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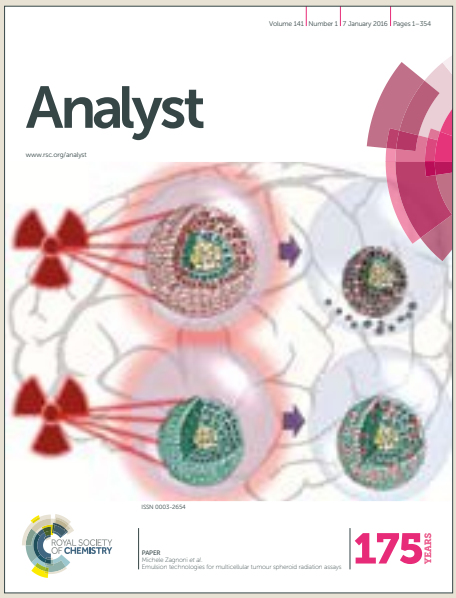

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ARTICLE

Rapid single-cell detection and identification of pathogens by using surface-enhanced Raman spectroscopy

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For the successful treatment of infections, real-time analysis and enhanced multiplex capacity, sensitivity and cost-effectiveness of the developed detection method are critical. In this work, surface-enhanced Raman scattering (SERS) was employed with the final aim of identification and discrimination of pathogenic bacteria, based on their detected SERS fingerprint at single-cell level. Several genera of bacteria that are found in most of the isolated infections in bacteraemia were successfully identified in less than 5 minutes without the use of antibodies or other specific receptors. The key element of the SERS direct detection platform is SERS substrate, which combines easy production at low costs with a high enhancement enabling single-cell detection. The innovative approach of detection required the *in situ* synthesis of silver nanoparticles (NPs), ensuring an intimate contact with the bacterial membrane. This protocol provided a good reproducibility of the single-cell SERS spectra and was successfully applied both on Gram-negative and Gram-positive microorganisms (*E. coli*, *M. morganii*, *E. lactis*, *L. casei*). Thus, a label-free SERS-based biosensor for pathogens detection was developed with low costs, minimal sample preparation, high-accuracy and a very short analysis time of less than 5 min, which is crucial for infection diagnose.

Keywords: SERS, single-cell detection, pathogens, identification

Introduction

Biosensors with integrated nanotechnology promise to address the analytical needs in practical pathogen diagnosis by offering clear advantages over the conventional culture-based assays such as high-throughput, low limit of detection, reduced sample volume required and the possibility of label-free detection. In addition, plasmonic biosensors allow easy-to-use, rapid testing (within 5-15 min)[1, 2] with superior sensitivity and multiplex capability.[3] The stable and reliable use of SERS for single-cell detection in real-life applications requires for a control of the stability of the plasmonic effect around nanostructures and hot-spots, thus limiting the fluctuations in SERS signal which hinder the reproducibility. This again demands for a better understanding of the origins of the highly enhanced single-cell signal.[4]

A great deal of research has gone into the development of Raman- and SERS-based biosensors for pathogenic microorganisms.[5-8] At least the most common uropathogens were detected by means of label-free SERS-based detection platforms and discriminated at strain level by using microarray immobilization of single bacterial cells coupled with *in situ* AgNPs synthesis and PCA data discrimination[7, 9-11] for Gram-negative bacteria; and SERS mapping by using silver dendrites[12] both for Gram-negative and –positive bacteria. Another successful approach for label-free SERS-based detection tested on *E. coli* method is incubating the bacteria with AgNPs.[13] This approach was not tested for Gram-positive bacteria and the required incubation time before the actual Raman measurement is rather long (3h).

In our previous studies on detecting bacteria at single-cell level, we were able to obtain highly reproducible and reliable SERS results by using the Bacteria@AgNP approach.[9-11, 14] However, this approach was significantly more reliable for Gram-negative bacteria than for the Gram-positive bacteria. Recently, a similar finding was reported by Wang et al.[12] They used silver dendrites as SERS-active medium and again, the results obtained were not so promising for Gram-positive bacteria, probably because the different surface structure of

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this group of bacteria does not facilitate colloid aggregation on its outer surface. Despite a large number of successful approaches, there is an ongoing discussion about the origin of the well-known fluctuations encountered in SERS microorganisms' membrane fingerprint spectra. Potential reasons may be photochemical effects in the proximity of the metal surface,[15] or the heterogeneous coverage of the membrane with the *in situ* synthesized SERS active substrate.

