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SURFACE-ENHANCED RAMAN SCATTERING
(SERS)-BASED DETECTION OF IONS

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“Grown-ups like numbers.

When you tell them about a new friend, they never ask questions about what really matters. They never ask: ‘What does his voice sound like?’ ‘What games does he like best?’ ‘Does he collect butterflies?’ They ask: ‘How old is he?’ ‘How many brothers does he have?’ ‘How much does he weigh?’ ‘How much money does he have?’ Only then do they think they know him.”

Antoine de Saint Exupéry, *The Little Prince*
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STRESZCZENIE

Przedmiotem niniejszej pracy jest opracowanie nowych czujników optycznych wykrywających jony, ze szczególnym uwzględnieniem czujników wykorzystujących nanostruktury metaliczne. W kręgu zainteresowań znalazły się kationy metali oraz aniony soli nieorganicznych jako cząsteczki chemiczne powszechnie występujące w środowisku w różnych rolach: od podstawowych elektrolitów w organizmach (kationy sodu, potasu, aniony chlorkowe), przez dodatki do żywności (azotany, węglany), po niepożądane zanieczyszczenia (kationy metali ciężkich, siarczany, fosforany). Ich detekcja jest istotna z punktu widzenia m.in. kontroli jakości produktów spożywczych, badania próbek środowiskowych, monitoringu odpadów przemysłowych, a także w szybkiej diagnostyce schorzeń.


W spektroskopii SERS, słabe z natury nieelastyczne rozproszenie światła – rozproszenie Ramana – ulega wzmożeniu dzięki lokalnemu wzmożeniu pola elektromagnetycznego przy powierzchni nanostruktur metalicznych. Umieszczenie
w tym obszarze cząsteczki, np. poprzez adsorpcję, skutkuje zwiększeniem intensywności jej widma ramanowskiego aż do poziomu obserwacji pojedynczych molekuł (tzw. single molecule SERS). Nanometryczne rozmiary takich czujników umożliwiają bezpośrednie dotarcie do nieosiągalnych do tej pory próbek, takich jak wnętrze komórki oraz jej poszczególne kompartmenty. Co więcej, zasięg wzmocnienia powierzchniowego w zjawisku SERS – ograniczający się do najbliższego otoczenia plazmonowej nanocząstki – daje nadzieję na przełamanie limitu dyfrakcyjnego i osiągnięcie rozdzielczości uwarunkowanej jedynie wielkością czujnika. Wszystkie te cechy, jak również optyczny charakter pracy, a zatem bezinwazyjne przekazywanie informacji o badanym środowisku, czynią nanoczujniki SERS szczególnie przydatnymi w zastosowaniach biologicznych. O ile jednak znanych jest już wiele układów opartych na spektroskopii SERS czułych na różnorakie parametry biologiczne (takie jak pH czy potencjał redoks), efektywne oznaczanie tą metodą jonów w stężeniach występujących w komórkach wciąż stanowi wyzwanie. Mimo że zagadnienie to jest pozornie zbadane – znany wszak równowagowe stężenia jonów nieorganicznych w komórkach – rozkład ich stężenia wewnątrz komórki, mechanizmy ich transportu oraz akumulacji w organellach są w dalszym ciągu obiektom badań. Opracowanie sensorów tego typu przyczyni się do rozwoju metod analitycznych i stworzy nowe możliwości w zakresie detekcji jonów, a co za tym idzie, zaburzeń ich gospodarki w organizmie i schorzeń nimi wywołanych.

Należy podkreślić, że detekcja kationów metali przy pomocy spektroskopii oscylacyjnej musi odbywać się w sposób pośredni. Jony te, jako jednoatomowe indywidualne chemiczne, nie posiadają żadnych modów drgań, są więc niewidoczne dla spektroskopii Ramana lub spektroskopii absorpcyjnej w podczerwieni. Obserwuje się natomiast wpływ jona na widmo cząsteczki reportera. Zmiany te dotyczą całkowitej intensywności widma, względnej intensywności pasm bądź położenia wybranych pasm. Opisany w pracy nanoczujnik na kationy działa w oparciu o widmo SERS anionu 2-merkaptoetanosulfonowego (MES) zaadsorbowanego na nanocząstkach srebra (Ag-MES). Pasmami markerowymi, czyli tymi, które służą do oznaczenia analitu, są dwie składowe przypisane
drganiami symetrycznym rozciągającym grup sulfonowych. Pasmo o niższej energii odpowiada drganom grup SO$_3^-$ otoczonych cząsteczkami rozpuszczalnika (pasmo 1), zaś druga składowa pochodzi od pasma drgań grup sulfonowych tworzących kontaktową parę jonową z kationem (pasmo 2). Położenie pasma 2 zależy od rodzaju oddziaływującego jonu – pozwala zatem rozpoznać kation – a jego intensywność względem pasma 1 wyznacza stężenie jonu. Położenie pasma 2 dla kationów bez silnej otoczkę hydratacyjnej zależy od energii ich wiązania elektrostatycznego z grupą sulfonową, zatem od powierzchniowej gęstości ładunku jonu. Opracowany nanosensor jest czuły na zmiany stężeń wszystkich badanych kationów (Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$, Fe$^{2+}$, Mn$^{2+}$, Co$^{2+}$), a dodatkowo rozwija najpierw cztery pierwsze z wymienionych kationów metali alkalicznych oraz metali ziem alkalicznych. Najniższy limit detekcji nanoczujnik wykazuje dla jonów wapnia: $10^{-8}$ M. Ponadto możliwe jest przy jego użyciu współznazczenie jonów magnezu i wapnia.

Test cytotoksyczności czujnika Ag-MES przeprowadzony na dwu liniach komórkowych: HeLa oraz CHO.K1 dowodzi, że w stężeniach poniżej 10 µg/ml jest on nietoksyczny dla komórek inkubowanych w medium komórkowym z nanosensorem przez 24 i 48 godzin. Na komórkach, które wchłonęły nanocząstki Ag-MES, można przeprowadzić mapowanie SERS, tj. rejestrowanie widm SERS z ustalonej siatki punktów na próbce. Na podstawie zebranych widm wyznacza się rozkład wewnątrzkomórkowego stężenie jonów, w tym przypadku potasu. Stężenia K$^+$ występujące w środku komórki przewyższają te w rejonach bliskich błonie komórkowej. Taki rozkład wynika z równowagi jonowej między wnętrzem i otoczeniem komórki oraz dynamiki zmian stężenia jonów wewnątrz komórek. Czujniki są wchłaniane przez komórkę na drodze endocytozy: umieszczane są wraz z medium w pęcherzykach zawieszonych w cytoplazmie – endosomach. Początkowo są zatem wystawione na kationy potasu w stężeniu charakterystycznym dla medium ([K$^+$]$_{medium}$ = 5 mM). Endosomy te przebywają w pobliżu błony komórkowej. W miarę upływu czasu dochodzi do dyfuzji endosomów we wnętrzu komórki, w trakcie której na drodze wymiany jonowej stężenie jonów w endosomach sięga wartości identycznych z tymi w cytoplazmie.
([K⁺]_{intracellular} = 150 \text{ mM}). Ten prosty eksperyment wykazuje, że opracowany nanoczujnik Ag-MES znajduje zastosowanie jako wewnątrzkomórkowy czujnik na kationy.

W pracy ujęte są także badania nad nanoczujnikami Ag-MES pokrytymi otoczką z krzemionki (Ag-MES@SiO₂). Taka warstwa stanowi barierę ochronną tak dla komórki – chroni biomolekuły przed dezaktywacją na powierzchni srebra oraz ogranicza dyfuzję jonów srebra do cytoplazmy – jak i dla czujnika: ogranicza adsorpcję cząsteczek na nanocząstekach metalu, co mogłoby zaburzyć sygnał rejestrowany od czujnika. Nanocząstki Ag-MES skutecznie pokryte 30-nanometrową otoczką SiO₂ wykazują aktywność w spektroskopii SERS. Zachowują także czułość na kationy. Gorszy limit detekcji jest rekompensowany podwyższeniem czułości: zmiana stężenia jonu o jeden rząd wielkości wiąże się z większą zmianą sygnału czujnika. Różnice między odpowiedziami analitycznymi czujników Ag-MES i Ag-MES@SiO₂ wywołane są różnym stopniem penetracji jonów przez warstwę krzemionki. Stężenie jonów docierających do metalowego rdzenia, gdzie następuje ich detekcja, jest mniejsze niż stężenie na zewnątrz otoczki w roztworze i jest tym większe, im łatwiej danym jonom pokonać barierę SiO₂. Zdolność penetracji SiO₂ zależy więc od wielkości jonu, jego energii solwatacji oraz czasu.

Przedstawiam także badania nad wytwarzaniem wysokowydajnych biokompatybilnych nanopodłoży SERS – nanogwiazdek złota pokrytych srebrem w otoczce z mezoporowatej krzemionki oraz magnetycznych nanocząstek janusowych: złota nanogwiazdka-nanocząstka Fe₃O₄ pokrytych srebrem. Złoto w porównaniu do srebra charakteryzuje się mniejszą toksycznością dla układów biologicznych. Niestety, nanostruktury złota wzmacniają rozproszenie ramanowskie w mniejszym stopniu niż nanocząstki srebra. Stąd też stosuję rozwiązanie polegające na pokryciu złotych nanogwiazdek cienką warstwą srebra, która nie zmienia znacząco toksyczności syntezowanych nanocząstek, a wpływa pozytywnie na wzmocnienie widma SERS.
Kolejnym problemem podjętym w pracy jest opracowanie sensora anionów wykorzystującego widma oscylacyjne SERS i IR. Jako receptor anionów stosuję pochodne 1,8-diamido-3,6-dichlorokarbazolu — związku, który od lat z powodzeniem jest przedmiotem badań grupy dr. Michała Chmielewskiego. Trzy atomy azotu we wnęce cząsteczki karbazolu są zaangażowane w tworzenie wiązań wodorowych z anionami. Uprzednio wykazane zdolności kompleksowania anionów siarczanowych (VI) w roztworze są zestawione z przeprowadzonymi przeze mnie badaniami nad receptorami karbazolowymi osadzonymi na metallicznym podłożu. Adsorpcja na powierzchni srebra i złota odbywa się dzięki funkcjonalizacji cząsteczki 1,8-diamido-3,6-dichlorokarbazolu poprzez wiązanie amidowe resztami kwasu liponowego zawierającego mostek disiarczkowy. Obecność atomów siarki pozwala na wytworzenie wiązania kowałencyjnego siarka-metal (Ag lub Au). Pochodne ujęte w projekcie różnią się modyfikacją przez drugie wiązanie amidowe: grupą t-butyłową, fenylową lub ponownie — resztą kwasu liponowego. Badania z wykorzystaniem spektroskopii SERS oraz absorbpcyjnej spektroskopii zewnętrznego odbicia w podczerwieni z modulacją polaryzacji (ang. polarization modulation infrared reflection absorption spectroscopy, PM-IRRAS) dowodzą, że receptory karbazolowe po adsorpcji na powierzchni zachowują zdolność wiązania jonów SO₄²⁻ z roztworów acetonitrylowych. Ponadto, obserwuje się różnice w powinowactwie badanych pochodnych względem anionów, które rośnie w kolejności: pochodna fenylowa < pochodna z resztą kwasu liponowego < pochodna t-butyłową.

Przedstawione badania prezentują możliwości zastosowania technik opartych na spektroskopii oscylacyjnej w zakresie detekcji jonów nieorganicznych. Optyczna droga przekazywania informacji decyduje o ich znikomej inwazyjności, a sprzężenie z nanostrukturami otwiera perspektywy dotarcia do wnętrza komórek i przyjrzenia się procesom tam zachodzącym z czułością sięgającą pojedynczych molekuł.

Rozprawa napisana jest w języku angielskim. Wyniki zostały opublikowane w dwóch czasopismach o zasięgu międzynarodowym: Sensors and Actuators B:
Chemical oraz Journal of Physical Chemistry C, a także w rozdziale w książce Optical Spectroscopy and Computational Methods In Biology and Medicine, wyd. Springer Netherlands. Kolejna oryginalna publikacja jest w zaawansowanej fazie przygotowań.
ABSTRACT

In this thesis, possibility of ion detection by means of surface vibrational spectroscopy techniques with emphasis on surface-enhanced Raman scattering (SERS) spectroscopy is addressed. Functionalization of metal nanoparticles (NPs) with 2-mercaptoethanesulphonate anion (MES), a reporter molecule with a cation-sensitive SERS spectrum, results in fabrication of a new, efficient cation nanosensor (Ag-MES). It can differentiate between various alkali and alkaline earth metal cations, which makes co-detection of ions possible. The lowest limit of detection reported here is achieved for calcium ions: \(10^{-8}\) M. Monitoring of cation concentration distribution inside living cells is demonstrated with use of the designed nanosensor. After encapsulation of the cation sensors in a thick silica shell (Ag-MES@SiO\(_2\)), nanoprobes of good temporal stability of the SERS signal are obtained. At the same time, the protective SiO\(_2\) layer does not hamper the sensing properties of the system. Synthesis of new multi-component plasmonic nanoparticles (silver-coated gold nanostars encapsulated in mesoporous silica and silver-coated gold nanoparticle-iron oxide Janus nanoparticles) with potential biological applications is also described. Furthermore, as anion receptors, a new class of diamidocarbazole derivatives adsorbed on metal substrates is used. Alongside SERS spectroscopy, PM-IRRAS measurements are employed to confirm binding properties of these compounds towards sulphate anions. Presented results enrich the scope of optical ion sensors described so far in the literature, in terms of both: i) alkali and alkaline earth metal cation and ii) inorganic anion detection by means of surface vibrational spectroscopy techniques. Due to the use of nanostructures, applicability of the described sensors is widened, as the detection can be performed directly and without damage in samples as demanding as living cells.
INTRODUCTION

The aim of this thesis is to obtain new optical sensors for ions, especially sensors using metallic nanostructures. Metal cations and inorganic anions were selected as the analytes, since they are widespread chemical species. They play various roles in our surroundings: as fundamental electrolytes in bodies of living organisms (e.g. sodium or potassium cations, chloride anions), food additives (nitrates, carbonates) and even pollutants (heavy metal cations, sulphates, phosphates). Detection of ions is essential in, just to name a few: quality control of food products, testing environmental samples, industrial waste monitoring, as well as rapid detection of diseases.

Due to the presence of ion in so many areas, lots of effort is put into development of diverse sensors. Apart from well-known electrochemical sensors, optical sensors gain popularity. In principle, information about the environment is in this case carried by a light beam. The analyte has an influence on interactions between the light and the reporter molecule (a molecule sensitive to the presence of a detected substance). Fluorescence spectroscopy, UV-Vis absorption spectroscopy and techniques based on oscillation spectroscopy are the main techniques employed for the optical detection. We live in a world driven by miniaturization: it comes as no surprise that analytical methods reach out to nanotechnology. This trend stimulates development of nanosensors based on surface enhanced Raman scattering (SERS). In SERS spectroscopy, intrinsically weak inelastic light scattering (Raman scattering) – is enhanced due to a giant increase of electromagnetic field intensity near the surface of metallic nanostructures. When placed in this area, a molecule is exposed to electric field of extremely high amplitude, which gives rise to a strong SERS signal – with sensitivity reaching down to a single molecule (so called single molecule SERS). More details on SERS spectroscopy, as well as PM-IRRAS spectroscopy, which accompanied SERS in research on anion detection, can be found in Chapter 1.
Nanometric size of such systems lets the researchers reach such unattainable places as the inside of a cell and its compartments. What is more, confinement of the SERS phenomenon to the direct vicinity of a metal nanoparticle gives promise to obtaining resolution much higher than diffraction limit, as it would be determined solely by the size of the nanoparticle (NP) itself. All these features, as well as optical character of work and, consequently, non-invasive way of passing information about the surroundings, make such sensors particularly useful in biological applications. However, while there are many known systems based on SERS spectroscopy which are sensitive to such biological parameters as pH value or redox potential, efficient ion determination in live cells with SERS still poses a challenge. Although this issue has seemingly been examined: equilibrium concentrations of inorganic ions are already commonly known, their distribution inside the cell, transport and accumulation mechanisms inside organelles have yet to be elucidated. Research on fabrication of such sensors will contribute to development of analytical methods already in use, as well as will open up new perspectives for detection of ions, and in consequence, of disorders in their metabolism paths as well as diseases caused by them. Chapter 2 contains current state of the art in the subject of optical nanosensors.

It is worth pointing out that metal cations, as one-atom species, are undetectable by vibrational spectroscopies. As they have no vibrational fingerprint, their detection must be made indirectly by observation of the impact they have on the signal of a reporter molecule. Changes may concern total intensities of the spectrum, relative intensities of some bands or the energy shift of bands. In the thesis, 2-mercaptoethanesulphonate anion (MES) is selected as a Raman reporter molecule. Two components of the $\text{SO}_3^-$ symmetric stretching vibrations are chosen to be marker bands – bands, whose changes are related to the presence of the analyte. Their intensity ratio is used to determine a cation concentration, while the energy shift of one of these two bands makes differentiation between cations possible. Chapter 3 presents the effort to use MES molecules adsorbed on silver nanoparticles as Ag-MES nanosensor. Its sensitivity towards heavy metal cations and more biologically relevant cations of alkali and alkaline earth metals
In Chapter 4, tests of performance of the Ag-MES nanosensor in biological systems can be found. Cells incubated in a cell medium containing the nanoprobe are proved to absorb Ag-MES NPs and the distribution of internal potassium concentration is deduced based on SERS mapping. Well studied system of Ag-MES NPs was employed in the further research on permeation of cations through a thick silica shell. Discussion on these results collected for silica covered Ag-MES nanosensors: Ag-MES@SiO$_2$ is given in Chapter 5. Synthesis of other types of biocompatible nanoparticles based on gold nanostars: i) Janus nanoparticles of gold nanostars and Fe$_3$O$_4$ and ii) gold nanostars encapsulated in mesoporous silica – both types additionally covered with a thin layer of silver – are included in Chapter 6. In case of Janus NPs, the effect of magnetic field on the intensity of SERS spectra is examined. Chapter 7 refers to anion detection with use of brand new type of carbazole derivatives deposited on metal surfaces. Surface-attached diamidocarbazoles are proved to be able to bind sulphates from the solution.

The scope of the thesis has already been published in two original articles (Sensors and Actuators B: Chemical and Journal of Physical Chemistry C) and one chapter in a book (Optical Spectroscopy and Computational Methods in Biology and Medicine, publ. Springer Netherlands); another article is being prepared for a prompt submission.
THEORETICAL SECTION
1 Surface vibrational spectroscopic techniques

SERS, the most important abbreviation in this PhD thesis, stands for Surface Enhanced Raman Scattering. Since it is the main experimental technique developed in the group I have been working in, improving my skills in this kind of spectroscopy and acquiring new ones was my main focus throughout all 5 years of my work as a PhD student. In the following chapter, I will try to explain where the enhancement effect in SERS spectroscopy originates from. Further in the dissertation, you will read an overview of applications where SERS spectroscopy was found to be useful as a sensing technique.

As another technique that I relied on during the anion detection studies was Polarization Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS), I incorporated a brief description of this technique as well. Both SERS and PM-IRRAS are surface vibrational spectroscopies, extremely potent in characterization of molecular layers (down to a monolayer) deposited on a metal surface. Even though origins of these techniques are different (SERS – Raman spectroscopy enhanced by plasmonic properties of the substrate, PM-IRRAS – infrared spectroscopy exploiting behaviour of polarized IR light upon reflection), information collected with both of them complemented each other and extended detection possibilities in the studied systems.

