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Prolonged Incubation with Glucose Induces Oligomerization and Aggregation of Hemoglobin: Role of Advanced Glycation End Product on Protein Structure

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Abstract

Non-enzymatic glycation is a post-translational modification of proteins that leads to protein crosslinking, aggregation, conformational changes, etc. Chemical reaction of sugars with proteins (Maillard reaction) leads to formation of glycated adducts, known as Advanced Glycation End Products (AGE). HbA1c, the major glycated hemoglobin, increases proportionately with blood glucose concentration in diabetes mellitus. Glycation-induced modification of hemoglobin is a major cause of oxidative stress in diabetic patients. In this study, we have investigated the effect of different concentrations of glucose on hemoglobin following long-term incubation (30 days) with the heme protein. Gel electrophoretic profile revealed the presence of high molecular weight oligomers and aggregated species of hemoglobin following incubation with glucose. MALDI-MS analysis also indicated the appearance of high molecular weight oligomeric species of hemoglobin following modification with glucose. Secondary structural analysis showed that glucose induces a change in native secondary structure of the protein from α-helix to β-sheet, particularly cross-β structure. Scanning electron microscopic imaging studies revealed the presence of amorphous protein aggregates. Considering the increased level of glucose in diabetes mellitus, the current study appears clinically significant, particularly in the context of understanding glycation-mediated complications and AGE formation in proteins under physiological conditions.

Keywords: Hemoglobin; Glucose; Advanced Glycation End Product; Mass Spectrometry; Scanning Electron Microscopy

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Introduction

Diabetes mellitus is a metabolic disorder characterised by increase in the concentration of blood glucose. Glucose has been reported to modify several proteins, namely, serum albumin [1], α-crystallin [2], collagen [3], low-density lipoprotein [4], hemoglobin [5] etc, through a non-enzymatic reaction process. The mechanism of non-enzymatic glycation involves Schiff base formation, followed by Amadori rearrangement [6], ultimately leading to the formation of Advanced Glycation End products (AGEs) [7]. Glycated hemoglobin (HbA_{1c}), has been reported to be modified at the N-terminus of the beta chain (valine) by glucose [6]. Diabetic patients have increased concentration of HbA_{1c} and characteristic hyperglycemia not found in normal healthy subjects [8]. Glycation-induced modification of hemoglobin has been reported in several studies [5, 9-16]. HbA1c is a potential source of free radicals and oxidative stress in diabetic condition as reported in some earlier studies [17, 18]. Glycation of hemoglobin has been suggested to induce free radical mediated oxidative damage to endogenous molecules.

Considering the clinical significance of hemoglobin glycation, in the current study, we have tried to gain further insight on AGE-induced modification of the heme protein. We investigated the effect of different concentrations of glucose (20, 30, 50 and 100 mM) on hemoglobin following long-term incubation with the heme protein (30 days). Our current findings indicate that glycation induced formation of high molecular weight oligomeric and aggregated species of hemoglobin due to possible protein crosslinking. Long-term glycation also induces a conformational change of the protein from native α-helix to β-sheet structure. Finally, electron microscopic studies indicated the presence of amorphous aggregates of glycated hemoglobin. Thus, glucose modification appears to significantly affect protein structure following long-term incubation. Considering the increased level of glucose in diabetic condition, the present findings appear clinically relevant in terms of understanding glycation-induced protein modification that may be associated with

pathophysiological complications including AGE-mediated (protein) conformational disorders.

Materials and Methods

Materials

Glucose and α-Cyano-Hydroxycinnamic Acid matrix (CHCA) were purchased from Sigma Chemical Company, USA. All other reagents were AR grade and purchased locally.

Methods

Separation of non-glycated hemoglobin (HbA0) from blood: Total hemoglobin (Hb) was isolated and purified from blood samples (collected from healthy human subjects) by method as described previously [19]. Glycated hemoglobin species (HbA_{1a1}, HbA_{1a2}, HbA_{1b} and HbA_{1c}) and non-glycated hemoglobin (HbA_0) were separated from Hb by cation exchange chromatography [6]. The concentration of HbA0 was determined from Soret absorbance using an extinction coefficient (ε_{415nm}) of 125 mM^{-1} cm^{-1} (heme basis) [20].

