($\tau_2 = 12.0$ s, the green curve) with the fluorescence lifetime $\tau_{\text{F}} = 4.0$ ns. A residual analysis is indicated in magenta dashed line.

lifetime gives the information on the average value of all the processes involved.

In the following, we will discuss one representative result of a FCS analysis, which was fitted with eq. (2), giving $\tau_1 = 7.8$ ms and $\tau_2 = 12.0$ s. We note strong deviations at shorter time lag, ($> 10^{-1}$ ms), ascribed to triplet blinking, while the minor deviations at medium time lag ($10^{-1} \sim 10^2$ ms) point to a more complex photophysics occurring from the triplet state, which may reflect, for instance, reorganization of the environment; finally, at long time lag ($> 10^2$ ms), the residuals converge to zero. Additional information can be obtained from the stretch parameters β ($\beta_1 = 0.62$, $\beta_2 = 0.32$); the latter allow to draw conclusions on how many processes are occurring in the corresponding time window. The first parameter $\beta_1 = 0.62$ is indicative of a large portion of short-time dynamics (τ_1). The second parameter shows only a minor contribution ($\beta_2 = 0.32$), reflecting the dominance of the short-time scale (triplet blinking) in the temporal emission behavior.

The histograms in Figure 5 show the distribution of the $\langle \tau_1 \rangle$ and $\langle \tau_2 \rangle$ for single (D-)hypericin in (D-)PVA and PMMA. The short-time fluctuation ($\sim$ ms) of hypericin in PVA shows a normal distribution with $\langle \tau_1 \rangle = 6.7$ ms, whereas the D-hypericin follows a Poisson distribution and yields an average lifetime of 5.0 ms. Such inverse deuterium isotope effect in residing time τ_1 is not expected, if deactivation by ISC to the ground state (GS) is concerned;^[32] this implies the involvement of other photophysical and/or -chemical processes from the triplet state, which are affected by deuteration, governed by τ_2 , exhibits a significant deuterium isotope effect with an increase of the residing time $\langle \tau_2 \rangle$, from 2.9 to 3.9 s, when going from H- to D-hypericin in (D-)PVA. As discussed before, this agrees with the expectation from the consideration on tunneling of a proton.

Changing the matrix to PMMA, the on-off triplet blinking behavior $\langle \tau_1 \rangle$ is largely suppressed, see Figure 2 (d); this may be due to the fact that the ISC rate in PMMA is much lower than in PVA, thus only a small number of hypericin molecules proceeds to the triplet manifold. For the long-time fluctuations, in comparison with PVA, tautomerization in PMMA appears to be less favored, consistent with the observations in the short-time scale (the average lifetime $\langle \tau_2 \rangle = 1.5$ s for H- and $\langle \tau_2 \rangle = 2.3$ s for D-hypericin in PMMA). We anyway note that, under certain circumstances, there is no apparent short/long timescale dynamics; this leads to a single exponential character with one component $\tau_{1/2} = \infty$. To indicate the probability of occurrence, we set all infinite fitting results to 20 ms and 40 s, respectively.

In order to rationalize the specific impact of the *intermolecular* interaction between (D-)hypericin and the polymer matrix, we conducted (TD)DFT calculations of the isolated hypericin molecule in methanol (using the implicit PCM model) and the explicit cluster model (hypericin embedded in the realistic polymer environment). The equilibrium geometries at the ground state (GS) and the transition state (TS) were obtained to investigate the stereochemistry and contributing geometrical & electronic factors (for further details see SI). Following our previous results on the tautomerization process,^[11–12] we focus on three dominating dihedral angles as indicated in Figure 1(d): the methyl (θ_{Me}) and hydroxyl (θ_{OH})

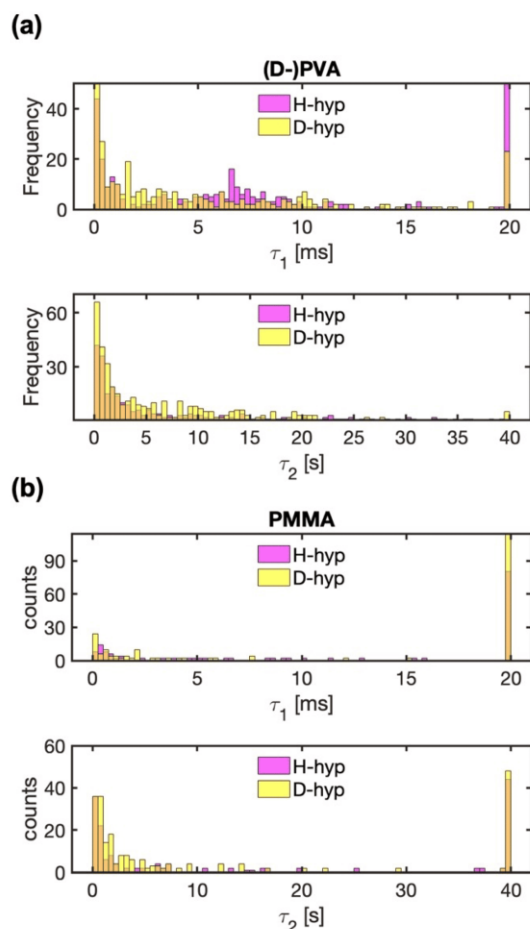


Figure 5. Histograms of the dynamic parameters for single hypericin molecules in (D-)PVA ($n=357$, $n=423$ for H- and D-hypericin) and in PMMA ($n=186$, $n=246$ for H- and D-hypericin). $\langle \tau_1 \rangle$ refers to the fast fluctuation dynamics, i.e. a triplet quenching process, while $\langle \tau_2 \rangle$ refers to the slow fluctuation dynamics, i.e. the tautomerization process. Orange bars represent the overlap.

groups at the *bay* region, which are responsible for the distortion of the aromatic parent backbone; and the hydroxyl group (θ_{OH}) at the *peri* region, where the proton transfer occurs at the TS. In a previous study, we showed a detailed picture of tautomerization pathways for the isolated molecule;^[11] i.e. without considering environmental effects. In a simple way, the latter can be treated in an implicit manner, for instance by PCM; this model however only considers polarizability effects. Therefore, it is essential to apply an explicit model with a realistic electronic environment. In fact, it can be seen in Table 2 that the hypericin geometry in PMMA is very similar to that in methanol, suggesting that PMMA itself serves as an 'innocent' solvent. In stark contrast, according to the calculations, hypericin is more distorted in PVA, which can be attributed to efficient *intermolecular* hydrogen bonding with the host polymer. In any case, the higher free volume in PVA due to the high water content may contribute to the experimental observations, an effect which was not considered in the calculations. From the energy point of view, the activation

Table 2. Descriptions of the calculated structure at the ground state (GS) and the transition state (TS) for single hypericin molecule in MeOH (PCM), and the cluster model using D3-B3LYP with the standard basis set 6-31G(d).

Dihedral angle (°)	MeOH (PCM)		PVA		PMMA	
	GS	TS	GS	TS	GS	TS
θ_{Me} (bay)	33	34	40	45	36	37
θ_{OH} (bay)	27	27	24	25	29	30
θ_{OH} (TS)	1	102	8	120	3	113

barrier for tautomerization is 0.18 eV higher in PMMA than in PVA, which indicates that tautomerization is more energetically favored in PVA in agreement with the experimental observations.

Conclusions
In conclusion, we have investigated the deuterium isotope effect on the photophysics and photochemistry of hypericin at the single-molecule level in different polymer matrices by a combined spectroscopic and computational approach. Two different polymers (PVA, PMMA) were used to investigate the influence of the local chemical environment. The hydroxyl group in PVA favors to form an H(D) bond with hypericin, giving rise to significant *intermolecular* solute-solvent interactions. In contrast, PMMA can be considered as an 'innocent' polymer host, as it does not provide such functionalities but benefits from photo-stabilization (a longer survival time) due to low oxygen exposure. Because of the high water content in PVA, this matrix has proven to be a good candidate to study the deuterium isotope effect on hypericin, and its decay mechanism of excited states. Deuterium increases the fluorescence lifetime, however the effect is shown to be small; this is due to the high triplet generation in hypericin, so that internal conversion, which is most affected by deuteration, is a minor pathway. Deuteration further decreases nonradiative decay of the triplet state as well as the tautomerization rate. All this can be rationalized by the decrease of energy spacing of vibrational quanta upon deuteration. Finally, in combination with the experimental observations, (TD)DFT calculations provide in-depth understanding of specific nano-environmental interactions between hypericin and the polymer matrix at the electronic level. In all, this work may pave the way to monitor and even control the photophysics and photochemistry of probe molecules at a single-molecule level.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: single molecule spectroscopy • tautomerization • deuteration • nano-environmental effect • density functional theory

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Donor–Acceptor–Donor Triads with Flexible Spacers: Deciphering Complex Photophysics for Targeted Materials Design

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The complex photokinetics of donor–acceptor–donor triads with varying flexible spacer lengths ($n = 4–10$ carbon atoms) are investigated in liquid and solid solution, as well as in crystals, by steady-state and transient fluorescence spectroscopy combined with computational studies. For the short spacer ($n = 4$) in a liquid solution, dynamic charge-transfer (CT) state formation with subsequent, efficient exciplex emission is observed, effectively competing with quenching through electron transfer (eT) via a radical ion pair. In a solid solution, a fluorescent CT static complex is formed upon freezing for all spacer lengths. This allows the observations of a former seminal report on stimuli-responsive high-contrast fluorescence on/off switching in films of the triads to be reassigned (Adv. Mater. 2012, 24, 5487), now providing a holistic picture on varying spacer length. In fact, external stimuli of the film by modulating the geometry of the CT complex, which results in on/off fluorescence switching (for $n > 4$) or in a change of the emission color ($n = 4$). The work thus demonstrates how in-depth analysis of complex photophysics can be put to practical use in materials science.

1. Introduction

All-organic π -conjugated materials have found immense interest for applications in energy or materials conversion and sensing, for instance in organic solar cells (OSCs), light emitting

diodes (OLEDs), field effect transistors (OFETs), sensors, or photocatalysts (OPCs). In the quest for novel, tailor-made materials, molecules with complex photophysical deactivation pathways are progressively explored, such as room temperature phosphorescence (RTP),^[1] thermally activated delayed fluorescence (TADF),^[2] non-Kasha emission,^[3,4] dual emission (DE),^[4,5] or exciplex formation.^[6] In many of these processes, the generation, spectroscopic signatures, and subsequent deactivation of energetically low-lying charge transfer (CT) states, formed between electron donor (D) and acceptor (A) moieties, is of central importance. This is in particular evident for DA interfaces in OSCs,^[7] for alternating DA structures in low-bandgap polymers in OSCs and OFETs,^[8] luminescent mixed-stacked co-crystals,^[9,10] TADF materials used in OLEDs^[2,11,12] or as OPCs.^[13,14]

as well as for fluorescence switching in stimuli-responsive materials.^[15,16,17] The generated CT states and their deactivation are a matter of ongoing discussions, as very significant dependencies are observed on the electronic properties of the D and A moieties, covalent versus noncovalent D–A linkage, and exact D–A positioning, as well as periodicity and

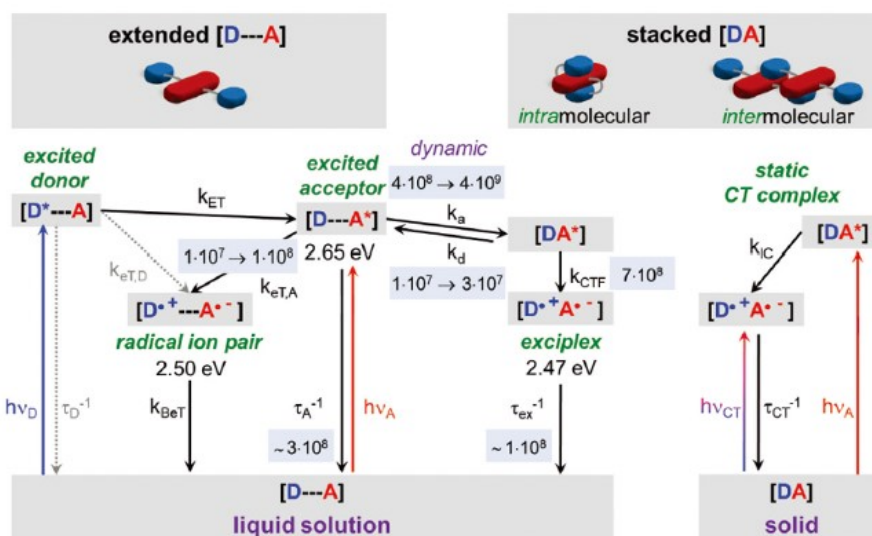
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Scheme 1. Deactivation scheme of the D-σ-A-σ-D triads **DAD_n** ($n = 4-10$) within the singlet state manifold under direct excitation of D or A for extended $[D^* \cdots A]$, which prevails in liquid solution over stacked $[DA]$. Under D excitation, A^* is instantaneously formed by resonant energy transfer (ET), which here outcompetes electron transfer (eT_D). From A^* , sequential electron transfer (eT_A) to form the radical ion pair $[D^{*+} \cdots A^{*-}]$, and subsequent quenching by back electron transfer (BeT) and vibrational relaxation, competes with A^* deactivation τ_A^{-1} (via fluorescence and IC). Alternatively, an exciplex $[D^* \cdots A^*]$ is formed via a pre-associated complex $[DA^*]$ with an association constant k_a , where the excitation is still localized on A, and which can re-dissociate with k_d ; from here, subsequent CT formation (k_{CTF}) and exciplex deactivation through τ_{ex}^{-1} (via fluorescence and IC) occurs. Energies of relevant states (in eV) are obtained from single point TD-DFT (PBE0). Orders of rate constants are given (light blue shaded numbers, in s^{-1}); indicating the evolution ($\rightarrow$) from $n = 4$ to 10, as resulting from photokinetic fitting (see Section 2: Results and Discussions). On the right, photoexcitation—and deactivation of the stacked $[DA]$ CT complex is shown, which is formed in solid solution or molecular solids in the ground state, either in an intra- or intermolecular fashion.

dimensionality of the D/A arrangement. Therefore, a detailed understanding of the underlying photophysics is of paramount interest for targeted materials design.

From a mechanistic understanding, the actual D–A distance within a pair (denoted as $[DA]$ in Scheme 1) is particularly important, as only at short distances the low-lying CT singlet state (S_1) can be effectively populated upon direct or indirect photoexcitation via the singlet state manifold. Such close D–A separations can be realized in an *intramolecular* fashion by direct covalent D–A linkage^[11,18] (including multiresonant structures^[19]), or by short conjugated or non-conjugated spacers (i.e., D- π -A,^[20] versus D- σ -A dyads^[18,21]), as well as in an *intermolecular* noncovalent fashion through D:A complexes. The generated CT state can be emissive (i.e., with pronounced fluorescence quantum yield Φ_F), if the radiative rate k_r can successfully compete with the nonradiative rate k_{nr} (the latter typically proceeds via internal conversion; IC, with subsequent vibrational relaxation, VR) or intersystem crossing (ISC) to the triplet manifold. The radiative rate k_r scales with the oscillator strength f_{CT} ,^[10,22] which depends on the overlap of the constituting molecular orbitals (MOs; or, in the possibly multiconfigurational description of the CT state, natural transition orbitals; NTOs) of D and A. This correlation with f_{CT} can be intuitively understood in a simple one-electron picture. In this framework, the CT character will be strong and f_{CT} therefore will be vanishing, if the highest occupied MO (HOMO) is localized entirely in D, and the lowest unoccupied MO (LUMO) is localized in A; otherwise partial CT, i.e., pCT, is observed with notable (or even rather large) f_{pCT} , as, e.g., in push–pull systems of organic chromophores (D- π -

A).^[20] The localization is mainly driven by the energetic offset of the MOs in the isolated D and A moieties,^[18,23] but also by MO symmetry,^[23] as well as connector type and position (e.g., meta versus para),^[18,24] Importantly, also geometrical factors—in particular the twist angle between D and A—decides on the amount of CT;^[2] such geometry change can also proceed dynamically during the excited state lifetime, as in compounds with twisted intramolecular CT (TICT) states.^[25] For certain D–A systems with very pronounced CT character of the S_1 - S_0 transition, ISC to the triplet manifold and subsequent reverse ISC from T_1 (due to the small exchange energy for the CT state) may give rise to TADF.^[12,11]

If the separation between the D and A moieties is too large, a CT state cannot be directly photoexcited. Instead, the CT state may be dynamically generated after photoexcitation of D or A, see Scheme 1. This is commonly termed as an exciplex,^[26,27] which may be created in mixed solutions of D and A molecules if the concentration is high enough to allow for the formation of an encounter complex, i.e., $[D^* \cdots A]$ or $[D \cdots A^*]$, during the lifetime τ of S_1 ; for this reason, pyrene is often utilized for excimer or exciplex formation, due to pyrene's very long τ .^[28] Exciplexes can also be formed in molecular D- σ -A dyads with a flexible spacer s (i.e., independent of the concentration),^[29,30] the relatively close separation also allows to use of chromophores with τ in the low nanosecond range.^[27,29,31–33] Effective exciplex formation commonly requires short spacers,^[32] in accordance with the “Hirayama” rule for excimers.^[34] It should be noted in this context, that the exciplex term is now often used for noncovalent D:A complexes in the solid state, e.g. for TADF materials

in OLEDs;^[6] strictly speaking, these species are not “classic” exciplexes, but rather intermolecular CT complexes, which, due to spatial confinement in the films, also exist in the ground state (see Scheme 1).