1.1 Surface enhanced Raman scattering

1.1.1 Raman scattering

The day I am writing this chapter, three US-based scientists: Rainer Weiss, Barry Clark Barish and Kip Stephen Thorne were announced as the Nobel Prize laureates in physics. Their contribution to the LIGO (Laser Interferometer Gravitational-Wave Observatory) experiment with the aim of detecting gravitational waves was appreciated. Despite being predicted in 1916 by Albert Einstein, first observations of gravitational waves – ripples in space-time left by merging of extremely heavy objects like black holes – were reported just two years ago, 14 September 2015 and only four more observations have been
confirmed so far.\textsuperscript{5–8} Detection of such an enigmatic effect as gravitational waves gives rise to a new branch of astronomy and brings hope for exploring the nature of the universe hidden in the yet unseen structure of space-time.

If we went 87 years back to 1930, we would witness another ground-breaking discovery of these times being awarded a Nobel Prize in physics – inelastic scattering of light. The laureate, Sir Chandrasekhara Venkata Raman\textsuperscript{*}, started contemplating on the origin of the blue colour of the Mediterranean Sea during one of his long cruises to Europe.\textsuperscript{9} This was the incentive to start the research on light scattering. His works were summed up in 1928 in the article \textit{A New Radiation} published in Indian Journal of Physics (journal he founded himself in Kolkata).\textsuperscript{10} In the paper, he described a new kind of light scattering measured by his student Kariamanickam Srinivasa Krishnan on ca. 80 different substances.\textsuperscript{11} The discovery demanded a set-up that might seem exotic for us, people working in well-equipped laboratories of 21\textsuperscript{st} century: to provide light, the most powerful and generally available light source was used, namely the Sun. The optics were made up by a telescope and a pair of complimentary filters. A bulb purified by vacuum distillation constituted what we would call a cuvette today. Previously considered as a kind of fluorescence (a phenomenon that is a nightmare for Raman spectroscopists to these days), it was identified as a separate effect due to its feebleness and polarization behaviour consistent with that of the ordinary, elastic (Rayleigh) scattering. The described effect was recognized as an optical analogue to the Compton effect (inelastic scattering of X-rays by electrons). The discovery was quickly acclaimed and called the Raman effect, regardless of the simultaneous reports on the same effect by two Russian scientists, Grigory Landsberg and Leonid Mandelstam.\textsuperscript{12} Just like the LIGO leaders awarded this year, Raman was waiting two years since the observation of the effect for the Nobel Prize in physics.

\textsuperscript{*} Names of Raman’s parents were Chandrasekhara Iyer (father) and Parvati Ammal (mother),\textsuperscript{9} which I mention to point out that Raman was actually not the surname but the given name of the scientist. This goes along with a South Indian tradition of adopting father’s name followed by your own name to form a full name of a person. Now imagine James’ (Maxwell’s) equations or Max-Julius (Born-Oppenheimer) approximation…
Raman scattering is an inelastic scattering of light by molecules, which means that scattered light has a different energy than the incident beam. The energy shift is independent of the initial radiation energy; it is, however, characteristic for each molecule. The energy transfer can occur between the light and rotational or vibrational states of the molecule. In this work, only vibrational Raman effect was used (rotational Raman effect can be observed only in gases so it is irrelevant to the project). Upon illumination with the electromagnetic wave of frequency $\nu_0$, the electron cloud of the molecule starts to oscillate with the same frequency. Consequently, as an oscillating electric dipole, the molecule is the source of an electromagnetic wave of the same frequency $\nu_0$ (Rayleigh scattering). In addition to that, a small portion of the scattered light exhibits a frequency modified with the frequencies of the molecular oscillations: $\nu_0 - \nu_{osc}$ and $\nu_0 + \nu_{osc}$ (Raman scattering: Stokes and anti-Stokes spectra, respectively). Full description of the phenomenon was developed by a Czech physicist, Georg Placzek. Placzek’s polarizability theory can be found in every course book on fundamentals of molecular spectroscopy alongside a quantum explanation of the Raman scattering, where the incident photon is annihilated and the scattered photon of different energy is created, resulting in vibrational excitation of the molecule.

It is worth pointing out that Raman spectroscopy is a kind of spectroscopy where we excite the spectrum with a beam of light of the energy that does not fit the molecular transition we observe. Information we receive relates to vibrational states of the molecule (which corresponds to the infrared spectrum of electromagnetic waves), but the light source is characterized with higher energies, ranging from the visible up to ultraviolet region. The biggest experimental obstacle is the low intensity of the Raman scattering. Most of the light illuminating the sample is scattered elastically. At the same time, intensity of the Raman scattering is approximately 3 orders of magnitude lower than the Rayleigh scattering\textsuperscript{13} and even 7-8 orders of magnitude lower than the incident light.\textsuperscript{14,15} In order to observe intrinsically pretty faint scattering, powerful light sources are required. First observations involved the Sun or gas-discharge lamps and the spectra
were collected on photographic films. Development of sensitive detectors (photomultipliers in the 40s and a charge coupled device in the 60s) alongside the appearance of lasers in the 60s vastly contributed to the revival of Raman spectroscopy. Not only did they increase the probability of inelastic scattering, but also improved the signal to noise ratio. They stimulated the development of new Raman-based techniques, especially non-linear Raman effects, such as hyper Raman scattering (HRS), stimulated Raman scattering (SRS) or coherent anti-Stokes Raman spectroscopy (CARS). Other techniques, as resonance Raman spectroscopy (RR) and surface enhanced Raman scattering spectroscopy (SERS) benefited from the introduction of lasers as well. Coupling Raman spectrometers with microscopes, especially confocal microscopes, was another milestone in the history of Raman techniques. That opened a broad field of Raman-based mapping and imaging, incredibly useful in analyzing content of heterogeneous samples with high spatial resolution. More recently, tip-enhanced Raman spectroscopy (TERS) combined advantages of Raman microscopy, SERS spectroscopy and AFM imaging to go beyond the diffraction limit in sample characterization.
1.1.2 Mechanisms of SERS

As it has already been stated earlier, Raman scattering is a weak effect – only 1 in $10^7$ scattered photons experience energy change during the process. Many of the Raman-based techniques aim at increasing the Raman signal. In SERS, presence of a molecule in the close vicinity of a plasmonic nanostructure is the key to observe the intensity gain. Interactions between incident light, the molecule and nanostructured metal substrate lead to an increase of sensitivity for molecular detection. Precise description of the enhancement origins is still a hot topic; as a phenomenon on the verge of physics and chemistry, it requires to join forces between various fields of science in order to explain the nature of SERS. Originally, experimental facts suggested that there were at least two different mechanisms responsible for the enhancement. Long-lasting debate reached a consensus about two main mechanisms of SERS: electromagnetic and chemical. Electromagnetic contribution is the dominating one, while the chemical effect is a minor component, especially pronounced in conjugated systems or systems with lone electron pairs.

Chemical theory elucidates SERS mechanism based on charge transfer (CT) between the Fermi level of the metal and electronic levels of the molecule (Figure 1). It can occur in both directions: from the highest occupied molecular orbital to metal and from the Fermi level of the metal to the lowest unoccupied orbital in the molecule. This way, electronic transition is facilitated by CT through vibronic coupling of molecular and metal energy levels. In this description, only real energy levels are involved. When the intermediate state in the scattering process is not a virtual but a real state, probability of the phenomenon increases, which is the basis of the resonance Raman effect, where incident line energy is tuned to electronic transitions in the molecule. This way, chemical mechanism of the SERS enhancement can be considered a type of resonance Raman scattering.\textsuperscript{16-19} Chemical effect is especially pronounced in the systems with lone pairs of electrons like aromatic ring, where coupling between molecular and metal states is the strongest.
Figure 1 Schematic presentation of the charge transfer theory, where the charge transfer happens a) between the Fermi level $E_F$ and a level created from an excited electronic level of the molecule and unoccupied levels from the conduction band of a metal $E_{CT}$; b) between the level of the adsorbed molecule $E_M$ and the Fermi level of the metal $E_F$.

Chemical theory treats the metal substrate only as a charge transfer intermediate that helps in efficient electronic transitions between states. Electromagnetic mechanism presents metal nanostructures as the essential part of the system and the source of the enhancement. When we start to explore the structure of metal and its interactions with electromagnetic waves, we need to make a distinction between effects connected with nuclei and with electrons. Such a separation is possible thanks to different scale of motion of these objects: excited nuclei move in a much slower pace than free carriers. Quantization of the vibration energies is made with help of different types of quasiparticles. Phonons represent excitation modes of a periodic lattice of nuclei. One kind of phonons – optical phonons – can couple with electromagnetic radiation. In fact, these are the lattice vibration that can be observed in Raman spectroscopy of the solid. However, they do not contribute to the SERS mechanism. In order to explain the enhancement, we should get acquainted with plasmons, associated with collective oscillations of the free electron plasma. The term was coined by Pines in 1956 in the review article where he writes:

“(…) The valence electron collective oscillations resemble closely the electronic plasma oscillations observed in gaseous discharges. We introduce
the term "plasmon" to describe the quantum of elementary excitation associated with this high-frequency collective motion (…)

Colloquially, what we tend to call plasmons are not quanta of excitation but the collective motions of the electron cloud itself. Plasmons localized at the metal surface are distinctive enough to separate them in a new type: surface plasmons. These longitudinal waves of charge density propagate along a metal-dielectric interface. They are excited only by transverse electromagnetic waves from the UV or visible range (depending on a metal) and, as such, they are less energetic than bulk plasmons. After interaction with light in resonance, an evanescent electromagnetic wave is generated. Its intensity both inside and – what is more important for SERS – outside the metal decreases exponentially with the distance from the interface. Additionally, when surface plasmons are confined in a metal nanostructure, we talk about localized surface plasmons and giant enhancement of electromagnetic field near the metal surface is observed. Molecules situated in the vicinity of the metal nanostructure are exposed to electric field of intensity exceeding the field from the incident beam. What is more, the light scattered by the molecule is enhanced by the same effect as well. This is how the origin of the enhancement in surface enhanced Raman scattering is explained. Since the effect depends strongly on the electric field intensity – it is proportional to $E^4$ – electromagnetic enhancement factors are high and estimated to reach up to $10^{11}$.

Metals have different plasmonic properties. If we approximate a nanostructure with a sphere smaller than the incident light wavelength, cross section for scattering is given with:

$$g = \frac{\varepsilon(\sigma) - \varepsilon_M}{\varepsilon(\sigma) + 2\varepsilon_M}$$

where $\varepsilon(\sigma)$ is a dielectric function of a metal which describes the response of the material to electric field of frequency $\sigma$ and $\varepsilon_M$ – relative permittivity of the surroundings (equals 1 for vacuum). Resonance is reached when $g$ has its singularity, i.e. $\varepsilon(\sigma) + 2\varepsilon_M \approx 0$. Since dielectric function is a complex-valued
function, it means that conditions for resonance in the resonant frequency $\omega_0$ based on real $Re[\varepsilon(\omega)]$ and imaginary $Im[\varepsilon(\omega)]$ parts of the dielectric function are as follows:

$$Re[\varepsilon(\omega_0)] \equiv -2\varepsilon_M$$

and

$$Im[\varepsilon(\omega_0)] \equiv 0.$$ 

$Re[\varepsilon(\omega)]$ and $Im[\varepsilon(\omega)]$ for different metals are plotted in Figure 2. If we want to select metals applicable as substrates in SERS spectroscopy, we should focus on near UV, visible and near infrared regions. Imaginary part of $\varepsilon(\omega)$ for palladium and platinum in the whole discussed range and aluminium for the visible and infrared wavelengths is too high, which theoretically makes these metals useless in SERS; however, there are works which show enhancement on substrates made of transition metals, including Pt and Pd.\textsuperscript{22–26} Of course, presented considerations are approximated and corrections need to be introduced to describe the problem more accurately. Also, according to the data, only excitations with the light of $\lambda_{\text{exc}}$ above ~600 nm are efficient for gold and copper. Optical properties of silver are the most favourable in the whole range of visible and near infrared wavelengths.

Indeed, these three metals: silver, gold and copper are utilized the most in SERS experiments. First reports on SERS spectra in 1974 by Fleischmann and co-workers involved a silver electrode.\textsuperscript{27} In the case of metal electrodes, nanostructures on the surface are formed in the oxidation-reduction cycle. For chemists, the simplest way to obtain metal nanostructures is, however, the synthesis of metal nanoparticles. After reducing a metal salt, a dispersion of metal nanoparticles is obtained. More advanced syntheses lead to fabrication of nanoparticles (NPs) of anisotropic shapes, core-shell NPs, where the one material is covered with a layer of another one, important e.g. in terms of its catalytic properties, Janus and patchy NPs – if their morphology consists of two or more separate phases, NPs with various functionalizations, controlled oligomers of NPs etc. Another possibility is to lithographically modify a metal surface, which
results in highly controllable periodic patterns. Tip enhanced Raman scattering (TERS) spectroscopy uses a tip of an STM or AFM probe made of a plasmonic material to enhance a Raman spectrum. It makes coupling of chemical and morphological characterization of the sample possible.

Enhancement of electric field is especially pronounced on the edges and tips of plasmonic nanostructures; gaps between closely situated NPs also exhibit higher than average electric fields. These characteristic areas are called hot spots and most of the collected SERS signal comes from them. In general: electromagnetic enhancement is not only an intrinsic feature of the NP material, but also depends

![Figure 2 Plots of the real part (top) and imaginary part (bottom) of dielectric function versus wavelength of the incident light for selected metals.](image_url)
on geometry of the system: shape and size of a NP, localization with respect to other NPs, adsorption sites of molecules on the surface and many others aspects.

Constant improvement of enhancement factors due to e.g. controlled nanoengineering of metal nanostructures resulted in a new branch of spectroscopy: single molecule SERS.\textsuperscript{29-32} Sensitivity reaching a single molecule opens new possibilities in studying physicochemical processes regarding molecules, as well as the mechanism of SERS enhancement itself.
1.2 Polarization Modulation-Infrared Reflection Absorption Spectroscopy

Another vibrational spectroscopy that was found to be useful in characterization of molecular layers on metal surfaces was Polarization Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS). In this technique, behaviour of an infrared (IR) beam at a smooth metal substrate is taken into account. Molecules at the interface are excited by IR light which is reflected by the metal. The thing is, IR light interacts not only with the molecules on the surface, but also with the medium (water, air). In order to minimize the contribution of the surroundings in the spectrum, changes of polarization of the light upon reflection must be considered. Two polarizations in relation to the plane of incidence are distinguished: if the electric field vector is oriented perpendicularly to the plane of incidence, the light is s-polarized. P-polarization means that vector $\vec{E}$ of electromagnetic wave is parallel to the plane of incidence. They act differently upon reflection by the surface, which determines their interaction with the molecular coating. S-polarized light reverses its phase. As a result, incident electric field ($E_i$) and reflected electric field ($E_r$) cancel each other out. On the other hand, component of the electric field parallel to the plane of incidence (p-polarized light) is enhanced upon reflection (Figure 3). The most beneficial conditions occur when the angle of incidence is close, but not equal, to the grazing angle. This way, only p-polarized beam is able to excite IR spectra of molecular species present on the reflecting interface as it has a non-zero amplitude. To sum up, both polarizations excite molecules in the medium but only p-polarization is absorbed by the surface molecules. If we want to obtain a surface spectrum without contribution from the surroundings, s-polarized light is subtracted from p-polarized reflected light. This is normalized by a mean value of the total reflection (sum of both polarizations divided by two):

$$\frac{\Delta I}{I} = \frac{I_p - I_s}{\frac{1}{2}(I_p + I_s)}.$$
Consequently, PM-IRRAS spectrum is excited by an electromagnetic wave with a well-defined direction of the electric field (combination of incident and reflected waves gives electric field perpendicular to the surface). The strongest bands are observed for those vibrations, for whom the angle between their transition dipole moment and the vector of electric field As a result, what vibrations can be observed with PM-IRRAS is determined by surface selection rules: only the modes which result in the dipole changes perpendicular to the metal surface will absorb radiation. This is the reason PM-IRRAS is a very potent method in determining the structure of molecular layers. Adsorption of molecules on a surface limits their degrees of freedom and enforces some degree of directional order. Differences between relative intensities of bands in the infrared spectra of randomly oriented material and IRRAS spectra of the ordered material indicate the orientation of molecules or their functional groups with respect to the surface.

Figure 3 Scheme of the electric field for s-polarized and p-polarized IR beam of light reflected from a metal surface; $E_i$ – incident electric field, $E_r$ – reflected electric field (materials of University of Guelph).
2 Optical sensors for ions - state of the art

Ions are the basis of most of the chemistry reactions and biochemical processes in our bodies. Sodium, potassium and calcium cations are widespread in our organisms and play a big role, especially as signal transducers that control the action of the nervous system and actuation of muscles. For example, low level of sodium, called hyponatremia, leads to, inter alia, muscle cramps, nausea and headache. Essentially, regulation of the electrolyte concentration is supervised by kidneys. Any deviation of the cation levels might be a symptom of a kidney failure. At the same time, they are also crucial to maintaining correct blood pressure. To say that they are in charge of the biochemical equilibrium of our bodies will not be an overstatement. Among transition metal ions, copper, iron and zinc are the most abundant. Again, incorrect intake of these elements can result in liver damage, organ failure and even lead to death.35 The same can be said about anions. Cell homeostasis depends on the efficient work of chloride channels in cell membranes, which also take part in transmission of action potential in neurons. Cystic fibrosis is one of the diseases that are caused by genetic defects in translation of chloride transport proteins.36 On the other hand, sulphates actively support the II phase of the drug metabolism (when xenobiotics are conjugated with charged species in order to produce more polar metabolites, which are easier to transport, and simultaneously to deactivate potentially toxic molecules).

Apart from biochemical functions, industrial role of ionic compounds cannot be overlooked. In fertilizers, you can usually find phosphates and nitrates. They accelerate the growth of the crops and make agriculture in less arable lands possible. However, only a fraction of the applied nutrients is ultimately utilized by the plants. The rest is accumulated in the soil and underground waters. The leftovers contaminate the surface waters. The biggest result is eutrophication. Due to concentration of high amount of nutrients in a reservoir, plants and algae have perfect conditions to overgrow. This leads to oxygen deficiency and reduction of water life. Other widespread example are nitrites, used as food additives, which
is currently a pretty controversial issue. Nitrites and nitrates (in form of sodium and potassium salts – labelled as E249-E252) preserve meat by inhibiting the growth of bacteria; it is not irrelevant that they enhance its red colour. This way nitrite preservatives improve the appearance of the meat products and extend time when it can be stored safely. However, during processing in high temperatures, e.g. frying, toxic nitrosamines are released. When digested, these compounds increase the risk of gastric and oesophageal cancer. Also, they oxidize iron ions in haemoglobin, which, in case of high intake of nitrites, can cause hypoxia, cyanosis and anemia.

Ionic compounds surround us in everyday life, which makes monitoring their levels in biochemical, medical and environmental samples necessary. This is where sensors come in handy. One of the fields that is being extensively developed nowadays are optical sensors. Fluorescence, UV-Vis or vibrational spectra provide information about a given parameter of the sample. Small invasiveness is the main advantage of the optical methods. Since the acquisition of a signal requires illumination and collection of a light beam without any preparation of the specimen, measurement leaves the sample intact. This strategy is very often coupled with the use of nanostructures. Metal nanoparticles of different plasmon resonance energies, luminescent quantum dots can be incorporated into the analytical process, which broadens the applications of optical sensors; not only do they reduce the amount of the sample needed for detection of the analyte, but also they can be successfully introduced to previously inaccessible environments, such as the interior of a living cell.

