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Aggregation and self assembly of non-enzymatic glycation of collagen in the presence of amino guanidine and aspirin: An *in vitro* study

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ABSTRACT

Non-enzymatic glycation of collagen has been used in modern biomaterials science. This paper deals with *in vitro* studies on the effects of amino guanidine (AG) and aspirin in the non-enzymatic glycation (NEG) of collagen using thermal, conformational, fluorescence, turbidity and powder XRD measurements. There is no significant change in the fluorescence emission spectra for different concentrations of AG treated NEG of collagen whereas the emission intensity decreases as the concentration of aspirin increases. Circular dichroism (CD) revealed the disappearance of the positive peak at 220 nm for glycated collagen in the presence of amino guanidine and aspirin suggesting the collapse of triple helical configuration. Nearly 15 °C decrease is observed in shrinkage temperature of glycated rat tail tendon (RTT) collagen fibres in the presence of aspirin. Powder XRD of glycated collagen nano-fibrils in the presence of amino guanidine reveals high crystalline nature and the enhancement of self assembly processes when compared to aspirin. To the best of our knowledge, this is the first report of powder XRD of the self assembly of collagen nano-fibrils without mineralization. Our experimental results suggest that in the non-enzymatic glycation of collagen both AG and aspirin play a pivotal role in the aggregation and self assembly processes. From the present study, it is possible to conclude that while AG significantly influences the self assembly processes, aspirin facilitates the aggregation processes.

1. Introduction

Collagen is the main protein of connective tissue in animals and most abundant protein in mammals making up 25% of total protein. Collagens are important proteins for the skin as they are essential for structure and function of the extracellular matrix in the dermis. In some tissues collagen may be dispersed as a gel that serves to stiffen the structure, as in the extracellular matrix or the vitreous humor of the eye. Collagen of bone occurs as fibres arranged at an angle to each other so as to resist mechanical shear from any direction. In other tissues, collagen may be bundled in tight parallel fibres that provide great strength, as in tendons. It is tough and inextensible with high tensile strength and is the main component of cartilage, ligaments and tendons [1,2]. It is responsible for skin strength and elasticity and its degradation leads to wrinkles that accompany aging [3]. The cross-linking of proteins that makes up the human body plays a role in aging process [4–9]. But for all individuals the consequences of protein glycation are involved in general aging phenomenon. They are also implicated in the pathology associated with diabetes, atherosclerosis and Alzheimer's disease [10–14].

Non-enzymatic glycosylation or glycation of collagen involves the addition of sugar molecules to the tertiary amino groups in collagen to form Schiff base adducts. Further chemical modification results in the formation of Amadori products (AP). A number of subsequent reactions result in the formation of advanced glycation end products (AGEs), some of which cross-link within and between proteins. Glycation and subsequent cross-link formation are thought to contribute to the complications seen in aging and diabetes mellitus. Non-enzymatic glycation can be studied in the presence of different sugars which are known to influence the physico-chemical properties and fluorophore production of the protein. In this present investigation we have used fructose as a glycating agent, since this sugar reacts with collagen at a faster rate than glucose. Fructose is closely involved not only in glycation but also in the polyol pathway [15–20]. The formation of new glycation-induced cross-links is slowed by several drugs and natural substances [21–24]. It has been reported that aspirin and amino guanidine inhibit the formation of pathological AGEs cross-links [25–34]. It has been previously shown that glycation and subsequent AGEs formation alters the molecular packing of collagen, both *in vivo* and *in vitro* [35–38]. Electron microscopy and synchrotron X-ray diffraction studies of NEG of collagen have also been carried out to study the surface charge distribution and interfibrillar spacing of collagen fibrils [39].

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Non-enzymatic glycation of collagen has been used in modern biomaterials science. Non-enzymatic glycation of collagen in the presence of glucuronic acid *in vitro* has been used to study for the self-organization of HAP nano-crystals on and within collagen fibrils [40–42].

Collagen, a prominent biopolymer is extensively used for tissue engineering applications, because its signature and physicochemical properties are retained in *in vitro* preparations. The physical stability of a protein depends on the protein aggregation or fibril formation. The *in vitro* self assembly of collagen molecules is an endothermic process and is usually studied by turbidity measurements, viscometry, fluorescence and X-ray scattering. Hence, in the present investigation the role of amino guanidine (AG) and aspirin on the non-enzymatic glycation of collagen *in vitro* were studied using thermal, conformational, fluorescence, turbidity and powder XRD measurements.

2. Materials and methods

2.1. Collagen solutions

Collagen solutions were prepared from freshly dissected 6-month-old male albino rat tails frozen at −20 °C, by acetic acid extraction and salting out with NaCl [43]. The purity of collagen preparation was confirmed by SDS–polyacrylamide gel electrophoresis. The concentration of collagen was determined from the hydroxyproline content [44]. The pH of the solution is around 3.0. The average molecular weight of collagen is 300,000 Da.

2.2. Preparation of collagen mixtures with fructose, amino guanidine and aspirin

Fructose, amino guanidine and aspirin used were purchased from Sigma Chemical Co., St. Louis, USA, and used as received. Glycated collagen was prepared by dissolving 1, 10 and 50 mM fructose with 1.5 mg/ml (w/v) of collagen solution. After 2 h of incubation with fructose, the glycated collagen solution was inhibited with 1, 5, 10 and 50 mM amino guanidine (AG) and aspirin, respectively. The glycated collagen solutions in the absence and presence of amino guanidine and aspirin were kept overnight at 4 °C for CD, viscosity and fluorescence measurements. Before all of these experiments, the solutions were allowed to equilibrate at room temperature.

2.3. UV–vis spectral studies

The UV absorption spectra for native, glycated collagen solution in the absence and presence of amino guanidine and aspirin were recorded using Shimaldzu UV-1700 (Pharmaspec) in the wave length range of 200–600 nm in quartz cuvette of 1 cm path length.

2.4. Fluorescence studies

The fluorescence experiments were carried out for glycated collagen solution in the absence and presence of amino guanidine and aspirin, respectively at the excitation wavelength of 280 nm. Cary Eclipse spectrofluorimeter was used to study the fluorescence measurements.