The specific signature of the exciplex is a bathochromically shifted, unstructured fluorescence with a prolonged lifetime; however, in most cases, exciplexes exhibit small Φ_F in solution, due to a combination of small k_r and high k_{nr} . In particular, for application in optoelectronics (OLEDs and sensors), it is therefore important to identify emissive dyads. The most important competitive path to exciplex formation after photoexcitation of D or A is photoinduced electron transfer (eT) to create a radical ion pair $[D^+ \cdots A^{\cdot-}]$,^[35] which is subsequently deactivated via back electron transfer (BeT) to the ground state (Scheme 1) or ISC.^[35,36] In the context of exciplexes, this process was mainly investigated for initial photoexcitation of A,^[27,29–32] where exciplex formation prevails in nonpolar environments, while eT dominates in polar solvents.^[35] For excitation of D, on the other hand, photoinduced eT_D was intensively investigated as a competitive pathway to resonant energy transfer (ET; see Scheme 1) in molecular dyads with conjugated spacers (molecular wires);^[37,38] these are in particular interesting as model systems for photosynthesis and organic electronics. In this context, $[D^+ \cdots A^{\cdot-}]$, generated by eT, is commonly termed as “charge-separated” (CS) state,^[37] in distinction to the CT state discussed above. The rate k_{eT} is usually thermally activated, as described, for instance, by the Marcus equation,^[37,39] and was shown to exhibit an exponential spacer length dependence, expressed by the attenuation factor β_{eT} .^[37] The latter depends on the electronic nature of D and A, as well as those of the linker, in particular on whether the linker consists of saturated hydrocarbons or conjugated strands, as well as on its specific geometry.

From the mentioned applications of molecular dyads and triads in optoelectronics, a seminal work by Kwon et al reported on D– σ –A– σ –D triads, based on a highly fluorescent *p*-distyrylbenzene-type A moiety, flexible, saturated hydrocarbon spacers of different chain length, and carbazole D moieties.^[15] Significantly, films of these triads were shown to exhibit high contrast fluorescence on/off switching under external stimuli (temperature and pressure). Based on time-dependent density functional theory (TD-DFT) calculations, this was attributed back then to the occurrence of stacked/extended conformations of the triad. However, the exact mechanism was not further pursued at this point. In the current work, we thus decided to tackle this challenge by an in-depth investigation of these triads in solution, and under variation of polarity, viscosity, and temperature. Combining (steady-state and time-resolved) optical spectroscopy with computational studies (TD-DFT, and molecular dynamics, MD, simulations), we decipher herein the complex photophysical deactivation pathways of the triads, with respect to eT, dynamic exciplex formation, and the generation of a static CT complex upon spatial confinement of the triads. Technologically relevant is our identification of an optimized system, which shows red exciplex emission with a considerable Φ_F of 14%. Furthermore, our results evidence a new scenario for the stimuli-responsive behavior in the films, which rationalizes also the different behavior with varying spacer length; this may inspire further work in stimuli-responsive materials as well as in exciplex-based OLED,

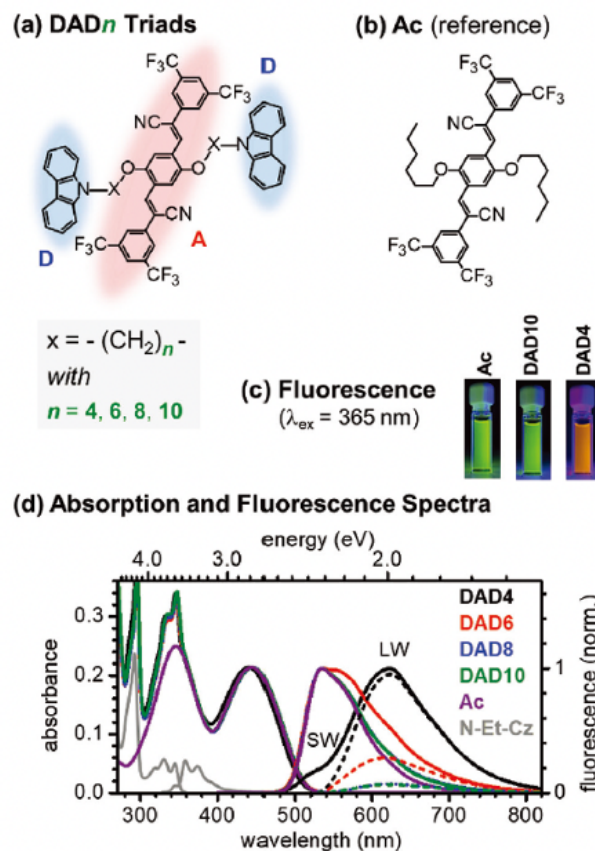


Figure 1. a,b) Molecular structures of the DAD n triads (a) and of reference Ac (b); c) fluorescence images of $CHCl_3$ solution; d) absorption (left) and fluorescence spectra (right) of the compounds in $10 \mu M$ $CHCl_3$ solution (DAD8 fluorescence is superimposed by DAD10, being almost identical). The dashed lines are the long-wavelength components, obtained by spectral decomposition. Absorption and fluorescence spectra of the donor *N*-ethyl-carbazole (N-Et-Cz) are shown for comparison.

relying on the subtle interplay of complex photophysical deactivation pathways.

2. Results and Discussion

The investigated DAD n triads consist of a luminescent dicyano-substituted *p*-distyrylbenzene (DCS) acceptor (A) core with four CF_3 groups in the *meta*-positions, to which two carbazole donor (D) moieties are covalently attached via alkoxy linkers of different chain length (with $n = 2, 4, 6, 8, 10$ carbon atoms) at the central ring of the DCS core, see Figure 1. The reference acceptor molecule (Ac, see Figure 1) owns the same structure as the acceptor moiety of the triads, but carries hexyloxy-groups at the central ring. The cyano-groups of the acceptor are located in the outer positions of the vinyne-unit. Such β -substitution commonly generates highly fluorescent molecules, in particular in the presence of alkoxy-substituents, while IC and ISC are rather minor pathways.^[40] It is reminded, that, on the contrary, DCS with α -substitution (in the inner vinyne position) are only very

Table 1. Spectroscopic data of the compounds in CHCl₃ solution at room temperature at short and long wavelength emission (SW, LW) with their corresponding emission maxima λ_{max} . Individual lifetimes (amplitudes) τ_i (A_i) are results of multiexponential fits, obtained at $\lambda_{\text{det}} = 536$ nm, 680 nm for SW and LW, respectively; τ_{PF} is the mean (intensity-weighted average) prompt lifetime (i.e., of the short components $\tau_{1,2}$), τ_{DF} is the delayed lifetime component, I_{DF} the respective fractional intensity, $\Phi_{\text{F,tot}}$ is the total fluorescence quantum yield, $\Phi_{\text{F,i}}$ are the quantum yields of the SW and LW components, obtained by spectral decomposition of the steady-state data in Figure 1 via the fractional intensities $x_{\text{SW,LW}}$.

		τ_1 (ns) ^{a)}	A_1	τ_2 (ns) ^{a)}	A_2	τ_{PF} (ns) ^{b)}	τ_{rise} [ns]	A_{rise}	τ_{DF} [ns]	A_{DF}	I_{DF} ^{c)}	$\Phi_{\text{F,tot}}$	$\Phi_{\text{F,i}}$	λ_{max} (nm)
Ac	SW	0.87	0.12	3.47	0.88	3.39						0.41		535
DAD10	SW	0.64	0.20	1.47	0.80	1.39			8.56	0.009	0.054	0.30	0.27	535
	LW	0.33	0.13	1.30	0.87	1.27	— ^{d)}		8.29	0.23			0.03	623
DAD8	SW	0.46	0.21	1.07	0.79	1.01			7.95	0.012	0.092	0.23	0.21	535
	LW	0.19	0.25	0.98	0.75	0.93	— ^{d)}		7.68	0.18			0.02	615
DAD6	SW ^{e)}	0.17	0.54	0.58	0.46	0.47			9.63	0.008	0.140	0.11	0.08	535
	LW	0.38	1.00	—	—	0.38	2.64	−0.21	9.51	0.25			0.03	620
DAD4	SW ^{f)}	0.07	0.99	1.17	0.01	0.21			9.28	0.003	0.26	0.16	0.02	535
	LW						1.50	−1.01	9.92	1.00			0.14	620

^{a)} The accuracy is limited by the IRF (ca. 50 ps); ^{b)} Intensity-weighted averaged lifetime; $\tau_{\text{PF}} = (A_1\tau_1^2 + A_2\tau_2^2)/(A_1\tau_1 + A_2\tau_2)$. Differences in τ_{PF} for SW and LW detection are within the experimental error of the setup; ^{c)} $I_{\text{DF}} = A_{\text{DF}}\tau_{\text{DF}}/(A_1\tau_1 + A_2\tau_2 + A_{\text{DF}}\tau_{\text{DF}})$; ^{d)} Contribution too small to be detected; ^{e)} $\lambda_{\text{det}} = 505$ nm; ^{f)} $\lambda_{\text{det}} = 524$ nm.

weakly luminescent due to efficient access to a conical intersection (CI);^[40,41,42] in solid environments, solid-state luminescence enhancement (SLE) is then observed.^[10,40]

2.1. Optical and Photophysical Properties

The absorption spectrum of Ac shows unstructured “double hump” features with maxima at 347 nm and 445 nm, respectively (Figure 1). This arises from excited state splitting of the DCS core due to the presence of the alkoxy functionalities in the central ring.^[40,43] The resulting states ($S_{1,2}$) are well described by one-electron excitations between the delocalized HOMO and HOMO-1, respectively, to the LUMO.^[40] The fluorescence spectrum in non-polar solvents (e.g., *n*-octane) is vibronically structured (vide infra); in the more polar solvent CHCl₃ it appears unstructured and somewhat bathochromically shifted (to 535 nm, see Figure 1), due to minor (but not insignificant) CT contributions in the emitting state (S_1), as discussed below. In accordance with the previous findings on β -DCS compounds,^[40,41] Ac is brightly fluorescent, emitting in CHCl₃ solution with a fluorescence quantum yield of $\Phi_{\text{F}} = 0.41$, see Table 1; in non-polar solvents (*n*-octane, toluene) Φ_{F} rises to 0.60. The high Φ_{F} of Ac is correlated to the low Franck–Condon energy (2.79 eV; 445 nm), which successfully inhibits access to the CI.^[40,41] The fluorescence decay of Ac in CHCl₃ (Figure 2) is nonexponential with a mean lifetime of 3.4 ns (in Table 1 denoted as prompt lifetime τ_{PF} in distinction from the delayed lifetime τ_{DF} observed for the exciplexes of the triads, vide infra). The non-exponential character of τ_{PF} pointing to significant geometry changes for Ac in the excited state (vide infra).

The absorption spectra of the DAD n triads in CHCl₃ solution are a simple superposition of those of the donor (carbazole) and acceptor (Ac) molecules alone,^[15] see Figure 1. Only for DAD4, a small hypsochromic shift of the low energy absorption band is observed. In particular, for none of the triads, any indication for additional absorption by a CT state is found. The fluorescence excitation spectra of all triads follow closely the absorption spectra

at all detection wavelengths (see Figure S8, Supporting Information). This is a direct proof^[44] that there are no major additional loss channels after photoexcitation of D $\rightarrow$ D* (in particular by fluorescence of D*, or by photoinduced eT_D^[37]); instead, A* is instantaneously prepared from D* by resonant (Coulomb-type) energy transfer (ET, through D* + A $\rightarrow$ D + A*), that means in equal manner as under direct photoexcitation of A, see Scheme 1.

Different from absorption, the fluorescence properties vary strongly among the triads. The fluorescence spectra of DAD10 and DAD8 are very similar to that of Ac, but show a slight

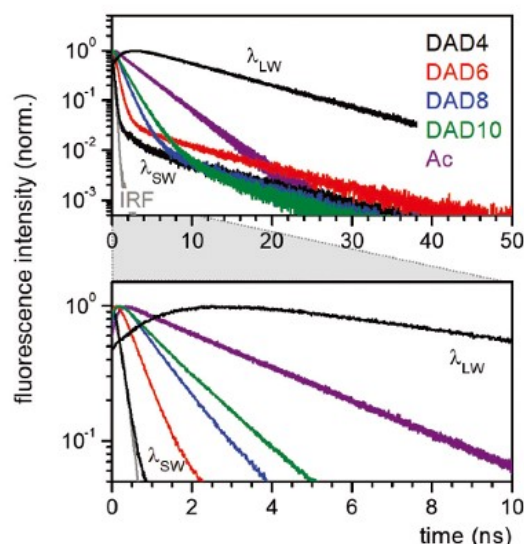


Figure 2. Fluorescence time traces of Ac and the DAD n triads in CHCl₃, detected at $\lambda_{\text{SW}} = 536$ nm (for $n = 4$, detection at $\lambda_{\text{SW}} = 524$ nm, $\lambda_{\text{LW}} = 680$ nm; for details see the Supporting Information) (top) and zoom into the time range up to 10 ns (bottom). The instrumental response function (IRF) is given as a gray line; details of the lifetime evaluations are given in Figure S10, Supporting Information.

broadening on the low-energy side (vide infra). At the same time, the prompt lifetime τ_{PF} is reduced strongly and monotonically with n versus Ac, particularly in polar solvents (see Table 1, and Table S6 in the Supporting Information). At the same time, Φ_{F} is also reduced compared to Ac, although not as much as τ_{PF} (vide infra). One scenario for the emission quenching is eT (see Scheme 1), as commonly observed for D- σ -A dyads.^[37] The different dependencies found for Φ_{F} and τ_{PF} as well as the time evolution of the emission features (vide infra), however, point to more complex photophysics.

This becomes in particular obvious for DAD4. In stark contrast to the triads with the long spacers, DAD4 shows only a shoulder at short wavelengths (SW, Figure 1) in CHCl_3 solution, corresponding to the spectral position of Ac, with a short lifetime (≈ 0.2 ns), and a small fractional quantum yield of $\Phi_{\text{F,SW}} = 0.02$, see Table 1. Instead, a new emission appears at long wavelengths (LW), centered at $\lambda_{\text{LW,max}} = 623$ nm, which dominates the spectrum ($\Phi_{\text{F,LW}} = 0.14$). DAD4 thus clearly exhibits dual emission.^[4] Accordingly, the emission color changes from green (Ac, DAD8,10) to orange (DAD4), see Figure 1. The fluorescence excitation spectra of DAD4, recorded at λ_{LW} and λ_{SW} , are identical (Figure S8, Supporting Information), demonstrating that the population of the LW state is independent of excitation; thus, SW and LW emission originate from the same absorbing species. In particular, no direct photoexcitation of a CT state is observed in the excitation spectrum recorded at λ_{LW} . Furthermore, no concentration dependence was found upon dilution (see Figure S12, Supporting Information); this in all points to an intramolecular exciplex between the D and A moieties as the source of the LW emission, i.e., a CT state which is only dynamically formed by folding of the spacer during the excited-state lifetime. This agrees with the observed broad features of the LW emission, as typically found for exciplexes.^[29–32] The dynamic nature of the exciplex formation is further confirmed by a fluorescence rise time of 1.5 ns in the transient fluorescence recorded at λ_{LW} (Table 1); the associated (delayed) decay time is long ($\tau_{\text{DF}} = 9.9$ ns) and exhibits the same amplitude, indicative of a consecutive reaction. The long decay evidences also a small oscillator strength of the emitting state, being consistent with a pronounced CT character of the exciplex. We note that the rise time of exciplex emission does not correspond to the decay time of the SW emission; this compels the introduction of an intermediary step in the photokinetics, vide infra. Significantly, a long lifetime component is equally found under detection at 524 nm (Table 1) and even at 505 nm detection, but only as a minor component. As the corresponding energy is higher than the spectral onset of the exciplex emission extracted from spectral decomposition (see Figure 1, and Figure S9, Supporting Information), this points again to more complex photokinetics, vide infra. Finally, DAD6 corresponds to an intermediate situation regarding the observed fluorescence in CHCl_3 (Figure 1), with significant broadening due to exciplex formation ($\lambda_{\text{LW,max}} = 616$ nm), being visible in the fractional $\Phi_{\text{F,LW}}$ as well as in the noticeable presence of the exciplex decay time τ_{DF} , see Table 1. The long decay component τ_{DF} can also be detected for long spacer length (DAD8, DAD10) in CHCl_3 when recorded at λ_{LW} (being somewhat shortened compared to DAD4, see Table 1); also here τ_{DF} is seen at λ_{SW} , but as a very minor component (Table 1). Spectral decomposition of the fluorescence spectra gives very similar exciplex feature as for

DAD4 with $\lambda_{\text{LW,max}} = 619$ nm, but as a contribution with much lower intensity, see Figure 1.

