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RESEARCH PAPER

Determination of L-selectin in blood plasma using DNA aptamer–based surface-enhanced Raman spectroscopy assay

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Abstract

In the human body, tumor cell occurrence can be indirectly monitored using the L-selectin concentration in the blood, since selectin ligands are present on the surface of tumor cells, and with tumor progression, a decrease in L-selectin levels can be expected and observed. In this study, we present a selective DNA-based surface-enhanced Raman spectroscopy (SERS) assay for the detection and determination of L-selectin in biological samples. Two calibration curves (linear in the 40–190 ng mL^{-1} region and exponential in the 40–500 ng mL^{-1} region) are fitted to the obtained SERS experimental data, i.e., the ratio of I_{732}/I_{1334} band intensities (LOQ = 46 ng mL^{-1}). Calculated determination coefficients are found to be $R^2 = 0.997$ for the linear region of the calibration curve and $R^2 = 0.977$ for the exponential region. Moreover, we demonstrate very good selectivity of the assay even in the presence of P- and E-selectin in a sample containing L-selectin. With our SERS assay, the L-selectin concentration in biological samples can be estimated directly from the calibration curves.

Keywords SERS · SERS assay · L-Selectin · DNA · Aptamer

Introduction

Selectin adhesion molecules play a prominent role in immune/inflammatory responses as well as at tumor metastatic and premetastatic sites of the diseases [1]. The selectin level has also been proven to be an indicator of the immunological processes in the pathophysiology of schizophrenia and major depression [2]. Noticeably, L-selectin is present at almost all leukocyte surfaces (T and B, NK, macrophage/monocyte, granulocyte cells), while P-selectin exists primarily on endothelium and platelet surfaces, whereas E-selectin occurs only on the endothelium [3, 4]. Moreover, selectins play a meditative role in T-cell recruitment to the lymph node and/or to the tumor sites, but also myeloid-derived cells to tumors. The neoplastic cells contain higher levels of selectin ligands in comparison to the non-neoplastic cells. That effect is due to the cancer-associated aberrant glycosylation that makes selectins potential targets for cancer therapy [5, 6]. Selectins are important in the inflammatory responses as they mediate the leukocyte-leukocyte interaction during inflammation [7]. The reduction in the L-selectin level on incoming leukocytes provokes that all those interactions are lost [8]. Therefore, with decreasing L-selectin levels, enhancing the metastasis and the poor prognosis for the cancer disease suffering individuals can be expected. As already shown, the amount of L-selectin in the tumor samples may decrease in ovarian cancer [9] or during high-grade urothelial carcinoma [10] progression as well as it can increase in the colorectal cancer [11] or lymphoma [12]. This has shown the observed changes in the selectins level to be a potential marker for cancer disease progression. In that light, it is essential to develop sensing systems to detect L-selectin in a wide range of concentrations, i.e., simultaneously at low (below 700 ng/mL) and high (above 1.5 mg/mL) levels to make early-stage diagnostics and further successful treatment possible [13]. Determining the L-selectin levels in various biological samples may assist in understanding the L-selectin clustering that can enhance T-cell receptor signaling, suggesting their more

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significant roles than just trafficking [14]. Multiple methods have already been applied to study L-selectin in biological samples, e.g., chromatographic [15], time-resolved luminescent spectroscopy [16] and SERS-based methodology [17]. Up to now, colorimetry-based ELISA is the most common commercially available assay for L-selectin determination. However, the ELISA assays exhibit numerous disadvantages such as the possibility of the bacterial and/or fungal contamination of either screen samples, reagents, and/or cross-contamination between reagents, which may cause erroneous results. That is not the case for surface-enhanced Raman spectroscopy (SERS) assays, as bacterial contamination gives completely different spectra to analytes, as any of moiety has own structure of signals that can be considered to be "fingerprints" of the moiety.

SERS attracted significant scientific interest due to its high selectivity and reproducibility. The most challenging in the SERS technique is the adequate substrate fabrication, which would allow for the non-destructive identification of different chemical and biological analytes. Commercially available substrates should ensure as much Raman signal enhancement as possible with its minimal value of at least 10^6; noticeably, currently the enhancement factor can reach as high as 10^{12} [18]. The SERS technique has been used to build ultrasensitive probes for biomedical and agro-food analysis applications such for detection: bacteria, viruses, fungal pathogen, circulating tumor cells, proteins, peptides, DNA, or neoplastic diseases [19, 20].

In this work, a DNA-based SERS assay for L-selectin detection is presented for the first time. The assay preparation used a DNA aptamer specifically designed to selectively capture L-selectin. Aptamers are short single-stranded RNA or DNA oligonucleotides with very high affinity for targets ranging from small ions to whole organisms. Their high selectivity and specificity of binding, as well as affinity, make them recognition units comparable with their properties to antibodies. Moreover, aptamers have a longer shelf life, simpler production, can be site-specifically labelled, homogeneously immobilized on the substrate surfaces [21], and possess low toxicity. Thus, in this respect, they can be a promising alternative to antibodies. As already demonstrated, aptamers may be used in lab-on-chip devices to sort, isolate, and detect tumor cells [21]. It has been proven that they specifically recognize, capture, and separate human glioblastoma (hGBM) cells [22, 23]. In this light, the specificity, selectivity, and sensitivity of the aptamer-selectin interactions are compared with recently published results obtained for the SERS-based L-selectin sensor using the antibody-selectin system. Particular attention is being paid to proving the possibility of using the fabricated L-selectin SERS assay for the L-selectin identification in blood samples.