2.5. Circular dichroism (CD)

Non-enzymatic glycosylation and their inhibition induced conformational changes of type 1 collagen solution was monitored by means of CD measurements using a Jasco J-715 spectropolarimeter and a quartz cell of 1 mm path length. The protein concentration was 2×10^{-6} M for the far UV region. The CD readings were expressed as the molar ellipticity. The molar ellipticity at 222 and 197 nm was measured and Rpn ratio (ratio of the positive to negative band) was calculated for glycated collagen in the absence and presence of amino guanidine and aspirin.

2.6. Viscosity measurements

The melting temperature (T_m, °C) of the acetic acid soluble glycated collagen in the absence and presence of amino guanidine and aspirin were estimated by measuring the viscosity at different temperatures. The viscosity measurements were based on the flow rate of collagen solution through the capillary of an Ostwald viscometer and all the results were presented as viscosity relative to 0.5 M acetic acid in deionised water. The viscosity was calculated from the relation $\eta = (t - t_0)/t_0$ where 't_0' is the flow time of buffer and 't' is the flow time for experiments. The measurements were carried out at 25–50 °C and accuracy in the temperature was ±0.05 °C. All the experiments were carried out till three subsequent readings in the series reached a constant value. Plots of relative viscosity as a function of temperature were made for all experiments. The temperature that corresponds to the half maximum viscosity is the melting temperature.

2.7. Fibril formation

Collagen fibril formation was initiated by mixing collagen with phosphate buffer (0.2 M) and sodium chloride (2 M) in an ice bath. The pH of the solution was adjusted to 7.4 with sodium hydroxide (1.25 N). The final composition in the mixture was collagen (1.5 mg/ml), phosphate buffer (0.02 M), and sodium chloride (0.13 M). A control did not contain fructose and amino guanidine. Turbidimetric analysis was made with Shimaldzu UV-1700 Pharmaspec having thermostatically controlled cuvette holders. The blank was run simultaneously which contained the same mixture, but the collagen solution was replaced with equal volume of 0.05 M acetic acid. Collagen solution was incubated with 10 mM fructose for 2 h and 10 mM amino guanidine was added to the above mixture. At 37 °C, the turbidity was measured as the optical density at 313 nm for collagen (1.5 mg/ml) and treated with 10 mM fructose, 10 mM fructose–10 mM amino guanidine and 10 mM fructose–10 mM aspirin (w/v), respectively. The fibril formation rate was represented by $T_{1/2}$ determined from the fibril formation curve. The time at which the turbidity was half the plateau value at 40 min was taken as $T_{1/2}$.

2.8. X-ray diffraction

To investigate the structure and crystallinity of the samples, the fibrils were analyzed with an X-ray diffractometer, XRD (Miniflux 11, Rigaku diffractometer, Cu Kα radiation (λ = 0.15418), Japan. After the fibril formation with collagen, the gels were spread on a glass plate and allowed to dry completely in dust free air-conditioned room. These fibrous samples were scraped from the glass plate and used for XRD measurements. Similarly, gels were formed with collagen in the presence of 10 mM fructose, 10 mM fructose–10 mM amino guanidine and 10 mM fructose–10 mM aspirin. The samples were scanned from 0 to 80° with a scan speed of 4°/min.

2.9. Differential scanning calorimetry (DSC)

Known amount of native rat tail tendon (RTT) collagen fibres (generally 5–10 mg) were immersed in 10 mM of fructose (in double distilled water) for 2 h. Some of the fructose treated rat tail tendons (RTTs) were washed thoroughly with double distilled

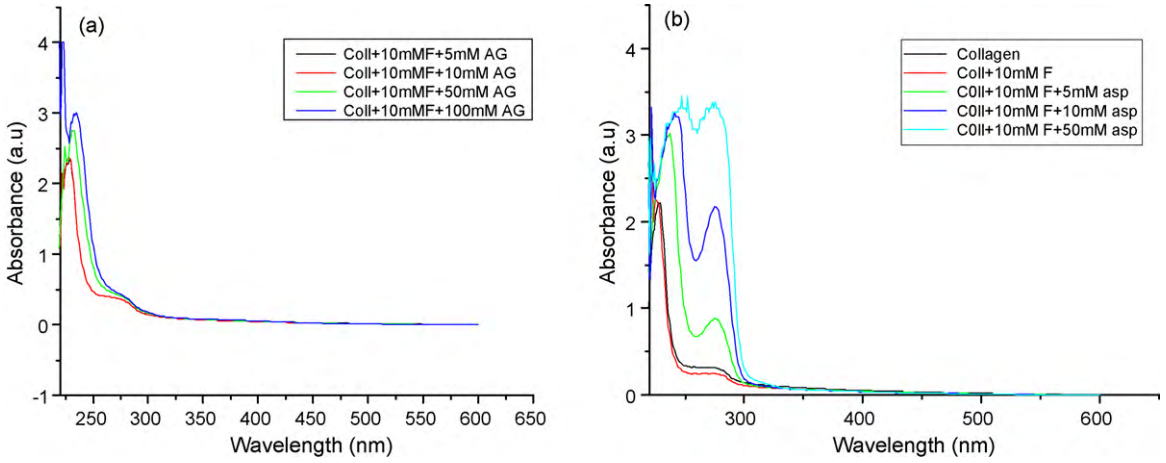

Fig. 1. Absorption spectra of (a) collagen in the presence of 10 mM fructose at various concentrations of amino guanidine and (b) collagen treated with 10 mM fructose and various concentrations of aspirin.

water and inhibited with 10 mM amino guanidine (in double distilled water) overnight. The soaked samples were blotted uniformly and hermetically encapsulated in aluminum pans. The samples were placed in a differential scanning calorimetric cell of a DSC 0200 V23.10 Build 79 instrument. The heating rate was maintained at a scanning rate of 3 °C/min. Before evaluation, the thermal runs were subtracted through analogous runs of an empty pan. DSC instrument was calibrated using indium as standard and fitted with intra-coolers. The degradation temperature for collagen to gelatin process was taken as a maximum peak height of the heating