The increasing significance of exciplex formation with decreasing spacer length n can be specifically visualized by time-resolved emission spectra (TRES, recorded in CHCl_3). As shown in Figure 3; the SW emission appears for Ac and all DAD n triads at $t = 0$ with a constant spectral position of 533 nm; only for DAD4, a hypsochromic shift to 530 nm is observed, in accordance with the shift of the absorption band (vide supra). The decay of the SW emission becomes increasingly faster with decreasing n . Subsequently, the LW emission rises and slowly decays. However, the contribution of LW depends much on n ; for instance, for DAD4, LW is already dominant after 3 ns, while for DAD6 the SW and LW emissions are seen with similar weights. At this delay time, LW is seen in DAD8 and DAD10 only as a small shoulder. Therefore, longer delay times are required to observe the LW features for these triads, as shown in Figure 3 for 20 ns. The appearance of the LW emission is particularly visible in the logarithmic TRES plots normalized at each wavelength (Figure 3, right-hand side), clearly showing the spectral drift from the SW to the LW emission over time for all triads, including $n = 8, 10$. The LW lifetime increases somewhat with the detection wavelength, which may hint at distributions of exciplex geometries

The overall photokinetics of inter- or intramolecular exciplexes are generally complex, and may largely differ depending on the exact composition of the system.^[29,35] In fact, for the present DAD n triads, after generation of A^* , the two competitive deactivation pathways to fluorescence, i.e., quenching by eT_A and exciplex formation, cannot be described as simple competitive reactions with a constant radiative lifetime of $[\text{D}\cdots\text{A}^*]$, as in this case a strict correlation of τ_{SW} and $\Phi_{\text{F,SW}}$ would be expected, in disagreement with the current results, see Table 1. Therefore, the direct extraction of the attenuation factor β_{eT} from the fluorescence quenching rate as a function of spacer length^[37,45,46] is not possible in the present case. Instead, the mismatch of τ_{PF} and $\Phi_{\text{F,SW}}$, together with the concurrent appearance of a delayed component in the SW band of DAD4 evidences exciplex dissociation under regeneration of A^* ,^[29,35] see Scheme 1. The underlying reaction kinetics,^[29] which were described in early publications,^[47,48] formally correspond to those used to analyze other thermally activated processes like excited state proton transfer^[47] or TADF.^[49,50] Additionally, the mentioned mismatch of the exciplex rise time and the decay time of the SW emission evidences an intermediate (pre-associated) species in the creation of the exciplex, noted as $[\text{DA}^*]$, i.e., where the excitation is still localized on A, see Scheme 1;^[35] from here the charge transfer state $[\text{D}^+\cdots\text{A}^*]$ is formed (k_{CTF}), which gives rise to the excimer emission. Furthermore, all rates are expected to vary among the different triads, in particular as a range of different triad conformations has to be anticipated. In fact, even the radiative deactivation from $[\text{D}\cdots\text{A}^*]$ is not expected to be constant for the various triads as the presence of the D moieties may impact the non-planarity of A; this, in turn, impacts the oscillator strength of the LE state, and with this the radiative rate. This is indeed observed in the quantum chemical calculations, which will be discussed hereafter. Moreover, also the formed exciplexes may adapt different conformations, including the formation with only one or two D moieties (D:A versus D:A:D). Therefore, the complexity of the photokinetics with a large number of rates and their variances impedes a

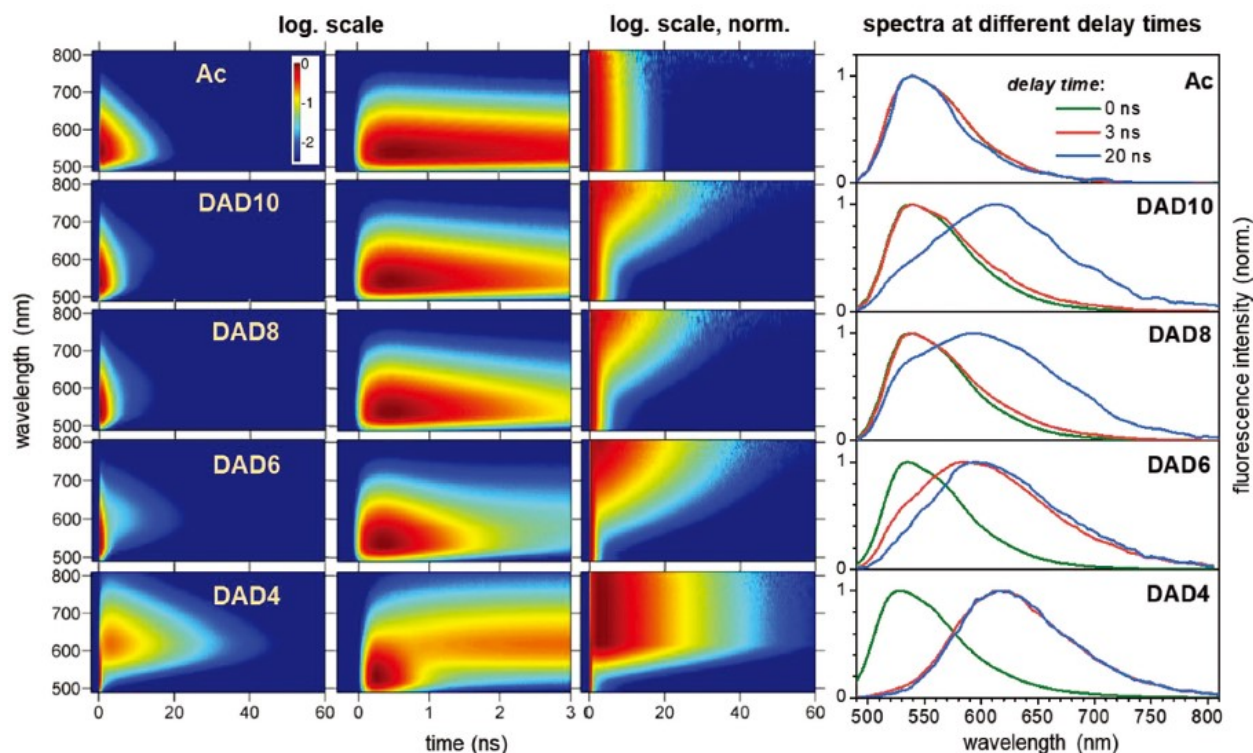


Figure 3. Time-resolved emission spectra (TRES) for the compounds under study in CHCl_3 , normalized to the overall maximum and on a logarithmic scale, zoom of the latter into the time range up to 3 ns, and normalized at each wavelength, on a logarithmic scale (columns from left to right); right: spectra at different delay times. The color scale applies to all the subfigures.

precise rate constant analysis of the current system. Nevertheless, the lifetime data ($\tau_{\text{p-}}$, τ_{DF}) and quantum yields (Φ_{F} , Φ_{SW} , Φ_{LW}) of the triads in CHCl_3 (Table 1) can be quite reasonably fitted by the above-mentioned reaction kinetics for a consecutive-competitive reaction with a reversible step,^[47,48] allowing to extract at least the order of magnitude for the processes, as given in Scheme 1. Accordingly, the excited state lifetimes of $[\text{D}\cdots\text{A}^*]$ and $[\text{D}^+\cdots\text{A}^{\bullet-}]$ are fairly constant for the triads with $\tau_{\text{A}^*}^{-1} \approx 3 \times 10^8 \text{ s}^{-1}$ and $\tau_{\text{ex}}^{-1} \approx 1 \times 10^8 \text{ s}^{-1}$ (however with somewhat varying rates for k_{r} , k_{nr}). The rate constant of quenching by eT increases from $k_{\text{eTA}} \approx 1 \times 10^7 \text{ s}^{-1}$ for DAD10 to $\approx 1 \times 10^8 \text{ s}^{-1}$ for DAD4 due to the shortened D–A separation, while the energy of the radical ion pair stays fairly constant according to the TD-DFT calculations (vide infra). Radical ion pair formation is however only a minor deactivation pathway in comparison with excimer formation $[\text{DA}^*]$. The latter increases from $k_{\text{a}} \approx 4 \times 10^8 \text{ s}^{-1}$ for DAD10 (corresponding to an association time of $t_{\text{a}} = 2.5 \text{ ns}$, see Table 2) to $\approx 4 \times 10^9 \text{ s}^{-1}$ for DAD4 ($t_{\text{a}} = 0.25 \text{ ns}$), due to the longer D–A separation (vide infra); on the other hand, the back reaction, i.e., excimer dissociation, increases only from $k_{\text{d}} \approx 1 \times 10^7 \text{ s}^{-1}$ to $\approx 3 \times 10^7 \text{ s}^{-1}$. The data essentially explain the experimental observations (Table 1) with a strong shortening of the prompt lifetime τ_{PF} with decreasing spacer length, along with a more efficient formation of the emissive exciplex $[\text{D}^+\cdots\text{A}^{\bullet-}]$. The latter is formed from $[\text{DA}^*]$ with a rate constant $k_{\text{CTF}} = 6.7 \times 10^8 \text{ s}^{-1}$; such two-step formation is in line with early suggestions by Weller.^[35]

In order to rationalize the extracted rate constant k_{a} for association, the rate is first estimated within a simple diffusion model, based on diffusion of free carbazole (Cz), bridging the distance between the extended and stacked form; the latter can be estimated from the DFT calculations (vide infra) as the shortest distance d_{min} to $\approx 1.2 \text{ nm}$ for DAD4 and 1.9 nm for DAD10. The diffusion coefficient D of Cz is known in water and acetonitrile.^[51,52] from which D in CHCl_3 can be interpolated by the Stokes–Einstein relation; using the viscosities of the solvents, this gives $D \approx 1.3 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$. Our MD simulations for free carbazole in CHCl_3 gave a very similar value with $D = 1.1 \pm 0.3 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, as obtained via the mean square displacement; see Table S4 in the Supporting Information. The minimum diffusion time is then calculated from $t_{\text{min}} = d_{\text{min}}^2 / (2xD)$ with $x = 1, 2, 3$ as the dimension of the diffusion. Calculating a reference value for free

Table 2. Exciplex association times (t_{a}) for DAD4 and DAD10 in CHCl_3 as determined from the experiment (exp), from a free diffusion model (3D), quasi-1D diffusion, and the results from MD simulations.

	DAD4	DAD10
$t_{\text{a,exp}}$	0.25 ns	2.5 ns
$t_{\text{a,3D}}$	0.18 ns	0.46 ns
$t_{\text{a,1D}}$	0.55 ns	1.4 ns
$t_{\text{a,MD}}$	$\approx 0.2 \text{ ns}$	$\approx 1 \text{ ns}$

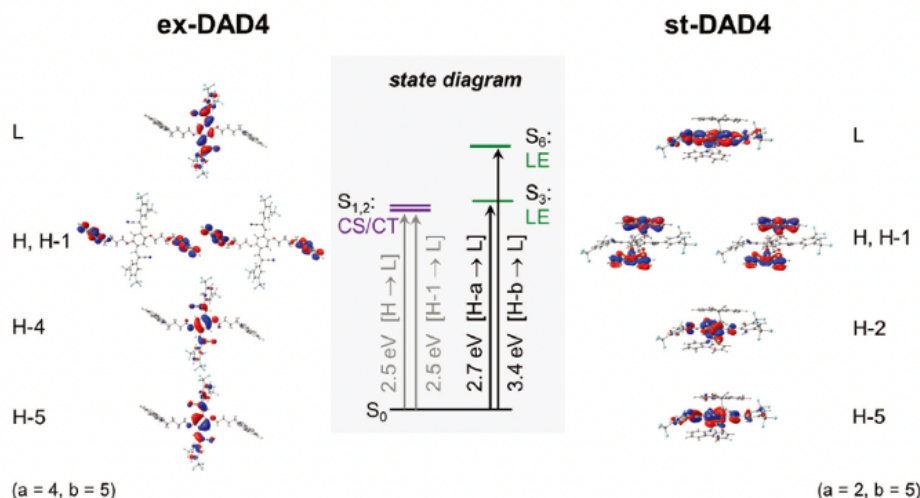


Figure 4. Simplified state diagram for the extended (ex) and stacked (st) forms of DAD4, displaying relevant electronic transitions, with the main contributions of the mono-electronic excitations ($S_{1,2}$: $\geq 98\%$; S_3 : $\geq 97\%$; S_6 : $\geq 85\%$) and the corresponding MO topologies (H = HOMO, L = LUMO). The energy difference between the two CS/CT states (S_1 , S_2) is < 0.05 eV; the two LE transitions ($S_{3,6}$) are mainly described by H-a $\rightarrow$ L and H-b $\rightarrow$ L, where $a = 4$, $b = 5$ for the ex-form, and $a = 2$, $b = 5$ for the st-form. Geometries optimized with the ω B97XD functional, electronic states calculated with the PBE0 functional.

(3D) diffusion of Cz, one gets 0.18 ns for DAD4 and 0.46 ns for DAD10, see Table 2. On the other side, assuming a quasi-1D trajectory due to the presence of the spacer, 0.55 ns for DAD4 and 1.4 ns for DAD10 is obtained. The results show indeed the same order of magnitude as the extracted association times reported above; nevertheless, the strong effect of spacer length, found in the experiment, is not predicted in the simple diffusion model. In order to gain atomistic insight into the association process, we performed MD simulations of the triads in CHCl_3 , giving a time constant t_a of ≈ 0.2 ns for DAD4 and ≈ 1 ns for DAD10 (Table 2), in reasonable agreement with the experimental values given above. It is noted that the difference between free 3D diffusion for DAD4 is very similar to the restrained diffusion; this is ascribed to small variations in energy by the torsional adjustments of the spacer configurations.

2.2. Quantum-Chemical Treatment

In order to gain further insight into the geometry, electronic structure, and optical excitations of the triads, (TD-)DFT calculations were performed, using the Gaussian16 program package.^[53] Geometry optimizations were carried out for two conformations: (i) fully extended (ex; imposing C_2 symmetry) and (ii) stacked form (st; C_1), to model the two extremes for D–A interactions, see Figure 4. As in particular the proper description of the stacked form requires the inclusion of dispersion, the ω B97XD functional was used for the DFT part. At this level of theory, the st-form is predicted to be more stable than the ex-form by ca. 1.5 eV (corresponding to a binding constant $\log K_a \approx 10$; somewhat varying on n), see Table S1 in the Supporting Information. In any case, it should be kept in mind that the very substantial energy difference between the two extreme cases does not necessarily reflect the dynamic situation in solution at room temperature. In the st-form, the two D moieties are forming a π -stacked

DAD sandwich structure with A; however, the exact positioning depends sensitively on n . In fact, for st-DAD4, the D moieties are located asymmetrically on top and bottom of the central β -cyano-styryl moiety of A (see Figure 4); on the other hand, for longer spacers, the D moieties are located on top/bottom of the outer α -cyano-styryl moieties of A (see Figure S1, Supporting Information). For this reason, the acceptor unit is significantly more twisted for $n = 4$ in comparison with $n > 4$, see Table S1 in the Supporting Information.

The correct TD-DFT treatment of excited states with (partial) CT character is a matter of ongoing discussions; in fact, it was shown that for states with only partial CT character (i.e., with considerable non-zero oscillator strength f_{CT}), standard DFT functionals like B3LYP or PBE0 tend to fail,^[54] due to the overdelocalization of the constituting wavefunctions. On the other hand, for states with very pronounced CT character (i.e., where the HOMO is localized in D and the LUMO in A, and, subsequently $f_{CT} \approx 0$), as in twisted D–A polyads or D– σ –A– σ –D triads, these functionals were shown to operate well.^[11,14,18,55] As the current triads fall in this category, we utilized here the PBE0 functional for the TD part (in implicit solvent, CHCl_3 , using the polarizable continuum model, PCM). This indeed reproduces the experimentally observed spectral features much better than, e.g., ω B97XD, M06-2X or CAM-B3LYP; in fact, the latter functionals predict the locally excited (LE) state as the lowest excited state (see Table S2, Supporting Information), in apparent disagreement with experiment. Within the PBE0 treatment, the overall description of the spectra is similar for the different spacer lengths n , and for both st and ex conformations, see Table S3 in the Supporting Information. The two intense bands in the experimental absorption spectrum (vide supra) are assigned to A-centered LE states; for instance, in ex-DAD4 they correspond to S_3 and S_6 with 2.7 eV and 3.4 eV, and with similar oscillator strength ($f_3 = 0.73$, $f_6 = 0.75$), in reasonable agreement with experiment, see Figure 1.^[56] On the other hand, two low-lying excited states ($S_{1,2}$) are found to

be of pronounced charge transfer character, located at ca. 2.5 eV with small oscillator strength ($f \ll 0.01$); for details see Table S2 in the Supporting Information. For the st-form, these states correspond to CT states, while in the ex-form they represent CS states (i.e., the radical ion pair), which cannot be directly photoexcited from S_0 due to the large D...A separation.^[57]

For the ex-form, a small increase of the LE state energy (S_3) is observed with increasing n ; at the same time, f_3 decreases somewhat. As the state description (almost exclusively HOMO-4→LUMO; see Table S2, Supporting Information) is the same for all n , the differences are ascribed to the topology of the MOs (Figure S3, Supporting Information), arising from (rather small) differences in the torsional angles within the vinylene units (Table S1, Supporting Information). This indicates that the presence of the D unit has a certain impact on the structure of A due to steric demands; in any case, the differences in the calculations are somewhat more pronounced than in experiment. The changes in the CS state ($S_{1,2}$) energy are more pronounced; in fact, for $n = 10$, the CS states are found somewhat above the LE state.

