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ABO blood groups' antigen–antibody interactions studied using SERS spectroscopy: towards blood typing†

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The article presents surface enhanced Raman scattering (SERS) technique associated with the principal component analysis (PCA) as a fast and reliable method for the study of interactions between the A, B, AB and O (abr. ABO) blood groups antigen and complementary monoclonal A and B antibodies. The possibility of simultaneous detection and differentiation within the ABO group was evaluated. Using 785 nm excitation wavelength, distinctive spectral changes among all types of the studied blood groups were found for mixtures of red blood cells (RBCs) with the A or B antibody. For PCA analysis, all the spectral data were divided into two main groups based on the type of antibody. The obtained PC scores in the area of antigen–antibody interactions (1311–1345 cm^{-1}) allow differentiation within blood groups with accuracy from 96% to 98%. Additionally, for this region the characteristic marker bands of specific antigen–antibody interactions in relation to both ABO system and antibody were established. The results show excellent segregation of the obtained data and the possibility to use SERS for determination of ABO blood group. Our study proves that SERS is one of the most sensitive techniques for investigations of biological samples and may be used as a new tool that provides one-step comprehensive and reliable medical diagnosis.

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Introduction

The studies regarding antigen–antibody interactions are based mainly on serology and the agglutination principle, as started in 1900 by Karl Landsteiner.[1] Currently, this is still a widespread method used both in manual and automated immunochemistry.[2] Typically, immunohematological tests need at least 30–45 minutes for identifying the blood group and antibody in the serum.[3] One has to bear in mind that sometimes even a few minutes are too long for a patient waiting for help at a medical center. Moreover, along with broadening the medical knowledge, the complexity of proper blood typing has been noticed and more deeply understood.[4,5] Interestingly, at present there are 33 blood group systems, including A, B, AB and O (abr. ABO), D, and Kell blood groups.[6] All blood groups are mainly due to many possible blood polymorphisms (depending also on the human genotype). There is still a high probability that not all human ethnic populations are known, thus not all possible antibody–antigen relations have been found and studied. The specific interactions between ABO blood group antigens and serum antibodies are important in biochemical analysis, clinical diagnostics, and environmental monitoring. The blood group antigens are sugars or proteins, and they are attached to various components in the red blood cell membrane. A person's DNA determines and holds the information for producing the type of antigen. Antigens of the bodies own cells are known as "self-antigens", and the immune system does not produce antibodies against them. However, the immune system will attack any red blood cells that contain other antigens. Moreover, any change in concentration of antibodies in body fluids may be a symptom of pathologies and various diseases. The antigen–antibody complex is formed based on weak and non-covalent interactions such as Ca^{2+}-bridges, hydrogen bonds or van der Waals forces, as well as hydrophobic and coulombic interactions. The way of binding between these two biomolecules strongly depends upon three-dimensional conformation and relative positioning of the chemical groups taking part in the interactions. Using elution techniques, it is possible to control, and even to break binding forces by changing ionic strength, pH, surface tension, dielectric constant, temperature, and the use of organic solvents.[7] However, due to the heterogeneity of the physical forces involved in binding, no single analytical technique finds universal applicability for all types of antibody–antigen bonds.[8] Therefore, a significant effort has been made in recent years to explore the potential of techniques

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such as surface plasmon resonance or atomic force microscopy[9] to study the antigen–antibody interactions on red blood cells. The majority of this research, however, is being pursued in laboratories and still need meaningful improvement before any applications are introduced in medical centers. For antigen–antibody interactions study, surface enhanced Raman spectroscopy (SERS) was successfully applied,[10] and at present, it is widely used as an analytical method to investigate biological molecules and materials. Noticeably, it can be applied to aqueous samples and samples under physiological conditions in a non-destructive manner. To the best of our knowledge, SERS has never been used to characterize interactions between the ABO blood group antigens and their specific serum antibodies.

The SERS technique exploits enhanced Raman scattering from molecules in close proximity to a nanostructured surface due to the coupling of metal surface plasmons with the oscillating electric field of the incident and scattered radiation.[11] It offers exceptional enhancement factors (EFs) for the intensity of the vibrational signals, 10^3 to 10,[14] which gives a possibility to observe even a single molecule.[12] Mainly due to high sensitivity, the SERS technique may be used as a tool in chemistry and biochemistry, forensic science, environmental research, and homeland security. However, for standard applications of SERS in biological and biomedical tests, SERS substrates should fulfil the following requirements: high enhancement factor, high stability upon exposure to air, and significant signal reproducibility (across a single substrate as well as between different substrates). Numerous promising materials have been investigated and claimed to be efficient SERS-substrates. The fabrication of SERS substrates includes electrochemically roughened electrodes,[13] colloidal particle arrays,[14] nanowire bundles,[15] nano-prisms,[16] nano-shells,[17] and nanoparticles.[18] Even though many different techniques have been applied for preparing the substrates, their reproducible fabrication remains a challenging task. In this respect, a promising approach has recently been proposed.[19] In this method, a silver–gold bimetallic surface was prepared by electrochemical deposition of gold over an electrochemically roughened silver surface.[20] This particular SERS substrate fulfils all the above mentioned requirements for biological, medical and analytical analyses. In addition, this SERS substrate was very stable under atmospheric conditions and exhibited strong Raman enhancement with high reproducibility of the SERS spectra. Recently, the use of this SERS substrate was successfully demonstrated to detect and identify bacteria cells from body fluids.[19]