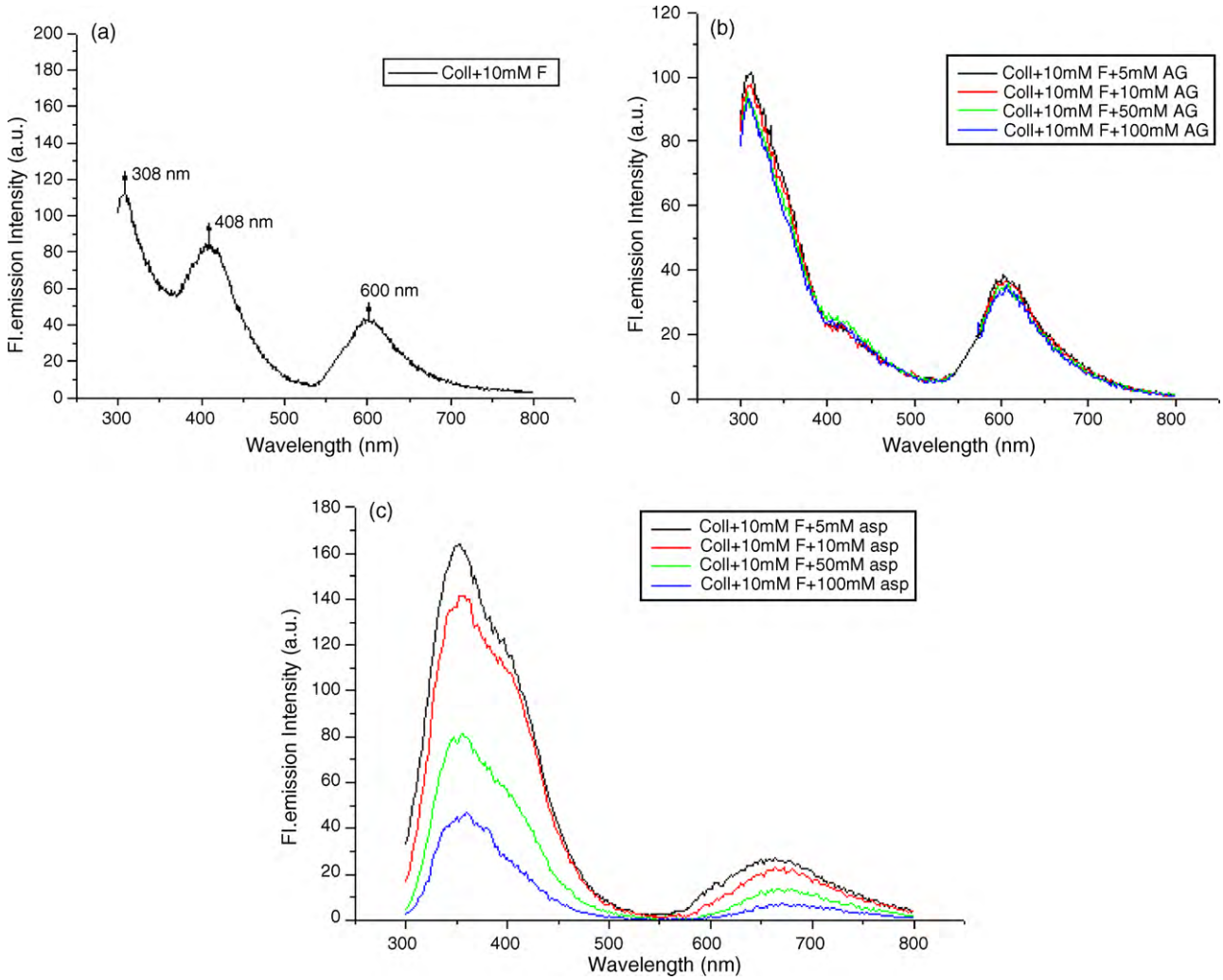

Fig. 2. Fluorescence emission spectra of (a) collagen in the presence of 10 mM fructose, (b) glycated collagen in the presence of various concentrations of amino guanidine and (c) aspirin. Emission at 280 nm.

endotherm. As aspirin is not soluble in water, the glycated RTT were inhibited with 1 mM aspirin in methanol and DSC thermogram was done.

2.10. Statistical analysis

For all experiments, one-way analysis of variance (ANOVA) was executed to determine the differences among glycated collagen in the absence and presence of amino guanidine and aspirin. A significant difference corresponds to $P < 0.05$ (confidence level >95%).

3. Results and discussions

3.1. Absorption spectra

Spectroscopy and fluorescence techniques are well suited for studying the chemical and physical properties of non-enzymatic glycation of proteins because of their sensitivity and non-destructive nature. The absorption spectra of glycated collagen in the presence of various concentrations of amino guanidine and aspirin are given in Fig. 1a and b, respectively. The absorption spectra show a broad peak in the region 250–280 nm for AG inhibited NEG of collagen. As seen from Fig. 1a, no significant difference is observed in the NEG of collagen at various concentrations of AG ($P > 0.05$). Aspirin inhibited NEG of collagen shows clear absorption peaks at 235 and 280 nm. A significant difference is observed at various concentrations of aspirin (Fig. 1b). The absorption spectra show the concentration dependence of the additives.

