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Exploring the antioxidant property of bioflavonoid quercetin in preventing DNA glycation: A calorimetric and spectroscopic study

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Abstract

Reducing sugars for example glucose, fructose, etc., and their phosphate derivatives non-enzymatically glycate biological macromolecules (e.g., proteins, DNA and lipids) and is related to the production of free radicals. Here we present a novel study, using differential scanning calorimetry (DSC) along with UV/Vis absorption and photon correlation spectroscopy (PCS), on normal and glycated human placenta DNA and have explored the antioxidant property of the naturally occurring polyhydroxy flavone quercetin (3,3′,4′,5,7-pentahydroxyflavone) in preventing the glycation. The decrease in the absorption intensity of DNA in presence of sugars clearly indicates the existence of sugar molecules between the two bases of a base pair in the duplex DNA molecule. Variations were perceptible in the PCS relaxation profiles of normal and glycated DNA. The melting temperature of placenta DNA was decreased when glycated suggesting a decrease in the structural stability of the double-stranded glycated DNA. Our DSC and PCS data showed, for the first time, that the dramatic changes in the structural properties of glycated DNA can be prevented to a significant extent by adding quercetin. This study provides valuable insights regarding the structure, function, and dynamics of normal and glycated DNA molecules, underlying the manifestation of free radical mediated diseases, and their prevention using therapeutically active naturally occurring flavonoid quercetin.
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Keywords: DNA; Nonenzymatic glycation; UV/Vis absorption spectroscopy; Differential scanning calorimetry; Photon correlation spectroscopy; Bioflavonoids; Antioxidant

Nonenzymatic condensation of the free carbonyl groups (C=O) of the reducing sugars such as glucose, fructose, mannose, etc., and their phosphate derivatives, with the free amino ($-NH_2$) residues of the biological macromolecules is known as glycation. This involves Schiff's base formation followed by Amadori/Heyns' rearrangement which happens during the disorder in the metabolism of reducing sugar molecules [1–3]. This early glycation is followed by irreversible advanced glycation end products (AGEs) which produce free radicals, the consequences of which can be cancer, heart disease, cataract, atherosclerosis, neurodegenerative disorder (e.g., Parkinson's disease, Alzheimer's disease), etc. Glycated DNA and protein have attained significant prominence in the modern world of medicinal biology due to their use as a scale in the long-term control of diabetes mellitus [4–7].

Flavonoids are a widely distributed group of polyphenolic compounds which are present in common plant based food items and beverages, e.g., onions, apples, tea, and red wine. These ubiquitous natural products possess a broad range of biological activities and important therapeutic applications (e.g., against cancers, tumors, allergies, cardiac problems, inflammation, AIDS, etc.) [8–10]. In this context, we emphasize on the novel use of naturally occurring flavonoid quercetin (3,3′,4′,5,7-pentahydroxyflavone, structure given in Scheme 1) as an antioxidant against glycation of human placenta DNA. The antioxidant activity of bioflavonoids is attributed to the presence of phenolic hydroxyl groups in the flavonoid structure [10,11]. We have chosen quercetin for the following reasons: (i) quercetin is the most abundant naturally occurring flavonoid in human diet, which is known to inhibit the activities of calcium

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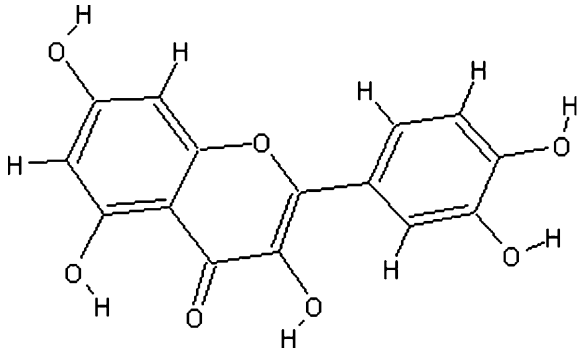


Scheme 1. Structure of Quercetin (3,3′,4′,5,7-pentahydroxyflavone).

phospholipid-dependent protein kinase [12], DNA topoisomerases [13], etc. (ii) Quercetin and other related flavonols are found to inhibit growth of leukemia cells, human gastric cancer cells, etc. [14], and (iii) quercetin is also well known for its antioxidant properties in preventing lipid peroxidation [15].