The concentration of some antigens and/or antibodies in biological fluids is very low and may range from 10^{-16} to 10^{-12} M, which is below the limit of detection of most techniques. Moreover, the specific antigen–antibody interactions take place in a complex physiological matrix containing proteins, peptides, and enzymes that may influence the analytical signal through non-specific interactions. Thus, proper sample preparation is crucial to obtain analytically valuable data. The strength of agglutination (reaction between particular RBC antigen and antibody) depends on two main factors: the antibody activity and the strength of the RBC antigen-binding site. For example, if the activity of the antibody is too high (*i.e.* lower dilutions), the excess antibodies form very small and unstable complexes. On the other hand, if the activity of antibody is too low, it will make normal RBCs unable to express strong agglutination.[21] Even without a noticeable agglutination-vivid existence of antigen–antibody interactions, the method proposed in this study based on the SERS technique may provide information about antigen–antibody complex formation. Moreover, this method, with its enormous signal sensitivity down to a single-molecule level and molecular fingerprint specificity is a unique, promising and powerful tool for study of disease markers in human serum,[22] whole blood, RBCs,[23] and hemoglobin.[24] Thus, in the light of all the issues mentioned above, the SERS technique seems to be the most promising to study interactions in the RBCs systems. It should be highlighted that, until now, no comparable data have been published that utilize the SERS technique for ABO study. In addition, in this study, the principal component analysis (PCA) was performed over the pre-processed SERS spectra in order to evaluate (a) the spectral differences among the ABO blood group systems and antigen–antibody interactions and (b) develop models allowing for discrimination and classification of the ABO group (ABO typing).[25]

Experimental

Sample preparation

RBCs sample preparations. A total of 160 blood samples from different patients (40 for each ABO blood system) obtained from the Regional Blood Donation Centre in Warsaw, Poland were examined. The following standard protocol was applied to draw and process blood: 4 mL of blood was collected by a venipuncture from healthy patients into vacutainer blood collection tubes containing EDTA as an anticoagulant. All samples were stored in a refrigerator at 8 °C until required measurements or additional separation steps. The RBCs were separated from blood by centrifugation at 1400*g* for 10 minutes in 2 mL vials. All the liquid above the RBCs was discarded. Then, the RBCs were washed three times in PBS (phosphate buffered saline) before re-suspending in saline (around 3 mL of PBS). A solution of washed RBCs (0.2 mL) was divided into aliquots (1 mL) and PBS saline (0.8 mL) was used to achieve the final concentration of 20% RBCs.

Preparation of serum antibodies. IgM antibodies of the ABO blood system, including anti-A (clone HEB-193) and anti-B (clone HEB-129), were obtained from Sigma-Aldrich Co, (Dorset, UK). At first, a series of antibody dilutions was prepared (1 : 1, 1 : 8, 1 : 64, 1 : 256, 1 : 512 and 1 : 1024) to find optimal conditions to study antigen–antibody interactions in the blood. All diluted antibody solutions were divided into small aliquots (around 0.5 mL) and stored at −20 °C. Before each measurement, a portion of antibody was defrosted (for around 5 minutes) and disposed after measurements.

Preparation of a mixture of RBCs with a known antigen system and serum antibodies. RBCs at 20% v/v concentration were mixed with each portion of prepared dilution of antibody in a 1 : 1 ratio. The same procedure was repeated for both A and

B antibodies. For SERS measurements, the suspension of RBCs with monoclonal antibody was applied onto the SERS substrate for 5 minutes. After this time, the SERS spectra were obtained (10 seconds for one single spectrum and 5 minutes for 20 spectra in the mapping mode). Contrary to immunochemistry methods, the prepared samples of RBCs with corresponding antibodies do not show a macroscopic agglutination reaction due to their trace amounts in tested samples. 5 μL of the prepared solution was applied to the SERS platform.

Preparation of silver–gold bimetallic surface

The silver–gold bimetallic surfaces were prepared through electrochemical deposition of nanostructured gold on an electrochemically roughened silver surface. The three-electrode electrochemical cell was filled with 0.4 mM solution of $HAuCl_4$ in 0.1 M $HClO_4$ and subsequently purged with argon for 30 min to remove air. A −80 mV potential was applied for 200 s and then the electrode was removed from the solution and washed with doubly distilled and deionized water to remove other ions from the surface. Finally, the electrode was dried with argon gas and used as a SERS substrate. Freshly prepared SERS substrates were used for further study. Fig. 1 presents the SEM images of a thus prepared SERS surface obtained with a FEI Nova NanoSEM 450 scanning electron microscope.

SERS measurements

The SERS measurements were performed using a Renishaw inVia Raman system equipped with 300 mW diode lasers as excitation sources emitting at 785 nm and 514 nm. After passing through a line filter, the light from the laser was focused with a 50× microscope objective (numerical aperture of 0.55, resulting in a lateral 4–5 μm spatial resolution) on a sample mounted on an *X*–*Y*–*Z* translation stage. The Raman-scattered light was collected using the same objective, but a holographic notch filter was used to block out Rayleigh scattering. 1800 and 1200 grooves per mm gratings were used to provide a 5 cm^{-1} spectral resolution. The SERS scattering signal was recorded using a 1024 × 256 pixel RenCam CCD detector. Typically, the SERS spectra were acquired with a 3 mW laser power at the sample. SERS spectra were obtained from 20 measurements at different places across the SERS surface using the mapping mode.