3.2. Fluorescence studies

Native collagen as well as AG treated collagen does not show any fluorescence. However, fructose treated collagen exhibits fluorescence due to Maillard reaction. The fluorescence emission spectra of collagen treated with fructose at the excitation wavelength of 280 nm are given in Fig. 2a. Three emission maxima are observed at 308, 408 and 600 nm, respectively. The fluorescence emission spectra of glycated collagen in the presence of various concentrations of amino guanidine at the same excitation wavelength are given in Fig. 2b. There are two emission maxima at 312 and 600 nm. There is no significant change in the fluorescence emission intensity at various concentrations of amino guanidine. Fig. 2c shows the fluorescence emission intensity of glycated collagen in the presence of various concentrations of aspirin. There is a significant decrease in the fluorescence emission intensity as the concentration of aspirin increases. This is indicative of the inhibiting ability of aspirin at various concentrations. The emission maximum is shifted to 353 and 662 nm for aspirin inhibited NEG of collagen. The emission maximum around 600 nm may be due to aggregation excimer. Takagi et al. [16] found that AG prevents the fructose derived AGE formation

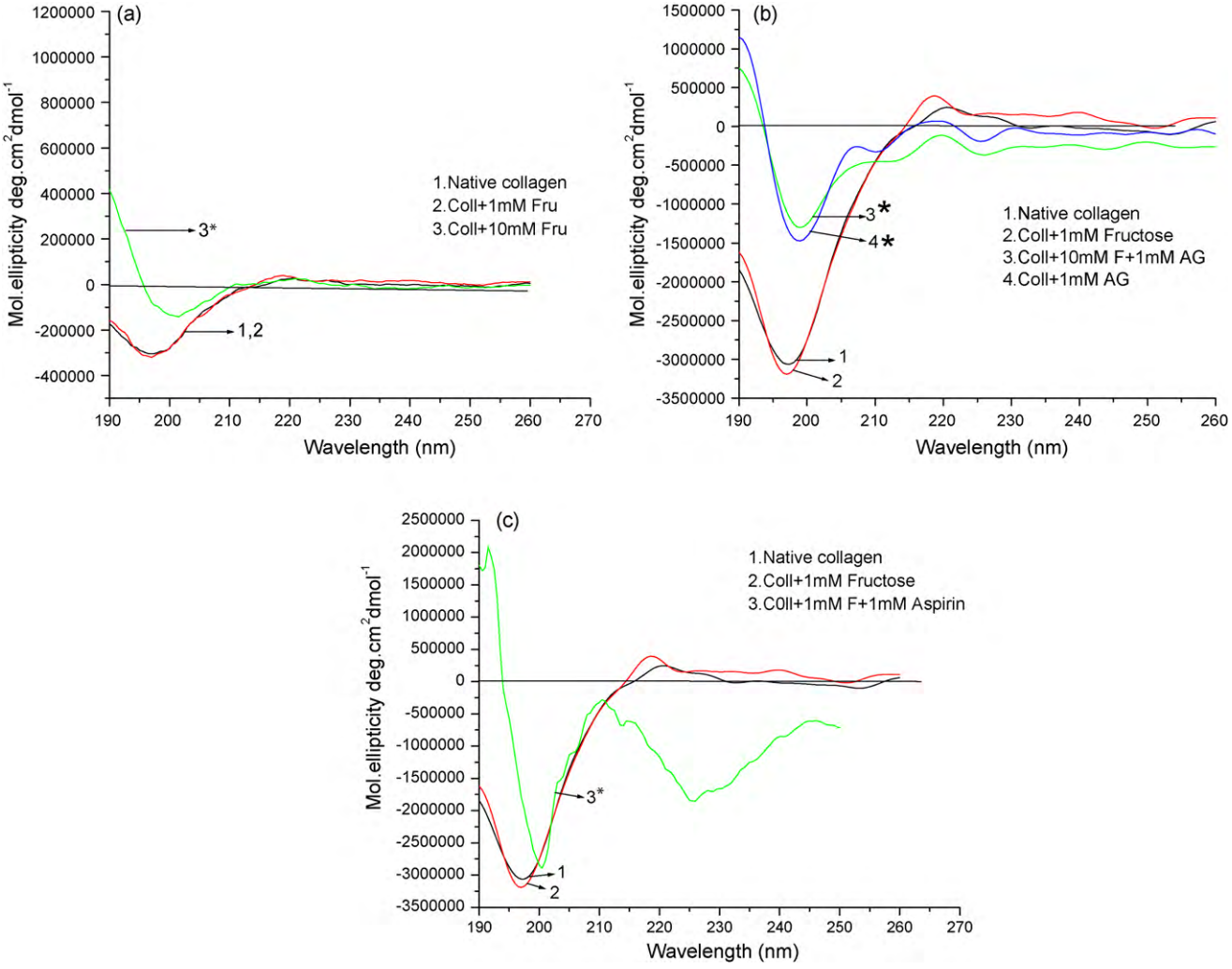

Fig. 3. (a) CD spectra of collagen treated with various concentrations of fructose, comparison of CD spectra of glycated collagen in the absence and presence of (b) amino guanidine and collagen treated with AG, and (c) aspirin. *$P < 0.05$ is significantly different for comparison of glycated collagen in the absence and presence of AG and aspirin.

Table 1
Molar ellipticity and Rpn ratio of collagen solution treated with fructose at different concentrations.

Specification	Molar ellipticity (° cm² dmol⁻¹)		Rpn ratio
Native collagen	197 nm	−303220	
	220.75 nm	28691.6	0.095
Coll-1 mM fructose	197 nm	−318508	
	220.75 nm	28691.6	0.09
Coll-10 mM fructose	200.86 nm	−137017	
	218.99 nm	37568.9	0.274

possibly through the trapping of the carbonyl group. Amino guanidine, being a nucleophilic hydrazine compound acts as a potent inhibitor of AGEs formation and cross-links with protein such as collagen. Aspirin, also a known inhibitor of non-enzymatic glycosylation, was effective in preventing the fructose-induced NEG of collagen. The significant change in the fluorescence spectra in the presence of AG and aspirin may be due to the structural differences of AG and aspirin. Fluorescence studies indicate that there is possible aggregation and inhibition of NEG of collagen in the presence of amino guanidine and aspirin.