Presumably, the SERS signal enhancement factor is directly influenced by the analyte adsorption efficiency, which can be improved by changing pH in the case of colloidal suspensions,[16] and the positioning at the plasmonic hot spot, which remains an issue, mainly in a biological milieu.

The key advantages that SERS-based biosensors are short assay times, a reduced sample volume, and a multiplex detection with high sensitivity and specificity. Since Efrima's group reported on producing *in situ* NPs through external (bacteria cell-wall) as well as internal (interior components mode) synthesis on bacteria,[17] the use of *in situ* synthesized Ag colloids for bacteria detection was demonstrated in several assays only in our group.[9-11, 14, 18] Recently, a label-free NIR-SERS detection and discrimination of bacteria after pre-treatment of bacterial cell membrane with disrupting agents was presented, yielding a sensitivity down to 10^3 CFU/ml and a measuring time of less than 5 min.[19] Latest studies underline the applicability of the *in situ* synthesized Ag colloids also in environmental research, for instance for the detection of bacteria in plant roots[20] and pesticide monitoring in edible leaves.[21] These results demonstrate the suitability of SERS-based non-invasive detection approach for identification and characterization of pathogens and their secreted metabolites. The *in situ* synthesis of Ag colloid ensures the structural integrity of the coated bacterial cells and thus helps to a rapid generation of spectral bacteria signatures with high sensitivity and specificity. This approach has been explored in several ways: by using bacteria treated with sodium borohydride to serve as a nucleating substrate for reduction of silver ions[22] or by subcellular fractionation of bacterial cells, prior to and subsequent to various silver treatments in order to access different regions of the cell[23]. SERS spectra of several bacterial growth media in dilutions were also reported.[24] However, when using in situ synthesized AgNPs for single-cell detection and the appropriate experimental conditions (low laser power, confocal irradiation), we haven't observed such contributions in the SERS results.

Most recently, Alula et al. used a reaction mixture treated at 55°C for generating AgNPs at the *Mycobacteria* bacterial cell wall followed by detection via SERS, but not at single-cell level[25].

In this work, we report a rapid (<5 min), label-free SERS-based detection at single-cell level, suitable for both, Gram-positive and Gram-negative microorganisms. For the practical applicability, sample preparation is crucial. We have previously clarified that by inducing a positive charge on the surface a glass slide, Gram-negative bacteria are successfully immobilized, without the need of a specific receptor.[10] However, this immobilization procedure based on electrostatic forces does not work for Gram-positive bacteria. Therefore, we propose in this study the use of microscope adhesion slides as an efficient tool for bacteria immobilization. A noteworthy outcome is the significant reduction of cost per slides (0.31 EUR/slide), the reduction of required assay time (<5 min), and the fact, that the time-consuming microarray preparation we proposed[26] earlier becomes completely obsolete.

Materials and methods

Preparation of bacterial cultures

In this study, nine bacterial strains, *Escherichia coli* TOP10 (OD=1.63), *E. coli* ROSETTA(DE3)pLysS (OD=1.68), *E. coli* BL21(DE3)pLysS (OD=1.53), *E. coli* XL1-Blue (OD=1.68), *E. coli* JM109 (OD=1.6), *Enterococcus lactis* CE-13 (OD=0.32) and *E. lactis* CE-39 (OD=0.48), *Morganella morganii* CS-102 (OD=0.38) and *Lactobacillus casei* ATCC 393 (OD=0.95) were used. *E. lactis* CE-13, *E. lactis* CE-39, and *M. morganii* CS-102 were isolated from environmental samples and were identified based on 16S rRNA molecular markers, using 27FB-1492R primer pair.[27]