PCA spectral preparation method/PCA data analysis

The SERS spectra were processed for principal component analysis (PCA) using a two-step approach. First, using the OPUS software (Bruker Optic GmbH 2012 version), the spectra were smoothed with the Savitzky–Golay filter, the background was removed using baseline correction (concave rubber band correction, number of iterations: 10, number of baseline points: 64), and then the spectra were normalized using a so-called Min–Max normalization (the area of porphyrin band around 796 cm^{-1} was used). All the data were transferred to the Unscrambler software (CAMO software AS, version 10.3), where PCA analysis was performed.

Results and discussion

SERS spectra of RBCs from different ABO blood groups

As can be seen in Fig. 1, the bimetallic SERS substrate was homogenous, showing crystalline nanostructures of the deposited gold layer with truncated edges. This feature guarantees the enhancing properties of these substrates.[20] The

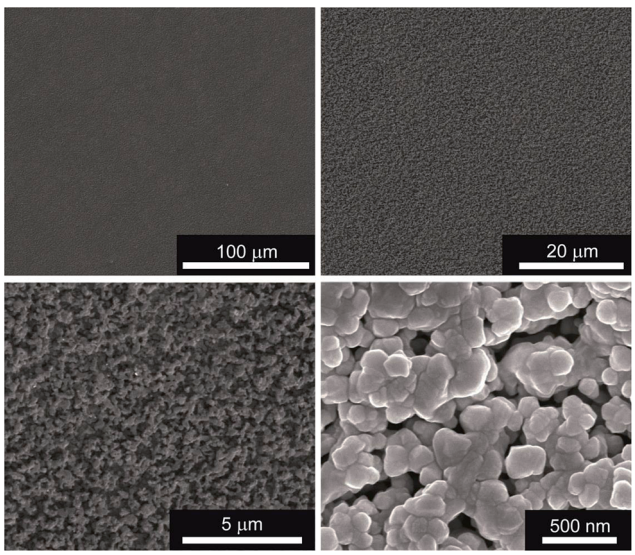

Fig. 1 SEM images of silver–gold bimetallic surface prepared by electrochemical deposition of gold over an electrochemically roughened silver surface.

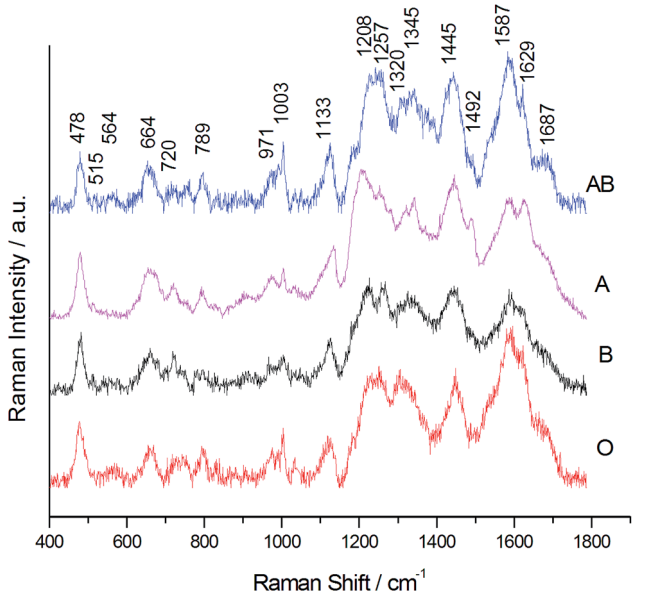

Fig. 2 Representative SERS spectra of RBCs of ABO blood groups taken with the 785 nm laser line. Experimental conditions: 5 mW of 785 nm excitation, 4 × 20 second acquisition time. The SERS spectra have been baseline-corrected and shifted vertically for better visualization. Each SERS spectrum was averaged from seven measurements at different places of the SERS platform.

calculated SERS enhancement factor for this substrate was 10^6 (see ESI†). Using these substrates, the SERS spectra were acquired for RBCs of each ABO blood groups and are presented in Fig. 2.

An empirical analysis of the SERS spectra of RBCs from different ABO systems exhibited the same common spectral features, but with some very small differences in the band positions, their relative intensity ratios and/or the appearance of new peaks. In order to overcome these hurdles with "hidden spectral changes," a principal component analysis (PCA) was performed over the pre-processed SERS spectra. The PCA analysis transforms a large number of correlated variables into a smaller number of uncorrelated variables called principal components (PC). The first component (PC1) accounts for as much of the variability in the data as possible. The general method of PCA is based on a model $X = \mathrm{TPT} + E$, where the X matrix is decomposed by PCA into two smaller matrices, one of scores (T) and other of loadings (P). PC scores are related to a linear combination of the original variables, which are orthogonal to each other. In addition, each PC accounts for the maximum variability of the data set. By plotting the principal component scores, for example, PC1 *vs.* PC2, similarities between the samples are revealed. PCA provides insight into the percentage of variance attributed to each PC and how many PCs should be kept to maintain the maximum information from the original data without adding noise to the current information. By analysing the plot of PC-loadings as a function of the variables (*i.e.* Raman shifts), one can indicate the most important diagnostic variables or regions related to the differences found in the data set. In this study, we applied the PCA1 loadings for all the collected spectra of ABO blood group taken together and their mixtures with A and B antibodies. This enabled investigation of the spectral variations to find the most significant modes contributing to the variance accounted for by this PC. Fig. 2 presents the SERS spectra obtained for ABO blood group without any antibodies. Based on collected data, the first principal component (PC1), which contained as much of the remaining variability in the analyzed data as possible, gave a 76% value of total variance. This percentage value is insufficient for medical diagnostics and clearly demonstrates

