Raman Spectroscopy-Based Sensing of Glycated Hemoglobin: Critical Analysis and Future Outlook

Rishikesh Pandey
G.R. Harrison Spectroscopy laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts, 02139, USA Email: pandey@mit.edu

Abstract
Glycated hemoglobin is a clinically established important biomarker that provides retrospective value of blood glucose concentration over the preceding 2-3 months. Owing to the biochemical specificity and multiplexing capability, Raman spectroscopy has emerged as a promising tool for detection and quantification of blood constituents in a label-free and non-destructive manner. Here, we critically review the Raman spectroscopy-based approach to detect and quantify this important biomarker. The potential of this spectroscopy-based approach and its possible clinical translation from the current optical bench will be also briefly discussed along with the future prospects.

Keywords : Raman Spectroscopy, Glycated Hemoglobin, Biomarker, blood constituents

Introduction

Diabetes mellitus is a chronic metabolic disorder due to the impairment of blood glucose regulation stemming from inadequate insulin secretion, or action or both. This disease is slowly becoming an epidemic which can be understood by the global figure of the people affected by diabetes which is estimated to increase from current number of nearly 382 million to 592 million diabetic patients by 2035 (1). Unfortunately, there is no well-established cure for the diabetes at present; and continuous blood glucose regulation through regular insulin injections and other related medications based on careful monitoring of blood-glucose levels are the only ways to maintain the blood glucose concentration. Glycated hemoglobin (HbA1c or A1c), a clinically established important biomarker for the diabetes monitoring, is a modified hemoglobin formed by non-enzymatic *in vivo* glycosylation (glycation) of hemoglobin in which glucose molecule chemically attaches to hemoglobin. The HbA1c value is a measure of the amount of glucose attached to N-terminal valine residue of the hemoglobin β-chains in red blood cells and provides retrospective values of blood glucose concentration over the preceding 60-90 days period, which is a direct function of the lifespan of erythrocytes. As Samuel Rahbar, one of the pioneers of HbA1c (or diabetic Hb as it was once known) measurements, states eloquently: “What was once thought to be artifacts of hemoglobin electrophoresis turned out to be the most reliable index of disease progression, and an indicator of long-term glycemic control in patients with diabetes, superior to oral glucose tolerance test” (2). HbA1c value is a superior functional metric of blood glucose concentration measurement as compared to the direct glucose concentration *c.a.* fasting plasma glucose (FPG) because (a) this is an averaged value and remains unaffected by the transient fluctuation due to life style changes (b) does not require over-night fasting, and (3) the secondary diabetic complication are known to correlate better with the HbA1c as compared to FPG. HbA1c is traditionally reported as a percentage of total hemoglobin; and the American Diabetes Association (ADA) has recently added HbA1c to screening for prediabetes ($5.7\% \leq HbA1c \leq 6.4\%$) and diabetes ($HbA1c \geq 6.5\%$)(3).

There are several non-photonic based methods, which are presently used in the clinical laboratories. These methods can be broadly clustered into two groups, one based on the exploiting the charge differences between HbA10 and HbA1c (ion-exchange chromatography, isoelectric focusing and

electrophoresis); and other on the basis of differing structural characteristics. The latter segment is comprised of affinity binding/chromatography and immunoassay. Immunoassays-based methods use poly- or monoclonal antibodies directed towards the glycated N-terminal group of the hemoglobin β-chain and present great promise in point-of-care diagnostics. However, immunoassays based approach shows substantive variance due to the precise specificities of the respective antibodies. Further, the methods and even protocols of HbA1c quantification vary from one clinical laboratory to other clinical laboratory and there is not a single method that is universally used globally. Clearly, there is an unmet need to develop accurate and objective methods of HbA1c detection and quantification preferably in a rapid and reagent-free manner.

