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Label-free spectrochemical probe for determination of hemoglobin glycation in clinical blood samples

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Abstract

Glycated hemoglobin, HbA1c, is an important biomarker that reveals the average value of blood glucose over the preceding three months. While significant recent attention has been focused on the use of optical and direct molecular spectroscopic methods for determination of HbA1c, a facile test that minimizes sample preparation needs and turnaround time still remains elusive. Here, we report a label-free approach for identifying low, mid and high HbA1c levels in hemolysate and in whole blood samples featuring resonance Raman (RR) spectroscopy and support vector machine (SVM)-based classification of spectral patterns. The diagnostic power of RR measurements stems from its selective enhancement of hemoglobin-specific features, which simultaneously minimizes the blood matrix spectral interference and permits detection in the native solution. In this pilot study, our spectroscopic observations reveal that glycation of hemoglobin results in subtle but reproducible changes even when detected in the whole blood matrix. Leveraging SVM analysis of the principal component scores determined from the RR spectra, we show high degree of accuracy in classifying clinical specimen. We envisage that the promising findings will pave the way for

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Author Contributions

RP, RRD, GLH and IB designed the research; GLH provided the blood samples and HbA1c values, RP acquired spectroscopic measurements; NL and LG contributed in devising experimental tool for spectroscopic measurement; SPS, CZ and RP contributed analytical tools for spectral analysis and interpretation; SPS, RP, CZ and IB analyzed the data; RP, SPS and IB wrote the manuscript with input from all the authors.

Competing Interests:

The authors declare no competing interests.

more extensive clinical specimen investigations with the ultimate goal of translating molecular spectroscopy for routine point-of-care testing.

Keywords

HbA1c; glycated hemoglobin; Raman spectroscopy; diabetes; label-free

1. Introduction

Diabetes mellitus is a chronic disease caused by impairment of blood glucose (BG) regulation due to inadequate insulin production by pancreas or ineffective usage of the secreted insulin. This chronic metabolic disorder is approaching epidemic proportions globally with an estimated increase from current number of nearly 382 million to 592 million diabetic patients by 2035 and potentially even higher.^{1,2} Unfortunately, due to the lack of any well-established cure, careful monitoring of elevated blood glucose levels is critical for avoiding severe secondary health complications. The direct measurement of BG values remains the “gold standard” and a number of research groups, including our own laboratory, have actively pursued the development of minimally invasive notably, electrochemical sensors^{3,4} and fully non-invasive glucose monitoring systems to reduce/eliminate the pain and inconvenience of finger prick measurements.^{5–10}

In addition and complementary to direct measurement of glucose, several other biomarker concentrations are known to correlate with glycemic control.⁸ Glycated hemoglobin (also known as HbA1c), which is formed by chemical attachment of glucose molecule to N-terminal valine residue of the hemoglobin β -chains via non-enzymatic *in vivo* glycation of hemoglobin, has been widely used as a clinical marker for diabetes monitoring and pre-diabetes screening.¹¹ Importantly, unlike fasting plasma glucose (FPG), HbA1c values provides an averaged glucose value unaffected by the transient fluctuation and show better correlation with secondary diabetic complications as compared to FPG.^{12,13}

Although several quantification methods, namely affinity chromatography, high-pressure liquid chromatography (HPLC) and immunoassays, are routinely used in clinical laboratories, HbA1c quantification often suffers from specificity concerns due to presence of hemoglobin variants.^{14,15} Innovations in the area of luminol chemiluminescence (CL)¹⁶ and in immunoassays (for instance, based on polydimethylsiloxane antibody microarrays)¹⁷ have sought to alleviate these limitations. Recently, photonic methods, and vibrational spectroscopy in particular, have drawn considerable attention in clinical diagnostics due to the promise of label-free, multiplexed and objective determination as well as the ability to gain molecular insights into the specific pathology.^{18–21} While non-invasive determination is not a requirement for HbA1c measurements, the potential for reliable, real-time and multiplexed protein glycosylation analyses provides a powerful incentive for development of Raman spectroscopy for point-of-care-testing²² and in-line process monitoring applications. To this end, Ishikawa and co-workers have proposed the use of label-free surface-enhanced Raman spectroscopy (SERS) for HbA1c determination;²³ while promising, the reproducibility concerns of SERS signals presents a major challenge. To allay these

reproducibility concerns, we have harnessed the “coffee ring” effect for analyte pre-concentration and shown the potential of drop coating Raman spectroscopy (DCDR) in quantifying HbA1c value in a hemolysate model.²⁴ Nevertheless, translating these findings to hemolysates obtained from human erythrocytes and, subsequently, to whole blood samples has proven to be difficult, principally due to the confounding scattering and fluorescence contributions from the other blood constituents.

