Discussion
Hyperglycemia plays an important role in the pathogenesis of diabetic complications by increasing protein glycation with the gradual build up of advanced glycation end-products (AGEs) in body tissues. Protein glycation and the formation of AGEs are accompanied by increased free radical activity by mitochondria that contribute toward the biomolecular damage in diabetes (Ahmed, 2005). AGE formation is an inevitable process in vivo and can be accelerated under pathological conditions such as oxidative stress. The increase in glycoxidation products in plasma and tissue proteins suggests that oxidative stress is increased in diabetes (Baynes and Thorpe, 1999; Ahmed et al., 2005). The majority of the glycated proteins in plasma of diabetic patients exist as Amadori products rather than in the more labile Schiff base form (Curtiss and Witztum, 1983; Cohen and Ziyadeh, 1994) and only a small part of the Amadori products undergo subsequent rearrangements to AGEs. Among AGE-structures, N\(^{\alpha}\)-carboxymethyllysine (CML) formation by oxidative cleavage of Amadori adducts represent a major pathway in vivo (Ahmed et al., 1986; Smith and Thornalley, 1992; Wells-Knecht et al., 1995). \(\cdot\)OH generated by Fenton reaction between Fe\(^{2+}\) and Amadori product-derived endogenous H\(_2\)O\(_2\) plays an important role in oxidative cleavage of Amadori compounds into CML (Nagai et al. 1997). These modifications on protein may lead to the formation of neoantigens which could in turn initiate autoimmunity.

Since amino groups of lysine residues are major sites for glycation of proteins, poly-L-lysine was chosen as a model compound for this study. It was modified with glucose and the resultant glycated poly-L-lysine was further modified with \(\cdot\)OH generated through Fenton reaction. High degree of Amadori product formation in the glycated poly-L-lysine was detected by NBT assay, which is specific for Amadori products but not for AGEs (Johnson et al., 1982; Lapolla et al., 2005). Our results showed higher yield of Amadori products in glycated poly-L-lysine than ROS-glycated poly-L-lysine. The yield got further increased when glycation was carried out in the presence of DTPA, a metal ion chelator which is known to inhibit glycoxidation and cross linking (Graf et al., 1984 and 1987). The chelator is also known to inhibit the development of AGE-specific fluorescence in proteins without significantly affecting the glycation of proteins (Fu et al., 1994).

The thiobarbituric acid (TBA) assay for glycated proteins has been used for providing structural evidence of hexose bound to hemoglobin (Ney et al., 1981). The
higher yield of HMF in glycated poly-L-lysine than in ROS-glycated poly-L-lysine is in agreement with the earlier finding (Tsuchiya et al., 1984).

The far-UV spectrum of poly-L-lysine exhibited a peak at 202 nm representing a randomly coiled form. This is in accordance with an earlier study (Rosenheck and Doty, 1961). However, hyperchromicity was observed in the case of glycated poly-L-lysine and ROS-glycated poly-L-lysine which could be due to structural perturbation in poly-L-lysine as a result of unfolding and aggregation of molecules upon glycation and -OH mediated oxidation. Glycation induced protein unfolding leading to aggregation has earlier been reported (Perry et al., 1987; Bucciantini et al., 2002).

ROS modification of glycated poly-L-lysine in the presence of various metal ion chelators and antioxidants was undertaken to ascertain the particular reactive oxygen species involved in the reaction. The reaction was significantly inhibited by EDTA, catalase and mannitol, whereas the effect of SOD was quite insignificant. This confirms the involvement of 'OH radical in Fe^{2+} and H_2O_2 mediated ROS modification of Amadori poly-L-lysine. The slightly lower inhibition by mannitol, as compared to catalase could be explained by the possibility that hydroxyl radical generated by Fenton reaction near metal-binding sites of glycated proteins can react readily with 2,3-dicarbonyl compound before they are quenched (Chevion, 1988; Chace et al., 1991; Kato et al., 1992).

