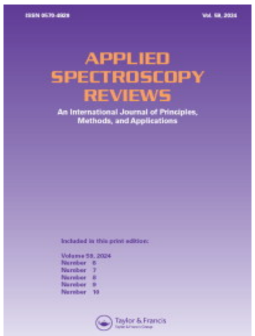

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Drop coating deposition Raman (DCDR) spectroscopy: fundamentals and potential applications

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ABSTRACT
Raman spectroscopy has developed significantly since its discovery and has become an important analytical vibrational technique for investigating the sample's chemical and structural properties. Today, great emphasis is placed on detecting low-concentrated samples of small volumes, which is a problematic task considering the weak intensity of the Raman signal. To improve the sensitivity significantly, resonance Raman spectroscopy and surface-enhanced Raman spectroscopy were employed. However, they face certain limitations and cannot generally be applied to any molecule. Here, we focus on drop coating deposition Raman (DCDR) spectroscopy that offers a general solution. DCDR lies in a droplet deposition of a liquid sample on an ideally solvophobic substrate where the subsequent drying process results in a preconcentrated deposit. After focusing on the preconcentrated dried parts in the deposit under the Raman microspectrometer, this offers high-quality classical Raman spectra even from a low-concentrated sample. Besides the overview of the method, its potential and the applications to biomolecules, biologically significant molecules and contaminants will be discussed.

KEYWORDS
Drop coating deposition Raman; DCDR; hydrophobic substrate; biomolecule; contaminant

1. Introduction

The Raman scattering phenomenon was discovered in 1928 and named after the physicist Sir Chandrasekhara Venkata Raman, who made the first recorded observation.[1,2] Raman was awarded the Nobel Prize for Physics in 1930 for this first experimental evidence. The phenomenon occurs when a sample is irradiated by incident light that is immediately scattered by molecules. Generally, the majority of the scattered light is elastic scattering (Rayleigh), which occurs at the same wavelength as the incident light. The Raman scattering is inelastic, shifting the frequency/wavelength of scattered radiation due to molecular vibrational motion. The Raman signal is very weak, at about a million times less intense than Rayleigh scattering (only one of 10^5-10^7 photons is scattered inelastically). Moreover, at the time of the first Raman experiments, only rudimentary instruments were available, and it was truly remarkable that such a weak phenomenon

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was detected. Nevertheless, the instrumentation gradually developed over time, making it possible to record Raman spectra of various compounds in liquid and solid phases.

However, despite the technical developments (e.g. using laser excitation sources and sensitive detectors), the maximal conversion of the incident laser photons into Raman signal is only about 10^8 due to the weak Raman scattering cross-section.[3] It is challenging to record Raman spectra from poor scatterers or low-concentrated materials, where relatively highly concentrated samples (more than 1 mM) and large amounts of analyte (more than 1 µg) are necessary. Concerning biological samples/molecules and contaminants (e.g. food and environmental contaminants), such high amounts do not correspond to physiological conditions and environmentally relevant concentrations.[3,4] Therefore, their detection is complicated.

To overcome the aforementioned obstacles, the Raman intensity is often increased using non-classical (enhanced) Raman spectroscopy such as resonance Raman spectroscopy (RRS) and surface-enhanced Raman spectroscopy (SERS) methods. RRS occurs when the incident laser light is near resonance with an electronic transition of the molecule and thus amplifies Raman scattering from a specific vibrational mode of the molecule in resonance with the excitation. It can enhance the Raman scattering cross-section by orders of magnitude. SERS is based on the presence of metal nanoparticles in colloid or in the form of roughened/nanostructured metal (plasmonic), very often silver and gold surface/platform, called "SERS-active" providing the electromagnetic enhancement due to the excitation of localized surface plasmons. For the molecule of interest adsorbed on such a platform (due to chemisorption or physisorption), the enhancement of the Raman signal over 10^5 can be achieved.[5] Used surfaces have an important influence on both Raman enhancement and spectral reproducibility. It has been widely and successfully employed in various applications over the past few decades. Even though the SERS method has brought many important discoveries, its use is often not straightforward and generally not applicable to any molecule. Moreover, both RRS and SERS have more restricted validity due to the problems with spectral alteration, increased background, strong fluorescence signal, and the propensity for photochemical damage.

Besides the above-mentioned enhanced methods, it would be advantageous to have another approach at one's disposal to increase the intensity of the Raman scattering when measuring classical (non-enhanced) Raman spectra. The drop coating deposition Raman (DCDR) method meets these requirements and overcomes the above-mentioned obstacles. As discussed further, the method benefits from a simple deposition of a small volume of liquid sample, which, when dried, creates a deposit suitable for measuring high-quality classical Raman spectra from the preconcentrated dried parts under the Raman micro-spectrometer. This review summarizes the state of the art of DCDR spectroscopy and demonstrates its potential and current applications on biomolecules and biologically significant molecules in relevant concentration ranges.