In the stacked forms (st-DAD n), the electronic situation is not very different from ex-DAD n ; in any case, the variations in energy are less pronounced. Remarkably, the oscillator strength of the LE-type $S_{3,6}$ states for $n = 4, 6$ are much smaller in the st-form compared to ex (e.g., $f_3 = 0.36$ for st-DAD4) due to reduced LCAO coefficients in the outer rings because of conformational constraints (Figure 4). Different from the ex-form, because of the close D–A separation in the st-form, the CT state can now be effectively populated after photoexcitation of A. The combined oscillator strength of the two low-lying CT states is calculated to be small (e.g., $f_{1+2} = 0.0033$ for st-DAD4, see Table S3 in the Supporting Information). This qualitatively rationalizes the long lifetime of the exciplex found experimentally (≈ 9.9 ns); however, a more quantitative treatment may require the inclusion of Herzberg–Teller coupling.

2.3. Solvent Effects on Fluorescence Quantum Yields and Lifetimes

The proposed dynamic nature of the exciplex formation in the current system should manifest itself in a pronounced dependency on environmental parameters, that is polarity and viscosity of the solvent, as well as temperature variation.

Polarity: To investigate the polarity effect, experiments were carried out in various solvents of similar viscosity, but varying polarity (Table 3), i.e., *n*-octane, toluene, CHCl_3 , and methanol (MeOH). It is emphasized that the analysis is complicated by the fact that also the SW emission (i.e., from A^*) is polarity dependent. In fact, also the emission of the acceptor alone, Ac, shows a systematic bathochromic shift with increasing polarity, i.e., from 515 nm in *n*-octane to 548 nm in MeOH (Figure 5). At the same time, the structured band shape in nonpolar *n*-octane evolves towards unstructured features in polar solvents, seconded by a systematic decrease of Φ_F from 0.6 in *n*-octane to 0.3 in MeOH; see Table 3. The decrease of (intensity-averaged) τ_F is less pronounced, while the decay is less exponential at higher polarity. All effects are attributed to a small but non-negligible partial CT character of the LE ($S_0 \leftrightarrow S_1$) transition in Ac (see Figure S3,

Table 3. Fluorescence quantum yields Φ_F and prompt (intensity-averaged) lifetimes τ_{PF} of Ac, DAD4, and DAD10 in different solvents (MeOH = methanol), with respective viscosities (mPa s, at 25 °C)^[60] and polarities (given in normalized $E_T(30)$ values, E_T^N).^[58]

		<i>n</i> -Octane	Toluene	CHCl_3	MeOH
Viscosity	η	0.508	0.560	0.537	0.544
Polarity	E_T^N	0.012	0.099	0.259	0.762
Ac	Φ_F	0.59	0.60	0.41	0.30
	τ_F [ns]	4.47	4.55	3.39	3.35
DAD10	Φ_F	0.48	0.33	0.30	0.02
	τ_{PF} [ns]	1.73	1.36	1.39	0.47
DAD4	Φ_F	0.33	0.22	0.16	0.06
	τ_{PF} [ns]	1.48	0.36	0.21	0.73

Supporting Information). This gives rise to a reorganization of the polar solvent shell after photoexcitation.^[58] In turn, the Ac structure in S_1 suffers from stronger reorganization with increasing polarity, which decreases $k_r = \Phi_F/\tau_F$. The geometry change also rationalizes the observed non-exponential fluorescence decay of Ac; details of this study will be given in a forthcoming paper.

Using the spectral positions of the Ac emission as references for SW emission of the triads, we can assign the spectrum of DAD4 in MeOH (556 nm) mainly to SW emission; at the same time, the emission is effectively quenched (with $\Phi_F = 0.02$; see Table 3), due to the stabilization of the radical ion pair.^[59] In any case, the spectrum is somewhat broadened in the low energy part in comparison with Ac, pointing to the presence of the exciplex, but to a very minor degree. This is consistent with the time traces, recorded in the LW region (i.e., at 680 nm), which shows a long, but weak component of ≈ 7.6 ns, see Figure S10 and Table S6 in the Supporting Information. This evidences the dominance of quenching by eT in polar solvents in line with former studies.^[35] In stark contrast, for lower polarity, the SW

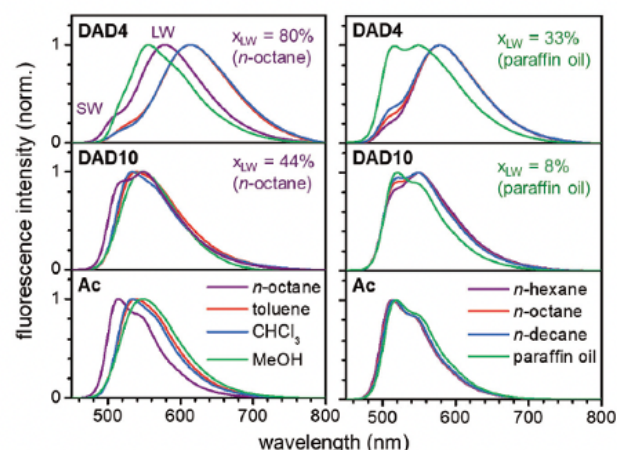


Figure 5. Fluorescence spectra of Ac, DAD10, and DAD4 in solvents of different polarity (left) and viscosity (right); fractional intensities of the long wavelength emission x_{LW} were obtained by spectral decomposition, see Figure S9 in the Supporting Information.

emission is seen only as a shoulder, so that the emission is dominated by the LW band; at the same time, a strong hypsochromic shift of λ_{LW} from 613 nm in toluene and CHCl_3 to 584 nm in *n*-octane is observed (with a spectral fraction of $x_{\text{LW}} = 80\%$, as determined by spectral decomposition, see Figure S9, Supporting Information). In all, the results demonstrate only a small polarity effect in exciplex formation, but a significant effect on the quenching pathway.

Viscosity. To investigate the viscosity effect, DAD4 (i.e., the triad with the highest tendency for exciplex formation) was dissolved in saturated hydrocarbons at ambient conditions with increasing viscosity, i.e., *n*-hexane ($\eta = 0.300$ mPa s), *n*-octane (0.508 mPa s), *n*-decane (0.838 mPa s),^[60] and paraffin oil (ca. 100 mPa s), see Figure 5. At low viscosities, a small increase of the SW intensity with η is observed, while the overall emission is dominated by the LW band. In paraffin oil, the LW band significantly decreases, and the spectrum is now dominated by the SW emission ($x_{\text{SW}} = 67\%$; as determined by spectral decomposition, see Figure S9, Supporting Information). This indeed proves that the dynamic exciplex formation is effectively suppressed in highly viscous media, as expected. Concomitantly, for DAD10, the LW intensity reduces from 44% in *n*-hexane (low viscosity, low polarity) to 8% in paraffin oil, see Figure 5 and Figures S9, S15 in the Supporting Information. In order to gain further insight into the dynamic exciplex formation, we repeated the MD simulations for DAD4 in paraffin oil. Indeed, the collapse of the extended to the stacked conformation is now extended to several nanoseconds, compared to ≈ 0.2 ns in CHCl_3 . Finally, due to the dynamic nature of exciplex formation, the process is completely inhibited in solid solutions, by embedding DAD4 at low concentrations in poly(methyl methacrylate; PMMA); in fact, only SW emission is observed in this case, see Figure S17 in the Supporting Information.

Temperature. The low temperature (LT) behavior of DAD4 and DAD10 in CHCl_3 at $T = 65$ K was investigated at different concentrations, see Figure 6 and Figure S13 in the Supporting Information. For DAD4 at $c = 1 \times 10^{-5}$ M, the emission spectrum shows a similar shape in comparison to room temperature (RT), dominated by the LW band, but being hypsochromically shifted by 0.13 eV vs RT, see Figure 6. The SW band, obtained by spectral decomposition, on the other hand, shows a small bathochromic shift. Importantly, the fluorescence excitation (FLE) spectrum shows a significant dependence on the detection wavelength λ_{det} ; for detection at λ_{SW} (500 nm), the spectrum is very similar to the FLE spectrum at RT, while under λ_{LW} detection (680 nm), the FLE spectrum exhibits a significant bathochromic shift. This is in stark contrast to the FLE at RT, which does not show a dependency on λ_{det} (vide supra), evidencing a fundamentally different scenario at RT and LT, that is dynamic exciplex formation in the liquid, but formation of static (i.e., ground-state) CT complexes in the frozen solution. The formation of the CT complex is already observed at $c = 1 \times 10^{-6}$ M (Figure S13, Supporting Information), evidencing an intramolecular scenario. On the other hand, a significant concentration dependence of the spectral features is observed (Figure S13, Supporting Information), so that at concentrations of 10^{-4} M and higher only LW emission is observed. This suggests the concomitant generation of intermolecular static CT complexes at higher concentrations. As dis-

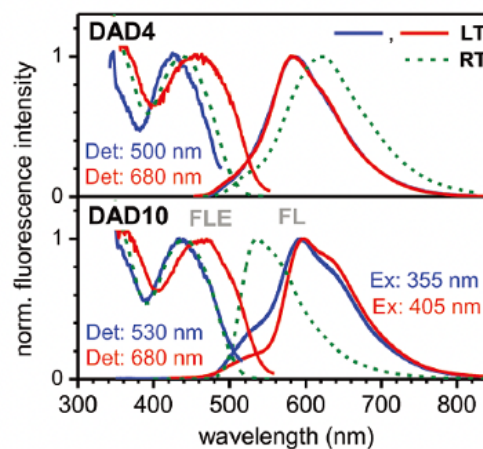


Figure 6. Fluorescence emission (FL, right) and excitation (FLE; left) spectra of DAD4 (top) and DAD10 (bottom) in CHCl_3 ($c = 1 \times 10^{-5}$ M) at low temperature (LT; FL at 65 K, FLE at 77 K; blue and red solid lines) under different excitation (detection) conditions for FL (FLE), $\lambda_{\text{ex}} = 355$ nm (blue), 405 nm (red), and $\lambda_{\text{det}} = 500/530$ nm (blue), 680 nm (red). FL and FLE spectra at room temperature (RT, green dashed lines, $\lambda_{\text{ex}} = 405$ nm) are shown for comparison.

cussed above, the exciplex at RT is formed in a dynamic manner from the ex-form, while the LT conditions evidently freeze out the st-form in the ground state, as well as intermolecular CT complexes. The CT complex is stabilized by 0.17 eV versus the ex-form, as extracted from the bathochromic shift of the FLE spectrum. The TD-DFT calculated bathochromic shift is significantly smaller (0.03 eV, see Table S3, Supporting Information), which may in part be related with the shortcomings of the linear response PCM used in this work. On the other side, also mutual polarization of the D and A moieties in the st-conformation will contribute to the bathochromic shift,^[10] which might be insufficiently described in the TD-DFT scheme. Such polarization, however, cannot be the source of the LW band shift upon cooling, as the LW band shows the opposite behavior, i.e., a hypsochromic shift. We thus rather infer that the latter originates from somewhat different intra- and/or intermolecular D–A arrangements, as the emission color should indeed sensitively depend on the exact mutual position of the moieties; this was in particular shown for excimers.^[28,34,61]

Turning from DAD4 to DAD10, the spectral changes in FL upon freezing are more drastic, as at RT conditions, dynamic exciplex formation in DAD10 was unlikely to occur due to the long spacer (vide supra). The decrease in solubility at LT conditions promotes intramolecular stacking already in the electronic ground state, giving rise to a static CT complex; this is apparently highly effective, so that the FL is now dominated by the LW band, see Figure 6. In any case, the fraction of LW is still significantly smaller compared to DAD4, reflecting the lower probability for stacking due to the longer spacer in DAD10. Concomitantly, shifting the excitation wavelength towards the red, the probability of exciting the st-form increases, due to the presence of the low-lying CT state; this enhances the LW fraction in the FL spectrum (i.e., compare the blue and red spectra in Figure 6). As in DAD4, also DAD10 shows a significant concentration dependence, evidencing the concomitant formation of intermolecular

CT complexes. In all, the LT studies demonstrate how dynamic exciplex formation can be transformed to a static, intramolecular CT complex by spatial confinement, and how spectroscopy allows to monitor these changes.

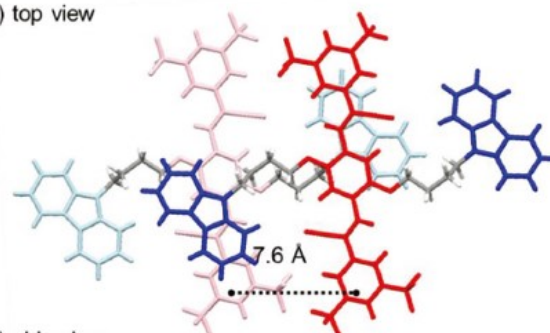
2.4. Emission of the Molecular Solids

Importantly, the current study allows also for a more nuanced view on the solid-state properties of the triads (i.e., powders or films). In fact, all DAD n triads were reported to show stimuli-responsive luminescence behavior, when applying pressure or temperature. While for $n = 4$ yellow/red fluorescence color switching was observed, for $n > 4$ fluorescence on/off switching occurred between pristine or annealed powder (“off” state) and ground powder (“on” state),^[15] see Figure 7. This was tentatively attributed back then to an intramolecular conformational change between the extended and the stacked form. Our current results, however, clearly evidence the emissive character of the CT complexes; in fact, the static CT complexes in frozen solution absorb and emit at the same spectral positions as the (pure) spin-cast films reported by Kwon et al.^[15] We therefore ascribe the emissive properties of spin-cast films and ground powders of all triads to the presence of static CT complexes. On the other hand, we have demonstrated in the current work the subtle competition between the fluorescence of the CT complex and fluorescence quenching by eT, in particular for small distance between the D and A moieties. Therefore, in the solid state, the effectiveness of exciplex emission will sensitively depend on the exact geometric positioning of D and A. It is thus evident that even small perturbations in the intra- and/or intermolecular arrangement of the dyads in the solid state, as induced by external stimuli, may generate effective fluorescence on-off switching by subtle balancing of the CT emission versus quenching through unfavorable D–A positioning.

The exact mechanism of fluorescence switching is however difficult to prove without structural information. However, crystallization of the materials turned out to be challenging for $n > 4$; we ascribe these difficulties to the enlarged conformational space with longer spacer length, which flattens the potential energy surface for intra- and intermolecular interactions. Fortunately, the growth of DAD4 single crystals could be achieved (see SI for details), whereas single crystal growth failed for the other triads. The DAD4 crystals fluoresces yellow under UV irradiation, very similar to the pristine powder sample, see Figure 7 and Figure S17 in the Supporting Information. Upon grinding the pristine powder, the color turns red, and turns back to yellow upon annealing at 120 °C. Here, the red color corresponds to that of the exciplex in solution, see Figure S17 in the Supporting Information. In fact, this behavior remained an open question in ref. [15] which can now, however, be answered in light of the current results. In the DAD4 crystal, the molecules adapt an extended conformation with a significantly twisted backbone of the acceptor unit (see Figure 7 and the SI for further details); in any case, it is less twisted than what was calculated for the free molecule, demonstrating the “twist elasticity” of the DCS motif.^[62] The triads are slipped along the short (x) axis of DCS by ≈ 7.6 Å, so that pronounced exciton formation between the DCS units can be neglected.^[10] Instead, the Cz (D) moieties are located above

DAD4

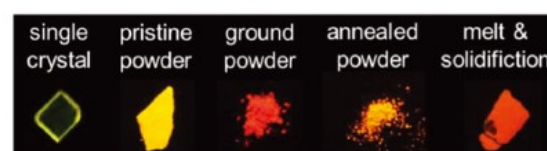
a) top view



b) side view



c) stimuli-responsive behavior



DAD10

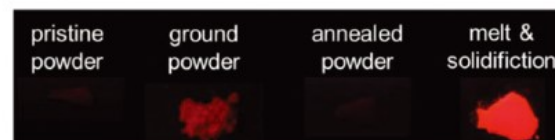


Figure 7. Solid-state properties of the molecular triads DAD n . Top: nearest neighbor arrangement in DAD4 crystals (CCDC 2 265 264) with D moieties in blue and A moieties in red; a) top view, b) side view, highlighting the sliding plane (green arrows); c) fluorescence images of the DAD4 single crystal and the powder samples and their stimuli-responsive behavior. Bottom: fluorescence on/off switching for $n > 4$; here exemplified for DAD10.

the vinylene unit of the DCS core (A) with a mean distance of 4.0 Å (see Figure S21 in the Supporting Information for details), providing sufficient π -overlap to form a D–A CT complex between the adjacent triads. The molecules form sliding planes (see Figure 7), which rationalizes the facile response to external stimuli. Hence, upon grinding, the relative position of D and A of the adjacent triads will change their position. Plausible is a positioning of Cz on the central ring of the adjacent DCS core, thus generating the same electronic situation which was predicted for the exciplex in an intramolecular fashion, but here in an intermolecular manner. It should be noted in this context that also powder samples of the acceptor alone (Ac) emit red, see Figure S19 in the Supporting Information. Therefore, it cannot be excluded that the red color of the DAD4 ground powder may originate from

DCS stacks generated through an x-slip of the molecules upon mechanical stimuli.

