Detection of hemoglobin variants using surface enhanced Raman scattering

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Abstract: Hemoglobin variants are abnormal hemoglobin molecules and some of them elude current methods of detection, making proper diagnosis rather difficult. We investigate SERS as an alternative for hemoglobin variant detection.

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1.   Introduction

Hemoglobinopathies are a group of disorders that affect red blood cells with abnormal hemoglobin (Hb). They can be divided into expression abnormalities, called *Thalassemias*, and structural disorders. Structural disorders are the result of aberrant hemoglobin molecules, i.e., hemoglobin variants. There are 1118 hemoglobin variants identified [1]; they are due to a point mutation in a globin gene which produces a molecule of hemoglobin that has a single amino acid substitution. They receive the name from where they were discovered, although the first ones were given letters from the alphabet. The most severe symptomatic hemoglobinopathies are the homozygous ones; heterozygous ones, also refered to as traits, tend to be milder or asymptomatic, but some have moderate clinical implications, especially traits of Hb S, the hemoglobin variant associated with sickle cell disease.

   Many of these variants do not have relevant clinical implications, but some like Hb S and Hb C do. Additionally, variants without clinical manifestations could cause substantial overestimation of HbA1c, the important marker for diabetes mellitus [2]. Hb variants are commonly identified using electrophoretic or chromatographic techniques, such as isoelectric focusing, high performance liquid chromatography (HPLC) and cation exchange HPLC. Neither electrophoretic nor chromatographic have enough sensitivity to detect Hb at low concentrations [3] and, since the principle of separation is based on differences in affinity and charge distribution, variants that are neutral elude these methods [2]. Mass spectroscopy (MS) has been used for Hb variant diagnosis as well, but still suffers from some important drawbacks: Due to low mass resolution MS is unable to differentiate among variants with few mass differences and MS is primarily a qualitative technique, so quantification of important Hb fractions like HbA1c and HbA2 is not possible [2]. We propose surface enhanced Raman spectroscopy (SERS) as an alternative for the detection of Hb variants that has the potential to differentiate them in a quantitative fashion.

2.   Methods

2.1.   Hemolysate protocol

Deidentified samples of whole blood from patients affected with hemoglobinopathies were obtained from ARUP Laboratories (Salt Lake City, Utah) under approved IRB protocol. Hemolysate was obtained the same day as follows. 2 ml of whole blood were diluted in 6 ml of PBS at room temperature. The solution was centrifuged at 600 g for 10 min, and the supernatant was discarded. Steps 1 and 2 were repeated at least twice. Pellet was diluted in autoclaved distilled water at 4 °C to fill a 15 ml centrifuge tube. The dilution stood for 15 min on ice. Samples were centrifuged at 2000 g for 15 min. The top 5 to 6 ml were collected and transferred to a new tube, while the rest was discarded.

2.2. Silver nanoparticles substrates

Silver nanoparticles aggregates were formed onto glass surfaces by the Tollens reaction described elsewhere [4, 5]. Briefly, solutions of silver nitrate, glucose and potassium hydroxide were separately prepared and mixed. Clean borosilicate cover slips were immersed in the solution allowing the silver aggregates to form on the surface for 120 seconds. Chemicals were bought from Sigma Aldrich (St Louis, MO).

2.3. Confocal micro-spectroscopy

A drop of hemolysate was placed onto the silver nanoparticles substrate and a coverslip was place on top carefully to avoid air bubbles. The SERS spectra were recorded using a WITec alpha300 microscopy system (WITec, GmbH, Ulm, Germany). The beam from a 488 nm Ar+ laser was focused onto the sample using a 60X objective (NA 0.80) at an excitation power of 0.5 mW on the sample. A 488 nm 'notch filter' was used to reject the elastic scattering, and a 600 grooves/mm diffraction grating was used to disperse the collected emission and obtain SERS spectra in the 500 to 4500 cm-1 spectral range using a charge couple device that was thermoelectrically cooled (Andor Technology, Belfast, Ireland). To acquire the SERS spectra, 5 to 6 scans of 25 μm by 25 μm (20 by 20 pixels) per sample were done, collecting 400 spectra per scan. Not all of the acquired spectra were surfaced-enhanced. Thus, a selection algorithm was implemented based on the spectral flatness, defined as the ratio of the geometrical mean to the arithmetical mean of the spectrum (eq. 1) [6].