Raman Spectroscopy- Based Approaches:

To address this unmet clinical need of reagent-free, real-time and automated detection and quantification, photonics based modalities offer a great choice(4). Our own laboratory has pioneered Raman Spectroscopy-based non-invasive blood glucose detection (5-8) but there are challenges associated with its successful clinical translation from the laboratory bench. Raman spectroscopy based approaches have also been recently proposed towards detection of alternative markers of glycemia *c.a.* HbA1c (9, 10) and glycated albumin(11). Raman spectroscopy is a vibrational spectroscopy technique based on the exchange of the energy between the molecule and an incident photon. When monochromatic light interacts with matter very few of the incident photons are scattered at slightly different wavelengths than the wavelength of the incident light. The inelastic scattering *i.e.* the energy exchange between incident photon and molecule is known as Raman effect and forms the basis of Raman spectroscopy(12). Importantly, the process of inelastic scattering is a reflection of vibrational transitions in the molecule and unique to the molecule under investigation. This enables Raman spectroscopy a highly specific probe providing unique chemical fingerprinting information. In other words two different molecules have two different Raman spectra; even an atomic substitution is known to affect the Raman spectrum of the parent molecule(13). The high specificity of the technique could be understood with the fact that same molecule in the different chemical environments(14) and even two polymorphic forms have different Raman signatures.

Surface-Enhanced Resonance Raman Spectroscopy: The first Raman spectroscopy based proof-of-concept detection of HbA1c was proposed by Ishikawa and coworkers(9). This work employed surface enhanced resonance Raman spectroscopy (SERRS) technique, a variant of Raman spectroscopy which combines two enhancements namely surface enhancement and resonance enhancement of the otherwise intrinsically weak spontaneous Raman signal (c.a. out of around 10^6 million incident photons, only 1 photon is a Raman-scattered photon). In the case of resonance Raman spectroscopy the wavelength of excitation is chosen such that it matches with one of the electronic transition(15). This increases the sensitivity (by as much as 10^6 times) and increases the selectively too because the vibrational modes coupled to that particular electronic transition are only enhanced. SERS on the other hand is a nano-optical and surface sensitive technique; and enhancement in the Raman signal originates due to the close proximity of the molecule under investigation to the rough metallic surfaces or metallic nanostructure(16). In the aforementioned work 532 nm laser was used as an excitation wavelength which is in resonance with the one of the absorption band of hemoglobin. For the surface enhancement silver nanoparticles (AgNPs) of 60 nm diameter has been used. In this proof-of-concept experiment both hemoglobin A (HbA) and HbA1c were incubated with AgNPs following which colloidal solutions were deposited on the slides via spin coating. It is observed that HbA1c exhibits a characteristic SERRS band at about 770 to 830 cm^{-1} (predominantly at 827 cm^{-1}). This band was

attributed to the glucosyl moiety attached to HbA1c and the origin of the band was rationalized by an *in-vitro* observation -a band at a similar wavenumber position appeared in HbA sample- when incubated with glucose. The SERRS spectra of HbA and HbA1c have been shown in the Figure 2. Interestingly, the author noticed different aggregation patterns of HbA and HbA1c when aggregated with silver nanoparticles and attributed to the presence of glucose moiety. This observation is important considering the separation of HbA1c from the mixture of HbA and HbA1c.

Drop Coating Deposition Raman Spectroscopy: The above SERRS based approach is novel and encouraging. However, as also noted by the authors, precise quantification of HbA1c using the above SERRS approach is difficult due to poor reproducibility and spurious background of SERRS spectrum(9). Our group has subsequently demonstrated an alternative route of HbA1c detection by exploiting preconcentration signal enhancement characteristics of drop coating deposition Raman (DCDR) spectroscopy (17) for the selective and sensitive detection of HbA1c(10). This approach is promising considering its reproducibility, reliability and no/minimal sample preparation requirement. Further, the experimental procedure is very simple and measurement can be easily performed using routine Raman instrumentation. In a typical DCDR experiment, a small volume of analyte solution (few microliters) is pipetted out and drop of the analyte is microdeposited/microprinted on a compatible substrate followed by air drying to make coffee ring pattern due to interplay between contact line pinning, solvent evaporation and capillary flow. The Raman spectrum is collected from the coffee ring pattern of the analyte. A typical photograph showing the drop coated and air-dried ring pattern of Hb and HbA1c mixture solution on the quartz substrate is illustrated in the Figure 3 (a).

The Raman spectra of Hb and HbA1c recorded from the coffee rings pattern of the sample using above approach show subtle but consistent differences as noted by the authors(10). This approach offers adequate specificity; and 100% classification accuracy (Fig 3(b)) is obtained when coupled with multivariate data analysis. Further DCDR modality is reported to provide high degree of prediction accuracy and precision; and limit of detection (LOD) for HbA1c is calculated to be 3.8 µM, which is 15 times smaller than the lowest absolute concentration of glycated hemoglobin found in the clinical samples.

