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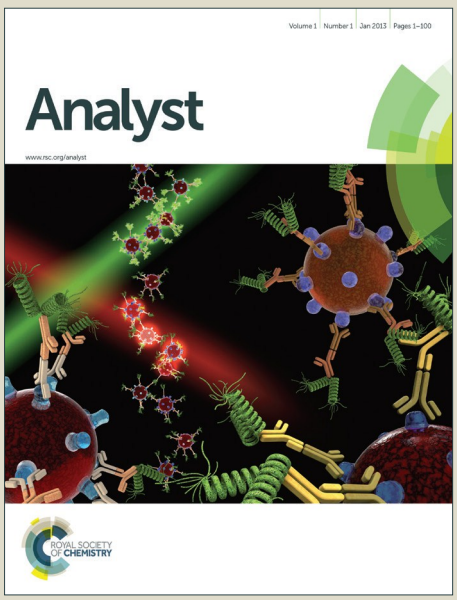

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Electrochemical redox signaling of hemoglobin in human whole blood and its relevance to *anemia* and *thalassemia* diagonsis†

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Abstract

A highly redox active human whole blood-carbon nanomaterial modified electrode, which showed a redox peak (E^o_{Blood} =-380 mV vs Ag/AgCl) similar to that of E^o_{Hb} of the hemoglobin (Hb; -370 mV vs Ag/AgCl) in the red blood cell, has been developed. Clinical relevance of it for direct electrochemical analysis of blood-hemoglobin content and its and *thalassemia* disease diagnosis were demonstrated.

Blood is a unique colloidal system that has no substitute and cannot be made or manufactured artificially. Electrochemical characteristic of blood refer oxidation/reduction (Redox, E^o_{Blood}) of the electro-active components, which are ubiquitous and are reflective of many important biological and clinical disorders. In general, following endogenous substances in the blood are responsible for the electrochemical characteristic; (i) Hemoglobin (Hb) in the red-blood cell (RBC), which contains redox active heme-Fe(III/II) site, (E^o_{Hb} = -370 ±20 mV vs Ag/AgCl in pH 7)[1-3] and (ii) reactive oxygen species (ROS)

such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl free radicals ($HO^\bullet$, $HO_2^\bullet$), singlet form of oxygen ($^1O_2$) and HO_2^- ions.[4] To use blood redox potential as a useful index in clinical diagnosis, since 1937, attempts have been made but it met with little success.[5-10] The conventional blood redox potential measurements are based on open-circuit potential (OCP) analysis of platinum or gold electrode along with silver-silver chloride or saturated calomel-reference electrode in a blood system. A positive shift observed in the OCP which is due to adsorption of blood-dissolved oxygen and ROS on the working electrode surface was referred as an electro-analytical parameter for the blood redox measurement. For instance, in 1967, Grosz and Farmer have measured the normal blood OCP value, -370 ±5 mV vs saturated calomel, after 20 h of exposure of a gold working electrode in a blood sample.[6] Similarly, in 1969, the same authors have extended their OCP studies for the thyroid disorder diagnosis, wherein the abrupt changes in the OCP values, -254 mV and -183 mV over the standard value (-370 mV) were noticed for the hypothyroid and hyperthyroid patients' blood samples.[7] It is noteworthy to mention that the metal oxides associated with Pt and Au change its composition seriously against time and hence it is difficult to get reproducible OCP data.[6,7] In 2014, Toh et al developed an electrochemical biosensor for Hemoglobin (Hb) in the RBC by immobilizing the lyophilized RBC and lyophilized blood on a unmodified glassy carbon electrode (GCE) surface with Nafion membrane.[10] Although cyclic voltammetric (CV) response of the above blood modified electrode showed electro-inactive behavior in nitrogen purged pH 3.5 phosphate buffer solution (PBS), an irreversible peak in oxygen saturated pH 3.5 PBS at a cathodic peak potential, E_{pc} -300 mV vs Ag/AgCl was noticed due to the electrochemical reduction of the dissolved oxygen to hydrogen peroxide.[10] In general, direct measurement of redox potential

of Hb-Fe(III)/Fe(II) which is buried in RBC is not easy and to the best of our knowledge it is never reported in the literature.