All bacterial strains were stored as stock cultures at -80°C in Luria Broth (LB) medium (Sigma-Aldrich, St. Louis, USA) containing glycerol (10% v/v) (AppliChem, Darmstadt, Germany). Before use, the stock culture of bacteria was thawed and propagated on agar plates with LB Broth medium, using serial dilution and then grown for 24h at 37°C. After incubation, a single colony of bacteria was transferred into 5 ml of sterile LB Broth, grown overnight at 37°C and stirred at 150 rpm. The overnight bacterial cultures were centrifuged at 4°C, 3824 g for 10 min and washed with 2 ml of sterile saline solution. This step was repeated three times in order to obtain the culture employed for the experiments. These steps are required to achieve completely standardized samples. This elaborate preparation method might be one reason for the high reproducibility of the achieved SERS spectra. We assume that the rather strong variations sometimes observed in the SERS analysis of bacteria may not only be caused by the sample preparation and analysis, but also by the high sensitivity of the bacteria to the specific cultivation conditions. After applying these preparation steps, SERS results indicate no contribution from compounds used in the process or released by the bacteria in the extracellular matrix, as the supernatant obtained from the third washing step was SERS tested and shows no signal (spectra included in Figure 4). From earlier studies[15] we know that our sample preparation leaves the bacteria intact, so that all further analyses were performed on living organisms. The OD was measured at 600 nm with the cell density meter, model 40 (Fisher Scientific, Pittsburgh, USA).

Silver NPs preparation

For the *in situ* (Bacteria@AgNPs) preparation of 1 ml final volume, 100 µl of silver nitrate (10^{-3}M) was added to 5 µl

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freshly washed bacteria and afterwards the reducing agent was rapidly added (900 µl). The reducing agent is hydroxylamine hydrochloride (12 mg solved in 90 ml of Millipore water), stabilized by adding 1.2 ml NaOH (1%). We successfully used this protocol for both, Gram-negative and Gram-positive bacteria. It requires for a total volume of reagents of only 100 µl/sample.

SERS measurements

The spectra were recorded with a confocal Renishaw inVia Reflex Raman Spectrometer covering a spectral range of 200 to 1800 cm^{-1} by using either the 532nm (up to 200 mW laser output) or 633 nm (up to 17 mW output power) excitation line. By using the 100× objective (Leica, NA 0.9, WD 3.4 mm) of the optical microscope, we recorded single-cell spectra and the results presented stem from individual acquisition without averaging or accumulation. The irradiance was determined for each laser source assuming an irradiated spot of 2µm in diameter (6.4kW/cm^2 for the 532 nm laser and 5.4kW/cm^2 for the 633 nm laser). These powers were selected to be low enough to avoid thermos-degradation.

For each sample, five replicates, i.e. five individual bacteria, were tested by using an optimum laser power (0.1% for 532 nm and 1% for 633 nm) and 10 s exposure time, thus avoiding sample burning. The experiments were repeated five times, each time on fresh biomass from independent cultures. Well-resolved Raman spectra were obtained for all the species investigated by using these experimental parameters. The sample substrates used were the Polysine™ Microscope Adhesion Slides (polyslides, Erie Scientific via VWR), providing an efficient bacterial cell adhesion.

Results and discussion

Reproducibility of the single-cell SERS spectra

Escherichia coli XL1-Blue was firstly used to reproduce the Bacteria@AgNPs approach,[9] demonstrating the applicability of our SERS *in situ* assay. Single-cell SERS spectra were recorded by using glass microscopic slide, irradiated with the 633 nm laser line (Figure 1).

In the following, the substrates used were the polyslides and the bacteria were irradiated with the 532 nm laser line. The excitation wavelength dependency of the SERS enhancement can be explained by the optical absorption of the silver colloidal suspensions. We already characterized the herein used AgNPs in our previous work; the particles feature a plasmon resonance band around 402 nm. Hence, excitation with the 532 nm laser line is favorable as compared to longer wavelengths (633 nm), being closer to the plasmonic band. Unfortunately, we do not have shorter wavelengths than 532 nm available on our instruments. In our earlier work, the *in situ* Ag colloid synthesis was performed in two steps, requiring in total at least 30 min.[9, 10] As we stated earlier, the effect of this incubation time is an increasing agglomeration of the SERS-active colloids, leading to a coupling of the plasmon resonances, which again leads to a red-shift of the main absorption. In consequence, we achieved in these earlier works the best SERS performance with the 633 nm excitation wavelength. However, as the aim of the current work was the optimization of the assay time, requiring for the omission of any incubation and agglomeration times, we achieve the best results with the 532 nm laser wavelength and within a minimum interaction time (<1 min). TEM images of the coated bacteria (see Figure S1 and S2) illustrate that also with this short incubation time, the AgNPs are generated selectively at the bacteria surface.