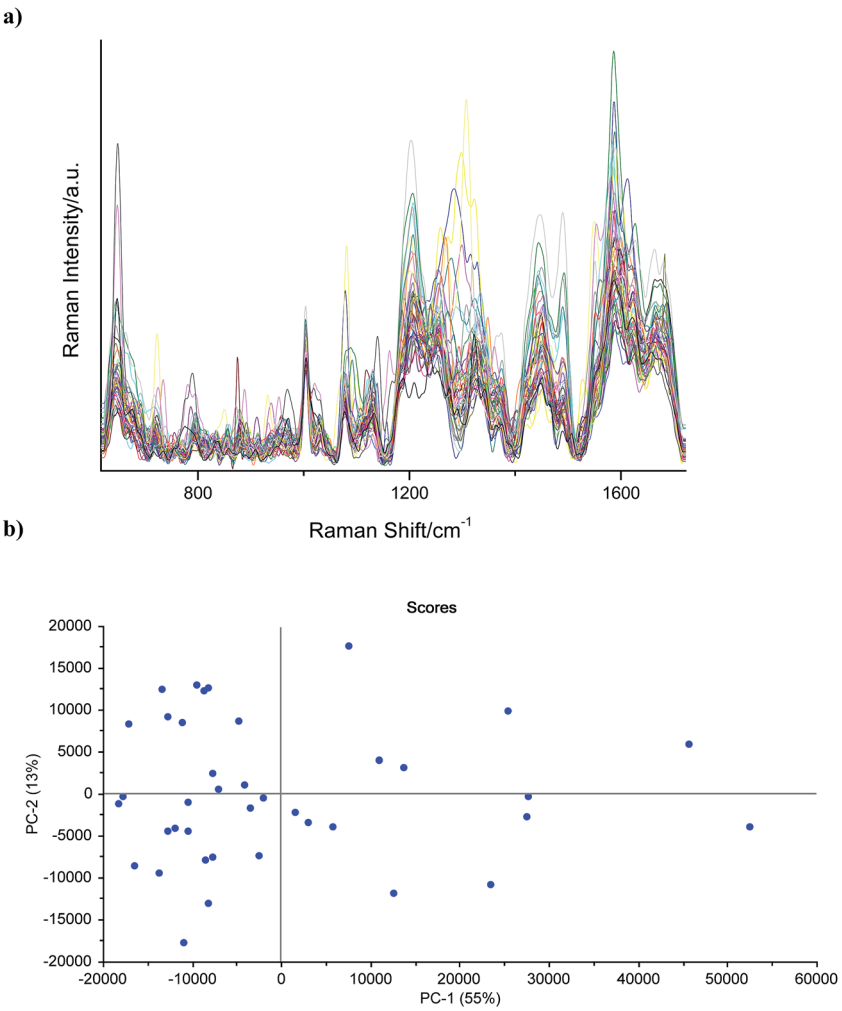

Fig. 3 Representative SERS spectra of RBCs recorded for one patient with the O blood group (a) and calculated two dimensional PCA scores plot component 1 and component 2 (b). Each SERS spectrum was averaged from 20 measurements in different places across the SERS surface using the mapping mode.

difficulties in recognition among the blood groups without applying complementary antibodies. To confirm this deduction, additional SERS experiments were performed. The SERS spectra were acquired at different spots for one sample obtained from one patient. As can be seen in Fig. 3, small changes in the Raman spectra may exist even for such a sample. Two-dimensional PCA scores plot for components 1 and 2 gave PC1 = 55% of the total variance and additionally indicated a reproducibility of the SERS signals.[26] It must be noted that this value calculated for SERS spectral data of one blood group is lower than the PC1 value obtained for SERS spectra data for all blood groups (PC1 = 77%). This PCA analysis clearly demonstrates that SERS data may vary depending on the blood group systems collected from one patient or many patients (differing in ABO blood group, gender, age, and health). Differences may also be caused by the morphological irreproducibility of the used SERS platforms. It must be noted that all observed differences among the collected spectra, both for the ABO system and among the spectra acquired for one sample, are possibly noticed only upon applying the SERS technique. Such a procedure is not possible with the small sensitivity of normal Raman spectroscopy.

