IMMUNOCHEMICAL STUDIES ON GLYCATED POLY-L-LYSINE: IMPLICATIONS IN DIABETES MELLITUS

ABSTRACT

THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

BY

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DEPARTMENT OF BIOCHEMISTRY
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Abstract
Nonenzymatic glycation and oxidation of proteins is increased in diabetes mellitus due to hyperglycemia and play an important role in the pathogenesis of the disease. Glycation primarily occurs at intrachain lysine residues of proteins and results in the formation of an early stage stable product as Amadori-modified proteins which undergo further irreversible chemical reactions to form advanced glycation end products (AGEs) with the involvement of free radicals. One of the pathways for formation of AGEs is oxidation of Amadori products through reactive oxygen species produced by transition metals. The accelerated accumulation of Amadori and AGE products in tissues of diabetic patients, particularly with secondary complications like nephropathy, retinopathy and atherosclerosis are accompanied by increased free radical activity that contributes to the biomolecular damage. Oxidative stress and oxidative damage to tissues are common end points of chronic diseases such as diabetes.

In the present study, poly-L-lysine was glycated non-enzymatically by incubating with glucose for 7 days. Glycated poly-L-lysine was further modified by hydroxyl radical (OH), the most reactive of the reactive oxygen species (ROS), generated by Fenton reaction. The modified poly-L-lysine showed conformational and structural changes on analysis by various colorimetric, spectral and immunological studies. The studies reveal remarkable changes in poly-L-lysine upon glycation and oxidation.

The colorimetric NBT assay showed significantly high yield of early glycation (Amadori) products in the glycated poly-L-lysine which was
confirmed by increased yield of 5-hydroxymethylfurfural (HMF) from Amadori products of glycated poly-L-lysine. ROS-glycated poly-L-lysine showed low levels of Amadori products and HMF while a negligible content was found in native poly-L-lysine. The same samples of modified poly-L-lysine were used for further studies.

Studies with specific and non-specific antioxidants and metal chelator on ROS-glycated poly-L-lysine showed inhibition of the ROS modification. The significant inhibition observed with EDTA, catalase and mannitol showed a definite role of hydroxyl radical, produced by Fenton reaction, in the ROS modification of glycated poly-L-lysine and AGE formation.

Glycated and ROS-glycated poly-L-lysine exhibited hyperchromicity as compared to unmodified poly-L-lysine in the far-UV spectra. These changes are indicative of structural perturbation in poly-L-lysine on modifications. Far-UV c.d. spectra of modified poly-L-lysine showed significant decrease in molar ellipticity together with a change in c.d. signal of modified poly-L-lysine as compared to native poly-L-lysine. The results reiterate the earlier observation of structural perturbation in modified poly-L-lysine due to alterations in mean residue weight and conformational changes induced by the modification.

The fluorescence studies exhibited reduced fluorescence intensity in the case of modified poly-L-lysine as compared to the native form. The fluorescence intensity of ROS-glycated poly-L-lysine was even less. The decrease in intensities of modified poly-L-lysine is due to loss of
intramolecular interactions between lysine side chains, a phenomenon responsible for the observed fluorescence of unmodified poly-L-lysine.

The studies on FT-IR and $^1$H-NMR spectra of modified poly-L-lysine showed characteristic bands and signals corresponding to chemical groups of Amadori products and $N^\epsilon$-carboxymethyllysine (CML), an advanced glycation endproduct as well as shift in bands and signals assigned for other chemical groups present in the native poly-L-lysine. These results confirmed the presence of Amadori products and CML in glycated and ROS-glycated poly-L-lysine respectively.

Native and both the modified samples of poly-L-lysine were used to induce antibodies in rabbits. Modified poly-L-lysine was found to be antigenic, producing high titre antibodies. The antigenic specificity of the induced antibodies was studied by direct binding ELISA, competition ELISA and band shift assay. The antigens exhibited a high degree of specificity for the induced antibodies. Anti-glycated and anti-ROS-glycated poly-L-lysine antibodies showed preferential recognition of glycated and ROS-glycated poly-L-lysine in a competition assay. Anti-glycated poly-L-lysine antibodies recognized Amadori specific epitopes present in glycated poly-L-lysine. The induced antibodies showed polyspecificity as they recognized other similarly modified lysine rich proteins, pointing towards generation of common epitopes in the modified lysine residues.

Sera of diabetes patients were probed for the presence of antibodies reactive with native, glycated, ROS-glycated and NaBH$_4$ reduced glycated forms of poly-L-lysine. Direct binding ELISA showed greater recognition of
the modified forms of poly-L-lysine samples as compared to the native form. Specific binding was ascertained by competition ELISA. Moreover, significantly higher recognition of Amadori-rich glycated poly-L-lysine over other modified forms of poly-L-lysine was observed in the sera of diabetes patients with and without secondary complications like nephropathy, retinopathy and atherosclerosis. The higher binding to glycated and ROS-glycated forms of poly-L-lysine over native poly-L-lysine of serum antibodies in diabetes patients suggests the involvement of modified poly-L-lysine in the production of autoantibodies in these patients. The antigenic epitope specificity of antibodies induced in rabbits and of the antibodies present in the sera of diabetes patients towards Amadori-rich glycated poly-L-lysine was confirmed by binding assay with NaBH₄ reduced glycated poly-L-lysine. Both the antibodies exhibited reduced binding to the NaBH₄ reduced poly-L-lysine thereby confirming the specific recognition of the Amadori product.

Human IgG is known to be rich in lysine residues. Glycation and ROS damage in the IgG from diabetes patients were detected using anti-glycated- and anti-ROS-glycated- poly-L-lysine antibodies as probes. IgG from different diabetic patients inhibited antibodies binding to their respective antigens demonstrating the presence of Amadori and AGE specific epitopes on IgG. The study is in conformity with the earlier reports that glycation and glycoxidation causes in vivo protein modifications. Glycation and oxidative damage in the diabetes IgG was further confirmed by a high degree of binding of the induced anti-glycated poly-L-lysine and anti-ROS-glycated poly-L-lysine antibodies to the in vitro glycated and ROS modified IgG from healthy subjects.
In conclusion, glycation and ROS modification of poly-L-lysine render it highly immunogenic generating antigen specific polyclonal antibodies. The modified poly-L-lysine (i.e. glycated and ROS-glycated forms) are representative models of the in vivo glycated and ROS-glycated damage of lysine residues of proteins resulting in autoantibodies induction in diabetes patients. Recognition of modified poly-L-lysine samples by serum antibodies from diabetes patients strongly points towards the involvement of glycated and oxidatively modified lysine rich proteins in the induction of autoantibodies in these patients. Amadori products, present as cyclic forms in the glycated poly-L-lysine, are the potential recognition epitopes of anti-glycated poly-L-lysine antibodies. The induced anti-glycated poly-L-lysine and anti-ROS-glycated poly-L-lysine antibodies detected early and advanced glycation mediated damage to the IgG of diabetes patients. It is, therefore, suggested that glycation and ROS modification of lysine rich proteins appears to play a major role in the production of autoantibodies in diabetes mellitus and in associated complications of the disease.
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Approved:

DR. MOINUDDIN (Supervisor)

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ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)
2008
Fed in Computer
Dedicated
To
My Parents
Certificate

I certify that the work presented in the thesis entitled "Immunochemical studies on glycated poly-L-lysine: implications in diabetes mellitus" has been carried out by Mr Nadeem Ahmad Ansari in the department of Biochemistry, under my direct supervision and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

Dr. Moinuddin
(Supervisor)
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<tr>
<td>A</td>
<td>Absorbance</td>
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<tr>
<td>AGEs</td>
<td>Advance glycation end products</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CML</td>
<td>N\textsuperscript{\textregistered}-carboxymethyllysine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylene triaminepentaacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FI</td>
<td>Fluorescence intensity</td>
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<tr>
<td>FT-IR</td>
<td>Fourier transformed infra-red</td>
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<td>HSA</td>
<td>Human serum albumin</td>
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<td>HMF</td>
<td>5-hydroxymethylfurfural</td>
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<tr>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>ICs</td>
<td>Immune complexes</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>KBr</td>
<td>Potassium bromide</td>
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<tr>
<td>MRE</td>
<td>Mean residual ellipticity</td>
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<tr>
<td>NaBH\textsubscript{4}</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitrobluetetrazolium</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>O\textsubscript{2}\textsuperscript{−}</td>
<td>Superoxide anion radical</td>
</tr>
<tr>
<td>\textasciitilde OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OD\textsubscript{280}</td>
<td>Optical Density at 280 nm</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for AGE</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
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<tr>
<td>TBS</td>
<td>Tris buffer saline</td>
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<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) amino methane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>Wavelength showing maximum absorbance</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
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Introduction
Proteins are the most important of the macromolecules of living substances. They are built up from a section of 20 different amino acids joined or linked by peptide bonds. Out of these 20 amino acids, 8 are essential amino acids. The product formed by a peptide bond between two amino acids is a dipeptide. The peptides containing 2-10 repeating amino acid residues (-NH-CH-CO) are referred to as oligopeptide. A polypeptide is formed by more than 10 amino acid residues while a protein has more than 100 residues. The proteins are thus polymers produced by linking together of a number of similar small units.

**Poly-L-lysine**

Poly-L-lysine is a homopolymer, consisting of many intrachain lysine residues and a large number of free ε-amino groups. Lysine is a base and an essential amino acid that the body needs for growth and tissue repair. It cannot be synthesized in the body and its breakdown is irreversible. It plays an essential role in the production of carnitine, a nutrient responsible for converting fatty acids into energy and helping to lower cholesterol. Lysine appears to help the body absorb and conserve calcium and it plays an important role in the formation of collagen. A unique property that lysine has is that it does not change its nitrogen with other circulating amino acids. O-Glycosylation of lysine residues in the endoplasmic reticulum or golgi apparatus is used to mark certain proteins for secretion from the cell. Lysine is involved in the browning reaction, or carmelization, in foods such as pastries, doughnuts, cookies and cereals. In this process, lysine and sugar become linked together in a way that makes lysine difficult for the body to absorb. It has been suggested that lysine may be beneficial for those with herpes simplex infections (Griffith et al., 1978). However, more research is needed to fully substantiate this claim. Lysine can help to alleviate the symptoms of cold sores. They help to speed up the healing process if taken immediately.

**Antigenicity of poly-L-lysine**

Polypeptides of single amino acids with absence of aromatic groups such as poly-L-lysine are not antigenic in any species of animals even though they may have molecular weight over 50 KD (Rao, 1972; Goldsby et al., 2003) However, polylysine with only 7 or 8 units to which is attached a single dinitrophenyl group is im-
munogenic in guinea-pigs giving rise to both antibody production as well as cellular
immunity as indicated by the delayed skin sensitization to the polypeptide (Green et
al., 1966; Stupp et al., 1971; Rao, 1972). The immunogenicity depends also on the
species and the strain of the animal used. Some strains of animals responded to
particular structure and other strains did not respond. For example, strain 2 of guinea-
pigs produced antibodies when immunized with dinitrophenyl poly-L-lysine (DNP-PLL) while strain 13 did not produce any antibody. Breeding experiments showed
that response to DNP-PLL is due to the so called poly-L-lysine gene which is present
in strain 2 and not in strain 13. Cross breeding experiments showed that this gene is
autosomal and dominant (Levine, 1969; Stupp, 1971).