2. Methods

2.1. Inspiration for the DCDR method

The development of the DCDR method was very likely inspired by the simple observation of drying a spilled liquid drop, such as a drop of coffee. Basic physical principles of drying were elaborated in the paper by Deegan et al.[6] published in the journal Nature in 1997. It described the phenomenon of the formation of an annular (ring-like) deposit known as a "coffee-ring" when a spilled drop of coffee evaporated on a solid surface. The authors stated the necessary conditions which had to be fulfilled for the formation of a "coffee-ring" deposit. These included a surface with a non-zero contact angle for the deposited liquid sample and the subsequent formation of a droplet contact line (at the air-liquid-solid interface) that is pinned to its initial position when dropped and dried. Although the evaporation process does not always result in the formation of a "coffee-ring" for each deposited droplet, this study emphasizes the effect of sample pre-concentration in the edge ring, which was later successfully applied in the DCDR method.[7]

In 2009, the paper by Larmour et al.[8] reminded and pointed out an idea about solvent removal that could be a universal mode for signal enhancement from dilute solutions. Solvent removal influences the increasing concentration of the solute. When the concentration of the solution increases, more molecules of interest (causing the inelastically scattered photons) enter the laser beam, and a higher signal is detected. As Raman scattering is proportional to concentration, the signal is expected to increase linearly with concentration. The authors calculated that if a 2-μl drop with a diameter of 1.56 mm shrinks by evaporation into the drop with a diameter of 100 μm, the signal will increase more than by a factor of 3000 $\times$. Further evaporation of the solvent to a 10 μm drop diameter will increase the concentration, and so will the Raman signal by at least a factor of 3×10^6 times. This approach is generally suitable for any solution and does not depend on the properties of diluted molecules or the surface used for deposition. Nevertheless, some types of surfaces could be more beneficial because they give rise to smaller, more compact deposits that provide higher signal.[9]

2.2. The principle of the DCDR method and fundamental features

The principle of the DCDR method (Figure 1) is straightforward and simple. It lies in the deposition of a small volume – a droplet – of solution or suspension of molecules of interest on a surface, later in the text, mentioned as substrate. The optimal substrate should be highly solvophobic in the range of used solvents. Since the studied sample is in an aqueous solution, in most cases, the preferred substrate is hydrophobic. Another commonly used solvent is ethanol. The deposition is then followed by the drying process, usually at room temperature. The sample deposited on the substrate is protected from dust by covering it with a Petri dish. A final preconcentration of the analyte occurs as a result of the complete drying process. The resulting dried samples create a pattern of different forms. Sessile droplets very often dry with the "coffee-ring" effect when the evaporating droplet has a pinned contact line, and the solvent that is evaporated from the periphery of the droplet is replenished by solvent from the interior. The resulting flow (streaming) can virtually carry all dispersed material to the edge, where a ring of sample molecules is formed. After the droplet deposition and its subsequent evaporation, the formed dried deposit is solvent-free. After putting the substrate under the Raman micro-spectrometer and focusing on the preconcentrated dried parts in the deposit, classical Raman spectra are measured. Besides dried deposits with the "coffee-

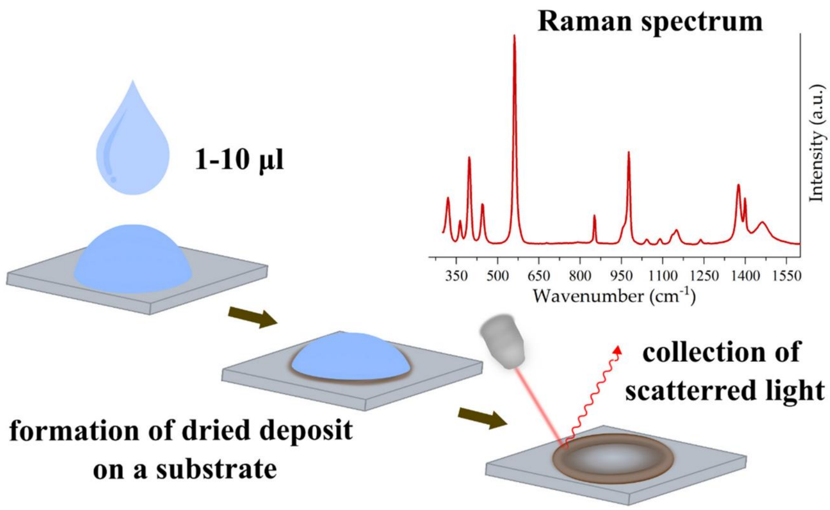

Figure 1. The principle of the DCDR method: the deposition of a droplet of solution or suspension on a solvophobic substrate followed by the drying process and the measurement of classical Raman spectra from the created deposit pattern.

ring" effect, other types of patterns formed after drying can be observed (Figure 2). Small spots with often spherical form are observed when sessile drops have less tendency to pin to the substrate during drying. Thus, the droplets will remain almost spherical as they shrink during evaporation. In addition, the formed pattern may be nonuniform and non-compact (composed of several parts, such as small isles). It can contain some amorphous parts, as well as tiny crystals, formed when the molecules in the drop tend to form self-assembling complexes *via* crystalizing.