Experimental

Chemicals and materials

L-selectin human protein (CD62L) and L-selectin antibody were obtained from Tocris Bioscience (Bristol, UK). L-selectin-specific aptamer (sequence: 5′-Biotin- GCG GTA ACC AGT ACA AGG TGC TAA ACG TAA TGG CGC C7-3′) was purchased from Metabion International AG (Martinsried, Germany). One strand of that oligonucleotide is modified with biotin in the 5′ end, while reverse strand is modified with aminolink in the 3′ end. TEA-trimethylamine as a solvent for DNA aptamer was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Phosphate-buffered saline (PBS) packs (10 mM, pH = 7.2) from Sigma-Aldrich (Dorset, UK) were used without further purification. Water (resistivity over 18 MΩ), purified using a Milli-Q plus 185 system, was used throughout the experiments.

Blood sample preparations

Our experiments used human plasma blood samples of a patient with adenocarcinoma pleural fluid (tumor tissue sample) (courtesy of the Department of Pneumonology, Oncology and Allergology, Medical University of Lublin, Lublin, Poland). Human plasma blood samples from 3 healthy patients (healthy tissue samples) were acquired from Regional Blood Center in Warsaw, Poland.

Samples were collected in the EDTA blood collection tube, and then 1.5 mL of blood is placed in a small 1 mL Eppendorf tube and centrifuged for 3 min at 1070 × g, at room temperature. Next the 5 µL of blood plasma is placed onto the SERS assay, after drying the next 5 µL is applied onto test (total 10 µL).

Additionally, all plasma samples were studied for L-selectin level using the commercial enzyme-linked immunosorbent assay (ELISA, Sigma-Aldrich), according to the Sigma-Aldrich procedure (e.g., used ELISA is sensitive to around 20 ng mL^{-1}). According to a protocol, first, the calibration curve was constructed, and samples were diluted 100 times.

Protocol of surface functionalization of DNA-based SERS assay

To produce the DNA-based SERS assay, the silicon platforms (5 mm × 5 mm) were fabricated according to data presented in the section "Surface-enhanced Raman spectroscopy (SERS) platform fabrication and characterization" (see Electronic Supplementary Material). As it is shown in the SEM images (see Figure S1 in ESI), the entire surface of the platforms is uniformly covered with nanocrystals with the

size in the range of 185 ± 65 nm. Additionally, the detailed procedure of microfabrication and statistical analysis (standard deviation, reproducibility, and stability) of the SERS platform was described in Szymborski et al. [24].

In presented experiments, one strand of used aptamer/oligonucleotides (reverse to these with biotin) is modified with aminolink in the 3′ end. When these oligos are immobilized on the SERS substrates (70 nm of Au/Ag alloy), these modifications allow the aminolink molecules to be close to the metal nanoparticle surfaces, facilitating strong surface-enhanced Raman scattering [25]. Additionally, when the DNA aptamer closes to form an interaction loop, biotin moiety binds to the surface to strengthen the binding of the aptamer to gold. Thus, the interaction loop of the aptamer may interact with L-selectins.

Briefly, the protocol procedure can be presented as follows (Fig. 1):

(1) Preparation of the SERS platform:
   – Au/Ag alloy (30:70) layer on the SERS surfaces was sputtered using PVD equipment.
(2) SERS platform surface functionalization:
   – Surface activation by ozone plasma using a corona treater (ETP, type: BD-20AV, Chicago, IL, USA). The setup consists of a high-voltage (HV) power supply connected to two electrodes, and one of these electrodes was electrically grounded. In the experiment, the distance between the sample and HV electrode was 11 mm, and the time of modification was 10 s.
   – The drop of 5 µL of the prepared DNA aptamer was deposited on the platform. Incubation was carried out in the fridge overnight. To preserve the humidity of the environment around the sample and avoid drying the solution that would yield the typical coffee ring effect, and consequent inhomogeneous distribution of the solutes, the prepared platform was protected using parafilm. The next day, the platform surfaces were subsequently rinsed with PBS to remove the aptamers. Then, the bovine serum albumin blocking of the free SERS surface sites was carried out by depositing a 5 µL of 2% BSA. Incubated at room temperature for 40 min, washed with PBS, and dried.
(3) Such freshly prepared aptamer-immobilized SERS platforms were used for L-selectin capture directly from the ready solution of L-selectin (for the calibration curve construction) and for the blood samples.