Non-enzymatic glycation of proteins/peptides

Non-enzymatic glycation is initiated by a nucleophilic addition reaction
between a free amino group of a protein and a carbonyl group from a reducing sugar
to form a freely reversible Schiff base. This reaction occurs over a period of hours and
once formed, the labile Schiff base rearranges to a more stable ketoamine
(fructosamine) or Amadori product (Horvat and Jakas, 2004; Lapolla et al., 2005)
which occurs as cyclic forms in equilibrium for added stability (Armbruster, 1987;
Yaylayan and Huyghues-Despointes, 1994) (Fig. 1). It is a classical covalent reaction
in which, by means of N-glycoside bonding, the sugar-protein complex is formed
through a series of chemical reactions described by a chemist, Maillard (Singh et al.,
2001).

Amadori-modified proteins as an early stage Maillard reaction product
undergo further reactions, involving dicarbonyl intermediates, such as 3-
deoxyglucosones (3-DG), giving rise to advanced glycation end products (AGEs)
which are also formed through other described pathways (Turk, 2001; Thornalley et
al., 2003) (Fig. 2). It was believed that the primary role in Maillard reactions was
exclusively played by high glucose concentration. However, in spite of the fact that
sugars are the main precursors of AGE compounds, numerous intermediate carbonyl
compounds (glyoxal, methylglyoxal, deoxyglucosones) formed through degradation
of Amadori products by oxidation and dehydration reactions, again react with the free
amino groups of proteins and act as propagators of non-enzymatic glycation reactions.
These compounds are much more reactive than the sugars from which they are
derived. Such intermediary products are also generated during glycolysis.
Glycation of proteins leads to the formation of reactive intermediates, crosslinking of proteins and the formation of brown and fluorescent polymeric materials (McRobert et al., 2003; Lapolla et al., 2005). Following reaction is described for glucose-dependent cross-linking of protein under physiological conditions (Eble et al., 1983):

\[
\text{GLUCOSE + PROTEIN} \rightarrow \text{GLUCOSE-PROTEIN} + \text{(PROTEIN)\textsubscript{2}} \text{PROTEIN}
\]

In the recent years extensive investigations have been made on the glycation of proteins exposed to high glucose concentrations, e.g. lens crystalline proteins (Stevens et al. 1978), insulin (Dolhofer and Wieland, 1979), proteins of erythrocyte membrane (Miller et al., 1980), bovine serum albumin (Arakawa and Timasheff, 1982), human serum albumin (Shaklai et al., 1984), enzymes (Coradello et al., 1982), high and low-density lipoproteins (Krittein et al., 1990), peripheral nerve myelin (Green, 1980), elastin (Baydanoff et al., 1994), collagen (Fu et al. 1994), IgM and IgG (Menini et al., 1993; Newkirk et al., 2003).

Glycation occurs primarily at intrachain lysine residues of proteins (Watkins et al., 1985). It takes place at ε-amino groups of lysine or hydroxylysine residues as well as at α-amino groups of amino terminal residues. The amino groups of arginine residues (Ledl and Schleicher, 1990) and that of histidine, tryptophan and cysteine residues (Munch et al., 1999) are also amenable to glycation. The valine residues have been identified as highly reactive sites in hemoglobin (Watkins et al., 1985). Specific lysine residues in hemoglobin and human serum albumin have also been identified as preferential sites of glycation in vivo. In hemoglobin, the most reactive lysine residues appeared to be located at carboxylate groups in the primary or three-dimensional structure of the protein, while in albumin the reactive lysine is adjacent to another lysine residue in the primary sequence.
Fig. 1. Scheme of the reaction of glucose and protein to form ketoamine (Amadori product).

Fig. 2. Scheme of potential pathways leading to AGE formation (GLO=glyoxal; MGO=methylglyoxal; 3-DG=3-deoxyglucosone; CML=carboxymethyllysine)

Source: Turk, 2001; Thornalley et al., 2003
Nonenzymatic glycation of proteins, peptides and other macromolecules has been implicated in a number of pathologies, most clearly in diabetes mellitus, normal aging and neurodegenerative amyloid diseases such as Alzheimer’s. Numerous studies have advocated a possible link between hyperglycemia/AGEs and the pathogenesis of diabetic complications such as retinopathy, nephropathy, neuropathy, and vasculopathy (Kimura et al., 1998).

**Early glycation product**

Measurement of early-stage glycation product (Amadori product) is routinely used to evaluate metabolic control in diabetic patients. The two parameters commonly used are HbA1c and glycated serum proteins (Ahmed, 2005). HbA1c is derived from the non-enzymatic reaction between glucose and the amino groups of valine and lysine of β-globin (John, 1997).

Glycated serum proteins are the products of the non-enzymatic reaction between the glucose and the free amino groups of serum proteins. Measurement of early glycation product expresses metabolic control during the last two weeks because the half-life of albumin and other serum proteins are close to 15 days (Schwartz, 1995; Furth, 1997; Goldstein et al., 2004). High level of Amadori products are formed through glucose mediated glycation of collagen, HSA and glutathione (Fu et al., 1994; Nagai et al., 1997; Linetsky et al., 2005).

**Advanced glycation end products (AGEs)**

N⁵-carboxymethyllysine (CML) and pyralline are non-fluorescent and non-cross-linking AGEs while pentosidine and crossline are fluorescent and cross-linking AGEs (Ahmed, 2005). CML was formed from oxidative degradation of N⁵-formyl-N⁵-fructoselysine (fFL) in glycated protein (Ahmed et al., 1986) or from a reaction of glyoxal with proteins (Jakus and Rietbrock, 2004). The browning of fFL incubation mixtures proceed to a greater extent under nitrogen than air, suggesting that oxidative degradation of Amadori adducts to form CML may have a role in limiting the consequences of protein glycation in the body. In air the concentration of the Amadori compound is more rapidly decreased by its conversion to CML and EA (erythronic acid) which are inert compounds (Ahmed et al., 1986). CML formation was also reported from hydroxyl radical mediated modification of Amadori products of proteins (Nagai et al., 1997) (Fig. 3).
Protein modification with AGE is irreversible, as there are no enzymes in the body that would be able to hydrolyze AGE compounds. These structures then accumulate during the lifespan of the protein on which they have been formed. Examples include all types of collagen, albumin, basic myelin protein, eye lens proteins, lipoproteins and nucleic acid. The major biological effects of excessive glycation include: inhibition of regulatory molecule binding, cross-linking of glycated proteins, trapping of soluble proteins by glycated extracellular matrix, decreased susceptibility to proteolysis, inactivation of enzymes, abnormalities of nucleic acid function and increased immunogenicity in relation to immune complex formation (Turk, 2001).

AGEs generate oxygen free radicals that may potentiate the development of atherosclerosis. Thus, AGEs might encourage aggregation of human platelets through oxidative stress (Bernheim et al., 2001). Protein glycation and AGEs are accompanied by increased free radical activity that contributes towards the biomolecular damage in diabetes. Receptors for AGEs (RAGE) are present on many cell types, particularly those affected in diabetes. Studies suggest that interaction of AGEs with RAGE alter intracellular signalling, gene expression, release of proinflammatory molecules and free radicals that contribute towards the pathology of diabetic complications (Watkins et al., 1985). Advanced glycation end products depress superoxide production by stimulated polymorphonuclear leukocytes. As superoxide plays an essential role in bactericidal activity, this polymorphonuclear leukocyte dysfunction may be a contributory factor to the increased prevalence and severity of bacterial infection seen in diabetic patients (Neglia et al., 1985). There is evidence of an association of age-associated diseases with AGEs and free radicals (Stachelin, 1997; Ligier et al., 1998).

**Glycation of poly-L-lysine**

The *in vitro* exposure of protein to glucose results in the non-enzymatic covalent attachment of glucose to lysine side chains in a manner that is observed *in vivo* (Brownlee et al., 1987). This process also occurs in individual with normal control of plasma glucose concentrations, but HSA is typically three times more glycated than the rest of the population in conditions of hyperglycemia (Bourdon et al., 1999) where, during periods of poor control of plasma glucose concentration, circulating levels of 25 mM glucose have been recorded.
Fig. 3. Scheme of the effect of hydroxyl radical modification on Amadori product.

Source: Nagai et al., 1997.
Glycated poly-L-lysine was taken as model compound for non-enzymatic glycation on proteins or enzymes. Poly-L-lysine exists as a random coil in solution at pH 7.4 and contains only $\alpha$-amino group and $\varepsilon$-amino groups as nonequivalent primary amino groups (Neglia et al., 1985). CML, an advanced glycation endproduct, was identified in hydrolysates of poly-L-lysine glycated in phosphate buffer under air (Ahmed et al., 1986). A yellow fluorescent compound, 2-(2-Furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) was generated from the acid-hydrolyzed Amadori products of poly-L-lysine, which had been incubated for 30 days with excess glucose in phosphate buffer, pH 7.5 at 37 °C (Njoroge et al., 1988).

**Free radical generation**

A number of processes *in vivo*, including the ‘respiratory burst’ of phagocytic cells, metal catalyzed substrate autoxidations, mitochondrial electron transfer and the reduction of hydroperoxides generate oxygen free radicals (Halliwell and Gutteridge, 1984). Free radicals and glycation are central to chronic diseases, degeneration and aging. One important source of free radicals is AGEs resulting from non-enzymatic glycation and oxidation of proteins and lipids (Thomas et al., 2005). AGEs generated oxygen free radicals may potentiate the development of atherosclerosis and might encourage aggregation of human platelets through oxidative stress (Hangaishi et al., 1998). Receptors for AGEs (RAGE) are present on many cell types, particularly those affected in diabetes. Studies suggest that interaction of AGEs with RAGE alter intracellular signalling, gene expression, release of proinflammatory molecules and free radicals that contribute towards the pathology of diabetic complications (Ahmed, 2005).

A rise in the oxygen free radical levels has two important effects: damage to various cell components and triggering the activation of specific signaling pathways NF-κB, ERK, JNK, NAPK, and PKC isoforms (Idris et al., 2001). Reactive oxygen species (ROS) generated during various metabolic and biochemical reactions have multifunctional effects that include oxidative damage to DNA leading to various human degenerative and autoimmune diseases (Hasan et al., 2003), moreover, also responsible for the elimination of invading pathogens (Khan et al., 2005).

ROS encompasses a variety of diverse chemical species including superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), alkoxyl ($RO^-$), peroxy ($ROO^-$), hydroxyl radicals (‘OH), and hydrochlorous acid (HOC1) (Jakus, 2000). ROS are highly
reactive and have extremely short half-lives. Most estimates suggest that the majority of intracellular ROS production is derived from the mitochondria. There are numerous mechanisms for generation of ROS \textit{in vivo} (Emerit \textit{et al.}, 1990).

**Superoxide anion radical**

The superoxide radical is generated within aerobic biological systems during both enzymatic and non-enzymatic oxidation. It is eliminated by conversion to H$_2$O$_2$ and O$_2$ by superoxide dismutase (Fridovich, 1978 and 1986). During glycation, Amadori products generate O$_2^-$ which is converted to H$_2$O$_2$ by non enzymatic dismutation reaction (Nagai \textit{et al.}, 1997). The finding that O$_2^-$ is produced by some enzymes and is efficiently scavenged by others (McCord and Fridovich, 1968 and 1969) led to the view that O$_2^-$ is an agent of oxygen toxicity. System generating O$_2^-$ has been observed to kill bacteria, inactivate viruses, damage enzymes and membrane and destroy animal cell culture (Fridovich, 1978; Halliwell, 1981).