Moreover, the results presented above evidently show that even with the support of the multivariate analysis, the difficulties in blood typing without studying antibody–antigen interactions are significant. Any infections, viruses or bacteria, which may have already entered the body fluids and tissues, could change the blood spectra. The use of the SERS technique for studying antigen–antibody interactions to differentiate between the ABO group blood is plausible even for one sample and can overcome many complications associated with inappropriate assignment of the observed bands in blood spectra, it may also allow for accurate distinction. To date, only one paper reports[27] a possibility to use SERS spectroscopy for ABO blood typing. The authors stated that with the help of a PCA-LDA (principal component analysis-linear discriminant analysis) model combined with ROC (receiver operating characteristics) analysis, it is possible to observe differences between A and B, A and O, B and O, AB and A, AB and B and, finally, AB and O blood groups with average sensitivities around 90%. The model proposed in the paper allows to differentiate only between two given groups of the ABO blood system by SERS spectroscopy, and thus cannot be easily applied for blood typing. Bearing in mind the application purposes, we went further in this study and intended to verify if the SERS technique may be exploited for the investigation of antigen–antibody interactions and finally, to carry out ABO blood typing with clinical accuracy.

SERS spectra of mixtures of RBCs with A and B antibodies of blood groups

Antibodies are part of the circulating plasma proteins known as immunoglobulins, which are classified by molecular size and weight and by several other biochemical properties. Most blood group antibodies are found either on immunoglobulin M (IgM) molecules or immunoglobulin G (IgG), but occasionally the immunoglobulin A (IgA) class may exhibit blood group

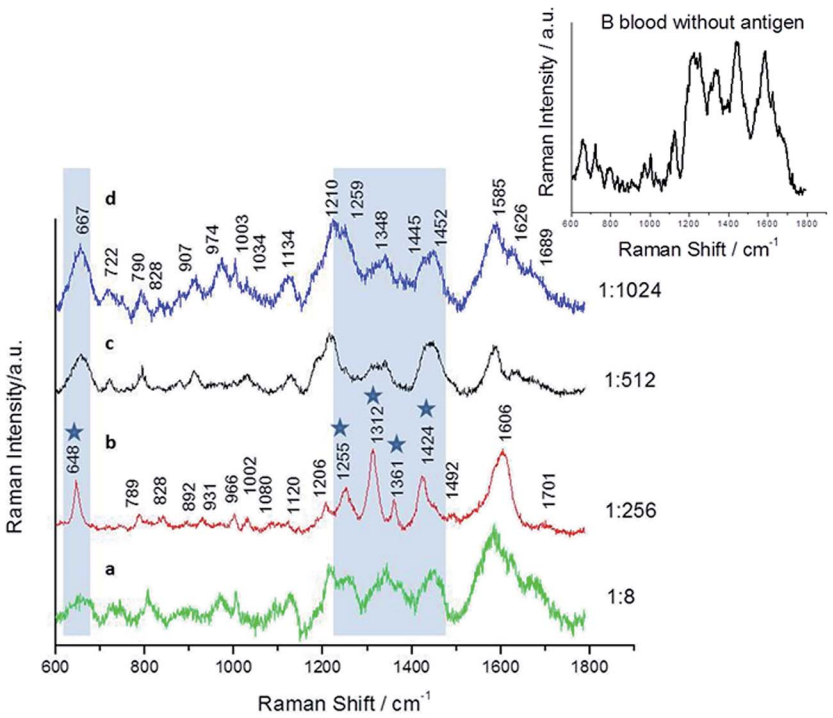

Fig. 4 SERS spectra show variations of the band intensity of RBCs antigen (B blood group)–antibody solution (mixed in a 1 : 1 ratio) depending on the added B antibody concentration. The most pronounced bands coming from globin vibrations in the antigen–antibody complex are marked by asterisks. The 1 : 256 antibodies dilution was chosen for further studies. Each SERS spectrum was averaged from 20 measurements in different places across the SERS surface using the mapping mode.

specificity. The IgG is composed of two light and two heavy chains. The four polypeptide chains are covalently held together by disulfide bonds.[28] Immunoglobulin M (IgM) forms biopolymers with five multiple immunoglobulins (monomers), also covalently linked with disulphide bonds. IgM is a strong complement activator and agglutinator due to its pentameric structure and it is called the primary response antibody.[29,30] Serum A and B antibodies are mainly IgM, but also IgG, and even IgA immunoglobulin. Group O people in particular tend to make more IgG anti-A and anti-B than the other groups.[30] A and B antibodies can be stimulated by exposure to A and B antigens on red cells, white cells or platelets, for example by injecting soluble A and B antigens and by some vaccinations that contain bacterial A- and B-like antigens. The A and B blood group antigens present on the RBCs surface and their complementary A and B antibodies in the serum may react with each other, leading to macroscopic agglutination, which is due to the antibody–antigen interactions and may be observed as red cells clumped together in the blood. These antigens are characteristic for each ABO blood group, and based only on the interaction between the RBCs and A or B antibodies, it is possible to distinguish each blood group of a sample. The antigen A exists on the RBCs surface of the A blood system. Thus, interaction may happen only with the A antibody. In the case of the B blood group, RBCs contain the B antigen on the surface, thus interaction only with the B antibody is expected. The AB blood group contains A and B antigens on the RBCs surface. Therefore, for the AB blood system, the antigen–antibody interactions are possible for both A and B antibodies. Contrary to AB, the RBCs of O blood system does not contain antigens against A and B antibodies.

