



SERS-based sensor for the detection of sexually transmitted pathogens in the male swab specimens: A new approach for clinical diagnosis

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ABSTRACT

The surface-enhanced Raman scattering (SERS) has been widely tested for its usefulness in microbiological studies, providing many information-rich spectra which are a kind of 'whole-organism fingerprint' and enabling identification of bacterial species. Here we show, previously not considered, the comprehensive SERS-chemometric analysis of five bacterial pathogens, namely *Neisseria gonorrhoeae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*, and *Haemophilus ducreyi*, all being responsible for sexually transmitted diseases (STDs). In the designed biosensor, the direct, intrinsic format of the spectroscopic analysis was adopted for the SERS-based screening of gonorrhea and chlamydiosis due to vibrational analysis of men's urethra swabs. Our experiments demonstrated that the applied method enables identification the individual species of the *Neisseria* genus with high accuracy. In order to differentiate the sexually transmitted pathogens and to classify the clinical samples of male urethra swabs, three multivariate methods were used. In the external validation the created models correctly classified the men's urethra swabs with prediction accuracy reaching 89% for SIMCA and 100% for PLS-DA. As a result, the developed protocol enables: (i) simple and non-invasive analysis of clinical samples (the collection of urethra swabs specimens could be carried out at different points of care, such as doctor's office); (ii) fast analysis (<15 min); (iii) culture-free identification; (iv) sensitive and reliable SERS-based diagnosis of STD. The simplicity of the developed detection procedure, supported by high sensitivity, reproducibility, and specificity, open a new path in the improvement of the point-of-care applications.

1. Introduction

Sexually transmitted diseases (STDs) refer to infections and syndromes caused by different microorganisms (bacteria, viruses, protozoa), passed through sexual contacts. It is estimated that each year around 214 million people struggle with STDs caused only by bacteria: *Chlamydia trachomatis* (127 million) and *Neisseria gonorrhoeae* (87 million) (J. Rowley et al., 2019). That makes STDs an epidemic and can lead to the numerous economic and health consequences (Owusu-Edusei et al., 2013).

Gonorrhea and chlamydiosis are the most common STDs caused by the Gram-negative *N. gonorrhoeae* and *C. trachomatis*, respectively. Although people diagnosed with chlamydiosis usually have no

symptoms, the gonorrhea progression patterns in men and women are significantly different – it is almost asymptomatic in women (only in some cases inflammation of the cervical canal and urethra are observed) in contrast to men, in the case of whom more than 90% experience mild to severe dysuria, and usually abundant purulent discharge (Bébéar and de Barbeyrac, 2009; Little, 2006; Stamm, 1999).

There are several methods that enable diagnosis of sexually transmitted infections, such as: syndromic approach, Gram staining, microbial culture, antigen detection test or nucleic acid amplification test (NAAT), including polymerase chain reaction (PCR). All these methods have some disadvantages and limitations, e.g., syndromic approach is difficult because each of the sexually transmitted infections require different therapy. Gram staining method is characterized by low

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detection rate, while immunoassay tests have low sensitivity (Dyck et al., 2001; Su et al., 2011). On the contrary, NAATs are characterized by high sensitivity and specificity, but the reagents required to perform the tests are expensive and the method itself is laborious and requires qualified staff (Hardwick et al., 2009; Su et al., 2011). Culture tests are also characterized by high level of specificity, but they are less sensitive than NAATs. Nonetheless, they are suitable for testing a vast majority of microbiological and clinical samples and thus they are considered as a 'gold standard' for gonorrhea diagnosis. Despite many advantages, the culture method has some drawbacks such as time-consuming incubation (≥ 48 h) or specific culture media. Also, the obtained bacterial colonies should undergo presumptive identification by Gram staining and oxidase or catalase test. In the case of positive result, confirmatory identification test should also be performed (fluorescent antibody test, coagglutination test, colourimetric test, API biochemical test including carbohydrate degradation test) (Olshen and Shrier, 2005; van Dyck et al., 1999). For these reasons, the fast, cost effective, and precise method of STDs detection is still desirable.

The surface-enhanced Raman scattering (SERS) has been applied as the powerful fingerprinting technique for the detection and characterization of whole-microorganisms such as bacteria and fungi (Lin et al., 2014; Mabbott et al., 2016). This technique offers many advantages including fluorescence quenching, short time of measurement, and low costs of analysis. All these features place the SERS technique among methods appropriate for biomedical applications (Hudson and Chumanov, 2009).

Even though several groups reported the successful detection and identification of bacteria using SERS-based assay (Bozkurt et al., 2018; Guven et al., 2011; Kamińska et al., 2015), the real application of this technique in clinical trials is still challenging due to the lack of the homogeneity and reproducibility of the SERS substrates, resulting in fluctuations of SERS responses. So far, the SERS analysis of only two sexually transmitted pathogens, namely *C. trachomatis* and *N. gonorrhoeae*, have been presented in terms of determining the molecular origins of particular bands in their SERS spectra and monitoring the extracellular dynamics and biochemical activity. The list of pathogens responsible for STD includes also *Ureaplasma urealyticum*, *Mycoplasma* spp., *Trichomonas vaginalis*, herpes simplex virus (HSV), human papillomavirus (HPV) and *Haemophilus* spp. (Holmes et al., 2008). The following work demonstrates an application of SERS technique for the diagnosis of sexually transmitted diseases (STDs). Our studies have been extended to the SERS measurements and chemometric analysis of the most common bacterial species causing STD: *N. gonorrhoeae*, *U. urealyticum*, *Mycoplasma hominis*, *Mycoplasma genitalium*, and *Hemophilus ducreyi*. Additionally, the results presented in this study reveal, previously not considered, rapid identification of *N. gonorrhoeae* in clinical samples, namely men's urethra swabs. The experiments can be performed both in the indirect and direct strategy. The analysis of spectral images of men's urethra swabs was performed with the use of PCA, partial least square discriminant analysis (PLS-DA), and soft independent modelling of class analogies (SIMCA).

2. Materials and methods

2.1. Bacteria strains and culture conditions

N. gonorrhoeae ATCC 70825/FA 1090, *Neisseria sicca* ATCC 9913, *U. urealyticum* ATCC 27618, *M. genitalium* ATCC 33530, *H. ducreyi* ATCC 33940 were purchased from American Type Culture Collection (ATCC), while *M. hominis* 50616T from Culture Collection University of Gothenburg (CCUG). The strains of *Neisseria meningitidis* ATCC 13102 and *Neisseria lactamica* ATCC 23790 were obtained from National Medicines Institute in Warsaw. The applied culture conditions are presented in Table S1 (Supplementary Materials).

