Autofluorescence characterization of advanced glycation end products of hemoglobin

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Abstract

This article describes the analysis of autofluorescence of advanced glycation end products of hemoglobin (Hb-AGE). Formed as a result of slow, spontaneous and non-enzymatic glycation reactions, Hb-AGE possesses a characteristic autofluorescence at 308/345 nm ($\lambda_{\text{ex}}/\lambda_{\text{em}}$). Even in the presence of heme as a quenching molecule, the surface presence of the glycated adduct gave rise to autofluorescence with the quantum yield of 0.19. The specificity of monoclonal antibody developed against common AGE structure with Hb-AGE was demonstrated using reduction in fluorescence polarization value due to increased molecular volume while binding. The formation of fluorescent adduct in hemoglobin in the advanced stage of glycation and the non-fluorescent HbA1c will be of major use in distinguishing and to know the past status of diabetes mellitus. While autofluorescence correlated highly with HbA1c value under in vivo condition ($r = 0.85$), it was moderate in the clinical samples ($r = 0.55$). The results suggest a non-linear relation between glycemia and glycation, indicating the application of Hb-AGE as a measure of susceptibility to glycation rather than glycation itself.

Keywords: Autofluorescence; Diabetes mellitus; Glycation; Hemoglobin; Polarization

1. Introduction

Diabetes mellitus is a metabolic disorder that is characterized by an elevation of fasting blood plasma glucose level caused by a relative or absolute deficiency in the hormone, insulin. Diabetes results in the poor maintenance of normal plasma glucose concentration that ranges between 70 and 100 mg/dL\textsuperscript{1}, and the elevated blood glucose concentration may reach upward of 500 mg/dL\textsuperscript{1} [1]. This state is also linked to other common health problems, such as obesity, polycystic ovarian disease, hyperlipidemia, hypertension and atherosclerosis [2]. The Diabetes Control and Complications Trial [3] was a landmark study and the flagship for a number of studies that established the value of intensive control of blood glucose to prevent the retinal, renal and neuropathic complications of diabetes. However, plasma glucose level is not a suitable parameter for monitoring glycemic control in diabetic individuals since it fluctuates very widely. Alternatively, measurement of glycated hemoglobin is widely used for routine monitoring of long-term glycemic status in patients with diabetes mellitus. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [4] gave the approved reference method for the measurement of HbA1c, in the human blood. There has been a suggestion to the effect that the measurement of hemoglobin advanced glycation end product (Hb-AGE) may provide a better index of diabetes [5]. As is well known, glucose reacts non-enzymatically with the protein amino groups to initiate a post-translational modification process known as glycation (Fig. 1). Advancement of glycation results in browning or Maillard reaction which comprises of two stages, early and advanced; proteins react with glucose to form a stable Amadori product (HbA1, in case of Hb) through Schiff base adducts, which is con-
verted upon further incubation, to advanced glycation end products (AGEs), some of which exhibit autofluorescence [6].

The autofluorescence nature of the AGEs may be a significant technique for both qualitative and quantitative identification during pathological complications. An age-related increase in fluorescence and brown pigment formation was observed in vivo with long-lived proteins such as lens crystallin, collagen and myelin [7]. Collagen AGE-specific fluorescence determination was performed by measuring emission at 440 nm upon excitation at 370 nm and the intensity known to increase with advances in diabetes [8]. Pentosidine, an intermediate adduct, is the major fluorophore formed during nonenzymatic browning of ribonuclease and lysozyme by glucose. Pentosidine was also observed to accumulate in tissue proteins with age and in diabetes [9]. The emission maximum for pentosidine has been observed at 385 nm when excited at 335 nm. Since the formation of fluorescent adduct in long-lived proteins due to aging cannot be distinguished from that due to disorder/disease, the entire focus was shifted towards the short-lived proteins like plasma albumin and hemoglobin. Also as spectroscopy based fluorescence determination has much higher sensitivity than the absorbance spectroscopy [10], fluorescence would be a better option for quick and accurate monitoring of AGEs.

Faucon et. al. [11] suggested an intrinsic fluorescence polarization study to analyze the changes in fluorescence polarization (P-value) during the self-association of melitin. As the measured change in the fluorescence polarization depends on the molecular size of the analyte [12], change in P-value could be of immense use to trace out the specificity of antigen and antibody binding especially Hb-AGE and MAb in our study. Our earlier report demonstrated the use of isoelectric focusing [13] in analyzing the variation in glycated hemoglobin fractions, HbA1c and Hb-AGE. The detailed exploration of autofluorescence nature, relative quantum yield and the effects of various parameters are dealt here. The specificity of the monoclonal antibodies towards Hb-AGE was studied by fluorescence polarization.