DCDR approach, due to the significant preconcentration during drying, usually leads to the acquisition of high-quality classical spectra. In addition, strong preconcentration permits the measurement from initially low-concentrated samples (lower than for classical Raman measurement measured directly from solution). It is enough to drop only a small initial volume, a droplet of a few microliters. This enables a several-fold improvement of the sensitivity when compared to classical Raman measurement from solution or suspension.[7,10–12]

Choosing a suitable substrate is crucial for optimizing a DCDR experiment. Hydrophobic substrates are preferred because the studied molecules are mostly in aqueous solution. Therefore, the substrate wettability, i.e. the extent to which a drop of deposited sample covers the surface, is relevant and affects both the drying process and the resulting appearance of the pattern. The wettability is described by a contact angle, the angle between liquid-solid and liquid-air interfaces. Generally, the pattern formed by the drying process is a result of the interplay among more factors, such as the type of molecular solution (or analyte), i.e. its chemical composition, concentration, molecular or particle size, charge, as well as the used solvent, and the selected substrate. All of these strongly influence the ability of sessile droplets to pin to the substrate and subsequently keep the contact line during drying. The resulting pattern is always a combination of both the properties of the analyte and the properties of the substrate. As presumed in the work of Larmour et al.[8], the smaller dried patterns with more

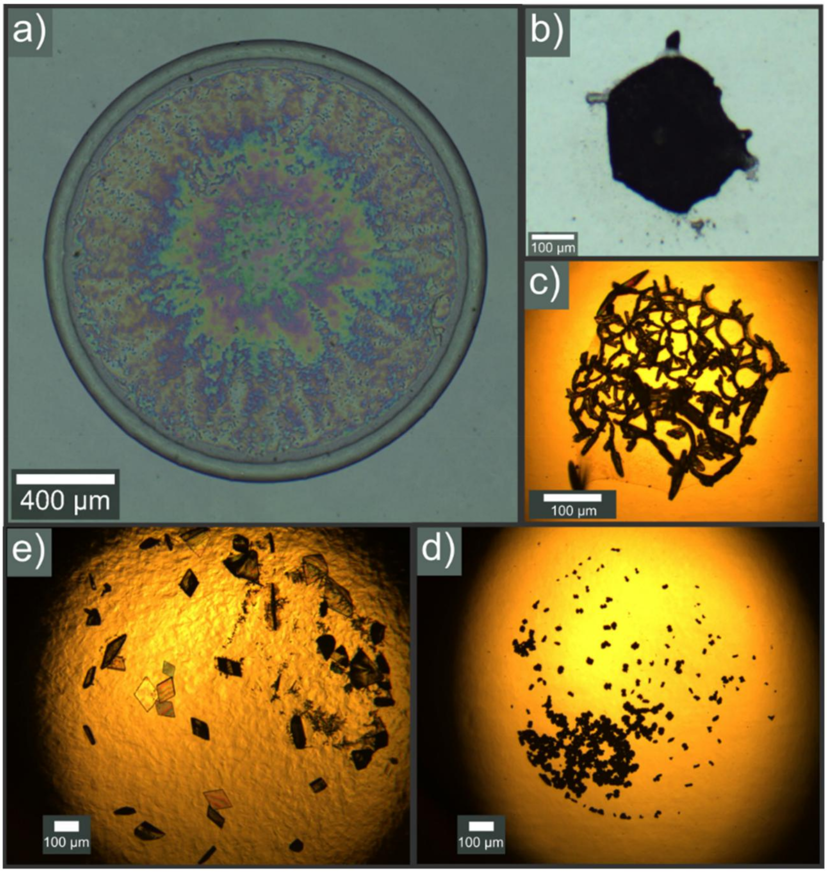

Figure 2. White-light microimages of dried deposit patterns. (a) DPPC, 1 mg/ml, (b) lactose, 1.2 mg/ml, (c) bentazone, 0.5 mg/ml, (d) picloram – 0.5 mg/ml, (e) melamine, 2 mg/ml.

preconcentrated samples give rise to a Raman signal of higher intensity. In this way, we can reduce the detection limit and significantly (several orders of magnitude) improve the detection sensitivity. At the same time, it is possible to extend the attractiveness and usefulness of Raman spectroscopy for any analyte, biomolecules and contaminants (at physiological conditions and environmentally relevant concentrations) that we are interested in.