2.2. Procedure of men's urethra swabs collection and preparation for SERS measurements

All the clinical samples (men's urethra swabs) were obtained from The Infant Jesus Clinical Hospital (Department of Dermatology and Venerology) in Warsaw. The samples were divided into two groups:

- 1) experimental group: samples from men diagnosed with STDs. In the case of all patients the infection was caused only by one type of bacterial pathogen.
- 2) control group: samples from healthy male volunteers.

The clinical samples were taken ~ 4 h after the patients had passed urine. In each case, the 10 μ L sterile inoculation loop was inserted into man's urethra in the depth of 2–3 cm and gently rotated for 5–10 s. The freshly collected samples were transferred into Eppendorf tubes® and immediately transported to the laboratory. The SERS measurements of clinical samples were performed in two variants:

- 1) indirect identification - the clinical samples were first inoculated on the appropriate agar medium (Table S1) and cultured. The inoculation was performed in the doctor's office immediately after the sample was taken. The bacterial colonies obtained after incubation were next investigated using the SERS method.
- 2) direct identification each clinical sample was mixed with 2.5 μ L of sterile saline solution (0.9% sodium chloride). Next, the 1 μ L of such mixture was placed onto SERS substrates and analyzed.

All the results obtained for clinical samples were confirmed by non-spectroscopic identification methods. Gonorrhea was confirmed by culture method (bacteria where cultured on Chocolate agar + PolyviteX™ vcAT3 agar (Biomerieux) in 37 °C and 5% CO₂ for 48h), Gram staining, oxidase test and biochemical methods, while chlamydiosis - by direct immunofluorescence test (MicroTrak *Chlamydia trachomatis* Direct Specimen Test, Trinity Biotech, Ireland) and fluorescence microscopy.

2.3. Procedure of bacteria preparation for SERS measurements

The preparation of bacteria cells for SERS measurements highly depends on the type of culture medium and the type of analyzed bacterial strain. Hence, two different preparation procedures were applied. In the case of *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. sicca*, and *H. ducreyi*, cultured on chocolate agar media, a few selected colonies of each strain were collected via inoculation loop and transferred to separate Eppendorf tubes® containing 100 μ L of 0.9% NaCl solution. Next, the samples were vortexed for 10 s and then centrifuged for 2 min at 1070 $\times$ g. For each sample the supernatant was removed, and the new portion of saline solution was added. Such rinsing procedure was repeated three times. The obtained pellet was resuspended in 10 μ L of saline solution and mixed. Finally, 1 μ L of the prepared solution was placed onto SERS substrate and left to dry for a few minutes.

M. genitalium, *M. hominis*, and *U. urealyticum* were cultured in liquid media. Next, 100 μ L of each mixture was separately transferred into Eppendorf tube® and centrifuged for 10 min at 1070 $\times$ g. Subsequently, the supernatant was discarded and mixed with 100 μ L of saline solution. The process was repeated two times. Finally, the obtained pellet was resuspended in 10 μ L of saline solution. The obtained mixture, in the amount of 1 μ L, was placed onto a SERS substrate and left to dry for a few minutes. Next, the samples were immediately analyzed via SERS. In each cultivated bacterial sample, the concentration of bacteria was at the level of 10⁸ cfu/ml, whereas in the clinical samples the concentration was at the level of 10⁶ cfu/ml (McFarland densitometer DEN-1B, Biosan).

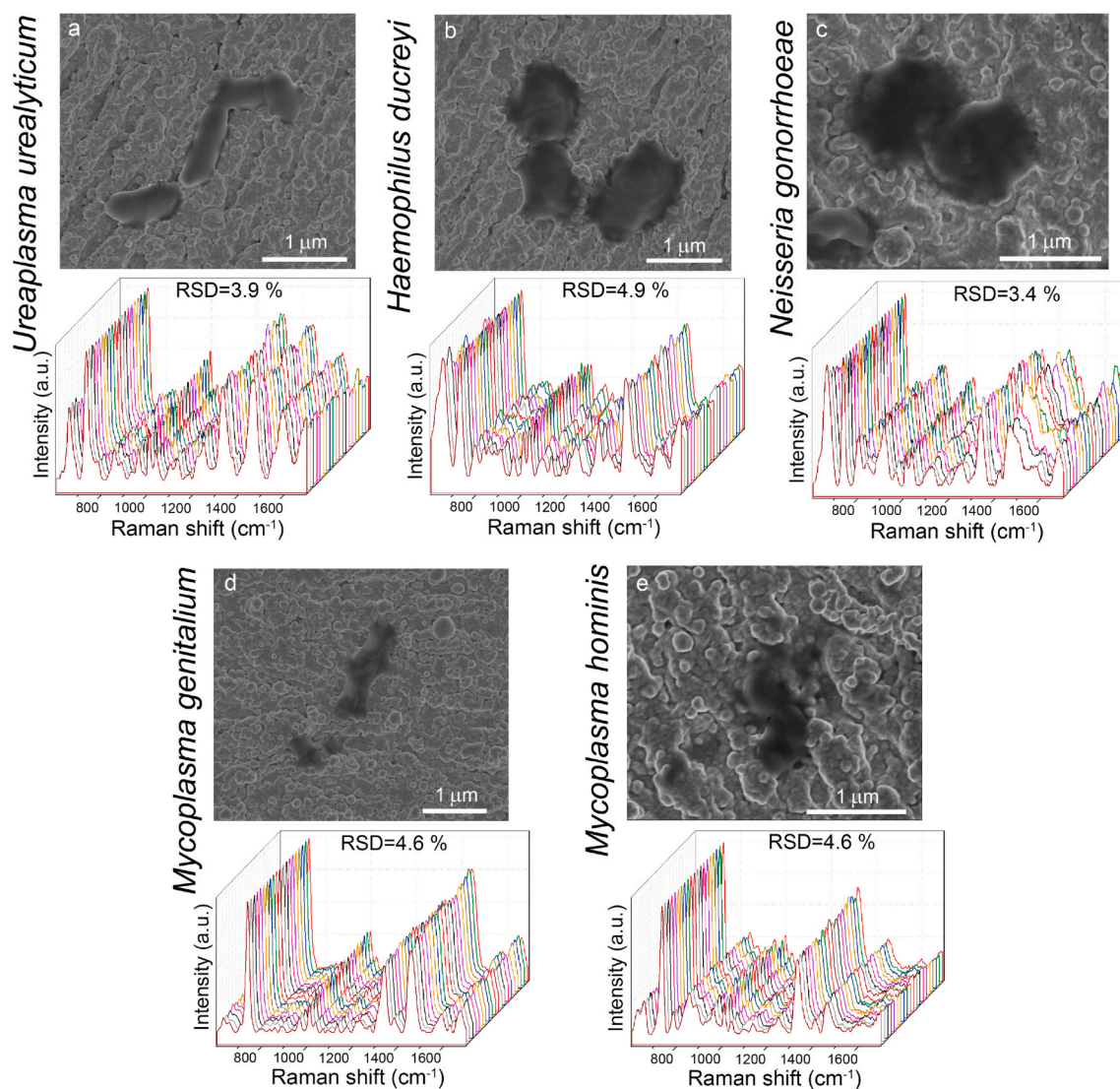


Fig. 1. The SEM images and SERS spectra with calculated RSD (relative standard deviation) for five investigated pathogens: *U. urealyticum*, *H. ducreyi*, *N. gonorrhoeae*, *M. genitalium*, and *M. hominis* placed onto the Si/Ag SERS platforms.