**Hydrogen peroxide and the hydroxyl radical**

Hydrogen peroxide is the second intermediate during the stepwise one-electron reduction of molecular oxygen. It may however also be generated directly via a two-electron reduction of molecular oxygen. Hydrogen peroxide is a stable molecule that is generated as an end product of a variety of oxidation reactions in living cells. It may, in fact, act as both oxidizing and reducing agents. In presence of transition metals, it is thought to give rise to peroxyl radicals and extreme reactive hydroxyl radicals via the Fenton reaction (O’ Brien, 1969).

$$\text{Fenton reaction} \quad \text{H}_2\text{O}_2 + \text{Fe}^{2+}/\text{Cu}^+ \rightarrow \text{Fe}^{3+}/\text{Cu}^{2+} + \cdot\text{OH} + \cdot\text{OH}$$

The hydroxyl radicals are a highly reactive oxidizing agent that can react with a wide variety of organic molecules. During glycation, \cdot\text{OH generated by Fenton reaction between Fe}^{2+} \text{ and Amadori product-derived endogenous H}_2\text{O}_2 \text{ plays an important role in oxidative cleavage of Amadori compounds into CML (Nagai \textit{et al.}, 1997). Photolysis by UV irradiation can directly split H}_2\text{O}_2 \text{ into two \cdot\text{OH and is another important way to produce hydroxyl radical (Masaki \textit{et al.}, 1995).}$$

$$\text{UV} \quad \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \cdot\text{OH}$$
Autoxidation and glycation

Glucose exists in equilibrium with their enediol, which can undergo autoxidation to form an enediol radical. This radical reduces molecular oxygen to generate the superoxide radical and becomes oxidized itself to a dicarbonyl ketoaldehyde that reacts with protein amino groups forming a ketoamine. Ketoamine are similar to, although more reactive, than Amadori products and participate in AGE formation (Ahmed, 2005).

Glycation is a major source of ROS that is generated by oxidative pathways of glycation (Rahbar and Figarola, 2003). Several studies support the idea that glycation and oxidation are closely linked processes; glucose autoxidation plays an essential role in non-enzymatic glycation of protein. In vitro glycated serum albumin showed possible interactions between glycation and oxidation (Traverso et al., 1997). Contents of the glycated serum albumin are an index of oxidative modification during glycation (Fu et al., 1992). In hyperglycemic conditions, most of the carbonyl compounds generated by glycation need oxidative steps in their formation. The protein dicarbonyl compounds can participate in AGE formation and referred to as glycoxidative products (Liggins and Furth, 1997).

ROS mediated protein oxidation

The oxidative protein modification by reactive oxygen species leads to a loss of physiological function, as in the case of covalent binding of reactive organic metabolites to proteins (Bradford and Allen, 1997). Amino acids can undergo oxidative damage if they interact with oxygen free radicals. Even peptide bonds are subject to oxidative modification by reactive oxygen species. As free radicals are capable of initiating chain reactions, cross-linking of soluble and/or membrane bound proteins, yield larger aggregates. The direct consequence is a change in higher order structure of the protein (e.g., in the quaternary structure), which in turn, results in a loss of biological function (Adams et al., 1999; Dhalla et al., 2000; Schoonover, 2001). Copper bound to biological molecules causes site-specific damage in the presence of hydrogen peroxide, and causes greater damage to biological molecules than does iron by the formation of hydroxyl radicals (Kocha et al., 1997).
The action of autoxidizing sugars on some soluble proteins may mark protein for complete intracellular proteolysis. Consequently on free radical generation by these sugars, proteins are glycosylated and fragmentation of proteins occur (Wolff and Dean, 1987).

**Degradation products of proteins**

Protein damage by radicals is critical in many biological processes. This damage may lead to functional inactivation (Willson, 1983), but usually the inactivated proteins are degraded, so that proteolysis forms a secondary defence after antioxidants. However, when the target proteins are critical for rapid homeostatic mechanisms (e.g., transport proteins) or when the proteolytic defense and/or other antioxidant defenses are overwhelmed, toxic events may ensue.

Protein glycation and oxidation are increased in cellular and extracellular proteins in diabetes. The physiologically damaged proteins are degraded and replaced by the cells (Thornally et al., 2003; Goldberg, 2003). Cellular proteolysis liberates the glycated and oxidized amino acids as free adducts. The plasma concentration and urinary excretion of the free adducts are increased excessively in Type 1 diabetes (Ahmed et al., 2005).

In the process of glycation, AGE peptides are released as degradation products, which partly occur through proteolysis of the matrix component commonly named glycotoxins. Glycotoxins (AGE peptides) are very reactive on entering blood circulation. In case they have not been eliminated through the kidneys, recirculating AGE peptides can generate new AGE products that react with other plasma or tissue components. At this stage, glycation becomes an autonomic process, which significantly accelerates the progress of the complications (Turk, 2001).

**Free radicals and AGE inhibitors**

Scavenging of free radicals by preventing radical formation and intercepting radical from further activity are done by antioxidants as key line of defense (Cotgreave et al., 1998). Antioxidant defenses are of two types. The defenses that directly scavenge \( \text{H}_2\text{O}_2 \) and \( \text{OH} \) are known as primary antioxidant defense. Secondary antioxidant defences consist of the repair mechanisms that act on biomolecules that have undergone oxidative damage.
The major antioxidants which are endogenous include superoxide dismutase (SOD) which removes $O_2^-$, catalase that converts $H_2O_2$ to $H_2O$ and $O_2$, and glutathione peroxidase, which helps with $H_2O_2$ removal and prevents hydroxyl radical (OH) formation (Halliwell and Gutteridge, 1999). Lipoic acid has the unique ability to regenerate several other antioxidants such as vitamin E and vitamin C (Packer and Coleman, 1999). Vitamin C, the major plasma antioxidant, is a scavenger of many ROS and reactive nitrogen species, and is capable of regenerating tocopherol from its radical (Maxwell, 1995; Halliwell and Gutteridge, 1999). Metal chelators ethylene diaminetetraaceticacid (EDTA) and diethylene triaminepentaaceticacid (DTPA) sequester the trace amounts of transition metal and hence inhibit oxidation (Wolff and Dean, 1987). Mannitol and benzoic acid are hydroxyl radical scavengers, which inhibit protein fragmentation (Hunt et al., 1988; Nagai et al., 1997). There are numerous other dietary sources of antioxidants (McDermott, 2000).

AGE inhibitors are of three types. First, carbonyl trapping agents like aminoguanidine that attenuate carbonyl stress. Second, metal-ion chelators like pyridoxamine, DTPA, which suppress glycoxidation reactions and third, crosslink breakers like thiazolium salts (ALT-711) that reverse AGE crosslinks. Chelators, sulphhydryl compounds, antioxidants and aminoguanidine inhibit formation of glycoxidation products, generation of fluorescence and cross-linking of collagen without significant effect on the extent of glycation of the protein (Fu et al., 1994). While use of aminoguanidine was discontinued because of its side effects, pyridoxamine and ALT-711 were shown to be promising AGE inhibitors (Reddy and Beyaz, 2006). The blocking of interaction between AGEs and RAGE by usage of soluble RAGE (sRAGE), which does not involve signal transduction, has therapeutic applications in animal models (Park et al., 1998; Pullerits et al., 2005).

**Autoimmunity**

In order to elicit an immune response, a molecule must be recognized as nonself by the biological system. When an antigen is introduced into an organism, the degree of immunogenicity depends on the degree of its foreignness; generally the greater the phylogenetic distance between two species, the greater the structural disparity between them. Bovine serum albumin is strongly immunogenic when injected into rabbit (Goldsby et al., 2003).
The lack of an immune response to self when responses to environmental antigens are retained is due to immunological tolerance. The role of tolerance, or lack of tolerance, is important to the understanding of autoimmune diseases and transplantation immunobiology (Goldsby et al., 2003). A loss of natural tolerance to self underlies all autoimmune diseases. Many more individuals develop autoimmune phenomena than autoimmune disease. Immune-mediated Type 1 diabetes mellitus results from an organ-specific autoimmune-mediated loss of insulin secreting β cells. This chronic destruction process involves both cellular and hormonal components detectable in the peripheral blood, months or even years, before the onset of clinical diabetes (Kukreja and Maclaren, 1999).

Amadori products of HSA are found to be immunogenic and Type 1 diabetic patients with retinopathy and nephropathy have higher Amadori albumin levels than did those without it (Cohen and Ziyadeh, 1994; Schalkwijk et al., 1999). Proteins containing AGEs are highly immunogenic and CML is one of the major epitopes recognized by anti-AGE antibodies (Reddy et al., 1995; Ikeda et al., 1996). The presence of CML-BSA antibodies in the serum of streptozotocin-diabetic rats as well as in a small number of diabetic patients have been reported (Shibayama et al., 1999). AGE can exert their immunogenicity, which demonstrate the presence of AGES-immune complexes (AGE-ICs) in the diabetic patients that may play a role in the atherogenesis (Turk et al., 2001). Interactions of AGE autoantibodies with AGEs as a continuously produced antigen result in the formation of AGE-ICs that may play role in diabetic complications (Jakus and Rietbrock, 2004). Autoantibodies against N\(^{\epsilon}\)-carboxyethyllysylsine (CEL) in human lens proteins are found in diabetic patients (Ahmed et al., 1997).

**Diabetes mellitus**

Diabetes mellitus is a syndrome characterized by disordered metabolism and hyperglycemia resulting from defects in insulin secretion, low levels of insulin or insulin resistance. In diabetes mellitus, low insulin levels prevent cells from absorbing glucose. As a result, glucose builds up in the blood. When glucose-laden blood passes through the kidneys, all the excess glucose cannot be absorbed. This excess glucose secreted in the urine, accompanied by water and electrolytes; ions required by cells to regulate the electric charge and flow of water molecules across the cell membrane.
This causes polyuria, polydipsia and weight loss as classical symptoms of the diabetes. These symptoms together with a random plasma glucose concentration ≥ 11.1 mmol/L (200 mg/dL) is sufficient for the diagnosis of diabetes mellitus although fasting plasma glucose is the most reliable and convenient test for identifying diabetes in asymptomatic individuals (Powers, 2005).

In diabetic patients, increased level of blood glucose correlates with increased formation of ketoamine. Measurement of blood glucose gives only the current glucose concentration as transient or short term indicator. The ketoamine value is the mean blood glucose concentration over preceding 2 to 3 weeks as medium term indicator while measurement of glycated hemoglobin (HbA1c) represents mean glucose concentration over a 6 to 8 week period as long term indicator. Though there is a strong correlation between elevated plasma glucose and HbA1c, the relationship between the fasting plasma glucose (FPG) and HbA1c in individuals with normal glucose tolerance and mild glucose tolerance is less clear and thus the use of HbA1c is not currently recommended for the diagnosis of diabetes (Powers, 2005).

The World Health Organization recognizes three main forms of diabetes mellitus: Type 1, Type 2 and gestational diabetes, which have different causes and population distributions. While, ultimately, all forms are due to the beta cells of the pancreas being unable to produce sufficient insulin to prevent hyperglycemia, the causes are different (Rother, 2007). Type 1 diabetes is usually due to autoimmune destruction of the pancreatic beta cells. Type 2 diabetes is characterized by insulin resistance in target tissues, this causes a need for abnormally high amounts of insulin and diabetes develops when the beta cells cannot meet this demand. Gestational diabetes is similar to Type 2 diabetes in that it involves insulin resistance; the hormones of pregnancy can cause insulin resistance in women genetically predisposed to developing this condition.