2.1 Detection of cations

Among cations, hydronium should be mentioned first. Organic chemists have been fighting for a long time to synthesize new pH-sensitive fluorescent molecules of high yield. On the website of Sigma-Aldrich, you can find around 50 different
fluorescent probes and indicators available commercially. However, their working range is rather narrow and usually spans 1-2 pH units. They are also prone to photobleaching, which negatively affects sensitivity of the probes whose readout bases on total intensity changes. Moreover, if used in a more complex matrix, like in case of intracellular research, their applicability is limited by intrinsic fluorescence of the sample. Uncontrollable diffusion and toxicity are other handicaps of fluorescent molecules in intracellular research. SERS nanoprobes offer a promising alternative. Vibrational spectra provide information about the chemical state of a reporter molecule, from which pH of the medium can be extracted. In the literature, 4-mercaptobenzoic acid (pMBA) is the most frequently mentioned molecule with a pH-sensitive SERS spectrum; other examples are 4-aminothiophenol (pATP), 4-mercaptopyridine, 3,5-dimercaptobenzoic acid or 3-amino-5-mercaptop-1,2,4-triazole, the latter described by the group of Prof. Bukowska. As far as pMBA is considered, its pH-responsiveness originates from the deprotonation of COOH groups (Figure 4). Intensity of a COO$^-$ symmetric stretching band at ca. 1423 cm$^{-1}$ is typically normalized to another band of a constant intensity (SERS spectrum of pMBA has two strong bands at 1080 cm$^{-1}$ and 1580 cm$^{-1}$) and used to measure the pH value of the surroundings. The working range of such nanodevices varies with respect to the used nanoparticles and is reported to be between: 5.5-8.0 in case of gold nanoshells-based sensor, 6.0-8.0 for gold nanospheres or 3.0-7.0 for silver nanoparticles. Use of a non-linear effect (surface enhanced hyper Raman scattering, SEHRS) expanded the range of the system to 2.0-8.0.

pMBA-based sensor was found to be useful in many aspects of intracellular sensing through SERS mapping (Figure 5). Kneipp et al. monitored pH dynamics in endosomes in cells, observing variations in pH with time. Balint et al. followed the apoptosis mechanism in photodynamic therapy. SERS nanosensors were embedded on silica microbeads, which were later moved within the sample by optical tweezers. Jaworska et al. observed changes in intracellular pH induced by tumour necrosis factor, which causes inflammation of endothelial tissue.
Needless to say, monoatomic cations do not exhibit any vibrational spectrum. If we aim at determining their concentration, we must monitor changes in the spectra induced by the presence of the analyte. In case of pH sensing, intensity of a marker band is given in relation to intensity of another band of relatively constant intensity in the SERS spectrum. This is also valid for detection of metal cations, however, other strategies are also in use. Mostly, variations in the total intensity of the SERS spectrum caused by aggregation of the nanosensors are considered. A big part of literature focuses on mercury(II) detection (Figure 6). It is a widespread pollutant which has a devastating effect.
on health of living organisms. Anaerobic microbes in the sediments of aqueous reservoirs transform it to methyl mercury, a strong neurotoxin. It undergoes bioaccumulation in marine organisms, which results in its high content in the flesh of predatory fish at the end of the aquatic food chain. When digested by humans, it increases the risk of e.g. heart attack, autoimmune diseases, even blindness and death in more acute poisoning. In many different sensors of mercury ion, increase of the cation concentration manifests by the decrease of the SERS signal intensity. Hg$^{2+}$ ions bind to a SERS active molecule which hinders an effective adsorption on nanoparticles, as it was demonstrated in 4,4’-dipyridyl-based sensor. Another mechanism causing intensity loss is incorporation of mercury atoms in the structure of metal nanoparticles; plasmonic properties of such an amalgam do not support SERS anymore. DNA-functionalized nanoparticles are an interesting design of a nanosensor of mercury(II). In presence of Hg$^{2+}$, DNA strand becomes folded. This draws the Raman-active molecule at the end of the strand close to the nanoparticle surface and then SERS might be observed; without mercury, reporter molecule is separated from the nanoparticle for the distance of DNA length and no SERS spectrum can be collected. Limit of detection of Hg(II) nanosensors goes as far down as single pM, and the system is often coupled with detection in microfluidic devices.

Another effect used in mercury(II) detection (albeit not limited to these cations) is cation-induced aggregation of the nanosensors. Surface of nanoparticles is functionalized with both a Raman reporter and a cation-complexing agent. Upon addition of metal cations, coordination complex forming between nanoparticles links them together. In such situation, hot spots are generated, which vastly increases the intensity of the acquired SERS spectrum. Apart from mercury(II), other ions detected this way include: copper(II), chromium(III), nickel(II), arsenium(III) or cadmium(II). At the same time, let us bear in mind that aggregation of nanoparticles may be monitored not only with SERS spectroscopy. The controlled formation of clusters of nanoparticles results in change of plasmonic
properties of the nanomaterial. This gives rise to a separate family of optical nanosensors: colorimetric nanosensors.\textsuperscript{70,71} Detection of e.g. lead(II),\textsuperscript{72–74} cadmium(II),\textsuperscript{72} chromium(III),\textsuperscript{75} nickel(II),\textsuperscript{67} copper(II)\textsuperscript{76} or mercury(II)\textsuperscript{72,77–80} can be carried out by UV-Vis measurements.

Optical nanosensors for lighter metal cations rely mostly on techniques other than vibrational spectroscopy. Monitoring simple intensity changes of a fluorescent dye attached to a polymer sphere,\textsuperscript{81} Förster resonance energy transfer (FRET)-based detection with use of CdTe quantum dots\textsuperscript{82} or colorimetric detection due to aggregation of functionalized nanoparticles\textsuperscript{83} are only some of the examples of nanosensors for calcium(II) described in the literature. These types of sensors usually suffer from really narrow working ranges and as their signal is based on the total intensity change, it cannot be internally normalized – this way

**Figure 6** Scheme of different working principles of a Hg\textsuperscript{2+} SERS nanosensor: A, B: based on a decrease of a SERS signal,\textsuperscript{57,59} C: DNA-based distance-dependent Hg(II) detection,\textsuperscript{60} D: based on cation-induced aggregation of nanoparticles.\textsuperscript{63}
it is prone to a higher error due to matrix background. Discussed disadvantages can be addressed by nanosensors based on vibrational spectroscopies, mostly SERS, which additionally provide information on the system on a molecular level.
2.2 *MES as a Raman reporter molecule for SERS sensing of cations*

In order to obtain efficient SERS-based nanosensors, two factors have to be taken into account: a suitable Raman reporter and SERS substrates characterized by high enhancement factors.\(^{37,84}\)

2-mercaptoethanesulphonate anion (MES) appears to be a perfect candidate for a Raman reporter. As a thiol, it has high affinity to both silver and gold. Formation of a strong chemical bond between sulphur and metal atoms of the surface facilitates efficient adsorption on metal nanostructures. This property will be exploited while functionalizing various metal nanoparticles with MES molecules.

MES is non-toxic; moreover, sodium salt of MES, MESNa (sodium 2-mercaptoethanesulphonate), shows biological activity. It acts as a protective agent against urothelial toxicity that appears during certain anticancer treatments.\(^{85}\) Acrolein, major metabolite of oxazaphosphorine-based anticancer drugs, like cyclophosphamide or ifosfamide, accumulates in bladder, where it causes haemorrhagic cystitis. This is a painful condition, which includes haematuria, haemorrhage and dysuria due to damage of bladder's transitional epithelium and blood vessels. Toxicity of acrolein is inhibited by glutathione. However, its level in the urinary tract is not sufficient to effectively protect from side effects of the treatment. MESNa, as a synthetic substitute of glutathione, helps in preventing from this condition to occur. It suppresses metabolic pathway of cytostatic drugs to acrolein and forms non-toxic complexes with the metabolites.\(^{86}\) MESNa also exhibits mucolytic properties. It breaks disulphide bridges in mucoproteins and, as a result, reduces viscosity of bronchial secretions. It can be applied in form of aerosol, bronchial lavage or inhalations without any side effects.\(^{87}\) As a chemotherapy adjuvant, MESNa is sold under names Uromitexan and Anti-Uron; as a mucolytic medicine, it can be found in Muco-fluid and Mistabron.
MES is known to biochemists as coenzyme M. It takes part in methyl-transfer metabolic reactions in microorganisms which live in low oxygen conditions. These microbes, called methanogens, populate habitats as varied as hot springs, digestive tracts of mammals or swamps. In order to survive in such hostile environment, they reduce carbon dioxide with hydrogen into methane. Methane formation is directly controlled by the level of coenzyme M, which is engaged in the last step of the carbon cycle.

![Structure of MESNa](image)

**Figure 7** Structure of MESNa (numbers denoting the atoms will be helpful in further band assignment) and gauche and trans conformers of MES anion upon adsorption on a metal surface.

MESNa on Ag and Au nanostructured electrodes exhibits a SERS spectrum of high intensity with well-defined bands. These features make the molecule suitable for studies of mixed thiol monolayers which mimic biological membranes, adsorption of proteins on protective thiol monolayers on metals or evaluation of SERS substrates efficiency. Band at 790 cm⁻¹
is the band of the highest intensity in the SERS spectrum of MESNa (Figure 8). It is attributed to the C(3)-S(4) stretching vibrations. Bands at 630 cm\(^{-1}\) and 700 cm\(^{-1}\) are assigned to the C(2)-S(1) stretching vibrations of the gauche and trans conformers, respectively (Figure 7).\(^{90,96}\) Their relative intensities change according to the arrangement of the molecules in the monolayer. Formation of the Ag-S(1) bond is proved by appearance of the band at 290 cm\(^{-1}\), which is absent from the bulk NR spectrum of MESNa. Moreover, two vibrational modes of the sulphonate group manifest in the SERS spectrum. Band corresponding to the antisymmetric stretching vibrations can be found at 1292 cm\(^{-1}\), while the band assigned to symmetric stretching vibrations is split into two components: at 1030 cm\(^{-1}\) and 1063 cm\(^{-1}\). Experimental and theoretical studies revealed that this split results from different local environment of the SO\(_3\)\(^-\) groups. Normal Raman spectrum of MESNa aqueous solution, where sulphonate groups are exposed mainly to water molecules, exhibits a band at 1030 cm\(^{-1}\). On the other hand, band at 1063 cm\(^{-1}\) dominates in the normal Raman spectrum of polycrystalline MESNa, where strong interactions between the anion and Na\(^+\) are expected. Additionally, in SERS spectra of MES, it was noticed that the intensity of the high-energy component varied upon transferring a MES-functionalized silver electrode between solutions of low and high concentrations of MESNa. Also, change of the cation from Na\(^+\) to Ca\(^{2+}\) influenced the position of the band. At the same time, theoretical simulations did not show any symmetry change upon ion pair formation, so this could not be the reason of the split. Based on these observations, two components of the SO\(_3\)\(^-\) symmetric stretching band at 1030 cm\(^{-1}\) and 1063 cm\(^{-1}\) were interpreted as vibrations of SO\(_3\)\(^-\) in the aqueous surroundings and forming a contact ion pair with the counter ion, respectively.\(^{91}\)
Figure 8 SERS spectrum of MESNa on an electrochemically roughened silver plate (own results). Excitation wavelength: 532 nm.
2.3 Detection of anions

Detection of anions with SERS spectroscopy should potentially be even easier than for cations. Except for halides, other inorganic anions have their own vibrational features so they can be monitored directly. Designed surfaces are used for detection of perchlorates, hypochlorites, cyanides, thiocyanates, chromates, and nitrates through direct monitoring of their SERS spectra. However, low Raman cross section makes direct detection of oxyanions in low concentrations a hard task. Therefore, many anion sensors need to rely on changes in spectra of reporter molecules induced by the presence of an ion. This is mainly the case for halides: chlorides and iodides, but also for nitrates. When it comes to the latter, detection can be also carried out based on an already known reaction. Common method to determine nitrites is the Griess reaction from 19th century. This test, used for a long time e.g. in forensics to detect traces of explosives results in formation of diazonium salt and pink colour appears in presence of NO$_2^-$ ions. The reaction can be followed by SERS spectroscopy, if Griess substrates are attached to silver nanoparticles (Figure 9). The diazo product manifests by new bands of N≡N bond vibrations. Use of an excitation wavelength resonant with the energy of electronic transitions of the diazonium dye additionally enhances its spectral features and leads to extremely low limit of detection, down to subpicomolar concentrations ($4 \times 10^{-13}$ M).
Among techniques, which are used to monitor anion binding, fluorescence and nuclear magnetic resonance (NMR) spectroscopies are predominantly reported. Fluorescence emission is a process of high quantum yield, which facilitates measuring the spectra and recognizing intensity differences upon anion addition. However, this strategy is limited only to fluorescent receptors. On the other hand, NMR spectroscopy is applicable to a broad range of organic molecules. Both of these methods are indirect – they rely on spectral changes induced by the analyte without direct observation of the anion signal. They also suffer from other disadvantages, like fluorescent background introduced by real-life samples or large facilities and complicated analysis of the origins of spectral changes in NMR. Sensors based on vibrational spectroscopy techniques potentially detect anions directly, as oxyanions have their own vibrational bands which can be measured upon incorporation into the receptor layer. As the position of the band in the spectra is specific for each anion, vibrational spectroscopies make differentiation between anions possible in case of non-selective receptors, which would be a tough task using other methods.
2.4 Carbazole derivatives as anion receptors

Anions play a crucial role in both biochemical and industrial processes. Recently, human activity increased nitrogen and sulphur levels in the environment.\textsuperscript{110} $\text{SO}_4^{2-}$ pollution results mainly from emission of gaseous $\text{SO}_2$. It originates from burning fossil fuels such as coal and petroleum. When in the atmosphere, it reacts with water droplets, eventually forming sulphuric acid. In turn, pH of precipitation increases and leads to acid rains, which enormously distort equilibrium in the ecosystem. It also has an impact on human health, as $\text{SO}_4^{2-}$ anions are the main form of sulphur intake in a liquid form.\textsuperscript{111} However, in comparison to cation sensors, detection of anions is still an emerging field. In aqueous solutions, they are known to have a strong solvation shell.\textsuperscript{112} This feature impairs electrostatic interactions with receptors, which makes anion sensing even more challenging.\textsuperscript{113,114}

![Figure 10 X-ray structure of diaminocarbazole derivative forming a complex with a sulphate anion in 2:1 stoichiometry.\textsuperscript{115}](image)
1,8-Diaminocarbazoles and its derivatives have been designed as efficient anion receptors.\textsuperscript{115–119} This class of electroneutral molecules bears three N-H bonds in the cleft, which favours anion complexation through hydrogen bonding. Also, as a rigid unit, carbazole moiety has better performance in anion recognition than receptors of more flexible structure.\textsuperscript{116} Substitution with electron-withdrawing atoms, like chlorine in 1,8-diamino-3,6-dichlorocarbazole, further facilitates anion binding. \textsuperscript{115} 1H NMR titration experiments have shown that diaminocarbazoles linked with different substituents by an amide bond form strong complexes with sulphate anions of varying stoichiometry in solution.\textsuperscript{115} Three-dimensional structures in crystals based on X-ray crystallography are also confirmed (Figure 10). These facts are a good incentive to test anion binding properties of the receptors based on diaminocarbazole derivatives attached to a metal surface.
RESULTS AND DISCUSSION
3 Ag-MES NPs: cation sensor based on SERS

3.1 Experimental

**Instrumentation** Raman spectra were collected on LabRAM HR800 (Horiba Jobin Yvon) Raman spectrometer with a charge-coupled device detector cooled by Peltier modulus. All the spectra were excited with a 532 nm Nd:YAG laser second harmonic line of a maximum light beam power (at the laser head) of about 100 mW. Holographic grating with 600 grooves/mm was used. The spectrometer was coupled with Olympus BX61 confocal microscope with a pinhole set to 200 µm. Backscattered light was collected through a 10× objective. Calibration of the system was performed with respect to 520 cm\(^{-1}\) silicon band.

**Chemicals** Magnesium chloride (99%), potassium chloride (99.5%) and silver nitrate (99.9%) were purchased from POCH, the rest of the metal chlorides (99%) as well as hydroxylamine hydrochloride (99.9%) and sodium 2-mercaptoethanesulphonate (98%) were purchased from Sigma–Aldrich. Ultrapure water (18 MΩ/cm\(^{-1}\)) was used to prepare all solutions.

**Sample preparation** Silver colloid which was exploited as a SERS substrate was synthesized according to Leopold and Lendl procedure.\(^{120}\) Ten millilitres of 1×10\(^{-2}\) M silver nitrate was added dropwise to 90 ml of 1.66×10\(^{-3}\) M hydroxylamine hydrochloride while stirring. Required pH value was achieved by adding 300 µl of 1 M sodium hydroxide. The whole solution was then stirred for 10 min. Extinction spectra of the obtained colloids exhibited peaks in the range between 404 and 417 nm. Resulting nanoparticles had a mean diameter of 29 nm (based on the statistics from TEM images). Functionalization of silver nanoparticles with sodium 2-mercaptoethanesulphonate (MESNa) was carried out by adding 1 ml of 1×10\(^{-2}\) M MESNa aqueous solution to 9 ml of the silver colloid. The mixture was subsequently centrifuged for 30 min and the supernatant was discarded in order to remove the unattached MES molecules. The test tube with the precipitate was filled with 45 mM hydrochloric acid solution and the suspension was centrifuged
once more. The precipitate of nanosensors was redispersed in water up to the initial volume of the sample. Stock solutions of metal chlorides varying in the range between $5 \times 10^{-9}$ M and 5 M with a step of one order of magnitude were used for the ion-concentration-dependent measurements. Test samples were prepared by adding 100 µl of the aforementioned metal ion solution to 400 µl of the nanosensors suspension obtained in the procedure described above. For Ca$^{2+}$ and Mg$^{2+}$ co-detection measurements, samples consisted of 400 µl of the nanosensor suspension, 90 µl of $5.56 \times 10^{-4}$ M (for final concentration $1 \times 10^{-4}$ M) or $5.56 \times 10^{-2}$ M (for final concentration $1 \times 10^{-2}$ M) Ca$^{2+}$ and 10 µl of Mg$^{2+}$ of the respective concentrations.

**Raman measurements and data processing** All spectra were collected for five accumulations, 50 s each. Spectra were baseline-corrected before further processing. In order to calculate intensity ratio of the chosen bands, absolute intensities in respective peak positions were read out. Up to four measurements were performed for each series in order to obtain statistical data. Error bars on all the figures reflect standard deviations of the respective measurements. Boltzmann function was fitted and plotted where needed.
3.2 Results and discussion

Figure 11 presents the SERS spectrum of MES adsorbed from $1 \times 10^{-3}$ M solution of MESNa on silver nanoparticles dispersed in water. Characteristic bands can be found: proof of Ag-S(1) bond formation at 290 cm$^{-1}$, dominating C(3)-S(4) stretching vibration band at 800 cm$^{-1}$, well pronounced C(2)-S(1) vibrations of the gauche and trans conformers (633 cm$^{-1}$ and 705 cm$^{-1}$), SO$_3^-$ asymmetric stretching vibration at 1298 cm$^{-1}$ and, most importantly, double band of SO$_3^-$ symmetric stretching (band 1 at 1040 cm$^{-1}$ and band 2 at 1066 cm$^{-1}$). Since these two components originate from vibrations of the sulphonate group interacting with water and the cations, respectively, their intensity ratio should depend on the cation concentration (Figure 12). For this reason, they were chosen to be marker bands in the designed nanosensor. Quantitative cation detection was accomplished based on $I_1/I_2$ intensity ratio as the analytical signal.