Hemoglobin (Hb), an iron-phorphyrin containing protein present in red blood cells, is a key system to transport oxygen to various parts of the body.[11] Normal levels of Hb in whole blood in different ages of human are; infant: 16-22 g dl^{-1}, adolescent: 12-14 g dl^{-1}, adult male: 14-18 g dl^{-1}, adult female: 12-16 g dl^{-1}. Abnormalities in the Hb contents are related to certain medical condition/disease like anaemia (Hb-deficiency), thalassemia (faulty/fragile function) and sickle cell anaemia (faulty shape) which are diagnosed with the blood hemoglobin clinical analysis. As per the world health organization, more than two million globally are anaemic and every year one million deaths are reported.[12,13] Similarly, more than 7% of the world population, (~ 250 million) are suffering from thalassemia and more than one million are estimated to acquire this in near future.[12,13] For the clinical diagnosis, several visual identification based method of analyses of hemoglobin, wherein, the colour of blood sample or derived hemoglobin is compared with the colour of a standard have been used.[14] But, these methods are not precise and are error prone.

In general, spectrophotometric method of analysis, wherein, blood hemoglobin is converted to cyanomethemoglobin by adding a mixture of potassium cyanide and ferricyanide and measuring the absorbance at 540 nm using calorimeter against a standard solution is an universally recommended procedure for the clinical Hb analysis.[15-17] The major disadvantage of this method are complication in conversion of all haemoglobin to cyanomethemoglobin and safety issues relating to the dead toxic cyanide reagent. Note that extra care needs to be taken while handling the cyanide reagent. To the best of our knowledge, direct electrochemical sensor has never been reported for Hb analysis so far. In this work, we report a new, simple and straight

forward electrochemical technique based on the redox peak of whole blood as a biomarker for analysis of the blood hemoglobin using a graphitized mesoporous carbon nanomaterial (GMC)-Nafion (Nf) chemically modified system, designated as GCE/GMC@Blood-Nf, in a deaerated neutral pH PBS. Electron-transfer behavior of the Hb-Fe(III)/Fe(II) redox site is tuned for the blood electrochemical analysis in this work.

In a typical preparation of GMC@Blood-Nf, GCE/GMC was first prepared by coating of 5μL of GMC (50 nm and 99.95% purity) -ethanol suspension (2 mg mL^{-1}) on a clean GCE surface and air dried at room temperature for 5±1 minutes. Then, a mixture of 8 μL of human whole blood (anticoagulant EDTA (electro-inactive compound) added and stored in refrigerator at 5±2^{o}C)) +2 μL of pH 7 PBS and 5 μL 1% Nf-ethanolic solution were successively drop casted on the GCE/GMC and air-dried for 20±2 and 5±1 minutes respectively (Scheme 1 and supporting information†) . Interestingly, CV of the blood-loaded electrode showed a well-defined redox peak, wherein, the magnitude of anodic/cathodic peak current ratio (i_{pa}/i_{pc}) is a unit, at an equilibrium potential, $E_{1/2}$ ($E_{pa}+E_{pc}/2$, where E_{pa} & E_{pc} are anodic and cathodic peak potentials)) = -380±2 mV vs Ag/AgCl and calculated peak-to-peak potential (_E_p = E_{pa}-E_{pc}) = 40±5 mV in a 10 minutes N_2 gas deaerated pH 7 PBS at a scan rate of 50 mV s^{-1} (Fig. 1A), unlike the previous report with irreversible response.[10] The above system has surface confined (plot of i_{pa} and i_{pc} versus scan rate is linear, Fig. 1B and 1C) and pH dependent electron-transfer characteristics (slope of plot E_{pa} vs pH is -30±5 mV s^{-1} ; Fig. 1D).