Optimal DNA concentration for SERS assay fabrication

These experiments were designated to find the best DNA concentration on the SERS substrate for aptamer-based SERS fabrication for L-selectin. To establish the optimal aptamer concentration for the L-selectin capture, a series of DNA dilutions (1200 µg mL^{-1}, 600 µg mL^{-1}, 300 µg mL^{-1}, 150 µg mL^{-1}) were used on four platforms during the functionalization step, prepared according to the protocol (Fig. 1). Then on such prepared four SERS platforms (functionalized using different concentrations of DNA) the 2.5 µL of 250 ng mL^{-1} of L-selectin were incubated for 30 min, and then 10 SERS signals were collected at one sample (see Figure S2; Electronic Supplementary Material). The presented SERS spectra are the averages from 10 SERS spectra and the ribbon-like shadow represents the standard deviation (SD). The most prominent band intensities of the L-selectin and also the best signal to noise ratio are observed in the spectra recorded from SERS platform fabricated using 600 µg mL^{-1} DNA. Spectra coming from the SERS platform covered with 300 ng $mL^{-1}$and 150 ng mL^{-1} DNA indicate decreasing

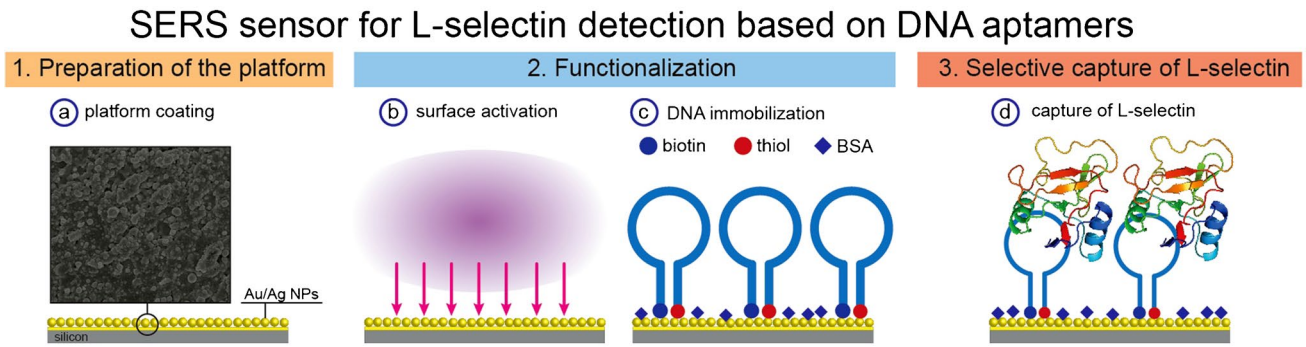

Fig. 1 The scheme shows the preparation of the DNA-based SERS assay in three steps: platform preparation (**1**) and the platform surface functionalization protocol (**2**) for selective detection of L-selectin (**3**). The first step involves coating the SERS platform with Au/Ag (**1a**). The second step contains activation of the platform surface with ozone plasma (**2b**) and immobilization the DNA aptamer and blocking by BSA the active sites on the surface of the SERS platform that are not covered by the aptamer (**2c**). As a final step, the scheme shows as prepared assay selectively capture L-selectin by means of a loop (**3d**)

intensities of all observed bands. On the other hand, the highest considered concentration of DNA (1200 μg mL^{-1}) did not significantly increase the intensity of the bands. Moreover, the measurement error is most significant for 1200 μg mL^{-1} DNA concentration. Therefore, the DNA concentration of 600 μg mL^{-1}, as the best for SERS platform functionalization, was chosen and used throughout the presented experiments. All observed bands for 250 ng mL^{-1} L-selectin captured on prepared DNA aptamer–based SERS platform are gathered and assigned in Table S1 (see Electronic Supplementary Material).

Surface-enhanced Raman spectroscopy (SERS)

All SERS measurements were performed using a handheld Bruker BRAVO Raman spectrometer (Bruker, Germany) equipped with a Duo LASER™ (700–1100 nm) system for fluorescence quenching Sequentially Shifted Excitation (SSE™) and CCD camera. The excitation of each laser power is 50 mW and the spectral resolution was 5 cm^{-1}. For each sample, the 10 single measurements were recorded (3 accumulations of 6 s measurement for each). The preprocessing of obtained SERS spectra was performed using the OPUS software (Bruker Optic GmbH, 2012 version). All spectra were cut in the range from 600 to 1700 cm^{-1}, smoothed with Savitzky-Golay filter (5 points), the background was removed using baseline correction (10 iterations and 64 points), and normalized using a so-called Min-Max normalization.

Establishing the DNA presence on the SERS substrate during functionalization step was realized by comparison of the SERS data recorded for a bare SERS substrate after plasma ozone cleaning and then for the same substrates after aptamer functionalization step (DNA and DNA + 6-MOH). For L-selectin detection, a 2.5 μL of carefully chosen concentrations of L-selectin (40, 60, 80, 100, 250, 500, 1000 ng mL^{-1} in PBS buffer) was incubated at room temperature for 30 min.