***In vitro* reaction of hemoglobin (HbA_0) with glucose:** HbA_0 (100 μM) was incubated with different glucose concentrations (20, 30, 50 and 100 mM) under sterile conditions for 30 days. For control experiments, HbA0 solution was incubated without glucose under identical conditions.

Polyacrylamide Gel Electrophoresis (PAGE): HbA_0 and glucose-incubated HbA_0 samples were subjected to native PAGE (10%) for 3 hr at constant voltage (60V). After electrophoresis, the gel was stained with Coomassie R250.

Matrix-assisted Laser Desorption Ionization-time of Flight (MALDI-TOF) mass spectrometric study: The mass spectra of control and glycated samples were recorded using a 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA) MALDI-TOF/TOF mass spectrometer (equipped with Nd /YAG laser; 355 nm). Samples were separately mixed saturated CHCA solution (prepared in 50% acetonitrile and 0.1% trifluoroacetic acid), loaded onto MALDI target plate and allowed to dry and crystallize. The mass spectra were recorded using the linear positive ion mode of MALDI-TOF MS.

CD study: CD spectra of control and glycated samples were recorded in a spectropolarimeter (Jasco 600) using 1 mm pathlength cuvette in the far UV region.

Fourier Transforms Infrared (FTIR) spectroscopy: FTIR spectra of samples were recorded in the transmission mode using Perkin-Elmer instrument (Spectrum BX Series, MA, USA) equipped with attenuated total reflection Diamond crystal cell. All spectra were recorded with DTGS (Deuterated Triglycine Sulfate) IR detector. 50 scans per sample were recorded at a resolution of 4 cm^{-1} with the wavenumber ranging from 2000-400 cm^{-1}. The acquired spectra were corrected for buffer and water vapour. Analysis of data was carried out using Origin Pro 8.5 software.

Scanning Electron Microscopy (SEM): The samples were subjected to scanning electron microscopy (SEM) imaging. Air-dried samples were gold coated and imaged in a scanning electron microscope (QUANTA 200) at low vacuum.

Results and Discussion

Gel electrophoresis

Gel electrophoretic profile of HbA0 incubated for 30 days with different concentrations of glucose is shown in Figure 1.

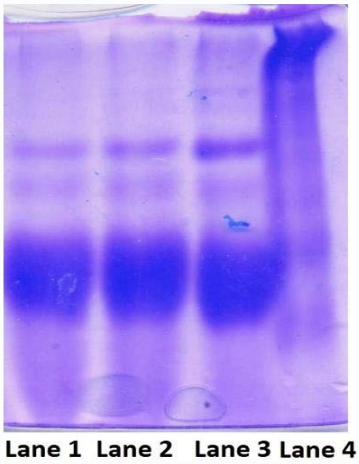

Figure 1: Gel electrophoresis of hemoglobin glycated with 20 mM (Lane 1), 30 mM (Lane 2), 50 mM (Lane 3) and 100 mM (Lane 4) glucose for 30 days.

While control HbA_0 appeared as a single band (data not shown), HbA_0 incubated with 20, 30and 50 mM glucose showed distinct appearance of high molecular weight bands, indicating formation of cross-linked oligomeric species. However, HbA_0 incubated with 100 mM glucose showed appearance of a smear throughout the lane, indicating both protein aggregation and possible degradation at higher glucose concentration. In a previous study, both glucose and fructose were found to induce crosslinking and aggregation of the heme protein on long-term incubation [21]. As proposed in that study, glucose-mediated AGE adducts appear to be responsible for the observed structural changes. Similarly, prolonged glycation of lysozyme with reducing sugars has been found to induce aggregation and oligomerization of the protein [22]. In a previous study, glyoxal was found to induce aggregation of bovine hemoglobin and AGE formation along with associated conformational changes [23].