3.3. Circular dichroism

Collagen adopts the polyproline-11-like helical conformation and exhibits CD spectra in solution that are characterized by a large negative peak. CD spectra of collagen solution treated with various concentrations of fructose in the absence and presence of amino guanidine and aspirin are given in Fig. 3a–c. There is no significant change in the CD spectrum of collagen solution treated with 1 mM of fructose as compared to the native ($P > 0.05$). This is attributed to the formation of aggregates. The collagen solution treated with 10 mM fructose shows decrease in molar ellipticity at 220 nm with an increase in value at 197 nm given in Table 1, showing that a part of the triple helix of native collagen has transformed into random coil configuration. However, these CD spectral shapes and peak position cannot be used conclusively to establish the presence of a triple helical conformation. Table 2 shows the CD parameter, Rpn, denoting the ratio of positive peak intensity to negative peak intensity (absolute values) was found to be useful in establishing the presence of triple helical conformation. The Rpn ratio for collagen treated with 10 mM fructose is ≈0.27. This also confirms that at higher concentrations of fructose a part of the triple helix of native collagen transformed to random coil configuration. The positive peak at 220 nm in the CD spectrum of glycated collagen in the presence of amino guanidine disappeared suggesting the collapse of triple helical configuration. Similar trend was observed for non-enzymatic glycation of collagen in the presence of aspirin and is given in Fig. 3c. Circular dichroism of the non-enzymatic browning products of poly-L-lysine and albumin was studied by Liang [45]. They have reported similar changes in CD parameters and reason out the same due to alterations in the intra- and inter-

Table 2
Comparison of molar ellipticity and Rpn ratio of collagen solution treated with fructose, fructose–AG and fructose–aspirin.

Specification	Molar ellipticity (° cm² dmol⁻¹)		Rpn ratio
Native collagen	197 nm	−3056264	0.085
	221 nm	248549	
Coll-1 mM fructose	197 nm	−3182424	0.126
	218 nm	400812	
Coll-1 mM fructose–1 mM AG	199 nm	−1304524	–
	219 nm	−103828	
Coll-1 mM fructose–1 mM aspirin	200	−285248	–
	–	–	

Table 3
Melting temperature of collagen treated with fructose, fructose–AG and fructose–aspirin.

Specification	Melting temperature (°C)
Native collagen	39
Coll-10 mM fructose	39
Coll-10 mM fructose–10 mM AG	36
Coll-10 mM AG	36
C-10 mM fructose–10 mM aspirin	37

molecular interactions. In the present case, it is suggested that the observed changes could be due to partial unfolding or alterations in inter- and intra-molecular interactions of collagen.

3.4. Viscosity measurements

The viscosity of a protein solution depends on intrinsic characteristics such as molecular mass, size, volume, shape, surface charge and ease of deformation. In addition, viscosity is influenced by environmental factors such as pH, temperature, ionic strength, ion type and shear conditions. The temperature that corresponds to half maximum viscosity is the melting temperature (T_m, °C). The melting temperature of glycated collagen in the absence and presence of amino guanidine and aspirin are given in Table 3 and was calculated from the relative viscosity vs. temperature curve given in Fig. 4. There is nearly 3 °C decrease in the melting temperature of glycated collagen in the presence of amino guanidine and aspirin. The melting temperature shifts almost identically for collagen and collagen–fructose solution after the addition of AG. This suggests that helix–coil transition of collagen and collagen–fructose solutions are affected by AG in a similar manner. Therefore, the intra-molecular interactions within the helix are not affected by the presence of fructose. The viscosity of solutions of dextrans with selected sweeteners has been studied and was reported that the molecular weight of polysaccharide is a dominating factor in controlling the rheology of the blends [46]. In the present study, the viscosity of glycated collagen solution decreases slightly in the presence of amino guanidine and aspirin as seen in Fig. 4. Statistical analysis revealed that $P = 0.5$, 0.14 and 0.22 for the viscosity of glycated collagen in the absence and presence of AG and aspirin, respectively. The hydration of collagen molecule is different causing the change in viscosity. The viscosity is maximum for collagen

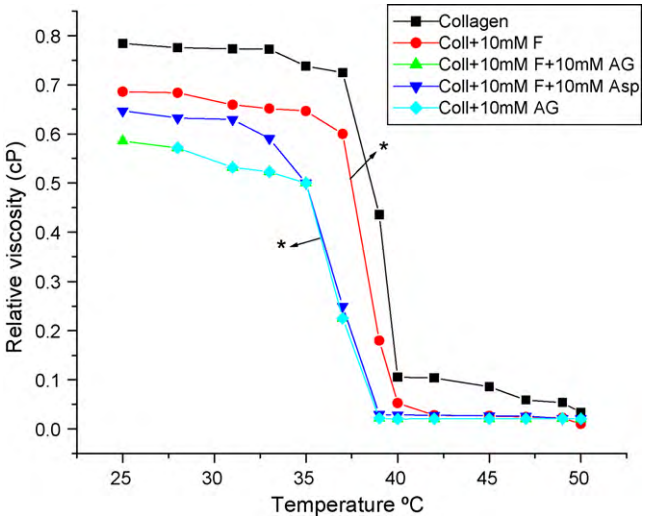

Fig. 4. Plot of relative viscosity vs. temperature of glycated collagen in the absence and presence of amino guanidine and aspirin and collagen treated with AG. *$P > 0.05$ is not significantly different for comparison of glycated collagen in the absence and presence of AG and aspirin.

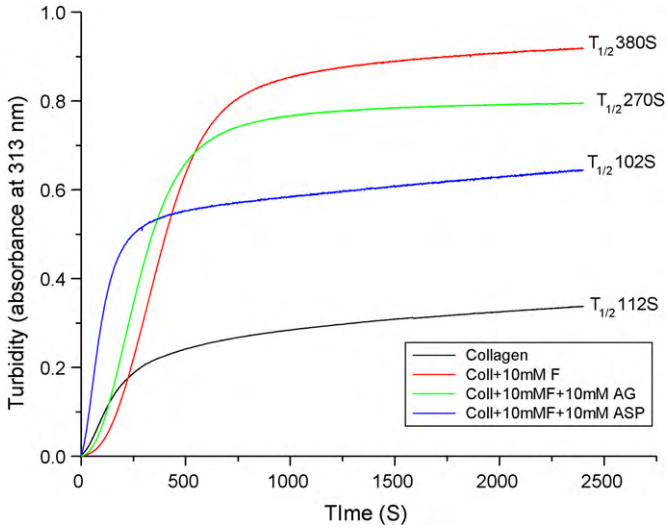

Fig. 5. Kinetics of fibril formation of collagen with fructose, fructose + AG and fructose + aspirin.

and it changes as $\eta_{collagen} > \eta_{collagen-fructose} > \eta_{collagen-fructose-AG}$. The decrease in the viscosity of glycosylated acid soluble collagen is indicative of *in vitro* AGEs.