Firstly, the Raman response of the polyslide was recorded and is shown in the Supplementary Material (Figure S3). There is no signal observed from the slide when it is covered with silver NPs, except the band at 240 cm^{-1}, ascribed to the metallic silver (Ag-Cl vibration). The polyslides we used to enable cell adhesion are efficient for both, Gram-negative and –positive bacteria. By reducing the spot volume, the sample is practically immobilized without the need of prior dehydration. The SERS experiments on different pathogens were assessed by stepwise reducing the total sample volume (bacterial and colloidal suspension) from 1 ml to 100 µl and the investigated spot volume to only 3 µl. A further reduction of the volume was tested without success, as the colloid is unstable in this case, probably due to an increased saline content in smaller volumes.

Figure 2A and 2B display the single-cell SERS spectra of several pathogens, recorded by using the polyslides as substrate for Bacteria@AgNPs approach, on Gram-positive and Gram-negative bacteria. The SERS spectra were processed by using a smoothing Savitzky-Golay function in OriginLab (OriginLab Corporation, Northampton, USA). The bacterial species exhibit similar SERS profile, with the precise position of some marker bands slightly varying between 724-728 cm^{-1}, 1046-1052 cm^{-1}, 1343-1349 cm^{-1}, 1413-1429 cm^{-1}, 1532-1536 cm^{-1}. In Figure 3, multiple raw single-cell SERS acquisitions are shown for each species; the corresponding assignment for the SERS bands is summarized in Table S1, which will be discussed in detail further down.

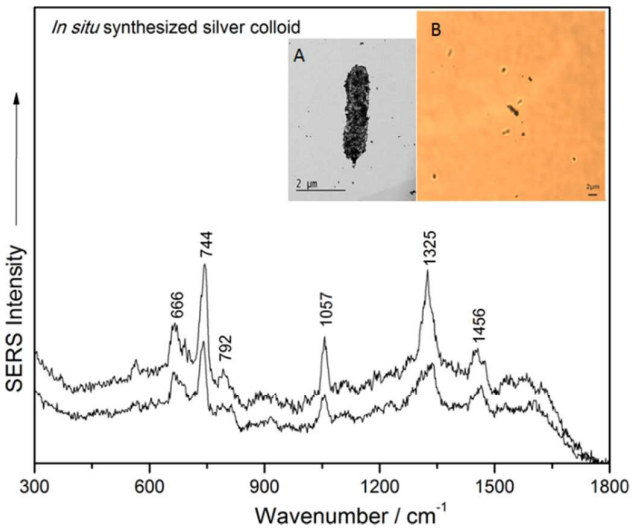

Figure 1. *Raw single-cell SERS spectra of E. coli XL1-Blue irradiated with 633 nm laser line. Inset – TEM micrograph (A) and 100× microscopic image (B) of E. coli, showing the in situ synthesized silver colloid coverage of the cell membrane.*

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Identification of pathogens based on single-cell SERS spectra

We were able to find SERS marker bands which are have been used for microorganisms' identification. These SERS bands are specific to the bacterial membrane components of the Gram-positive microorganisms (658 cm^{-1} - guanine, 731 cm^{-1} – glycosidic ring, 823 cm^{-1} - tyrosine, 1323 cm^{-1} and 1346 cm^{-1} - proteins, etc.) and of the Gram-negative pathogens, respectively, which are slightly shifted from one species to another (661/655 cm^{-1} - guanine, 736/738/728 cm^{-1} - adenine, 927/929/923 cm^{-1} – ring breathing vibration, 1151/1166 cm^{-1} - lipids, 1324/1328 cm^{-1} - adenine, 1467/1453/1455 cm^{-1} - saturated lipids). In all SERS spectra, a medium to high and sharp band appears in the range 1046-1052 cm^{-1}. These might originate from amine bond vibrations which have a strong affinity for adsorbing at the Ag surface and are selectively enhanced in SERS when perpendicularly oriented to the surface[28].