In a very recent communication, we have shown with photon correlation spectroscopy (PCS), how the dynamics of DNA is strongly affected by glycation [16]. In the present work, we have explored in detail for the first time, using PCS and differential scanning calorimetry (DSC), the novel and therapeutically important antioxidant property of quercetin in preventing the structural and dynamical variations of glycated DNA to a considerable extent. Electronic absorption spectroscopic studies have been performed on normal and glycated DNA in order to observe the incorporation of sugar molecules in the latter case. With the PCS and DSC we studied the structural differences between normal and glycated DNA in absence and presence of quercetin exploiting their relaxation profiles [17] and melting temperatures [18], respectively.

Materials and methods

Genomic human placenta DNA, 100× Tris–HCl (1.0 M Tris–HCl, pH ≈ 8, containing 0.1 M EDTA) buffer, and NaCl were obtained from Sigma–Aldrich (USA). Quercetin, fructose, and glucose-6-phosphate were purchased from Aldrich. Milli-Q water was used for all the experiments. All samples were used without further purification. The concentration of DNA was kept at ~10–20 μg/ml in 10× Tris buffer with a NaCl concentration of 100 mM.

We have chosen the reducing sugars fructose (Frc, 3 g/100 ml) and glucose-6-phosphate (Gl 6-P, 4 g/100 ml) to glycate the placenta DNA and these concentrations of the sugars have previously been shown [1,19,20] to correspond to the minimum concentration for an in vitro glycation of proteins, DNA, etc. We also prepared a concentrated stock solution of quercetin in spectrograde methanol. Small aliquots from this solution were added to DNA in buffer solution to give a final concentration of ≈30 μM (the final methanol concentration in the buffer was <1%). Frc and Gl 6-P were separately added to DNA, both in absence and presence of quercetin, and kept for 24 h in room temperature and 48 h in 4 °C. These solutions were then used for experimental studies.

Absorption spectrophotometry. Cary 500 spectrophotometer was used to record the absorption spectra of the normal and glycated DNA solutions. The absorbance of DNA in absence and presence of the two sugars was studied and the ratio of the absorption intensities of DNA at 260 nm (maximum absorption of DNA):310 nm (baseline) was used to observe the formation of glycated DNA. This ratio was determined, rather than the absolute intensity at 260 nm, in order to avoid any erroneous interpretation due to instrumental error and unusual scattering from samples. In presence of quercetin, which like other flavonoids is likely to intercalate between DNA [21,22], such comparison cannot be done as the absorbance of quercetin (quercetin in buffer absorbs maximally at ≈370 nm [23,24]) interferes with the absorption at 310 nm.

Photon correlation spectroscopy. With photon correlation spectroscopy (PCS) the macromolecular dynamics [17,25,26] is directly investigated. The dynamical features can be coupled to the structure and size of the scattering molecules and used to describe processes that bring changes to the macromolecular sizes and shapes [27]. The PCS measurements were performed using the homodyne technique with a frequency doubled Nd–Vanadate laser (532 nm) and a correlator (ALV-5000/FAST). The measurements were carried out at 25 °C over the time range 10^{-8} to 10^{3} s with a tunable output power of 0.4–0.8 W depending on the light scattering signal from each sample. It has been confirmed that the output power of the laser does not affect the observed relaxation functions of the samples. In the present study, we have used Siegert's relation [28].