![SERS spectrum](image_url)

**Figure 11** SERS spectrum of $1 \times 10^{-3}$ M MESNa on silver nanoparticles (excitation wavelength: 532 nm). Red asterisks mark two components of SO$_3^-$ symmetric stretching vibration band (at 1040 and 1066 cm$^{-1}$), which are chosen to be marker bands of the system. Blue dots mark bands which are assigned to C-S (sulphur bound to the surface) stretching vibrations of a gauche and a trans conformer of MES (at 633 and 706 cm$^{-1}$ respectively). Ag-S stretching vibration band is marked with an arrow.
Figure 12 Schematic presentation of the nanosensor working principle: MES anions on the surface of a silver nanoparticle (Ag NP) interact with both solvent molecules and cations present in the medium. The higher the cation concentration is, the higher the intensity of the second marker band ($I_2$) is recorded in comparison to the intensity of the first marker band ($I_1$).}

Important issue that needed to be addressed was the presence of Na$^+$ ions in the initial structure of MES monolayer adsorbed from the MESNa solution. Directly after functionalization of AgNP with MES, SERS spectrum disclosed both marker bands (Figure 13A). In order to get rid of the interference from Na$^+$ ions interacting with the monolayer, MES-functionalized silver nanoparticles (Ag-MES NP) were centrifuged and resuspended in HCl (Figure 13B). Optimal concentration of the acid was determined to be 45 mM. Upon resuspension in HCl of lower concentrations, intensity of the band 2 did not decrease, which suggested that Na$^+$ have not been washed off of the monolayer. Higher concentrations of the acid led firstly to dramatic decrease in the SERS intensity and consequently to precipitation of the nanoparticles on the walls of the test tube due to aggregation. Band 2 was
fully eliminated after centrifugation of Ag-MES NP in HCl and resuspension in water (Figure 13C). This procedure completed the fabrication of the SERS cation nanosensor.

![SERS spectrum image](image)

**Figure 13** Changes in the SERS spectrum of MESNa on silver nanoparticles during the procedure of obtaining the nanosensor: (A) SERS spectrum of MESNa, (B) SERS spectrum of MESNa with HCl added, (C) SERS spectrum of MESNa with HCl added after centrifuging, removing the acid and redispersing the nanosensor in water. Spectra were shifted for clarity. Excitation wavelength: 532 nm.

Response of the nanosensor was first tested on the heavy metal cations such as Co$^{2+}$, Fe$^{2+}$ and Mn$^{2+}$. In Figure 14 one can see SERS spectra collected from Ag-MES nanosensors exposed to Co$^{2+}$ cations in concentrations in the range $1 \times 10^{-8} - 1$ M. In the series, **marker bands 1** and **2** occurred at 1040 and 1051 cm$^{-1}$, therefore a shift of the cation-sensitive **band 2** was seen in comparison to exposure to intrinsic Na$^+$ cations. As the spectra in Figure 14 were not normalized, a nonmonotonic change in total SERS intensity throughout the series could be observed. This effect, with a maximum at $1 \times 10^{-3}$ M CoCl$_2$, was consistent with the results obtained for other studied cations. SERS enhancement depends on the number of hot spots created in the gaps between nanoparticles, thus on the aggregation degree of NPs. Addition of the electrolyte leads to electrostatic stabilization of the NP suspension.$^{121,122}$ Initial negative charge of Ag-MES NPs is neutralized by interactions with the counterion. These interactions reduce
electrostatic repulsion between NPs and stabilize the distance between them. The closer to the optimal size gap, the higher SERS intensity is achieved. Further increase of the electrolyte concentration beyond the optimal conditions induces ongoing aggregation. These big clusters display red-shifted plasmon resonance which is not favourable for SERS enhancement in the measurement conditions. As a result, SERS intensity drops in higher salt concentration due to the loss of effectively enhancing plasmonic nanoparticles. This effect occurred in the described system; UV-Vis spectra of Ag-MES exposed to varying concentrations of CoCl₂ are plotted in the Figure 15. As long as [Co²⁺] did not exceed 1×10⁻⁴ M, no influence on the UV-Vis spectrum was revealed (spectra overlapped). Formation of the small aggregates containing several NPs cannot be followed by extinction measurements, even though they may play crucial role in SERS enhancement.¹²³ Increase of the cation concentration was followed by appearance of a new broad band between 700-800 nm, which was the proof of significant aggregation. Additionally, hydrodynamic radius for Ag-MES NPs monitored by dynamic light scattering (DLS) also increased in higher ion concentrations, which showed gradual formation of bigger aggregates (compare Table 1 and Figure 15). Studies on other cations led to similar observations. These conclusions agree with reports of the dependence of the SERS enhancement on different cationic and anionic aggregating agents.¹²⁴
Figure 14 The SERS spectra of MES anions in the presence of Co$^{2+}$ ions. Cation concentration in [M] is given for each spectrum. Red lines show positions of the ion-sensitive marker bands (1040 and 1051 cm$^{-1}$). Spectra were shifted but not scaled, $\lambda_{exc} = 532$ nm.

Table 1 Mean radii of Ag-MES nanoparticles determined by DLS for samples without cations and with different concentrations of Co$^{2+}$.

<table>
<thead>
<tr>
<th>Concentration of Co$^{2+}$ added to Ag-MES NPs</th>
<th>Ag-MES NPs radius (from DLS)/nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c_{Co} = 0$</td>
<td>22.67</td>
</tr>
<tr>
<td>$10^{-8}$ M</td>
<td>22.04</td>
</tr>
<tr>
<td>$10^{-7}$ M</td>
<td>22.59</td>
</tr>
<tr>
<td>$10^{-6}$ M</td>
<td>22.84</td>
</tr>
<tr>
<td>$10^{-5}$ M</td>
<td>22.72</td>
</tr>
<tr>
<td>$10^{-4}$ M</td>
<td>22.28</td>
</tr>
<tr>
<td>$10^{-3}$ M</td>
<td>52.27</td>
</tr>
<tr>
<td>$10^{-2}$ M</td>
<td>105.3</td>
</tr>
<tr>
<td>$10^{-1}$ M</td>
<td>117.7</td>
</tr>
<tr>
<td>$10^{0}$ M</td>
<td>108.4</td>
</tr>
</tbody>
</table>
Figure 15 UV-Vis spectra of the Ag-MES nanosensor suspension upon addition of Co$^{2+}$ ions in different concentrations and the spectrum of 0.5M CoCl$_2$ solution. The band at 520 nm corresponds to d-d transition of cobalt complexes in solution.

Poorly controlled NP aggregation, which greatly affects SERS intensity, has always been a substantial obstacle to application of SERS-based nanodevices in quantitative measurements. In the presented sensing system, ion concentration had an impact not only on the spectral features, but also on the total SERS intensities of the Raman molecule. This issue was overcome by selecting two marker bands and analysing their intensity ratio. In case of any aggregation-driven fluctuations of total intensities, $I_1/I_2$ will remain unaffected and might be treated as internal normalization of the analytical signal. Marker bands are indicated in Figure 14 to highlight their behaviour. Initially, before the exposure to cations, no sign of band 2 was recognized in the spectrum. As the concentration of Co$^{2+}$ increased, the ion-sensitive band gained intensity accordingly. This trend was illustrated in Figure 16, where $I_1/I_2$ was plotted against cation concentration. Sensor response varied for Co$^{2+}$ presence down to $\sim 10^{-6}$ M. Below this value, cation concentrations were indistinguishable based on the SERS spectra of MES.

The following ions, Fe$^{2+}$ and Mn$^{2+}$, generated changes in the collected spectra comparable to those for Co$^{2+}$. In case of Fe$^{2+}$, position of the band 2 was exactly the same as for Co$^{2+}$ (1051 cm$^{-1}$); Mn$^{2+}$ caused a shift to 1055 cm$^{-1}$. Sensor response for all three studied heavy metal ions exhibited a similar trend (Figure 16),
as well as the range of \( \text{I}_1/\text{I}_2 \) values (between 0.6 and 1.7) and the limit of detection (\( \sim 10^{-5} \) M for \( \text{Fe}^{2+} \) and \( \text{Mn}^{2+} \)). To conclude, each of the studied transition metal cations is detectable with the designed Ag-MES nanosensor; however, the sensor is not selective enough to make a distinction between them.

\[ \text{Performance of the sensor was also checked for representatives of biologically relevant cations of group I (Na}^+ \text{ and K}^+ \text{) and group II (Mg}^{2+} \text{ and Ca}^{2+} \text{) elements. For this set of cations, shifts of band 2 were much more differentiating (Table 2). Since the interactions between the cations and SO}_3^- \text{ groups from the monolayer are of electrostatic nature, the energy of the ion-sensitive band should be affected by the surface charge density of the ion. Positions of the band of sulphonate groups forming contact ion pair with the studied cations are collected in Table 2. They were confronted with calculated values of parameter A which is proportional to the surface charge density of the ion: charge of the ion (Z) divided by its ionic radius squared (r\(^2\)). Figure 17 presents a plot of the band 2 position versus parameter A. There was a clear linear dependence for three studied cations:} \]
Na\textsuperscript{+}, K\textsuperscript{+} and Ca\textsuperscript{2+}. As transition metals and Mg\textsuperscript{2+} did not match the trend, they must behave in the system in a different way. This divergence can be explained firstly by other than electrostatic nature of the interactions: for transition metal ions, coordinate bond with the sulphonate groups might be formed. As such, their impact on the vibrational energy of SO\textsubscript{3}\textsuperscript{-} would be independent of the ionic surface charge density. Another issue that must be taken into account is solvation of the cations that occurs in the aqueous solution. Both magnesium and transition metal ions have considerably higher hydration enthalpies than the rest of the studied cations (Table 2). It means that water molecules which are arranged in a hydration shell are bound strongly to the cation and release from the hydration shell requires high energy. This impedes direct interactions with the monolayer. In comparison to the cations forming a linear trend in Figure 17, band 2 in presence of Co\textsuperscript{2+}, Fe\textsuperscript{2+}, Mn\textsuperscript{2+} and Mg\textsuperscript{2+} exhibited rather similar and low energies, which implies that aforementioned cations do not lose their hydration shell while interacting with sulphonate (solvent-separated ion pairs).

**Table 2** Comparison of the parameters describing the investigated cations: experimental position of the cation-sensitive marker band 2, ionic radius,\textsuperscript{125} the A parameter denoting surface charge density of the ions and hydration enthalpy.\textsuperscript{126}

<table>
<thead>
<tr>
<th>Cation</th>
<th>Position of the ion sensitive marker band 2/cm\textsuperscript{-1}</th>
<th>Ionic radius /nm</th>
<th>A = Z/r\textsuperscript{2}/nm\textsuperscript{2}</th>
<th>Hydration enthalpy /kJ mol\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co\textsuperscript{2+}</td>
<td>1051</td>
<td>0.0745</td>
<td>360</td>
<td>-1996</td>
</tr>
<tr>
<td>Fe\textsuperscript{2+}</td>
<td>1051</td>
<td>0.078</td>
<td>329</td>
<td>-1946</td>
</tr>
<tr>
<td>Mn\textsuperscript{2+}</td>
<td>1055</td>
<td>0.083</td>
<td>290</td>
<td>-1841</td>
</tr>
<tr>
<td>Na\textsuperscript{+}</td>
<td>1066</td>
<td>0.102</td>
<td>96</td>
<td>-409</td>
</tr>
<tr>
<td>K\textsuperscript{+}</td>
<td>1060</td>
<td>0.138</td>
<td>53</td>
<td>-322</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>1076</td>
<td>0.100</td>
<td>200</td>
<td>-1577</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>1048</td>
<td>0.072</td>
<td>386</td>
<td>-1921</td>
</tr>
</tbody>
</table>
In addition to cation recognition based on band 2 shifts, concentration determination based on SERS band intensities was studied as well. Spectral responses of the Ag-MES nanosensor in K⁺ solutions of concentrations in the range $1 \times 10^{-9} - 1$ M are displayed in Figure 18 as an example. As described for Co²⁺, total intensity variations occurred in the series. Once again, they correlated well with aggregation process monitored by UV-Vis spectra (Figure 19). Behaviour of the marker bands was also assessed. Higher amount of K⁺ cations able to interact with MES monolayer manifested in the spectrum as expected: by intensity increase of the 1060 cm⁻¹ marker band. Plots of $I_1/I_2$ versus cation concentration for sodium, potassium, calcium and magnesium can be found in Figure 20. Limits of detection for alkali metal ions fell at $\sim 10^{-4}$ M for Na⁺ and $\sim 10^{-5}$ M for K⁺. Contrast between alkaline earth metal ions was more pronounced (compare Figures 20B and 20C). LOD (limit of detection) for Mg²⁺ was the lowest among all the analytes: $\sim 10^{-3}$ M. On the other hand, Ca²⁺ could be determined in concentrations from the range $10^{-8} - 10^{-2}$ M. Although there are reports on other optical calcium nanosensors,⁸³,¹²⁷ their responses rely mainly on aggregation-activated changes in the UV-Vis spectrum. Obtained working range makes Ag-MES sensor much more universal in comparison.
If we look closely to the Figure 20A, we can notice that the slope of the curve in the working range is steeper for Na\(^+\) than for K\(^+\). This means that concentration increment of one order provokes more pronounced changes in I\(_1\)/I\(_2\) for sodium cations. Such behaviour indicates stronger interactions between sodium and sulphonate groups. In fact, comparing responses for all studied cations, strength of the interactions deduced from the slopes of calibration curves appears to decrease in the order: Ca\(^{2+}\) > Na\(^+\) > K\(^+\) > Mn\(^{2+}\) ≈ Fe\(^{2+}\) ≈ Co\(^{2+}\) > Mg\(^{2+}\). This correlates perfectly with **band 2** positions (see Table 2) and complements previous conclusions about the relationship between the cation-anion bond strength and the position of the ion-sensitive band.

**Figure 18** Spectral differences for MES anions under the influence of K\(^+\) ions in the medium. **Right:** SERS spectra showing changes in the total intensity throughout the series. **Left:** changes in the range 1000-1100 cm\(^{-1}\). Cation concentration in [M] is given for each spectrum. **Red lines** show positions of the ion-sensitive marker bands. Spectra were scaled and shifted for clarity, excitation wavelength: 532 nm.
Apart from the investigated bands of the sulphonate group vibrations, there is another pair of bands which exhibits concentration-dependent intensity behaviour. Presence of cations in the solution had also an impact on relative intensities of the bands at 633 and 705 cm\(^{-1}\) assigned to C(2)-S(1) vibrations of the gauche and trans conformers of MES. This effect was previously reported for MES monolayers on silver electrodes exposed to sodium and potassium salts.\(^{90}\) Figure 21 presents intensity ratio of these two bands (I\(_g\)/I\(_t\)) plotted versus cation concentration. This approach did not affect LOD for Mg\(^{2+}\) (10\(^{-3}\) M). Na\(^+\) and K\(^+\) could be detected in about one order lower concentrations (10\(^{-5}\) M and 10\(^{-6}\) M, respectively). On the other hand, performance of the sensor in low concentrations of Ca\(^{2+}\) worsened, with LOD reaching 10\(^{-6}\) M. For all the presented cations, linear working range reached higher concentrations (up to 1 M), as opposed to the considered SO\(_3^−\) bands, where plateau was observed in more concentrated analyte solutions. To sum up, even though not useful for distinction of cations, I\(_g\)/I\(_t\) can be considered an auxiliary parameter to the intensity ratio of the bands of sulphonate group vibrations in ion detection.
Figure 20 Concentration dependence of the intensity ratio of bands 1 and 2 for metal ions: A: Na$^+$ (dots) and K$^+$ (triangles), $I_1$ – intensity at 1040 cm$^{-1}$, $I_2$ – intensity at 1066 cm$^{-1}$ for Na$^+$ or 1060 cm$^{-1}$ for K$^+$; B: Mg$^{2+}$, C: Ca$^{2+}$, $I_1$ – intensity at 1040 cm$^{-1}$, $I_2$ – intensity at 1048 cm$^{-1}$ for Mg$^{2+}$ or 1076 cm$^{-1}$ for Ca$^{2+}$. 
**Experimental section**

**Ag-MES NPs: cation sensor based on SERS**

**Figure 21** Intensity ratio of C(2)-S(1) stretching vibration bands of gauche ($I_g$) and trans ($I_t$) conformers against the concentration of: Na$^+$ (red squares), K$^+$ (green dots), Mg$^{2+}$ (blue triangles) and Ca$^{2+}$ (orange stars). $I_g$ – intensity at 633 cm$^{-1}$, $I_t$ – intensity at 705 cm$^{-1}$.

Studies on simultaneous detection of several cations were carried out in two-component solutions of Mg$^{2+}$ and Ca$^{2+}$, since the bands positions shift significantly for these two ions (1048 and 1076 cm$^{-1}$, respectively). Keeping the [Ca$^{2+}$] constant ($1 \times 10^{-4}$ M in one series, $1 \times 10^{-2}$ M in another), [Mg$^{2+}$] was increased from $1 \times 10^{-9}$ M to $1 \times 10^{-1}$ M. Behaviour of sensor responses for Mg: $I_{1041}/I_{1048}$ and for Ca: $I_{1041}/I_{1076}$ was investigated. Calibration curve for Mg with Ca$^{2+}$ in $1 \times 10^{-4}$ M greatly overlapped with the original sensor response in interferent-free series (Figure 22a). This level of interference enabled detection on Mg$^{2+}$. Higher concentration of the interferent ($1 \times 10^{-2}$ M) introduced significant deviation from the reference Mg series.

When it comes to treating Mg$^{2+}$ as an interferent in Ca$^{2+}$ sensing, it was monitored how much Ca$^{2+}$ readout ($I_{1041}/I_{1076}$) varied compared to reference measurements in respective calcium solutions (shown in Figures 22b and c). Concentrations of magnesium up to $1 \times 10^{-4}$ M did not disturb the response for $1 \times 10^{-4}$ M of calcium (Figure 22b). Higher level of interference affected the intensity ratio of the marker bands making determination of calcium concentration
impossible. In Figure 22c, similar analysis for more concentrated solutions of calcium (1×10⁻² M) was presented. In this case, the sensor was more resistant to magnesium presence: concentrations up to 1×10⁻² M did not impair calcium detection.
Figure 22 Data collected from samples containing both Ca$^{2+}$ and Mg$^{2+}$ in the solution: a) with $10^{-2}$ M (triangles) and $10^{-4}$ M (dots) of interfering Ca$^{2+}$, squares depict the readout in solution containing Mg$^{2+}$ solely, b) sensor response for $10^{-4}$ M Ca$^{2+}$ plotted against concentration of interfering Mg$^{2+}$, red dashed line marks the reference response for $10^{-4}$ M Ca$^{2+}$ without Mg$^{2+}$ in the solution, c) sensor response for $10^{-2}$ M Ca$^{2+}$ plotted against concentration of interfering Mg$^{2+}$, red dashed line marks the reference response for $10^{-2}$ M Ca$^{2+}$ without Mg$^{2+}$ in the solution. $I_1$ – intensity at 1040 cm$^{-1}$, $I_2$ – intensity at 1048 cm$^{-1}$ for Mg$^{2+}$ or 1076 cm$^{-1}$ for Ca$^{2+}$. 
3.3 Conclusions

In the section above, studies on Ag-MES NPs were described. Summing up the results:

- Ag NPs labelled with MES molecules were demonstrated to work as an novel and efficient cation sensor;
  - two pairs of marker bands were selected, with sulphonate group bands reflecting both concentration changes and type of a cation;
  - selecting intensity ratio of the ion-sensitive bands worked as internal normalization of the signal, making the sensor response independent of total intensity fluctuations;
  - distinction between cations was feasible for alkali and alkaline earth metal cations (Na⁺, K⁺, Mg²⁺ and Ca²⁺);
    - best LOD was achieved for Ca²⁺: 10⁻⁸ M;
    - possibility of co-detection of Ca²⁺ and Mg²⁺ was shown;
  - conclusions on the nature of interactions between the cations and sulphonate group from the monolayer were drawn: electrostatic interactions occur for the cations with relatively lower hydration enthalpies, coordinate bonds and hindered by hydration shell for the cations with higher hydration enthalpies.
4 Ag-MES NPs in intracellular SERS

4.1 Experimental

**SERS measurements** SERS measurements were performed with an InVia Reflex Raman system (Renishaw) comprising a microscope (Leica), a 785 nm laser excitation source (nominal output 260 mW) and a spectrometer equipped with a 1200 grooves per mm diffraction grating and a front-illuminated Peltier-cooled CCD detector (1024 pixels × 512 pixels). Spectra were collected for 1 second each, accumulated 5 times and averaged before further processing.