Chemometric analysis

Firstly, to find the optimal condition for SERS-based platform fabrication and for L-selectin, the SERS data (concerning all steps recognized on Fig. 1) were recorded and then analyzed using principal component analysis (PCA) method implemented in the Unscrambler software (CAMO software AS, version 10.3, Norway). PCA reduces the dimensionality of a data set by projecting each data point onto only the first few major components in order to obtain lower dimension data while maintaining the greatest variability in the data (correlated variables to a smaller number of uncorrelated variables). The general pattern of the PCA model is: $X = T P^T + E$, where matrix X consists of two smaller matrices, T- with scores and P- with loadings.

In order to differentiate the SERS data, multi-factor models of discrimination between the two classes of the studied groups are used using the supervised method partial least squares (PLS) regression (Unscrambler software; CAMO software AS, version 10.3, Norway). This method is characterized in that one or more Y variables are modeled simultaneously, exploiting the benefits of possible correlations or collinearities between Y variables. In this method, the matrix X is decomposed as in the PCA method ($X = T P^T + E$) and a comparable investigation is performed for Y by making a matrix results, U and loads, Q ($Y = UQT + F$). The objective of PLS is to demonstrate all the constituents forming X and Y so that the residuals for X block, E, and the residuals for Y block, F, are around equal to 0. An inner relationship is additionally built that relates the scores of X block to the scores of the Y block.

Results and discussion

The DNA-based SERS assay for the L-selectin detection: reproducibility, sensitivity, detection limit, specificity, and selectivity

Reproducibility

In order to determine the calibration curve, the SERS data with L-selectin captured from the solution concentration in the range of 40 to 500 ng mL^{-1} (precisely 40, 60, 80, 100, 250, 500 ng mL^{-1}) were used. Each dilution was placed onto active-SERS substrate and left for 30 min at room temperature to assure L-selectin-aptamer interaction. Then, the SERS spectra were acquired (see Figure S3, Electronic Supplementary Material). The spectra show characteristic features with the most intensive bands at 732 cm^{-1}, 1324 cm^{-1}, and 1572 cm^{-1} indicating that vibrations of adenine, phospholipids or nucleic acids, and phenylalanine have the greatest impact on the obtained SERS spectra. Moreover, these bands show a shift in plasma samples to 726 cm^{-1} and 1332 cm^{-1} and additionally the band at 1582 cm^{-1} drastically decreases in intensity in healthy subjects. Therefore, to correlate the concentration of L-selectin with the spectral changes observed in SERS data, two band intensities were chosen: 732 cm^{-1} and 1324 cm^{-1}. In Table S1 (see Electronic Supplementary Material), we gathered the assignment of the SERS bands characteristic for L-selectin. The reproducibility of SERS spectra were calculated based on the acquired spectra using mapping mode (10 SERS spectra across the sample). In Figure S3 (Electronic Supplementary

Material), the ribbon-like shadow along each average spectrum represents the standard deviation of less than 15%. It should be mentioned that we have also studied the L-selectin captured from its solution at the concentration of 1000 ng mL^{-1}. However, in comparison to SERS data recorded for 500 ng mL^{-1}, the bands observed in the SERS spectrum seemed to exhibit a bit lower intensities, while the intensities were expected to grow. Thus, we concluded the prepared DNA aptamer–based SERS assay probably begins to be oversaturated. Therefore, we decided, in further analysis, to not include the SERS data recorded for 1000 ng mL^{-1} L-selectin.

Calibration curve: sensitivity

Based on the SERS data presented in Figure S3 (Electronic Supplementary Material), the ratio I_{732}/I_{1324} was calculated for each sample and the ratio dependence versus L-selectin levels is presented in Fig. 2A. The exponential calibration curve was fitted for all tested L-selectin concentrations (40, 60, 80, 100, 250, 500 ng mL^{-1}) and the coefficient of determination (R^2) was calculated to be 0.997. As spectra taken at L-selectin concentration of 500 ng mL^{-1} are different in the intensity than the SERS data recorded for lower concentrations (Figure S3), the linear fitting is considered for the SERS data excluding the points I_{732}/I_{1324} calculated for