$$g_2(q,t) = 1 + \sigma|g_1(q,t)|^2 \tag{1}$$

using a set of Kohlrausch–Williams–Watts (KWW) functions [29]

$$g_1(q,t) = \sum_{i=1}^{3} S_i \exp(-t/\tau_i)^{\beta_i} \tag{2}$$

to analyse and describe the normalized autocorrelation functions ($g_2(q,t)$) of the scattered intensity obtained from the PCS measurements on normal and glycated DNA (both in absence and presence of quercetin). $g_2(q,t)$ is related to the structural relaxation function $g_1(q,t)$ by Eq. (1). Here σ is the instrumental coherence factor (ideally close to one), q is the scattering vector, and τ is the typical relaxation time; $0 < \beta \leqslant 1$, is the stretching parameter; S is the relaxation strength and i is the index of the relaxation function. The scattering vector q is inversely proportional to the measured length scales and also a function of the scattering geometry; $q = (4\pi n/\lambda)\sin(\theta/2)$ [30], where n, λ, and θ are the refractive index, wavelength of the incident light, and the angle between incident and scattered light, respectively.

Regularized inverse Laplace transformation (RILT) [17,31] was also used to analyze the measured intensity time correlation function and obtain a relaxation time distribution function ($A(\tau)$). The RILT analysis uses the calculation algorithm REPES, incorporated in the GENDIST analysis package. In this way, $g_1(q,t)$ and $A(\tau)$ were studied at various scattering angles (50°, 90°, and 130°) for both pure and glycated DNA solutions in absence (studied at all three angles) and presence of quercetin (studied at the angle 90°). All the experiments were repeated three times to check the consistency of the results.

Differential scanning calorimetry. Differential scanning calorimetry (DSC) measurements were performed with TA instruments, DSC Q 1000. Melting studies of all the samples of DNA were carried out at the heating rate of 10 °C/min between 20 and 90 °C. Hermetic pans with a sample volume of about 18 μl were used for all measurements. The reference cell was an empty hermetic pan with which the instrument was previously calibrated. Each set of experiments was repeated three times in order to check the reproducibility of the data. The program Universal analyses 2000 was used to determine the transition temperature in each case.

Results

We have performed absorption spectroscopic studies on DNA in absence and presence of sugars and the ratio of the intensities at 260:310 nm (I_{260}/I_{310}), which is described under Materials and methods, is observed in each case. As is given in Table 1, I_{260}/I_{310} decreases for DNA in presence of sugars.

Table 1
A summary of the calorimetric and spectroscopic properties of normal and glycated DNA in absence and presence of quercetin as an antioxidant

Sample	DNA	DNA + Frc	DNA + Gl 6-P	DNA + quercetin	DNA + quercetin + Frc	DNA + quercetin + Gl 6-P
I_{260}/I_{310}	38.5	10.88	10.4	—	—	—
T_m (°C) around	81	78	77	81	81	80
β at 90° [16]	1	0.6	0.7	1	0.97	0.86
τ (ms) ~ at 90° [16]	4	5, 0.4, 0.0034	5, 1.25, 0.0034	1.4	2	5, 0.7

—, Here I_{260}/I_{310} cannot be obtained as the absorption of quercetin interferes at 310 nm [23,24].

We performed photon correlation spectroscopy (PCS) measurements on the normal and glycated forms of the human placenta DNA: the structural relaxation functions $g_1(q,t)$ for $\theta = 50°$ are presented in Fig. 1. The relaxation functions are fitted by Eq. (2), which is described under Materials and methods, and is shown by the solid line. As shown in Fig. 1, the addition of sugar changes the main relaxation process of normal DNA (•••) with stretching parameter $\beta = 1$ (i.e., a single exponential) to a stretched exponential with $\beta \sim 0.93$ and 0.8 for DNA with Frc (○○○○) and Gl 6-P (++++), respectively. Thus, the single, main relaxation of pure DNA with relaxation time $\tau \sim 11.6$ ms is transformed to a broad distribution in presence of Gl 6-P which consists of a main $\tau \sim 11$ ms and two new relaxation processes on a considerably faster time scale with $\tau \sim 1$ ms and 8.6 μs. For DNA with Frc, the main τ is ~15.8 ms with another broad relaxation of very low intensity having τ mainly ~21 μs.