Different substrates were continuously tested from the beginning of the DCDR experiments. More experimental results showed that a polished stainless steel overlaying with a hydrophobic layer was the most promising. SpectRIM™ substrate, commercially produced by Tienta Sciences, was successfully employed in many published works. It consisted of a $3 \times 1 \times 0.1$ inch plastic pad in which an optically flat stainless steel plate coated with an ultra-thin (50 nm) hydrophobic layer was placed. It provided a contact angle of 122° for a drop of water. The substrate was chemically inert and appeared ideal for DCDR spectroscopy for dilute solutions in polar solvents. However, the SpectRIM™ Slide is no longer commercially available. Another substrate that is currently at disposal is a µRIM™ from BioTools. This substrate is less hydrophobic than SpectRIM™ Slide, with a contact angle of 108°.

Another big group of substrates are the noncommercial home-made ones profiting from the hydrophobic character of smooth fluorocarbon coating with contact angle in the range of 108°-120°. They represent a very promising platform for DCDR spectroscopy. This class of hydrophobic substrates can be additionally improved to create nanostructured platforms with metal nanoparticles to increase the hydrophobicity even more.[9,13,14] Metal nanoparticles are sandwiched in plasma polymerized fluorocarbon films and prepared by magnetron sputtering. The presence of nanoparticles, depending on the type of metal (copper or silver), their size and amount, leads to a significant increase in the roughness of the surface and so in the contact angle of the deposited droplet. It was shown that surface roughness is a key parameter adjusting the wettability.[14] In this way, not only highly hydrophobic but also super-hydrophobic substrates with a contact angle of at least 150° can be produced.[13] Super-hydrophobic substrates thus can cause the formation of smaller dried patterns with better preconcentration.[15] Despite the use of metal nanostructures, the enhancement of the Raman signal from deposited molecules is caused only by a better preconcentration on the roughened surface, and the contribution of the SERS enhancement to the resulting signal is excluded due to the long distance from the metal nanostructures.

The other noncommercial and often used DCDR substrates include polished CaF_2 plates, silanized glass surfaces, quartz slides, gold foil, aluminum foil, thiol-modified gold-coated glass etc.[16–18] A polytetrafluoroethylene tape as a low-cost hydrophobic substrate was also tested.[19] The contact angles of these substrates vary in the range of 80°-120°. With a higher contact angle, the preconcentration of the analyte is generally more efficient.[9,17]

As for the Raman spectra measurement, there are several crucial points regarding substrates and measurement conditions. Any used substrate should have a minimal background signal in the spectral region of interest, otherwise, the subsequent signal subtraction of this signal may not be straightforward. It is also necessary to properly adjust the intensity of the incident laser beam, as the sample may be photodamaged, especially when the sample layer is very thin. The presence of the fluorocarbon layer is advantageous because when thin, it shows no apparent Raman or fluorescence signal and thus provides the spectra from dried deposits of good quality. Furthermore, the polished stainless steel performs as a mirror and significantly increases the incoming signal by reflecting it to the objective. Similarly, all the substrates with mirror-like surface (e.g. gold and aluminum foils) profit from its presence. For substrates with the background signal from glass surfaces, a gold coating can be used to reduce it efficiently. For polished CaF_2 plates, the single Raman band at $322\,cm^{-1}$ can be easily removed by subtraction methods. Although, CaF_2 band is mostly outside the spectral region of interest. Based on experience to date, a stainless steel plate coated with a thin hydrophobic fluorocarbon layer appears to be the most suitable substrate for DCDR spectroscopy.

3. Results and discussion: Beginnings, first results and our work

In the first DCDR experiments, emphasis was placed on the research of important biological molecules, such as amino acids, peptides and proteins.[7,18,20–25] Later, due to the promising results, the studies were extended to investigate other molecules, molecular mixtures and complex solutions.[10,11,26–31]

The first encouraging results were published by Zhang et al.[7] in 2003 on samples of lysozyme and human insulin proteins. Their work combined Raman measurements with chromatography and mass spectrometry to demonstrate proteomic sensing. The authors were driven by an effort to detect the proteins derived from a chromatographic (HPLC) fraction by classical Raman or by following the Raman acquisition by mass spectroscopic techniques (MALDI-TOF). Since the concentrations needed for the classical Raman spectroscopy from solutions are higher than those issued from chromatography and needed for mass spectroscopy, the possibility of following up on it was challenging. However, the work of Zhang et al.[7] reported the potential to reproducibly measure high-quality classical Raman spectra from femtomole amounts of only micromolar concentrations of protein samples when deposited and dried on a hydrophobic substrate. The observed results showed a more than 1000-fold increase in the sensitivity of classical Raman spectroscopy from protein solutions. An important aspect of the DCDR experiments was the observation that the DCDR spectra were virtually identical to those measured from protein solutions at higher concentrations. In addition, it turned out that proteins maintain their structure from solution after the drying process so their native structure is not disrupted. All this indicated a potential benefit of DCDR spectroscopy in proteomic diagnostics.