We propose to leverage the selective signal enhancement of resonance Raman (RR) spectroscopy for detection of hemoglobin and its glycated derivative. We hypothesize that this would enable differentiation of blood specimens with low (below 7%), mid (between 7% and 10%) and high (above 10%) A1c values without necessitating drying of the specimen to achieve pre-concentration. The latter would facilitate measurements in the solution form and mitigate the risk of variable readings over a coffee ring-type drying pattern that can limit the applicability of DCDR in the clinic. In this pilot study, we seek to couple the measurements of RR profiles with support vector machines (SVM) in order to map the subtle differences in the spectra to prediction of class label (low, mid and high). Segmentation into these categories by sampling small volumes of the specimen offers the requisite clinical information to inform therapeutic decision-making, as espoused in a recent report on differentiation of diabetic and non-diabetic populations by Villa-Manríquez et al.²⁵

2. Methods and Data Analysis

Methods

Anonymized leftover whole blood samples were collected from the clinical pathology laboratory of Beth Israel Deaconess Medical Center, Boston. The whole blood samples were collected in sodium EDTA tubes. For the hemolysate study, 100 µl of the whole blood sample was aliquoted and centrifuged at 112 RCF for 10 mins at 25 °C. The supernatant was removed and 10µl of the Millipore water was added to lyse the erythrocytes. For reference, HbA1c values on the same blood samples were measured using immunoassays with Tina-quant A1c Gen 2 assay kit (Roche Diagnostics) by Dr. Gary Horowitz (Beth Israel Deaconess Medical Center, Harvard Medical School). Table 1 lists the number of specimen belonging to each of the three classes (low, mid and high), and the range, mean and standard deviation of HbA1c reference values for each class.

Raman spectroscopic measurements

For the hemolysate study, 1 µl of the lysed RBC sample was placed on a quartz cover slip to enable scanning measurements in an upright geometry. We used a home-built microscopy system for Raman data acquisitions, and an excitation wavelength of 532 nm was chosen to utilize the resonance effect. The UV-vis absorption spectrum of HbA1c (Fig. S1) was used to converge on this excitation wavelength. Raman spectra of the whole blood and lysed RBC were recorded in the backscattering configuration with a free space Raman microspectroscopy system consisting of optical microscope (BX41, Olympus), 750mm focal length spectrograph (Acton Spectra-Pro SP-2750, Princeton Instruments) equipped with TE-cooled CCD camera (PIXIS 400 BR, Princeton Instruments). The power at the sample was held constant at 5 mW and a total of 9 spectra were co-averaged with integration time of

10s. Both excitation and collection were performed using the same 40× objective. The recorded spectra were corrected for the presence of cosmic rays before further data analysis and interpretation. In addition to elimination of spectra that showed intensity saturation, a student's t-test employing Mahalanobis distance function was used to reject spectra with 95% probability of being spectral outliers ($p < 0.05$). Finally, a total of 198 spectra (75-low, 77-mid and 46-high), from the hemolysate samples of the 40 subjects, were used for further analysis. Likewise, a total of 227 spectra (92-low, 82-mid and 53-high) from whole blood aliquots were used.