Diabetes can cause many complications. Acute complications of diabetes include diabetic ketoacidosis (DKA) and hyperglycemic hyperosmolar state (HHS). Hypoglycemia is most commonly caused by drugs used to treat diabetes mellitus or by exposure to other drugs, including alcohol. Serious long-term or chronic complications include cardiovascular disease, chronic renal failure, retinal damage, nerve damage and microvascular damage, which may cause impotence and poor healing. Poor healing of wounds, particularly of the feet, can lead to gangrene, which
may require amputation. Around 4 million deaths per year are caused due to conditions directly or indirectly associated with diabetes.

**Epidemiology**

According to the World Health Organization (WHO), at least 177 million people worldwide suffer from diabetes in 2000. Its incidence is increasing rapidly and it is estimated that by the year 2030, this number will rise to >360 million. The International Diabetes Federation (IDF) estimated about 194 million people worldwide to have diabetes in 2003 and is expected to increase to about 333 million by 2025. Diabetes mellitus occurs throughout the world, but is more common (especially Type 2) in the more developed countries. The greatest increase in prevalence is, however, expected to occur in Asia and Africa, where most patients will likely be found by 2030. The major risk factors of diabetes mellitus are age, obesity, lack of exercise, along with a strong genetic predisposition.

For at least 20 years, diabetes rates in North America have been increasing substantially. In 2005, there are about 20.8 million people with diabetes in the United States alone. According to the American Diabetes Association, there are about 6.2 million people undiagnosed and about 41 million people that would be considered prediabetic.

India now being termed the “diabetes capital of the world” as it leads the world with largest number of diabetic subjects. WHO report shows that 32 million people in India had diabetes in the year 2000 and is expected to increase to about 80 million by 2030. The International Diabetes Federation (IDF) estimated the total number of diabetic subjects to be around 35.5 million in India in 2003 and this is further set to rise to 73.4 million by the year 2025.

**Type 1 diabetes mellitus**

Type 1 or insulin dependent diabetes mellitus, also known as juvenile onset diabetes mellitus is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas, leading to a deficiency of insulin and hyperglycemia. The main cause of this beta cell loss is a T-cell mediated autoimmune attack (Rother, 2007). A number of antibodies have been detected as the markers of autoimmune diabetes. These include islet cell autoantibodies (ICA), insulin
autoantibodies (IAA) (Atkinson et al., 1992), glutamic acid decarboxylase 65 (GAD65) autoantibodies (Jun et al., 2002), antibodies to tyrosine phosphatases IA-2 and IA-2β (Lan et al., 1996; Lu et al., 1996). In over 90% of the cases, Type 1 diabetes mellitus is immune mediated and in less than 10% of the cases it is idiopathic. Most affected people are otherwise healthy and of a healthy weight when onset occurs. Sensitivity and responsiveness to insulin are usually normal, especially in the early stages.

It usually occurs in younger age group with highest incidence in 10-14 years age-group. The incidence of Type 1 diabetes shows little bias upto 15 years of age, but more males are diagnosed in early adult life. The lower prevalence of IAA in adolescent females implies sex specific modulation of the autoimmune process during puberty (Williams et al., 2003). The principal treatment of Type 1 diabetes, even from the earliest stages, is replacement of insulin combined with careful monitoring of blood glucose levels using blood testing monitors. Without insulin, diabetic ketoacidosis can develop and may result in coma or death. Emphasis is also placed on lifestyle adjustments (diet and exercise) though these cannot reverse the loss.

Genetic susceptibility to Type 1 diabetes mellitus involves multiple genes. The major susceptibility gene for Type 1 diabetes mellitus is located in the HLA region on chromosome 6. Polymorphisms in the HLA complex account for 40 to 50% of the genetic risk of developing Type 1 diabetes mellitus. This region contains genes that encode the class II MHC molecules. Most individuals with Type 1 diabetes mellitus have HLA DR3 and/or DR4 haplotype (Powers, 2005).

Even in the presence of the above predisposing factors the pathogenic process might not get initiated. Detailed studies have shown that a toxic or infectious insult is required for triggering the autoimmune destruction of β-cells of pancreas. Coxsackie-B virus, mumps virus, congenital rubella and cytomegalovirus and more recently hepatitis C virus have been shown to be related to Type 1 diabetes in this regard (Forrest et al., 1971; King et al., 1983; Pak et al., 1988; Karjalainen et al., 1998; Chen et al., 2005). Molecular mimicry has been closely linked to Type 1 diabetes mellitus in this respect where an autoimmune response is developed either against pancreatic antigen or against molecules of B cells resembling the viral protein.
Type 2 diabetes mellitus

Type 2 or non-insulin dependent diabetes mellitus also known as adult onset diabetes mellitus is the most common form of diabetes mellitus. It is also characterized by hyperglycemia and abnormalities of insulin resistance or reduced insulin sensitivity, combined with reduced insulin secretion and excessive hepatic glucose production. The defective responsiveness of body tissues to insulin almost certainly involves the insulin receptor in cell membranes. The autoantibodies of Type 1 diabetes mellitus especially GAD may also occur in up to 10% of adults initially classified as Type 2 diabetes in a condition known as Latent Autoimmune Diabetes in Adults (LADA) or Type 1.5 diabetes. The disease process in LADA patients is similar to that in Type 1 diabetes in that they share some HLA genetic susceptibility and some Type 1 diabetes associated autoantibodies (Agardh et al., 2005).

In the early stage the predominant abnormality is reduced insulin sensitivity, characterized by elevated levels of insulin in the blood. At this stage hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver. As the disease progresses the impairment of insulin secretion worsens and therapeutic replacement of insulin often becomes necessary.

The pathogenesis of insulin resistance is currently focused on PI-3 kinase signalling defect, which reduces translocation of GLUT 4 to the plasma membrane. Another emerging theory proposes that increased levels of free fatty acids, a common feature of obesity, may contribute to pathogenesis. Free fatty acids can impair glucose utilization in skeletal muscles, promote glucose production by the liver and impair β-cell function (Powers, 2005).

Type 2 diabetes may go unnoticed for years because visible symptoms are typically mild, non-existent or sporadic and usually there are no ketoacidotic episodes. However, severe long-term complications can result from unnoticed Type 2 diabetes, including renal failure due to diabetic nephropathy, vascular disease (including coronary artery disease), vision damage due to diabetic retinopathy, loss of sensation or pain due to diabetes neuropathy and liver damage from non-alcoholic steatohepatitis.

A heterogeneous group of factors are involved in the pathogenesis of this form of diabetes. Obesity has a strong predisposing factor for the development of insulin
resistance and hence Type 2 diabetes mellitus (Bogardus et al., 1985). Moreover, visceral obesity and not subcutaneous disposition of fat has been held responsible for giving rise to insulin resistance (Kissebah et al., 1982). Genetic predisposition is also a strong predisposing factor (Byrne et al., 1996).

Asian Indians are more prone to Type 2 diabetes and premature coronary artery disease due to “Asian Indian Phenotype”. The phenotype refers to certain unique clinical and biochemical abnormalities in Indians which include increased insulin resistance, greater abdominal adiposity i.e., higher waist circumference despite lower body mass index, lower adiponectin and higher high sensitive C-reactive protein levels (Mohan et al., 2007).

Type 2 diabetes is a polygenic disorder with multiple genes located on different chromosomes contributing to its susceptibility and numerous environmental factors interact with these genes to produce the disorder. Single gene defects like maturity onset diabetes of the young (MODY) cause only a minority of cases of Type 2 diabetes. There are certain genes which predispose Indians to diabetes while other genes (for example Pro 12 Ala polymorphism of PPAR gamma gene) which afford protection against diabetes and insulin resistance to Caucasians, do not appear to protect Indians (Radha and Mohan, 2007). Individuals with a parent with Type 2 diabetes mellitus have an increased risk of diabetes; if both parents have Type 2 diabetes mellitus, the risk is approximately 40%. The concordance of Type 2 diabetes mellitus in identical twins is between 70-90% (Powers, 2005).

Environmental factors such as nutrition and physical activity further modulate phenotypic expression of the disease. Genetic defect in insulin secretion or action may not manifest itself unless an environmental effect or another genetic defect such as obesity is superimposed (Shervin, 2001).

**ROS in diabetes mellitus**

The overproduction of ROS lowered antioxidant defense and alterations of enzymatic pathways in humans with poorly controlled diabetes mellitus (Jakus, 2000). Diabetic individuals may exhibit elevated levels of iron and free copper ions (Cutler, 1978; Mateo et al., 1978), which in the presence of glycated proteins *in vitro* have been shown to generate free radicals (Hunt, 1994; Nagai et al., 1997). The accumulation of glycated material in tissues that contain free copper ion, contribute to
the generation of free radical mediated damage. The formation of α-dicarbonyl compounds is known to be an essential step for the cross linking of proteins and subsequent free radical generation (Rahbar and Figarola, 2003). In the presence of oxidative stress, glycation of proteins by methylglyoxal is enhanced. This may underlie the link of glycation and oxidative stress with diabetic complications, and may also contribute to the pathological processes of ageing (Harman, 1998).

**Hyperglycemia in the pathogenesis of diabetic complications**

In diabetic subjects, hyperglycemia leads to glycated and advanced glycated products and is widely recognized as the major cause of diabetic secondary complications (Table 1) due to overgeneration of ROS (Cai and Kang, 2001; Palm et al., 2003).

**Diabetic retinopathy**

It is characterized by increased proliferation of blood vessels, haemorrhages and infarction affecting retina of the eye accompanied by thickening of the capillary basement membrane and increased permeability of the capillaries. AGEs have been detected in retinal blood vessel walls and contribute towards vascular occlusion and increased permeability of retinal endothelial cells causing vascular leakage (Beisswenger et al., 1995; Chibber et al., 1997). AGEs exert their effect on microvascular endothelial cells and pericytes by upregulating levels of their RAGE mRNA (Tanaka et al., 2000). AGEs may cause loss of pericytes and death of endothelial cells in diabetic retinopathy. Retinal cells exposed to AGEs cause upregulation of vascular endothelial cell growth factor (VEGF), which in turn, stimulates angiogenesis and neovascularization, and accounts for the pathogenesis of proliferative retinopathy (Yamagishi et al., 2002). AGEs stimulate secretion of IL-6 from human retinal cells which can induce angiogenesis by increased expression of VEGF (Nakamura et al., 2003).

Fructoselysine, an initial Amadori adduct in collagen in Type 1 diabetic patients was independently associated with retinopathy (McCance et al., 1993). Elevated concentrations of Amadori albumin in animal have been implicated in the development of diabetic retinopathy (Clements et al., 1998).
**Diabetic nephropathy**

It is clinically manifested by proteinuria progressing to renal insufficiency and histopathologically by mesangial expansion and glomerular basement membrane thickening. Hyperglycemia and AGEs increase the release of transforming growth factor-β (TGF-β) and may contribute towards thickening of basement membrane, altered filtration and ultimately loss of glomerular function (Monnier *et al.*, 1992). Serum AGE levels reflect the severity of diabetic nephropathy and their measurement can predict the histopathological conditions (Berg *et al.*, 1997).

Circulating serum AGE level is so markedly increased in patients with diabetic nephropathy and renal insufficiency that it cannot be cleared by the kidneys (Turk, 2001). Serum AGEs include both serum proteins that have been modified by advanced glycation and low molecular weight AGE peptides (Shimoike *et al.*, 2000). Increased concentrations of serum AGEs in nephropathy could account for the rapid development of atherosclerosis (Bucala and Vlassara, 1995). Some studies have shown that advanced nephropathy develops in transgenic mice that over-express RAGE in vascular cells (Yamamoto *et al.*, 2001). Fructoselysine (Amadori product) in collagen was also independently associated with nephropathy in Type 1 diabetic patients (McCance *et al.*, 1993).