3.5. Fibril formation

Collagen molecules form fibrils or fibril bundles in tissues and can be extracted by acid solubilisation. Acid soluble collagen molecules self assemble and form fibrils under physiological conditions [47–51]. The rate of fibril formation $T_{1/2}$ (s) was calculated from turbidity curve for glycated collagen in the absence and presence of AG and aspirin and is given in Fig. 5. $T_{1/2}$ is larger and the fibril formation is slower in the presence of fructose and fructose–AG as compared to native collagen. Fibril formation is faster and $T_{1/2}$ values are smaller for glycated collagen in the presence of aspirin. The increase in OD in the case of collagen–fructose and collagen–fructose–AG can be attributed to the amount of prenucleic fibrillar collagen assemblies. This increase in the turbidity plateau may also be due to increase in the particle size.

3.6. XRD analysis

Hierarchical self assembly of nano-fibrils in mineralized collagen and apatite formation on collagen fibrils have been studied and reported in literature [52–56]. It has been reported that self-organization of HAP nano-crystals on and within collagen fibrils was intensified by carboxymethylation which is the major product of the *in vitro* NEG between glucuronic acid and collagen [42]. XRD pattern obtained for reconstituted collagen fibrils with fructose, fructose–amino guanidine and fructose–aspirin are given in Fig. 6. The narrow peak in between 31 and 32 of 2θ is common for native and treated samples. The sharp peaks indicate the high crystalline nature of the native and treated analogue. The percent crystallinity was calculated relative to native collagen. The intensity of the reflections decrease for collagen fibrils treated with fructose, which may be due to the reduction in the crystallinity when compared with native. The increase in the intensity of the peaks for collagen fibrils treated with fructose–AG show the high crystalline nature of fibrils and the enhancement of self assembly process in the presence of AG. Whereas there is a slight increase in the intensity of reflection for collagen fibrils treated with fructose–aspirin when compared to that of native. This can be explained in terms of the increase in the particle size of the collagen fibrillar assembly. It

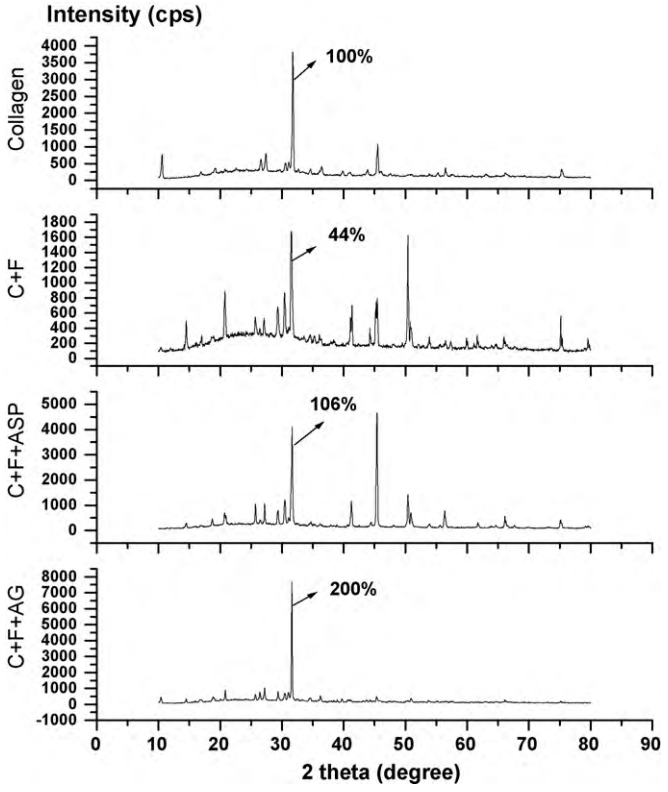

Fig. 6. X-ray diffraction pattern of native, fructose and fructose + AG and fructose + aspirin treated reconstituted collagen fibrils.

is very difficult to predict the structure of this macromolecule from the 2θ and '*d*' values given in Fig. 7.

3.7. Differential scanning calorimetry (DSC)

DSC is considered a useful method for assessing glucose-mediated cross-linking *in vitro* with non-physiological glucose concentrations. The *in vivo* use in biological samples is limited due to the lack of sensitivity. However, DSC remains a quick and well defined method as compared to enzymatic digestibility. This method is based on the determination of the biopolymer degra-

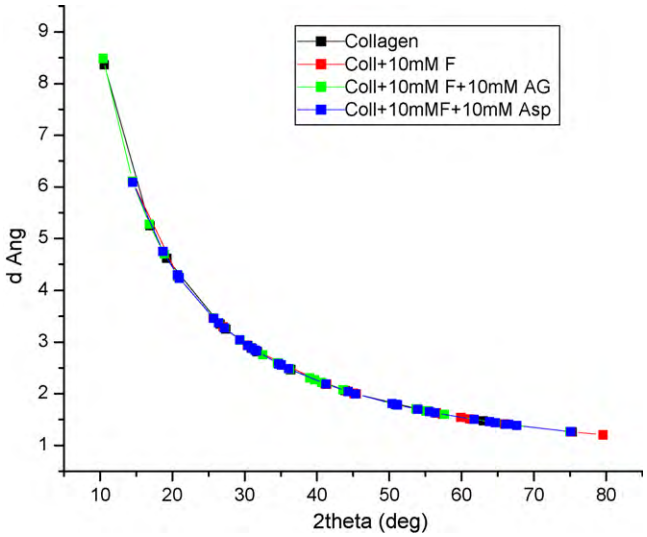

Fig. 7. 2θ and '*d*' spacing values of collagen, treated with fructose, fructose + AG and fructose + aspirin.