MADI-MS Results

Mass spectrometry has been widely employed to study and analyse protein glycation, as reported in several studies [24-26]. MALDI-MS analysis of HbA_0 glycated with 30and 50 mM glucose is shown in Figures 2-5. Control HbA_0 generally exhibits two peaks with m/z values 15,126 Da and 15,868 Da that correspond to α and β chains, respectively, as reported in a previous study (data not shown) [27]. As shown in the spectral pattern (Figure 2), HbA0 glycated with 30 mM glucose showed two peaks with m/z values 15,146 Da and 15,898 Da, indicating modifications of α and β chains of the heme protein, respectively. The m/z values 7573 Da and 7947 Da indicate the doubly charged ionic species of corresponding α and β chains. In addition, a minor peak with m/z 16,524 Da indicating presence of high molecular specie was also observed. Spectral analysis of glycated HbA_0 at higher m/z range revealed the presence of high molecular weight species with subsequent broadening of peak, indicating possible protein crosslinking and oligomerization on long-term incubation with glucose (Figure 3). The extent of modification of α and β chains of HbA_0 appears to be higher at 50 mM glucose in comparison with the lower glucose concentration as observed in Figure 4. Similar to that of 30 mM glucose, high molecular weight species were also observed at a higher glucose concentration indicating considerable formation of cross-linked oligomeric species (Figure 5). Thus, mass spectrometric study provides further insight and confirmation at molecular level on glucose mediated oligomerization and formation

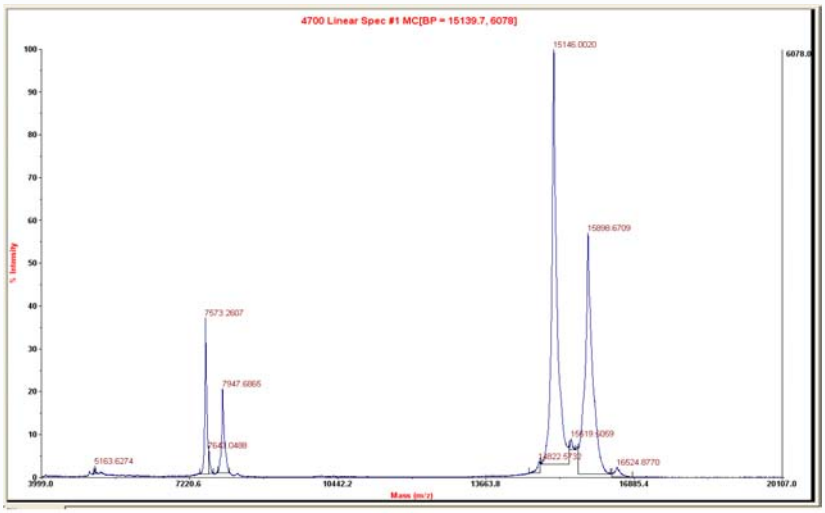

Figure 2: MALDI-MS analysis of hemoglobin glycated with 30 mM glucose recorded at 3999-20107 m/z range.