**Diabetic atherosclerosis**

It is characterized by deposition of atherosclerotic plaques in arterial walls and myocardial infarction. Glycated LDL may be responsible for the hyperlipidemia and accelerated foam cell formation in diabetic patients (Lopes-Virella *et al.*, 1988). Diabetic patients have high LDL-AGE levels and their reduced serum clearance. Increased oxidation of LDL leads to increase in this atherogenic oxidized LDL in diabetes during glycation and AGE formation (Bucala, 1997). Glycated HDL has an indirect role in atherosclerotic plaque formation in which AGEs have been detected (Hedrick *et al.*, 2000). AGEs promote these plaques through secretion of several cytokines including insulin-like growth factor-I (IGF-I) and platelet-derived growth factor (PDGF) (Vlassara, 1996).
TABLE 1

Role of AGEs and AGE receptors in the pathogenesis of diabetic complications.


<table>
<thead>
<tr>
<th>Diabetic atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular tissue AGE accumulation → protein crosslinking → oxidative damage</td>
</tr>
<tr>
<td>Increased vascular matrix → thickening and narrowing of lumen</td>
</tr>
<tr>
<td>Increased endothelial cell permeability and procoagulant activity → thrombosis</td>
</tr>
<tr>
<td>Mononuclear cell chemotaxis/activation → cytokine and growth factor release</td>
</tr>
<tr>
<td>Increased macrophage uptake of AGE-LDL → atheroma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diabetic kidney disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased mesangial matrix secretion</td>
</tr>
<tr>
<td>Increased basement membrane deposition</td>
</tr>
<tr>
<td>Increased vascular permeability</td>
</tr>
<tr>
<td>Increased growth factor secretion</td>
</tr>
<tr>
<td>Glomerular hypertrophy → glomerulosclerosis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diabetic retinopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased cell permeability → vascular leakage and retinal damage</td>
</tr>
<tr>
<td>Increased vessel wall thickening → occlusion → retinal ischemia → neovascularization</td>
</tr>
<tr>
<td>Increased intravascular coagulation → occlusion → retinal ischemia → neovascularization</td>
</tr>
</tbody>
</table>
A study on diabetic mice showed that deficiency of apolipoprotein E (apo E) promotes atherosclerosis and increased expression of RAGE and AGEs (Park et al., 1998). Approximately 2% to 5% of apo B in the plasma of diabetic persons are glycated, compared with about 1% in the plasma from nondiabetic control subjects (Turk, 2001).

**Objectives of the present study**

Non-enzymatic glycation of proteins with generation of free radicals occur by covalent interaction between sugar and free amino groups of proteins. The early and advanced glycation end products are known to be involved in the pathogenesis of diabetes and associated secondary complications. These products and free radicals are known to cause severe protein damage resulting in major structural alterations, which is implicated either through their functional disability or these being highly immunogenic.

Since glycation of proteins primarily occurs at intrachain lysine residues, the present study was planned to study the effect of glucose and reactive oxygen species (‘OH) on poly-L-lysine, a homopolymer of lysine residues. It was first modified with glucose to obtain a high yield of Amadori products (an early glycation product) and then the glycated poly-L-lysine was subjected to hydroxyl radical (‘OH) generated by Fenton reaction. Native and modified poly-L-lysine were then characterized by various biochemical, biophysical and immunological studies.

Antigenicity of native and modified poly-L-lysine was probed by inducing antibodies in rabbits and assessed by direct binding ELISA. The specificity of the induced antibodies was evaluated by competition ELISA and gel retardation assay. The serum of patients with diabetes mellitus contains a variety of autoantibodies that react with a variety of protein and nuclear antigens. In order to assess the possible role of glycated poly-L-lysine and ROS-glycated poly-L-lysine in diabetes and associated secondary complications, serum samples of diabetes patients were investigated for the presence of antibodies against native and modified forms of poly-L-lysine. The polyclonal anti-glycated- and anti-ROS-glycated-poly-L-lysine antibodies could be used as a probe to detect glycation and ROS modification of lysine rich proteins in diabetes patients as well as in healthy subjects.
Materials & Methods
MATERIALS

Poly-L-lysine, Millipore filter (0.2 μm), diethylene triaminepentaacetic acid (DTPA), catalase, superoxide dismutase (SOD), bovine serum albumin (BSA), protein A-Sepharose CL-4B (5 ml pre-packed column), agarose, coomassie brilliant blue G-250, anti-human and anti-rabbit IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, Tween-20, Freund’s complete and incomplete adjuvants were purchased from Sigma Chemical Company, U.S.A. D-Glucose and sodium borohydride (NaBH₄) were obtained from Merck (India). Acrylamide, bisacrylamide, ammonium persulphate, sodium dodecyl sulphate (SDS) and nitrobluetetrazolium (NBT) were from SRL Pvt. Ltd., India. N,N,N’N’-Tetramethylethylenediamine (TEMED) was obtained from BDH Chemicals Ltd., England. Polystyrene microtitre flat bottom ELISA plates and modules were purchased from NUNC, Denmark. Silver nitrate, hydrogen peroxide, methanol, isopropanol, formaldehyde, glacial acetic acid and glycerol were from Qualigens Fine Chemicals, India. All other chemicals/reagents were of the highest analytical grade available from commercial sources.

Collection of sera and blood samples

Blood samples from diabetes mellitus patients were collected from outdoor and indoor patients under treatment at the Jawaharlal Nehru Medical College Hospital, A.M.U., Aligarh. Samples from age/sex matched healthy individuals were used as normal control. Sera, separated from the blood samples, were heated at 56 °C for 30 min. to inactivate complement proteins and stored in aliquots at -20 °C with 1% sodium azide.

METHODS

Protein estimation by Bradford (dye-binding) method

This assay is based on colour change observed when an acidic dye binds hydrophobically to protein having positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue colour is observed (λₘₐₓ = 595 nm).
Dye preparation

100 μg of coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added to it. The resulting solution was diluted to a final volume of 1.0 litre and filtered through a Whatman No. 1 filter paper to remove undissolved particles.

Protein assay

To 1.0 ml solutions, containing 10-100 μg polypeptide, 5.0 ml of dye solution was added and the contents were mixed by vortexing. The absorbance was read at 595 nm after 5 min., against a blank reagent.

Modification of poly-L-lysine by glucose

For the preparation of high contents of glycated Amadori product but an extremely low level of AGEs, poly-L-lysine (1mg/ml) was incubated for 7 days at 37 °C with 50 mM glucose in 20 mM sodium phosphate buffer (pH 7.4) in the presence of 1 mM diethylenetriaminepentaacetic acid (DTPA) (Nagai et. al., 1997). The sample was then extensively dialyzed against sodium phosphate buffer (pH 7.4) and stored at -20 °C before use. Poly-L-lysine dissolved in the same buffer and incubated in the above described conditions was used as control.

Same procedure was adopted for the glycation of IgG, HSA and histone.

Modification of glycated poly-L-lysine by reactive oxygen species (ROS)

Glycated poly-L-lysine was incubated for 1 hr at 37 °C with 10mM anhydrous FeCl2 in the presence of 10 mM H2O2 in 20 mM sodium phosphate buffer (pH 7.4), (Nagai et al, 1997). Excess of hydrogen peroxide was removed by extensive dialysis at 4 °C against phosphate buffered saline (PBS). This condition would be favourable for the generation of 'OH radical.

Glycated IgG, HSA and histone were also modified by ROS as mentioned above.

Effect of quenchers on ROS-glycated poly-L-lysine

The effect of individual free radical quenchers like SOD (an O2− scavenger) at 500 units, EDTA (a metal chelator) at 5 mM, catalase (an H2O2 scavenger) at 500
units and mannitol (an OH scavenger) at 100 mM, on Fe$^{2+}$ induced ROS modification of Amadori poly-L-lysine was determined by carrying out the reaction in the presence of specific quencher.

**Amadori product / ketoamine estimation**

The level of Amadori product in the native and modified poly-L-lysine was determined by an established colorimetric procedure (Johnson *et al.*, 1982) using nitroblue tetrazolium (NBT) with minor modification. Native, glycated and ROS-glycated poly-L-lysine samples (100 µl) were added to the wells of 96 well microtitre plate in duplicate. 100 µl of NBT reagent (250 µM in 0.1 M carbonate buffer, pH 10.35) was added to each well and incubated at 37 °C for 2 h. The reduction rate of NBT was monitored by increase in absorbance at 525 nm. The content of Amadori products was determined using an extinction coefficient of 12640 cm$^{-1}$ M$^{-1}$ for monoformazan (Mironova *et al.*, 2005).

**Determination of 5-hydroxymethylfurfural (HMF)**

The thiobarbituric acid (TBA) reaction for determining the formation of 5-hydroxymethylfurfural (HMF) from Amadori products of glycated poly-L-lysine was employed as described previously (Fluckiger and Winterhalter, 1976), with slight modifications. Briefly, 1 ml each of native and modified poly-L-lysine samples were mixed with 1 M oxalic acid and incubated at 100 °C for 2 hr (10). The protein from the assay mixture was removed by precipitation with 40% trichloroacetic acid. TBA (0.05 M) was added to protein free filtrate and incubated at 40 °C for 40 min. The colour was developed and amount of HMF was calculated using molar extinction coefficient value of $4 \times 10^4$ at 443 nm for HMF.

**Spectrophotometry**

Absorbance (A) of native and modified poly-L-lysine samples was recorded in the wavelength 190-400 nm range on a Shimadzu UV-1700 spectrophotometer using 1 cm-path length cuvette. The increase in absorbance (hyperchromicity) was calculated using the following equation

$$% \text{Hyperchromicity} = \left( \frac{A_{\text{modified poly-L-lysine}} - A_{\text{native poly-L-lysine}}}{A_{\text{modified poly-L-lysine}}} \right) \times 100$$
Fluorescence measurements were performed on Shimadzu RF 5301-PC spectrofluorophotometer. The fluorescence spectra were recorded at 25 ± 0.1°C in a 1 cm-path length cuvette with a slit width of 5 nm. The native and modified poly-L-lysine samples were excited at different wavelengths (290-410 nm) and the emission spectra were recorded in the range (300-500 nm) to obtain the maximum intensity peak. Loss of fluorescence intensity (FI) was calculated using the following equation

\[
\text{% Loss of FI} = \left( \text{FI}_{\text{native poly-L-lysine}} - \text{FI}_{\text{modified poly-L-lysine}} / \text{FI}_{\text{native poly-L-lysine}} \right) \times 100
\]

Circular dichroism spectropolarimetry

CD of native and modified poly-L-lysine was measured on a Jasco J-810 spectropolarimeter equipped with a temperature controlled sample cell holder. Spectra were taken with a scan speed of 20 nm/min at a response time of 1 sec. Each spectrum was the average of two scans. Far-UV CD spectra were taken at protein concentration of 3.0 µM with a cell of 1 mm path length. For each sample, buffer was used as blank.

The results in mean residual ellipticity (MRE), expressed in deg.cm².mol⁻¹, as

\[
\text{MRE} = \text{CD} / (10 \times n \times l \times C_p)
\]

where CD is in milli-degree (mdeg), n is the number of amino acid residues, l is the path length of the cell and C_p is the mole fraction.