2.4. Preparation of SERS substrates

The SERS-active silicon-based substrates covered with silver (the Si/Ag SERS platforms) were prepared in few steps. First, a clean silicon wafer was mechanically cut into 3 mm × 3 mm pieces. Subsequently, the substrates were physically subjected to laser ablation with a femto-second laser and sputtered with 100 nm Ag layer. Detailed preparation procedure is described in another publication (Szymborski et al., 2021).

2.5. SERS spectroscopy

Bruker's BRAVO spectrometer equipped with Duo LASER™ (spectral range 700–1100 nm) and a CCD camera was used for all the SERS investigations. The application of a double-laser system allowed for automatic removal of the background fluorescence. The laser power was 100 mW for both lasers and the spectral resolution was 2–4 cm⁻¹. In order to show the reproducibility of the results, about 30 single measurements were performed for each bacteria strain and for each clinical sample. The time needed to record a single spectrum of bacteria or clinical sample was 18 s (3 accumulations of 6 s measurement).

2.6. Post-processing of SERS spectra

The SERS spectra were postprocessed using the OPUS software (Bruker Optic GmbH, 2012 version). For this purpose, the following manipulations were applied: smoothing (Savitzky Golay Filter: 5 points), baseline correction (concave rubberband correction; number of iterations: 6, number of baseline points: 6), cutting (in the range from 600 to 1700 cm⁻¹), and normalization (Min-max normalization). Next, the spectra were analyzed (PCA, PLS-DA, and SIMCA) in the Unscrambler program (CAMO software AS, version 10.3, Norway). For more information about applied chemometric methods, see Supplementary Materials – Section S1.

3. Results

3.1. SERS-based analysis of sexually transmitted pathogens

The SEM images presented in Fig. 1 reveal the surface of the SERS-active platforms coated with bacterial cells (*U. urealyticum*, *H. ducreyi*, *N. gonorrhoeae*, *M. genitalium*, and *M. hominis*). The size of the crystallites of SERS support (which usually appear, according to the SEM images, as agglomerates with size ranging from 60 nm to 100 nm) corresponds to

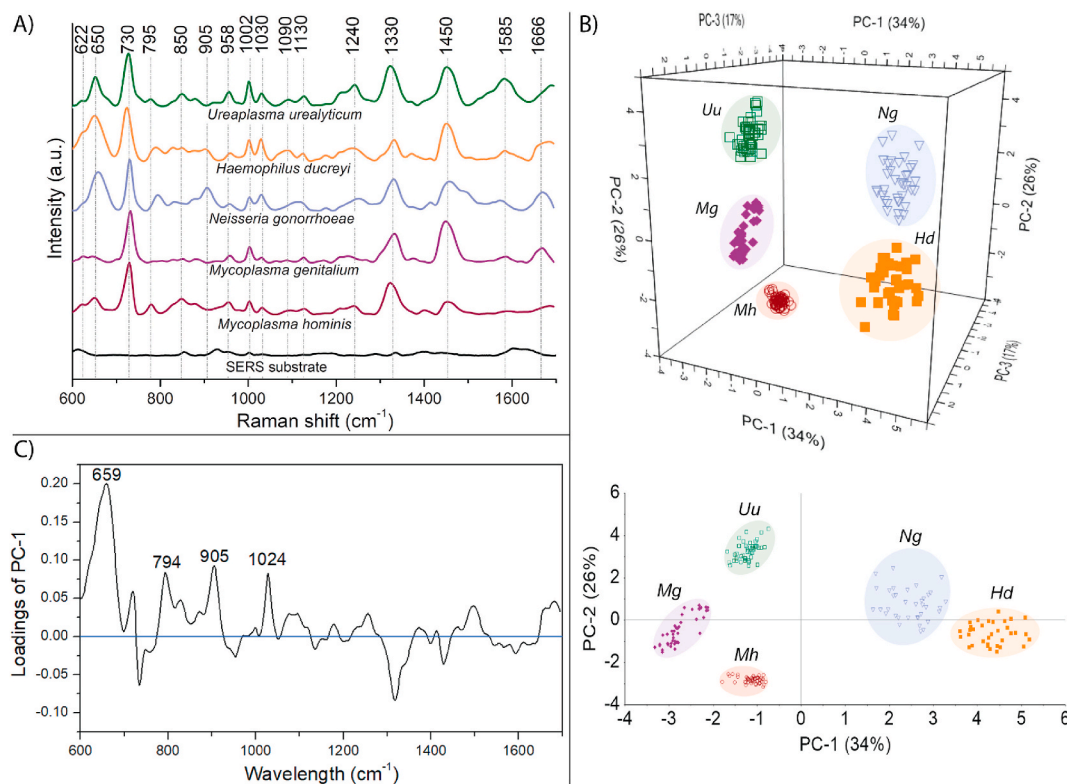


Fig. 2. The SERS spectra (A), PCA (B), and loadings plot of PC-1 (C) obtained for five investigated, sexually transmitted pathogens: *U. urealyticum* (*Uu*), *H. ducreyi* (*Hd*), *N. gonorrhoeae* (*Ng*), *M. genitalium* (*Mg*), and *M. hominis* (*Mh*).

the optimal size of nanostructures (50–70 nm) for the LSPR resonance. The enhancement factor (EF) calculated for 10^{-6} M p-MBA measured on Si/Ag platform reached the value of 10^8 . As can be noticed, *H. ducreyi* has a shape of coccobacillus, while *N. gonorrhoeae* has well-defined coccal morphology (Goldman and Prabhakar, 1996). In turn, *U. urealyticum*, *M. genitalium*, and *M. hominis* belong to Mollicutes - a class of the smallest living prokaryotes characterized by the absence of a cell wall and round or coccobacillary shape (*U. urealyticum* and *M. hominis*) or irregular/coccal shape (*M. genitalium*) (Roberts and Kenney, 1987; Tully et al., 1983).