**Cytotoxicity test** Cells were cultured on a 96-well plate. Cytotoxicity was performed with an MTT assay (containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MTT solution was added to the culture medium at final concentration 0.5mg/ml. After 4 hours of incubation, the plate was analysed by Elisa reader: absorbance at 560 nm and the reference at 650 nm were read.

**Fluorescence imaging** HUVEC cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) cell medium containing foetal bovine serum and penicillin–streptomycin to final concentrations of 10% and 1%, respectively. A suspension of Ag NPs or Ag-MES NPs at a final concentration of 1 µg/ml was added to the medium. After incubation overnight, the cells were washed and fixed using formaldehyde at a final concentration of 4%. Cells were stained with DAPI (Invitrogen) before imaging. The imaging was performed on a Zeiss Cell Observer microscope using an oil 63x objective. Filters for 405 nm to capture DAPI and 561 nm to capture NP reflection (false coloured in green in Figure 25) were applied.
4.2 Results and discussion

Applicability of silver nanoparticles functionalized with MES molecules (Ag-MES) as intracellular cation nanosensors was tested. Detection of ions was possible thanks to two SERS marker bands of MES assigned to SO$_3^-$ stretching vibrations. Different cations were distinguished based on the position of one of the bands (e.g. 1066 cm$^{-1}$ for Na$^+$, 1076 cm$^{-1}$ for Ca$^{2+}$). Moreover, its intensity normalized to the intensity of the second marker band at 1040 cm$^{-1}$ provided information about the cation intensity.$^{128}$ Before the measurements, calibration of the nanosystem was carried out. SERS calibration curve for K$^+$ (marker band at 1058 cm$^{-1}$) is plotted in Figure 23. In the intracellular studies, I$_{1058}$/I$_{1040}$ (I$_2$/I$_1$) was calculated as the sensor response, which is the inverse of the response evaluated so far. Working range of the sensor went down to 10$^{-5}$ M, with readout between 0.4 and 1.6 in concentrations 10µM-0.1M.

![Graph](image)

*Figure 23 Calibration curve of the Ag-MES nanosensor towards K$^+$.*

Viability and uptake test

Cytotoxicity of Ag-MES nanoparticles was tested on two cell lines: HeLa and CHO.K1. Both 24h and 48h viability test were carried out (Figure 24). MES-coated nanoparticles turned out to be more toxic to CHO cells.
Use of Ag-MES NPs in concentrations higher than 10 µg/ml resulted in significant decrease in cell viability, reaching around 40% and 20% at 20 µg/ml after 24h and 48h, respectively. Aqueous suspension of NPs was added to the medium and therefore a water-only control measurement at the same final volume as the highest NP concentration was included (1/4 H2O). At this proportion of water, CHO cells showed lower cell viability so we could not prove that the toxicity noted towards CHO cells at 20 µg/ml was only due to the silver. However, we can estimate that this proportion of water caused a decrease in approximately 30% viability. At the same time, HeLa cells remained more or less intact, regardless of NP concentration. Viability did not go below 90% even after 48 h exposure to 20 µg/ml AgNP.

Uptake of Ag-MES NPs was confirmed with confocal microscopy imaging. Figure 25 presents microscopic images showing DAPI (4',6 diamidino-2-phenylindole) stained HUVEC cells which were incubated overnight with a suspension of Ag NPs (Figure 25A) or Ag-MES NPs (Figure 25B) at a final concentration of 1µg/ml. The following day the cells were washed and fixed using formaldehyde. In Figure 25 the outline of the cells is seen as a slight change of contrast. DAPI is a fluorescent dye that binds to DNA. Therefore, high emission indicates DNA rich regions inside the cells, mainly the nuclei, which helps localize cells. Introduced nanoparticles cannot be followed by fluorescence – additional functionalization of both Ag NPs and Ag-MES NPs could change their properties i.e. ability to penetrate the cell membrane. Therefore, their presence is monitored by light reflection from NPs (marked green in Figure 25). Both non functionalized and MES-functionalized Ag nanoparticles were detected inside the cells. Substantial number of nanoparticles visible in the imaged focal plane confirmed that Ag NPs and Ag MES NPs enter the cells and can be used in SERS mapping.
Figure 24 Viability of two cell lines: HeLa and CHO.K1 upon 24 and 48h exposure to Ag-MES nanoparticles.
Figure 25 Confocal microscope images of DAPI-stained HUVEC cells incubated with A non-functionalized Ag NPs B Ag-MES NPs. Blue emission comes from DAPI-stained DNA-rich regions, green spots mark reflection from the nanoparticles.

SERS mapping

Live CHO-K1 cells were loaded with Ag-MES NPs. Figure 26 I presents the optical image of the cells. Central area with three cells was selected for SERS mapping. In Figure 26 II, total intensity of the most intensive band in the SERS spectrum of MES at 795 cm\(^{-1}\) was mapped. SERS signal of high intensity was present in the areas where the cells were located. It proves that the sample was rinsed properly after the incubation and not many Ag-MES NPs were left outside the cells. In Figure 26 III, ratio of the MES marker band intensities is shown in a green scale. The ion-sensitive band had the energy of 1059 cm\(^{-1}\), which is characteristic for potassium ions. White/grey areas (spectrum C) depict spots with no SERS spectrum of MES.
**Figure 26** I Optical image of CHO-K1 cells loaded with Ag-MES SERS nanosensors
II SERS map of the total intensity of the C-S band at 795 cm\(^{-1}\) between 763 and 820 cm\(^{-1}\)
III SERS map of the MES marker bands 1 and 2 intensity ratio, spots where no Raman reporter spectrum was collected are marked in white, regions with MES spectra are green; legend shows intensity ratio of two marker bands. Spots A and B represent places with high concentration of ions (light green), low concentration of ions (dark green) inside the cells, respectively and spectrum in the spot C outside the cell shows no characteristic bands of MES (white).
Detection of potassium was expected, as intracellular $\text{K}^+$ concentration is the highest among the main electrolytes. High intensity ratio of the Raman reporter bands (spectrum A) indicates high concentration of the cation and was mainly seen in the middle of the cells. At the same time, low concentration of the cation (spectrum B) was mostly measured at the border of the cells. Nanosensors pass through the membrane by endocytosis and remain in endosomes. When endosomes are formed from the cellular membrane, composition of the endosomal lumen represents the composition of the medium outside the cell (concentration of $\text{K}^+$ in a standard DMEM medium is around 5 mM). Therefore, nanosensors trapped in these endosomes detect lower concentration of potassium. As time goes by, equilibrium is set and ions inside endosomes are found in regular intracellular concentrations (around 150 mM).$^{129}$ Those endosomes had enough time to be transported towards the middle of the cell, which is why the signal of nanosensors from central parts of the cells indicates higher concentrations of $\text{K}^+$. These results prove that MES acts as an efficient Raman reporter in biological conditions and Ag-MES nanosensors have potential to be applied in intracellular SERS mapping.
4.3 Conclusions

- Ag-MES nanoparticles were shown to enter the cells.
- CHO.K1 cell line was sensitive to Ag-MES NPs, which resulted in decreased viability upon exposure to higher concentrations of the nanoparticles. Incubation in various concentrations of nanosensors did not cause any significant changes in HeLa cells viability.
- Ag-MES nanosensors were shown to be applicable to intracellular SERS mapping of biologically relevant cations. Differences in the detected K⁺ concentration inside cells are related to the dynamics of the ion concentration inside the endosomes.
5 Ag-MES@SiO₂ NPs: testing silica permeability to metal cations

5.1 Experimental

**Instrumentation** Raman spectra were collected on LabRAM HR800 (Horiba Jobin Yvon) Raman spectrometer with a charge-coupled device detector cooled by Peltier modulus. All the spectra were excited with a 532 nm Nd:YAG laser second harmonic line of a maximum light beam power (at the laser head) of about 100 mW. Holographic grating with 600 grooves/mm was used. The spectrometer was coupled with Olympus BX61 confocal microscope with a pinhole set to 200 μm. Backscattered light was collected through a 50x objective. Calibration of the system was performed with respect to 520 cm⁻¹ silicon band. Transmission electron microscopy images were collected with a Zeiss Libra 120 microscope, with a LaB6 cathode, equipped with OMEGA internal columnar filters and a CCD camera. The samples were prepared by deposition of the suspension on 400-mesh nickel grids coated by the Formvar layer and left to dry.

**Chemicals** 2-Propanol (99.7%), magnesium chloride (99%), potassium chloride (99.5%), and silver nitrate (99.9%) were purchased from POCh; other metal chlorides (99%) as well as hydroxylamine hydrochloride (99.9%), sodium 2-mercaptoethanesulphonate (98%), and tetraethyl orthosilicate (98%) were purchased from Sigma-Aldrich. Ammonia solution (25%) was provided by Chempur. Ultrapure water (18 MΩ·cm⁻¹) was used to prepare all solutions.

**Sample Preparation** Full procedure for synthesis of MES-functionalized cation nanosensor is described in Chapter 3. In brief, silver nanoparticles synthesized according to Leopold and Lendl procedure¹²⁰ were functionalized with MESNa (sodium 2-mercaptoethanesulphonate) by mixing the solutions in a ratio of 1:9 (1×10⁻² M MESNa aqueous solution to Ag colloid). After centrifugation, the nanosensors were redispersed in water up to the initial value. The final concentration of Ag-MES NPs is estimated to be 1.12 nM. Next, the cation nanosensors were covered with a layer of silica. A volume of 10 mL of the nanosensor suspension was added to 40 mL of isopropanol under stirring.
Subsequently, 960 µl of ammonia and 24 µl of tetraethyl orthosilicate were added. The mixture was kept for 15 min at room temperature and then at 4°C. After 2 h, the mixture was taken out of refrigerator and centrifuged three times. Finally, the sample was redispersed in water up to the initial volume of the used nanosensor suspension. SiO$_2$ encapsulation of bare AgNPs was carried out analogously. In order to keep the AgNPs concentration constant in both experiments, 1 mL of water was added to 9 mL of the AgNPs suspension. After centrifugation, NPs were redispersed in water up to 10 mL. Then, silica encapsulation procedure was applied. Metal cations were introduced to the Ag-MES@SiO$_2$ samples as chloride salts. Stock solutions of salts (100 µl, 0.5 M) were added to 400 µl of the Ag-MES@SiO$_2$ suspension. For concentration-dependent series, KCl stock solution was used to obtain concentrations in the range $5 \times 10^{-7}$ to $5 \times 10^{-1}$ M differing by 1 order of magnitude and those were added to the Ag-MES@SiO$_2$ suspension in volumes as described above.

**Raman Measurements and Data Processing** All spectra were collected for five accumulations, 50 s each. Spectra were baseline-corrected before further processing. In order to calculate intensity ratio of the chosen bands, intensities in corresponding peak positions were read out.
5.2 Results and discussion

Growth of the silica layer on previously described Ag-MES NPs using a modified Stöber method was monitored with UV-Vis spectroscopy (Figure 27). Changes in extinction spectra were controlled throughout 20 hours of reaction. In the beginning, the extinction peak occurred at 404 nm for uncoated Ag NPs. Upon modification with MES molecules, nanoparticles exhibited a peak at 408 nm. Once the silica coating initiated, plasmon energy experienced a gradual red-shift seen after 15 minutes already ($\lambda_{\text{max}(15\text{min})}=409$ nm). After longer time: 2, 6 and 20 hours, peak position did not change significantly – in fact, after 20 hours $\lambda_{\text{max}(20\text{h})}=422$ nm which was the same as the position after 2 hours. However, broadening of the whole band was observed. As this could indicate aggregation of nanoparticles in times exceeding 2 hours, Ag-MES@SiO$_2$ NPs obtained in 2h reaction were further studied.

![Extinction spectra of Ag colloid (orange), Ag-MES colloid (purple) and Ag-MES@SiO$_2$ (blue). $\lambda_{\text{max,Ag}}=404$ nm, $\lambda_{\text{max,A g-MES}}=408$ nm, $\lambda_{\text{max,Ag-MES@SiO$_2$}}=422$ nm. Spectra were normalized to 1.0 with respect to the maximum extinction. Inset: changes in UV-Vis spectra in the span of 20 hours, the shift of the peak maximum suggests a gradual growth of the silica layer. Numbers in the legend indicate the time of synthesis in hours.](image)

TEM images of the samples confirmed formation of silica-encapsulated silver nanoparticles (Figure 28). Bare nanoparticles (without the silica shell) were not
detected. Also, only a few silica nanospheres without silver inside were found. Based on the statistics on 126 Ag-MES@SiO$_2$ nanoparticles, mean diameter of the silver core was determined to be 30.47 ± 8.84 nm, while SiO$_2$ shell was 30.35 ± 4.76 nm thick in average (for histograms, see Figure 29). The mean diameter of Ag nanoparticles did not change significantly during the process compared to bare Ag nanoparticles. It shows that silver was not affected by ammonia present in the reaction mixture. If silver had not been protected in any way, NH$_3$ would have caused severe etching. Therefore, it might be concluded that compact monolayer of MES molecules was sheltering the metal core from the destructive effect of ammonia.

In order to demonstrate that MES functionalization played the crucial role in protection of the silver core, synthesis of the silica layer was also carried out using bare Ag nanoparticles not covered with MES molecules. The course of reaction was different this time, as there was considerably less product recovered after the centrifugation. Despite being dispersed in 5 times smaller volume of water, intensity of the UV-Vis spectrum of the final sample decreased in comparison to Ag-MES@SiO$_2$ (Figure 30A). This suggested that the majority of the silver nanoparticles was dissolved by NH$_3$. Additionally, the plasmon band was observed at 410 nm, which ruled out the existence of a thick silica layer on the surface of the remaining Ag NPs. Conclusions drawn based on the extinction spectrum were confirmed by TEM images (Figure 30B). The sample consisted mostly of silica particles without any visible metal core. While the surface of nanoparticles was not functionalized prior to the reaction, silver was etched and was present only as really small nanoparticles stuck to the walls of the SiO$_2$ beads. Overall, MES functionalization was verified as the essential factor in silica layer formation: mostly as a protection layer against ammonia, but also as a stabilizer that prevents the NPs from aggregating during the reaction in isopropanol and as an agent promoting silica growth on the metal surface.
Experimental section

Ag-MES@SiO$_2$ NPs: testing silica permeability to metal cations

**Figure 28** TEM images of Ag-MES@SiO$_2$ nanoparticles at different magnifications.
Figure 29 Histograms of the A: silver core diameter, B: SiO₂ layer thickness based on 126 Ag-MES@SiO₂ nanoparticles.

Figure 30 A: Comparison between the extinction spectra of Ag@SiO₂ obtained with use of MES-coated Ag nanoparticles (Ag-MES@SiO₂, blue spectrum) and MES-free Ag nanoparticles (Ag@SiO₂, green spectrum). B: TEM images of Ag@SiO₂ nanoparticles obtained with use of MES-free Ag NPs.
Modification of the Ag-MES sensor with a silica layer can facilitate the use of the system in biological applications, by: i) increasing its biocompatibility through separation of the metal core and biological matrix, ii) protecting Raman reporter molecules from undesirable interaction with biomolecules. However, silica encapsulation can hinder or even block sensing properties of the probe as well. Inability to collect the SERS spectrum using Ag-MES@SiO₂, significant changes in the spectrum of the Raman reporter caused by interactions with the protective layer or insufficient permeation of the ions through a thick SiO₂ shell are some of the hurdles that may occur in such nanomaterials.

SERS spectrum of MES for Ag-MES@SiO₂ NPs is displayed in Figure 31. Characteristic bands of the Raman reporter were still recognized in the spectrum. Some differences between the spectra collected from Ag-MES and Ag-MES@SiO₂ were however noticed. The first one concerned the marker bands: band 1 and band 2. In the SERS spectrum of MES on Ag-MES@SiO₂, the band assigned to the SO₃⁻ vibrations was situated at 1035 cm⁻¹ and stood out from other bands in terms of intensity. However, in case of Ag-MES, the band consisted of two components corresponding to the sulphonate groups interacting with water (band 1 at 1040 cm⁻¹) and with cations (band 2 at 1066 cm⁻¹ for the sodium salt, MESNa). Rinsing with HCl, which was applied to remove band 2 before cation detection with use of Ag-MES, was therefore not needed for Ag-MES@SiO₂. Absence of the cation-sensitive band in the SERS spectrum from Ag-MES@SiO₂ NPs implied that creation of MES-Na⁺ ion pairs was hampered by the silica layer. Instead of interacting with the monolayer of the metal surface, Na⁺ cations were probably incorporated in the silica structure, forming a glass-like layer around the silver core. What is more, band 1 found in the spectrum from Ag-MES@SiO₂ NPs was much more intensive with respect to the C-S stretching vibration band at 795 cm⁻¹ than in the spectrum from Ag-MES. This behaviour was connected to the structural changes in the monolayer, which influenced the I₇₀₅/I₆₃₀ intensity ratio (corresponding to the relative number of trans and gauche conformers of MES, respectively). There was a clear correlation between the values of I₇₉₅/I₁₀₃₅ and I₇₀₅/I₆₃₀, where the increase of the former coincided with the increase
of the latter (Figure 32). MES molecules were more likely to appear as trans conformers when \( \text{SO}_3^- \)–cation ion pairs formed, which decreased electrostatic repulsion between the anions in the monolayer. This correlation was observed for all SERS spectra of MES exposed to different cations.\(^{91}\)

**Figure 31** SERS spectra of: MES molecules adsorbed on the silver core of Ag-MES@SiO\(_2\) (bottom), MESNa adsorbed on Ag NPs before the exposure to 0.045 M HCl solution with two clearly visible components of the \( \text{SO}_3^- \) stretching band (middle) and MES molecules adsorbed on Ag NPs after the exposure to 0.045 M HCl solution with only one component of \( \text{SO}_3^- \) stretching band left (top). Middle spectrum of Ag-MES before \( \text{H}^+ \) was normalized to the 795 cm\(^{-1}\) band intensity with the scaling factor of 0.25 to illustrate differences in relative intensities of the most prominent band at 795 cm\(^{-1}\) and the \( \text{SO}_3^- \) symmetric stretching band.
Figure 32 Intensity ratio of symmetric stretching C-SO$_3^-$ vibrational band in the SERS spectrum of MES to the marker band at 1035 cm$^{-1}$ plotted against the intensity ratio of C-S bands for trans and gauche conformers for different samples. The plot shows that these two values are proportional. Since a change of $I_{\text{trans}}/I_{\text{gauche}}$ indicates structural changes in the monolayer, such a relation suggests that $I_{\text{C-SO}_3^-}/I_{\text{marker}}$ is also influenced by the structure of the monolayer.

Spectra collected from the studied nanomaterial showed good stability over time. In the span of 2 hours, no major fluctuations of SERS intensities of the two bands at 795 cm$^{-1}$ and 1035 cm$^{-1}$ were detected (Figure 33). However, subsequent measurements after almost 5 days revealed a decrease of the monitored intensities. Within the next several hours, both bands also exhibited good reproducibility. These observations were supported by the time evolution of the Ag-MES@SiO$_2$ UV-Vis spectra (Figure 34). Up to 120 minutes after the synthesis, the extinction maximum was constant. However, blue shift was seen already after two days. It was accompanied by the decrease in the peak intensity. This could be the sign of gradual etching of the SiO$_2$ layer, which resulted in a change of the local dielectric constant around the plasmonic core.\textsuperscript{132}
Experimental section

Ag-MES@SiO₂ NPs: testing silica permeability to metal cations

Figure 33 Integrated intensities of the two most intensive SERS bands of MES on Ag-MES@SiO₂, that is 795 and 1035 cm⁻¹ (blue squares and red triangles, respectively), plotted against the time after the synthesis. Notice a break between 150 and 6800 min (over 4 days) on the time axis.

Figure 34 Temporal changes in UV-Vis spectra of Ag-MES@SiO₂; time after the synthesis (in minutes) is given in the legend.