The presence of multiple excitation and emission bands in fluorescence spectra of poly-L-lysine is in line with an earlier study (Homchaudhuri and Swaminathan, 2001) on concentrated solutions of lysine, indicating the involvement of multiple states and/or multiple species. Since poly-L-lysine lacks any aromatic residue that can contribute to fluorescence, the origin of the observed fluorescence can be attributed to intramolecular interactions between side chains of lysine residues in solution (Homchaudhuri and Swaminathan, 2001 and 2004). The excitation wavelength of poly-L-lysine at 290 nm has been used to evaluate the changes in the fluorescence intensity of modified poly-L-lysine at the emission wavelength of 325 nm. The decrease in fluorescence intensity in the modified poly-L-lysine could be attributed to modification of free amino groups in lysine side chains. The increase in emission intensity of ROS-glycated poly-L-lysine at 365 nm could be due to
fragmentation of the polypeptide, as a result of ROS exposure and also due to availability of lysine-lysine pairs as a result of oxidation of glycated poly-L-lysine.

It is apparent from the far-UV c.d. spectra that poly-L-lysine exists in random coil structure at pH 7.4 in the described conditions while modified poly-L-lysine showed significant changes in ellipticities at 217 nm and 200 nm. The decrease in ellipticity of modified poly-L-lysine at 217 nm was due to alteration of mean residue weight of poly-L-lysine on glycation as supported by earlier study of far U.V. c.d. on glycated poly-L-lysine (Liang, 1990). The increase in ellipticity of modified poly-L-lysine at 200 nm could be due to alteration in the original conformation of poly-L-lysine on glycation and -OH mediated oxidation of glycated poly-L-lysine.

Results on FT-IR spectra clearly showed the position and intensity of characteristic bands in poly-L-lysine and modified poly-L-lysine. The shifting and increase in intensity of the amide 1 bands in modified poly-L-lysine indicate a change in the peptide group state (Rozenberg and Shoham, 2007) as a result of glycation and oxidation of reactive amino groups in glycated poly-L-lysine and ROS-glycated poly-L-lysine respectively. The C–N group specific band in the spectra of glycated poly-L-lysine and ROS-glycated poly-L-lysine could be due to exposure of these buried groups on denaturation-cum-unfolding of poly-L-lysine after the modifications. The appearance of band at 1702 cm⁻¹, corresponding to carbonyl (C=O) group of ketones in the spectra of glycated poly-L-lysine, confirmed the formation of ketoamines (Amadori product) in the glycated poly-L-lysine (Wnorowsky and Yaylayan, 2003; Yaylayan and Locas 2007). The presence of more prominent and intense band of methylene (CH₂) group in ROS-glycated poly-L-lysine spectrum is reported for carboxymethylated arginine or lysine (Cardenas et al., 2004). Carboxymethylation of lysine is known to generate CML, an advanced glycation end product. The shifting and increase in band intensity of ε-NH₂ groups in glycated poly-L-lysine and ROS-glycated poly-L-lysine could be due to attachment of glucose moiety during glycation and further modification during oxidation of side chain ε-amino groups respectively. These results are in conformity with the earlier investigations that absorption of poly-L-lysine due to side chain NH₂ groups, extensively studied in multiple peptide model systems, is generally found at 3400-3100 cm⁻¹ (Susi, 1969; Rozenberg et al., 2005).

In the ¹H-NMR spectrum of poly-L-lysine the different chemical shifts of ε-methylene (ε-CH₂) protons and other protons indicate the presence of different lysine
residues in poly-L-lysine. This difference is due to distinct pK values of the lysine residues (Tressi et al., 2002). The NMR analysis clearly shows the glycation in modified poly-L-lysine. This was confirmed by the ε-methylene protons chemical shift differences between the modified and unmodified counterparts of poly-L-lysine and presence of the proton signals of glucose (Gruetter et al., 1996) in glycated poly-L-lysine. The appearance of singlet resonance signal of methylene proton of glucose in the spectra of ROS-glycated poly-L-lysine has been reported in the spectrum of CML (Delatour et al., 2006).

The antigenicity of native and modified poly-L-lysine was ascertained by experimental induction of antibodies in rabbits. Both the modified forms of poly-L-lysine were found to be extremely potent antigens inducing high titre antibodies in the animals. However, immunization with native poly-L-lysine could result only in a weak antigenic response. The antibody titre was checked through direct binding solid-phase immunoassay. The non-antigenicity of poly-L-lysine may be due to its homopolymeric nature and lack of aromatic residue (Rao, 1972; Goldsby et al., 2003). However glycation as well as ROS modification of glycated poly-L-lysine results in structural changes/conformational alteration thus imparting a highly antigenic characteristic to the poly-L-lysine. Amadori products of HSA and proteins containing AGEs have been reported to be highly immunogenic in experimental animals with CML as one of the major epitopes recognized by anti-AGE antibodies (Reddy et al., 1995; Ikeda et al., 1996; Schalkwijk et al., 1999).