FT-IR and NMR Spectrometry

FT-IR spectra of the samples were obtained on a Shimadzu 8201-PC spectrometer with a resolution of 4 cm⁻¹. Samples to be analyzed on FT-IR spectrophotometer were first lyophilized and prepared as KBr pellets.

¹H NMR spectra were recorded on a Bruker DRX-300 MHz FT NMR spectrometer. All samples for NMR spectroscopy were first lyophilized and prepared using DMSO as a solvent. The chemical shifts in parts per million (ppm) are expressed with respect to tetramethylsilane (TMS) as a reference.

Reduction of glycated poly-L-lysine with NaBH₄

Glycated poly-L-lysine was reduced with 200–300-folds molar excess of sodium borohydride (NaBH₄) in phosphate buffer, pH 8.0, using a previously described procedure (Njoroge et al., 1988). Excess borohydride was destroyed by slow addition of IN HCl.
Immunization schedule

The immunization of random bred, female New Zealand white rabbits was performed as described previously (Dixit et al., 2005). Briefly, rabbits were immunized intramuscularly at multiple sites with 100 μg of each of native and modified poly-L-lysine, emulsified with an equal volume of Freund’s complete adjuvant. The animals were boosted in Freund’s incomplete adjuvant at weekly intervals for 6 weeks with the same amount of antigen. Test bleeds were performed 7 days post boost and antibody titre was determined. Blood was taken out from the ear vein and serum separated from the blood (preimmune and immune) was heated at 56 ºC for 30 min to inactivate complement proteins and stored at -20 ºC with 0.1% sodium azide as preservative.

Isolation of IgG by Protein A-Sepharose

Immunoglobulin G (IgG) was isolated by affinity chromatography on Protein A-Sepharose CL-4B column (Dixit et al., 2005). Serum (0.5 ml), diluted with an equal volume of PBS, pH 7.4 was applied to the column, previously equilibrated with the same buffer. The wash through was recycled 2-3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride and neutralized with 1 ml of 1.0 M Tris-HCL, pH 8.5. The fractions (3 ml) were read at 251 and 278 nm. The IgG concentration was determined considering 1.40 OD_{280} = 1.0 mg/ml. The isolated IgG was dialysed against PBS, pH 7.4 and stored at -20 ºC with 0.1% sodium azide.

ELISA

The following reagents and buffers were prepared in distilled water and used in ELISA.

Antigen coating buffer
50 mM carbonate-bicarbonate, pH 9.6

Tris buffered saline (TBS)
10 mM Tris, 150 mM NaCl, pH 7.4

Tris buffered saline-Tween-20 (TBS-T)
20 mM Tris, 150 mM NaCl, 2.68 mM KCl, 0.05% Tween-20, pH 7.4
Carbonate-bicarbonate buffer

15 mM sodium carbonate, 35 mM sodium bicarbonate, 2 mM MgCl, pH 9.6

Substrate

500 μg p-nitrophenyl phosphate in 1 ml carbonate-bicarbonate buffer.

Enzyme linked immunosorbent assay (ELISA) was carried out on polystyrene plates with slight modification (Ali and Alam, 2002). Polystyrene polysorp immunoplates were coated with 100 μl of native or modified poly-L-lysine (10 μg/ml) in antigen coating buffer for 2 h at 37 °C and overnight at 4 °C. Each sample was coated in duplicate and half of the plate devoid of antigen coating served as control. Unbound antigen was washed twice with TBS-T and unoccupied sites were blocked with 150 μl of 2% fat free milk in TBS for 4–6 h at 37 °C. After incubation the plates were washed three times with TBS-T. Test serum, serially diluted in TBS-T, was added to each well (100 μl/well) and was left for 2 h at 37 °C and overnight at 4 °C. After incubation, the plates were washed thrice with TBS-T and bound antibodies were assayed with anti-rabbit (or anti-human, for human autoantibodies) alkaline phosphatase conjugate in TBS. After incubation at 37 °C for 2 h, the plates were again washed three times with TBS-T and twice with distilled water and were subsequently developed using p-nitrophenyl phosphate as substrate. The absorbance (A) of each well was monitored at 410 nm on an automatic microplate reader. Results have been expressed as mean of A<sub>test</sub>−A<sub>control</sub>.

Competition ELISA

The antigenic specificity of the antibodies was determined by competition ELISA (Habib et al., 2005). Varying amounts of inhibitors (0–20 μg/ml) were mixed with a constant amount of antisera or IgG. The mixture was incubated at room temperature for 2 h and overnight at 4 °C. The immune complex thus formed was coated in the wells instead of the serum. The remaining steps were the same as in direct binding ELISA. Percent inhibition was calculated using the formula:

\[
\text{Percent Inhibition} = 1 - \left( \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \right) \times 100
\]

Band shift assay

For the visual detection of antigen antibody binding and immune complex formation, gel retardation assays were performed (Dixit and Ali, 2004). Immune
complexes were prepared by incubating constant amount of native and modified poly-L-lysine with varying amounts of affinity purified diabetes IgG or immune IgG in PBS for 2 h at 37 °C and overnight at 4 °C. One-fourth of sample dye was added to the mixture and electrophoresed on 8% SDS-PAGE for 6 h with 80 V at 4 °C. The gels were visualized using silver nitrate staining.

**SDS-PAGE**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed by the tris-glycine buffer system of Laemmli (1970) using slab gel electrophoresis apparatus. Concentrated stock solution of 30% acrylamide containing 0.8% bisacrylamide, 1.0 M Tris (pH 6.8 and 8.8) and 10% SDS were prepared and mixed in appropriate proportion to give the final required percentage. Samples were prepared in the sample buffer containing 1% (w/v) SDS, 10% (v/v) glycerol, 0.0625 M Tris, pH 6.8 and 1% bromophenol blue as tracking dye. Electrophoresis was performed in electrophoresis buffer containing 0.025 M Tris and 0.2 M glycine till the tracking dye reached the bottom of the gel.

**Silver nitrate staining**

After electrophoresis the protein bands were fixed by rapidly immersing the gel in a mixture of 40% methanol and 13.5% formaldehyde for 10 min with instant shaking. The gel was washed twice with distilled water each time for 5 min. This was followed by incubation in 0.02% sodium thiosulphate for 1 min and two times washing with distilled water at an interval of 20 sec. The washed gel was then immersed in 0.1% silver nitrate solution for 10 min and then rinsed with distilled water and the bands were developed with 280 mM solution of sodium carbonate containing 0.5% formaldehyde and 0.02% sodium thiosulphate. After the appearance of brown colored band, the reaction was stopped by transferring the gel to a mixture of 10% acetic acid and 30% methanol solution for 5 min. It was again washed 4-5 times with distilled water and finally stored in distilled water.
Results
Determination of Amadori products

NBT reactive ketoamine in a mixture of glucose and –NH₂ containing biomolecules is a standard and reliable chemical parameter to confirm early glycation products. High content of Amadori product was prepared by 7 days incubation of poly-L-lysine at 37 °C with 50 mM glucose in the presence of 5 mM DTPA in 20 mM sodium phosphate buffer (pH 7.4). ROS modification of glycated poly-L-lysine was done by incubating glycated poly-L-lysine for 1 hr at 37 °C with 5 mM anhydrous FeCl₂ in the presence of 10 mM H₂O₂ in 20 mM sodium phosphate buffer (pH 7.4). As shown in Fig. 4, glycated poly-L-lysine yielded high content of ketoamine (9.4±0.8 mM) while ROS-glycated poly-L-lysine had a very low ketoamine content (1.8±0.4 mM). Native poly-L-lysine showed almost a negligible amount of ketoamine.

Determination of 5-Hydroxymethylfurfural

5-Hydroxymethylfurfural (HMF) that might have formed from hydrolysis of the Amadori product of glycated poly-L-lysine was estimated as thiobarbituric acid (TBA) reactive substance for the presence of glucose (hexose) moiety on glycated poly-L-lysine (Fig. 5). Loss in HMF content was observed for ROS-glycated poly-L-lysine. The HMF content in glycated poly-L-lysine and ROS-glycated poly-L-lysine were calculated to be 10.4±1.0 μM and 1.7±0.4 μM respectively while the poly-L-lysine without glucose in the reaction mixture, taken as control, had almost negligible level of HMF.

FAR-UV Spectra

As shown in Fig. 6, the native and modified poly-L-lysine gave characteristic peak at 202 nm. Upon glycation and ROS modification, hyperchromicity was observed. Glycated poly-L-lysine and ROS-glycated poly-L-lysine samples respectively exhibited 59.2% and 78.9% hyperchromicity. The ROS-glycated poly-L-lysine samples showed a new broad peak centered at 278 nm. Absorbance and percent hyperchromicity values of poly-L-lysine and modified poly-L-lysine are summarized in Table 2.
Fig. 4. Level of Amadori product as ketoamines in poly-L-Lysine (PLL), glycated poly-L-lysine (Glycated PLL) and ROS-glycated poly-L-lysine (ROS-glycated PLL).
Fig. 5. Level of HMF in poly-L-lysine (PLL), glycated poly-L-lysine (glycated PLL) and ROS-glycated poly-L-lysine (ROS-glycated PLL).
Fig. 6. Ultraviolet absorption spectra of poly-L-lysine (---), glycated poly-L-lysine (---) and ROS-glycated poly-L-lysine (---).
**TABLE 2**

**UV absorption data on poly-L-lysine and modified poly-L-lysine**

<table>
<thead>
<tr>
<th></th>
<th>Wavelength (nm)</th>
<th>Absorbance</th>
<th>Percent Hyperchromicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-L-lysine</td>
<td>202</td>
<td>0.52</td>
<td>~</td>
</tr>
<tr>
<td>Glycated poly-L-lysine</td>
<td>202</td>
<td>0.84</td>
<td>59.2</td>
</tr>
<tr>
<td>ROS-glycated poly-L-lysine</td>
<td>202</td>
<td>1.71</td>
<td>78.9</td>
</tr>
</tbody>
</table>
Quenching studies on ROS-modified glycated poly-L-lysine

The ROS modification in the present study is a result of production of hydroxyl radical, a powerful oxidant. Its \textit{in vitro} generation was confirmed by the use of quenchers of various radicals. We exploited SOD (O$_2^-$ scavenger), EDTA (metal ion chelator), catalase (an H$_2$O$_2$ scavenger) and mannitol (OH scavenger) to study their quenching effect on the oxidation of glycated poly-L-lysine. When ROS modification was carried out in the presence of each of these quenchers, certain degree of inhibition of the modification was observed with individual quenchers (Fig. 7). SOD had no significant inhibitory effect while both catalase and mannitol appeared to be strong inhibitors of the ROS modification causing 78.6\% and 75.1\% quenching respectively. EDTA also exhibited a marked inhibition (82.1\%) of the ROS modification.

Fluorescence Studies

Fluorescence emission profiles of poly-L-lysine excited at different wavelengths (290-370 nm) were recorded to find out emission wavelength of maximum intensity which could be exploited to monitor changes in the modified poly-L-lysine. The fluorescence intensity of poly-L-lysine was maximum at an emission wavelength of 324 nm upon excitation at 290 nm. The modification of poly-L-lysine did not alter its wavelength of maximum emission. Emission profiles of poly-L-lysine and their modified counterparts have been presented in Fig. 8. Glycation of amino groups of lysine residues in poly-L-lysine and ROS modification of glycated poly-L-lysine were evident from 42.0\% and 68.3\% loss of intensity respectively. There was a slight increase in emission intensity of ROS-glycated poly-L-lysine at 365 nm. The fluorescence characteristics of poly-L-lysine and modified poly-L-lysine have been presented in Table 3.