In recent studies, the band used for detection and/or discrimination of the pathogens is either the 1332 cm^{-1} band assigned to the CH deformations in proteins,[12] either the 730 cm^{-1} band assigned to the adenine and glycosidic ring stretching vibration.[10, 18] The "cathedral bands" (>1300 cm^{-1}), sometimes discussed in the context of Raman on biological material, can be assigned to photo-thermal degradation present at 1360 cm^{-1} and 1560 cm^{-1}, respectively,[29] and hence, should not be interpreted, nor should they be generated at all. High laser power and long exposure times result in photo-dissociation of the molecules situated at SERS "hot spots" and conduct to fluctuating carbon contributions within the SERS spectra.[30] Figure S4 displays the effect of long laser exposure on *Aeromonas* bacteria, prepared by using *in situ* synthesized AgNPs. Significant differences in the spectral profile were observed when irradiating for more than 30s. Overheating the sample and thermo-degradation can be avoided by careful optimization and adjustment of the experimental parameters (minimizing the laser power, reducing acquisition exposure time, defocusing, using an immersive objective and water to avoid local heating of the metallic NPs, etc.).

The influence of the growth medium used for cultivation on the SERS signature of bacteria

In a recent publication, Premasiri et al.[31] suggested that the SERS spectra of bacteria are caused by starvation conditions and reflect secreted metabolic substances rather than cell wall components. They proposed that bacterial SERS spectra are usually due to small purine-like molecules such as adenine, hypoxanthine, guanine, uric acid and adenosine monophosphate (AMP), all metabolites of purine degradation as starvation response. They enriched cell supernatant by approximately an order of magnitude before analysis, revealing SERS spectra which are astonishingly similar to bacteria SERS spectra.

In order to assess if this hypothesis holds when using our *in situ* generated SERS colloids, we tested the sensitivity of our approach to influence on the organisms' growth conditions. A high sensitivity to these conditions would enable to distinguish effects such as the suspected excretion of substances upon starvation. In an earlier study, we could already demonstrate the discrimination between the different growth phases of *E. coli* by using the Bacteria@AgNPs approach.[10] Now, we were

