CHAPTER 6
(Result and Discussion)
6.1 GLYCATION ASSAY USING NANO SENSORS

6.1.1 Systematic Colour Development With Glycation

Fig. 6.1.1 GNP solutions synthesized using fructosylated hemoglobin (withdrawn on different days) as template. The figure shows a systematic increase in the intensity of pink color at pH 7 (upper panel) and a blue to pink transition at pH 5 (lower panel) with progress of the reaction.

The formation of graded colors at two different pH values (see Fig. 6.1.1) confirms the spectroscopic finding described in Fig. 6.1.2a and Fig. 6.1.2b. Fig. 6.1.3 clearly illustrates that GNPs of small size predominates in the solution when AGEs are present in higher population as described by the TEM images.
6.1.2 Behaviour of Plasmon Bands

Fig. 6.1.2(a) shows the dependence of the plasmon resonance intensity (lighter line) and $\lambda_{\text{max}}$ (darker line) on the number of days of incubation, i.e. on the extent of glycation, as a result of GNP seeding at pH 7. The inset in the figure shows the UV-Vis absorbance spectra taken on different days.
To assess the possibility of GNP-based detection of the process, GNP seeding was performed at different stages of glycation. It was observed that the size of the particles as well as their plasmon resonance peak responded systematically to the formation of AGEs. Both the Fig. 6.1.2a and 6.1.2b clearly indicates the time course of shift of these two parameters, at two different pH, the inset showing the gradual shift of the GNP spectrum.

Fig. 6.1.2b Results of similar measurements carried out at pH 5.0.
6.1.3 Particle size distribution from TEM

Fig. 6.1.3 The two upper panels show TEM images of GNP samples synthesized using Hb templates incubated for 1 (left) and 12 (right) days in 0.05 M fructose solution. The lower panel shows the size distribution of GNPs obtained by analyzing these two TEM images.

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The particle size of the GNPs determined from TEM studies is shown in Fig. 6.1.3. Electron micrographs of nanoparticles synthesized on Hb templates taken from the 1st and 12th days of incubation are shown in the left and right upper panels of Fig. 6.1.3, respectively. The study reveals that smaller-sized particles are formed in aliquots withdrawn from the GNP solution formed using the 12th day protein as seed, whereas slightly larger particles were formed in the aliquots from the GNP formed in the 1st day. The number of particles, however, varied more significantly, the total particle number per field being much higher in case of the sample containing AGEs.
6.1.4 CD Measurement

Fig. 6.1.4 CD spectra of Hb before and after synthesis of GNPs (at pH 7). Aliquots drawn on the 0th day and 8th days (control and glycated hemoglobin) were used for the measurement of secondary structure in the range 190-250 nm.

The secondary structure of fructosylated Hb before and after synthesis of GNP is shown in Fig. 6.1.4. While the secondary structure of the unglycated sample changed significantly before and after seeding by gold nanoparticle, no such change was observed with glycated samples subjected to glycation for a period of eight days.
6.1.5 The FTIR Analysis

Fig. 6.1.5 FTIR spectrum of Hb and fructosylated Hb (FHB) in presence and absence of GNP seeding. The GNP seeded spectra clusters to identical amide I transmittance region.
Fig. 6.1.5 shows FTIR spectrum of Hb and fructosylated Hb (FHb) in presence and absence of GNP seeding. The GNP seeded spectra clusters to identical amide I transmittance region.

The FTIR spectrum for hemoglobin, its extensively glycated form (at which AGE formation has occurred) and Hb seeded with GNP shows some interesting features. The amide I peak is affected as a result of glycation as well as GNP seeding. While both factors reduce the transmittance of the amide peak, the effect of GNP is independent of the glycation state of the protein template. Similar GNP induced fixation of secondary structure is also observed in the CD data.

This chapter deals with the new method developed for detection of advancement of glycation reaction using nanoparticles. How Gold nanoparticles used by seeding technique detects the progress of the reaction is illustrated in the sections 6.1.1-6.1.5.
6.2 DISCUSSION

Noble metal nanoparticles directly conjugated to globular proteins has been reported earlier (78). In this work the direct conjugation method has been used to develop a bioassay for non-enzymatic glycation of proteins. It is shown that nanoparticles formed on conformational templates of proteins can be helpful in tracking protein modification caused by glycation. Advanced glycation is accompanied with formation of AGEs. It is not clear, however, whether the changes observed are due to glycation related structural changes in the protein or due to accumulation of AGEs that is associated with the process.

Our results indicate that glycation induced seeding of GNP shows a pH effect. At normal pH, the plasmon resonance shifts towards lower wavelength maxima (towards intense red color), implying formation of smaller particles. At lower pH, the progress of glycation does not shift the plasmon resonance maxima appreciably. In both cases however the particle number increases with progress of glycation. Such increase in intensity of the plasmon band (79) could be either due to multiple layers of clusters, or may be simply due to higher number of seeding of particles. Alterations due to fructosylation, such as formation of β-sheet or cross-linked small peptides, may help in the formation of such clusters.

The FTIR data indicated that the –NH₂ group is probably the seeding site. Fructosylated Hb molecules are evidently more accessible for GNP seeding. The presence of GNP seemed to fix the structure in a stable state that is independent of its glycation condition. This follows from both the FTIR and CD data, according to which the presence of nanoparticles forces the protein into a structure independent of its state of glycation.

Hb and fructosylated Hb sample incubated for 10 days shows that the amide I peak is masked in the latter. The reaction between free –NH₂ group and the reducing end of the sugar undergoes significant changes and leads to specific masking of N-H bond (80). Synthesis of GNP on the protein template also diminishes the peak signal. This can be explained in the following way: Seeding of GNP is facilitated by the presence of amino group, which has a reducing property. Therefore the amino group used for seeding is not available to produce signal and is masked as well.