Data analysis

In order to assess the discrimination ability based on RR spectral signature, the blood samples were classified into three groups based on their reference HbA1c values. The first group consists of blood samples with HbA1c values $<7\%$ and termed as low. Additionally, we defined two categories with A1c values between 7% and 10%, and above 10% as mid and high, respectively. Spectra were baseline corrected by fitting a 3rd order polynomial function and area normalized. Mean centering of the spectral data was performed for Principal Component Analysis (PCA), and the generated principal component scores and loading were used to gain insights into the key spectral features as well as the inter- vs intra-group variability. PCA employs a linear transformation of the data into a new set of axes such that the largest variance lies on the first axis and decreases thereafter for each successive axis. This analytical approach is widely used to classify a group of spectra into similar classes and just a few principal components are capable of segmenting observations.^{26,27} To view the differences among the classes, radial visualization maps were constructed using the Radviz tool in the Orange data mining toolbox.²⁸ Here, we employed the scores of select PCs obtained from subjecting the entire spectral dataset to PCA. Specifically, guided by the Vizrank algorithm,²⁹ the PCs were selected to maximize class separation. In the radial visualization plot, the scores corresponding to a spectrum regulate the position of the corresponding data point relative to the PC anchors. Furthermore, classification algorithms were derived using the support vector machines (SVM) framework. SVM was employed as it is particularly suitable in handling ill-posed problems, such as those encountered in our RR spectral dataset, furnishes unique global models and enables non-linear classification.^{30,31} The aforementioned PC scores were used as inputs for development of the SVM-based classification algorithm. Classification accuracy of PC-SVM algorithm was evaluated using leave-one-spectrum-out (LOSO) and leave-one-patient-out (LOPO) cross-validation. Additionally, we also analyzed the data on a per-patient basis by training on calibration data set and testing on an independent validation dataset. The external validation set included 4, 4 and 2 randomly selected samples from 'low', 'mid' and 'high' classes, respectively. After repeating this 10 times, correct and incorrect classifications were summarized.

3. Results and Discussion

The objectives of this pilot study are: (a) to test the viability of RR spectroscopy in selectively enhancing the spectroscopic signature of hemoglobin and its glycated variant (Fig. 1(A)–(B)) show the crystal structures of glycated hemoglobin, HbA1c; and hemoglobin, HbA); and (b) to assess the diagnostic feasibility of RR spectroscopy-based

differentiation of HbA_{1c} groups in clinical samples - without requiring drying or any other alteration of the native solution form. To achieve these objectives, we recorded RR spectra from hemolysates obtained from the centrifuged erythrocyte component (Fig. 1(C)) as well as whole blood sample.

Our group has previously demonstrated the potential of near-infrared spontaneous Raman spectroscopy for HbA_{1c} quantification in model hemolysate samples created by mixing pure hemoglobin and A_{1c} in physiologically relevant concentrations.²⁴ Yet, the use of 785 nm excitation for measurements of the clinical specimens shows considerable non-analyte specific spectral variability, which we attribute to the presence of myriad contributors present in blood. Additionally, the sensitivity of spontaneous Raman signals acquired from the blood specimens was found to be inadequate in making accurate determinations thereby leading to our selection of 532 nm for RR measurements.

Another notable feature of the current proof-of-concept study is the use of categorical classifiers for differentiating patients with diabetes from at-risk and normal cases. While glycation is usually represented as a percentage, therapeutic decision-making is based on reference intervals and clinical decision limits. In particular, the American Diabetes Association (ADA) endorses HbA_{1c} as a diagnostic test for diabetes at a specific cut-off value.³² Individuals with a slightly lower HbA_{1c}, but higher than normal levels, are considered to be at 'increased risk' for diabetes as well as cardiovascular disease, and are counseled about effective strategies, such as weight loss and physical activity, to lower their risks. We reason that accurate discrimination of such glycemic states, based on the spectral profiles recorded from the whole blood and hemolysate specimen, would offer value in aiding treatment decisions. A recent study using spontaneous Raman spectroscopy adopts a similar approach, namely categorical classification of glycated hemoglobin levels between healthy volunteers (HV), well-controlled diabetic patients (WCDP) and not controlled diabetic patients (NCDP).²⁵

Spectroscopic Measurements in Hemolysate Samples

To address the issues of spectral variability and inadequate signal-to-noise ratio, an excitation wavelength of 532 nm was chosen to exploit the increased sensitivity and selectivity due to resonance effect of hemoglobin.³³ Fig. 2 shows the RR spectra acquired from hemolysate specimens, separated according to their respective A_{1c} reference values into three classes (low, mid and high). Here, we provide the mean RR spectra along with the ± 1 standard deviation, where the spectra have been normalized, background corrected and offset for visualization purposes. The spectra exhibit bands associated with the porphyrin macrocycle and proteinaceous component of hemoglobin molecule. Prominent Raman peaks at 744, 1123, 1161, 1218, 1298, 1333, 1365, 1577 and 1626 cm^{-1} were observed (detailed assignment is provided in Table S1). Among these bands, the spin state marker region (1500–1650 cm^{-1}) and pyrrole ring stretching region (1300–1400 cm^{-1}) are reported to have resonance at 532nm excitation.^{34–36} In prior work, Brewster *et al.* have used 2D correlation moving windows plots to deduce specific wavelength regions that are affected by glycosylation of the protein.³⁷ Also, glycation-induced changes to the hemoglobin molecule

have been reported to decrease the α -helix content³⁸ and weaken the heme–globin interaction.³⁹