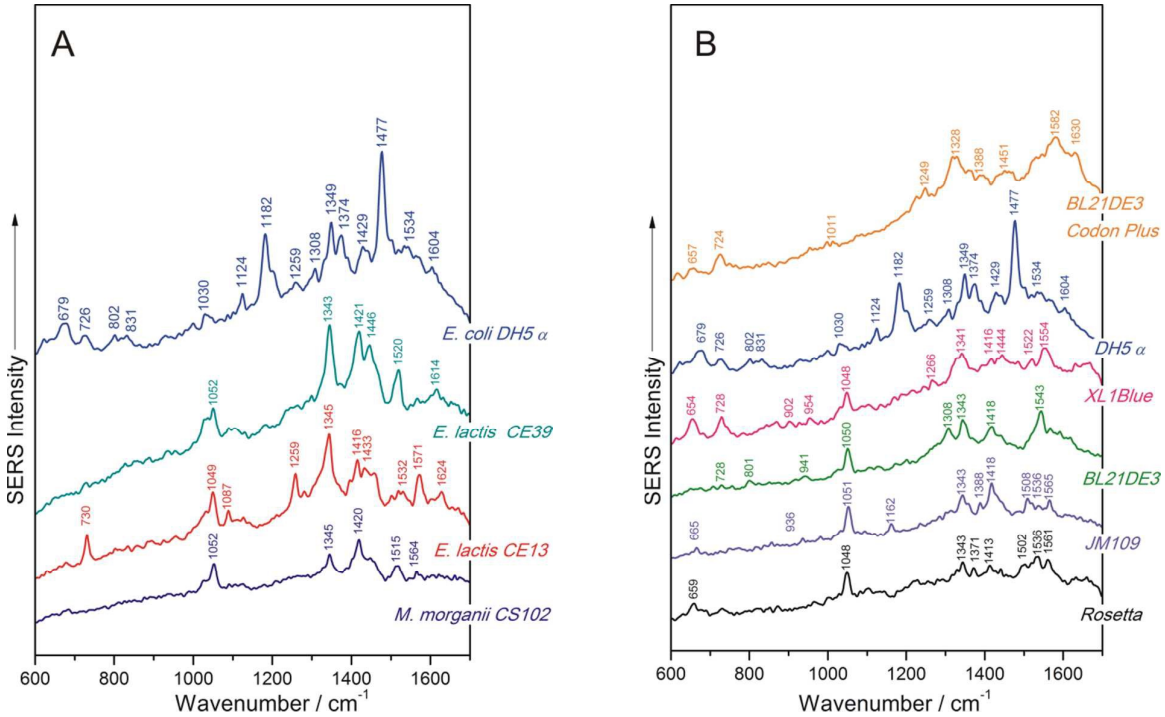

Figure 2. *Single-cell SERS spectra of several pathogens compared to an E. coli strain (A) and of different strains of E.coli (B), respectively, recorded by using the polyslides as substrate for Bacteria@AgNPs, irradiated with the 532 nm laserline.*

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looking for the influence of slightly different growth media upon SERS spectral features. We compared the SERS signatures of *E. coli* bacteria grown in culture media sterilized in two distinct ways. Additionally, the supernatant obtained from each centrifugation step was tested by SERS. *E. coli* ROSETTA (DE3)pLysS were grown in autoclaved LB Broth medium and in filtered LB Broth medium. It was verified that both sterilization treatments are successful and no contamination was identified.

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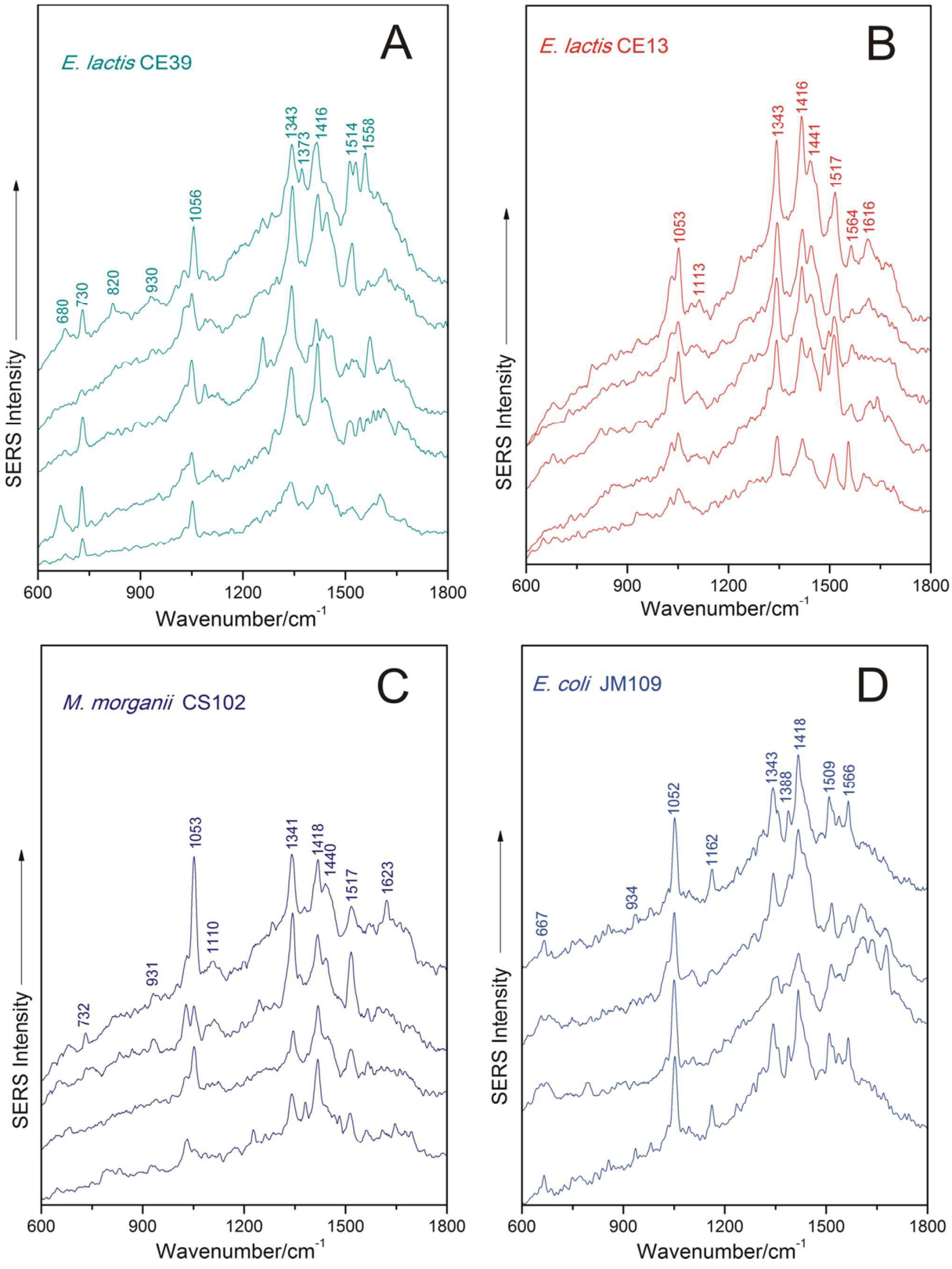