We have also performed PCS studies on normal and glycated DNA at angles 90° and 130°. For the angle 90°, the values of β as well as τ are presented in Table 1 which clearly shows that the structure of DNA is significantly altered when glycated. When the angle was increased to 130°, we observed a substantial decrease of the β value to 0.67 for pure DNA, with a main $\tau \sim 2.6$ ms (and another $\tau \sim 33.6$ ms). Fig. 2 presents the structural relaxation ($g_1(q,t)$) and relaxation time distribution functions $A(\tau)$ of glycated DNA at angle 130°. Here, the $g_1(q,t)$ profiles are even more stretched with $\beta = 0.33$ and 0.54 for DNA with Frc (○○○○) and Gl 6-P (++++), respectively

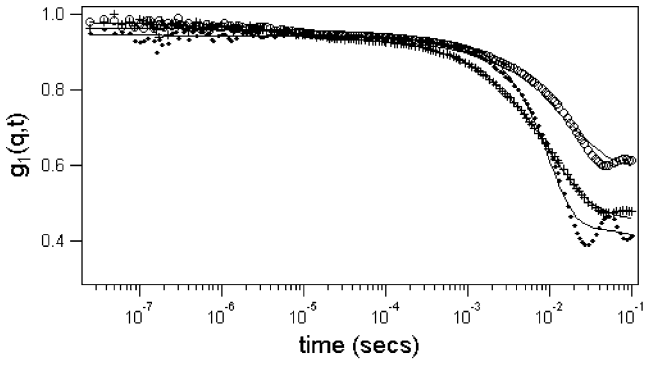


Fig. 1. Structural relaxation functions ($g_1(q,t)$) obtained for normal (•••) and glycated (with fructose (○○○○) and glucose 6-phosphate (++++)) human placenta DNA at 298 K at a scattering angle $\theta = 50°$. The solid lines are fitted curves using Eq. (2), given under Materials and methods.