Multivariate Analysis for Classification of Spectra in Hemolysate Samples

To determine whether such glycation-induced structural changes may be present, we employed multivariate analysis of the spectral profiles recorded from the hemolysate specimens. PCA is implemented without any prior information about the spectra under consideration and is related to the least-squares regression approach.⁴⁰ We used PCA to offer a transformed view of the spectral dataset to explain deviations from the average spectral profile. Using PCs in place of the recorded spectral data as input for the classification model also aids in suppressing noise, autofluorescence background (that may remain despite baseline subtraction) and reduce possibility of spurious correlation-driven predictions. As per standard protocol, data are mean centered prior to the transformation and the mean spectrum is used as a base comparator.

Fig. 3A shows the PC loadings that account for the maximum variance in the spectral data while Fig. 3B gives the radial visualization map, based on the corresponding PC scores, depicting the inter vs intra-group clustering behavior. Here, the scores provide sufficient information to assign the recorded spectra into the classes whose biochemical distinctions may be explained by the profiles of the loading vectors. In particular, the PC loadings reveal that the principal sources of differences between the three groups stem from the expected vibrational RR bands pertaining to hemoglobin and its modification due to non-enzymatic glycation. Clear separation among pooled diabetic and non-diabetic populations in Fig. 3B suggests the high sensitivity of the technique to subtle structural differences in hemoglobin – especially given that the measurements are made in lysed erythrocytes (and not in pure chemicals).

Encouraged by the higher inter-group RR spectroscopic differences in relation to the intra-group variability, we developed SVM based classification models to assess the predictive power of the proposed approach. Specifically, the PC scores were used as inputs for the SVM model and the model was tested using LOSO cross-validation. The results of the LOSO analysis are tabulated in the confusion matrix (where the diagonal elements represent accurate predictions) presented in Table 2(A). Evidently, spectra belonging to all three classes, low, mid and high, can be discriminated with 98% accuracy. We also conducted analyses on a patient-basis: first, by conducting leave-one-patient-out cross-validation; and second, by testing on an independent validation set comprised of data from 10 subjects (4 low, 4 mid and 2 high). In the former, spectra belonging to a particular specimen (subject) were held out for validation and the rest of the data was used for training the classifier. This procedure is repeated till each of the subjects is held out once. In the second case, an independent set constituted by spectral data recorded from 10 subjects was used for testing the classifier. To obtain a robust estimate, the classification accuracy was obtained by averaging ten such independent iterations. The results of both the leave-one-out cross-validation and external validation are provided in Table 2(B) and Table 2(C). Average classification accuracy of 100% and *ca.* 89% were obtained for the two respective cases

indicating that RR spectroscopy, when coupled with multivariate analyses, offers a feasible route to identification of A1c levels from hemolysate measurements.

Spectroscopic Measurements in Whole Blood Specimens

We subsequently carried out the RR spectroscopic measurements on whole blood samples. The average Raman spectra along with standard deviations obtained from three groups are shown in Fig. 4. Major contributions from hemoglobin and a smaller feature from tryptophan (747 cm^{-1}) Raman bands were observed. The overall spectral profiles are similar as that of hemolysate specimens. This is expected as in RR measurements the spectral contribution of non-resonating components, such as serum proteins, are largely minimized in comparison of the constituents that are in resonance with the excitation wavelength. This represents a considerable advantage for high throughput measurements, as it reduces the need for extensive sample pre-processing as is often required in other analytical approaches.

Multivariate Analysis for Classification of Spectra in Whole Blood Samples

Similar to the hemolysates, we performed PCA to assess the feasibility of discrimination among the whole blood samples on the basis of glycated hemoglobin concentration differences (Fig. 5). Clusters of the three groups were observed in the PC scores plot, albeit with a slightly larger overlap than was observed in the case for the hemolysates. This is not entirely surprising, as the glycation-induced differences in the hemoglobin content may be masked by the presence of myriad whole blood constituents. To evaluate the prediction performance of this spectral dataset, we used the PC inputs for developing a SVM-based classification algorithm and tested it using a LOSO cross-validation approach (Table 3(A)). With respect to the hemolysate findings, reduced average accuracies of 81% in discrimination of three groups (low, mid and high) were observed. The reduction in discrimination accuracy is primarily attributed to the presence of spectral interferents in whole blood with respect to the lysed RBC specimens, which is rich in hemoglobin. This is evidenced by the observed differences in the loading factors plot (Fig. 3 and Fig. 5), where the PC loadings derived from the whole blood specimen spectral data exhibit more features of other constituents and reduced signal-to-noise ratio.