For the longer spacers, no structural data are available, as all attempts to grow the corresponding crystal did not succeed. In any case, it is evident from the negligible emission in the pristine powder (as well as after thermal annealing) that the thermodynamically stable mutual arrangement of the D and A moieties in this case do not provide sufficient π -overlap to allow for an emissive CT state, but quenches fluorescence through unfavorable D:A positioning. Mechanical force then displaces the molecules along the sliding axis, which allows for sufficient π -overlap to generate red emission, see bottom of Figure 7. In all, the current findings provide an important complement to the work by Kwon et al.,^[15] the current results may inspire further investigations in stimuli-responsive materials relying on the subtle interplay of complex photophysical deactivation pathways. Moreover, this work may be relevant in the fundamental understanding for the occurrence of emissive exciplexes in OLEDs^[6] or reconsideration of organic exciplex lasers.^[63]

3. Conclusions

A holistic picture on the complex photophysical pathways of D- σ -A- σ -D triads with varying flexible spacer lengths ($n = 4$ –10 carbon atoms) was established in liquid and solid solution by combining (steady-state and time-resolved) optical spectroscopy with TD-DFT and MD computational studies. In liquid solution, the competitive dynamic CT state formation with subsequent exciplex emission versus (minor) quenching by electron transfer (eT) via a radical ion pair has been quantified, identifying an optimized system with 14% exciplex fluorescence quantum yield. In solid solution, a fluorescent static CT complex is formed upon freezing/solidification; this is assigned also as the fluorescent phase in the film samples. Thermal stimulus of the film (annealing) either: i) breaks the CT complex, leading to on/off fluorescence switching by effective fluorescence quenching (for $n > 4$), or ii) modulates the geometry of the CT complex, which results in a change of the emission color ($n = 4$). In all, the study emphasizes the imperative for a holistic, detailed understanding of compounds with complex photophysics, as achieved here, in order to reach the ultimate goal of targeted design of novel materials in exciplex-based applications in sensing, OLEDs or lasing.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

exciplexes, fluorescence, photophysics, organic optoelectronics, stimuli-responsive materials

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Higher Order Cavity Modes for SERS in a Gold Nanosphere-on-Mirror Configuration

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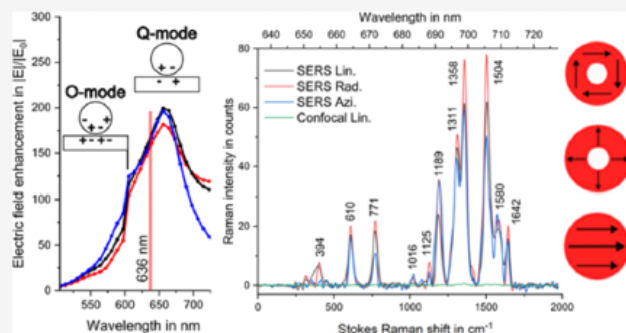
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ABSTRACT: Nanoparticle-on-mirror (NPM) configurations have demonstrated enormous enhancements toward Raman scattering due to a highly confined and amplified electromagnetic field within the nano/picocavity between the gold nanoparticle and the underlying ultrasmooth gold film. While the dipolar gap mode has been often referred to in view of its high Raman enhancement, we contend that higher order plasmonic modes such as the quadrupole mode can contribute significantly at the commonly used Raman exciting wavelength of around 633 nm. This finding is supported by experimental results obtained by exciting the NPM configuration in the focus of a linearly, radially, and azimuthally polarized laser. We demonstrate that both in-plane and out-of-plane electric fields are suitable for enhancing the Raman scattering of Rhodamine 6G (R6G) molecules sandwiched in the NPM configuration making use of the field distribution of the higher order gap mode, as confirmed by boundary element method simulations. Further, significant variations in the polarization-dependent optical response can be seen for the investigated individual NPMs, which are attributed to variations in the individual NPM geometries. As the probe molecule for our studies is Rhodamine 6G (R6G), Raman spectra show peak-specific enhancement factors, which correlate with the tensor properties of individual vibrational modes.



1. INTRODUCTION

Investigating the optical properties of organic molecules deposited on metal surfaces is of great interest to improve the understanding of their applicability in technical solutions. To compensate for low signal intensities from small quantities of substance, the molecules can be placed on or in proximity to plasmonic substrates for surface-enhanced Raman scattering (SERS).^{1,2} Typically, rough gold or silver surfaces, specifically tailored plasmonic nanostructures, or nanoparticle-on-mirror (NPM) configurations are used as the substrates.^{3,4} To achieve a high electromagnetic field, the plasmonic nanostructure needs to be excited by the incident laser radiation by matching the localized surface plasmon resonance (LSPR) to the laser wavelength.^{5–7} Significantly enhanced local electromagnetic fields can be generated at the so-called plasmonic “hot spots”, i.e., at sharp edges, or in nano/picometer gaps. Particularly in the NPM configuration, the incident electric field excites the LSPR of the nanoparticle, which induces charge density shifts at the surface of the smooth gold substrate underneath that can be described in the formalism of mirror dipoles. These oscillating dipoles interfere with each other in the NPM geometry to form

coupled gap-modes. A plethora of gap-modes can exist depending on the geometric properties of the gap, which have been shown to strongly influence the resonance wavelengths and susceptibility to polarization of NPM configurations.^{8,9} Resonant excitation of gap-modes results in a strong and highly localized electric field in the gap region that leads to enormous electromagnetic enhancement of optical signals originating within the gap.^{10,11} In SERS, the Raman signal enhancement depends on the spectral overlap of both the incident radiation and the scattered radiation with the plasmonic resonances of the metallic nanostructure. In turn, the Raman intensity of a certain vibrational mode depends not only on its intrinsic Raman tensor, but also on how much the incident field is enhanced at the location of the molecule and in turn how much the scattered

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field which is energetically shifted with respect to the incident radiation is enhanced.^{12,13}

The influence of gap geometries on plasmonic gap-modes has been of great interest for theoretical analysis and experimental work in the recent years, highlighting the impact of polarizability changes on gap-mode responses.^{14–16} It has been shown that the SERS enhancement factor can vary for different excitation polarizations in NPoM systems.⁴ This was recently applied to obtain polarization-dependent dark-field spectra, SERS spectra and intensity maps of a nanocube-on-mirror system where the signal under radial polarization strongly outweighed that under azimuthal polarization.⁹ However, the applicability of higher order plasmonic modes (i.e., multipolar modes) of single nanosphere-on-mirror configurations and their SERS performances under different excitation polarizations remains seldomly discussed.

The target of this work is to explore the applicability of a higher order gap-mode in SERS, with the main focus on the polarization-dependent optical responses of single NPoM configurations. We experimentally measure SERS spectra of Rhodamine 6G (R6G) molecules on gold films (ultrasmooth and rough films), and NPoM substrates. We use focused azimuthally (APDM) and radially (RPDM) polarized doughnut modes and linearly polarized beams to compare the polarization-dependent SERS performances at the higher order plasmonic gap mode resonances. We analyze dark-field-scattering spectra of single NPoMs to reveal the higher order gap-modes and perform boundary element method (BEM) simulations to obtain their local field distributions. We further discuss the peak-specific SERS enhancement considering the electric field distribution and the Raman polarizability tensors of R6G vibrational modes calculated using density functional theory (DFT).

2. EXPERIMENTAL AND COMPUTATIONAL DETAILS

2.1. Sample Preparation. Following previous works,^{4,17,18} ultrasmooth gold films were fabricated by electron-beam physical vapor deposition of gold with an evaporation rate of 0.15 nm/s and a chamber pressure of 2×10^{-6} mbar on a polished silicon (1,1,0) wafer. In this work, the gold film thickness is 100 nm. To evaluate the roughness of the sample surface, an atomic force microscope (AFM) was used (tapping mode, Veeco Nanoscope III system). The root-mean-square (RMS) of the silicon wafer surface roughness was determined as 0.154 ± 0.026 nm, while that of the evaporated gold film was 2.250 ± 0.113 nm. To achieve the ultrasmooth gold surface, the evaporated gold layer is template-stripped off from the underlying silicon wafer using UV-activated glue after curing for 24 h. The roughness of the revealed gold film surface after template stripping was determined as 0.341 ± 0.033 nm, representing a significant improvement of surface smoothness compared to the evaporated gold layer.

As the probe molecule, Rhodamine 6G, a well-studied organic molecule with high Raman scattering cross section was used. It has the absorption maximum at 530 nm and the emission maximum between 540 and 570 nm.^{19–21} An ethanolic R6G solution with a concentration of $1.0 \times 10^{-6} \frac{\text{mol}}{\text{L}}$ was prepared, of which 5 μL were evenly distributed on a $3 \times 3 \text{ mm}^2$ gold surface area by spin-coating at 2000 rpm for 10 s. This leads to a maximum of $\sim 10^3$ R6G molecules in a single NPoM region, corresponding to submonolayer coverage. No spacer layer was used between the R6G and the gold film to guarantee an

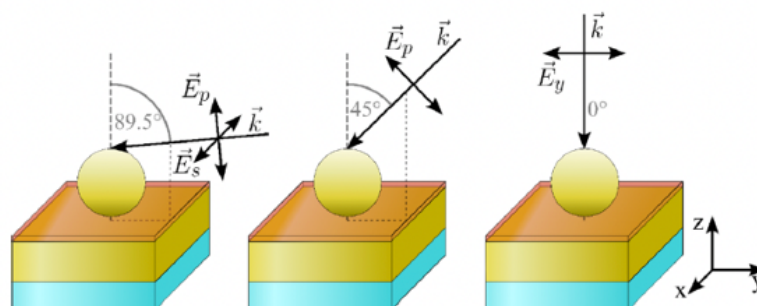
optimally flat adsorption surface. Gold nanospheres with an average diameter of 115.18 ± 11.78 nm (determined by scanning electron microscopy (SEM) of 107 nanoparticles) were used for creating the NPoM configuration. The Raman-active stabilizing surfactant was removed by repeated heating of the nanoparticle solution to 80 °C and centrifugation at 500 rpm. The suspension was diluted by a factor of 5 in each repetition, resulting in a factor of 125 dilution at the end. This is beneficial to reduce the probability of multimer or cluster formation on the sample. The cleanliness of the nanoparticles was proved by their optical spectra where only gold photoluminescence was visible and no Raman features from the surfactants were observable. A volume of 5 μL surfactant-free gold nanoparticle suspension was drop cast onto the R6G layer. The solvent was left to evaporate swiftly at 60 °C.

A rough gold film was used as the reference SERS substrate, which was prepared by electron-beam physical vapor deposition of gold with an evaporation rate of 0.15 nm/s and a chamber pressure of 2×10^{-6} mbar through a copper grid mask (grid edge length: 60 μm) onto a glass surface. The resulting gold film contained periodically ordered gold patches with rough edges, serving as a plasmonically active platform for SERS. The introduction of organic molecules followed the method described above, however without further gold nanoparticle deposition.

2.2. Confocal Optical Microscope Setup. Optical spectroscopy and imaging based on the Stokes-Raman scattering signals were conducted in a home-built optical microscope. It was equipped with a 636 nm CW laser for the Raman scattering measurements. Laser focusing and optical signal collection were done using a parabolic mirror of NA = 0.9986 (in air).^{22–24} The image intensities were recorded by a PerkinElmer SPCM-AQR-13 avalanche photodiode. All spectra were recorded using an Acton SpectraPro 300i spectrometer coupled to a Roper Scientific LN/CCD-1340/100-EB camera. A 600 lines/mm grating was selected for the Raman scattering measurements. All Raman scattering spectra were recorded with an acquisition time of 30 s for 10 repetitions. For all Raman measurements with the 636 nm CW laser, the laser intensity was determined as 36 μW directly in front of the parabolic mirror. It was adjusted with optical density filters for each laser polarization to ensure a similar irradiation intensity in the focal field for the differently polarized modes. Radially and azimuthally polarized higher order laser modes were generated by converting a linearly polarized laser beam with a home-built mode-converter consisting of four quarters of a lambda-half waveplate. To obtain a high quality of the polarization state, a 20 μm pinhole was used to spatially filter the modified beam. The quality of the higher order laser modes was monitored by inspecting the photoluminescence images of gold nanocones that were excited with the different excitation polarizations (see Figure S1a–c).²⁵ Such nanocones act as quasi point-like probes for the local electric field distribution in the focus and can be selectively excited by in-plane or out-of-plane electric field components.²⁶

2.3. Dark-Field Scattering Setup. The dark-field-scattering spectra were recorded using a commercial Zeiss Axio Scope.A1 microscope equipped with a Zeiss Epiplan-Neofluar 100x/0.9 air objective in reflection geometry where the illumination is provided by an unpolarized white light halogen lamp. Spectra were acquired using an Andor SR-303i-B spectrograph equipped with an Andor iDus 416 CCD camera. A 150 lines/mm grating was used to collect the scattered light in a range of 500 to 1000 nm. All spectra were obtained by

Scheme 1. Incidence Angles in the y - z -Plane and Respective Electric Field Vectors in the Plane Wave Approximation Used in the BEM Simulations^a



^aDepicted are 89.5° s- and p-polarized incident plane waves (left), a 45° p-polarized plane wave (middle), and a 0° y-polarized plane wave (right). The sample plane is defined as the x - y -plane. The electric field vectors of s-polarized plane waves are perpendicular to the y - z -plane, i.e., parallel to the sample plane, irrespective of the incidence angle. Those of p-polarized plane waves are parallel to the y - z -plane, with varying components E_y and E_z depending on the incidence angle relative to the optical axis (z)

integrating over 5 s and averaging 5 times. Correction of the spectra is achieved by subtracting the background from the raw data and normalizing by the lamp spectrum minus the dark current of the CCD camera. Background spectra were taken from an uncovered glass slide or a smooth gold film, respectively, directly in the vicinity of the corresponding particle, while the lamp spectrum was recorded by back reflection of the source light from a Teflon plate.

2.4. Computational Methods. The BEM simulations were performed using the MNPBEM-toolbox v.17²⁷ for the calculation of scattering spectra, charge density distributions, electric field distributions as well as field enhancement. The first simulation geometry consists of a gold sphere of 120 nm diameter located 2 nm above a 100 nm thick gold film with complex refractive index.²⁸ This height was chosen to adapt the scattering behavior in the simulation to the experiment. The glass substrate is modeled in the lower half-space with the refractive index of glass $n_{\text{glass}} = 1.5$ while the upper half-space is chosen as air with $n_{\text{air}} = 1$. The second geometry includes an approximated R6G layer in the gap with a thickness of $d = 1.5$ nm (not the entire gap is filled due to numerical restrictions) and refractive index $n = 1.9$.²⁹ Scattering cross sections are obtained by integrating the far-field projection of the Poynting vector over a spherical detection surface enclosing the particle system and normalizing by the incident power. Electric field maps are recorded in the plane parallel to the substrate in the center of the gap. To approximate the different local electric field orientations using radially, azimuthally, and linearly polarized laser excitation in the experiment, excitation angles of 89.5, 0, and 45°, respectively, were used. Incidence for all cases is defined with respect to the optical axis (z) in the y - z -plane of the system (see Scheme 1). To mimic the purely transverse electric field distribution in the focus of an azimuthally polarized laser, a 0° y-polarized plane wave and an 89.5° s-polarized plane wave were used, which showed similar results. The electric field distribution at the location of a NPoM in the focus of a radially polarized laser was simulated by an 89.5° p-polarized plane wave representing the dominant longitudinal electric field component (see discussion and Figure 4 in Results). For the focus of a linearly polarized laser, a 45° p-polarized plane wave was chosen to represent the combination of a transversal and longitudinal electric field component in the focus. The BEM simulation is limited to plane wave excitation and cannot perfectly represent the real electric field distribution in the focus of the respective

polarizations. For azimuthal polarization, the electric field vector is limited to one transverse direction and does not represent the real situation with all transverse electric field vectors simultaneously. However, it is a reasonable estimation considering that the NPoM is much smaller than the azimuthal focal field, where a plane wave approximation comes close to the local experimental situation in the focal field. Likewise, for the focus of a radially polarized laser, the weak transverse mode is ignored when approximating the electric field distribution with a longitudinal plane wave. Since the E_{xy} electric field component in the focal field of a radially polarized laser is distributed around the center of the focal region, while the E_z component is strongest directly at the center, an exclusively out-of-plane approximation of the plane wave comes close to the experimental situation in the focal field, too. The approximation thus only models the dominant local electric field orientations at the point of highest field strength in the focus. It does not reproduce the spatial variation and therefore the position dependence of the NPoM excitation, making it of limited quantitative predictive power.