Figure 3. *Reproducible raw single-cell SERS spectra of E. lactis CE39 (A), E. lactis CE13(B), M. morganii CS102 (C) and E.coli JM109 (D), respectively.*

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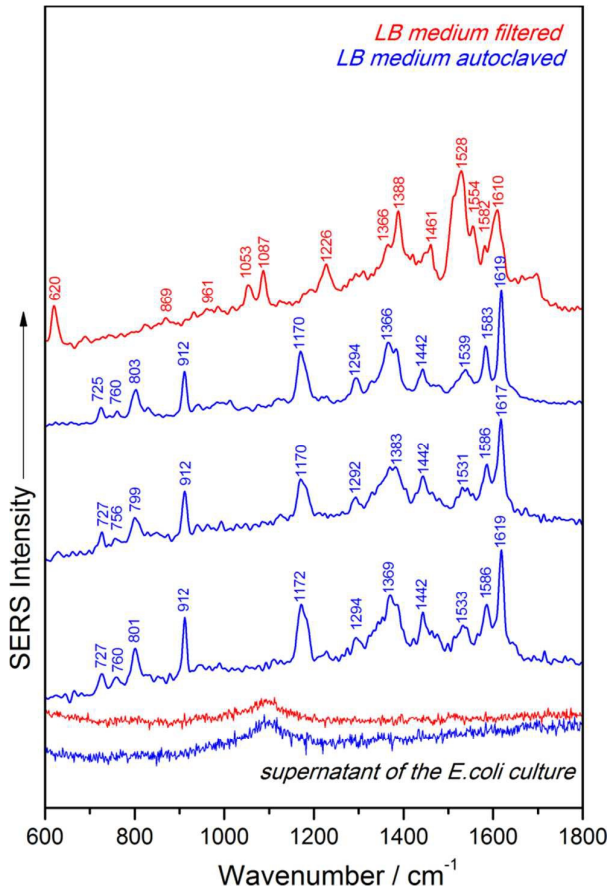

Figure 4. *Single-cell SERS spectra of E. coli ROSETTA* (DE3)pLysS *showing the influence of different sterilization pretreatments of the growth media used for cultivation (532 nm excitation wavelength).*