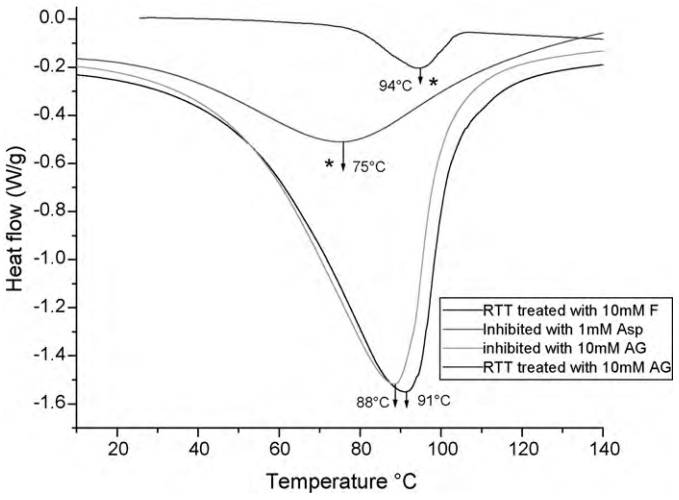

Fig. 8. DSC thermogram of glycated RTT and inhibited with AG and aspirin and RTT treated with AG. $^{*}P < 0.05$ is significantly different for comparison of glycated RTT collagen fibre and inhibited with aspirin and treated with AG.

dation temperature (T_d). The change in T_d can provide adequate reference for the cross-linking and tissue strength. DSC thermograms of glycated RTT collagen fibre in the absence and presence of AG and aspirin and RTT treated with AG are given in Fig. 8. It is an endothermic process. Incubation of RTT with 10 mM fructose and 10 mM AG showed an increase in collagen cross-linking expressed as increase in shrinkage temperature of 91 and 94 °C, respectively. It has been reported that glycation was exploited to stiffen and strengthen tissue equivalents for tissue engineering [57]. Glycation-induced matrix stability in the rabbit Achilles tendon has also been reported [58]. There is nearly 3 ± 1 °C and 15 °C decrease in shrinkage temperature of glycated collagen fibres in the presence of 10 mM AG and aspirin, respectively. The experimental results suggest that aspirin is a good inhibitor than amino guanidine in the thermal properties of NEG of collagen for *in vitro* studies.

4. Conclusion

The present study explores the effect of AG and aspirin on the non-enzymatic glycation of collagen *in vitro* using thermal, spectral and XRD methods. Two emission maxima are observed for both AG and aspirin inhibited NEG of collagen. There is no significant change in the fluorescence emission spectra at different concentrations of AG treated NEG of collagen. The emission intensity decreases as the concentration of aspirin increases. The emission maximum around 600 nm may be due to aggregation excimer. The positive peak at 220 nm of CD spectrum of glycated collagen in the presence of amino guanidine and aspirin disappeared suggesting that the triple helical configuration has collapsed. The decrease in viscosity of glycosylated acid soluble collagen in the absence and presence of amino guanidine and aspirin is indicative of *in vitro* AGEs. Aspirin treatment produces drastic changes in the thermal properties of fructose treated rat tail tendon collagen fibre. Powder XRD results show the high crystalline nature of fibrils and the enhancement of self assembly processes in the presence of AG than aspirin. To conclude, in the non-enzymatic glycation of collagen both AG and aspirin play a pivotal role in the aggregation and self assembly processes. However, AG significantly influences the self assembly of collagen nano-fibrils while aspirin facilitates their aggregation processes. Studies of this kind create a new basis for the practical use of Maillard reaction products in materials science and biomedicine.