2. Experimental

2.1. In vitro synthesis of Hb-AGE

Bovine hemoglobin (Sigma Co., St. Louis, USA), d(+)-glucose, and 0.4 mol sodium phosphate buffer (pH 7.4) [14] were used for the in vitro experiment. Two different hemoglobin samples were prepared one with 5 mmol glucose and another with 20 mmol glucose to mimic the normoglycemic and hyperglycemic conditions, respectively, while the control solution lacked glucose. Hemoglobin at a concentration of 100 mg mL⁻¹ [13] was dissolved in 0.4 mol sodium phosphate buffer (pH 7.4) and filter-sterilized by using 0.22 (µm Millipore filter. The samples were incubated in rotary shaking water bath at 37 °C for 120 days under sterile and dark conditions. Intermittent sampling was done to check for microbial contamination. The samples taken at the regular intervals were gel filtered in column packed with Sephadex G-25, to remove the unbound glucose and used for fluorescence analysis.

2.2. In vivo study

Approval was obtained from the Institute Review Board of the All India Institute of Medical Sciences, New Delhi, India, for animal experiments. Healthy male Wistar rats weighing 200-250 g and 6 months old, procured from the experimental animal research facility, were used as the experimental animal model. The rats were fed with ad libitum pelletite (Hindustan Lever Ltd., Mumbai, India) and water. All approved animal care protocols were followed [15]. Diabetes was induced in rats with single intraperitoneal injection of Streptozotocin (60mgkg⁻¹ body weight) dissolved in 10 mol citrate buffer (pH 4.5). The control rats received an equivalent volume of citrate buffer in the same manner. The blood glucose concentration of control rats remained less than 150 mgdL⁻¹ throughout the experimental time (13 weeks), whereas the glucose level of diabetes induced rats rose up to 350-550 mg dL⁻¹. The blood samples (0.5 mL) were collected from the orbital veins of diabetic
rats at an interval of 7 days and centrifuged at 4000 g to remove the plasma and buffycoat. The sample was hemolyzed and centrifuged to remove the red blood cells ghosts. The trend of autofluorescence values formed over the period of 13 weeks was studied, as the half-life of rat RBCs is only 75 days.

2.3 Clinical study

Blood samples were obtained from patients above 30 years of age with type 2 diabetes (category I) from the Internal Medicine ambulatory care department and diabetes research clinic. The patients were on various combinations of antihyperglycemic treatment and free from severe end-organ damage and acute systemic illnesses. Informed consent was obtained from all the subjects. One milliliter of venous blood was obtained from human subjects after an overnight fast, and the sample was processed in a similar manner as in rats. The total glycated hemoglobin was separated from the hemolysate by phenylboronate affinity chromatography [13] followed by the cation exchange chromatographic separation to separate HbA1c and Hb-AGE. Simultaneously, fluorescence analysis was also carried out.

2.4 Fluorescence analysis

The LS 50B Luminescence spectrometer (Perkin-Elmer) controlled from a personal computer using the Perkin-Elmer Fluorescence Data Manager (FLDM) was used for measuring the fluorescent signal. Fluorescence intensity standards (Perkin-Elmer) were used to calibrate and monitor the performance of the instrument. Fluorescence values of the glycated hemoglobin samples were measured at a protein concentration of 1.0 mg/mL. This dilution was made using 0.4 mol sodium phosphate buffer (pH 7.4). The prescan was carried out using the full range of monochromator limits from 200 to 900 nm. Based on the results obtained from the prescan, the emission scan was carried and all the parameters were adjusted to obtain the optimum intensity. The excitation and emission slit widths for both prescan and emission scan were initially changed with the values, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 nm. Based on the highest intensity values, optimum slit width values were screened and used for further analysis.

2.5 Removing the Rayleigh and Raman scattering

To remove the Rayleigh and Raman scattering effects, an emission spectrum of blank solution was recorded and it was subtracted from the emission spectrum of sample. Since Rayleigh scattering is largely a random effect, the scatter peaks in the two spectra may not be identical. Hence, the spectra were normalized before performing the background subtraction. When a spectrum is normalized, each data point is multiplied by a normalization factor. After normalization, normalized blank spectrum was subtracted from the normalized sample spectrum. In the resultant spectrum, major peaks were identified by changing the threshold values.

2.6 Effect of various parameters on fluorescence

To study the effect of different concentrations of buffer, in addition to 400 mmol, 5 and 200 mmol sodium phosphate buffer (pH 7.4) were used to analyze the autofluorescence. Similarly, Kreb’s Ringer phosphate (pH 7.4) buffer was also used to compare the effect of different buffer on fluorescence. For the purified fraction of Hb-AGE, effect of varying pH on the fluorescence intensity and quantum yield was studied by using different buffers to cover the wide range of pH.