Circular dichroic (c.d.) spectra

Far-UV c.d. spectral profile used for rapid determination of protein’s secondary structure is a useful technique for quick assessment of conformation. The amide chromophore of the peptide bonds in proteins gives c.d. spectra in far-UV range (200-250 nm). The characteristic far-UV c.d. properties of proteins were
exploited to look into the secondary structure of poly-L-lysine at pH 7.4 and changes obtained upon glycation and hydroxyl radical modification. The far-UV c.d. profile of poly-L-lysine and its modified counterpart is shown in Fig. 9. Poly-L-lysine showed a far-UV c.d. spectrum characteristic of a random coil. Poly-L-lysine showed a positive c.d. at 217 nm and a negative c.d. at 200 nm. The c.d. spectrum of modified poly-L-lysine showed significant changes in c.d. intensity and signals at 217 nm and 200 nm when compared to that of native poly-L-lysine. Glycated poly-L-lysine showed a negative c.d. both at 217 nm and 200 nm while ROS-glycated poly-L-lysine showed a negative c.d. at 217 nm and a positive c.d. at 200 nm. Both modified poly-L-lysine showed a significant decrease in c.d. intensity at 217 nm and a significant increase in c.d. intensity at 200 nm. Mean residual ellipticity (MRE) values of non-modified and modified poly-L-lysine at 217 nm and 200 nm are presented in Table 4. These findings indicate alteration in the original conformation of poly-L-lysine and a partial destruction of the secondary structure of poly-L-lysine upon modification.

IR Spectra

The FT-IR spectra of poly-L-lysine (Fig. 10a) and modified poly-L-lysine (Fig. 10b and c) were determined by the position and intensity of bands of amide and other characteristic groups present in their structures. The band near 1062 cm⁻¹ in the poly-L-lysine spectrum corresponding to C–N bond of amines was more prominent and broad peaked in the spectra of glycated and ROS-glycated forms of poly-L-lysine. The band at 1625 in poly-L-lysine, related to amide I was shifted to 1653 and 1655 cm⁻¹ in glycated and ROS-glycated forms of poly-L-lysine respectively. The band at 702 cm⁻¹ corresponds to keto (C=O) group of Amadori products in glycated-poly-L-lysine. The band at 1158 cm⁻¹ in the spectra of ROS-glycated poly-L-lysine can be assigned to methylene (CH₂) group of AGEs. The band at 3391 cm⁻¹ related to the side chain ε-amino groups in poly-L-lysine was shifted to 3399 and 3401 cm⁻¹ with change of intensity in glycated and ROS-glycated counterparts of poly-L-lysine respectively. The important diagnostic IR bands corresponding to poly-L-lysine and modified poly-L-lysine are compiled in Table 5.
Fig. 7. Effect of free radical scavengers on ROS induced modification of glycated poly-L-lysine. The $O_2^-$ scavenger (SOD, 500 units); metal chelator (EDTA, 5 mM); $H_2O_2$ scavenger (Catalase, 500 units); $OH^-$ scavenger (Mannitol, 100 mM) were used.
Fig. 8. Fluorescence emission spectra of poly-L-lysine (——), glycated poly-L-lysine (---) and ROS-glycated poly-L-lysine (— — —). Excitation wavelength was 290 nm.
Fig. 9. Circular dichroic spectra of poly-L-lysine (——), glycated poly-L-lysine (--) and ROS-glycated poly-L-lysine (———).
### TABLE 3

**Fluorescence characteristic of poly-L-lysine and modified poly-L-lysine**

<table>
<thead>
<tr>
<th></th>
<th>Intensity (A.U.)</th>
<th>Percent Loss in Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-L-lysine</td>
<td>92.3</td>
<td>–</td>
</tr>
<tr>
<td>Glycated poly-L-lysine</td>
<td>53.5</td>
<td>42.0</td>
</tr>
<tr>
<td>ROS-glycated poly-L-lysine</td>
<td>29.3</td>
<td>68.3</td>
</tr>
</tbody>
</table>

Excitation and emission wavelengths were 290 and 324 nm respectively.

### TABLE 4

**CD characteristic of poly-L-lysine and modified poly-L-lysine**

<table>
<thead>
<tr>
<th></th>
<th>MRE at 217 nm (deg.cm².mol⁻¹)</th>
<th>MRE at 200 nm (deg.cm².mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-L-lysine</td>
<td>+86.27</td>
<td>–2124.28</td>
</tr>
<tr>
<td>Glycated poly-L-lysine</td>
<td>–17.92</td>
<td>–384.29</td>
</tr>
<tr>
<td>ROS-glycated poly-L-lysine</td>
<td>–25.76</td>
<td>+154.61</td>
</tr>
</tbody>
</table>
Fig. 10. Infrared spectra of poly-L-lysine (a), glycated poly-L-lysine (b) and ROS-glycated poly-L-lysine (c).
<table>
<thead>
<tr>
<th></th>
<th>N–H</th>
<th>C–N</th>
<th>C=O</th>
<th>ε-NH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-L-lysine</td>
<td>1625</td>
<td>1062</td>
<td>−</td>
<td>3391</td>
</tr>
<tr>
<td>Glycated poly-L-lysine</td>
<td>1653</td>
<td>1062ᵇ</td>
<td>1702</td>
<td>3399</td>
</tr>
<tr>
<td>ROS-glycated poly-L-lysine</td>
<td>1655</td>
<td>1062ᵇ</td>
<td>−</td>
<td>3401</td>
</tr>
</tbody>
</table>

ᵇ = broad band

TABLE 5

Characteristic IR bands of poly-L-lysine and modified poly-L-lysine in wavenumbers (cm⁻¹)
NMR Spectra

Resonance signals can be identified and characterized in $^1$H-NMR spectrum of poly-L-lysine (Fig. 11a) and modified poly-L-lysine (Fig. 11b and c), recorded in DMSO. In poly-L-lysine spectrum, the signals at $\delta = 2.72$ ppm and $\delta = 3.16$ ppm should be assigned to the chemical shifts of $\varepsilon$-methylene ($\varepsilon$-CH$_2$) protons. The assignment of chemical shifts of $\beta$, $\gamma$, $\delta$-methylene protons is inferred from multiplicity of the resonance signals within a range of 1.10-1.92 ppm. The multiplicity of the signal is due to the J coupling between adjacent methylene protons. The signal at $\delta = 2.5$ ppm is due to DMSO.

Determination and assignment of glycated lysine residue in glycated poly-L-lysine and oxidation of glycated poly-L-lysine in ROS-glycated poly-L-lysine were undertaken by direct comparison of changes in the NMR spectra before and after modification. In the modified poly-L-lysine, the chemical shifts of $\varepsilon$-methylene protons were found at $\delta = 2.8$ ppm and $\delta = 3.0$ ppm with their multiple signals while the shift of $\alpha$-methylene proton appeared at $\delta = 4.2$ ppm with multiple signal. In the spectrum of glycated poly-L-lysine the resonance signals within the range of 3.38-3.62 ppm are assigned to the hydrogen atoms of glucose while with ROS-glycated poly-L-lysine these proton signals become more intense at $\delta = 3.62$ ppm due to oxidation of glycated poly-L-lysine. The appearance of other multiple and singlet resonance signal at $\delta = 4.89$ ppm in the spectra of glycated poly-L-lysine and ROS-glycated poly-L-lysine respectively should be assigned to chemical shift of the methylene proton of glucose. The important $^1$H- NMR chemical shifts corresponding to poly-L-lysine and modified poly-L-lysine are presented in Table 6.

Antigenicity of native and modified poly-L-lysine

The antigenicity of native and modified poly-L-lysine was determined by inducing antibodies in rabbits against native poly-L-lysine, glycated poly-L-lysine and ROS-glycated poly-L-lysine. The antigenic specificity of induced antibodies was assayed by direct binding and competition ELISA. Binding of these antibodies was further ascertained by band shift assay.
Fig. 11. $^1$H-NMR spectra of poly-L-lysine (a), glycated poly-L-lysine (b) and ROS-glycated poly-L-lysine (c).
**TABLE 6**

Characteristic $^1$H-NMR chemical shifts (ppm) of poly-L-lysine and modified poly-L-lysine

<table>
<thead>
<tr>
<th></th>
<th>Lysine Component</th>
<th>Glucose Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_\varepsilon(CH_2)$</td>
<td>$C_{\beta,\gamma,\delta}(CH_2)$</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>2.72$^{s}$, 3.16$^{s}$</td>
<td>1.10-1.92</td>
</tr>
<tr>
<td>Glycated poly-L-lysine</td>
<td>2.80$^{m}$, 3.00$^{m}$</td>
<td>–</td>
</tr>
<tr>
<td>ROS-glycated poly-L-lysine</td>
<td>2.80$^{m}$, 3.00$^{m}$</td>
<td>–</td>
</tr>
</tbody>
</table>

$s$ = single signal  
$m$ = multiple signal
Antibodies against native poly-L-lysine

Native poly-L-lysine showed very low titre antibodies (<1:400) by direct binding ELISA (Fig. 12). The binding of pre-immune serum was almost similar to that of immune serum at low titres. In competition ELISA an inhibition of 28.3% was obtained at 20 μg/ml of the immunogen (Fig. 13).

Antibodies against glycated poly-L-lysine

Glycated poly-L-lysine antiserum showed high titre antibodies (> 1: 12800) by direct binding ELISA. However, the binding of pre-immune serum was of low magnitude (Fig. 14). In competition ELISA, a maximum of 75% inhibition was observed at 20 μg/ml and 50% inhibition was achieved only at 7.2 μg/ml of the immunogen (Fig. 15).

Purification and characterization of glycated poly-L-lysine immune IgG

Immunoglobulin G was isolated from pre-immune and immune rabbit antiserum of glycated poly-L-lysine by affinity chromatography on Protein-A Sepharose column (Fig. 16). The purity of IgG was evaluated by SDS-polyacrylamide gel electrophoresis in the absence of a reducing agent. The purified IgG migrated as a single band on 7.5% polyacrylamide gel upon electrophoresis (Fig. 16 inset).

Direct binding ELISA of the purified anti-glycated-poly-L-lysine IgG showed strong reactivity towards its immunogen (Fig. 17). However, the pre-immune IgG showed negligible binding.

When glycated poly-L-lysine was treated with NaBH₄, the reduced glycated poly-L-lysine showed an inhibition of 62.5% at 20 μg/ml in comparison to non-reduced glycated poly-L-lysine which caused 85.3% inhibition at 20 μg/ml in the activity of anti-glycated poly-L-lysine IgG (Fig. 18).