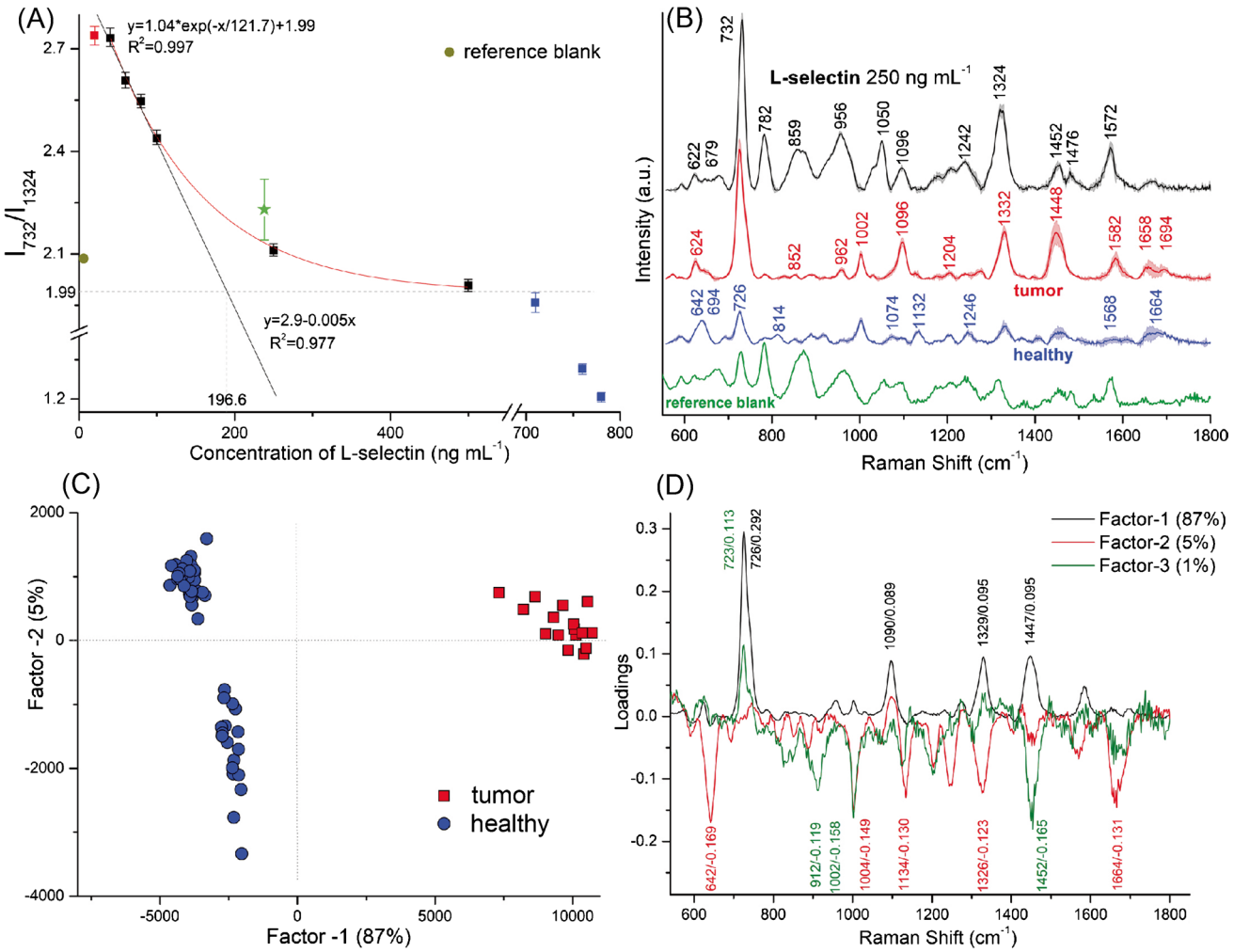

Fig. 2 The calibration curve established based on the I_{732}/I_{1324} band intensity ratio and the L-selectin concentration in the analyzed samples. The green star represents a mix of 250 ng mL^{-1} of L-E-P-selectins (in concentration ratio 1:1:1). The SERS data of representatives: 250 ng mL^{-1} L-selectin and one tumor (red squares) and three healthy (blue dots) tissues. The olive dot represents the I_{732}/I_{1324} band intensity ratio calculated for blank reference (SERS platform after functionalization step) without L-selectin. **A** The SERS data gathered for three samples attached on a DNA aptamer–immobilized SERS platforms: 250 ng mL^{-1} L-selectin, tumor, and healthy plasma samples. Shadows represent the standard deviations, which was found to be less than 15%. **B** The principal component analysis for L-selectin assay captured from plasma of tumor and healthy subjects. **C** The 2D plots of three factor loadings calculated for tumor and healthy samples (**D**)

500 ng mL^{-1}, but also 250 ng mL^{-1} of L-selectin. For this linear curve, the coefficient of determination (R^2) was calculated to be 0.977.