The antigenic specificity of affinity purified anti-glycated poly-L-lysine IgG and anti-ROS-glycated poly-L-lysine IgG was ascertained by competition ELISA and band shift assay. The results reiterate that the induced antibodies are immunogen specific and recognizing the modified epitopes on poly-L-lysine. The in vivo generation of autoantibodies can be explained as a result of hyperglycemia wherein glucose and reactive oxygen species may induce modifications in certain proteins generating neoeptopes against which the antibodies are raised. Autoantibodies directed against intracellular proteins have been found to be serological markers of diabetes disease (Makino et al., 1995).

The induced antibodies showed appreciable recognition for glycated IgG, HSA and histone. These molecules caused an inhibition of 42.7% to 55% in the activity of experimentally induced anti-glycated poly-L-lysine antibodies. These
results clearly point towards epitope sharing between the glycated poly-L-lysine and glucose modified IgG, HSA and histone. While anti-ROS-glycated poly-L-lysine antibodies inhibited in the range of 34% to 52.6% by ROS-glycated counterparts of IgG, HSA, histone and ROS-poly-L-lysine. However native counterparts of the above mentioned proteins did not cause appreciable inhibition in the activities of anti-modified poly-L-lysine antibodies. The induced antibodies against the modified forms of poly-L-lysine are found to be polyspecific and cross reactive with a variety of inhibitors having common antigenic determinants. These results explain that one of the factors for the in vivo induction of antibodies in diabetes mellitus is hyperglycemia induced glycation of certain proteins as well as their oxidative modification by the reactive oxygen species generated under stress.

Amadori products have been found in the tissues of diabetic rats as well as in diabetic patients (Myint et al., 1995; Schalkwijk et al., 1999; Jaleel et al., 2005). AGEs are also reported to be accumulated in the tissues of diabetic patients (Kume et al., 2005). The presence of anti-CML-BSA antibodies has been reported in the serum of streptozotocin-diabetic rats as well as in a small number of diabetic patients (Shibayama et al., 1999). In the present study, we have investigated the possible role of native and modified (glycated, ROS-glycated and NaBH₄ reduced glycated) forms of poly-L-lysine in diabetes mellitus. Serum antibodies from diabetic patients, when analyzed in competitive binding assay, showed highest recognition of glycated poly-L-lysine. Maximum inhibition in the antibody activity was caused by Amadori-rich glycated poly-L-lysine, followed by ROS-glycated poly-L-lysine or NaBH₄ reduced glycated poly-L-lysine and least with the native poly-L-lysine. Thus circulating autoantibodies in the sera of diabetic patients showed preferential recognition of the epitopes on modified poly-L-lysine as compared to the native form.

The presence of autoantibodies against native and modified forms of poly-L-lysine in the sera of diabetic patients having secondary complications (nephropathy, retinopathy and atherosclerosis) was also investigated. Earlier studies have shown the presence of autoantibodies directed against AGEs in diabetic patients having secondary complications (Rahbar and Figarola, 2003). Higher autoantibodies against AGEs have been reported in patients with the renal failure than in normal subjects or diabetic subjects without renal failure (Shibayama et al., 1999). Amadori products of glycated serum proteins and Amadori albumin have been reported to be associated
with glomeruli of diabetic patients having nephropathy (Cohen and Ziyadeh, 1994; Sakai et al., 1996). Elevated concentrations of Amadori albumin in animals have been implicated in the development of diabetic retinopathy (Clements et al., 1998). In our studies maximum recognition of Amadori-rich glycated poly-L-lysine followed by ROS-glycated poly-L-lysine and NaBH₄ reduced glycated poly-L-lysine was observed in the sera of diabetic patients with nephropathy. Similarly high recognition of Amadori-rich glycated poly-L-lysine was observed in the sera of diabetic patients with retinopathy and atherosclerosis. Recognition of native poly-L-lysine was the least.