$$flatness = \frac{\sqrt[n]{\prod_n x_n}}{\frac{\sum_n x_n}{n}} \tag{1}$$

where n is the the number of points per spectrum. Using this criterion, only between 0.1 % and 2 % of the total number of spectra per sample were selected. Selected SERS spectra were normalized to the total integrated emission and averaged, such that each sample was represented by one spectrum. Spectra were analyzed using principal component analysis (PCA) and classification of various Hb variants was explored using support vector machine (SVM) classification [7]. The result from the SVM analysis is given in terms of the miss rate (eq. 2) which represents the percentage of samples incorrectly classified. Both PCA and SVM computation were done with the *The Unscrambler X* software [8].

$$Miss\ Rate = \frac{Number\ of\ miss\ assigned\ cases}{Total\ number\ of\ cases} \times 100 \tag{2}$$

3. Results and discussion

Hb variants are structurally different, thus, SERS is expected to be able to discriminate among various Hb variants. This could successively help the diagnosis of those variants that elude other techniques. To prove the ability of SERS to discriminate Hb variants, samples from patients affected with Hb E, Hb S and Hb C and traits FE and FS were analyzed. Example of the PCA results are shown in Figure 1A, where the principal components (PCs) PC1 and PC2 obtained from Hb C and Hb S are plotted. The results obtained from the SVM classification are shown in Table 1. For the case of hemoglobin E and traits FS and FE a visible separation is clear from Figure 1B but more samples are required for proper analysis and conclusion.

In addition to the measurements performed using the Tollens substrate, measurements on standard human hemoglobin using colloidal silver nanoparticles were done. The silver colloid was prepared according to published protocol [9]. In this case an aggregation agent is required to maintain a short distance between

Table 1. SVM results for hemoglobin variants. The 'miss rate' is defined in equation 2.

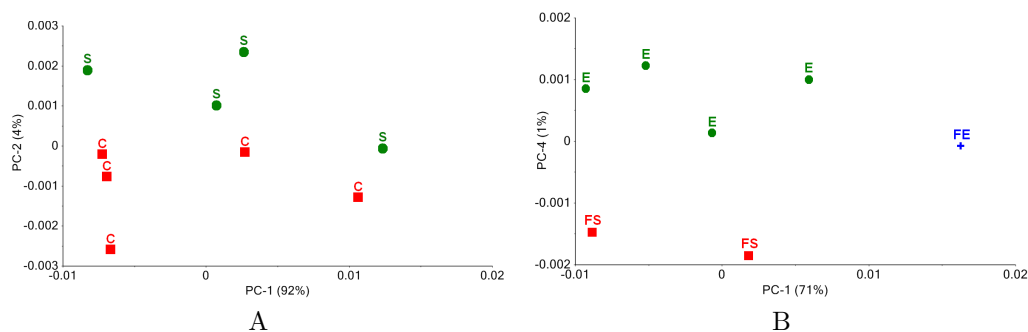| Data Set	No. False Cases	Total Cases	Miss Rate
Hb E, Hb C	1	9	11%
Hb E, Hb S	1	8	12.5%
Hb S, Hb C	1	9	11%
Hb E, Hb C, Hb S	3	13	23%

Fig. 1. Scores plot **A:** PC1 vs. PC2 for Hb variant S (green) and Hb variant C (red) and **B:** PC7 vs. PC3 for Hb E(green) and traits FS(red) and FE(blue).

the analyte and the metal nanoparticles. We used the agent reported by Han *et al.* [10]. It was evident that SERS was not observed using a silver colloid (data not shown), possibly because single silver nanoparticles are not as good for enhancement of the Hb Raman spectrum. Although low concentrations of hemoglobin were used a similar spectra to the bulk Hb Raman were obtained. Failed SERS measurements have been also reported in ref. [11]. These results suggest that SERS measurements from hemoglobin using colloidal silver nanoparticles in solution are not feasible.

We proposed SERS as an alternative for Hb Variant detection and we obtained excellent results. We were able to discriminate among Hb variants S, C and E and, traits FS and FE showed promising preliminary results although more samples are required for better analysis. Further studies are currently being done to evaluate SERS as a quantitative technique for Hb variant detection.

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