Moreover, the capacity of the DCDR to act as a segregation method was also shown by the effective separation of proteins from the other components, such as some impurities (mostly fluorescent ones) and buffer compounds (obviously salts).[32] The segregation effect is produced by convective streaming occurring during the evaporation and subsequent precipitation of components in different regions on the substrate. The streaming is a prominent driving force for the observed tendency of proteins and lipids (in the form of liposomes) to accumulate in an edge ring of dried patterns. In addition, crystallization may also contribute to segregation, as compounds with higher nucleation and crystal growth rates tend to deposit separately from compounds with lower crystallization rates. Different solubility of the solution's components also plays an important role in the segregation process. However, for example, components with a strong affinity for each other will not tend to segregate, and thus, the segregation process does not work universally for every two- or multi-component analyte. As mentioned later, the potential profit from the segregation of individual components can be useful for understanding the interactions in complex samples.

In addition to proteins, lipids were another group of biologically important molecules that were studied by the DCDR method.[33–38] Since 2010, these studies have been carried out mainly in our laboratory. Lipids perform several functions in the cell, but in particular, they are the main structural components of living membranes (cell and organelle). This task is provided mainly by phospholipids. We studied lipids organized in liposomes (spherical self-enclosed structures formed by curved lipid bilayers) as a liposomal suspension. Due to their structure, liposomes are often used as suitable model membrane systems. The homogeneous liposomal suspension (in liposome dimension as well as lipid composition), when deposited on a hydrophobic substrate, dries to similar patterns as proteins studied so far. It has an overall round shape form with a "coffee-ring" edge ring.[37] The size of the formed pattern, the diameter, as well as the width of the edge ring varies depending on the type of lipid, its concentration in the deposited drop and the lipid composition, i.e. whether they consist of only one type of lipid or a lipid mixture. The differences in patterns were observed for 1,2-distearoyl-sn-glycero-3-phospocholine (DSPC) (pure) compared to asolectin (mixture) samples.[37] At the same initial deposited concentrations, the diameter of the DSPC dried pattern was higher than that for the asolectin sample. The formed edge rings had a width of about 65-70 µm. Moreover, when comparing DSPC and asolectin dried patterns, less asolectin is transported to the edge during the drying as the formed ring is less distinct. In the case of DSPC liposomes, the membrane is composed of lipids with fully saturated carbohydrate chains (with 18 carbons), so it is rigid. Since the phase transition temperature of DSPC is 54 °C, the DSPC suspension is in an ordered gel phase at room temperature. The studied asolectin is a natural lipid extract from soybeans and is composed of various types of lipids with approximately equal proportions of lecithin, cephalin and phosphatidylinositol and other phospholipids, as well as polar lipids in small amounts. As it is a mixture of various lipids, the phase transition is broad, and the temperature cannot be precisely determined. Moreover, liposomes formed by asolectin differ from those formed by only one type of lipid, as that is a model system closer to and mimics natural/real membranes.

As for Raman measurement, the classical Raman spectroscopy directly from liposomal suspension is possible only with high initial concentrations of lipids, obviously more than tenfold higher than are needed for DCDR. For the classical Raman measurement of DSPC liposomes from suspension, it had about 120 times higher concentration than the one found to be optimal for the DCDR measurement (0.3 mg/ml).[37] Such a low concentration is sufficient to form a thin layer pattern and obtain high-quality and highly reproducible DCDR spectra, which was confirmed by Raman mapping. Moreover, comparing the classical Raman spectra from aqueous suspension of liposomes and their DCDR spectra, the spectral features were the same in both cases. This demonstrates that the measured samples maintained their phase even after drying, liquid crystalline and gel one for asolectin and DSPC, respectively. As a result, DCDR spectroscopy provides Raman spectra of lipids using initial concentrations of at least two orders of magnitude lower than those needed for classical Raman spectroscopy in aqueous suspensions.[37]