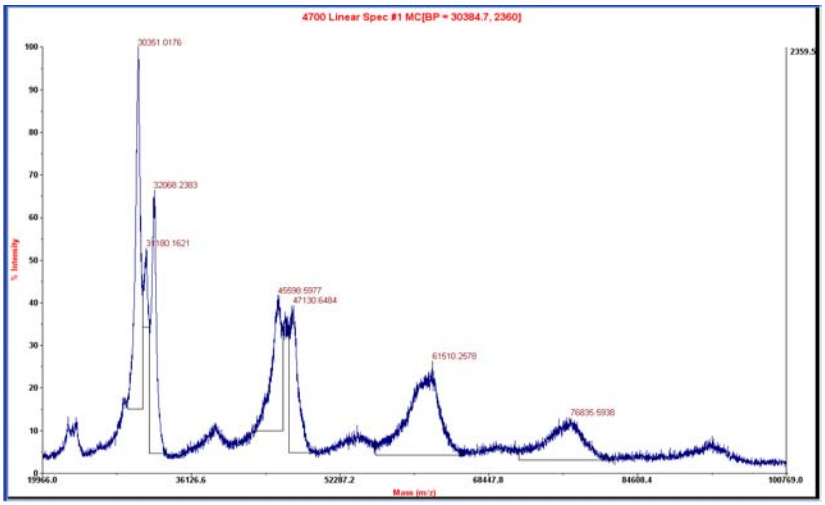

Figure 3: MALDI-MS analysis of hemoglobin glycated with 30 mM glucose recorded at 19966-100769 m/z range.

of high molecular weight species of HbA_0, thereby supporting the results of gel electrophoresis.

CD Spectroscopy

The CD spectra of control and glycated HbA_0 is shown in Figure 6. While CD spectrum of control Hb indicated a predominantly α-helical structure, Hb glycated with 50 mM and 100 mM glucose showed a substantial reduction in α-helical content. This indicated that glucose induced an alteration in native secondary structural conformation of protein following long-term incubation. The observation is consistent with some previous studies, where glucose or fructose was found to alter the native structural conformation of hemoglobin, favouring formation of β-sheet structure [21, 28] preferably at higher incubation time. Increase of β-sheet has been found to be associated with protein aggregation in several studies, because β-sheet generally provides a better environment for intermolecular interactions required for aggregation. In an earlier study, glycation of myoglobin by glucose was also found to alter the secondary structure of the protein resulting in lowering of α-helical content [29]. Glycated hemoglobin (HbA_{1c}) was found to exhibit a decrease in α-helicity, in comparison to non-glycated hemoglobin (HbA_0) as previously reported [30].

FTIR Spectroscopy

Other than CD analysis, FTIR spectroscopy provides sensitive information on protein secondary structural content [24]. FTIR analysis was employed to detect the possible presence of β-sheet structure in HbA_0 glycated with 50 mM or 100 mM glucose (Figure 7A, B). As shown in each spectrum, the peak at 1612 cm^{-1} in the amide I range indicated the presence of amyloid cross-β sheet structure [31]. Additionally, the smaller peaks observed in amide II region (1506 cm^{-1}, 1524 cm^{-1}) are also characteristic of β-sheet conformation [32]. Thus, our FTIR results indicate that long-term glycation of hemoglobin induced formation of cross-β structure indicating its amyloid nature.

SEM analysis

SEM imaging studies are widely used for detecting the nature and morphology of protein aggregation [33-35]. SEM imaging of HbA_0 glycated with glucose is shown in Figure 8. While SEM analysis of control HbA_0 did not show any aggregate (data not shown), HbA_0 glycated with 50 mM or 100 mM glucose revealed the presence of

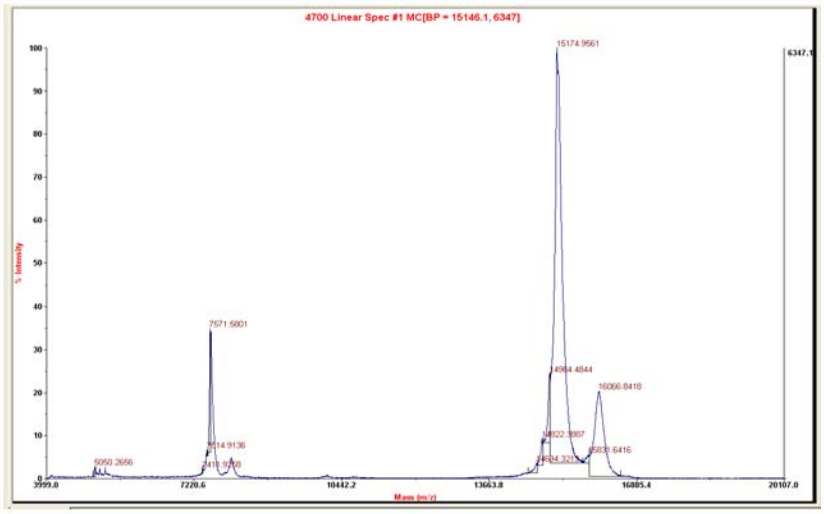

Figure 4: MALDI-MS analysis of hemoglobin glycated with 50 mM glucose recorded at 3999-20107 m/z range.