Apart from the sensitivity and selectively which is clearly offered by DCDR of HbA1c detection and quantification, the reproducibly is very critical. Authors have performed Raman microscopy studies over a segment of the annular ring to investigate the reproducibility of measurements. In the Figure 4, 2D Raman maps over 44µm X 44 µm for both Hb and Hba1c are illustrated in the figure. Authors have noted high degree of consistency between point-to-point measurements and point-to-point deviation at any radial distance is less than 5%.

Closing remarks and Future Outlook

Raman spectroscopy presents a promising route towards reagent-free and real-time detection and quantification as demonstrated in the hemolysate model. Author envisions seeing Raman spectroscopy based automated high-throughput HbA1c sensors in clinical laboratories in future. However, there is a long way to go especially given the stringent clinical requirements both in terms of sensitivity and robustness of glycated hemoglobin measurement. It is noteworthy that the studies reviewed here on HbA1c detection have been performed as a proof-of-concept on hemolysate model, and the intermediate and immediate next step towards its realization in the clinical setting would be to demonstrate its validity on the actual hemolysate samples obtained from a wide range of known HbA1c concentrations. Hemolysate samples are obtained from lysing the erythrocytes and latter is derived from centrifugation of whole blood. Because of the fact that erythrocytes significantly outnumbers leukocytes and thrombocytes in the whole blood; and hemoglobin forms 95% of

erythrocytes dry mass, therefore, it is expected that non-hemoglobin components would not significantly confound the Raman signals from the glycated and nonglycated hemoglobin. After successful validation on the hemolysate samples, the final critical step will be to test its validity and efficacy on the actual blood samples encountered in the clinical setting. Raman measurement on this sample type requires lysing but centrifugation is not required. It is anticipated that the extent of confounding signals will be more in the case of blood lysate as compared to hemolysate sample especially due to serum proteins (e.g. albumin, globulin and transferrin *etc.*). The good news is that the physiological concentration of hemoglobin is relatively high as compared to the serum protein concentration and talking together the strong Raman signal from the hemoglobin (both nonglycated and glycated) one can predict that the selectivity, prediction accuracy and precision would not adversely affected. Further, since the Raman spectra of the possible blood constituents (contributing to the confounding signal) are well known in the literature and therefore can be mathematically subtracted after proper normalization. The DCDR experiment has been carried out at 785 nm excitation and research is underway to acquire Raman signal using 532 nm which is anticipated in principle to show clearer differences between Hb and HbA1c Raman spectra especially given the difference absorption profiles/absorbance at 532 nm. This will also reduce the relative confounding signal originating from non-resonant species. However, this would come at the cost of increased fluorescence, which may be corrected during spectral processing step. The development of this type of suitable optical tool would represent not only a key advance in clinical diagnostics but would also provide fresh impetus to the existing diagnostic arsenal of the pharmaceutical industry. Yet, significant efforts need to be made to advance these preliminary findings in order to display the method's clinical viability as a potential HbA1c sensing modality.