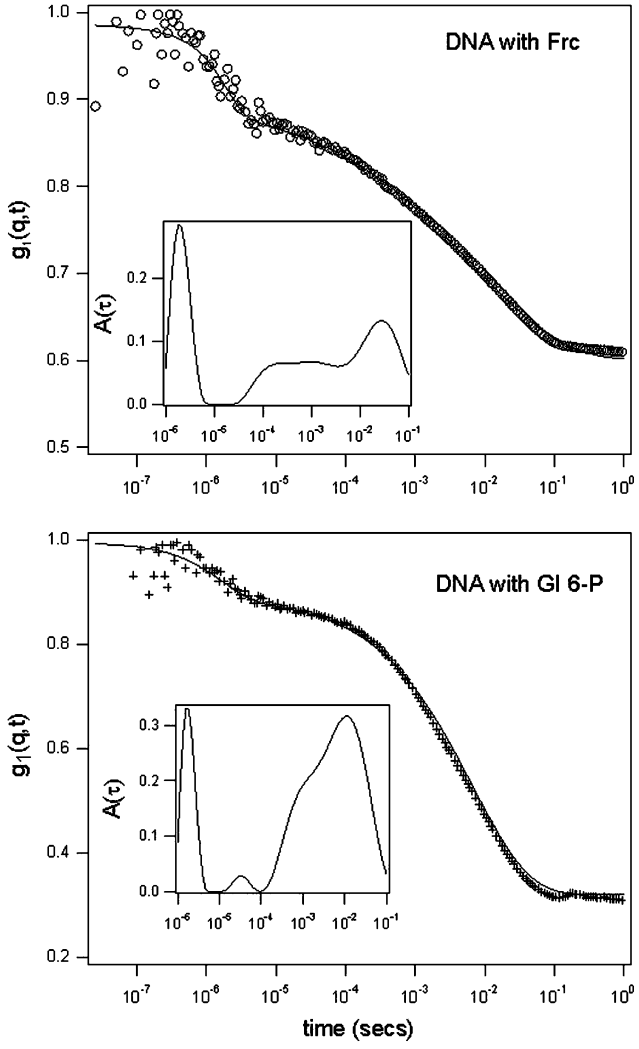


Fig. 2. Structural relaxation functions ($g_1(q,t)$) obtained for glycated (with fructose (○○○○) and glucose 6-phosphate (++++)) human placenta DNA at 298 K at a scattering angle $\theta = 130°$. The solid lines are fitted curves using Eq. (2), given under Materials and methods. Inset: relaxation time distributions ($A(\tau)$) of the same samples of glycated DNA under the same condition. The x axis unit is same for the main figure and the inset.

(Fig. 2). The $A(\tau)$ profiles are therefore very broad for both the glycated DNA samples with the relaxation times 1.8 μs, 0.16, 1, and 29 ms for DNA with Frc and 1.6, 34 μs, 1, and 11.6 ms for DNA with Gl 6-P.

The DSC curves for melting of pure DNA (–) and glycated DNA (with Frc, …. and Gl 6-P, ----) with increasing temperatures are shown in Fig. 3. The broad melting profile of pure DNA suggests that the base composition of

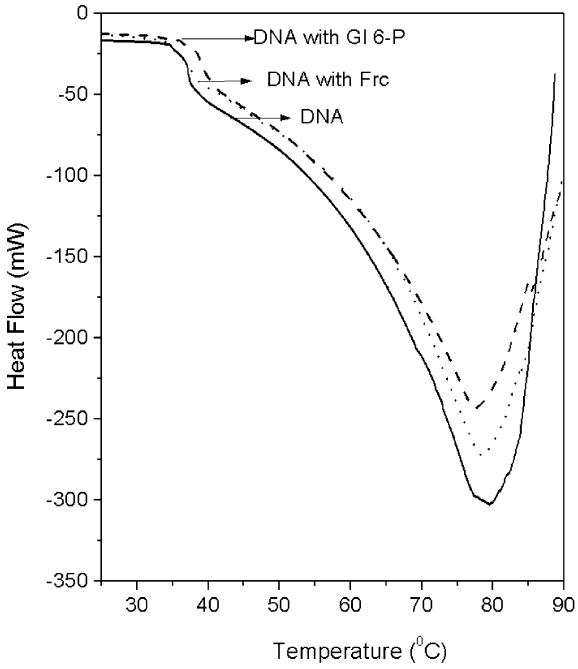


Fig. 3. Thermal unfolding and melting profiles of human placenta DNA at various conditions: pure DNA (solid line) and DNA with Frc (dotted line) and Gl 6-P (dashed line). Heating rate = 10 °C/min.

human placenta DNA is heterogeneous [18]. We observed appreciable differences in the melting profiles of both the glycated DNA in comparison to pure DNA. The melting temperature (T_m) decreases when the DNA is glycated (see Fig. 3 and Table 1).

To explore the role of the bioflavonoid quercetin in the glycation of DNA, we have added sugar (Frc and Gl 6-P) to DNA in presence of quercetin and carried out PCS and DSC studies. Fig. 4 presents the structural relaxation functions $g_1(q,t)$ and relaxation time distributions $A(\tau)$ (inset) of glycated DNA with quercetin at the scattering angle 90°. By comparing with the relaxation parameters given in Table 1, it is evident that the structural relaxation function and the corresponding relaxation time distribution of glycated DNA with quercetin are reasonably similar to that of normal DNA, regarding the shape of the main relaxation. Nevertheless, some minor differences are still observed; the existence of the broad minor peak in case of Gl 6-P added DNA and a minor contribution of the fast process in both the glycated DNAs, with quercetin.