The 3D plots in Fig. 1 demonstrate the reproducibility of SERS spectra of five investigated bacteria species, each measured on a single SERS platform. The relative standard deviation (RSD) of the band at around 1330 cm^{-1} ranged from 2.6% to 4.9% in relation to the intensity of the same band in the averaged plot (Fig. S1). Such an outstanding reproducibility is crucial for performing SERS-based classification.

Fig. 2A presents the averaged SERS spectra of i) the implemented SERS substrate and ii) all investigated sexually transmitted pathogens which demonstrate the SERS-based response of the biomolecules which are in close proximity to the mentioned SERS substrate. Therefore, these SERS images of bacteria are dominated by the bands assigned to: (i) the molecules present in the cell wall and/or cell membrane, e.g., flavin-containing compounds (riboflavin, flavin adenine dinucleotide - FAD), phospholipids, proteins and to (ii) the metabolic secretions of the cells, e.g., metabolites of purine degradation (guanine, adenine, adenosine monophosphate, xanthine, hypoxanthine, and uric acid) (Mosier-Boss, 2017). In the case of living cells, the effect strongly differs from this observed for single molecules in terms of Raman signal enhancement (as bacterial cells are many times bigger than molecules and they cannot reach the single hot spots). Still, the results obtained for bacteria allow to differentiate even closely related species.

As one can observe, every spectrum is dominated by the bands at around 730 , 1330 , and 1450 cm^{-1} , which are attributed to adenine-related compounds (FAD, AMP, ADP, ATP, NAD, and RNA), CH_3CH_2

wagging mode in purine bases, and CH_2 bending mode in phospholipids, respectively (Kubryk et al., 2016). The strong band can be observed at around 650 cm^{-1} can be assigned as C–C twisting of tyrosine or ring-breathing mode of guanine. Despite the common spectral features which can be detected for all investigated bacteria, the distinctive spectroscopic dissimilarities between them can be clearly observed. For example, in the SERS spectra of *M. hominis* and *M. genitalium* the band at ca. 650 cm^{-1} is very weak in comparison to the same band in other spectra of sexually transmitted bacteria. The low intensity of this mode could be explained by the reduced amount of extracellular DNA in the biofilm matrix of *Mycoplasma* spp. (Chao and Zhang, 2012). Additionally, while comparing the region between 1640 and 1680 cm^{-1} in the spectra of *M. hominis* and *M. genitalium*, one may notice different contributions of the amide I band, which arises from C=O stretching, out of phase C–N stretching, N–H bending and/or from the C–C–N deformations in proteins (Siddhanta and Narayana, 2012). Other unique features are, e.g., the intense band at around 905 cm^{-1} in the case of *N. gonorrhoeae*, the shoulder band at ca. 622 cm^{-1} in the spectrum of *H. ducreyi*, and the intense signals at 1240 and 1585 cm^{-1} in the *U. urealyticum* spectroscopic image. The spectrum of SERS substrate is completely devoid of any intense bands, hence no related interference affects the spectrum of bacterial cells. The tentative band assignments for all the investigated bacteria are presented in Table S2.

To summarize, although the SERS spectra of five studied bacterial pathogens exhibit many similarities, they differ in the relative intensity and/or position of many bands. These differences can be easily observed and are connected with metabolic secretions of the cell (components of biofilm matrix), its biological structure (proteins, lipids, carbohydrates, polysaccharides, etc.) and biochemical activity.

In the present work, the discrimination among five studied bacterial strains was obtained by applying PCA. As one can observe, the 2D-PCA and 3D-PCA plots exhibits five separate groups corresponding to five analyzed pathogens (Fig. 2B). The calculated scores for all studied bacteria indicate that PC-1 and PC-2 are responsible for 60% of the