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Figures

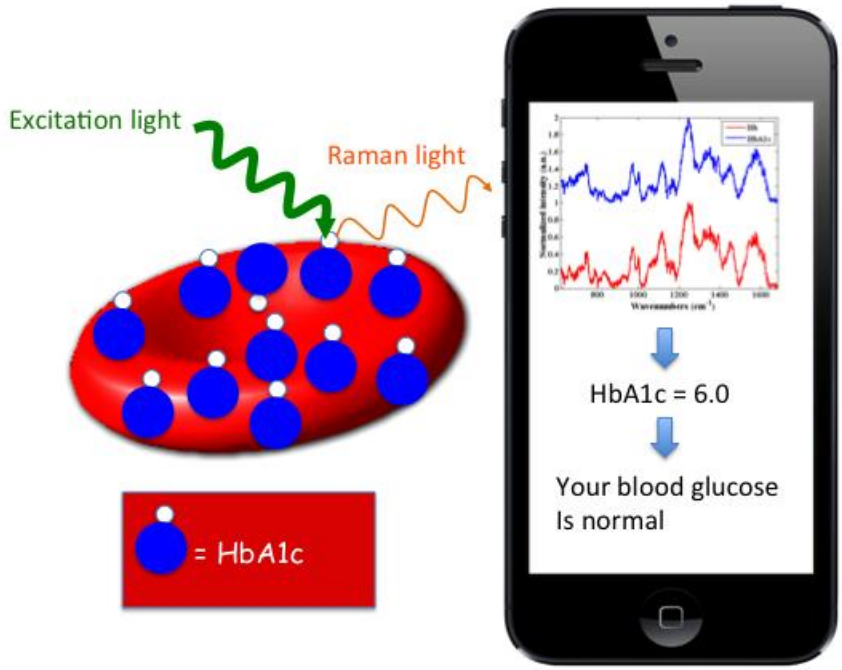

Figure 1. Graphical Abstract

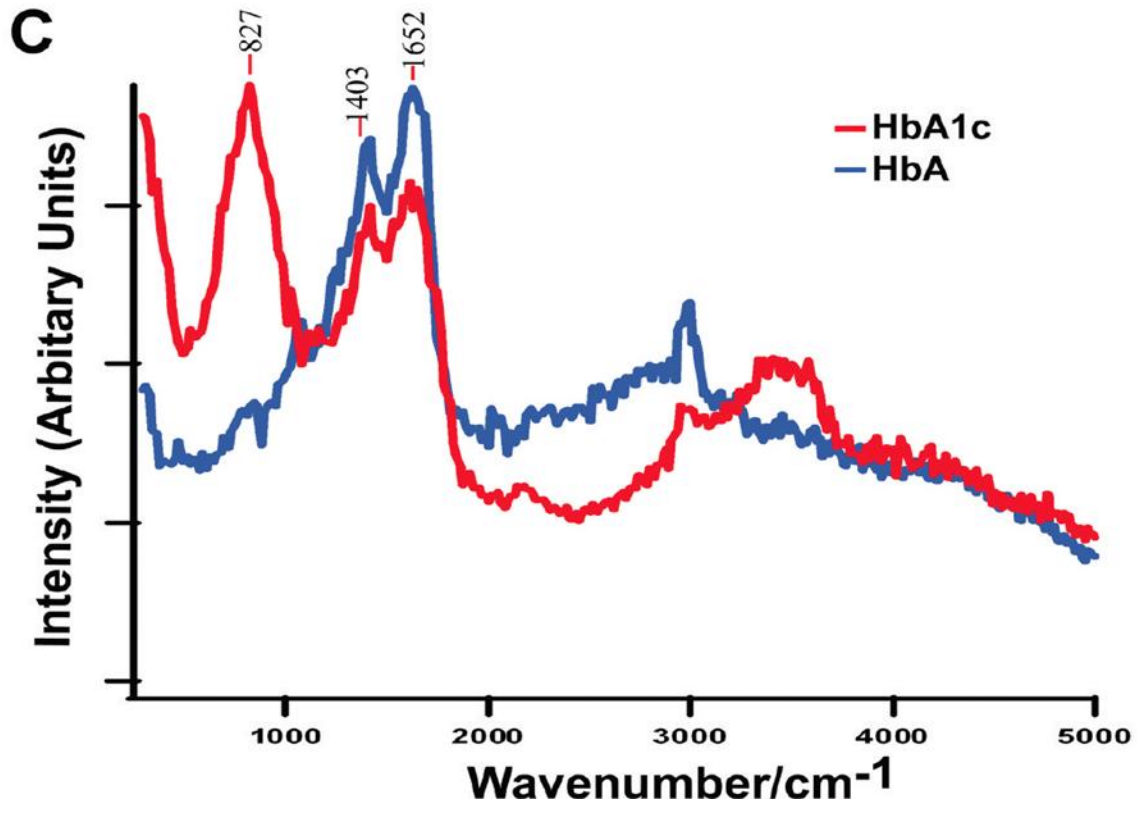

Figure 2. SERR spectra of HbA and HbA1c (Reproduced with permission from ref [9]).