Density functional theory (DFT) calculations were performed to obtain the equilibrium geometry of R6G at the ground state without any symmetry restrictions in ethanol, using the polarizable continuum model (PCM). The D3-B3LYP functional and 6-311+G(d) basis set were employed as implemented in the Gaussian 16.³⁰ Energy minima were confirmed by the absence of imaginary frequencies in the vibrational calculations. Raman spectra were computed on the full structure with a frequency scaling factor of 0.9680.³¹ In details, the calculated absorption spectrum well reproduced the experimental results, giving an offset of 0.32 eV for the absorption maximum. The offset is defined as the energy difference between the experimental absorption maximum $\lambda_{\text{max}} = 530$ nm (2.34 eV)³² and the calculated first transition from S_0 to S_1 at $\lambda = 466$ nm (2.66 eV). Therefore, the (pre)resonance Raman spectra are expected to provide a reliable description for a defined excitation wavelength. To study the influence of the adsorption on the smooth gold film, the calculation was repeated with a D3-B3LYP functional and 6-31+G(d) basis set. Here, the R6G molecule was placed in varying geometries on a flat gold surface consisting of two layers of gold atoms. The frequency scaling factor was empirically determined as 0.9520.³³

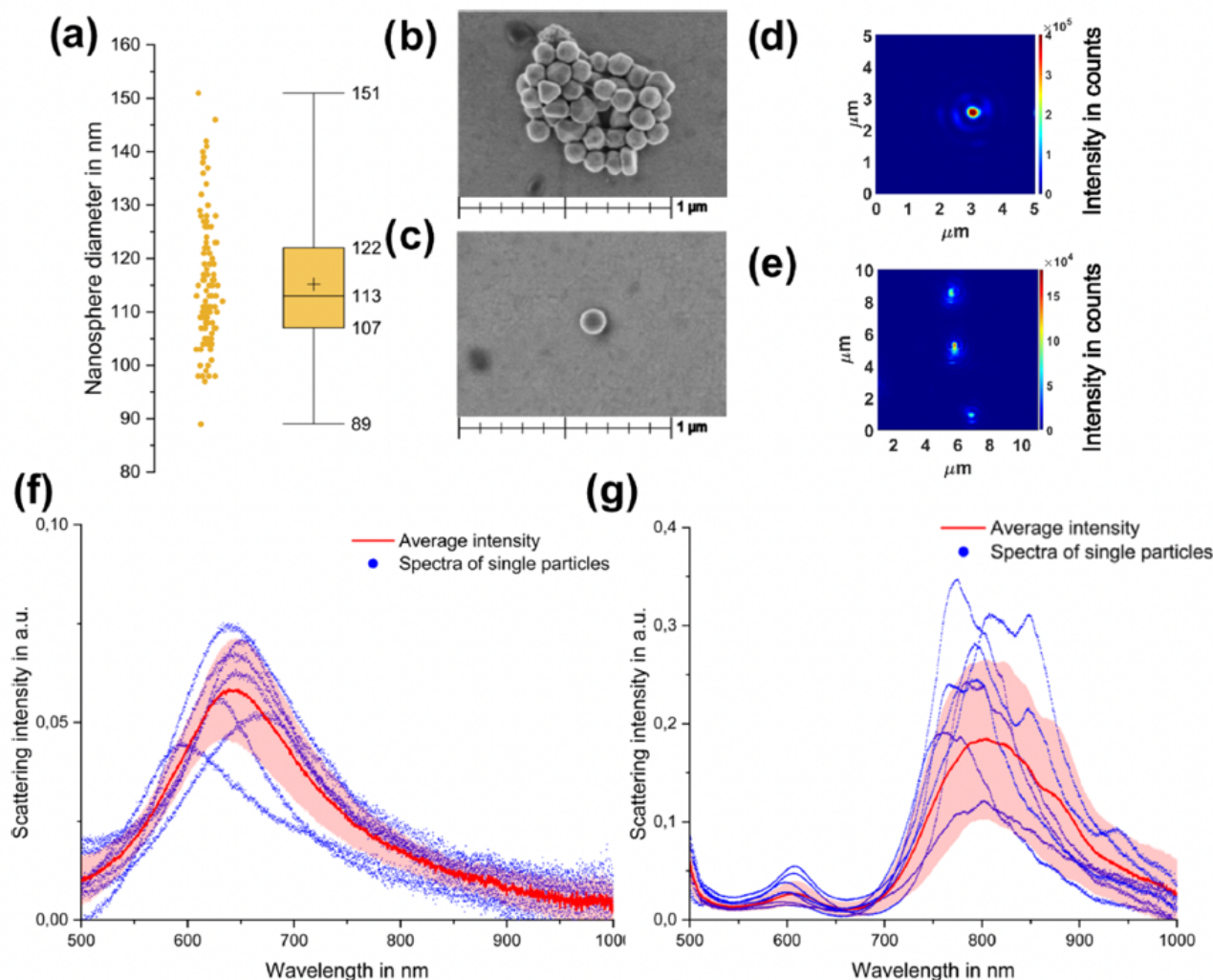


Figure 1. Dark-field scattering spectra with size and geometry distribution of the used nanospheres. (a) Boxplot covering a statistical size distribution of 107 single nanospheres determined by SEM. A median diameter of 113 nm is found, while 90% of all nanoparticles lie within 98–136 nm. For asymmetric particles, the longest axis was measured. (b, c) Scanning electron micrographs of nanospheres. Widely differing geometries are shown in a large cluster (b), while the desired configuration is shown in (c). (d, e) Photoluminescence scans of single NPoMs using a radially polarized laser beam with $\lambda_{\text{ex}} = 636$ nm. (f) Dark-field scattering spectra of a reference of single gold nanospheres deposited on glass without added R6G. Selected spectra of a variety of single particles (blue scatter plots) and the scattering intensity averaged over all nanospheres (red line) including its standard deviation (pale red area) with a single intensity maximum around 643 nm. (g) Dark-field scattering of single gold nanospheres deposited on ultrasmooth gold, no R6G added. The plot includes spectra of a few selected NPoM varieties (blue scatter plots) as well as the average of all sampled spectra (red line) with standard deviation (pale red area). A weak scattering peak can be seen at $\lambda_{\text{max}} = 607$ nm. A strong scattering peak is found ranging from $\lambda = 700$ –950 nm that typically consists of several narrow peaks. On average, the maximum of the strong scattering peak is found at $\lambda_{\text{max}} = 804$ nm, while a shoulder at $\lambda = 874$ nm persists.

3. RESULTS AND DISCUSSION

3.1. Morphology and Scattering Behavior of Nanoparticles. The shape and size of nanoparticles are decisive factors for their plasmonic properties.^{8,14–16,34–38} Therefore, we first characterized the morphology of the nanospheres by spin-coating the suspension onto an ultrasmooth gold film and imaging them in a HITACHI SU8030 scanning electron microscope (SEM) at 1 kV acceleration. The median diameter of the nanospheres was found to be 113 nm, with a tendency of the distribution to be considerably larger than 100 nm (Figure 1a). Further, the particles showed varying geometries (Figure 1b), including faceted gold particles, elongated rod-like structures, pyramidal particles, and more. Perfectly round nanospheres could be found occasionally (Figure 1c). These

different particle morphologies, resulting in varying NPoM cavity geometries,^{6,35,39,40} are expected to influence the sensitivity to excitation polarizations of single NPoM configurations in the SERS enhancement. In the photoluminescence images, individual gold nanoparticles were clearly visible, showing Airy-disk like patterns when their sizes were smaller than the laser focus (Figure 1d,e).

It is well-known that matching the LSPR of nanoparticles used in SERS to the laser excitation wavelength, the emission wavelength of the sample, or in optimal cases an overlap of both, leads to a stronger signal enhancement.^{5–7,34,41} To study the LSPR properties of the sample, dark-field optical spectra were collected from the surfactant-free nanospheres that were deposited on a glass surface (Figure 1f) and an ultrasmooth

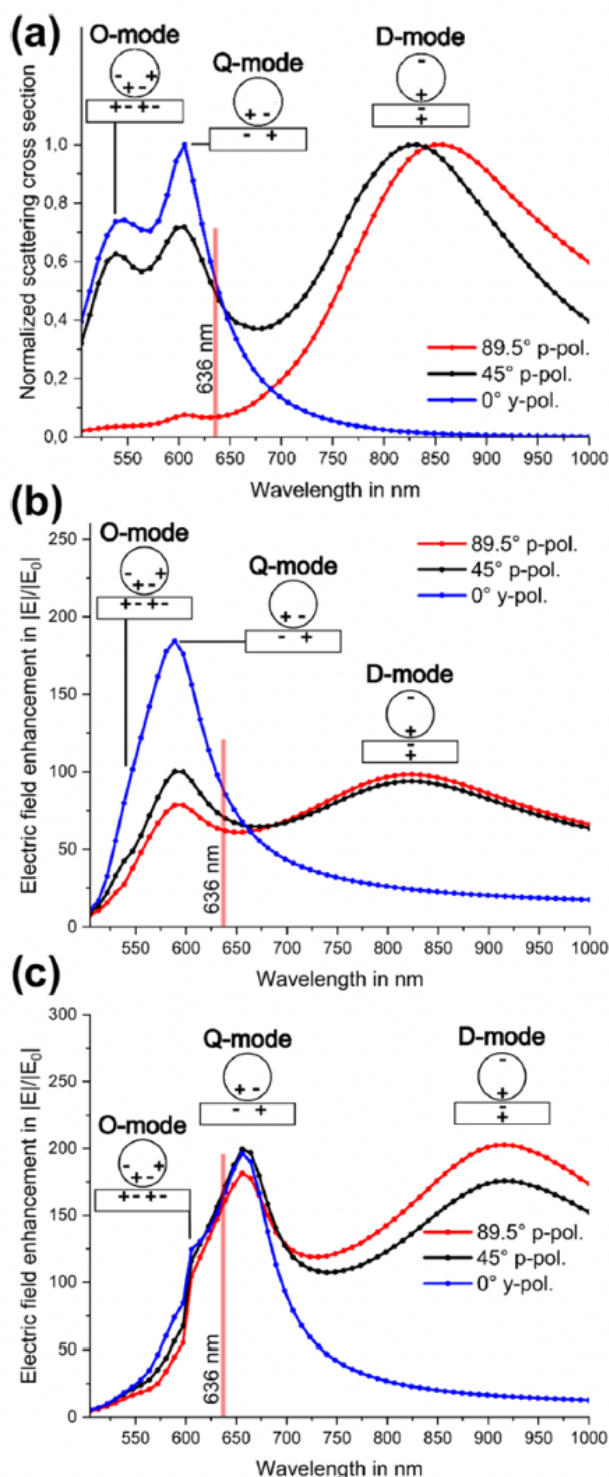


Figure 2. Boundary element method simulation of the scattering cross section and the electric field enhancement of an ideal nanosphere-on-mirror configuration with sphere diameter $d = 120$ nm and gap width of $h = 2$ nm above the gold surface. (a) Shows the normalized scattering cross sections from 500 to 1000 nm for 0° y-polarized excitation, 89.5° p-polarized excitation, and 45° p-polarized excitation under incidence in the y - z -plane, approximating azimuthally, radially and linearly polarized excitation, respectively. (b) Maximum electric field enhancement of the nanocavity system in the x - y -plane ($z = 0$) in the range of 500–1000 nm wavelength for 0° y-polarized excitation, 89.5° p-

Figure 2. continued

polarized excitation, and 45° p-polarized excitation under incidence in the y - z -plane, approximating azimuthally, radially and linearly polarized excitation, respectively. The locations of maximum electric field enhancement in the x - y -plane at the laser wavelength depend on the used excitation polarization and are depicted in Figures S2, S3. (c) Maximum electric field enhancement in the range of 500–1000 nm for the NPoM system including a R6G layer. Excitation is shown for a 0° y-polarized, 89.5° p-polarized, and 45° p-polarized plane wave.

gold film (Figure 1g), respectively. In both cases, no R6G molecules were used to (i) prevent spectral influence of R6G absorption or fluorescence and (ii) make the cavity systems as homogeneous as possible. For the nanoparticle-on-glass configuration, single resonance peaks were found at about $\lambda = 643$ nm that were close to our excitation laser wavelength of 636 nm. This resonance peak varied in the range of $\lambda = 600$ –690 nm depending on the selected nanoparticle (Figure 1f). This observation agrees well with the fact that particles of different geometries are present at the sample surface, which interact with the incident light differently.^{15,39,40} For the NPoM configuration (Figure 1g), the dark-field spectra were more complex. The dominant scattering peaks appeared in the range of $\lambda = 750$ –950 nm, i.e., they were red-shifted compared to the single scattering peaks in the nanoparticle-on-glass configuration. The large variations in the scattering peak maxima agreed well with the literature, where it was shown before that the resonances of NPoM configurations are sensitive to the size and shape of the nanoparticle,^{15,39} the substrate underneath,^{42,43} and especially of the gap widths, and therefore the individual geometry of the formed cavity.^{8,16,35,43} The averaged spectral maximum (highlighted as the red curve with the standard deviation given as a shadow) of these scattering peaks was located at $\lambda = 804$ nm, with a shoulder at $\lambda = 874$ nm. Blue-shifted from this dominant peak, a less intense scattering peak appeared at around $\lambda = 607$ nm, whose position does not vary much from particle to particle. Notably, this scattering peak has an energy that is closer to our excitation laser of 636 nm than that of the dominant peak. Therefore, great attention has been paid to understanding the origins of these scattering peaks.

As shown in the BEM simulations of the NPoM system in Figure 2, three characteristic modes are expected in the investigated range. A perfect sphere with a diameter of 120 nm at 2 nm distance above the gold film is assumed, although we emphasize that some deviations are expected in practice due to the nanoparticle geometry variations. Scattering resonances are observed at $\lambda = 538$, 602, and 832 nm (Figure 2a) with varying electric field enhancements (Figure 2b). The resonances at 602 and 832 nm are well in line with our experimental dark-field scattering results (Figure 1g), however the peak at 538 nm cannot be resolved properly in the unpolarized dark-field scattering experiment. From a numerical evaluation of the surface charge density distributions, the nature of the modes can be clearly deduced. The dominant peak at 832 nm is attributed to a vertical dipolar mode that together with its image dipole forms a vertical dipolar gap mode (D). The mode at 602 nm can be interpreted as a lateral dipolar gap mode, forming a quadrupolar mode (Q) around the gap with its image dipole. The mode near 538 nm is composed of a quadrupolar mode in the nanosphere and its image in the gold film and is denoted as an octupolar mode (O). Previous theoretical or experimental