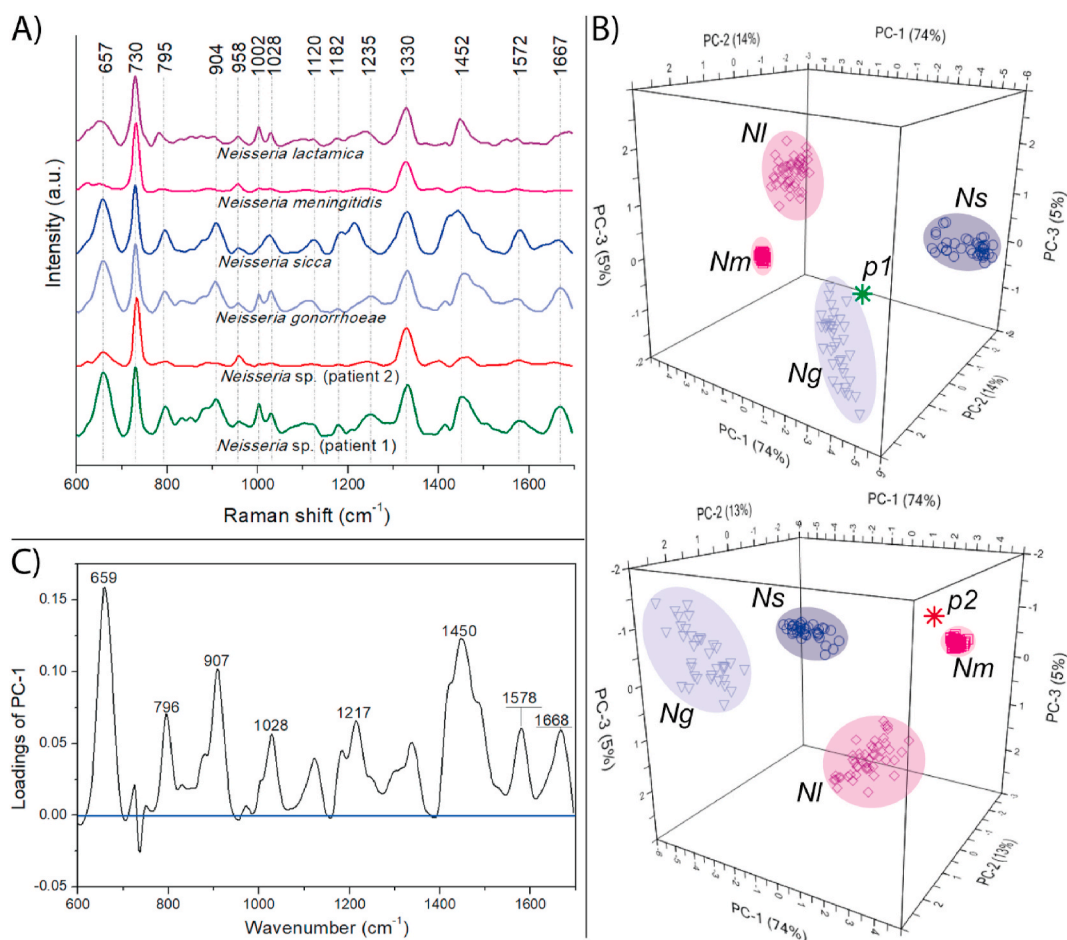


Fig. 3. The SERS spectra (A), 3D-PCA (B), and loadings plot (C) performed for two *Neisseria* strains detected in urethra swabs of two male patients: Patient 1 (p1) and Patient 2 (p2), and for type strains of *N. gonorrhoeae* (Ng), *N. meningitidis* (Nm), *N. lactamica* (Nl), and *N. sicca* (Ns).

variance in the dataset (Fig. 2B, bottom), and together with PC-3 – for 77% (Fig. 2B, top). Interestingly, the PC-1, which defines the direction of the greatest variance in the data, distinctly divides the class of Mollicutes (*U. urealyticum*, *M. genitalium*, and *M. hominis*) from the rest of analyzed bacteria (*H. ducreyi* and *N. gonorrhoeae*). According to the PC-1 loading plot (Fig. 2C), the most weighted variables located at 659 cm⁻¹ (protein, guanine, and thymine), 794 cm⁻¹ (cytosine, uracil), 905 cm⁻¹ (tyrosine), and 1024 cm⁻¹ (phospholipids, carbohydrates) are most liable for differentiation among analyzed species.

3.2. Indirect identification of bacterial strains presents in clinical samples

As was mentioned in the introduction, microbial culture is a ‘gold standard’ method for diagnosis of gonorrhea in men. Thus, in most laboratories it is used as a reliable method. However, the identification of cultured colonies and the subsequent confirmation of the obtained results should be performed. Since taxonomic differences between members of *Neisseria* genus are not significant, the identification of *N. gonorrhoeae* may be problematic (Alexander and Ison, 2005; Ng and Martin, 2005). Hence, in our study we would like to propose an alternative SERS-PCA approach of fast identification of bacteria species isolated from man’s urethras and grown on chocolate as an indirect method of gonorrhea diagnosis.

To confirm the potential of the proposed biosensor for the gonorrhea detection, the spectra of unknown strains detected in two different samples of men’s urethra swabs were compared with the spectra of four different *Neisseria* species (*N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, and *N. sicca*). Opposite to *N. gonorrhoeae*, the rest *Neisseria* species

colonize especially upper respiratory tract, but their presence in the genital tract was also confirmed (Faur et al., 1975; Wilkinson, 1952). Fig. 3A depicts the obtained SERS results.

The SERS spectra analysis, calculated reproducibility of recorded signals (Fig. S1) and SEM images (Fig. S2) of all *Neisseria* species have been described in Section 2 of the Supplementary Materials. It may be concluded that each *Neisseria* sp. spectrum has conspicuous features which are crucial for taxonomic affiliation.

In the next step, to confirm the practical applications of SERS-based analysis in the STDs diagnosis, two different bacterial strains detected in two different samples of men’s urethra swabs were measured via SERS. The results are labeled as ‘Patient 1’ and ‘Patient 2’ in Fig. 3A and are represented respectively, by green (p1) and red (p2) asterisks in Fig. 3B. As can be noticed, the spectrum obtained for the bacterial strain detected in clinical sample of Patient 1 practically overlaps in the whole fingerprint region (600 - 1700 cm⁻¹) with the spectrum of *N. gonorrhoeae*, while the spectrum of the bacteria found in urethra swab of Patient 2 with the spectrum of *N. meningitidis*. In order to improve the empirical analysis and differentiation of the recorded SERS results, the spectra were analyzed via PCA. The applied chemometric method confirmed the visual assignment. The Unscrambler program located the green asterisk (p1) in close proximity to the points representing *N. gonorrhoeae* (Fig. 3B, top), while the red asterisk (p2) was found among points representing *N. meningitidis* (Fig. 3B, bottom). The PC-1 together with PC-2 and PC-3 explained 93% (Fig. 3B, top) and 92% (Fig. 3B, bottom) of the total spectral variance for the investigated strains. The SERS identification of unknown pathogens isolated from clinical samples were confirmed by genetic analysis of the 16S rRNA gene sequencing (primers: 27F, 1492R). Based