Calibration curve: limit of detection

Based on the SERS data, the calibration curves were fitted in two clearly distinguished concentration ranges: first 40–190 ng mL^{-1} as linear fitting and second, working for 40–500 ng mL^{-1} concentrations of L-selectin, as exponential function. The exponential fitting to the experimental points, the same as observed for the antibody-based L-selectin SERS sensor [17] is presumably due to progressing saturation of the SERS assay. Thus, the amount of the DNA-captured L-selectins is strongly determined by the assay topography and the number of available binding sites. Previously presented antibody-based L-selectin SERS sensing platform allowed for observing changes in plasma samples in the L-selectin concentration range of 80–450 ng mL^{-1} with coefficient of determination $R^2 = 0.88$ for linear and $R^2 = 0.85$ for exponential fitting [17]. However, when using the DNA aptamer to build the SERS assay, the L-selectin is selectively captured from the sample, and thus the fit of the two curves presented above to the measurement points is much more accurate. This is emphasized by the obtained values of the determination coefficient, i.e., $R^2 = 0.977$ for the exponential curve (40–500 ng mL^{-1} region) and $R^2 = 0.997$ (40–190 ng mL^{-1} region) for the linear curve. Moreover, for linear fitting, the limit of detection was calculated (LOD) on the basis of obtained data as equal 18 ng mL^{-1}. Taking into account the possibility of Raman spectra recognition the lowest possible L-selectin concentration as 40 ng mL^{-1}, in the studied sample (see Figure S3 in SI), is considered as the most proper for presented DNA-based SERS assay. For that concentration and for the 1324 cm^{-1} band, the calculated signal to noise ratio (SNR = S/SD, where S is the average peak height and SD is the standard deviation of the peak height) is very low, 2.79. It should be highlighted that determination of the limit of detection (LOD) is important, but to provide information on the ability of the method to determine very low concentrations of the analyte calculation of the limit of quantification (LOQ) should be performed. Thus, LOQ estimation gave value of 46 ng mL^{-1}. Therefore, 46 ng mL^{-1} L-selectin is the limit of quantification of the presented technology. In conclusion, based on the presented two curve calibrations and LOQ calculation, the aptamer SERS assay for L-selectin detection is suitable for studying L-selectin levels in plasma samples in the range of 46–500 ng mL^{-1}. In the concentration range of 46–190 ng mL^{-1} L-selectin, both linear and exponential curve-based equations can be considered, while for higher concentrations, only the exponential fitting should be considered. The lower concentration may be studied using commercially available ELISA tests for the detection of L-selectins in the range of e.g. 0.625–4.0 ng mL^{-1}, 1–20 ng mL^{-1} or 1–58 ng mL^{-1}, and 1.6–100.0 ng mL^{-1} (see Table S2 in Electronic Supplementary Material). The relationships between L-selectin concentration and absorption/optical density, on which the results are based, are not linear, and are presented on a logarithmic scale. Moreover, because exact conditions may vary from assay to assay, a standard curve must be established for every run. Additionally, using ELISA, the serum or plasma samples must be diluted, and thus it is not possible to accurately calculate the L-selectin level for higher L-selectin concentration, on the basis of the determined curve (determined curves differ at low and high concentrations). The presented SERS assay makes possible the determination of L-selectin in clinical samples without any dilution step, which simplifies the whole sample preparation procedure and reduces the possibility of errors to occur. Herein, the levels of L-selectin in the tested plasma samples were determined by the ELISA with its sensitivity up to 20 ng mL^{-1}. However, the obtained data were above the sensitivity of the used test. At the same time, based on the SERS spectra (Fig. 2A) for plasma samples, the I_{732}/I_{1324} ratio was calculated. Coordinates of the points are correct only in the sense of Y-value (I_{732}/I_{1324}). Similar to ELISA, the calculated data (I_{732}/I_{1324}) are outside the range of the presented curves—we cannot determine unambiguously the X-value (L-selectin concentration) for the studied samples. Thus, in this particular case, the assay only allows for qualitative analysis, while being able to accurately classify for other clinical samples that contain amounts of L-selectin within the sensitivity area of the presented approach. However, SERS assay is superior if we take into account time and cost associated with additional reagents, what nowadays are important factors, particularly in the view of increasing importance of green chemistry (see Table S2).

Calibration curve: specificity and selectivity

The selectivity of the proposed method was studied in the presence of the P- and E-selectins. The functionalized SERS assay was dipped for 30 min in the mixture of 250 ng mL^{-1} of each L-, P-, and E-selectins in the PBS buffer in ratio 1:1:1. Figure S4 (Electronic Supplementary Material) presents the SERS spectrum acquired for such prepared sample along with 250 ng mL^{-1} L-selectin spectrum. Over the whole recorded area, the SERS signals exhibit standard deviation (SD) of less than 15% for both shown spectra. There is no obvious differences between these SERS spectra; observed features are almost identical for both presented data. Moreover, taking into account the intensities of bands at 732 cm^{-1} and 1324 cm^{-4} (spectra of L-, E-, and P-selectins together; Figure S4—green plot) and based on the exponential fitting of experimental data shown in Fig. 2A, the

I_{732}/I_{1324} ratio (herein 2.23 value, while for 250 ng mL^{-1} L-selectin spectrum, this value is counted as 2.11), the L-selectin concentration in the analyzed sample was calculated as 250 ± 14 ng mL^{-1} (5.7% of difference between them). Thus, the above-described comparison and analysis give clear and unambiguous information that only L-selectin molecules are detected using the prepared and functionalized SERS platform. Thus, elaborated DNA aptamer SERS assay for L-selectin detection proves a good specificity and excellent selectivity.