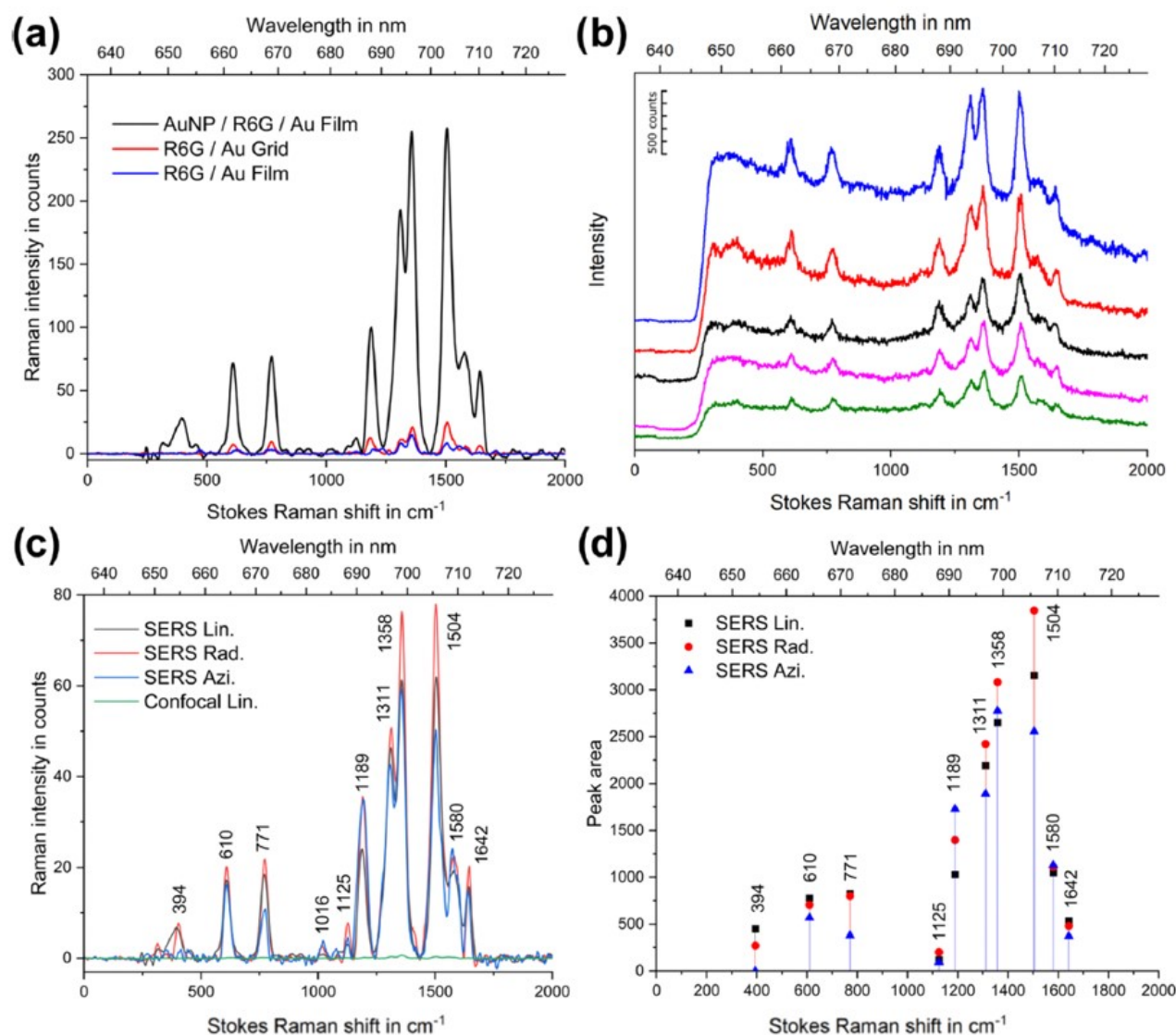


Figure 3. Surface-enhanced Raman spectra for different substrates and laser polarizations with $\lambda_{\text{ex}} = 636$ nm, $t_{\text{aq}} = 30$ s per spectrum, averaged from 10 acquisitions, and $36 \mu\text{W}$ laser power. (a) Averaged background-adjusted Raman spectra of Rhodamine 6G deposited on ultrasmooth gold (blue), rough gold (red) and in a nanoparticle-on-mirror system (black) using a radially polarized laser. (b) Representative Raman spectra of Rhodamine 6G in different nanocavities using radial polarization. Spectra are offset in y -direction for visual clarity. Strong differences in the gold photoluminescence background and Raman enhancement can be observed. (c) Averaged background-adjusted Raman spectra of Rhodamine 6G in nanoparticle-on-mirror configuration for linear (black), radial (red) and azimuthal (blue) polarization, compared to a confocal measurement without nanoparticles introduced onto the sample (green). (d) Comparison of averaged integrated peak areas for linear (black), radial (red) and azimuthal (blue) polarization. While radial polarization leads to the strongest enhancement on average, peak-specific polarization-dependencies emerge.

studies have reported similar scattering behaviors for NPoM configurations^{9,10,39} or plasmonic nanosphere dimers.^{40,44}

Following the simulation results, we assign the plasmonic modes that were experimentally observed in our dark-field spectra as follows. The red-shifted scattering peaks, e.g., the ones at around 804 nm, were due to the dipolar coupled mode^{10,45} or cavity plasmon.^{4,46} In this spectral region, most studies only report a single red-shifted mode or do not further elaborate on the individual subpeaks,^{4,9,39} although it has been suggested that it includes the contributions from both dipolar and quadrupolar modes that behave differently depending on the polarization of the excitation source,^{4,10} and the nanoparticle geometry.³⁵ Considering the fact that a plethora of nanoparticle geometries are present in our sample, and unpolarized light was used for the

dark-field scattering measurements, we attribute individual subpeaks appearing in the broad scattering continuum in the range of $\lambda = 750\text{--}950$ nm to the individual dipolar cavity mode, or a coupled dipolar mode with different multipolar modes generated in NPoMs of specific geometries.^{4,8,47} The peaks at shorter wavelengths, at around 607 nm, were attributed to the Q-mode.^{10,45} In previous work, this resonance is sometimes also referred to as transverse mode,^{35,46} or higher order cavity mode.^{45,48} This quadrupolar resonance that has good overlap with the excitation laser wavelength for the Raman experiments is of great importance for the present study. It has been only rarely used for SERS,¹⁵ and without detailed investigations of the excitation polarization influences.

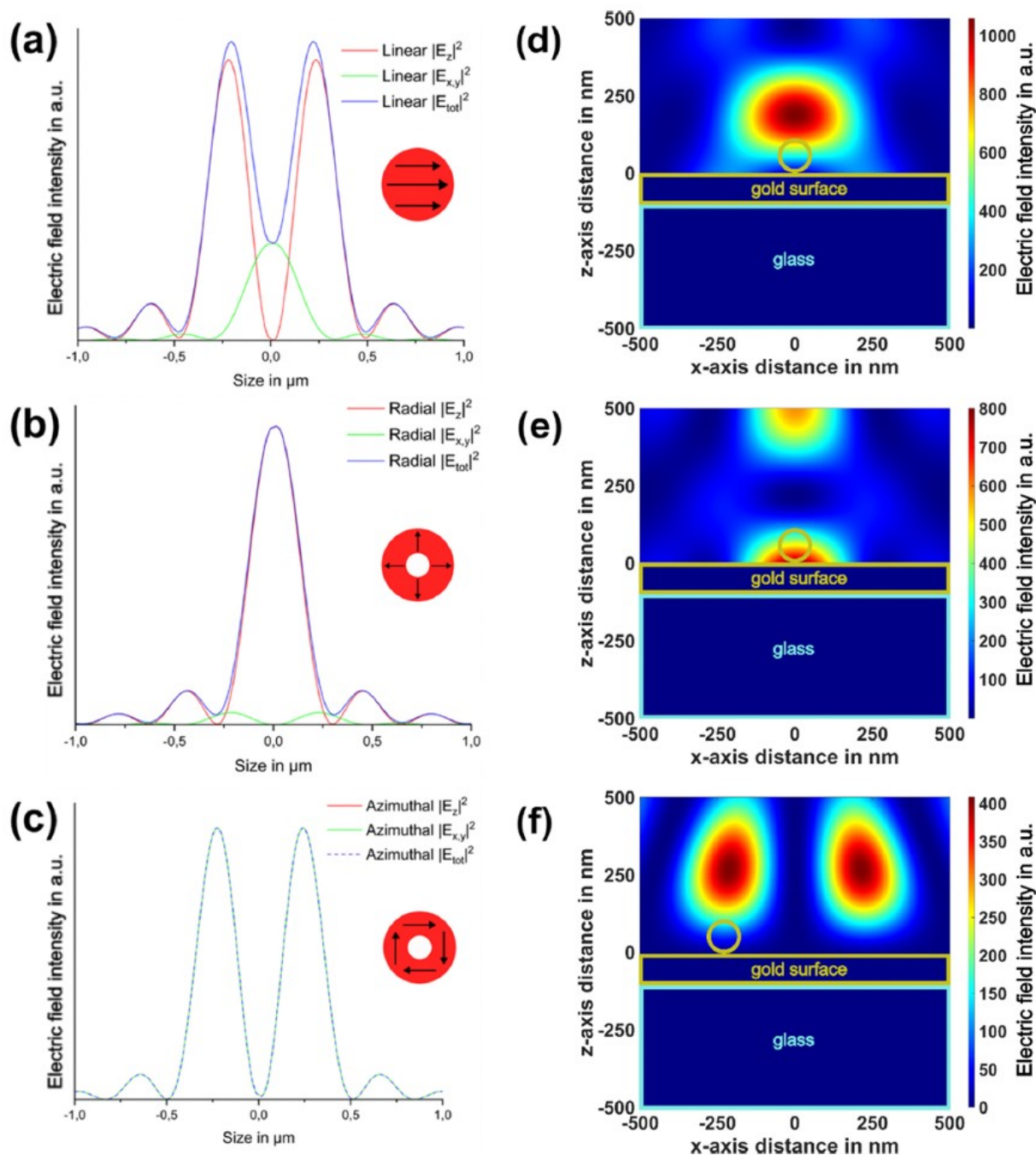
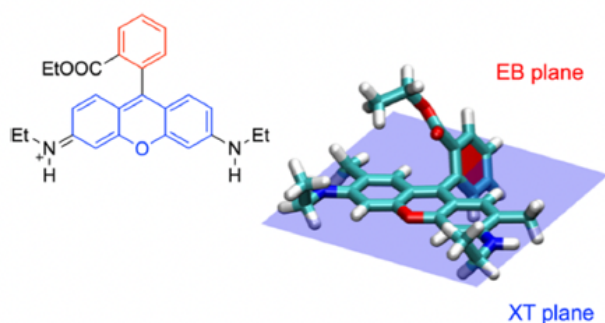


Figure 4. Calculated electric field intensities of longitudinal and transversal components as a result of interference of incident plus reflected components in the focal region of a parabolic mirror of NA = 0.9986 for a linearly, radially, and azimuthally polarized laser with $\lambda = 636$ nm above a gold surface with no nanoparticle present. The circular red insets with the black arrows indicate the polarization of the incident beam. (a) Shows that the focal field of a linearly polarized laser beam at $z = 0$ is composed of in-plane (E_{xy}) and out-of-plane (E_z) electric field components. In the focal region of the parabolic mirror on a gold surface, the field intensity of the E_z component is significantly stronger than that of the E_{xy} component. (b) Shows that the focal field of a radially polarized laser beam at $z = 0$ consists of a very strong out-of-plane electric field component (E_z) in the center of the focus, accompanied by a very weak in-plane electric field component just outside the center. (c) Shows that the focus of an azimuthally polarized laser beam at $z = 0$ is composed of strictly in-plane electric field components that constitute the total electric field in the focus. (d) Electric field intensity distribution in the x - z plane along the optical axis through the focal region of the linearly polarized laser beam. The circle sketches the gold nanosphere and shows that it is not in the maximum of the focus field. (e) Section through the focal field distribution along the optical axis showing the total electric field intensity formed by a radially polarized laser beam. The circle indicates the gold nanosphere and shows that the maximum electric field intensity can excite effectively the plasmon oscillation in the NPoM. (f) Electric field intensity distribution in a section through the focal region along the optical axis formed by an azimuthally polarized laser. The real scale inset gold nanosphere shows that the electric field intensity on the NPoM is considerably lower.

Scheme 2. Structural Formula of Rhodamine 6G^a

^aThe molecule is composed of a planar xanthene (XT) chromophore (blue, XT-plane), to which two ethylamine groups and one ethyl benzoate (EB) group are attached, and it is positively charged. In ethanolic solution, the ethylamine groups are oriented as shown here. In adsorbed geometry on gold, they face upwards out of plane, with the C–N bonds oriented in the XT-plane. The planar benzene of the ethyl benzoate group forms another plane (EB-plane), which is tilted with respect to the XT-plane.

The intensity of each resonance is strongly influenced by the orientation of the incident electric field. The Q-mode is excited for all excitation polarizations, but the D-mode is strictly related to vertically oriented electric field components and is not excited by in-plane excitation (Figure 2a). At the resonant excitation of the Q-mode, a strong electric field enhancement is observed in the sample plane for the plane wave approximation, which contains a small contribution of the O-mode (Figure 2b). It should be noted that the Q-mode, which is otherwise assumed to be nonradiative for symmetry reasons, has a significant contribution to the scattering behavior.¹⁰ This can be explained by the symmetry-breaking effect of coupling to the gold film, where part of the plasmonic excitation is transferred via surface plasmon polaritons into the film and by radiating into the far-field. We note that also due to the deviations of our nanosphere geometries from a perfect sphere, we must consider the influence of cavity symmetry breaking in our NPoM hotspots. This can lead to a quadrupolar mode that is not canceled out by destructive interference in the gap³⁹ in our individual nanocavities. The D-mode that is not resonantly excited in this work leads to a slightly higher electric field enhancement in the sample plane using the high-angle approximation (Figure 2b). Some spectral overlap with the excitation laser is still given, which suggests a notable contribution of the D-mode when using a linearly or radially polarized laser. However, experimentally the dark-field scattering spectra indicate that the Q-mode and D-mode are clearly separated (Figure 1g), hence the resonance excited in our SERS measurements appears to be predominantly the Q-mode. As the electric field distributions of the different field components for the Q-mode indicate (see Figures S2, S3), the magnitude of the E_z component appears to be 3 to 6.1 times larger than that of the x - y -component. A dominant dipolar electric field component is present along the symmetry axis of the cavity with a strong and highly confined electric field enhancement outside the gap center for p-polarized 89.5° incidence, while a zero-node is found at the gap center (Figure S2a–d, middle). For y-polarized 0° incidence, a dominant electric field component in the y - z -plane of the cavity results in a very strong electric field enhancement in a two-lobed pattern around the gap center with a zero-enhancement node directly in the center of the cavity (Figure S2a–d, left).

While this model represents the sample configuration used for the dark-field scattering experiments (no R6G added) well, it is necessary to include a dielectric spacer layer in the gap for the SERS experiments. When a R6G molecular layer is considered by a refractive index of $n = 1.9$,²⁹ a red-shift of the Q-mode resonance to $\lambda = 656$ nm is observed (Figure 2c), which matches the excitation wavelength of our laser even better compared to the case with no dielectric layer. The electric field enhancement of the Q-mode seems to be similar irrespective of 0° y-polarized, 89.5° p-polarized or 45° p-polarized incidence (Figure S3d). Although the ratios of the out-of-plane to in-plane electric field component are altered with the dielectric spacer layer included, the E_z component is still 2.3 to 4.0 times larger than the E_{xy} component, and the quadrupolar nature of the resonance remains (Figure S3a–c). It should be noted that while the Q-mode is near-resonantly excited by the incident laser at $\lambda = 636$ nm (Figure 2c) and therefore dominates the excitation enhancement, some residual spectral overlap with the D-mode is still given. From the experiments (Figure 1g), this overlap seems to be negligible. The Stokes-shifted Raman signal emitted at longer wavelengths (in the range of ~650 to 720 nm) however decreasingly overlaps with the Q- and increasingly overlaps with the D-mode, i.e., the D-mode is expected to additionally contribute to the observed enhancement of the far-field emission.

These simulations imply that although the NPoM cavity is oriented out-of-plane on the sample, high field enhancement can be achieved by both in-plane and out-of-plane excitation, delivering high signal enhancement for SERS. Next, we exploit these properties of the Q-mode by harnessing it for SERS enhancement.