Table 1 Tentative assignments of SERS signals obtained from interactions between the antigen on RBCs of known ABO blood systems and A or B antibodies. Vibrations: n – valence, d – deformation, g – deformation (out of plane), stretch – stretching

SERS band positions [cm^{-1}]	Vibrational mode	Symmetry of vibration	Assignment
648	C–S stretch[31]	B_{1g}	Globin and cellular components (cysteine)
667	d(pyrrole)[32]	A_{1g}	Globin
722	$d(COO^-)$[31]	B_{1g}[33]	Globin and cellular components (amino-acids)
790	n(pyrrole breathe), n_6	B_{1u}[34]	Porphyrin
828	$g(C_mH)$	B_{1u}[34]	Porphyrin
907	C–C stretch[31]		Globin and cellular components (glutamic acid, isoleucine, threonine, lysine)
931	(COO^-)stretch[34]		Globin
966	C–C stretch[35,]		Globin and cellular components (proteins)
974	d(pyrrole)[32]	E_u[32]	
1003	Indole asymmetric ring breathe[31,36]	A_1	Globin and cellular components (phenylalanine)
1034	In plane ring CH deform[31]		Globin and globin and cellular components (phenylalanine)
1080	$=C_{2vinyl}H$[32]	A_{2g}[32]	
1120	Stretch (pyrrole half-ring) asymmetric[35]	A_{2g}[32]	Globin and cellular components (proteins)
1210	$d(C_mH)$, n_{13} (ref. 37) or n_{42}	B_{2u}[34]	Porphyrin
1255	CH_2 wagging[31] $d(CH_2/CH_3)_6$ (ref. 33, 35 and 38)		Globin (glutamic acid) and cellular components (proteins, lipids: amide III)
1312	CH_2 wagging[38]		Globin and cellular components (phenylalanine, glutamic acid, serine, methionine, histidine)
1348	CH_2 scissoring[31] CH_3 deformation[31]		Globin and cellular components – glutamic acid, aspartic acid, asparagine, glutamine; alanine, leucine, valine, isoleucine
1361	Stretch (pyrrole half-ring) symmetric[32]	A_{1g}[32]	Globin
1424	$n(C_\alpha C_m)$ symmetric?[36]	A_{2g}[32]	
1445	$d(CH_2/CH_3)$	B_{2g}[32]	Globin[38] and porphyrin[36]
1494	CH and CC ring	A_{1g}	Tryptophan[31]
1585	$n(C_\alpha C_m)$ asymmetric, n_{37}	E_u[32]	Phenylalanine, tyrosine[37]
1606	Stretch $(C=C)_{vinyl}$	A_{2g}[32]	Phenylalanine, tyrosine[37]
1626	Stretch $(C=C)_{vinyl}$ asymmetric	B_{1g}[32]	Phenylalanine, tyrosine[37]
1689, 1701			Amide I

The activity of an antibody affects the agglutination strength, therefore there is a need to find optimal conditions, *e.g.*, the suitable concentration of RBCs antigen and particular antibody for their complex formation. Taking into account the serological tests for the assessment of blood group antigen strength,[21] a series of dilutions of A and B antibodies in a PBS buffer was prepared (such as 1 : 8, 1 : 256, 1 : 512, and 1 : 1024) and mixed with RBCs of a known group in a 1 : 1 ratio. Only for one antibody titration (1 : 8), the macroscopic agglutination was observed. For more diluted antibodies (1 : 256, 1 : 512, and 1 : 1024), no vivid reactions were detected. In the SERS signals, we expected to find spectral changes corresponding to antigen–antibody complex formation, such as shifting and/or relative changes in intensities of the RBCs vibrations related to their globulin and cellular components, and the appearance of new bands due to complex formation. Based on the obtained results, the most appropriate antigen–antibody dilution was selected (1 : 256). The corresponding SERS spectra, presented in Fig. 4, evidently show that the observed SERS signals varied depending on the added antibody concentration and for a specific antibody dilution (1 : 256), these vibrations were more pronounced. The spectra taken for a mixture of RBCs with 1 : 8, 1 : 512 and 1 : 1024 (see Fig. 4a, c and d) antibodies titrates are almost the same. The main recorded SERS spectral features are due to RBCs porphyrin vibrations (790, 828, 1210, 1445, and 1626 cm^{-1}) and signals assigned to proteins and lipids (966, 1120, 1255 and 1585 cm^{-1}). This is due to the fact that at the low antibody concentration (1 : 512 and 1 : 1024), only few antigens on the RBCs surfaces are bound to the antibody and cannot change the RBCs vibrations. On other hand, the highest antibody concentration (1 : 8) yields the RBCs surfaces completely covered by excess antibodies. In addition, the A and B antibodies vibrational bands were very weak compared to the RBCs signals (see Fig. 1S, ESI†), which results in almost the same SERS spectra for antibodies with 1 : 8, 1 : 512 and 1 : 1024 concentrations. Only for the 1 : 256 dilution (Fig. 4b), there were noticeable spectral changes as new bands at 648, 1255, 1312, 1361, and 1424 cm^{-1} appeared. These bands are marked by asterisks in Fig. 4b and correspond to RBCs antigen–antibody complex formation. The proposed assignments of the SERS vibrations observed for RBCs and their complexes with A and B antibodies are shown in Table 1. Based on these results, all the SERS spectra presented in this manuscript were collected for the mixture of 20% of RBCs and antibodies titres of 1 : 256 (1 : 1 ratio).