2.7 Fluorescence quantum yield

The relative quantum yield (QY) of intrinsic fluorescence nature of Hb-AGE was measured with quinine sulfate as the standard, using the formula

\[
\Phi(\epsilon) = \frac{\Phi(u) q(s) A(s)}{F(u) q(\epsilon) A(\epsilon)}
\]

where, \(F(\epsilon)\) is the relative fluorescence determined by integrating the area beneath the corrected fluorescence spectrum, \(q\) is the relative photon output of the source at the excitation wavelength (taken directly from the curve), and \(A\) the absorbance. \(\Phi(u)\) is standard, using the formula

\[
(\text{mol Quinine sulfate in } 1.0\text{N sulfuric acid.})
\]

2.8 Fluorescence polarization

The fluorescence polarization (P-value) was studied using in-built polarization accessory in the spectrofluorimeter, by adding the MAb (donated by Prof. R. Bucala, Picower Institute, New York) to the purified fraction of Hb-AGE. Polarization value was calculated using the equation,

\[
P = \frac{I_{vv} - I_{vh}}{I_{vv} + I_{vh}},
\]

where, \(I_{vv}\) = emission intensity of vertically polarized light parallel to the plane of excitation and \(I_{vh}\) = emission intensity of horizontally polarized light perpendicular to the plane of excitation. The P-values were directly obtained from the FLDM software. Also, their corresponding fluorescence intensity values were recorded to study the quenching effect of MAb binding with Hb-AGE.

3 Results and discussion

The fluorescence analysis of glycated hemoglobin fractions showed the emission peak at 345 nm when excited at 308 nm. This was found to be different from its unglycated counterpart. Native hemoglobin exhibited weak fluorescence due to the presence of (3-37 tryptophan, with emission peak at 330 nm when excited maximally at 280 nm. Interestingly, the emission peak was found to shift to 345 nm after a period of 60 days of incubation. Simultaneous analysis showed that...
HbA1c was formed after 30 days of incubation, while the autofluorescence was observed only after Maillard reaction. The prescans (Fig. 2a and b) were analyzed and the peaks were identified to differentiate between HbA1c and Hb-AGE. The characteristic emission peak was observed for Hb-AGE at 345 nm having the excitation at 308 nm whereas HbA1c failed to exhibit this autofluorescence nature. The range of slit widths from 2.5 to 15 nm is optimized for fluorescence scattering.

![Diagram](image-url)

Fig. 2. (a) Prescan spectra of HbA1c (a: excitation scan; b: emission scan); (b) prescan spectra of Hb-AGE (a: excitation scan; b: emission scan).
analysis. As the reduced slit width produced very low intensity, increased slit widths resulted in the broadening of emission peaks. The combination of 2.5 nm slit width for excitation and 5.0 nm slit width for emission yielded the maximum intensity and narrowed peak. Based on this, both excitation and emission scans were carried out. Fig. 3 shows the contour plot of excitation and emission wavelengths for Hb-AGE. The maxima were clustered around 308 and 345 nm for excitation and emission, respectively. The fluorescence obtained for Hb-AGE is found to be of stokes effect (red shift) since the wavelength of the emission peak is 15 nm more than that of hemoglobin. The lowering of this energy level can be attributed to the structural modification. As the glycation process leads to both HbA1c and Hb-AGE, having different isoelectric and autofluorescence phenomena, the chances of variation in their glycated adduct cannot be ruled out.

Absolute measurement of quantum yields and determination of the absolute energy distribution of a fluorescence spectrum are difficult to make experimentally, and are infrequently done in biochemistry. In practice, the quantum yields are usually determined by comparison of the fluorescence emission of the species of interest with a standard having a known quantum yield. Because the quantum yield is proportional to the area under a fluorescence band in the spectrum, relative quantum yields can be obtained by comparing the peak areas (or heights) under identical excitation conditions. The mean QY of Hb-AGE isolated from the in vitro sample was calculated to be 0.194±0.005. The studies on the effect of various pH shows that the fluorescence intensity was maximum at the pH 9.0 with the corresponding highest QY of 0.211. This supports the earlier finding of increased QY of intrinsic fluorescence of hemoglobin due to tryptophan in the alkaline condition [16]. Between the two buffers tested, no significant variation was observed in the fluorescence intensity values. Similarly, no remarkable variation was observed on the fluorescence intensity due to the variation of buffer concentration from 5 to 400 mmol.

The increase in fluorescence polarization (P-value) was noticed during the addition of MAb to the purified solution of Hb-AGE with corresponding reduction in its fluorescence intensity (Fig. 4). The increased P-value could be attributed to the increased molecular volume and decreased Brownian motion due to Hb-AGE and MAb binding. Similarly, quenching of fluorescence intensity by MAb binding supported the antigenic property of the fluorescent moiety formed due to glycation of Hb.