3.2. Surface-Enhanced Raman Scattering. After analyzing the nature of the plasmonic modes present in our NPoM configuration, we continue with the experimental Raman measurements of R6G molecules deposited on a rough gold film, ultrasmooth gold film, and in a NPoM cavity. The weakest Raman intensity of R6G is found on the ultrasmooth gold film (blue spectrum, Figure 3a) that can be barely extracted even after acquisition times of more than 5 min. Although on the rough gold grid film (red spectrum, Figure 3a) particularly strong Raman intensities of R6G can be observed on some spots, the average intensity is significantly weaker than that from the NPoM configuration (black spectrum, Figure 3a). We emphasize that for the comparison in Figure 3a, all measured SERS spectra were averaged for the respective sample configuration. We note that only few spots on the rough gold grid film showed stronger enhancement than that from the NPoM hotspots. This observation indicates that the Q-mode is significantly involved in the excitation of the gap mode responsible for Raman scattering. Notably, as shown in Figure 3b, the level of enhancement differs from particle to particle for the NPoM configuration even with the same excitation polarization, which can be well-understood considering the spectral variability of the Q-mode resonance wavelength and strengths revealed in Figure 1g, which we attribute to varying NPoM geometries. A possible influence by varying molecular adsorption geometries in the experiments cannot be completely excluded, though the calculated most thermodynamically stable configuration of R6G is the xanthene(XT)-plane lying down on the gold surface (Figure S4). We note that all rotational orientations of the adsorbed R6G molecules within the x - y -plane are equally possible, and therefore expect an average optical response across all angles within the sample plane. We

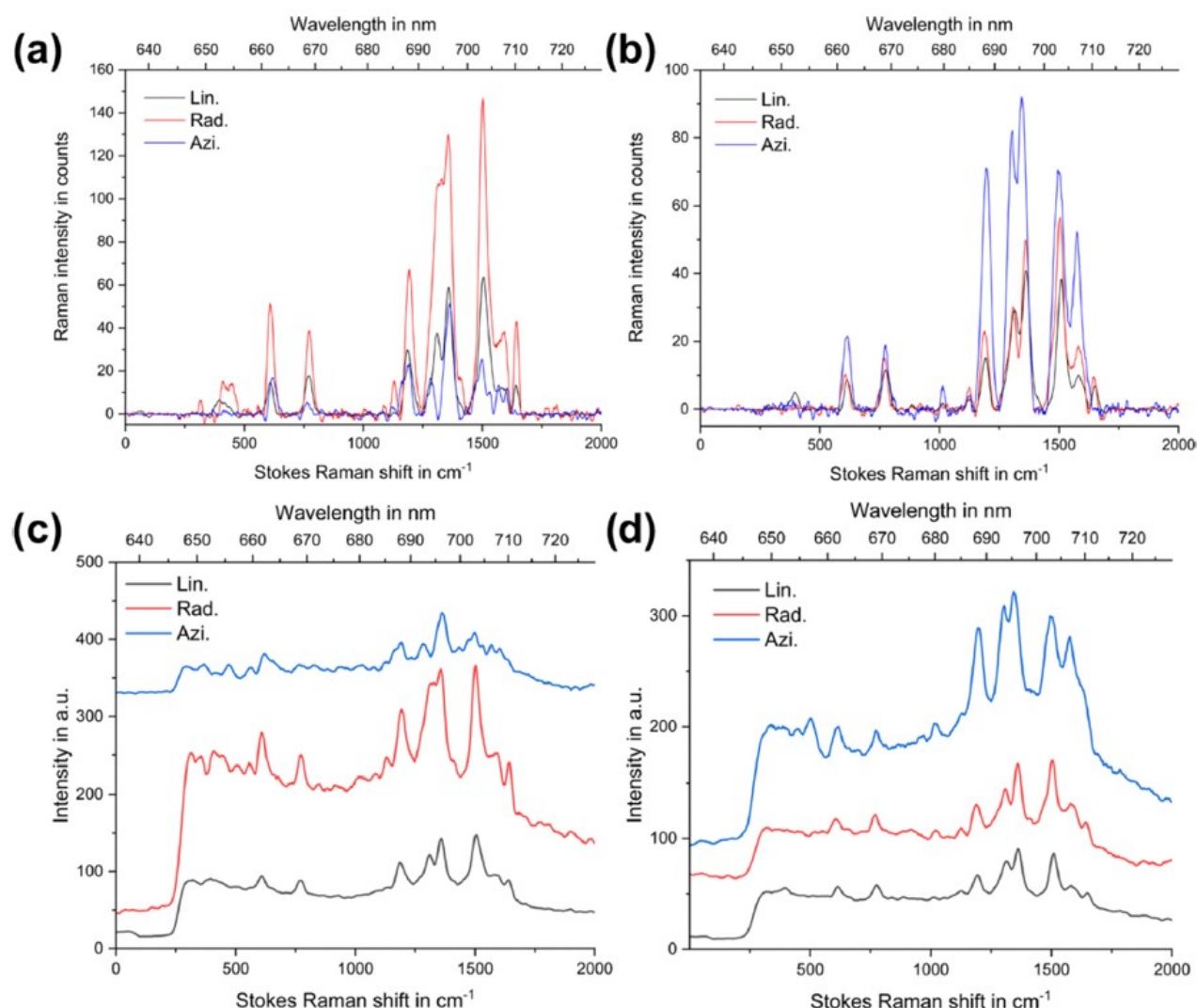


Figure 5. Polarization-dependent Raman spectra recorded on selected hotspots with $\lambda_{\text{ex}} = 636$ nm, $t_{\text{aq}} = 30$ s per spectrum, averaged from 10 acquisitions, and 36 μW laser power. (a)–(b) Averaged background-subtracted Raman spectra of Rhodamine 6G deposited in two individual nanoparticle-on-mirror configurations for linear (black), radial (red) and azimuthal (blue) polarization. Large discrepancies in the enhancement factors for different excitation polarizations are observed depending on the individual hotspot. (c)–(d) Unprocessed Raman spectra of individual hotspots shown in (a)–(b), respectively. The polarization-dependent Raman enhancement is accompanied by a polarization-dependent enhancement of the photoluminescence background.

estimate this average of statistical angular rotation in the sample plane to be similar on different nanoparticle spots, due to the considerable number of molecules per hotspot. As future work, molecular thin films with well-ordered molecular orientations could be used to clarify this point.

Since the simulated electric field distribution for the Q-mode has distinct contributions in the x – y -plane and the z -direction, we therefore continue to study the impacts of the excitation polarizations on the SERS enhancement by the Q-mode. As shown in Figure 3c, on average, excitation with a radially polarized laser (red) leads to the strongest enhancement, which is followed by that from linear polarization (black) and last the azimuthal polarization (blue). The enhancements for all three polarizations are significant as compared to the confocal reference spectrum taken from the ultrasmooth gold film (green). This shows that the used laser polarizations are all well-suited to excite the NPoM system. According to our BEM

simulations, the maximum field enhancement for all laser polarizations at $\lambda = 636$ nm (not on maximum resonance) is approximated to be similar when the dielectric R6G spacer layer is present.

To understand the excitation polarization-dependent SERS results shown in Figure 3c, we consider not only the electric field enhancement factors in the gap induced by the Q-mode, but also the electric field distributions in the focus of our parabolic mirror. The electric field distributions of a linearly, azimuthally, and radially polarized laser beam focused onto a gold surface by a high NA parabolic mirror differ significantly. The focal field of a linearly polarized laser beam (Figure 4a) consists of longitudinal (out-of-plane perpendicular to the sample plane, z -direction) and transversal (in-plane, x - and y -direction) electric field components in the focus with spatially separated intensity maxima, where the maximum intensity of the out-of-plane component at $z = 0$ in the x – y sample plane ($|E_z|^2$) is 2.9 times

stronger than the maximum intensity of the in-plane component ($|E_{xy}|^2$). For a radially polarized beam (Figure 4b), the longitudinal electric field intensity ($|E_z|^2$) is 24.6 times higher than that for the maximum transverse electric field ($|E_{xy}|^2$) at $z = 0$. The focal field intensity of an azimuthally polarized laser beam (Figure 4c) contains no longitudinal electric field components ($|E_z|^2$), it is strictly composed of a transverse electric field intensity ($|E_{xy}|^2$).⁴⁹ These distinct spatial distributions of the field intensities in the focal fields of differently polarized lasers need to be considered for the excitation of the NPoM configurations in the focus. A radially polarized laser will lead to the highest electric field intensity in the sample plane in the center of the focus (Figure 4d), followed by the linearly polarized laser (Figure 4e). For the SERS measurements with a radially or a linearly polarized laser, the NPoM was positioned in the center of the focus for excitation. The electric field intensity of the azimuthally polarized laser is zero in the center of the focus, it is instead distributed symmetrically on a ring around the optical axis. Hence, for excitation with an azimuthally polarized laser (Figure 4f), the NPoM was positioned to the side of the optical axis below the intensity maximum of the ring-shaped field distribution. Furthermore, we note that the in-plane electric field component E_{xy} has a node on the flat gold surface due to the reflection, and the longitudinal field component E_z has a maximum on the optical axis directly on the flat gold surface.

For a radially polarized laser mode, most of the total electric field intensity is concentrated on the center of the focus (Figure 4b), which is directly in the sample plane (Figure 4e). The total electric field intensity in the focal field of a linearly polarized laser is the strongest around the optical axis. Furthermore, due to the reflection of the incident electric field at the smooth gold surface, the incident and the reflected horizontal field components cancel at the gold surface and form an intensity maximum about 200 nm above the sample plane (Figure 4d). Likewise for azimuthal polarization, the total electric field intensity is distributed along the donut-shaped intensity profile (Figure 4c), and the maximum of the total electric field intensity is located approximately 250 nm above the sample plane (Figure 4f). Therefore, the far-field laser excitation field at the NPoM position is the highest for a radially polarized laser beam, followed by the linearly and then the azimuthally polarized laser beams. Considering a similar electric field enhancement $\frac{|E|}{|E_0|}$ of the Q-mode for each laser polarization, we attribute the strongest experimental SERS found for a radially polarized laser (Figure 3c, red) to the higher exciting laser beam focus field (far-field) at the NPoM position in the focus. Likewise, a weaker SERS enhancement for a linearly polarized (Figure 3c, black) and then an azimuthally polarized laser beam (Figure 3c, blue) can be attributed to their weaker focus far-field at the NPoM position in the focus, respectively.

In addition to the observed polarization-dependent total enhancement differences, we note that the enhancement for each individual Raman peak depends on the excitation polarization (Figure 3d). For polarization-dependent enhancement we consider the ratio of the out-of-plane and in-plane electric field components of the Q-mode excited at $\lambda = 636$ nm with respect to the sample plane quantified as $\frac{|E_{z,max}|}{|E_{x,max}| + |E_{y,max}|}$ for all three polarizations, including the dielectric R6G layer (see Figure S3a–c). The out-of-plane to in-plane electric field ratio differs for the computed plane wave approximations and amounts to 4.0 for 89.5° p-polarized incidence (radial), 3.0 for

45° p-polarized incidence (linear) and 2.3 for 0° y-polarized incidence (azimuthal). Although this ratio changes for the different simulated excitation polarizations, the out-of-plane electric field component is in all cases much higher than the in-plane electric field component.

The Raman peaks at 610 cm^{-1} (aromatic C–C–C in-plane bending), 771 cm^{-1} (C–C–C aromatic in-plane bending and C–C–H out-of-plane bending) and 1642 cm^{-1} (aromatic C–C stretching) experience the strongest enhancement excited by the focal field of the linearly polarized laser beam, very closely followed by the focal field distribution created by the radial polarization (with the radial polarization yielding the highest peak intensities). For the peaks at 1311 cm^{-1} (aromatic C–C stretching, C–N–H in-plane bending) and 1504 cm^{-1} (aromatic C–C stretching), radial polarization induces significantly stronger enhancement than that of the linear polarization, while azimuthal polarization leads to the weakest enhancement. For 1358 cm^{-1} (aromatic C–C stretching, C–N–H bending), radial polarization causes the strongest enhancement, followed by azimuthal and then linear polarization. At 1189 cm^{-1} (aromatic C–C in-plane stretching), azimuthal polarization causes significantly stronger enhancement than those of the radial polarization and linear polarization. The same trend is seen at 1580 cm^{-1} (aromatic C–C stretching), though less significant. To understand these specific enhancement factors for individual Raman peaks, we performed DFT calculations of the vibrational modes for an ethanolic solution of R6G and for R6G adsorbed on a smooth gold film. The comparison of the Raman calculations for the free molecule versus that on the gold surface revealed a significant dependency of frequencies and intensities on the surrounding conditions due to the modification of the local chemical environment, in particular for those modes which exhibit admixing of C–N–H and C–C–H bending motions in the amino groups (see Figure S4 for details). Furthermore, R6G shows “clusters” of Raman-active modes with very similar frequencies, but different vibrational patterns; therefore, many of the experimentally observed lines must be considered as multicomponent signals. As both factors inhibit a precise assignment for these modes, we concentrate in the analysis on modes which solely contain aromatic in-plane bending or stretching either in the XT or in the ethyl benzoate (EB) units of R6G (see Scheme 2), and, at the same time, do not show clustering.

We follow the suggested adsorption geometry of R6G onto silver and gold surfaces with the XT part face-on onto the film, both nitrogen atoms of the ethylamine groups close to the surface and the respective ethyl-groups as well as the EB group bending out of plane.^{50,51} DFT calculations allow us to correlate molecular orientations with SERS experiments,^{12,13} which we do here for the averaged spectra in Figure 3c,d. Most of the experimentally observed Raman lines represent clusters of several Raman-active modes and show complex, ambiguous nuclear displacement patterns. Notable exceptions are the vibrational modes at $\nu_1 = 394 \text{ cm}^{-1}$ and $\nu_2 = 610 \text{ cm}^{-1}$ with a significant component perpendicular to the XT-plane, and the mode at $\nu_3 = 1189 \text{ cm}^{-1}$ with exclusive nuclear displacements within the XT-plane. In fact, while ν_3 shows higher Raman intensity with the azimuthal polarization than with the radial polarization, the situation inverts for the Raman modes ν_1 and ν_2 (Figure 3c,d). This is indeed in line with the model of R6G molecules lying flat with their XT moiety on the gold surface.

A closer look at single NPoM hotspots instead of their total average reveals unique nanocavity optical responses. While

radial polarization leads to the strongest overall SERS enhancement, exactly the opposite can be observed on some individual hotspots. For few hotspots, azimuthal (Figure 5a,b) or linear polarization (Figure S5a) leads to the strongest signal enhancement, while some respond indifferently to the laser polarization (Figure S5b). Furthermore, polarization-dependent changes of the relative intensities of the R6G Raman scattering on individual hotspots can be observed, which is most evident for the vibrational modes at $\nu = 1311$ and 1580 cm^{-1} , but is not consistent for each respective polarization when comparing individual nanoparticles. Further investigations with systematically varied particle geometries will be performed to conclude the geometry-dependent SERS response. When considering the raw data without background-subtraction (Figure S5c,d), it becomes apparent that there are polarization-dependent optical responses also in the photoluminescence for individual nanocavities. These phenomena are due to the variations in nanoparticle shape, size and gap distance^{8,15,16,35,39} for individual NPoM hotspots. This has been revealed by previous dark-field-scattering measurements showing different Q-mode resonance intensities for varying NPoM configurations excited by different excitation polarizations.³⁹ Similar work was recently done for larger gold nanospheres and silver nanocubes on ultrasmooth gold with similar geometry-specific polarization susceptibilities and the excitation of particular antenna modes depending on the laser polarization.⁹ Possible chemical enhancement due to charge redistribution that occurs for R6G adsorbed on gold substrate is expected to be similar over large areas. This assumes that on an ultrasmooth gold surface, where the prevalence of lattice disorders or defects is largely negligible, the usual discrepancies of electric surface charge density distributions⁵² are not present and therefore different R6G molecules should adsorb onto the gold film uniformly, as has been suggested in a recent theoretical study for differently charged gold surfaces.⁵¹ We therefore propose that the main contribution to signal enhancement in Figure 3a is from the electromagnetic enhancement by exciting the Q-mode of the NPoM system resonantly, which is effectively possible using linear, radial and azimuthal polarizations.

4. CONCLUSIONS

Our experimental results demonstrate that the excitation of NPoMs for enhanced Raman scattering can be achieved through the resonant excitation of the multipole Q-mode. In this case, both in-plane and out-of-plane electric field components are suitable for exciting the Q-mode and enhancing the Raman scattering of R6G molecules adsorbed in the NPoM configuration. These findings can be well-explained by the mode-analysis performed by BEM simulations for NPoM configurations with and without R6G adsorbates. For all three approximated excitation polarizations (azimuthal, radial, and linear polarizations), the simulations show that the out-of-plane electric field component of the Q-mode is much higher than the in-plane electric field component. This distinct electric field distribution heavily impacts the polarization-dependent SERS enhancement at the NPoM. Particularly, individual Raman peaks are shown to be enhanced differently, which is in line with the tensor properties of the DFT-calculated R6G vibrational modes and the local field distribution. We note that while the role of the Q-mode is dominant in the SERS excitation enhancement, both Q-mode and D-mode contribute to the enhanced emission of the Stokes-shifted optical signal into the far-field. Further, the different polarization-dependent SERS

enhancement at individual NPoM hotspots highlights that the implementation of higher order plasmonic modes (i.e., Q-mode) is also sensitive to the nanoparticle shape, size and gap distance. A quick and simple change of the incident laser polarization leads to significant differences of NPoM system polarizability, which could be exploited for applications in other systems by tailoring the nanocavity response through their geometry. A further investigation with more uniform plasmonic particles to eliminate particle-specific variations and correlative microscopy to extract their exact morphology seems promising to further elucidate polarization-dependent cavity mode effects for possible applicability in other research challenges.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcc.4c03962>.

Additional experimental details for optimizing laser beam polarizations, and supplementary Raman spectra. BEM simulation results of electric field distributions of the Q-mode with and without dielectric spacer. DFT-calculation of the discussed R6G vibrational modes (PDF)

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Author Contributions

F.S. fabricated the R6G samples, performed the confocal Raman, SERS and reference measurements, processed all experimental results and wrote the manuscript. L.L. and F.S. performed the

dark-field scattering measurements. L.L. fabricated the gold cone reference sample, performed the BEM simulations, and contributed to the manuscript. L.L. and M.F. contributed significantly to discussions regarding the plasmonic configuration. L.W. performed the DFT calculations for the R6G system and contributed to the manuscript, supervised by J.G. and A.J.M. J.G. and A.J.M. obtained funding, participated in writing the manuscript and contributed significantly to the scientific discussion. D.Z. conceived the project, obtained funding, participated in writing the manuscript, coordinated the scientific discussion and provided overall direction. All authors discussed the results and commented on the manuscript.

Notes

The authors declare no competing financial interest.

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