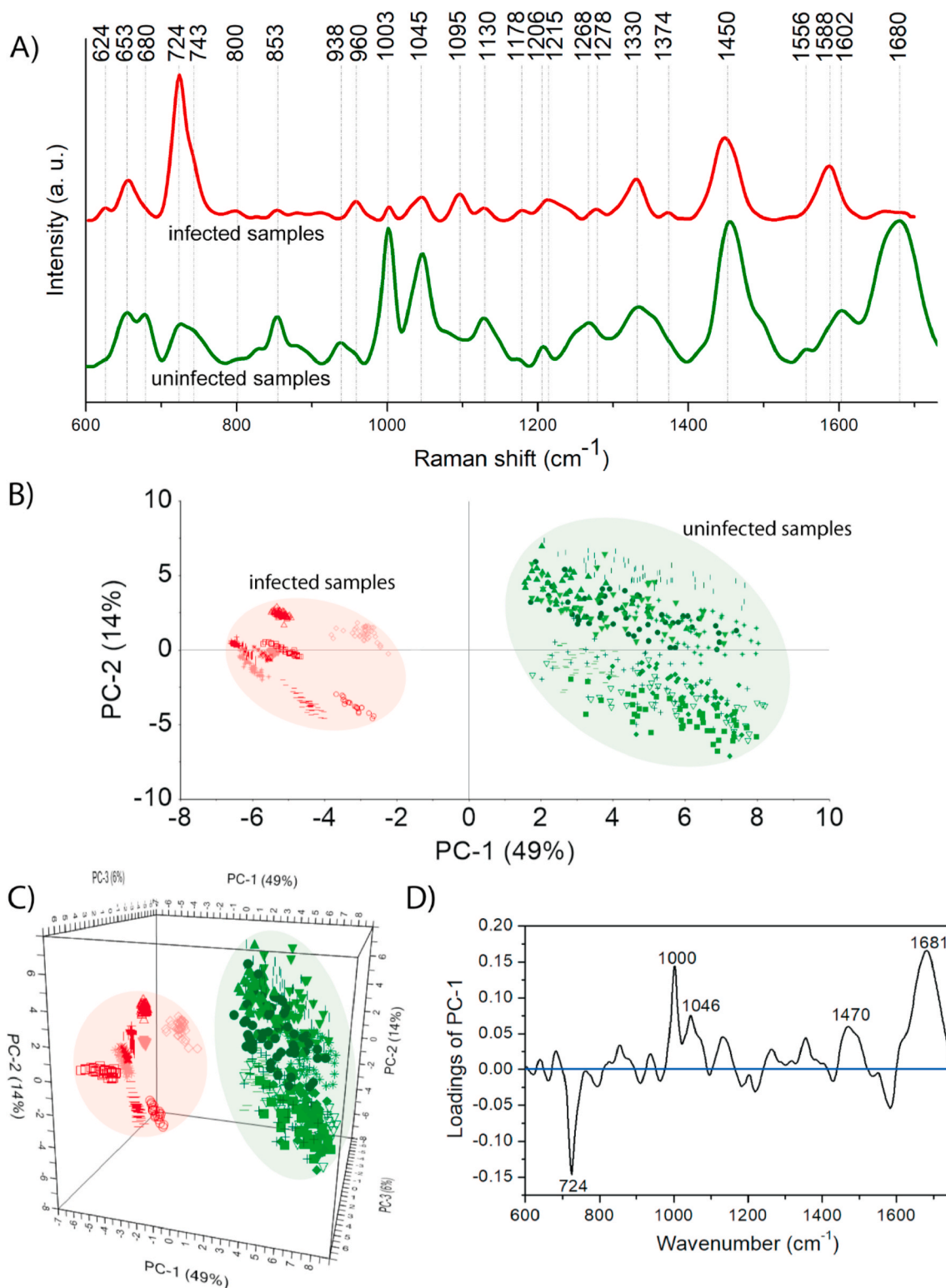


Fig. 4. The averaged SERS spectra (A), 2D-PCA (B), 3D-PCA (C), and loading plot (D) calculated for 20 different samples of male urethra swabs: 10 from control group (signed as 'uninfected samples' and marked with green) and 10 from experimental group (signed as 'infected samples' and marked with red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

on the loading plot (Fig. 3C) the most prominent bands, which strongly influenced the obtained group separation, are located at 659, 796, 907, 1028, 1217, 1578, 1450, and 1668 cm^{-1} and are highly correlated with the bands observed in the SERS spectra (Fig. 3A). These results clearly indicate that the accuracy of the identification of unknown pathogens detected in men's urethra swabs is very high and thus reliable. This

makes the proposed method competitive to currently used techniques applied in gonorrhea diagnosis (Table S3); (Moyes et al., 2009; Spence et al., 2008; Wunschel et al., 2020).

3.3. The direct SERS-based analysis of men's urethra swabs

To validate the potential of the SERS-based biosensor in clinical applications, the direct measurements of urethra swabs (infected and uninfected) were performed.

In our approach a unique SERS spectral fingerprints of infected and uninfected specimens allow for their binary differentiation. Additionally, the results obtained from infected samples served to identify the type of pathogen which caused an infection. The analysis may be performed almost immediately after obtaining a spectroscopic image, as the proposed identification method is label-free and the additional recognition elements such as Raman reporters or specific antibodies are redundant. Moreover, in the case of all infected samples it was observed that the concentration of bacteria was very high ($\sim 1.5 \times 10^6$ cells/mL) (Priest et al., 2017). Such a number of bacterial cells was sufficient to obtain a very strong SERS signal, as the method is characterized by ultra-high sensitivity proven by the multiple studies performed for a single molecules. As a result, the time-consuming step of bacteria culture could be eliminated. Due to the spectroscopic properties of our developed SERS platform, it is possible to detect very low concentrations of bacterial cells in the investigated sample. The obtained limit of detection (LOD) for *N. gonorrhoeae* was at the level of 10^2 cfu/mL (Fig. S3).

Here, for the first time, the direct spectroscopic analysis of the men's urethra swabs was performed in order to facilitate and accelerate the diagnosis of gonorrhea and chlamydia. For this purpose, 20 samples of male urethra swabs were collected: 10 from men diagnosed with gonorrhea or chlamydia (experimental group) and 10 from healthy male volunteers (control group). It should be highlighted that the biochemical composition of the secretions from male urethra is very complex. In the case of healthy individuals, it consists of urea, creatinine, uric acid, ammonia, and mineral salts (chlorides, phosphates, carbonates). The swab consists also of cells such as leukocytes (neutrophilic granulocytes), and epithelial cells (pseudostratified columnar epithelium, stratified squamous epithelium), which are built of, among others, glycolipids, phospholipids, cholesterol and various surface and transmembrane proteins. Additionally, as the men's urethra has a frequent contact with semen, the components such as: albumins, putrescine, spermine, spermidine, cadaverine, lipids, proteins, prostaglandins, steroid hormones, cholesterol, globulins, fructose, vitamins B₁₂ and C, citric acids, acid phosphatase, fibrinolysis, hyaluronidase, mucus may also be found in urethra swab (Horner et al., 2016; Owen and Katz, 2005).

As one can notice, the differences between the averaged SERS spectra are significant. This includes bands positions and intensities in the whole fingerprint region. The spectrum representing healthy subjects is dominated by the bands at 1003, 1045, 1450, and 1680 cm^{-1} which can be assigned to various biochemical compounds present in clinical sample (see Table S4) (Dong et al., 2015; Neugebauer et al., 2010). In turn the infected clinical sample reveals very intensive SERS band at 730 cm^{-1} which is absent or weak in the case of uninfected specimens. Moreover, the infected samples can be characterized by other unique features at 653, 796, 960, 1090, 1375, and 1585 cm^{-1} , which highly correspond to the SERS fingerprints of reference bacteria species (see Fig. 2, Table S2). This clearly indicates high contribution of bacterial cells to the overall SERS pattern of infected urethra swabs. For more information regarding SERS analysis and recorded signals reproducibility see Section 3 and Figs. S4A and B in Supplementary Materials.