The time dependent formation of Hb-AGE under in vitro condition showed a characteristic sigmoid profile (Fig. 5). The curves obtained for the glucose concentrations shifted proportionately, from 5 to 50 mmol. These curves demonstrate a non-linear response with an abrupt but small rise in advanced glycation at 7 weeks. Subsequently at 11 weeks of incubation the increase is substantial, which finally reaches...
threshold at 17 weeks. The threshold intensity varies linearly with the concentration of glucose. Concomitantly, change in color was noticed from red to dark brown after 2 months of incubation, which perhaps indicated the advanced stage of glycation (Maillard reaction). At different glucose concentrations, the shape of the curves remains the same, indicating that saturation of hemoglobin was not achieved in this in vitro model.

Similarly, the in vivo experiments carried out with the rats showed the fluorescence behavior at 308/345 nm, proving the formation of fluorescence adduct, after 2 months of hyperglycemic condition. The values of Hb-AGE demonstrated better correlation (r = 0.85) with the already established marker HbA1c (Fig. 6). Earlier report on the rat experiments by Odetti et al. [17] demonstrated the use of glycated collagen-linked fluorescence (370/440 nm) and oxidation-related fluorescence (356/460 nm) as a marker for diabetes mellitus. They have reported a very good exponential correlation (r = 0.993). Inspite of many earlier reports on correlation between collagen post-translational modifications and presence of diabetic complications [18-20], these suffered the drawbacks of tedious extraction procedures in addition to the accumulation of collagen-AGEs due to aging. A variety of experimental studies suggest that AGES play several critical roles in the structural and functional alterations which result from their accumulation in tissues and on serum proteins during normal aging and at an accelerated rate in diabetes [21].

Clinical samples analyzed for their Hb-AGE content by fluorescence correlated well with the established standard method of HbA1c measurement in diabetics (r = 0.55) (Fig. 7) while the samples from normal subjects failed to show correlation (r = 0.02). As compared to the in vivo sample analysis, the correlation coefficient between Hb-AGE and HbA1c in clinical samples was lower. This was probably due to the fact that the group of clinical sub-
jects in this study was under a treatment regimen and hence their level of HbA1c was lower than 10%. A non-linear relationship between glycemia and glycation is established from the data shown in Figs. 6 and 7. There is an abrupt formation of Hb-AGE above 8% of HbA1c in humans and 11% of HbA1c in rats. Below this level of the glycemia index there is very little Hb-AGE and absolutely no increase in Hb-AGE with HbA1c. Partly, this may be attributed to the variations in the lifetime of the erythrocyte and because formation of Hb-AGE follows that of HbA1c. Additionally, it may be speculated that susceptibility of HbA1c to be converted to Hb-AGE may vary in different individuals. An earlier study [22] reported that the fluorescence of human optical lens is dependent on age, non-enzymatic glycation by glucose or glycotoxins in tobacco smoke.

AGEs have been linked to many sequelae of diabetes, renal disease and aging. To detect AGE levels in human tissues and blood samples, a competitive enzyme linked immunosorbent assay (ELISA) has been widely used for research work. As no consensus or standard research method for the quantification of AGEs currently exists, nor a universally defined AGE unit available, the comparative quantification of AGEs between research laboratories is problematic and restricts the usefulness of inter-laboratory clinical data [6]. Hence an alternative efficient method would be useful to fix the standard analytical method for Hb-AGE. Autofluorescence, as proved in this study to be common for in vitro, in vivo and clinical glycated hemoglobin, will be of much use in qualitative and quantitative analysis.

4. Conclusions

Hb-AGE formed during the advanced stage of glycation exhibits characteristic autofluorescent behavior \( \lambda_{\text{ex}}/\lambda_{\text{em}} = 308/345 \text{ nm} \) which could be of major use in hastening its qualitative and quantitative estimation. Due to the increased sensitivity of fluorescence analysis, the Hb-AGE formed at an advanced stage of diabetes can be used to assess the stage of diabetes development. Though the quantum yield is comparatively lower, the presence of AGE adduct on the surface of hemoglobin molecule suppresses the fluorescence due to tryptophan. Also the alkaline environment increases the fluorescence intensity of Hb-AGE molecule. Autofluorescence \( (308/345) \text{ nm} \) run was found to be the common phenomenon for the glycated hemoglobin formed under in vitro and in vivo conditions and also from the clinical subjects. Until a study of the temporal correlation between blood glucose and HbA1c/Hb-AGE has been made, we must assume that HbA1c is the better index of integrated glycemia (blood glucose concentration). Research is in progress to elucidate the molecular structure of Hb-AGE by NMR, MALDI-TOF-MS and to develop the optic fiber-based immunosensor for detection of Hb-AGE using immobilized monoclonal antibodies.

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