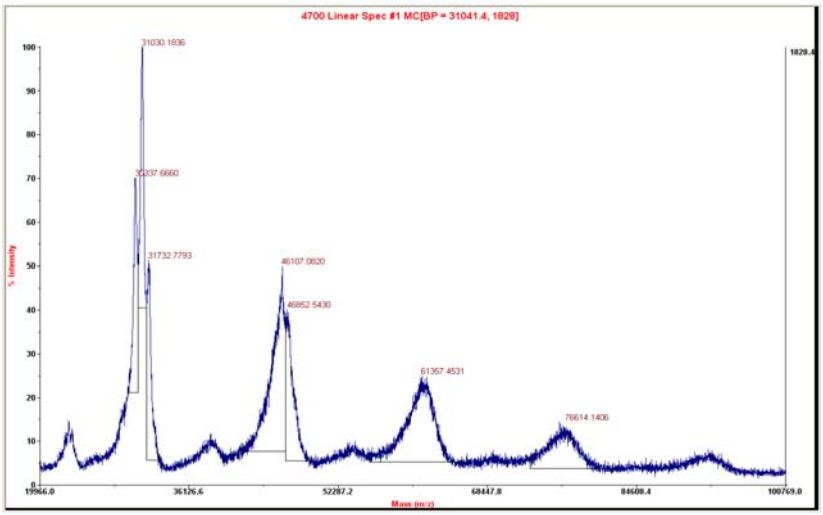

Figure 5: MALDI-MS analysis of hemoglobin glycated with 50 mM glucose recorded at 19966-100769 m/z range.

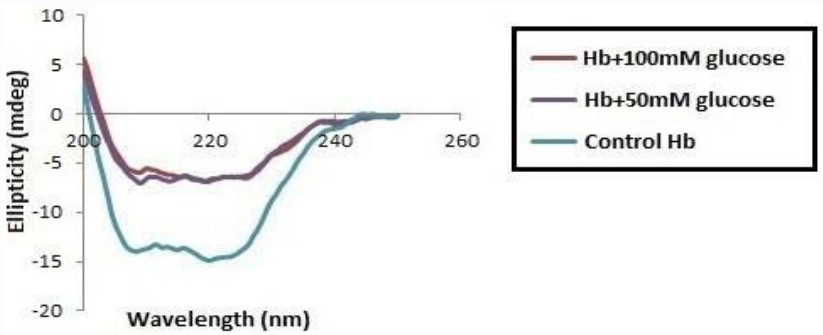

Figure 6: Far-UV CD spectra of control hemoglobin ( ), hemoglobin glycated with 50mM glucose ( ) and 100 mM glucose ( ) for 30 days.

amorphous type of protein aggregates (Figure 8A-C). As revealed by FTIR study described above, the said samples showed the presence of cross-β structure, indicating the possible amyloid nature of glucose-induced protein aggregates.

Thus, from the above experimental observations, we find that prolong incubation with increasing concentration of glucose appears to induce oligomerization and aggregation of the heme protein, hemoglobin. Covalent modification of lysine and arginine residues may induce partial unfolding and aggregation of proteins. AGE adducts may influence the overall structure of proteins leading to aggregation [36-38] and pathological complications.