As no drastic variations in the SERS spectra from Ag-MES@SiO₂ NPs over time were detected, no loss of the Raman reporter molecules from beneath the silica shell might be assumed. Permeability of the SiO₂ layer to MES molecules was tested by adding MESNa to the suspension of Ag-MES@SiO₂ NPs (final concentration of MESNa: 2×10⁻³ M). As can be seen in the Figure 35, this affected the SERS spectral features. The band at 795 cm⁻¹ grew significantly and relative intensities between the band of trans and gauche conformers at 705 cm⁻¹
and 630 cm\(^{-1}\) inverted. When we realize that the cation-sensitive band 2 also appeared in the spectrum, we can conclude that all these changes were caused by the sodium cations from the added salt which penetrated silica. Interactions between the cation and the anions on the surface led to the structural adjustment of the monolayer and the expected spectral changes (compare considerations above). At the same time, intensity of the asymmetric stretching vibration band of the SO\(_3^-\) group at 1300 cm\(^{-1}\), which does not depend on the conformation of the molecule, remained almost unaffected. This shows that large MES anion did not permeate the silica layer alongside the cations and did not adsorb on the silver core.

**Figure 35** SERS spectra of MES encapsulated in Ag-MES@SiO\(_2\): before (solid line) and after the addition of 2 \(\times\) \(10^{-3}\) M MESNa molecules to the Ag-MES@SiO\(_2\) colloid (dashed line).

Cation permeation through the silica shell observed in the experiment was extensively addressed in the next step. Presence of the studied cation under the SiO\(_2\) layer near the metal core was assessed based on the SERS spectra of MES. Response of Ag-MES@SiO\(_2\) was to some extent similar to the trend observed for Ag-MES nanosensor. Once cations were present in the solution, band 2 appeared alongside band 1. Its position varied depending on the studied cation. Also, the higher the ion concentration was, the bigger the intensity gain of band 2 relative to band 1 was detected. These effects indicate that the cation was able to penetrate
the structure of silica and interacted electrostatically with MES anions adsorbed on the Ag nanoparticle inside the shell. Nevertheless, if we compare the response of both systems: Ag-MES and Ag-MES@SiO$_2$ exposed to the analyte in the same concentration: $1 \times 10^{-1}$ M, we can conclude that the marker bands behaved differently in each case (Figure 36). **Band 2**, assigned to the sulphonate groups which create ion pairs with cations, grew noticeably less in the spectra collected from silica-covered nanostructures. While the ion concentration in the solution was equal in both experiments, silica layer introduced a kind of barrier that separated cations and the Raman reporter. MES molecules interacted only with those cations which went through the SiO$_2$ coating. Deprotonated, negatively charged silanol groups in the nanopores of the shell most likely supported this form of transport. Therefore, in the SERS spectra from Ag-MES@SiO$_2$ we see the “effective” concentration of the ions present by the ion-responsive monolayer at the Ag core. The results were consistent for all the considered cations: Na$^+$, K$^+$, Mg$^{2+}$ and Ca$^{2+}$ but the intensity alterations of band 2 due to the silica shell looked different for each of them.

Quantitative analysis of the spectra in Figure 36 brought information about the “effective” concentration of the cations inside the nanostructure. Intensity ratio of the marker bands: $I_1/I_2$ studied for Ag-MES NPs in Chapter 3, was associated with the concentration of the ions interacting directly with MES anions on the surface of Ag NPs. We can expect that the Raman reporter gives the same SERS response for the same amount of the analyte present at the Ag core for Ag-MES and Ag-MES@SiO$_2$, regardless of the ion concentration outside silica. Calculations based on that assumption are gathered in Table 3. The response of Ag-MES@SiO$_2$ nanosensor ($I_1/I_2$) was related to the calibration curve of the respective cation for Ag-MES and the concentration corresponding to this value was determined. This way, the **effective** concentration of the ion was evaluated. Obviously, for each cation, it was lower than $1 \times 10^{-1}$ M, concentration outside the protective shell. The effective concentration increased in the following order: Mg$^{2+} < K^+ < Na^+ \approx Ca^{2+}$. Except for magnesium, this trend corresponds to the decreasing ionic radii of the cations (0.138 nm for K$^+$, 0.102 nm for Na$^+$).
and 0.100 nm for Ca\textsuperscript{2+}).\textsuperscript{125} Even though it was easier for smaller cations to permeate the silica, the smallest cation of the set – magnesium – did not pass through the SiO\textsubscript{2} coating in the biggest amount. On the contrary, its effective concentration was the lowest among the tested analytes. Magnesium ion is known to be surrounded with a strongly bound hydration shell – its hydration energy is high in comparison with the rest of the listed cations. Therefore, water molecules stuck to the cations so firmly that efficient transport of magnesium through the silica structure was inhibited.

![Figure 36](image)

**Figure 36** Differences in the behaviour of the ion-sensitive SERS bands of MES in the $10^{-1}$ M solutions of four metal cations: K\textsuperscript{+}, Na\textsuperscript{+}, Mg\textsuperscript{2+} and Ca\textsuperscript{2+} for monolayers adsorbed on: bare Ag NPs (a: red spectra) and on the Ag core of Ag@SiO\textsubscript{2} (b: blue spectra). The position of the higher-wavenumber marker band characteristic for the respective ion is indicated in the figures.

Comparison between the response of Ag-MES and Ag-MES@SiO\textsubscript{2} nanosensors was carried out not only in a relatively high concentration of $1 \times 10^{-1}$ M
but over a broader range: $1 \times 10^{-7} - 1 \times 10^{-1}$ M. Behaviour of both systems exposed to $K^+$ ions is presented in Figure 37. Marker bands disclosed a similar trend throughout the whole range: $I_2$ was less intensive in case of Ag-MES@SiO$_2$. It manifested as higher values of $I_1/I_2$ in the concentration-dependent sensor response plot (Figure 37c), especially for lower concentrations. As a result, the calibration curve for silica-coated nanosensors had a steeper slope: increase of the concentration of one order of magnitude was followed by a bigger change in the sensor response. Thus, Ag-MES@SiO$_2$ can be said to exhibit improved sensitivity towards cations.

**Table 3** $I_1/I_2$ intensity ratios for Ag-MES@SiO$_2$ exposed to $1 \times 10^{-1}$ M of the respective ion and the estimation of the effective concentration of the ion at the surface of the Ag core. Concentration was determined based on the calibration curves obtained for Ag-MES NPs$^{128}$.

<table>
<thead>
<tr>
<th>Cation</th>
<th>$I_1/I_2$ on Ag-MES@SiO$_2$</th>
<th>Effective concentration /M</th>
<th>Ionic radius$^{125}$/nm</th>
<th>Hydration enthalpy$^{126}$/kJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>0.86</td>
<td>$1.5 \times 10^{-2}$</td>
<td>0.100</td>
<td>-1577</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>0.84</td>
<td>$3.5 \times 10^{-2}$</td>
<td>0.102</td>
<td>-409</td>
</tr>
<tr>
<td>K$^+$</td>
<td>0.67</td>
<td>$4.6 \times 10^{-3}$</td>
<td>0.138</td>
<td>-322</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>1.48</td>
<td>$&lt;1.8 \times 10^{-4}$ (under LOD)</td>
<td>0.072</td>
<td>-1921</td>
</tr>
</tbody>
</table>
Figure 37 Comparison between the SERS spectra of MES from (a) Ag-MES and (b) Ag-MES@SiO$_2$ nanoprobes in KCl solutions of $10^{-1}$-$10^{-7}$ M; negative logarithm of $K^+$ concentration is indicated in the legend. Spectra were baseline corrected and shifted. (c) Comparison between Ag-MES (red triangles) and Ag-MES@SiO$_2$-based (blue squares) sensor response.
Figure 38 Time changes in the ion-sensitive region of the SERS spectra of MES for Ag-MES@SiO\textsubscript{2} exposed to K\textsuperscript{+} ions of 10\textsuperscript{-1} M (left) and 10\textsuperscript{-4} M (right) for a prolonged time. The spectra were baseline corrected and normalized with respect to the lower-frequency band (I\textsubscript{l}) so that the direct comparison between intensities of the bands assigned to MES interacting directly with the cation was possible.

As the transport through the silica coating is a dynamic process, the situation might evolve in time. Results described above were obtained shortly after introducing the cation in the sample. Temporal measurements shed some light on the kinetics of the silica permeability to ions. Since some cations were already detected by the Ag core, it was proved that some instant equilibrium between inner and outer side of the shell was set. In the span of several hours, the effective concentration of the cation was rising in a rate depending on its total amount in the sample. When a high concentration of K\textsuperscript{+} was applied (1×10\textsuperscript{-1} M), originally strong band 2 rose significantly after 3 hours (Figure 38); its intensity remained unchanged after a prolonged time. Here, the flow of ions through SiO\textsubscript{2} continued at a fast pace over short time, driven by the high content of K\textsuperscript{+} in the solution outside of the shell. On the other hand, lower concentration of the ion (1×10\textsuperscript{-4} M) induced more subtle temporal changes in the marker bands outline. Band 2 kept growing...
slowly over 15 hours, as this concentration was too low to force a high flow of the ions continuously in time. Due to this effect, sample analysis requires a meticulous choice of conditions: calibration and concentration determination need to be carried out in the same time frame so that the results can be relatable.
5.3 Conclusions

Studies on Ag-MES@SiO$_2$ NPs can be summed up in several points:

- adsorbed MES molecules facilitated growth of the silica layer on the Ag core, mainly as a protective layer against dissolving in ammonia, but also as a stabilizing agent in isopropanol;
  - SERS signal collected from Ag-MES@SiO$_2$ NPs was stable in time;
  - relatively thick silica shell of ~30 nm was penetrated by various metal cations, as proved by changes in the SERS spectra of MES;
  - amount of ions reaching the encapsulated core increased over time;
  - different cations displayed different ability to move across the protective coating; the transport was easier for smaller ions;
  - factors other than size also played a role in the process, seen on the example of the reduced mobility of strongly hydrated magnesium cation;
  - silica coating did not impair the ability of the nanosensor to determine cation concentration; differences in the SERS response between Ag-MES and Ag-MES@SiO$_2$ resulted in the improved sensitivity of the latter.
6 Developing new plasmonic nanosubstrates for intracellular SERS

6.1 Experimental

All the chemicals were purchased in Sigma Aldrich in at least 99% purity. Transmission electron microscopy (TEM) images were obtained in a field-emission high-resolution Philips JEOL JEM-2100F electron microscope, at an acceleration voltage of 200 kV. Samples for TEM analysis were prepared by drop-casting the diluted dispersions of nanoparticles holey carbon-coated Cu mesh grids (400 mesh) and air-dried at room temperature. SERS measurements were performed with an InVia Reflex Raman system (Renishaw) comprising a microscope (Leica), a 785 nm laser excitation source (nominal output 260 mW) and a spectrometer equipped with a 1200 grooves per mm diffraction grating and a front-illuminated Peltier-cooled CCD detector (1024 pixels x 512 pixels). Spectra were accumulated 5 times and averaged before further processing.

6.2 Results and discussion

6.2.1 Silver-coated gold nanostars

The aim of the project was to synthesize plasmonic nanoparticles to apply in intracellular SERS. As a Raman tag, 2-mercaptopethanesulphonate (MES) molecule was used. Gold nanoparticles, due to their biocompatibility, have broad applications in biodetection.\textsuperscript{133} In order to overcome intrinsically weak enhancing properties of gold, several designs of gold-silver nanosystems were tested as well. Functionalized nanoparticles with the biggest enhancement factor were introduced into live cells and their applicability as a cation sensor was determined.

Gold nanostars (AuNS) were produced in a standard citrate-free synthesis.\textsuperscript{134} pH of 10 ml of 0.25 mM HAuCl\textsubscript{4} was adjusted with 5 µl of 1M HCl. Subsequently, 500 µl of Au seeds (Turkevich method) were added. Under vigorous stirring, 100 µl
of 3 mM AgNO₃ and 50 µl of 100 mM ascorbic acid (AA) were injected simultaneously. Immediate colour change from red to blue was observed, which proved that the stars synthesis reaction was completed. To prevent the stars from aggregating, the mixture was left stirring for 5 minutes with 100 µl of 100 mM cetyltrimethylammonium bromide (CTAB). The stars were centrifuged (20 min, 2000 rpm) to get rid of unreacted reagents.

Obtained nanostars were further covered with silver. Equal volumes (between 2 and 8 µl) of 0.1 M solutions of AgNO₃ and AA were added to 2 ml of AuNS under vigorous stirring. Subsequently, 4 µl of 25% ammonia was injected. Minor change in the colour of the suspension was observed, which suggested that the Ag layer was formed (Ag@AuNS). Nanoparticles were cleaned by centrifugation after 5 minutes of reaction. UV-Vis spectra of the samples obtained with various volumes of the reactants (AgNO₃ and AA) are presented in Figure 39. The addition of higher amount of AgNO₃ induced bigger blue shift of the extinction maximum. In the most extreme case, almost 200 nm shift was achieved. Such changes in the UV-Vis spectra were caused by the growth of a silver layer on Au stars. It was confirmed with TEM images (Figure 40): the nanostars were more and more round and branches were harder to discern as the amount of AgNO₃ used in the synthesis increased.

<table>
<thead>
<tr>
<th>Sample</th>
<th>λₘₐₓ/μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>701</td>
</tr>
<tr>
<td>2</td>
<td>544</td>
</tr>
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<tr>
<td>4</td>
<td>521</td>
</tr>
<tr>
<td>5</td>
<td>517</td>
</tr>
<tr>
<td>6</td>
<td>513</td>
</tr>
<tr>
<td>7</td>
<td>512</td>
</tr>
<tr>
<td>8</td>
<td>513</td>
</tr>
</tbody>
</table>

**Figure 39** UV-Vis spectra of Ag@AuNS samples. Name of the sample reflects the volume of AgNO₃ and AA added during the synthesis (in µl).
Ag@AuNS were tested as SERS substrates. Two different Raman tags were used: p-mercaptobenzoic acid (pMBA) and 2-mercaptoethanesulphonate (MES). As an indication of the SERS enhancement obtained on the nanostructures, intensity of 795 cm\(^{-1}\) band was used in case of MES. For pMBA, intensity of 1580 cm\(^{-1}\) band was normalized to 880 cm\(^{-1}\) band of ethanol spiked in the same amount in all samples (\(I_{\text{pMBA}}/I_{\text{standard}}\)). In Figure 41, SERS intensities are plotted against the volume of AgNO\(_3\) used to synthesize respective samples of Ag@AuNS.

*Figure 40* TEM images of A AuNS, B-D Ag@AuNS synthesised with use of: B 3 µl, C 5 µl and D 7 µl AgNO\(_3\).
Thickness of Ag shell had an influence on the intensity of the spectra for both Raman tags. Gold nanostars covered with Ag layer with use of 6 µl of AgNO₃ yielded the strongest SERS spectra. Further increase of Ag shell thickness above the optimal value led to decline in SERS enhancement, probably due to the absence of the gold branches in the structure of nanoparticles.

**Figure 41** SERS intensities of MES (blue squares, y left scale) and pMBA (red triangles, y right scale) on Ag@AuNS with varied amount of Ag. λₑₓc =785 nm.

The main goal was to obtain SERS-active branched gold nanostars with silver tips, all within mesoporous silica shell. Gold nanoseeds were synthesized according to the Turkevich procedure. Before the synthesis of the silica coating, citrates were exchanged for CTAB molecules, which later acted as a template for pores in silica. SiO₂ layer was grown as follows: 32.64 ml of 100 mM CTAB, 528.36 ml of water and 216 ml of ethanol were mixed together. pH was adjusted to the value of around 10 by adding 1.6 ml of 5% ammonia. Next, 23 ml of 2 mM AuNP in 50 mM CTAB was poured into the mixture. In the end, 640 µl of TEOS was added dropwise. The solution was heated to 60°C. After two hours, it turned turbid due to formation of silica. The reaction was complete after 42 hours. Nanoparticles were centrifuged (20 min, 8500 rpm, 35°C) and then redispersed in 1 M HCl in ethanol to get rid of CTAB from the silica pores. This was followed by three more cleaning steps with H₂O.
Since the silica layer is not stable in water, calcination was performed. Firstly, the sample was left in the oven in 60°C until the solvent evaporated. Then, the seeds were heated for 2 hours in 200°C. During calcination, some silica shells melted together, preventing from complete redispersion in ethanol, therefore several steps of sonication in ethanol were applied. During transferring to water, nanoseeds were concentrated two times. Silica coated seeds of high uniformity (Figure 42) were used in further synthesis.

![Figure 42 TEM images of silica coated gold nanoseeds.](image)

Au@mSiO$_2$ (gold nanoseeds encapsulated in mesoporous silica) were branched analogously to the stabilizer-free nanostars synthesis. Branches are grown after simultaneous addition of AgNO$_3$ and ascorbic acid (AA) to nanoseeds in acidic solution of HAuCl$_4$. Applying initial conditions of the synthesis led to uncontrolled secondary nucleation of gold (and as a result, gold stars) outside of silica. Therefore, in our synthesis, concentration of HAuCl$_4$ and the volume of Au@mSiO$_2$ were changed. Parameters for the discussed series are given below:

- 10 ml of HAuCl$_4$: 5, 7 and 10 µl of 0.1263 M Au$^{III}$ solution were diluted to 10 ml, resulting in 0.06 mM, 0.09 mM and 0.13 mM, respectively (samples Au5, Au7, and Au10);
- 10 µl 1M of HCl;
- 666 µl of Au@mSiO$_2$ seeds;
- 100 µl 3 mM AgNO$_3$;
- 50 µl 0.1 M AA.

The mixture changed the colour immediately from red to blue after the addition of AgNO₃ and AA. Thanks to adjusting synthesis conditions, secondary nucleation was remarkably limited in these samples, especially for the one with the lowest concentration of Au³⁺ used. Even for Au7 and Au10, potato-shaped, hollow nanostructures were the main structures observed outside silica instead of free nanostars (Figure 43). Size of the gold stars inside silica increased with the amount of Au³⁺. The sample with the highest concentration of HAuCl₄, Au10, developed the biggest stars inside SiO₂, which led to progressing disintegration of the shell (Figure 43C).

Figure 43 TEM images of the samples A Au5 B Au7 C Au10 before purification.
Even though the size of AuNS@mSiO$_2$ was satisfactory, secondary nucleation free nanoparticles outside silica still posed a problem. In order to get rid of them, two step purification was performed. Firstly, the sample was left until a part of the nanoparticles precipitated. The supernatant was then centrifuged shortly under low speed (5 minutes, 1600 rpm) to get rid of the rest of big free gold clusters. Remaining nanoparticles were stabilized with CTAB solution (Figure 44).

![TEM images of the samples A Au5 B Au7 C Au10 after purification.](image)

**Figure 44** TEM images of the samples A Au5 B Au7 C Au10 after purification.

AuNS@mSiO$_2$ were subsequently covered with silver in the same way silver coating is obtained on gold nanostars. Equal volumes of 2 ml of 0.1 M solutions of silver nitrate and ascorbic acid were added to nanoparticles under vigorous
stirring. Subsequently, 4 µl of 25% ammonia was injected. Minor change in the colour of the suspension could be observed, which suggested formation of Ag layer. Stirring speed was then decreased. Nanoparticles were cleaned by centrifugation after approximately 10 minutes of reaction (2x15 minutes, 3000 rpm). First striking observation revealed by TEM images (Figure 45) is that nanoparticles in the sample Au7 lost their silica coating during the process. Again, it was probably caused by the increasing size of metallic core over the disintegration point. In addition to Ag-coated stars, there was also a significant number of more spherical nanoparticles, which most likely were just overcoated stars. In sample Au5, where mSiO₂ layer survived the experiment, some silica-free nanoparticles could be seen. In further experiments, Ag concentration must be meticulously adjusted to prevent from overgrowing golden stars with too much silver.