Given the reduced classification accuracy for the whole blood samples, we sought to understand how such discrepancies mapped to a patient-specific classification, i.e. whether the spectral misclassifications were uniformly distributed across all samples or arose from data points belonging to only a few samples. Hence, we conducted a leave-one-patient-out study, where the spectra from each human subject was kept aside for validation while the data from the rest of the subjects was used to develop the calibration model. This accurately mimics the clinical situation, where classification on a per patient basis is meaningful (rather than a per spectrum basis). The results from this analysis are presented in Table 3(B). In the event that the spectral assignment of a blood sample is split between the classes such that the membership probability of the sample is not higher for any specific class, it is categorized as an “unclassified” sample (In clinical practice, samples for which there is no definitive spectral diagnosis would be designated as unclassified or equivocal and additional Raman measurements made in the hope of obtaining more definitive results). We observe that the overall differentiation accuracy is significantly better on a per individual basis (93%)

indicating that the spectra of only a few blood samples were skewing the classification results. Furthermore, most of these samples (as well as the misclassified ones) belong to the mid class, which can be attributed to the closely related A1c values for those on the boundary with the low and high classes. Similar to the hemolysate case, we also conducted patient-level analyses using an independent validation set featuring spectra from 10 individuals and using the rest as inputs for classifier development. The promising independent test set prediction results (Table 3(C)), while lower than the leave-one-out cross-validation accuracy values, further reinforces the viability of RR spectroscopy as an augmentative tool for sample differentiation based on HbA1c value even when measurements are carried out on whole blood specimen – a vital step in its translation to clinical practice.

Spurious Correlation Study

Finally, we also explicitly examined the presence of any spurious correlations in the spectral data set by performing a negative control study for both hemolysate and whole blood sample. Here, randomly chosen HbA1c class labels were assigned regardless of the true identity of the blood sample prior to the development of the PC-SVM derived decision algorithms. We obtained an overall accuracy of classification of 33.3% averaged over 1000 iterations with standard deviations *ca.* 9% for both the hemolysate and whole blood samples. These values are in line with the random likelihood of selection of the true class label (1/3) and verify that our derived decision algorithms are based on reproducible variations in the vibrational spectra of the hemoglobin and its glycated counterpart.

4. Conclusion

The present pilot study underscores the promise of a label-free direct optical spectroscopy platform for diabetic screening based on HbA1c values in a point of care (POC) setting. The lack of need to form an optimal drying pattern or use of plasmonic nanoparticles to impart the signal enhancement further facilitates integration with clinical practice and reduces non-analyte specific variability. This is the first reported demonstration of the differentiation of both hemolysate and whole blood specimen based on glycated hemoglobin content without any additional sample preparation. Moreover, this preliminary RR spectroscopy results should not be interpreted as representing the best possible signal-to-noise ratio or lowest detection limits that are likely to be obtained after further system optimization. In our ongoing and future studies, we plan to test its classification ability in a larger sample cohort as well as to quantify the HbA1c values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Structure of HbA1c. The alpha and beta subunits are colored in magenta and blue, respectively, and are shown in cartoon representation. The heme groups are shown in sticks with C, N and O atoms colored in green, blue and red, respectively. The alpha-D-glucose molecules are shown in sticks with carbon atoms colored in yellow (downloaded from RCSB protein data bank (PDB ID 3B75)). (B) Structure of HbA. The alpha and beta subunits are colored in limegreen and blue, respectively, and are shown in cartoon representation. The heme groups are shown in stick representation with the C, N and O atoms colored in green, blue and red, respectively (downloaded from RCSB protein data bank (PDB ID 3HHB)). (C) Schematic of RR spectroscopy-based label-free detection of glycated hemoglobin in hemolysate and whole blood samples.

Figure 2.

Resonance Raman Spectra recorded from hemolysate samples of A. Low (HbA1c<7%), B. Mid (7% HbA1c 10%), and C. High (HbA1c>10%) classes. The spectra are baseline corrected and vertically offset for visualization purposed. The dark line represents the mean and the accompanying shaded area the standard deviation of the spectral dataset for each group.

Figure 3.