References

[1] M.G. Patino, M.E. Neiders, S. Andreana, B. Noble, R.E. Cohen, Implant. Dent. 11 (2002) 280–285.
[2] L.C. Abraham, E. Zuena, B. Perez-Ramirez, D.L. Kaplan, J. Biomed. Mater. Res. B: Appl. Biomater. 87B (2008) 264–285.
[3] S. Tomohiko, K. Takuji, H. Kawada, H. Kazulik, J. Dermatol. Sci. 53 (2009) 77–79.
[4] V. Monnier, in: J.W. Baynes, V.M. Monnier (Eds.), The Maillard Reaction in Aging, Diabetes and Nutrition, vol. 1, Alan R. Liss, New York, 1989, pp. 1–22.
[5] A.J. Bailey, T.J. Sims, N.C. Avery, C.A. Miles, Biochem. J. 296 (1993) 489–496.
[6] V.M. Monnier, A. Cerami, Science 211 (1981) 491–493.
[7] C.J.A.L. Mentink, M. Hendriks, A.A.G. Levels, B.H.R. Wolffenbuttel, Clin. Chim. Acta 321 (2002) 69–76.
[8] M.J.C. Kent, N.D. Light, A.J. Bailey, Biochem. J. 225 (1985) 745–752.
[9] J.W. Baynes, Exp. Gerontol. 36 (2001) 1527–1537.
[10] H. Vlassara, Anal. Med. 28 (1996) 419–426.
[11] G. Munch, R. Schenzel, C. Loske, A. Wong, N. Duraney, J.J. Li, H. Vlassara, M.A. Smith, G. Perry, P. Reiderer, J. Neural Transm. 105 (1998) 439–465.
[12] C.A. Colaco, M.D. Ledesma, C.R. Harrington, J. Avila, Nephrol. Dial. Transplant. 11 (1996) 7–12.
[13] Z. Makita, K. Yanagisawa, S. Kuwajima, et al., J. Diabetes Complicat. 9 (1995) 265–268.
[14] J. Hadley, N. Malik, K. Meek, Micron 32 (2001) 307–315.
[15] J.D. McPherson, B.H. Shilton, D.J. Walton, Biochemistry 27 (1988) 1901–1907.
[16] Y. Takagi, A. Kashiwagi, Y. Tanaka, T. Asahina, R. Kikkawa, Y. Shigeta, J. Diabetes Complicat. 9 (1995) 87–91.
[17] C.G. Schalkwijk, C.D.A. Stehouwer, V.W.M. vanHinsbergh, Diabetes Metab. Res. Rev. 20 (2004) 369–382.
[18] V.M. Monnier, B.A. Vishwanath, K.E. Frank, C.A. Elmets, P. Dauchot, R.R. Kohn, N. Engl. J. Med. 314 (1986) 403–408.
[19] A. Schmith, J. Schmith, G. Münchi, J. Gasic-Milancovic, Anal. Biochem. 338 (1995) 201–215.
[20] H. Kato, D.B. Shin, F. Hayase, Agric. Biol. Chem. 51 (1987) 2009–2011.
[21] N. Matsuura, T. Aradate, C. Sasaki, H. Kojima, M. Ohara, J. Hasegava, M. Ubukata, J. Health Sci. 48 (2002) 520–526.
[22] J. Zhang, M. Slevin, Y. Duraisamy, J. Gaffiny, C.A. Smith, N. Ahmed, Biochim. Biophys. Acta: Mol. Basis Dis. 1762 (2006) 551–557.
[23] D.K. Yue, S. McLennan, D.J. Handerman, L. Delbridge, T. Reeve, J.R. Turtle, Diabetes 33 (1984) 745–751.
[24] M. Sensi, M.G. De Rosse, F.S. Celi, A. Cristina, C. Rosati, D. Perrett, D. Andreani, U. Di Mario, Diabetologia 36 (1993) 797–801.
[25] G.N. Rao, E. Cotlier, Biochem. Biophys. Res. Commun. 151 (1988) 991–996.
[26] T.S. Kern, R.L. Engerman, Diabetes 50 (2001) 1636–1642.
[27] M. Oimomi, N. Igaaki, T. Ohara, M. Sakai, T. Nakamichi, F. Hata, S. Baba, Kobe J. Med. Sci. 35 (1989) 255–259.
[28] J.T. Skamarauskas, A.G. Mckay, J.V. Hunt, Free Radic. Biol. Med. 21 (1996) 801–812.
[29] M. Brownlee, H. Vlassara, A. Kooney, P. Ulrich, A. Cerami, Science 232 (1986) 1629–1632.
[30] H. Klandorf, Q. Zhoq, A.R. Sams, Poultry Sci. 75 (1996) 432–437.
[31] I. Misur, B. Turk, Croat. Chem. Acta 79 (2001) 455–465.
[32] P.R. Odetti, A. Borgoglio, A. Pascale, R. Rolandi, L. Adezati, Diabetes 39 (1990) 796–801.
[33] S. Swamy-Mruthini, K. Green, C.C. Abraham, Exp. Eye Res. 62 (1996) 505–510.
[34] P. Urios, A.-M. Borsos, J. Garaud, S. Feing-Kwang-Chan, M. Sternbey, Int. Congr. Ser. 1245 (2002) 413–414.
[35] G.B. Sajithlal, P. Chithra, G. Chandrakasan, Biochim. Biophys. Acta 1407 (1998) 215–224.
[36] N.S. Malik, S.J. Moss, N. Ahmed, A.J. Furth, R.S. Wall, K.M. Meek, Biochim. Biophys. Acta 1138 (1992) 222–228.
[37] N.S. Malik, K.M. Meek, Age Aging 25 (1996) 279–284.
[38] S. Tanaka, G. Avigad, B. Brodsky, E.F. Eikenberry, J. Mol. Biol. 203 (1988) 495–505.
[39] J. Hadley, N.S. Malik, K.M. Meek, Micron 32 (2001) 307–315.
[40] H. Ehrlich, T. Hanke, C. Fischer, U. Schwarzenbolz, T. Henle, R. Born, H. Worch, J. Membr. Sci. 326 (2008) 254–259.
[41] H. Ehrlich, T. Hanke, C. Fischer, A. Frolov, T. Langrock, R. Hoffmann, U. Schwarzenbolz, T. Henle, R. Born, H. Worch, Int. J. Biol. Macromol. 44 (2009) 51–56.
[42] H. Ehrlich, T. Hanke, C. Fischer, A. Frolov, T. Langrock, R. Hoffmann, U. Schwarzenbolz, T. Henle, R. Born, H. Worch, J. Biomed. Mater. Res. B. Appl. Mater. 92 (2010) 542–551.
[43] G. Chandrakasan, D.A. Torchia, K.A. Piez, J. Biol. Chem. 251 (1976) 6062–6067.
[44] J.F. Woessner Jr., Arch. Biochem. Biophys. 93 (1961) 440–447.
[45] J.N. Liang, Int. J. Biol. Macromol. 12 (1990) 273–277.
[46] J. Mazurkiewicz, K. Rębilas, Eur. Food. Res. Technol. 213 (2001) 470–473.
[47] D. Stamov, M. Grimmer, K. Salchert, T. Pompe, C. Werner, Biomaterials 29 (2008) 1–14.
[48] A. Veis, K. Payne, in: M.E. Nimni (Ed.), Collagen, Biochemistry, vol. 1, CRC Press, Boca Raton, FL, 1988.
[49] J.-H. Bradt, M. Mertig, A. Teresiak, W. Pompe, Chem. Mater. 11 (1999) 2694–2701.

[50] M. Rasponti, M. Viola, M. Sonaggere, M. Enrica Tira, R. Tenni, Biomacromolecules 6 (2007) 2087–2091.
[51] J.F. White, J.A. Werkmeister, J.A. Darby, T. Bisucci, D.E. Brik, J.A.M. Ramsahw, J. Struct. Biol. 137 (2002) 23–30.
[52] W. Zhang, S.S. Liao, F.Z. Cui, Chem. Mater. 15 (2003) 3226–3321.
[53] E.K. Girija, Y. Yokagawa, F. Nagata, J. Mater. Sci.: Mater. Med. 15 (2004) 593–599.
[54] J. George, Y. Kuboki, T. Miyata, Biotechnol. Bioeng. 95 (2006) 404–411.
[55] M. Otsuka, T. Kuninaga, K. Otsuka, W.I. Higuchi, J. Biomed. Mater. Res. B: Appl. Biomater. 79 (2006) 176–184.
[56] L. Yang, F. Ye, R. Xing, B. Zhang, Q. Ren, Supramol. Chem. 20 (2008) 761–763.
[57] T.S. Girton, T.R. Oegema, R.T. Tranquillo, J. Biomed. Mater. Res. 46 (1999) 87–92.
[58] G.K. Reddy, L. Stehno-Bithel, C.S. Enwemeka, Arch. Biochem. Biophys. 399 (2002) 174–180.