Aptamer-based SERS assay for L-selectin in use for biological sample analysis

Additionally, the possibility to use such prepared aptamer-based SERS assay for L-selectin determination from biological samples were evaluated. The SERS data for plasma from individual with adenocarcinoma and three plasma samples from healthy volunteers, in relation to L-selectin captured on DNA SERS assay, are presented in Fig. 2B. The most significant difference among the presented spectra (assay with captured 250 ng mL^{-1} of the L-selectin, healthy and tumor tissue plasma samples) are due to the band intensities, that is the biggest for L-selectin, then for tumor and the lowest for healthy subjects. Usually, for plasma with a tumor, lower band intensities are observed (worse spectrum quality compared to the spectrum of a healthy person). However, the reverse spectral behavior observed in this case is justified. In the plasma of a person with tumor, there are tumor cells that capture some amount of L-selectins, and therefore the decreasing of the L-selectin in a plasma is observed (I_{732}/I_{1324} ratio is higher). However, with cancer progressing, the tumor cells (with L-selectins) appear in the plasma and can be attached to the L-selectin assay giving in a result very good quality of SERS data (better signal-to-noise ratio). Thus, SERS spectrum for the tumor plasma sample shows higher band intensities compared to spectrum of healthy samples. On the other hand, the SERS spectrum of reference blank is a DNA spectrum (assay after functionalization step) that was specifically prepared to capture L-selectin, and thus the SERS data of the reference blank is more comparable to the SERS spectrum of L-selectin than that of plasma, since it contains the same molecules, i.e., the corresponding nucleic acid groupings. In Fig. 2B, the most intense bands observed at 1096 cm^{-1}, 1332 cm^{-1}, 1452 cm^{-1}, and 1572 cm^{-1} occur in both L-selectin and tumor subject. However, these bands are slightly shifted in the spectrum of the tumor plasma sample. In the spectrum of healthy tissue plasma sample, these bands show a very low intensity. The most intensive bands observed in the spectra of three analyzed samples (L-selectin, plasma taken from healthy persons and with tumor) are depicted and assigned accordingly in Table S1 (see Electronic Supplementary Material).

Moreover, taking the intensities at 726 cm^{-1} and $1_{332}/1_{334}$ cm^{-1} bands (for healthy or tumor subjects, respectively), that are corresponding with the intensities I_{732}/I_{1324} ratio of the calibration curve, the selectin level from the plasma samples taken from three healthy patients and one sample from individual with adenocarcinoma can be determined. However, for the subjects in the experiments conducted, the intensity ratios obtained fall outside the range of the data unambiguously indicating healthy subjects and one sick person. In other cases, when we deal with a person, for example, undergoing treatment, the ratio of intensities for selected bands will be found on the calibration curve and which will allow us to observe the results of treatment. The calculated I_{732}/I_{1324} ratio and the corresponding values of L-selectin can be read directly from the calibration curve presented on Fig. 2A (blue dots for healthy and red squares for tumor subjects).

Chemometric analysis

Along the experiments, chemometric analyses in a form of principal component analysis (PCA) as well as partial least squares (PLS) were performed. PCA transforms a large number of original Raman data into a smaller number of uncorrelated variables called principal components (PCs). Analysis of the calculated scores may show how the studied data are related to each other despite envisioned empirical lack of any spectral changes. In other words, based on the calculation of any changes, differences or similarities found in the SERS data set that influence computed differentiation can be found in relation to Raman shifts. Further, to differentiate the SERS spectra, multi-factor models of discrimination between the two classes of the studied groups usually can be used. The most common application is the partial least squares (PLS) method. This method is characterized in that one or more Y variables are modelled simultaneously, exploiting the benefits of possible correlations or collinearities between Y variables. The details of chemometric analyses are in the Supplementary Information. Firstly, to check if DNA is interacting with the prepared SERS platform, the multivariate calculation in a form of unsupervised principal component analysis (PCA) was performed using SERS data gathered for DNA-covered SERS assay at different concentrations: 300 µg mL^{-1}, 600 µg mL^{-1}, 1200 µg mL^{-1}, and 24,000 µg mL^{-1} and compiled with data based on SERS spectra of platform after a first-step SERS assay preparation (Fig. 1; platform coating); scores named *Platform*. In Fig. S5 (Supplementary Information), the *Platform* scores are in large distance from scores named *DNA* (24,000 µg mL^{-1}; the highest DNA concentration) obtained from platform after a second step of functionalization (Fig. 1; immobilization step). Also, the other scores obtained based on the SERS signals of platforms covered with 300 µg mL^{-1},

600 μg mL^{-1}, and 1200 μg mL^{-1} DNA in a second step are in distance from *Platform* scores indicating the platform surface activation and interaction with applied DNA along the procedure of SERS assay fabrication. Thus, we can be sure that according to the applied procedure, DNA aptamer and BSA are attached on the SERS platform. On the other hand, using prepared DNA aptamer–immobilized SERS platforms allows to catch and detect the L-selectin from all applied solution, as resultant scores of their spectra are in the middle of scores calculated for a clean SERS platform (*Platform*) and prepared SERS assay using the most concentrated DNA aptamer (DNA, 24,000 μg mL^{-1}). Such positioning of scores with captured L-selectins can be understood by similarities between data recorded for clean platform and functionalized platform. Based on that data, but also on that presented in Figure S2 (Electronic Supplementary Material; the best signal to noise ratio), the 600 μg mL^{-1} DNA aptamer concentration was chosen as the most optimal for the DNA aptamer–based SERS assay fabrication.