Besides the liposomes composed of pure phospholipids or their mixtures, we have also studied the changes in liposomes containing cholesterol.[35] Cholesterol is a sterol-ringed lipid important in biosynthetic pathways, especially in mammalian organisms, as it acts as a precursor for the biosynthesis of steroid hormones. Although it does not form the membrane itself, it is an important structural component. Cholesterol helps to maintain the proper membrane heterogeneity in both membrane leaflets, its overall stability and the necessary permeability. When mixed with phospholipids, molecules of cholesterol insert between the phospholipid molecules so that their polar hydroxyl group is close to the head group of phospholipids and thus significantly affect the membrane structure and fluidity. The presence of cholesterol in the liposomes influences the dried patterns and induces changes in the dimension and thickness of the edge ring. The growing amount of cholesterol leads to a diminishing of pattern size as well as the increase of the edge ring thickness. This was confirmed for both homogenous liposomes composed of pure 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and mixed asolectin liposomes. Moreover, a higher amount of cholesterol (20%) in DPPC liposomes caused the formation of a compact flat pattern without the edge ring. Measured DCDR spectra contained characteristics of both, phospholipid and cholesterol. The presence of cholesterol in the liposomes can be revealed in the DCDR spectra already at 5% amount. As the proportion of cholesterol increases in liposomes, so does its signal in the spectra. The measured Raman spectra showed that both liposomes without cholesterol and with an increasing proportion of cholesterol after drying retain their initial phase from a liquid suspension, i.e. gel phase for DPPC and liquid crystalline for asolectin samples. Additionally, spectral mapping was also used to examine the edge ring of the lipid dried patterns, and it demonstrated the formation of homogenous rings providing reproducible DCDR spectra with maximum intensity originating from the central part of the ring structure.

We[9] then utilize homogeneous DPPC liposomal suspensions to quantify the DCDR signal improvement by comparing the smooth fluorocarbon (C:F) surfaces and nanostructured ones with increased hydrophobicity but identical surface chemical composition. The smooth C:F thin films were prepared by magnetron sputter deposition of 40 nm thick polytetrafluoroethylene (PTFE) on the polished Si wafers. Nanostructured surfaces were prepared by embedding a controlled number of nanoparticles between two 20 nm thick C:F layers. The resulting surface roughness and increased hydrophobicity were reached by adjusting the size and the number of nanoparticles embedded in the C:F film. Drying the liposomal suspension on nanostructured surfaces led to a well-defined "coffee-ring" pattern with a lower diameter of the resulting rings when compared to the pattern on the smooth C:F coating (pictured in Figure 3), which means a

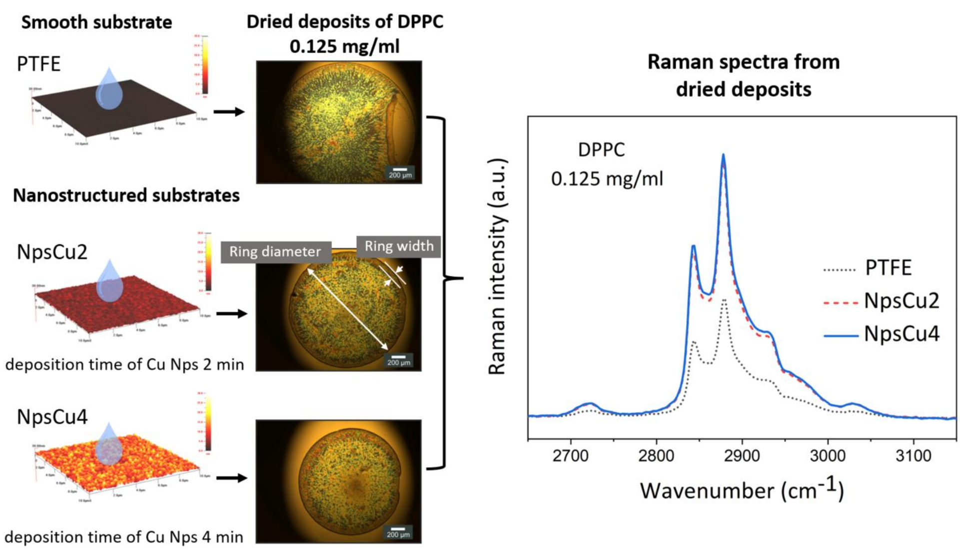

Figure 3. DCDR signal improvement for DPPC liposomal suspension by employing nanostructured substrates with embedded Cu nanoparticles in the PTFE layer. The deposition time set to deposit Cu nanoparticles was 2 min and 4 min. The longer deposition of nanoparticles leads to higher roughness and thus more hydrophobic substrate.

better accumulation of lipid molecules in the ring. After measuring DCDR spectra, two times higher Raman signal intensity was acquired from a sample dried on a nanostructured substrate than from a dried pattern on smooth fluorocarbon film, without compromising the profile of recorded Raman spectra. It was shown that the intensity in DCDR spectra could be significantly increased only by changing and adjusting a substrate roughness.

We[33,38] also did the DCDR study on the interaction of the complex of the cationic copper(II) 5,10,15,20-tetrakis(1-methyl-4-pyridyl) porphyrin (CuTMPyP) and phosphorothioate modified oligonucleotide with DPPC liposomes. In the first experiment, we prepared a suspension composed of CuTMPyP and liposomes. The DCDR spectra contained both the lipid and porphyrin spectral features, but upon the treatment by factor analysis, it was demonstrated that the distribution of CuTMPyP throughout the dried pattern was not homogenous. In the next experiment porphyrin/oligonucleotide complex was added to the liposome suspension. The drying process caused a separation of liposomes with bound complex CuTMPyP/oligonucleotide in the formed edge ring, and the complex remained in the pattern's central part. Moreover, it was possible to distinguish the spectral changes caused by the drying process from the changes caused by the interaction of CuTMPyP with the DPPC liposomes. These were attributed to the perturbation of lipid chain order as well as porphyrin interaction with the lipid head.