The PCA multivariate statistical analysis has been applied for all the SERS data. As mentioned before, it is possible to separate the points reflecting the uninfected clinical samples (control group) from the infected ones (experimental group). Thus, the created model is suitable for clinical applications, as it can facilitate the diagnosis of ongoing STD based on very fast SERS test. The PC-1, PC-2, and PC-3 reached together almost 70% in PCA (see Fig. 4B–C). This result should be interpreted as a satisfactory, as the analyzed samples were collected from different patients, in the case of whom the composition of urethra swab strongly

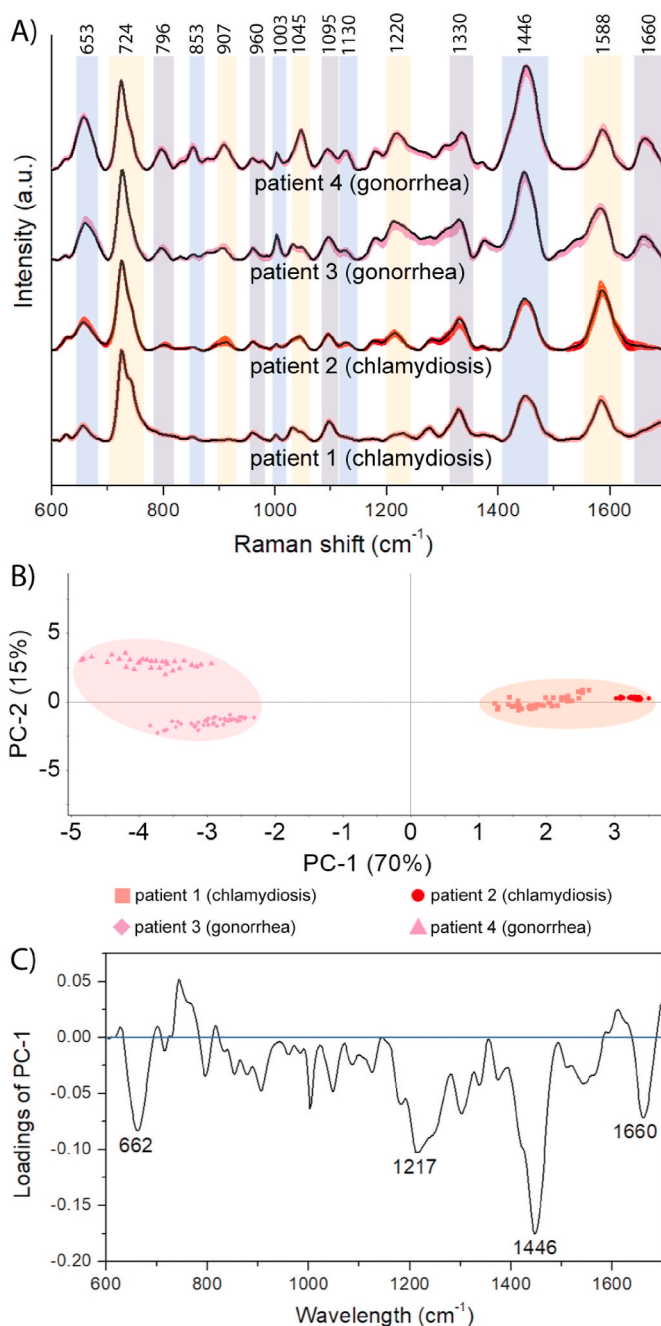


Fig. 5. The SERS spectra (A), 2D-PCA (B), and loading plot (C) obtained for four infected male urethra swabs collected from patients diagnosed with chlamydia and gonorrhea. For each specimen at least 30 single spectra were superimposed along with the averaged spectrum (black).

depends on age, diet, health condition, and other individual factors. These differences are noticeable, especially for uninfected samples (Fig. S4C). The infected samples are strongly dominated by the bands characteristic for bacterial cells, in the case of which the EF is always higher than in the case of uninfected bodily fluids. Thus, the contribution of urethra swab components in the SERS spectra of infected samples is significantly reduced and the differences between them are smaller than for uninfected specimens.

The differences between the spectra of the analyzed samples are reflected in loadings of the PC-1 (Fig. 4C). The variables with the highest loading values, i.e. 724, 1000, 1046, 1470, and 1681 cm^{-1} are the most important for diagnostic purposes. The first one is attributed to the main marker band of the bacterial cell (see Figs. 2 and 3), while the remaining

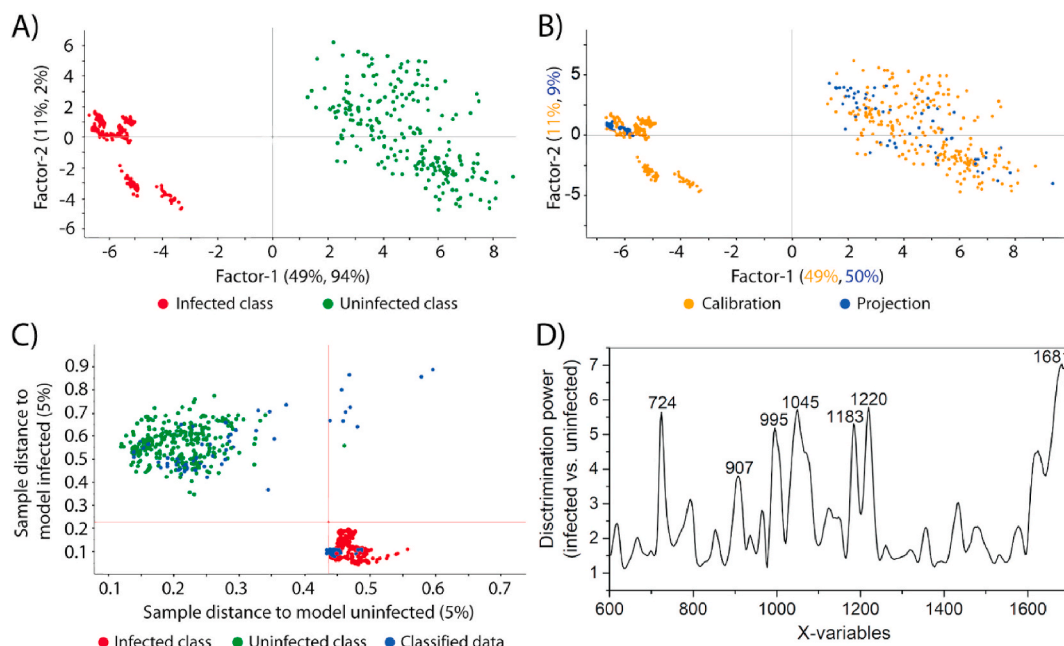


Fig. 6. The results of PLS1-DA analysis in the form of: (A) scores plot (LV1 vs LV2) for eight infected and eight uninfected samples of men's urethra swabs and (B) projection of tests samples (blue dots) onto the prepared PLS1-DA calibration model (orange dots). The results of SIMCA analysis in the form of: (C) Coomans' plot for infected and uninfected samples and (D) sample discrimination power on X-variables between infected and uninfected classes. For each analyzed sample about 30 spectra were used to create the calibration model. The green and the red clusters refer to all infected and uninfected samples, respectively (training set), while the blue points to the samples of the test set. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ones are characteristic for bodily fluids from urethra.