Blood stored in the refrigerator for 6 months showed activity as that of the fresh sample (Fig. S1†). Twenty continuous and three discontinuous (three days) CV responses of the blood patterned electrode showed a relative standard deviation (RSD) value 2.1% and 4.7% (based on base-line corrected anodic peak current, i_{pa} value; Fig. 1A, curve a) respectively indicating good

stability and reproducibility of the modified electrode. In order to understand the role of the graphitized mesoporous nanocarbon, different carbon materials like bare GCE, graphite nano powder (GNP), iron impurity containing-multi-walled carbon nanotube (MWCNT) and functionalized-MWCNT (f-MWCNT), purified-MWCNT (p-MWCNT) and single walled carbon nanotube (SWCNT) were subjected to blood-chemically modified electrode preparation as in the Fig. 1A and Fig. S2†. Additional experiments were also carried out in the absence of GMC, (Fig. 1A, curve c) and Nafion (Fig. S3†) and with commercial Hb (5 μL of 20 mg mL^{-1} Hb-pH 7 PBS drop coated; Fig. 1A curve b) as controls for the GCE/GMC@Blood-Nf in N_2 purged pH 7 PBS. Except with the GCE/GMC@Hb-Nf control, all other systems showed either poor or unstable redox peak responses only. This observation indicate denaturation or non-compatibility of the blood-Hb site with those carbon nano-materials. It is likely that the porous sites of graphitized mesoporous interact selectively with the RBCs and enhance the stable and facile electron-transfer behaviors (Scheme 1). To understand the interaction between the GMC and blood-Hb, GMC@Blood-Hb-Fe(II)-Nf system was subjected to Transmission electron-microscope (TEM), Raman, UV-vis and FTIR spectroscopic characterizations (Scheme 1E and F & Fig. 2B-D). Scheme 1E and F are

TEM images of GMC and GMC@Blood samples. Light colored aggolomorated hallow spheres of GMC, very light spots and dark spots spread over hollow spheres with GMC@Blood samples were noticed. These observations suggest strong adsorption of the blood on the porous structure of GMC. Due to complex structure of blood it is difficult to predict the chemical structure and its intereaction with GMC from the TEM images. A gold screen-printed modified electrode was used as an underlying system for the Raman spectroscopic measurements. Fig. 2A is a comparative Raman spectroscopic responses of GMC@Blood and unmodified GMC systems

showing D and G bands at 1380 and 1560 cm^{-1} corresponding to the graphitic lattice vibration mode (hexagonal sp^2 carbons) and disordered graphitic structure (sp^3 carbons) respectively.[18,19] The ratio between D band and G bands, I_D/I_G for GMC@Blood (0.213) is lesser than that of the value for the unmodified GMC (0.369). A π-π interaction between the porphyrin units of Hb and graphitic units of GMC is the possible reason for the decrement.[20] UV-vis spectrum of a pH 7 PBS diluted blood without and with addition of 1% Nafion showed qualitatively similar absorption peak at $\lambda = 406\pm2$ nm (Fig. 2C), which is due to Soret absorption band of Heme group in the blood-Hb.[21] However, about 20% reduction in the quantitative absorption intensity was noticed with the diluted-blood sample after addition of the Nafion solution. This observation suggests partial denaturation of the blood-heme protein possibly due to the Nafion's sulphonic acid groups (Fig. 2B). For FTIR characterization, GMC@Blood-Nf film pealed-off from the chemically modified electrode using a doctor's needle (1mm × 4 cm) mixed with KBr as a pellet was subjected to the analysis. Amongst two stretching frequencies of Hb (RBC)[22] carbonyl group (1631 cm^{-1}; amide-I) and N-H bending/C-N group (1535 cm-1; amide-II), the amide-I carbonyl functional group's vibration frequency of GMC@Blood-Hb is shifted to 1658 cm^{-1} indicating specific interaction of GMC with the hemoglobin in the RBC (Fig. 2C). This new GCE/GMC@Blood-Nf patterned electrode is useful for some critical clinical applications.