Much work was then devoted to the study of smaller molecules of different origin, character and properties. Djaoued et al.[39] focused on a domoic acid (tribasic amino acid containing a proline ring, structurally related to glutamic acid, with a side chain having a conjugated double bond moiety and a hind carboxylic acid) that is naturally present in the marine ecosystem in different locations in the world and recognized as a neurotoxin. Poisoning can cause short-term memory loss, nausea, breathing difficulty and coma. The study demonstrated that DCDR spectroscopy can be applied for rapid detection down to 25 ng of domoic acid per ml (0.025 ppm).[39] Dingari et al.[29] demonstrated an application of DCDR spectroscopy on the detection and quantification of glycated albumin, an important glycemic marker for long-term diabetes monitoring. Using this approach, it was possible to accurately discriminate glycated albumin from the unglycated variant, even at low µM concentrations. The reached limit of the detection for glycated albumin is nearly 4 times lower than the minimum physiological concentrations encountered in practice.[29]

Dipicolinic acid, another interesting small molecule, was studied in our laboratory.[11] It is a component of bacterial spores of Bacillus species and represents ~10% of their dry weight. The spores are dormant and extremely resistant to different environmental stresses. Dipicolinic acid is a crucial component of the spore core responsible for this resistance. Using the DCDR approach, it was possible to detect dipicolinic acid from a 2-µl drop of 5×10^{-7} M deposited concentration, which is equivalent to approximately 90 Bacillus anthracis spores.[11] Other molecules with biological importance studied by the DCDR approach include acetylsalicylic acid (a substance with antipyretic, anti-inflammatory and cardioprotective properties), riboflavin (vitamin B2, important in the living cell functioning) and methylene blue (used in different areas of biology and medicine for therapeutic and diagnostic purposes) [31]. The found DCDR detection limits are 10^{-6}, 10^{-7}, and 10^{-8} M of deposited concentrations for riboflavin, acetylsalicylic acid, and methylene blue, respectively. These detection limits are comparable to or even better than those attained by the SERS method by means of commercial silver SILMECO substrate. For example, for acetylsalicylic acid, no SERS spectra were obtained from the concentrations used for the DCDR approach, probably due to weak adsorption on the silver surface. For riboflavin, a concentration of 10^{-5} M was the last concentration providing a SERS spectrum, and this is one order of magnitude lower than in the case of DCDR measurements. The SERS spectrum of methylene blue can be obtained from 5×10^{-9} M concentration, which is only twice lower than the DCDR approach. Thus, it can be concluded that the sensitivity of DCDR could be comparable to or even better than that of SERS spectroscopy.[31] Using the DCDR approach, it was possible to improve the Raman detection sensitivity of porphyrins by a factor of 10^5 and thus, the spectra from ~20 nM initial (deposited) concentrations of cationic CuTMPyP and anionic copper(II) 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrin were obtained. Protoporphyrin IX, known as a marker in the clinical diagnosis of cancer, was also detected from a deposited concentration of 10 nM, and although a fluorescence background also appeared, clear Raman bands of this molecule were observed in the spectra.

The relevant group of studied molecules represented food and agricultural contaminants. In our study[30] selected contaminants such as food contaminant melamine (known as an illegal food additive of infant formula in China in 2008 and pet food in 2004 and 2007), fungicide thiram (an animal repellent to protect fruit trees and used as well to prevent deterioration from harvested crops in storage or transport from fungal diseases), and herbicides bentazon (detected in groundwater as well as in surface water, applied aerially on food crops to control the spread of weeds occurring amongst the food crops) and picloram (systemic herbicide used for broadleaf weed control in pasture and rangeland, wheat, barley, oats, and woody plant species, can contaminate groundwater and surface water) were detected by DCDR approach whereas classical Raman spectra directly from stock solutions of these substances were impossible to obtain under the same experimental conditions.[30] In addition to that, no reported SERS study for bentazone and picloram was found in the literature for comparison. The lowest detected concentrations were determined as 6.4 μM, 0.31, 20 and 2 μM for deposited concentrations of melamine, thiram, bentazon and picloram aqueous solutions, respectively. DCDR approach was shown to be a powerful tool for the detection of these contaminants at concentrations relevant to food/groundwater contamination.