3.3.1. The direct identification of bacterial strains presents in male urethra swab

Since gonorrhea and chlamydia are one of the most prevalent STDs, in the next step we demonstrate the potential of SERS-PCA approach to differentiate between clinical samples (man's urethra swabs) infected with different etiological agents (*N. gonorrhoeae* or *C. trachomatis*). As one can notice in Fig. 5 all the SERS spectra of men's urethra swabs infected with sexually transmitted bacteria exhibit many spectroscopic similarities, e.g., the bands at 734, 1330, 1446, and 1588 cm^{-1} . Nonetheless, the spectral differences also can be noticed. This refers to, e.g., the band at 1660 cm^{-1} which is present in the case of gonorrhea and absent in the spectrum of chlamydia.

The PCA results in the form of 2D projection calculated for analyzed urethra swabs (Fig. 5B) clearly prove that clinical samples of patients infected with *N. gonorrhoeae* or *C. trachomatis* can be distinguished from each other. The PC-1 and PC-2, which are the most diagnostically significant, explain together 86% of the variance in the data. The loadings plot (Fig. 5C) designated that 662, 1217, 1446, 1660 cm^{-1} can be markers band responsible for such differentiation. Interestingly, most of them refer to the spectra of bacterial pathogens.

These results indicate that the developed SERS-based sensor can be applied for the diagnosis of STDs caused by different etiological agents (*N. gonorrhoeae*, *C. trachomatis*). Table S5 summarizes the comparison of SERS, NAATs and cultivation methods in terms of selected aspects of STDs diagnosis (Locksmith, 1997; Whiley et al., 2006).

3.3.2. The validation of sexually transmitted pathogens detection approach

The main goal of the performed PCA was to recognize the most characteristic spectral features among the studied bacterial pathogens and to identify the similarities and differences which are responsible for their classification. As the PCA is based on unsupervised learning algorithm, it can be used as pre-selection tool for supervised classification methods. In the present work, the PLS-DA and the SIMCA models were built to improve the classification of man's urethra swabs to proper

groups. These types of analysis require two steps: calibration and validation. In this study to create PLS1-DA classification model, the total of 480 spectra obtained from eight infected and eight uninfected patients were used for calibration (training set). Then 120 single spectra recorded for the remaining two infected and two uninfected samples were used for validation (test set). Fig. 6 depicts the results of PLS1-DA and SIMCA analysis for the SERS data.

The score plot of PLS1-DA model i) indicates that only two latent variables are needed to describe the differences, ii) presents the distinct separation between the considered classes (infected and uninfected samples) by means of Factor-1. The first latent variable (LV1) explains 94% of the variance in the block Y with 49% of the spectral data (X matrix) (Fig. 6A). Next, such created and evaluated model was used to classify the external samples. Fig. 6B presents the projection of all external spectra onto this model. As one can observe, the predictive values were always very close to 1. The low value of standard deviation (0.04–0.31) ensures that the model is robust and provides correct classification between infected and uninfected samples of male urethra swabs (Table S6). The results show 100% accuracy of the classification, as all the samples were properly assigned to suitable groups.

In SIMCA analysis the calculated distance between two analyzed models was 9.3, which means that classes are excellently distinguished. The significance level that defines classification limit equaled 5%. The Coomans' plot (Fig. 6C) shows the distance of each tested sample to 'infected' and 'uninfected' PCA models. The red and green points correspond to the samples from the calibration model, while the blue points indicate classification samples. The red lines designate the limit value for the occurrence of similarities in each group thus identifying the given sample. In the presented SIMCA model only 13 out of the 120 samples were misclassified giving 89% accuracy. The variable responsible for group separation is located at 1681 cm^{-1} with the discrimination power of 7.04. Other important variables located at 724, 907, 995, 1045, 1183, and 1220 cm^{-1} reached the value of 5.7 (Fig. 6D). These results are consistent with the loading plot obtained for the PCA model. Section 4 of the Supplementary Materials reveals the subsequent and deep analysis of Fig. 6.

In summary, this study proves that the proposed SERS-based sensor together with an appropriate chemometric methods can be applied in fast discrimination between infected and uninfected male urethra swabs. This in turn may facilitate the quick diagnosis of STD in the future.

4. Conclusion

The results of spectroscopic and chemometric experiments demonstrated in this work prove that it is possible to differentiate *N. gonorrhoeae* (with 77% of accuracy in PCA) from other bacterial species responsible for STDs, namely *M. hominis*, *M. genitalium*, *U. urealyticum* and *H. ducreyi*. Additionally, it was proved that *N. gonorrhoeae* can be differentiated from other *Neisseria* strains with the accuracy of $92.5 \pm 0.5\%$. Importantly, the results were confirmed in the indirect analysis for *Neisseria* spp. obtained from clinical samples. In turn, direct approach for STDs diagnosis allows to eliminate the time-consuming cell culture step and thus enables fast, label-free, and reliable identification of sexually transmitted pathogens in very small volume of urethra swab. As a result, the time needed to diagnose STD can be reduced to 15 min, which are sufficient to collect the sample, prepare it for SERS experiment, measure and analyze with chemometric method. The use of both nonsupervised (PCA) and supervised (PLS-DA, SIMCA) methods allowed for characterization, differentiation and classification of clinical samples with the prediction accuracy reaching 100% for PLS1-DA and 89% for SIMCA. The significantly reduced time of the analysis and the simplified procedure while maintaining the same sensitivity make a SERS-based sensor highly competitive with very complex NAAT procedures currently applied for STDs diagnosis.

In the future, the integration of such biosensing platform with a small, portable Raman spectrometer could lead to the development of a handheld point-of-care device, which would enable the diagnosis of STDs in extremely short time.

CRedit authorship contribution statement

Sylwia M. Berus: Methodology, Validation, Investigation, Writing – original draft. **Monika Adamczyk-Popławska:** Resources, Investigation. **Beata Młynarczyk-Bonikowska:** Resources, Investigation. **Evelin Witkowska:** Writing – original draft, Visualization, Methodology. **Tomasz Szymborski:** Data curation, Software. **Jacek Waluk:** Supervision. **Agnieszka Kamińska:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2021.113358>.

Compliance with ethical standards

All experiments were performed in compliance with the relevant laws and institutional guidelines. The protocol of the study was approved by the Ethics and Bioethics Committee of Cardinal Stefan Wyszyński University in Warsaw. Informed consent was obtained from all patients.

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