As a proof of concept, following analyses were performed with normal and anaemia patients' blood samples as GCE/GMC@Blood-Nf patterned electrodes; (i) Measurement of whole blood redox potential value (E^o_{blood}): $E_{1/2}$ value measured for the GCE/GMC@Blood-Nf patterned electrode using $E_{pa}+E_{pc}/2$ is considered as a E^o value of the whole blood system (E^o_{blood}). Based on the Fig. 1A result, E^o_{blood} value of the normal blood sample is -380 mV vs Ag/AgCl. Note that the E^o_{blood} value obtained from the CV result (Fig. 1A) closely matching with

the value, -370 mV vs saturated calomel measured for a normal blood sample by the OCP using gold working electrode after 24 h exposure time.[6] The Blood-Hb-Fe(III)/Fe(II) redox site is responsible for the redox value. (ii) Measure of blood-hemoglobin content: charge under the CV redox peaks (*Q*, either anodic or cathodic) of GMC@Blood-Nf patterned electrode can be taken as a measure of blood-Hb content. A set of experiments were conducted with different hemoglobin content blood samples (#1—#5), 8, 9.1, 11.2, 13.5 and 15 g dl^{-1} as in (Fig. 3A). The blood samples used in this study were collected with the help of VIT University's Health Care Centre diagnostic laboratory after getting consent from Institutional Ethical Clearance Committee for Human Studies of VIT University, Vellore, India [Ref. No. VIT/IECH/020/Jan.24.2015]. Fig. 3A is CV response of various [Hb] containing blood samples modified electrodes in N_2 purged pH 7 PBS. The Hb content in test samples was discretely measured by standard clinical procedure at VIT University's health care centre. Fig. 3B is a plot of *Q* (anodic charge from CV result) *vs* hemoglobin content (clinical result) showing a linear line response. In Fig. 3A, the redox potential shows deviations within the limit (5-10mV) for the [Hb]= 8-15 gm dL^{-1} samples, accompanied with the significant difference in the peak charge and peak current. This observation is an evidence for the possibility of development of simple Hb sensor using the present technique. (iii) Diagnosis of hemoglobin related diseases based on the stability of the redox peak with respect to time: A thalassemia diseased [23] blood sample from a 7 month old infant admitted in Government Hospital, Chennai was subjected to electrochemical studies along with normal blood sample as in Fig. 3C and Fig. 3D. Triplicate of the blood samples converted to GCE/GMC@blood-Nf chemically modified electrodes and were analysed by the CV at three different days in N_2 purged pH 7 PBS solution at v = 50 mV s^{-1}. Unlike a healthy human blood patterned electrode which showed stable CV redox peak responses

measured at 3 days (Fig. 3C; slight current alteration observed in the cathodic peak side is due to fraction of dissolved oxygen in the working cell), continuous reduction in the peak current response was noticed with the thalassemia diseased blood sample (Fig. 3D). The measured redox potential (E^o) of thalassemia diseased blood (Fig. 3D) on day 1 is -310 mV, day 2 is -329 mV, day 3 is -313.5 mV vs Ag/AgCl, whereas, in case of normal blood sample, redox potential value for all three consecutive days are constant -380±20 mV. Since the infant was undergoing blood transfusion (an injection of a volume of blood, previously taken from a healthy person, into a patient) the increased level of hemoglobin content observed here could be due to the transfusion therapy. Beside, on storing the blood in refrigerator (3 days) and studying the redox behavior of the sample, we found that the thalassemia blood lost about 50% stability unlike the normal blood which was stable for all three days with nearly identical E^o value. It is likely that under the electrified condition the Hb in the diseased blood undergoes denaturation and hence decrease in the CV responses. Note that anemia is a condition, whereas thalassemia is a disease with faulty Hb. Thus, changes in the E^o is unique with thalassemia and are acceptable.