In the next step, the chemometric analysis in a form of partial least squares (PLS) calculation was performed over the collected SERS spectra for SERS assay–captured L-selectin in the following concentrations: 40, 60, 80, 100, 250, and 500 ng mL^{-1} (Figure S6, Electronic Supplementary Information). As we have already observed, lowering of whole band intensities in a SERS data of 500 ng mL^{-1} L-selectin, appearance of the respective PLS scores in the area of scores calculated from SERS data of 40, 60, and 80 ng mL^{-1} L-selectins is understandable—the intensities of the corresponding SERS data of those L-selectin concentrations are also similar (Figure S3, Electronic Supplementary Information). Thus, the calculated scores form groups of points that are close to each other indicating similarities between the samples (250 ng mL^{-1} and 100 ng mL^{-1} vs. 500, 80, 60, and 40 ng mL^{-1}). Moreover, there is an *X*-axis (Factor-1) dividing the more concentrated in L-selectin samples (100 and 250 ng mL^{-1}) from those less ones (40, 60, 80 ng mL^{-1}). This observation does not apply to the 500 ng mL^{-1} L-selectin level, for which it is likely that a steric obstacle has already occurred and the prepared SERS assay has been overloaded. Additionally, taking into account the difficultness in SERS spectra analysis of healthy and tumor plasma sample recognition, we intended to check if recorded SERS data really spectrally differ. In that purpose, the PLS analysis was used. Presented data prove (Fig. 2C) the existence of differences between healthy and tumor plasma, as calculated scores characterized by those spectra are divided by *X*-axis. In addition, the obtained results indicate that Factor-1 carries 87%, Factor-2 5%, and Factor-3 1% of the variance between the analyzed plasma samples. Moreover, the calculated scores of healthy and tumor plasma samples are at a fairly large distance that allows for accurate discrimination between analyzed samples. Thus, using the PLS analysis of SERS data, it is possible to make satisfactory differentiation and recognition between plasma samples of tumor and healthy individuals. Moreover, as Factor-1 counts for 87% of differentiation between samples, based on the Factor-1 loadings (Fig. 2D), it is possible to indicate the most prominent bands that strongly influenced obtained differentiation, i.e., 726, 1090, 1329, and 1447 cm^{-1}. These variables are the most weighted and important for obtained discrimination, and can be correlated with SERS spectra (Fig. 2B). First three variations can be correlated with DNA, nucleic acids, and phospholipid vibration observed in SERS spectra of both healthy and tumor subjects. Interestingly, the variable 1447 cm^{-1} is associated with L-tryptophan/tryptophan vibrations observed at 1448 cm^{-1} in SERS spectrum and is directly related to the level of L-selectin in the tested samples and to the development and progression of cancer. Tryptophan is an essential amino acid that represents a major metabolic pathway, promotes tumor cell intrinsic malignant properties, and restricts antitumor immunity. Increase in the intensity of the bands corresponding to tryptophan vibration may be considered as the first sign of tumor in the studied sample.

Conclusions

In this study, we presented a selective DNA-based surface-enhanced Raman spectroscopy assay for the detection and determination of L-selectin in biological samples. In healthy individuals, L-selectin level ranges from 700 to 1500 ng mL^{-1} and the commercially available L-selectin tests allow to detect L-selectin in the lower region (ELISA are sensitive to around 100 ng mL^{-1}). However, for tumor subjects, the L-selectin concentration is decreasing with progressing tumor. Therefore, it is important to develop L-selectin sensor/assay for early-stage tumor diagnosis, with lower L-selectin levels observed in healthy individuals. Using fabricated (herein DNA-based SERS) assay, it is possible to evaluate the selectin level in the lower region of concentration, then for healthy subjects, in less than 10 min (total time of procedure, including the time for sample preparation). Elaborated assay may also be used to measure the L-selectin level during patients' treatment. What is also very important, the use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Thus, using other assays not related to antibodies, as presented in the manuscript, DNA-based SERS assay for L-selectin detection, is of great importance for proper analysis of data especially for individuals with tumor. The presented assay may find application as a scientific tool, but we strongly believe in its high

potential for biotechnological and medical applications for studying the development of benign and malignant cancers from the blood plasma samples.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-023-05110-x.

Declarations

Ethics approval Measurement for clinical samples was performed in compliance with the relevant laws and institutional guidelines. The protocol of study was approved by the Ethics and Bioethics Committee of Cardinal Stefan Wyszynski University in Warsaw (Poland): Opinion number 13/2019.

Source of biological material Plasma samples from patients with tumor were collected according to standard best practice and ethics and bioethics guidelines. Informed consent was obtained from all subjects.

Plasma samples from patients with tumor are gathered by the Department of Pneumonology, Oncology and Allergology, Medical University of Lublin, (Lublin, Poland). Human plasma blood samples from 3 healthy patients (healthy tissue samples) were acquired from Regional Blood Center in Warsaw, Poland.

Conflict of interest The authors declare no competing interests.

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