*In vitro* glycated albumin (Amadori adducts) has been shown to stimulate tumor necrosis factor-α gene expression and modulate nitric oxide synthase activity in endothelial cells (Amore et al., 1997) and is associated with other activities (Cohen and Ziyadeh, 1994) in glomerular mesangial cells that are possibly involved in the pathogenesis of nephropathy. Interaction of Amadori albumin with specific receptors that can induce receptor-mediated responses (Predescu et al., 1988) and accumulation of immunocomplexes of AGEs and their autoantibodies in glomeruli might contribute to the diabetic pathogenesis (Shibayama et al., 1999).

The straight chain of the Amadori adduct, ketoamine, undergoes cyclization to a more stabilized hemiketal furanose or pyranose ring structures (Armbruster, 1987; Yaylayan and Huyghues-Despointes, 1994). NaBH₄ is effective in the reduction of Amadori products, but not of AGEs. In this study reduced form of glycated poly-L-lysine showed impaired recognitions of the induced anti-glycated poly-L-lysine antibodies as well as of the autoantibodies from diabetic patients sera due to change in the structure of Amadori products as a result of reduction by NaBH₄ leading to elimination of keto group of the ketoamine. This results in shifting the existing equilibrium between the linear configuration and the furanose or pyranose ring structures more towards the linear configuration (Schalkwijk et al., 1999). The results suggest that the ring structures responsible for the antigenic epitope of Amadori-rich glycated poly-L-lysine are essential for recognition of the molecule by the antibodies.

IgG has greater number of lysine residues than other serum proteins like albumin and red blood cells and hyperglycemia and/or oxidative stress can cause AGE-damaged IgG. Advanced glycation of IgG and other immunoglobulins has been shown to occur in serum of diabetic patients (Kaneshige, 1987; Kalia et al., 2004).
Recognition of the IgG from diabetes patients by our induced anti-glycated- and anti-ROS-glycated-poly-L-lysine antibodies clearly shows the presence of glycated and ROS modified epitopes on the IgG from diabetes patients. However, presence of glycated as well as ROS modified epitopes on the diabetes IgG is further corroborated by the recognition of in vitro glycated normal human IgG by our experimentally induced antibodies against glycated- and ROS-glycated-poly-L-lysine. On the other hand the native (i.e. unglycated IgG) from the healthy human subject was poorly recognized by the induced antibodies.

Based on the above studies the following conclusions can be drawn:

1. The glycated poly-L-lysine, formed upon incubation of poly-L-lysine with glucose, was found to contain high concentration of early glycation (Amadori) products.

2. Hydroxyl radical modification of the glycated poly-L-lysine was achieved by 'OH radical generated in the Fenton reaction.

3. The modifications cause major biochemical and biophysical changes in poly-L-lysine resulting in hyperchromicity and decreased fluorescence intensity in the spectral analysis.

4. Both the glycated and ROS-glycated poly-L-lysine were found to be highly immunogenic in experimental animals despite the native form of poly-L-lysine being non-antigenic.

5. Induced antibodies against modified poly-L-lysine forms were highly specific for the respective antigens. However, they also showed cross reactivity with glycated and ROS-glycated forms of certain lysine rich proteins.

6. Less recognition of NaBH₄ reduced glycated poly-L-lysine by the induced anti-glycated poly-L-lysine antibodies clearly show that the induced antibodies are predominantly formed against the Amadori components of glycated poly-L-lysine.

7. Autoantibodies from diabetic patients, with or without secondary complications, showed higher recognition for Amadori-rich glycated- and ROS-glycated-poly-L-lysine than the unmodified form. The antibodies from
normal healthy subjects showed negligible binding to either forms of poly-L-lysine as well as to the native form.

8. Higher recognition of autoantibodies in the diabetes patients by Amadori-rich glycated poly-L-lysine as compared to the ROS modified glycated poly-L-lysine or the NaBH₄ reduced glycated poly-L-lysine provides ample evidence that the major population of autoantibodies in diabetes patients is directed against the intact (unmodified/analysis) Amadori components of the glycated poly-L-lysine.

9. Furanose or pyranose (cyclic) forms of Amadori product are the potential epitopes responsible for higher recognition of glycated poly-L-lysine.

10. Recognition of IgG from diabetes patients by anti-glycated- and anti-ROS-glycated-poly-L-lysine antibodies points towards the presence of glycation and ROS induced damage in diabetes IgG.