Sample with the biggest golden cores, therefore with the largest surface available for Ag atoms, was used to determine distribution of atoms in the nanostructure. It was done by EDX mapping. Figure 46 presents a STEM image and a detailed distribution map of gold and silver in the nanoparticle. Mapping showed that gold was indeed the major component of the structure (Figure 46B and D). Silver, however, was spotted only at the surface of the nanoparticle (Figure 46C). It was localized mostly in the cavities between the branches. It is worth pointing out that this result is distorted by several factors. First, signal of a chosen element is proportional not only to its amount but also to the thickness of the sample. When the beam comes through the branches of nanostars, it collects data mostly about the dominant element, which is gold. Silver, present only as a thin layer, is less prominent. This way, higher probability to detect Ag occurs in hollow spaces near the core of the gold star. Another aspect is the heating of the sample. Collecting an EDX map presented in Figure 46 took ca. 40 minutes. Throughout all this time, the sample was exposed to the electron beam. In such conditions, Ag atoms are prone to thermodiffusion and change location. This makes determination of its initial localization by STEM difficult. In order to verify distribution of elements in the examined structure, further experiments should be carried out.
Unfortunately, many steps of the synthesis and loss of the nanomaterial throughout the procedure made the control of the final NP concentration and the acquisition of reliable SERS spectra with use of these NPs extremely difficult. Therefore, more effort needs to be put to obtain conclusive results of the SERS enhancement of the nanostructures, as well as to produce NPs of better quality.

Figure 45 TEM images of the samples A Au5 B Au7 C Au10 after Ag coating.
Figure 46 A STEM image of a selected Ag-coated AuNS@mSiO₂ nanoparticle for sample Au10, B EDX map presenting distribution of silver (red) and gold (green), C distribution of silver in the nanoparticle, D distribution of gold in the nanoparticle.
6.2.2 SERS performance of Janus gold-iron oxide nanostars covered with silver

The aim of the study was to cover Janus plasmonic-magnetic nanoparticles with a thin layer of silver. Initially, each nanoparticle consisted of a magnetic sphere made of iron oxide and a plasmonic gold nanostar (Janus magnetic nanostars, JMNS). After growing the silver shell (JMNS@Ag), the nanoparticles were functionalized with a Raman tag so that their efficiency as substrates in surface-enhanced Raman scattering (SERS) spectroscopy could be evaluated. Comparison between JMNS and JMNS@Ag was made without magnetic field and after magnetic concentration.

**Characterization of the nanomaterial**

JMNSs were synthesized according to the previously described procedure.\textsuperscript{136} Nanoparticles used in this study (batch JR64) had flakey morphology (Figure 47a), which makes them slightly different than the usual spikey nanostars. Mean diameter of nanoparticles was 59.8±11.0 nm (statistics on more than 200 nanoparticles, Figure 47b).

![TEM image of JMNSs and their distribution of size.](image)

*Figure 47 a) TEM image of JMNSs and b) their distribution of size.*
JMNSs were covered with silver in the same way silver coating was obtained on gold nanostars. Since Ag coating of PVP-stabilized JMNSs was proved to be unsuccessful, nanoparticles were stabilized with CTAB. Equal volumes of 0.1 M solutions of silver nitrate and ascorbic acid were added to nanoparticles ([Au0] = 0.24 mM, diluted around 3 times from the initial concentration of the batch) under vigorous stirring. Subsequently, 4 µl of 25% ammonia was injected. Minor change in the colour of the suspension could be observed, which suggested formation of Ag layer. Nanoparticles were cleaned by centrifugation after 5 minutes of reaction. Samples were called JMNS@xAg, where x is the volume of AgNO3 and ascorbic acid (AA) used in the reaction of Ag coating.

TEM images of JMNS@Ag unveiled no drastic change neither in nanoparticles morphology (Figure 48) nor in the mean diameter of nanoparticles (59.8±7.6 nm; however, the statistics included only 27 nanoparticles, therefore the error is higher). Ag layer obtained on the surface was too thin to be detected. Moreover, despite cleaning the samples, some secondary nucleation of Ag in the solution was observed in the form of small nanoparticles in the background. This effect can be eliminated in further experiments by e.g. changing the kinetics of the reaction through cooling the reagents or decreasing concentrations of used solutions.

*Figure 48* TEM image of JMNSs@6Ag.
Even though Ag coating could not be directly seen on the collected TEM images (without e.g. EDX mapping), changes in extinction spectra proved it indirectly. UV-Vis spectra of the JMNS@Ag samples are presented in Figure 49. As the amount of silver used in the reaction was increased, the maximum of the plasmon band shifted towards shorter wavelengths and this trend was reproducible among different series of JMNS@Ag synthesized on different days. Change in the plasmonic properties of JMNSs confirmed that Ag was deposited on the surface of the nanoparticles. However, when 10 µl of AgNO₃ was used in the reaction, the plasmonic band broadened and the background intensity drastically increased. This suggests that uncontrolled aggregation of the nanoparticles occurred.

![Figure 49](https://example.com/figure49.png)

**Figure 49** UV-Vis spectra of JMNS@Ag. Volume (in µl) of added AgNO₃ and AA is indicated in the legend. Spectra are normalized to unity in the maximum of the peak. Table: wavelength of the maximum for every spectrum.

<table>
<thead>
<tr>
<th>Volume of AgNO₃ and AA/µl</th>
<th>Wavelength of the maximum/nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>743</td>
</tr>
<tr>
<td>2</td>
<td>701</td>
</tr>
<tr>
<td>4</td>
<td>673</td>
</tr>
<tr>
<td>6</td>
<td>657</td>
</tr>
<tr>
<td>8</td>
<td>645</td>
</tr>
<tr>
<td>10</td>
<td>647 (broad)</td>
</tr>
</tbody>
</table>

**SERS experiments**

Obtained JMNS@Ag were tested as SERS substrates. For this purpose, nanoparticles were functionalized with a Raman tag, p-mercaptopbenzoic acid (pMBA). It is characterized by a strong SERS spectrum with well-defined bands.
at 1080 and 1580 cm\(^{-1}\) corresponding to the \(v_{12}\) and \(v_{8a}\) ring vibrations (Figure 50).\(^{38}\) Based on the intensity of the spectrum, comparison between JMNS and JMNS@Ag was carried out. All spectra were excited with 785 nm laser, 5 acquisitions with integration time 5 seconds each were averaged to give a final spectrum. Two concentrations of pMBA were applied: 1 mM and 10 µM.

**Higher concentration of the Raman tag**

*Effect of silver coating*

Firstly, enhancement of the spectrum due to the silver coating was examined. Intensity of the SERS band of 1 mM pMBA at 1080 cm\(^{-1}\) was normalized with respect to the ethanol band at 880 cm\(^{-1}\) which might be treated as an internal reference (ethanolic solution of pMBA was added in the same amount to all the samples in the series). For JMNSs + 1 mM pMBA, this “normalized intensity” was equal to 1.57. At least two times more intensive SERS spectra were collected with JMNS@Ag samples, reaching maximum of 4.29 for JMNS@2Ag (Table 4). Typically, extinction of nanostructures is the factor determining their SERS enhancement factor. Here, extinction of JMNS@Ag in 785 nm was lower than for JMNS. The opposite, unexpected behaviour of SERS intensity suggests that the effect is caused by the silver coating of nanostars.
Figure 50 SERS spectra of 1 mM pMBA adsorbed on JMNS (black line) and JMNS@2Ag (red line). Most prominent bands of pMBA are marked with circles, the band of ethanol used for normalization of the spectra is marked with a rhombus.

Table 4 Intensities of the pMBA band at 1080 cm\(^{-1}\) normalized to the ethanol band at 880 cm\(^{-1}\) (\(c_{pMBA} = 1\) mM) and enhancement factors (EF) with respect to Ag-free nanoparticles. In the samples called JMNS@\(x\)Ag, \(x\) denotes ml of AgNO\(_3\) and AA used in the synthesis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(I_{pMBA}/I_{EtOH})</th>
<th>EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMNS</td>
<td>1.57</td>
<td>1</td>
</tr>
<tr>
<td>JMNS@2Ag</td>
<td>4.29</td>
<td>2.73</td>
</tr>
<tr>
<td>JMNS@6Ag</td>
<td>3.83</td>
<td>2.43</td>
</tr>
<tr>
<td>JMNS@8Ag</td>
<td>3.68</td>
<td>2.34</td>
</tr>
<tr>
<td>JMNS@10Ag</td>
<td>3.33</td>
<td>2.12</td>
</tr>
</tbody>
</table>

Effect of magnetic confinement

The following step of the research was to assess the performance of the JMNS@Ag nanostructures in presence of magnetic field. Each sample (nanoparticles coated with pMBA) was introduced into a capillary and two SERS
spectra were collected: before and 30 minutes after placing a magnet near the solution (Figure 51). In this time, nanoparticles formed magnetically driven aggregates. As listed in Table 5, intensity of the pMBA band at 1080 cm$^{-1}$ is substantially higher for JMNS@Ag compared to JMNS both without a magnet ($I_{\text{cap}}$) and with a magnet ($I_{\text{mg}}$). It supports conclusions from the previous section about the influence of the silver layer on increasing SERS efficiency of the nanostructures. Ratio of these two intensities is called magnetic enhancement factor (MEF) and it says how many times higher the signal is after magnetic aggregation. Surprisingly, it turned out that the biggest gain was observed for JMNSs. Silver-coated samples exhibited lower MEFs and the optimal value was obtained for JMNS@6Ag. This might be caused by quenching of E-field in hot spots due to proximity of branches with different orientations. As the nanostars have flakey structure, flat surfaces make it easier for nanoparticles to get closer to each other in the aggregate. Additional shell on the surface of nanoparticles might have an impact on this effect, however, the role of silver in this case should still be investigated.

**Table 5** Intensities of SERS spectra of pMBA before and after magnetic aggregation based on the intensity of the band at 1080 cm$^{-1}$(c$_{\text{pMBA}}$ = 1 mM).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$I_{\text{cap}}$</th>
<th>$I_{\text{mg}}$</th>
<th>MEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMNS</td>
<td>1185</td>
<td>46456</td>
<td>39</td>
</tr>
<tr>
<td>JMNS@2Ag</td>
<td>4225</td>
<td>70673</td>
<td>17</td>
</tr>
<tr>
<td>JMNS@6Ag</td>
<td>3856</td>
<td>78187</td>
<td>20</td>
</tr>
<tr>
<td>JMNS@8Ag</td>
<td>4284</td>
<td>40594</td>
<td>9</td>
</tr>
<tr>
<td>JMNS@10Ag</td>
<td>3286</td>
<td>40529</td>
<td>12</td>
</tr>
</tbody>
</table>
Figure 51  Comparison between SERS spectra of 1mM pMBA on JMNS (right window) and JMNS@6Ag (left window) in two situations: before (black spectra) and after magnetic aggregation (red spectra). Both windows have the same intensity scale.

**Lower concentration of the Raman tag**

Since the main purpose of SERS measurements with magnetically confined nanosubstrates is nanodetection, the same experiments were carried out with JMNS@Ag functionalized with a lower concentration of the Raman tag. Final concentration of pMBA was 10 µM. Once again, after taking normalization with respect to the ethanol band into account, SERS spectra of pMBA collected from JMNS@Ag were more intense than from nanoparticles without silver coating (Table 6); JMNS@2Ag was the sample characterized by the biggest gain (over 2 times).

In this situation, influence of magnetic concentration on SERS intensity was also more pronounced. For JMNSs, spectrum of pMBA was as high as 150 more intensive after the use of a magnet. As it was in the case of higher concentration of the Raman tag, JMNS@Ag exhibited lower MEFs (Table 7). However, this time
it rose with the amount of AgNO$_3$ used in the preparation of nanoparticles (except for JMNS@4Ag where no significant increase of the spectrum intensity was observed). It is worth pointing out that for some measurements in this series partial decarboxylation of pMBA was detected.$^{38}$ Conditions applied in these measurements: low surface concentration of the molecules and high laser power favoured the process. This effect might have disturbed the final results.

**Table 6** Intensities of the pMBA band at 1080 cm$^{-1}$ normalized to the ethanol band at 880 cm$^{-1}$ ($c_{pMBA} = 10$ µM) and enhancement factors (EF) with respect to Ag-free nanoparticles.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$I_{pMBA}/I_{EtOH}$</th>
<th>EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMNS</td>
<td>4.19</td>
<td>1</td>
</tr>
<tr>
<td>JMNS@2Ag</td>
<td>9.00</td>
<td>2.14</td>
</tr>
<tr>
<td>JMNS@4Ag</td>
<td>6.29</td>
<td>1.50</td>
</tr>
<tr>
<td>JMNS@6Ag</td>
<td>7.53</td>
<td>1.80</td>
</tr>
<tr>
<td>JMNS@8Ag</td>
<td>7.07</td>
<td>1.69</td>
</tr>
</tbody>
</table>

**Table 7** Intensities of SERS spectra of pMBA before and after magnetic aggregation based on the intensity of the band at 1080 cm$^{-1}$ ($c_{pMBA} = 10$ µM).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$I_{cap}$</th>
<th>$I_{mg}$</th>
<th>$MEF$</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMNS</td>
<td>481</td>
<td>72735</td>
<td>151</td>
</tr>
<tr>
<td>JMNS@2Ag</td>
<td>2942</td>
<td>70700</td>
<td>24</td>
</tr>
<tr>
<td>JMNS@4Ag</td>
<td>1344</td>
<td>3605</td>
<td>3</td>
</tr>
<tr>
<td>JMNS@6Ag</td>
<td>1433</td>
<td>78028</td>
<td>54</td>
</tr>
<tr>
<td>JMNS@8Ag</td>
<td>615</td>
<td>45991</td>
<td>75</td>
</tr>
</tbody>
</table>
6.3 Conclusions

6.3.1

- Gold nanostars were successfully coated with a silver shell of different thickness.
- Intensity of SERS spectra for both tested Raman tags reach maximum for the Ag@AuNS of the same Ag thickness. This proves that gold branches which disappear under the thicker Ag shell play the biggest role in SERS enhancement.
- Excess of Au\textsuperscript{III} in the synthesis of AuNS@mSiO\textsubscript{2} lead to secondary nucleation of Au in a form of irregular empty potato-like nanoparticles.
- Silica shell has a tendency to disintegrate due to the excessive growth of the AuNS inside the shell.
- EDX map shows that silver-coated AuNS@mSiO\textsubscript{2} consist mainly of Au with Ag detected in the hollow spaces between the branches near the core of the star.

6.3.2

- Silver coating of JMNS was proved based on the shift in UV-Vis spectra,
- JMNS@Ag were demonstrated to be better SERS substrates than JMNS, exhibiting even over 3 times higher enhancement factors,
- gain in SERS intensity achieved after magnetic concentration turned out to be higher for Janus particles without silver coating, which may be due to additional mechanism of quenching hot-spot activity by Ag layer,
- after decreasing the concentration of the Raman tag to 10 µl, MEFs went higher, which makes the nanostructures promising substrates for use in SERS nanodetection.
7 Carbazole derivatives: towards quantitative detection of anions

7.1 Experimental

Preparation of the samples for PM-IRRAS measurements Au supports for PM-IRRAS (polarization modulation infrared reflection absorption spectroscopy) experiments were obtained by subsequent sputter coating of thin layers of Cr (5 nm) and Au (25 nm) on a microscope glass slide cleaned in ethanol under sonication. Derivatives of diamidocarbazoles were adsorbed on Au substrates from $10^{-3}$ M solutions in ethanol for 2 days.

For sulphate concentration dependent measurements, tetrabutylammonium sulphate (TBA)$_2$SO$_4$ solutions in acetonitrile were used. PM-IRRAS measurements were carried out after immersing the Au slide covered with the layer of respective diamidocarbazole derivative in acetonitrile solution of (TBA)$_2$SO$_4$ in the range $10^{-6} – 10^{-1}$ M for 5 minutes, rinsing with a solvent and leaving it to dry. Measurements in water were carried out analogously. Aqueous solutions of (TBA)$_2$SO$_4$ were prepared from 50% (TBA)$_2$SO$_4$ in water, solutions in acetonitrile were prepared from dried (TBA)$_2$SO$_4$.

To monitor direct interactions of sulphates with the Au surface, the Au coated slide which was not functionalized with carbazole derivatives was immersed in $10^{-2}$ M aqueous solution of (TBA)$_2$SO$_4$.

FT-IR spectra of carbazoles were measured in KBr pellets with ca. 1% w/w content of the studied compound.

Details of PM-IRRAS measurements PMIRRAS experiments were performed on the Thermo Nicolet 8700 spectrometer equipped with the external table–top optical mount, an MCT detector cooled with liquid nitrogen, a photoelastic modulator (PEM) (PM-100 Hinds Instrument, Hillsboro, OR) and a synchronous sampling demodulator (SSD) (GWC Instruments, Madison, WI). The spectrometer was coupled with an ATR (Attenuated Total Reflectance) accessory equipped with a diamond crystal. The IR spectra were acquired with the PEM set for the half-wave retardation at 1500 cm$^{-1}$. The angle of incidence was
set at 83°. Typically, 1000 scans were averaged per single spectrum, and the resolution was set at 4 cm\(^{-1}\).

**Details of SERS measurements** Raman spectra were collected on LabRAM HR800 (Horiba Jobin Yvon) Raman spectrometer with a charge-coupled device detector cooled by Peltier modulus. All the spectra were excited with a 633 nm He-Ne laser. Holographic grating with 600 grooves/mm was used. The spectrometer was coupled with Olympus BX61 confocal microscope with a pinhole set to 200 \(\mu m\). Backscattered light was collected through a 50x objective. Calibration of the system was performed with respect to 520 cm\(^{-1}\) silicon band. The samples for monitoring receptor-anion interaction were prepared by drop casting of 1x10\(^{-3}\) M solution of the receptor-anion complex (1:1 ratio) in acetonitrile and measured during drying. NR spectrum of (TBA)\(_2\)SO\(_4\) was collected from a deposited drop of 50% salt solution in water, SERS spectrum of (TBA)\(_2\)SO\(_4\) was recorded from a silver electrode dipped in 10\(^{-3}\) M salt solution in acetonitrile. Silver support for SERS measurements was prepared by electrochemical roughening of polycrystalline Ag plate in 0.1M KCl solutions. Five oxidation-reduction cycles between -0.3 and 0.3 V vs. Ag/AgCl were applied at sweep rate 5 mV/s.
7.2 Results and discussion

**PM-IRRAS experiments**

*Interpretation of the PM-IRRAS spectra*

We studied a family of new derivatives of diamidocarbazole which act as anion receptors. Amide bonds were used in the molecules to attach two substituents, one of which is a lipoyl group (Figure 52). Such a design provided strong binding of the receptor molecules to the Au surface through a sulphur-metal bond. The second substituent was a t-butyl, phenyl or lipoyl group. Performance of the receptors immobilized on the Au substrate in aqueous and acetonitrile solutions of (TBA)$_2$SO$_4$ was compared.

![Structure of the studied diamidocarbazole derivatives.](image)

**Figure 52 Structure of the studied diamidocarbazole derivatives.**

In order to check monolayer formation on Au and to monitor binding of sulphate anions to different diamidocarbazole-based anion receptors immobilized on the gold substrate, PM-IRRAS spectra were collected. Spectra of the studied monolayers formed on the Au surface exhibit similar features to these observed in the infrared spectra of the respective bulk samples (Figure 53). However, differences in relative intensities of the bands can be noticed; this may be easily explained in terms of the surface selection rules that apply to IRRAS spectra. The surface selection rule limits observable bands corresponding to infrared-active vibrations to those, whose transition dipole moment is perpendicular or inclined to the metal surface. Consequently, the vibrational modes with the biggest component of transition dipole moment perpendicular
to the Au surface will exhibit the highest relative intensity. This is, however, strongly dependent on the orientation of the receptor monolayer with respect to the Au surface, which is in turn determined by the intermolecular interactions between molecules in the monolayer. All studied compounds show differences between IR and PM-IRRAS spectra which indicate the all compounds form ordered layers on the gold surface.