The melting profiles of DNA containing quercetin (solid line), DNA-quercetin-Frc (....) and DNA-quercetin-Gl 6-P (----), with increasing temperatures are shown in Fig. 5. It is evident that the unfolding patterns of DNA-Frc and DNA-Gl 6-P return to a state similar to that of pure DNA in presence of the antioxidant quercetin which agrees well with our studies using PCS (Fig. 4). Furthermore, it is worthwhile to mention that the unfolding and complete melting of pure DNA is similar in presence and absence of quercetin.

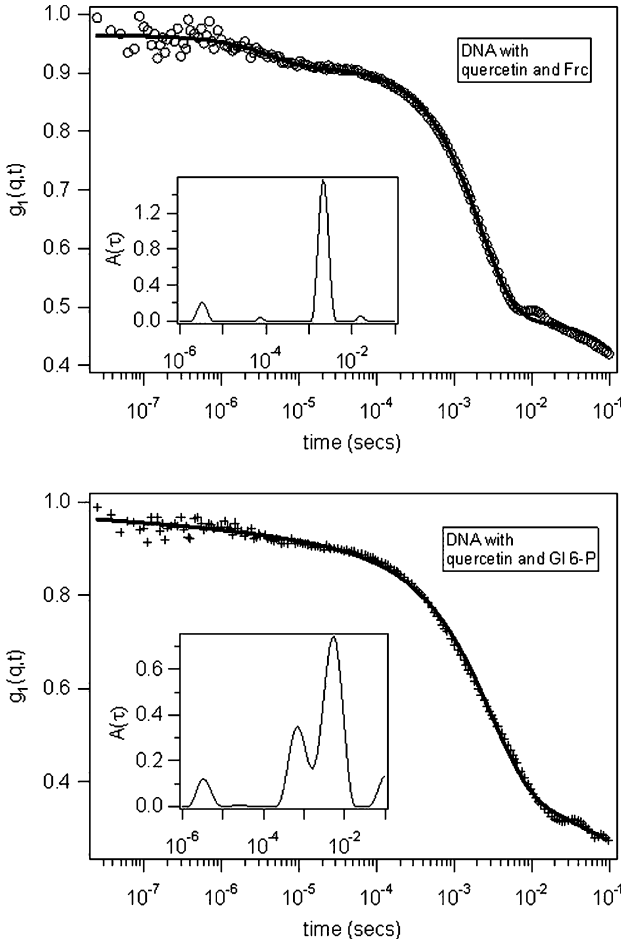


Fig. 4. Structural relaxation functions ($g_1(q,t)$) obtained for the glycated (with fructose (○○○○) and glucose 6-phosphate (++++)) human placenta DNA in presence of the bioflavonoid quercetin, at 298 K and at a scattering angle $\theta = 90°$ and the solid line is the fitted curve drawn using Eq. (2), given under Materials and methods. Inset: relaxation time distributions ($A(\tau)$) of the same samples of DNA under the same condition. The x axis unit is same for the main figure and the inset.

Discussion

From Table 1 the decrease in the ratio of I_{260}/I_{310} for DNA in presence of Frc and Gl 6-P with respect to that of pure DNA suggests the following picture: the sugar molecules have been incorporated inside the two bases of a DNA base pair. The $-NH_2$ groups of the DNA bases adenine (A), guanine (G), and cytosine (C) (Scheme 2) are the possible sites for the reaction with the free carbonyl (–C=O) group of the sugar molecules. The presence of sugars in the DNA (e.g., A-sugar-T, G-sugar-C) shields the bases and makes them less accessible to absorb UV light resulting in a decreased absorption intensity. This is demonstrated in the Scheme 2 where the free –C=O group of Frc molecules attack the free $-NH_2$ group of the bases of DNA.