(A) The first three principal components corresponding to the entire RR spectral dataset. (B) Radial visualization plot based on scores of the corresponding principal components. The low, mid and high samples are indicated by red squares, green triangles and blue circles, respectively.

Figure 4.

Resonance Raman Spectra of whole blood samples of A. low (HbA1c<7%), B. mid (7% HbA1c 10%), and C. high (HbA1c>10%) classes. The spectra are baseline corrected and vertically offset for visualization purposed. The dark line represents the mean and the accompanying shaded area the standard deviation of the spectral dataset for each group.

Figure 5. (A) Five principal components with highest discriminative power for HbA1c groups. (B) Radial visualization plot based on scores of the corresponding principal components. The low, mid and high samples are indicated by red squares, green triangles and blue circles, respectively.

Table 1

Table of number of human subjects and their HbA1c value range along with mean and standard deviation split into the groups, low, mid and high.

A1c Values	Number of subjects	Range of A1c values	Mean	Standard Deviation
A1c < 7% (Low)	17	4.5 – 6.9	5.78	0.79
7% A1c 10% (Mid)	16	7.4 – 9.6	8.40	0.67
10% < A1c (High)	7	10.8 – 14.1	12.77	1.11

Table 2

Confusion matrix for leave-one-spectrum-out (LOSO) cross-validation analysis of PC-SVM resonance Raman decision algorithm in hemolysate samples. (B) Confusion matrix for leave-one-patient-out (LOPO) cross-validation analysis of PC-SVM resonance Raman decision algorithm in hemolysates. (C) Confusion matrix for external validation analysis of PC-SVM resonance Raman decision algorithm in hemolysates.

A				
Leave-one-spectrum-out				
Reference class	Resonance Raman spectroscopy prediction			
	A1c < 7% (Low)	7% A1c 10% (Mid)	10% < A1c (High)	
A1c < 7% (Low)	74(99%)	0	1	
7% A1c 10% (Mid)	0	77(100%)	0	
10% < A1c (High)	0	1	45(98%)	

B				
Leave-one-patient-out				
Reference class	Resonance Raman spectroscopy prediction			
	A1c< 7% (Low)	7% A1c 10% (Mid)	10% < A1c (High)	Unclassified
A1c < 7% (Low)	17 (100%)	0	0	0
7% A1c 10% (Mid)	0	16(100%)	0	0
10% < A1c (High)	0	0	7 (100%)	0

C				
Independent test predictions				
Reference class	Resonance Raman spectroscopy prediction			
	A1c<7% (Low)	7% A1c 10% (Mid)	10% < A1c (High)	
A1c < 7% (Low)	35/40 (87.5%)	5/40	0/40	
7% A1c 10% (Mid)	0/40	38/40 (95%)	2/40	
10% < A1c (High)	0/20	3/20	17/20 (85%)	

Table 3

(A) Confusion matrix for leave-one-spectrum-out (LOSO) cross-validation analysis of PC-SVM resonance Raman decision algorithm in whole blood samples. (B) Confusion matrix for leave-one-patient-out (LOPO) cross-validation analysis of PC-SVM resonance Raman decision algorithm in whole blood samples. (C) Confusion matrix for external validation analysis of PC-SVM resonance Raman decision algorithm in whole blood samples.

A				
Leave-one-spectrum-out				
Reference class	Resonance Raman spectroscopy prediction			
	A1c < 7% (Low)	7% A1c 10% (Mid)	10% < A1c (High)	
A1c < 7% (Low)	86(93%)	5	1	
7% A1c 10% (Mid)	12	62(76%)	8	
10% < A1c (High)	6	8	39(74%)	

B				
Leave-one-patient-out				
Reference class	Resonance Raman spectroscopy prediction			
	A1c < 7% (Low)	7% A1c 10% (Mid)	10% < A1c (High)	Unclassified
A1c < 7% (Low)	16(94%)	1	0	0
7% A1c 10% (Mid)	1	12(86%)	1	2
10% < A1c (High)	0	0	6(100%)	1

C				
Independent test predictions				
Reference class	Resonance Raman spectroscopy prediction			
	A1c < 7% (Low)	7% A1c 10% (Mid)	10% < A1c (High)	
A1c < 7% (Low)	32/40 (80%)	7/40	1/40	
7% A1c 10% (Mid)	7/40	31/40(77.5%)	2/40	
10% < A1c (High)	1/20	3/20	16/20(80%)	