Figure 53 Comparison between IR spectra of bulk samples and PM-IRRAS spectra of monolayers on Au for the studied diamidocarbazole derivatives.
Detection of $SO_4^{2-}$ anions in acetonitrile solutions

Series of PM-IRRAS spectra were recorded after immersing the Au-receptor samples in acetonitrile solutions of $(TBA)_2SO_4$ with gradually increasing concentration of the sulphate anions (Figure 54). As may be seen in Figure 54 there are some differences in the spectral changes provoked by sulphate anions between the samples. The spectra of t-butyl and phenyl derivatives (Figure 54a and b) reveal several common changes after exposing the substrate to sulphate solutions.
Figure 54 PM-IRRAS spectra of the studied diamidocarbazole receptor monolayers: a) t-butyl, b) phenyl, c) lipoyl derivative on Au before (blue spectrum, denoted as 0) and after the exposure to SO$_4^{2-}$ anions in different concentrations in acetonitrile (green spectra: i: $1 \times 10^{-6}$ M, ii: $1 \times 10^{-5}$ M, iii: $1 \times 10^{-4}$ M, iv: $1 \times 10^{-3}$ M, v: $5 \times 10^{-3}$ M, vi: $1 \times 10^{-2}$ M, vii: $5 \times 10^{-2}$ M, viii: $1 \times 10^{-1}$ M).

First, some changes in relative intensities of the receptor bands are clearly visible. As may be seen in Figure 54a and b, in the case of receptors substituted with one lipoyl group (R=t-butyl and R=phenyl ring), initially strong amide I band at 1645 cm$^{-1}$ becomes weaker and considerably broader after exposing the receptor monolayer to the sulphate solutions. The broadening of the band suggests that interactions with the anions and/or the solvent result in reorientation of some receptor molecules at the Au surface, which introduces a certain degree of disorder in the diamidocarbazole layer. However, for the receptor containing two lipoyl anchors (Figure 54c) no significant changes in the amide I band intensity can be seen throughout the series. It is consistent with the expected monolayer structure, as the receptor orientation is stabilized by both, the presence of two lipoyl anchors and possibility of π-stacking interactions between neighbouring carbazole rings in the monolayer, which make the structure of the monolayer more rigid.

The second clearly visible change in the series in Figure 54 is the appearance of a new feature whose intensity increases when the concentration of the sulphates
in the solution goes higher. This band is very broad and strongly asymmetric with a well-defined maximum about 1150-1165 cm\(^{-1}\) and probably consists of at least two strongly overlapping components (one around 1090-1100 cm\(^{-1}\) and the second around 1150 cm\(^{-1}\)). For sulphate concentrations higher than 5×10\(^{-3}\) M the band maximum shifts to lower frequencies down to 1090 cm\(^{-1}\) at 1×10\(^{-1}\) M. The new band emerging upon interaction of the receptor molecules with the sulphate solutions can be ascribed to the asymmetric stretching (\(\nu_3\)) mode of the SO\(_4^{2-}\) anion bound to receptor molecules. It is well known that symmetry reduction of the sulphate anion manifests the most clearly and strongly in the asymmetric stretching vibration band. In our case this may be caused by nonequivalent hydrogen bonding of oxygen atoms with NH groups and/or by interaction of SO\(_4^{2-}\) anion with the surface, which provoke splitting of triply (F) degenerated \(\nu_3\) mode. For unbound SO\(_4^{2-}\) anions belonging to the \(T_d\) point group this mode is active in the infrared spectrum, giving rise to a strong band around 1100 cm\(^{-1}\).\(^{138}\) As may be seen in Figure 55, which shows the ATR (attenuated total reflection) spectrum of (TBA)\(_2\)SO\(_4\), the band corresponding to the \(\nu_3\) mode of the unperturbed anion of \(T_d\) symmetry appears at 1090 cm\(^{-1}\). As may be inferred from the group theory considerations, this band splits into two or three components in the case of \(C_{3v}\) and \(C_{2v}\) symmetry respectively, which are situated between 1050 and 1150 cm\(^{-1}\) in the case of \(C_{3v}\) and between 1050 and 1250 cm\(^{-1}\) in the case of \(C_{2v}\) symmetry.\(^{138,139}\) The strong upward shift of the \(\nu_3\) band upon trapping the sulphate anion by the receptor molecules implies lowering of the anion symmetry from \(T_d\) to \(C_{3v}\) as a result of hydrogen bonding with three NH groups of diamidocarbazole units. Such symmetry lowering was observed for sulphates adsorbed on various solid substrates.\(^{138-141}\)

In this case (\(C_{3v}\) symmetry) the \(\nu_3\) mode splits into one two-fold degenerated E mode (higher frequency) and one symmetric \(A_1\) mode (lower frequency). Both vibrations are infrared active. Furthermore, it is expected that the totally symmetric \(\nu_1\) mode (around 960 - 990 cm\(^{-1}\)), that is forbidden under \(T_d\) symmetry is activated in both \(C_{3v}\) and \(C_{2v}\) symmetry groups. This band however is hardly visible in the presented spectra. While discussing the IRRAS spectra one must take into
account the surface selection rules. The \( A_1 \) mode has a dipole moment change parallel to the symmetry axis (\( z \)), while the \( E \) mode has a dipole change in the \( xy \) plane. Lack (or very low intensity) of the \( \nu_1 \) modes may indicate almost parallel orientation of the main symmetry axis of the \( \text{SO}_4^{2-} \) anion with respect to the Au surface.

However, it has to be noted that spectral changes due to symmetry lowering similar to those described above can also be observed in case of adsorption of \( \text{SO}_4^{2-} \) anion on the Au support not covered with organic adlayer. In order to prove that the observed sulphate band belongs to the anions which interact with the receptor molecules and not to the anions bound directly to the Au surface, PM-IRRAS spectrum of \( \text{(TBA)}_2\text{SO}_4 \) adsorbed on a non-fuctionalized Au-covered slide was recorded. As may be seen in Figure 55, a broad peak appears at 1101 cm\(^{-1}\). Although the shift and considerable broadening of the band as compared to the spectrum of “free” anions in the salt is observed, it is not as great as the shift of the \( \nu_3 \) band for anions trapped by the receptor molecules. This suggests that the interaction of the anion with the receptor molecules is stronger than that between the anion and the Au surface, or that the receptor molecules form a very tight adlayer on the Au surface which makes the contact between Au and the anion not possible.

![Figure 55](ATR spectrum of 50% (TBA)_2SO_4 in water and PM-IRRAS spectrum of Au surface immersed in 1x10^-2 M aqueous solution of (TBA)_2SO_4)
Quantitative data

Intensity of the emerging \(\text{SO}_4^{2-}\) band can be used to determine concentration of sulphates. However, fluctuations in the total intensity of recorded PM-IRRAS spectra are observed. To avoid such interference, internal calibration of the signal must be introduced. It was performed by calculating the intensity ratio of the sulphate band \(I_1\) at 1165 cm\(^{-1}\) and the amide I band \(I_2\) at 1645 cm\(^{-1}\). \(I_1/I_2\) was plotted as a function of a negative logarithm of sulphate concentration. Such an internal calibration brings more credible information about the number of sulphate anions in relation to the receptor molecules on the substrate in case of changes in total spectral intensity. Dependence of the intensity ratio of marker bands on the negative logarithm of the sulphate concentration in the range between \(10^{-6}\) M and \(10^{-1}\) M was plotted in Figure 56. Plots for all three kinds of receptors show two separate ranges, where fitted linear trends exhibit different slopes: at high concentrations the slope is steeper than for low concentrations. It suggests two different types of interactions between amidocarbazoles and anions. When the concentration is low, we can expect to observe spectral manifestation of direct interactions of the receptors with the sulphate anion. However, due to the limited number of carbazole moieties on the gold surface, there is a critical concentration of anions when the system reaches its capacity. Hydrogen-bonded \(\text{SO}_4^{2-}\) ions attract TBA\(^+\) counterions to neutralize net charge in the monolayer. It leads to a subsequent formation of TBA\(^+\)-\(\text{SO}_4^{2-}\) layers, thus to a crystalline-like structure at higher concentrations of the salt. This is in good agreement with what can be seen in the spectra i.e. a gradual shift of the asymmetric stretching \(\text{SO}_4^{2-}\) band from about 1150-1165 cm\(^{-1}\) to 1090 cm\(^{-1}\), which is characteristic of non-H-bonded sulphate anions. For the highest concentrations, the spectral features that can be ascribed to the carbazole layer become weaker and the total spectral pattern is dominated by contribution from the spectrum of \((\text{TBA})_2\text{SO}_4\) (compare ATR spectrum of \((\text{TBA})_2\text{SO}_4\) in Figure 55).

In order to compare the binding properties of the carbazole receptors adsorbed on the surface, we compared response plots for all three receptors only in the low
concentration range (Figure 57). Steepness of the line is related to the increase of the number of bound anions upon concentration increment of one order, which correlates with the binding affinity of a respective receptor. The steepest slope is observed for t-butyl derivative. Therefore we can conclude that the binding affinity to $\text{SO}_4^{2-}$ anions increases in order: phenyl $<$ lipoyl $<$ t-butyl. This goes along with previous reports that derivatives with aliphatic substituents bind anions stronger than aromatic analogues.\textsuperscript{115,116} It is worth pointing out that even if the data obtained for the phenyl derivative vary between two separate series (phenyl 1 and phenyl 2 in Figure 56), most probably because of different quality of the monolayers, the slope is reproducible, which is illustrated in Figure 57.
Figure 56 Changes in the intensity ratio of the chosen bands in PM-IRRAS spectra of the carbazole derivatives with respect to the sulphate anion concentration.
**Figure 5** Comparison between the performances of different carbazole receptors in the low concentration range.

**Detection of SO₄²⁻ anions in aqueous solutions**

PM-IRRAS spectra of the carbazole layers in contact with aqueous solutions of SO₄²⁻ are presented in Figure 58. Concentration series for t-butyl and phenyl derivatives do not vary significantly (Figure 58a and b). The band characteristic for SO₄²⁻ asymmetric stretching vibrations did not appear in the spectra even at the highest concentrations of the sulphate solutions. However, the first striking observation is that after exposure to even very dilute aqueous solutions of (TBA)₂SO₄, a new band at 1735 cm⁻¹ emerges. It is accompanied by a decrease of the amide I band intensity at 1645 cm⁻¹ with concomitant appearance of new bands around 1200 cm⁻¹ (1180 and 1198 cm⁻¹). The new band at 1735 cm⁻¹ can be ascribed to the C=O stretching vibration of the COOH groups. The other ones are most probably due to C-O or C-C stretching vibrations of the lipoyl acid. Therefore these results indicate hydrolysis of the amide bond in at least part of the receptor molecules and creation of the carboxyl groups instead. As seen in Figure 58a and b, in case of derivatives substituted with t-butyl and phenyl groups most of the spectral features remain unchanged throughout the spectral
series. So, a considerable number of the receptor molecules is left intact. Different situation is observed for lipoyl derivative exposed to SO$_4^{2-}$ (Figure 5c). Here, sulphates in concentrations of $1\times10^{-6}$ and $1\times10^{-5}$ M cause substantial intensity increase of the bands at 1180, 1198 cm$^{-1}$ and 1735 cm$^{-1}$. However, these bands, along with the whole spectrum, decrease in $10^{-4}$ M SO$_4^{2-}$. The rigid structure of the layer is closely related to presence of two lipoyl groups acting as anchors for the receptor. If hydrolysis of the amide bond occurs, it disturbs the initial orientation of the molecules. Subsequently, both amide bonds may break, as in this molecule they are equivalent. This, in turn, results in detachment of numerous carbazole rings from the lipoyl groups attached to the surface which is observed as a decrease in total intensity of the spectra above $1\times10^{-4}$ M SO$_4^{2-}$.

Generally, the hydrolysis may occur on the amide bond linking the t-butyl/phenyl/lipoyl group to the rest of the molecule or on the amide bond linked to the lipoyl group. The similarity of the spectral features (new bands at the same positions independent on the receptor molecule) that arise upon hydrolysis points to the latter situation.

It is worth stressing that in contrast to the acetonitrile solutions, no evidence was found for binding of the sulphate anions to the receptor molecules in the case of aqueous solutions. It is connected with the extremely high hydration enthalpy for SO$_4^{2-}$ anions (1080 kJ mol$^{-1}$),$^{126}$ which is considerably higher than the energy of hydrogen bonds between NH groups of carbazole unit and the sulphate ions.
Experimental section

Carbazole derivatives: towards quantitative detection of anions

a)

![Graph a)

b)

![Graph b)
Carbazole derivatives: towards quantitative detection of anions

c)

Figure 58 PM-IRRAS spectra of the studied carbazole derivatives: a) t-butyl b) phenyl c) lipoyl on Au before (blue spectrum, denoted as 0) and after exposure to SO$_4^{2-}$ anions in different concentrations in H$_2$O (green spectra: i: $1 \times 10^{-6}$ M, ii: $1 \times 10^{-5}$ M, iii: $1 \times 10^{-4}$ M, iv: $1 \times 10^{-3}$ M, v: $5 \times 10^{-3}$ M, vi: $1 \times 10^{-2}$ M, vii: $5 \times 10^{-2}$ M, viii: $1 \times 10^{-1}$ M).

SERS experiments

In order to confirm binding of sulphate anions by surface-attached derivatives of diamidocarbazoles, SERS experiments were carried out using electrochemically roughened silver supports. After dropcasting the carbazole-anion complex solution on the electrode, SERS spectra were recorded in time. Immediately after depositing the solution, weak SERS spectra of the monolayer were observed (Figure 59c, e and g). After several minutes, a new band at 975/976 cm$^{-1}$ appeared, which was consistent for all the examined receptors (Figure 59d, f and h). This band can undoubtedly be ascribed to the Raman-active totally symmetric stretching vibration of the SO$_4^{2-}$ anion ($\nu_1$). To rule out that the observed SERS band is due to sulphate anions directly interacting with the Ag support through chemisorption on the Ag electrode, the SERS spectrum of (TBA)$_2$SO$_4$ adsorbed from the solution in acetonitrile was recorded (Figure 59b). As may be seen in Figure 59, both: a general spectral pattern and the SO$_4^{2-}$ band position at 970 cm$^{-1}$, which is considerably lower than that found in the SERS spectra of the receptor-bound
anion, confirm an assignment of the SERS band at 975 cm\(^{-1}\) to the sulphate anions interacting with the receptor molecules. Moreover, in the normal Raman spectrum of (TBA)\(_2\)SO\(_4\) deposited on a glass support (Figure 59a), the \(\nu_1\) band is situated at 978 cm\(^{-1}\), which is a higher wavenumber in comparison to the spectrum of the sulphate anion interacting with the carbazole moiety. Significant changes in the positions of the \(\nu_1\) band in these three situations confirm that a new feature seen at 975 cm\(^{-1}\) in the SERS spectra of the Ag/receptor/SO\(_4^{2-}\) system originates from neither SO\(_4^{2-}\) species chemisorbed directly on silver nor (TBA)\(_2\)SO\(_4\) salt crystals precipitated on the electrode, but due to anions trapped by the receptor molecules attached to the Ag surface.

Feasibility of observing the SERS signal from the sulphate anions interacting with the carbazole derivatives immobilized on the surface of the Ag nanostructures opens a promising perspective for construction of a highly sensitive nanosensor of SO\(_4^{2-}\) anions, which may be important in variety of environmental applications.
Figure 59  a – NR spectrum of 50% (TBA)$_2$SO$_4$ in water, b – SERS spectrum of (TBA)$_2$SO$_4$ adsorbed on acetonitrile, c, e, g – SERS spectra of monolayers of t-butyl, phenyl and lipoyl receptor, respectively, on Ag, d, f, h – SERS spectra of respective monolayers after complexing sulphate anions.
7.3 Conclusions

Results of the research on carbazole-based anion receptors can be summed up in the following points:

- binding properties of a new class of anion receptors based on carbazoles are demonstrated,
- carbazole derivatives retain their receptor properties upon adsorption on a metal surface,
- low concentrations of sulphates in acetonitrile are bound by receptors directly,
- in higher concentrations of the salt, growth of (TBA)_2SO_4 layer over the carbazole-functionalized Au surface is induced,
- binding affinity to sulphates increases as follows: phenyl < lipoyl < t-butyl derivatives,
- in aqueous solutions of the (TBA)_2SO_4 salt, partial hydrolysis of the receptor molecules is observed, which impairs their binding properties.
FINAL CONCLUSIONS

To sum it up, Ag-MES nanoparticles were shown to be useful in SERS-based cation detection. Alkali and alkaline earth metal ions, as well as transition metal cations were detected with different limits of detection, reaching down to $10^{-8}$ M for Ca$^{2+}$. Except for heavy metal ions, the sensor was able to distinguish between various cations. Possibility of co-detection of Ca$^{2+}$ and Mg$^{2+}$ was demonstrated. These properties were made use of when applying the sensor in the study of intracellular ion detection. Spatial distribution of potassium was revealed inside CHO.K1 cells, originating from dynamics of K$^+$ concentration in endosomes over time.

Silica encapsulated nanosensors were shown to retain their SERS activity. Various cations penetrated the silica shell with different efficiency, depending of the ion size and hydration energy. Ag-MES@SiO$_2$ nanosensors exhibited improved sensitivity to concentration changes compared to Ag-MES nanosensors, as well as high temporal stability.

Research on a new type of biocompatible plasmonic nanoparticles was also presented. They were based on gold nanostars: silver coated gold nanostars encapsulated in mesoporous silica and magnetic-plasmonic Janus nanoparticles of gold nanostars and iron oxide nanoparticles, also coated with a layer of silver. Due to small amount of silver in the structure, nanoparticles of this type stay biocompatible and their SERS enhancing properties are improved. What is more, aggregation in magnetic field was possible for the studied Janus NPs, which additionally increased the SERS signal.

Binding properties of a new class of carbazole-based anions receptors were demonstrated. Diamidocarbazole derivatives, previously shown to bind sulphates in solution, were proved to preserve their ability to interact with these anions after being adsorbed on the surface of Au substrate. Studies based on surface vibrational spectroscopies (PM-IRRAS and SERS) revealed that three studied carbazole receptors: with a t-butyl, phenyl and lipoyl substituent linked by the amide bond
to the carbazole moiety, bind sulphates from low concentration solutions (up to $1 \times 10^{-3}$ M $\text{SO}_4^{2-}$). As expected, higher concentrations induced growth of (TBA)$_2\text{SO}_4$ layer over the carbazole-functionalized Au surface. Binding affinity to sulphates was shown to increase in the following order: phenyl < lipoyl < t-butyl. While this kind of behaviour was observed in acetonitrile solutions, no proof of receptor-anion interaction was seen in aqueous solutions of sulphates. In aqueous environment, partial hydrolysis of amide bonds in the receptor molecules was revealed, which led to decomposition of the active site and, in case of the lipoyl derivative, even detachment of the carbazole moiety from the surface-anchored receptor.

Obtained results show how powerful surface vibrational spectroscopy techniques are in ion detection. Presented high-sensitivity nanosensors for cations (both Ag-MES and silica-covered Ag-MES@SiO$_2$) extend the field of already described SERS nanosensors, which is even more valuable due to the fact that alkali and alkaline earth cation detection with SERS spectroscopy has not been previously investigated comprehensively. Demonstration of use of these nanosensors for intracellular mapping gives a promise to application of the system for bioanalytical purposes. Comparison between Ag-MES and Ag-MES@SiO$_2$ sheds light on the influence of the protective layers commonly used for nanoparticles on their sensing performance. Research on multicomponent nanoparticles illustrates how easily modification of nanosystems can lead to improvement of their performance e.g. under magnetic field, but also shows potential obstacles in synthesis of new plasmonic substrates. Described studies on anion sensors build a bridge between efforts of organic chemists focused on design of new anion receptors and detection of chemicals with vibrational spectroscopy.
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