We observed a decrease in the main τ values of pure DNA when the scattering angle was increased from 50° to 90° and 130°. The main τ values of the pure DNA were 11.6, 4 and 2.6 ms for the above angles, respectively. The

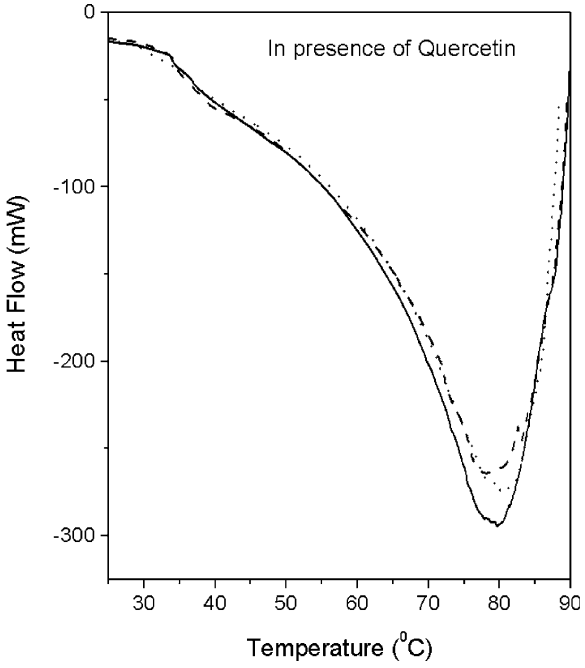
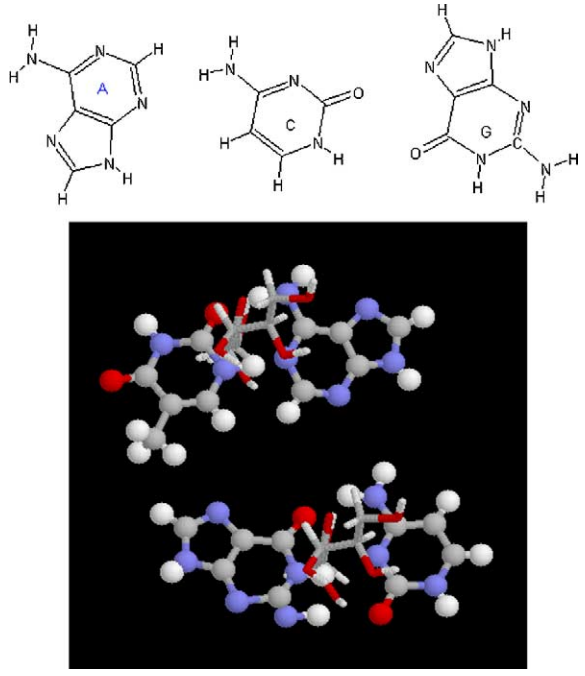


Fig. 5. Thermal unfolding and melting profiles of human placenta DNA in the presence of the antioxidant quercetin at various conditions: DNA with quercetin (solid line), DNA with quercetin containing Frc (dotted line) and Gl 6-P (dashed line). Heating rate = 10 °C/min.



Scheme 2. Top: Structures of DNA bases adenine (A), cytosine (C), and guanine (G) having free $-NH_2$ groups to take part in the glycation reaction. Bottom: A model of DNA base pairs (up T = A and below G ≡ C) containing sugar fructose inside them.

decrease of τ with increasing θ (scattering angle) value is the expected behavior for a normal translational motion of the DNA molecules [16,17,27]. For the angle 130°, we also observed a substantially increased stretching (the β value decreased to 0.67) of the relaxation process for pure DNA and obtained two values of τ, the main was at 2.6 ms and another at 33.6 ms. This is because of the following reason: at the higher angle 130° internal dynamics plays an important role in the structural relaxation and time distribution profiles. The non-exponential decay at higher angles indicates the influence of the internal dynamics [17].