In conclusion, we report a novel protocol for patterning of human whole blood as carbon nanomaterial@Blood-Nafion thin film electrode that showed a well-defined redox peak corresponding to the E^o of hemoglobin-Fe(III)/Fe(II) species buried in the RBC. This newly developed human blood patterned electrode shows appreciable linearity and clear proof of concept about the increment of redox peak charge and current against increase in the hemoglobin concentration. The blood redox potential measurement is highly useful for real-time monitoring of hemoglobin content and diagnosis of certain faulty hemoglobin based diseases. In future, we propose to extend this work with screen-printed electrode as a base to modify the blood system and for clinical and biomedical applications.

Acknowledgements

The authors acknowledge the Department of Science and Technology -Technology System Development Scheme, Dr. Paul Ramesh, Cardiothoracic surgeon, Apollo hospital, Chennai, Dr Narmada, Pediatrician, Nalam hospital, Vellore, India and Professor Radhasaraswathi, Biomolecules & Genetics, VIT University for their valuable support to this work and the Institutional Ethical Clearance Committee for Human studies (Ref. No. VIT/IECH/020/Jan.24.2015).

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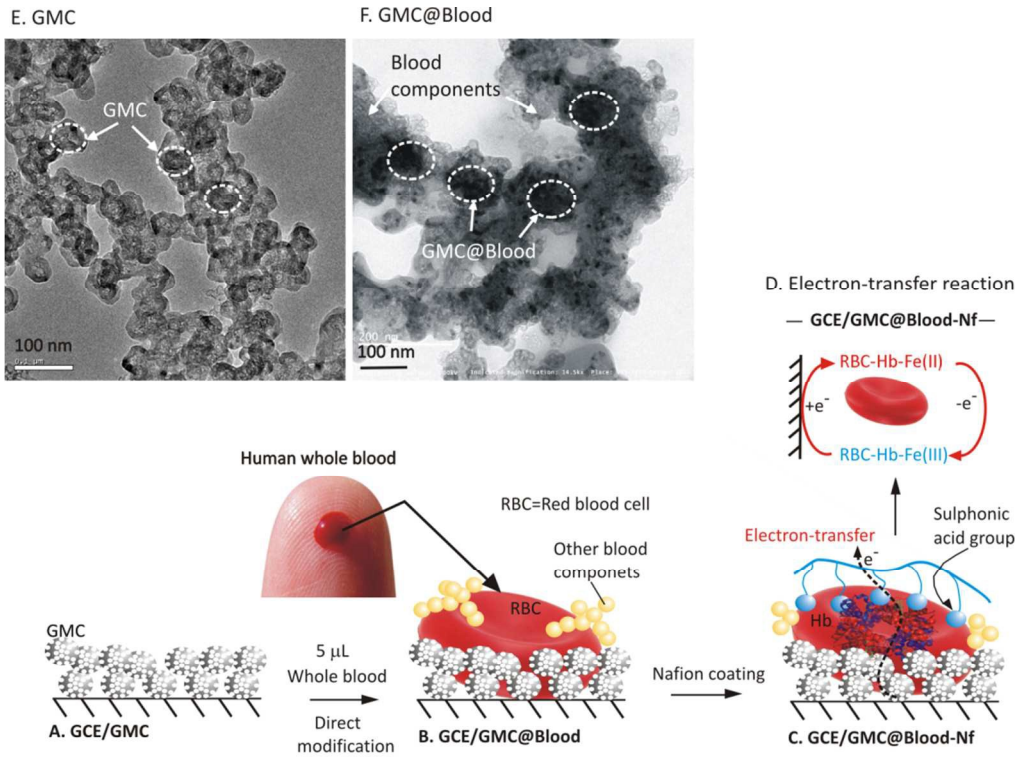
Figure captions