In the next step, the SERS spectra for RBCs of known ABO blood group systems mixed with A or B antibodies were obtained using two laser wavelengths: 514 and 785 nm (Fig. 2S, ESI†). These experiments were performed to establish the optimal experimental conditions for studying antigen–antibody interactions. Using the 514 nm wavelength, the applied approaches evidently show no evident spectral changes between the A, B, AB and O antigens on RBCs surfaces upon the specific interaction with antibodies. The spectrum acquired with this laser excitation line reveals mainly a mixture of A_{1g} and A_{2g} porphyrin modes, largely enhanced relative to the other excitation wavelength, which indicates resonance enhancement. Using 785 nm, a strong B_{1u} mode enhancement of the vinyl modes relative to the other ones was detected. For this laser wavelength, there is a possibility to notice differences among the spectra of RBCs from a known blood group and those acquired for RBCs mixed with different antibodies (A or B antibody). This was the reason for using the 785 nm laser wavelength in this study. In addition, for this wavenumber, the reproducibility of all recorded SERS spectra (each ABO blood system with A or B antibody) was calculated with a Savitzky–Golay second derivative method (see Fig. 4S and Table 1S in ESI†). The lowest value of the calculated average correlation coefficient was 0.90, which is sufficient enough for analytical applications.

The PCA analysis allows for segregating of different antigen–antibody interactions into separate clusters,[39] which subsequently makes it possible to differentiate among ABO blood groups.[40] At first, the analysis was performed in the spectral region between 650 and 1700 cm^{-1}, wherein the RBCs vibrational bands are expected. As can be seen in Table 2, for the whole region, the PCA technique enables us to satisfactory differentiate among the ABO systems only in a case of their mixtures with A and B antibodies. For the ABO system, the first component (PC1) gave the score 76% of the total variance, which is definitely insufficient for blood group typing. In the case of ABO samples with their monoclonal antibodies, PC1 gave the score 94% and 98% for A and B antibody, respectively. These results clearly demonstrate that using the SERS technique, the differentiation within the ABO system is possible in

Table 2 *X*-Explained variance values (based on Raman shifts as variables) obtained from PCA scores analysis. To point to the best area for differentiating the obtained Raman data the highest PC1 scores are in bold

Studied sample	Range/cm^{-1}	PC-number	*X*-Explained variance (%)
ABO blood group	650–1700	PC1	76
		PC2	16
	750–1050	PC1	75
		PC2	9
	1150–1350	PC1	69
		PC2	27
	1400–1700	PC1	**93**
		PC2	5
A antibody mixed with ABO	650–1700	PC1	94
		PC2	4
	750–1050	PC1	95
		PC2	3
	1150–1350	**PC1**	**96**
		PC2	3
	1400–1700	PC1	92
		PC2	5
B antibody mixed with ABO	650–1700	PC1	98
		PC2	1
	750–1050	PC1	96
		PC2	2
	1150–1350	**PC1**	**99**
		PC2	0
	1400–1700	PC1	98
		PC2	1