From Figs. 1, 2, and Table 1, we observed that for all the three values of θ (50°, 90°, and 130°), the stretching parameter β decreases for the glycated DNA compared to pure DNA. β is a measure of the width of the decay in the structural relaxation functions. $\beta = 1$ correspond to the single exponent decay predicted for diffusive process, while smaller β ($0 < \beta < 1$) gives a broader and more stretched decay. The non-exponential decay is the manifestation of a heterogeneous distribution of relaxation processes each decaying exponentially and gives rise to the broad relaxation time distribution functions. For DNA with Gl 6-P, the relaxation time τ, corresponding to the main τ of pure DNA, are 11, 5 and 1 ms for the angles 50°, 90°, and 130°, respectively, while for DNA with Frc the corresponding times are 15.8, 5, and 1 ms. The existence of a very fast relaxation process (on the μs time scale) for the glycated DNA suggests that the DNA molecules have undergone fragmentation and this fast relaxation is then due to a very small moiety which can move very fast [16,32].

The melting temperature of the glycated DNA decreases in comparison to pure DNA (Fig. 3, Table 1), which can be due to the following reason: the presence of the sugar molecules between the DNA base pairs (e.g., Scheme 2) destabilizes their H-bonds which in turn make the DNA unstable and are responsible for the decrease in the T_m value for the glycated DNA. So, incorporation of sugar molecules inside DNA causes substantial changes in its structure which agrees with our findings from PCS measurements.

The potentiality of quercetin as a therapeutic agent against glycation of DNA can be well understood from Figs. 4 and 5. The structural relaxation function ($g_1(q,t)$) and the corresponding relaxation time distribution ($A(\tau)$) of glycated DNA in presence of quercetin (as presented in Fig. 4) is dramatically altered from that in absence of the flavonoid and the relaxation parameters match to a large extent with the profile of normal DNA (see Table 1). The structural relaxation functions of DNA with quercetin and sugar (as in Fig. 4) are less stretched ($\beta \sim 0.97$ for Frc and 0.86 for Gl 6-P as compared to 0.60 and 0.70, respectively, without quercetin, Table 1), and consequently the corresponding peak in $A(\tau)$ becomes sharper and more like that of pure DNA. Yet, some minor processes on other time-scales do exist in glycated DNA in presence of quercetin (Fig. 4), although the intensities of these processes are substantially lower compared to those observed in glycated DNAs in absence of the bioflavonoid. The reason for these alterations compared to normal DNA can be found in the literature [21,22] where it is known that flavonoids bind

intercalatively between DNA base pairs. This intercalation is most likely responsible for the observed differences.

From Fig. 5, it is evident that in the presence of the antioxidant quercetin, the changes in the DSC profiles of DNA by Frc and Gl 6-P are less, which suggest that quercetin inhibits the binding of the sugar molecules with DNA to a considerable extent which supports our results from Fig. 4.

The present comparative study on normal and glycated DNA with and without quercetin, using calorimetric and spectroscopic approaches, strongly suggests that the changes in the structure of DNA due to glycation is significantly reduced in the presence of quercetin. The mechanism of inhibition of quercetin against glycation of DNA is unknown, but probably the hydroxyl group of the antioxidant (structure given in Scheme 1) binds to the reacting site (carbonyl group) of sugars, and thereby blocking the site responsible for the reaction with DNA. The intercalation of quercetin between the DNA base pairs can also to a great extent prevent the glycation of DNA. It is likely that quercetin scavenges free radicals produced during the reaction between DNA and Frc or Gl 6-P via $ROH + {}^{\bullet}OH \rightarrow RO^{\bullet} + HOH$ (ROH is the antioxidant quercetin), thereby preventing the damaging effects of free radicals (e.g., the hydroxyl radical ·OH). We hope this preliminary study on quercetin with glycated DNA will form a baseline for fruitfully utilizing the potentialities of various naturally occurring flavonoids as therapeutic agents against the diabetic abnormalities.

Acknowledgments

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