Figure 1 (A) Continuous CV responses of GCE/GMC@blood-Nf (a), GMC@Hb-Nf (commercial hemoglobin (Hb)) (b) and GCE/blood-Nf (c), (B) scan rate effect of GCE/GMC@blood-Nf in N_2 purged pH 7 phosphate buffer solution, (C) Nature of electron-transfer behaviour: Plot of anodic peak current (i_{pa}) and cathodic peak current (i_{pc}) versus scan rate for the CV response of GCE/GMC@Blood-Nf in nitrogen purged pH 7 phosphate buffer solution. (D) Effect of solution pH (N_2 purged) on the CV of GCE/GMC@blood-Nf

Figure 2 (A) Raman spectroscopy, (B) UV-vis and (C) FTIR characterization of various blood modified systems.

Figure 3 CV responses of (A) different [Hb] content blood samples modified GCE/GMC-Blood-Nf (triplicate) and its respective *Q* vs [Hb] plot. (B). Three discontinuous days CV responses of normal- (C) and (D) faulty-RBC containing blood (Thalassemia diseased) modified electrodes in N_2 purged pH 7 PBS at v= 50 mV s^{-1}.

Scheme 1



Scheme 1. (A-C) Cartoon for the preparation of blood patterned electrode (GCE/GMC@Blood-Nf) using graphitized mesoporous carbon (GMC) and Nafion (Nf), (D) proposed electron-transfer behavior and (E & F) TEM images of GMC (E) and GMC@Blood (F).