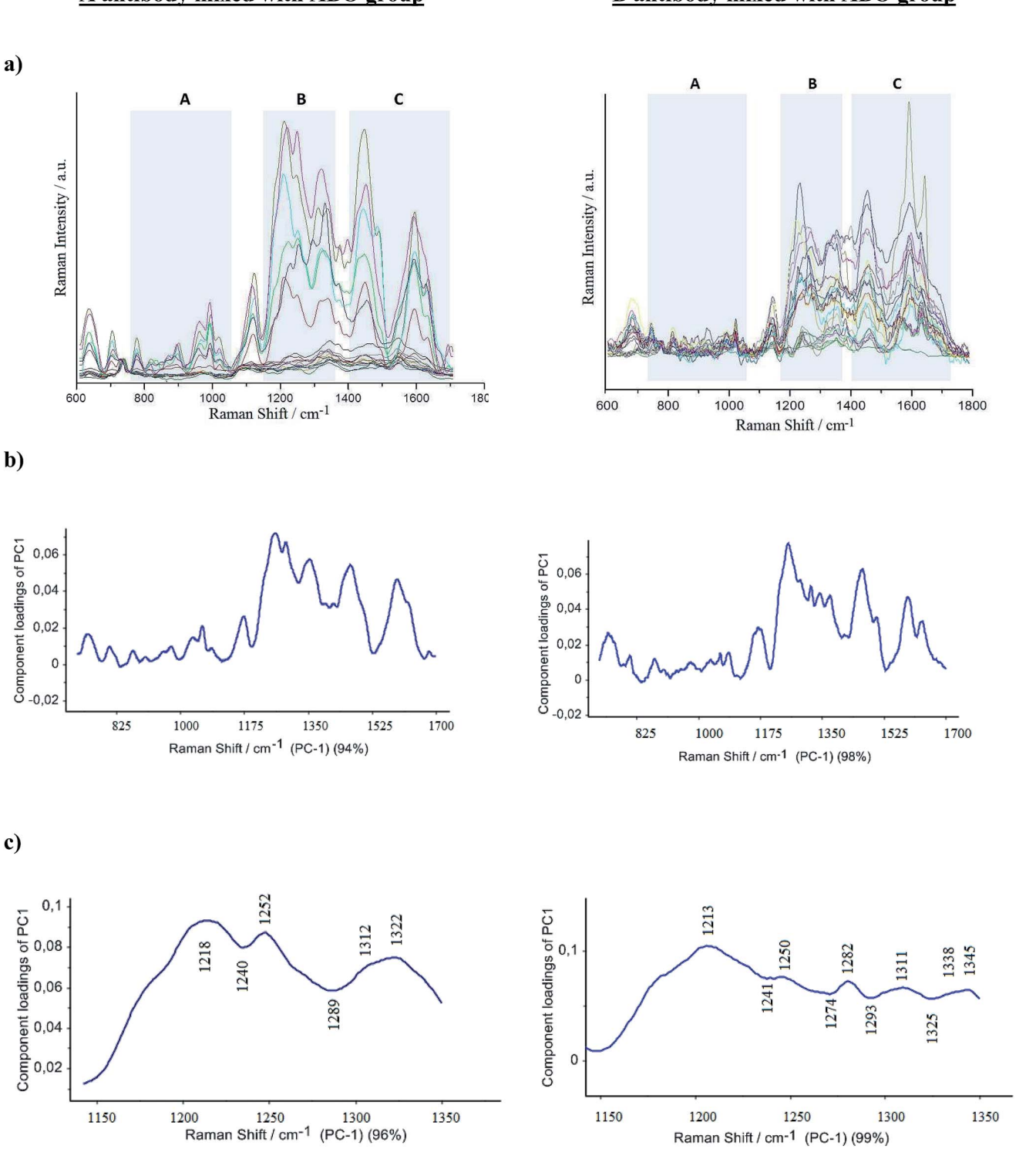

Fig. 5 (a) SERS spectra after mathematical treatment in OPUS software; prepared for PCA statistical analysis (smoothing, baseline correction and normalization). The frames show the spectral regions wherein the main differences among spectra are located. (b) One dimensional PCA loadings plot performed for the 625–1700 cm^{-1} region. (c) The PCA loadings plot performed for the B region showing the most sensitive diagnostic bands.

the case of antigen–antibody complex formation. Moreover, to improve the convergence of the calculated data, three different regions were selected in the next step within the analysed area (A: 750–1050 cm^{-1}, B: 1150–1350 cm^{-1}, and C: 1400–1700 cm^{-1}), which correspond to the main differences in the SERS spectra (Fig. 5 and 3S†). As can be seen in Fig. 5, the obtained

data clearly demonstrate that the B area is the most appropriate for differentiating ABO blood groups. For this particular region (Table 2), the PC1 for ABO blood group without and with A and B antibodies gave the scores of 69%, 96% and 99% of the total variance. These results show an excellent segregation of the analysed data only for blood samples with their specific antibody. It should be highlighted that using the first component, which gave the dominant account for the maximum variance in data, the adequate discrimination within the ABO blood system might be successfully performed. In the B region, the bands at 1240, 1252, cm^{-1}, and a broad band at 1322 cm^{-1} with a small shoulder at 1312 cm^{-1} (RBCs of all ABO groups mixed with A antibody) or bands at 1325, 1338, and 1345 cm^{-1} (RBCs with B antibody) correspond to globin and cellular components vibrational modes. Two other observed bands may be assigned to a different group, porphyrin vibration modes (1218 or 1213 and 1289 or 1274–1293 cm^{-1}). Moreover, the analysis enabled us to find the wavenumber in the spectrum that contributes to the largest inter-spectral variance,[41] validating the identification of the marker bands of the obtained spectral data, which are due to antigen–antibody interactions. These marker bands were located in the 1311–1345 cm^{-1} region and showed shifts that depended on the different antibodies, which furthermore allows for more precise distinguishing among the ABO blood group system.

In addition, the validation of the proposed method for blood typing was performed (see ESI, Fig. 5S†). In the first step, the PCA analysis for A-antibody mixed with RBCs of all ABO systems (80 SERS spectra from 4 patients) was used to build the PCA model. Then, the additional data of the test sample (external blood sample with a known AB group) were introduced into this model. The PC score calculated for this test sample was located in the cluster of model PC scores corresponding to the AB blood group. These results present the analytical potential of the SERS technique combined with PCA analysis towards blood differentiation.

Conclusions

The specific antigen–antibody interactions in ABO blood groups were extensively studied by the SERS technique using two laser wavelengths, 514 nm and 785 nm. The optimal conditions for antigen–antibody complex formation study using bimetallic SERS surfaces were established. Upon applying PCA analysis in the area of antigen–antibody interactions (1311–1345 cm^{-1}), the main marker bands were found. These bands show frequency shifts in dependence on both, the type of ABO system and the added antibody. The obtained results, combined with PCA analysis clearly demonstrate that it is possible to differentiate ABO system by SERS spectroscopy. However, for the purpose of direct use of the proposed method for practical applications, a larger data matrix must be created for PCA analysis. Thus, further studies based on large population of clinical samples are in progress. Moreover, recent medical work suggests close relationships between the blood groups and the morbidity from cancer (stomach, pancreas, and colon) diseases.[42] Therefore, the presented studies may contribute to an improvement of medical analysis and support developing new methods for an early-stage clinical diagnosis using SERS spectroscopy.

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