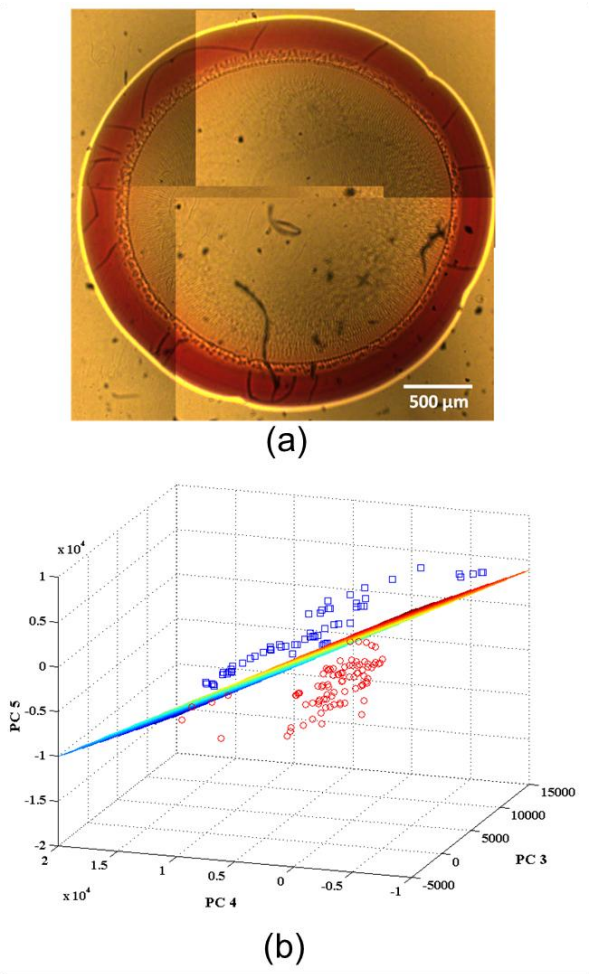

Figure 3. (a) Photograph illustrating the DCDR pattern. (b) Score plots corresponding to PCs 3, 4, and 5 for the spectral dataset acquired from the single-protein Hb and HbA1c drop-coated rings. Red circles: Hb and blue squares: HbA1c samples respectively (Reproduced with permission from ref. [10]).

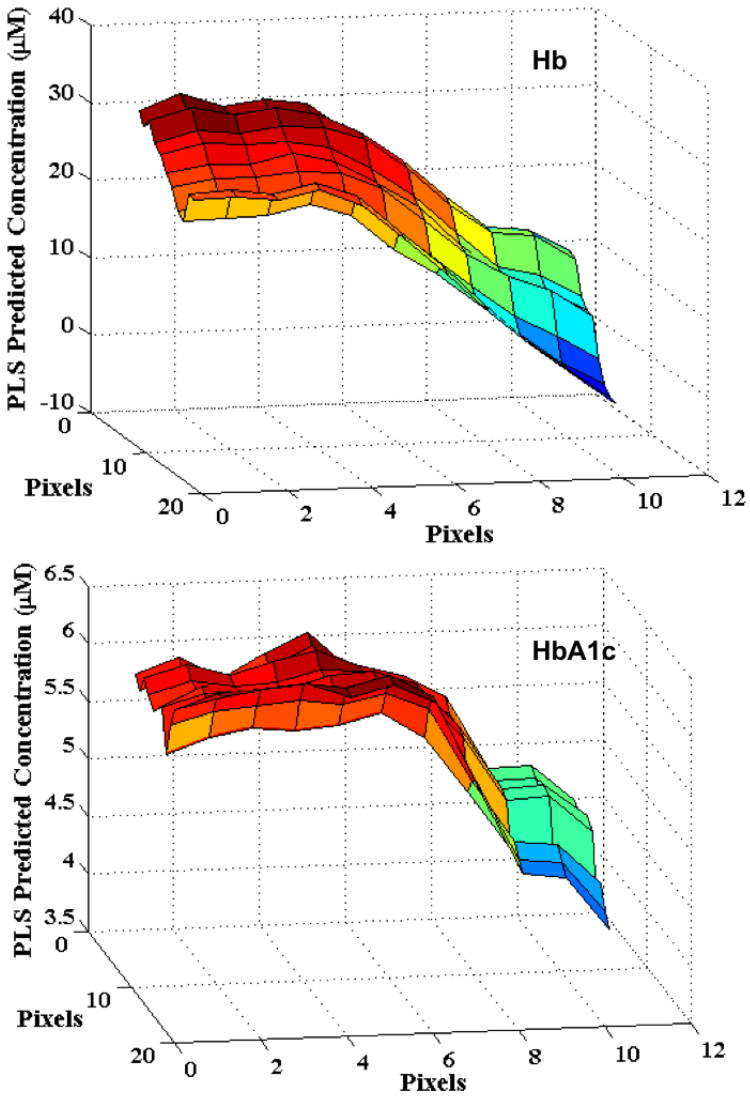

Figure 4. 2D spatial Raman mapping of drop-coated Hb (a) HbA1c (b). (Reproduced with permission from ref. [10]).