Figure 1

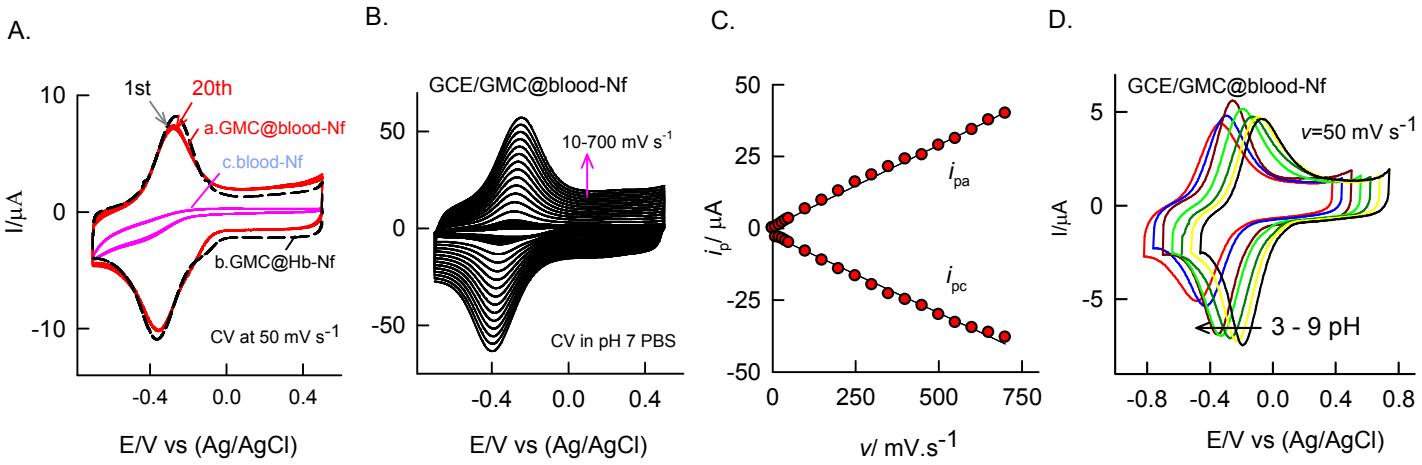


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Figure 2

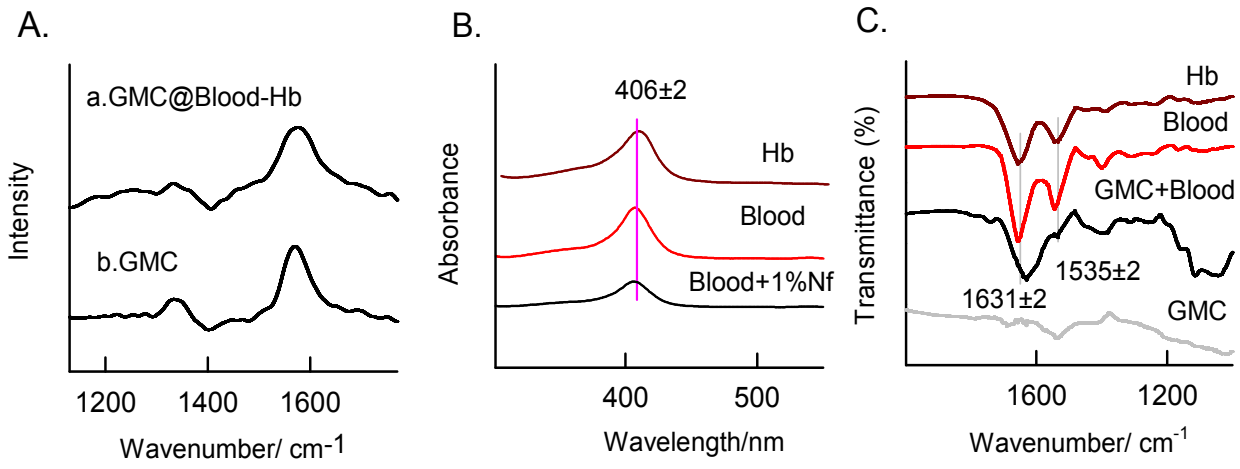
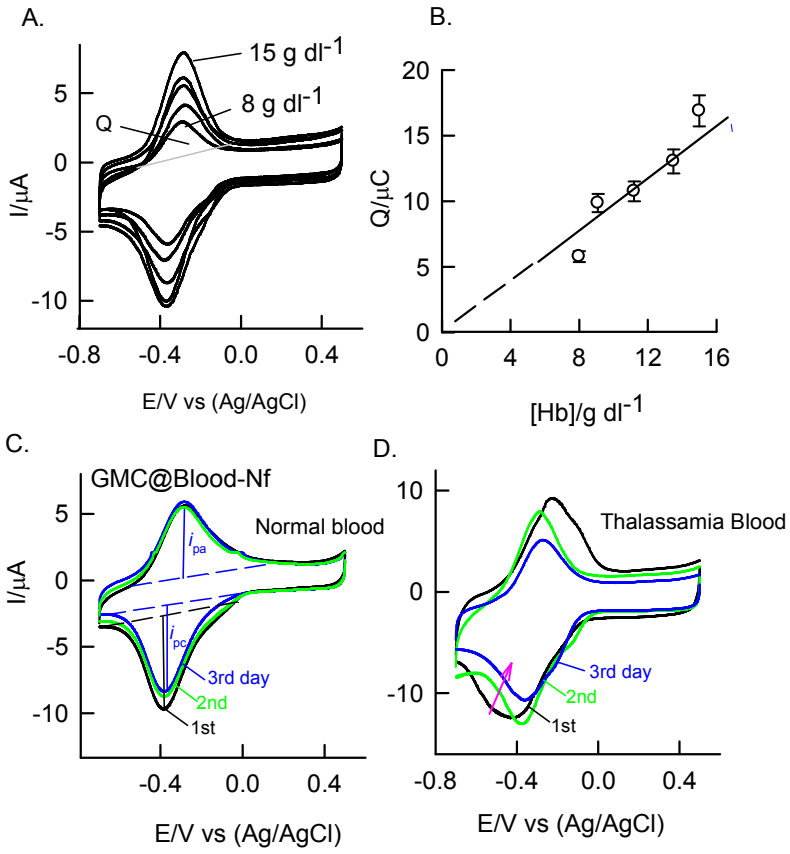


Figure 2. (A) Raman spectroscopy, (B)UV-vis and (C) FTIR characterization of various blood modified systems.

Figure 3



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