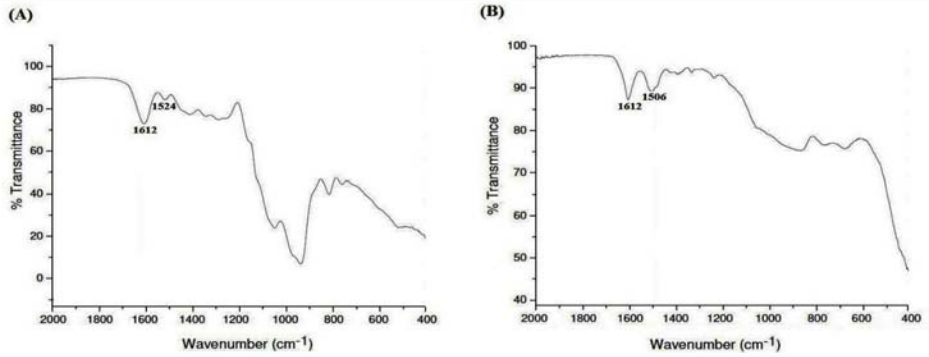

Figure 7: FTIR spectra (2000-400 cm^{-1}) of hemoglobin glycated with 50 mM glucose (A) and 100 mM glucose (B) for 30 days.

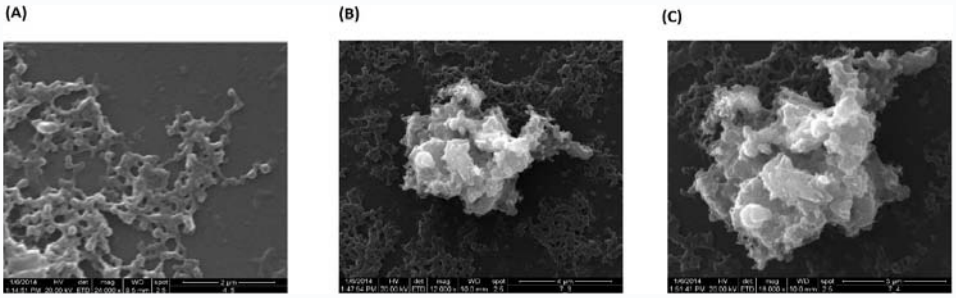

Figure 8: SEM imaging of hemog lobin glycated with 50 mM glucose (A) and 100 mM glucose (B, C) recorded at different fields and magnifications.

Methylglyoxal-induced modifications of arginine residue of insulin (Arg-46) or cytochrome c (Arg-92) have been reported to cause conformational changes and aggregation of the proteins [39, 40]. Protein glycation including carboxymethyllysine formation has been reported to induce protein cross-linking in several studies [21, 41, 42]. Detection of cross-β sheet structure indicates amyloid nature of protein aggregates. Methylglyoxal was reported to induce amyloid-like aggregation of the heme protein myoglobin through the formation of several AGE adducts [24]. On the other hand, prolonged glycation of hen egg white lysozyme with glucose, fructose or ribose induced formation of non-amyloidal structures [22]. In this study, we find that glucose induces aggregation of hemoglobin at high concentration(s) and longer incubation time. Long-term incubation of hemoglobin with high concentration of glucose or fructose was reported to induce aggregation of the heme protein, as reported previously. On the other hand, short-term incubation of several proteins namely insulin, cytochrome c and histone H2A with high concentrations of methylglyoxal induces protein aggregation [39, 40, 43]. Thus, both concentrations of the glycating agent and incubation period of the reaction mixture appear to control the extent and nature of modification. Overall, this study adds further to previously published reports on hemoglobin glycation particularly in terms of understanding AGE-induced protein structural changes. Usage of mass spectrometry provides sensitive information on the extent of glycation-induced protein oligomerization. It will be interesting to detect and identify the specific AGEs responsible for the observed structural changes in future studies.

Conclusion

In the current study, long-term incubation with glucose was found to induce oligomerization and formation of high molecular weight aggregates of hemoglobin. Glycation-mediated aggregation of hemoglobin was associated with a significant reduction in native α-helical content of protein with a concomitant increase of β-sheet, particularly cross-β sheet, characteristic of amyloid protein aggregates. Glycation-derived AGE adducts appear to be play significant role in the observed structural changes of the protein.

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Conflict of Interest

The author declares that there is no conflict of interest associated with the study.

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