Furthermore, concerning melamine contamination, our laboratory also focused on melamine detection from intentionally adulterated infant formula. Firstly, we applied the DCDR approach to pure milk infant formula to discover that the drying process induced the spatial separation of main milk constituents like carbohydrates and lipids (Figure 4). Lipids tended to accumulate in the ring structure, as discussed before, for liposomal suspensions, and the carbohydrates formed the thin film layer in the central part of the ring pattern. Spatial separation was confirmed by the acquired classical Raman spectra from the dried pattern. We observed the same spatial separation of lipids and carbohydrates even for the melamine-blended infant formula, detecting melamine only in the central thin layer with the sugars. Melamine detection was possible due to the distinct Raman band belonging to the breathing vibration of melamine at the position of 681 cm^{-1}. We assumed that melamine glycation by carbohydrates in the

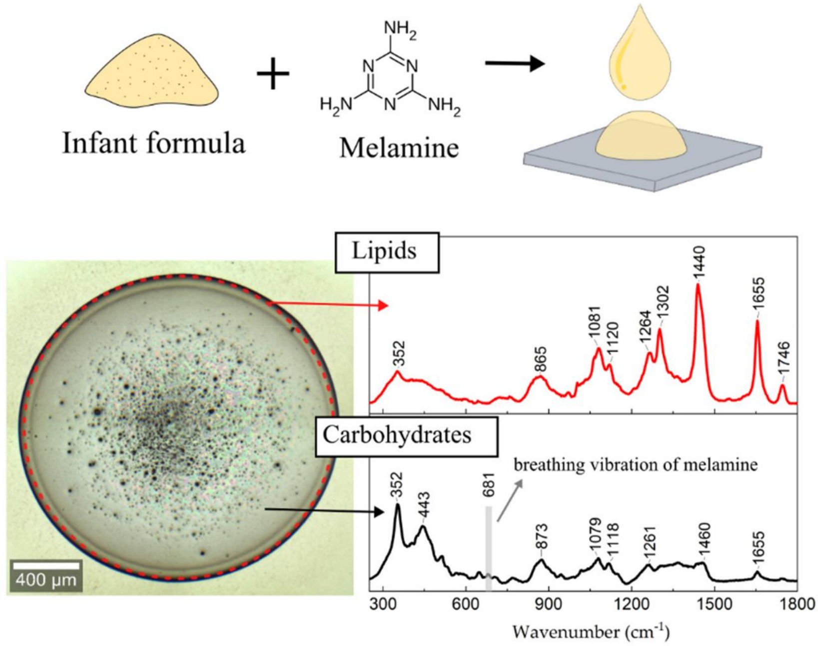

Figure 4. The spatial separation of lipids and carbohydrates in the dried deposit pattern of melamine adulterated milk solution by using the DCDR method with melamine detection only from lipid ring structure.

formula played a crucial role in accumulating melamine molecules inside the ring, making its detection possible even for relevantly low concentrations for melamine contamination. These findings are relevant for the future utilization of DCDR spectroscopy, which can profit not only from analyte preconcentration but also from the chemical reaction potential of molecules of interest and their drying-induced spatial separation in the deposit.

4. Conclusion

Raman spectroscopy, based on the phenomenon of inelastic light scattering, has undergone significant development and has become an important analytical vibrational technique. Nowadays, it is well-established for investigating the chemical and structural properties of a sample in both liquid and solid phases. From the beginning, it has gradually branched out so much that currently, it includes several special methods that are successfully used in many different applications. Today, a strong emphasis is placed on high-quality detection of samples with small volumes and low concentrations. Resonance Raman spectroscopy and surface-enhanced Raman spectroscopy meet this requirement, although they face certain limitations, like spectral alteration, increased spectral background, propensity to photochemical damage, etc. Moreover, neither of the mentioned methods is generally applicable to any molecule.

This review focuses on drop coating deposition Raman (DCDR) spectroscopy that aspires to overcome these problems and increases the intensity of the Raman scattering when measuring classical (non-enhanced) Raman spectra. This is ensured by the deposition of a small volume – a droplet – of liquid of interest on a solvophobic substrate, leading after the evaporation process to the preconcentration of the analyte into the dried deposit. Measuring spectra from selected positions throughout the deposit provides high-quality and reproducible classical Raman spectra, even from small volumes of low-concentrated samples.

The DCDR method has already been applied for the detection and studies of properties of biomolecules, biologically significant molecules, and various food and environmental contaminants at relevant concentrations, both physiological and environmental. The principal advantages of this method are the analyte preconcentration, the chemical reaction potential of the monitored molecules in the droplet, and the subsequent spatial separation (segregation) of interacting/non-interacting components induced by drying. In addition, the optimization of hydrophobicity of used substrates, for example, by tuning surface roughness using nanoparticles of various numbers and sizes deposited in the PTFE film, is promising for its implementation in routine sensing applications.

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Disclosure statement

The authors declare no conflict of interest.

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