Figure 4 shows the SERS signature of *E. coli* bacteria and the Raman signal of the supernatant obtained after centrifugation of the bacteria suspension. While the constituents of the medium used for cultivation feature no relevant signals, the signature of the bacteria cultivated by using these two media differ significantly, depending on the pre-treatment of the medium used for cultivation. These media contain high concentrations of nutrients and therefore, we presume that metabolic changes occurring during the growth are influencing the SERS signature via their influence on the membrane composition of the bacteria. These results underline the fact that our *in situ* prepared colloids grant sufficient sensitivity to monitor even slight differences in the bacterial membrane composition induced by external factors (growth phase, nutrition supply, sterilization treatment of the growth medium, etc.) based on single organism analyses. Despite this high sensitivity, we did not find any indication for the theory of Premasiri et al. The spectral variations induced by the growth conditions do not hinder the discrimination of bacteria at strain level. Major SERS marker bands are identified in each case by chemometric analysis.

Principal Component Analysis on the SERS single-cell spectra

While the spectra of different organisms feature some evident differences, a chemometric approach is required to classify accurately and automatically different pathogens based on the single-cell SERS spectra. Principal Component Analysis (PCA) was employed as unsupervised multivariate analysis tool for spectral data management. Before the PCA analysis, pretreatments such as baseline correction and normalization were applied to each raw SERS spectra. By summing the first three principal components, we obtained an explained total variance of 56% for the full range of SERS spectra. However, the variables corresponding to the spectral range between 585-767 cm^{-1}, and those corresponding to 1320-1690 cm^{-1} interval respectively, have more significant contribution to the principal components of our analysis than the rest of the variables (as seen in Figure S5 and in the loadings from Figure S6). We performed a new principal component analysis taking into account just the above mentioned variables. There was no significant change regarding the distribution of the samples. The two clusters (Gram-negative in red and Gram-positive in green – Figure 5) kept their previous location on the 3D score plot. Samples belonging to Gram-positive group were placed in the negative direction for PC-1, negative direction for PC-2, and were evenly distributed along the PC-3 axis. Most of the samples belonging to the Gram-negative group were distributed along the positive direction for both PC-1 and PC-2, except some of them, which fell into the negative direction of PC-1. Their distribution on the PC-3 axis is also evenly in both directions. On the other hand, the explained total variance has increased up to 63%, as pictured in Figure 5. Even if Gram-positive samples are more dissipated than Gram-negative ones, they do not interfere with each other.

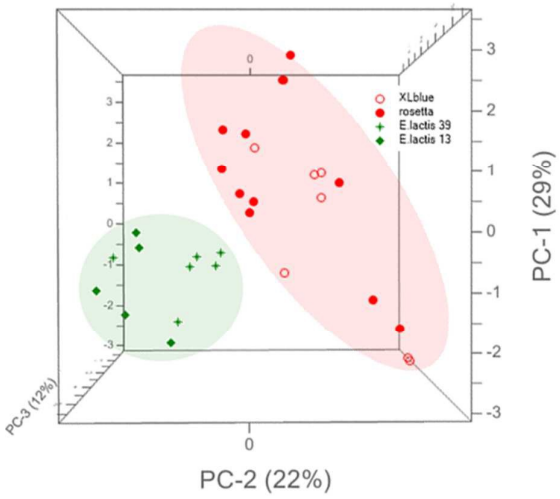

Figure 5. *PCA scores 3D plot showing the grouping of two Gram-positive (E. lactis CE13 and CE39, respectively) and two Gram-negative species (E. coli ROSETTA* (DE3)pLysS *and E. coli XL1Blue).*

Conclusions

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A rapid label-free SERS-based biosensor for pathogens detection and identification was successfully tested for both Gram-positive and –negative bacteria by using low cost, accessible polyslides, featuring assay time below 5 min. It was found that for SERS analyses with such short incubation times of the SERS-active nanoparticles, the best excitation wavelength is 532 nm, while after longer incubation times, leading to nanoparticle agglomeration, a better signal to noise ratio is achieved with an excitation wavelength of 633 nm. The reproducibility and reliability of the SERS results were demonstrated and the influence of growth media pretreatment before cultivation on the bacterial SERS signatures was revealed. The model strains selected for investigation represent common human pathogens. The approach has to be reliable both for the Gram-negative and Gram-positive bacteria, in single or multi-species detection cases.

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