Band shift assay

The binding of glycated poly-L-lysine with affinity purified anti-glycated poly-L-lysine IgG was ascertained by band shift assay. Increasing concentrations of the IgG were incubated with a constant amount of the antigen for 2 hr at 37°C and overnight at 4°C. With the increasing concentration of the antibodies a proportional
Fig. 12. Direct binding ELISA of poly-L-lysine with preimmune (■) and immune (▲) sera. Microtitre plates were coated with native poly-L-lysine (10 μg/ml).
Fig. 13. Inhibition ELISA of anti-poly-L-lysine immune (●) and preimmune (■) sera with native poly-L-lysine. Microtitre plates were coated with native poly-L-lysine (10 μg/ml).
Fig. 14. Direct binding ELISA of glycated poly-L-lysine with preimmune (■) and immune (▲) sera. Microtitre plates were coated with glycated poly-L-lysine (10 μg/ml).
Fig. 15. Inhibition ELISA of anti-glycated poly-L-lysine immune (●) and pre-immune (■) sera with glycated poly-L-lysine. Microtitre plates were coated with glycated poly-L-lysine (10 μg/ml).
Fig. 16. Elution profile of anti-glycated poly-L-lysine IgG on Protein-A Sepharose affinity column. *Inset:* SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Fig. 17. Binding of affinity purified anti-glycated poly-L-lysine immune IgG (▲) and preimmune IgG ( ■) to glycated poly-L-lysine. Microtitre plates were coated with glycated poly-L-lysine (10 μg/ml).
Fig. 18. Inhibition ELISA of anti-glycated poly-L-lysine IgG binding to glycated poly-L-lysine. The inhibitors were glycated poly-L-lysine (●) and NaBH₄ reduced glycated poly-L-lysine (■). Microtitre plates were coated with glycated poly-L-lysine (10 µg/ml).
Fig. 19. Band shift assay of anti-glycated poly-L-lysine IgG binding to glycated poly-L-lysine. Lane 1 contains anti-glycated poly-L-lysine IgG (10 μg). Lanes 2-5 contain increasing concentration of IgG (10, 20, 30 and 40 μg) incubated for 2 hr at 37 °C and overnight at 4 °C with a constant amount of antigen (10 μg).
increase in the formation of high molecular weight immune complexes resulted in the retarded mobility, as visualized by shifting of bands towards the wells in the electrophoretic analysis (Fig. 19).

**Antibodies against ROS-glycated poly-L-lysine**

ROS-glycated poly-L-lysine antiserum showed high titre antibodies (> 1: 12800) by direct binding ELISA. However, the binding of pre-immune serum was of low magnitude (Fig. 20). In competition ELISA, a maximum of 70.8% inhibition was observed at 20 μg/ml and 50% inhibition was achieved only at 8 μg/ml of the immunogen (Fig. 21).

**Purification and characterization of ROS modified glycated poly-L-lysine immune IgG**

Immunoglobulin G was isolated from pre-immune and immune rabbit antiserum of ROS-glycated poly-L-lysine by affinity chromatography on Protein A-Sepharose column (Fig. 22). The purity of IgG was evaluated by SDS-polyacrylamide gel electrophoresis in the absence of a reducing agent. The purified IgG migrated as a single band on 7.5% gel (Fig. 22 inset).

Direct binding ELISA of the purified ROS-glycated poly-L-lysine IgG showed strong reactivity towards its immunogen (Fig. 23). Pre-immune IgG from rabbit, as negative control, showed negligible binding.

**Band shift assay**

The binding of ROS-glycated poly-L-lysine with affinity purified anti-ROS-glycated poly-L-lysine IgG was ascertained by band shift assay. Increasing the concentration of IgG, incubated with constant amount of antigen for 2 hr at 37°C and overnight at 4°C, resulted in a proportional increase in the formation of high molecular weight immune complexes as visualized by gradually increased band intensity near the wells in polyacrylamide gel electrophoresis (Fig. 24).
Fig. 20. Direct binding ELISA of ROS-glycated poly-L-lysine with preimmune (■) and immune (▲) sera. Microtitre plates were coated with ROS-glycated poly-L-lysine (10 μg/ml).
Fig. 21. Inhibition ELISA of anti-ROS-glycated poly-L-lysine immune (●) and preimmune (■) sera with ROS-glycated poly-L-lysine. Microtitre plates were coated with ROS-glycated poly-L-lysine (10 μg/ml).
Fig. 22. Elution profile of anti-ROS-glycated poly-L-lysine IgG on Protein-A Sepharose affinity column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Fig. 23. Binding of affinity purified anti-ROS-glycated poly-L-lysine immune IgG (▲) and preimmune IgG (■) to ROS-glycated poly-L-lysine. Microtitre plates were coated with ROS-glycated poly-L-lysine (10 μg/ml).
Fig. 24. Band shift assay of anti-ROS-glycated poly-L-lysine IgG binding to ROS-glycated poly-L-lysine. Lane 1 contains anti-ROS-glycated poly-L-lysine IgG (10 μg). Lanes 2-5 contain increasing concentration of IgG (10, 20, 30 and 40 μg) incubated for 2 hr at 37 °C and overnight at 4 °C with a constant amount of antigen (10 μg).
Fig. 25. Inhibition ELISA of anti-glycated poly-L-lysine IgG binding to glycated poly-L-lysine. The inhibitors were native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and ROS poly-L-lysine (▼). Microtitre plates were coated with glycated poly-L-lysine (10 μg/ml).
Fig. 26. Inhibition ELISA of anti-glycated poly-L-lysine IgG binding to glycated poly-L-lysine. The inhibitors were native human IgG (○), glycated human IgG (●) and ROS-glycated human IgG (▲). Microtitre plates were coated with glycated poly-L-lysine (10 µg/ml).
Fig. 27. Inhibition ELISA of anti-glycated poly-L-lysine IgG binding to glycated poly-L-lysine. The inhibitors were native HSA (○), glycated HSA (●) and ROS-glycated HSA (▲). Microtitre plates were coated with glycated poly-L-lysine (10 μg/ml).
Fig. 28. Inhibition ELISA of anti-glycated poly-L-lysine IgG binding to glycated poly-L-lysine. The inhibitors were native histone (○), glycated histone (●) and ROS-glycated histone (▲). Microtitre plates were coated with glycated poly-L-lysine (10 μg/ml).
<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Maximum % inhibition at 20 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native poly-L-lysine</td>
<td>18.0</td>
</tr>
<tr>
<td>ROS-poly-L-lysine</td>
<td>25.3</td>
</tr>
<tr>
<td>Glycated poly-L-lysine</td>
<td>85.3</td>
</tr>
<tr>
<td>ROS-glycated poly-L-lysine</td>
<td>29.0</td>
</tr>
<tr>
<td>Native human IgG</td>
<td>20.1</td>
</tr>
<tr>
<td>Glycated human IgG</td>
<td>55.0</td>
</tr>
<tr>
<td>ROS-glycated human IgG</td>
<td>26.8</td>
</tr>
<tr>
<td>Native HSA</td>
<td>18.6</td>
</tr>
<tr>
<td>Glycated HSA</td>
<td>48.3</td>
</tr>
<tr>
<td>ROS-glycated HSA</td>
<td>28.5</td>
</tr>
<tr>
<td>Native Histone</td>
<td>16.3</td>
</tr>
<tr>
<td>Glycated Histone</td>
<td>42.7</td>
</tr>
<tr>
<td>ROS-glycated Histone</td>
<td>24.6</td>
</tr>
</tbody>
</table>
Fig. 29. Inhibition ELISA of anti-ROS-glycated poly-L-lysine IgG binding to ROS-glycated poly-L-lysine. The inhibitors were native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and ROS poly-L-lysine (▼). Microtitre plates were coated with ROS-glycated poly-L-lysine (10 µg/ml).
Fig. 30. Inhibition ELISA of anti-ROS-glycated poly-L-lysine IgG binding to ROS-glycated poly-L-lysine. The inhibitors were native human IgG (○), glycated human IgG (●) and ROS-glycated human IgG (▲). Microtitre plates were coated with ROS-glycated poly-L-lysine (10 μg/ml).
Fig. 31. Inhibition ELISA of anti-ROS-glycated poly-L-lysine IgG binding to ROS-glycated poly-L-lysine. The inhibitors were native HSA (○), glycated HSA (●) and ROS-glycated HSA (▲). Microtitre plates were coated with ROS-glycated poly-L-lysine (10 μg/ml).
Fig. 32. Inhibition ELISA of anti-ROS-glycated poly-L-lysine IgG binding to ROS-glycated poly-L-lysine. The inhibitors were native histone (○), glycated histone (●) and ROS-glycated histone (▲). Microtitre plates were coated with ROS-glycated poly-L-lysine (10 µg/ml).
**TABLE 8**

Antigenic specificity of anti-ROS-glycated poly-L-lysine antibodies

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Maximum % inhibition at 20 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native poly-L-lysine</td>
<td>16.5</td>
</tr>
<tr>
<td>ROS-poly-L-lysine</td>
<td>34.0</td>
</tr>
<tr>
<td>Glycated poly-L-lysine</td>
<td>35.3</td>
</tr>
<tr>
<td>ROS-glycated poly-L-lysine</td>
<td>82.6</td>
</tr>
<tr>
<td>Native human IgG</td>
<td>19.8</td>
</tr>
<tr>
<td>Glycated human IgG</td>
<td>25.2</td>
</tr>
<tr>
<td>ROS-glycated human IgG</td>
<td>52.6</td>
</tr>
<tr>
<td>Native HSA</td>
<td>16.1</td>
</tr>
<tr>
<td>Glycated HSA</td>
<td>22.7</td>
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<tr>
<td>ROS-glycated HSA</td>
<td>45.3</td>
</tr>
<tr>
<td>Native Histone</td>
<td>18.0</td>
</tr>
<tr>
<td>Glycated Histone</td>
<td>22.1</td>
</tr>
<tr>
<td>ROS-glycated Histone</td>
<td>40.7</td>
</tr>
</tbody>
</table>
Antibodies against native and modified poly-L-lysine in the sera of diabetes patients

Sera from diabetes patients were tested for binding to native and modified poly-L-lysine (glycated poly-L-lysine and ROS-glycated poly-L-lysine) by direct binding and competitive ELISA. Thirty four serum samples of patients suffering from uncontrolled hyperglycemia (both types of diabetes mellitus) for long duration were used in the study.

Serum antibodies from diabetes patients showed appreciable binding to modified forms of poly-L-lysine as compared to the native form (p<0.001). Similar results were obtained with the sera of diabetic patients having secondary complications (Fig. 33). However, glycated poly-L-lysine showed greater binding than ROS-glycated and NaBH₄ reduced glycated forms of poly-L-lysine to serum antibodies in diabetes patients. The average absorbance at 410 nm with the sera of diabetes patients sera (without secondary complications) binding to native poly-L-lysine, glycated poly-L-lysine, ROS-glycated poly-L-lysine and NaBH₄ reduced glycated poly-L-lysine was 0.10±0.04, 0.63±0.07, 0.54±0.09 and 0.29±0.05 respectively. The average observed absorbance with the sera of diabetes patients (with secondary complications) binding to native poly-L-lysine, glycated poly-L-lysine, ROS-glycated poly-L-lysine and NaBH₄ reduced glycated poly-L-lysine was 0.11±0.04, 0.72±0.08, 0.52±0.07 and 0.33±0.02 respectively. No appreciable binding was observed with the normal subjects.

Competition ELISA of 22 diabetic patients’ sera which showed strong binding to modified forms of poly-L-lysine was carried out to analyze the specific recognition of circulating autoantibodies in diabetic patients for native and modified poly-L-lysine. The autoantibodies showed remarkably higher recognition for modified forms of poly-L-lysine than the native form (p<0.001) in all the sera tested. The maximum inhibition of serum antibodies by native, glycated, ROS-glycated and NaBH₄ reduced glycated forms of poly-L-lysine was observed in the range of 13% to 20.8%, 42% to 62.4%, 40.4% to 61% and 22.8% to 40.9% respectively (Figs. 34-55). The inhibition data of serum antibodies in diabetic patients has been summarized in Table 9.
Fig. 33. Direct Binding of serum antibodies from diabetes patients to native poly-L-lysine (■), glycated poly-L-lysine (■) and ROS-glycated poly-L-lysine (■) and NaBH₄ reduced glycated poly-L-lysine (■). Serum samples from normal human subjects served as control. The microtitre plates were coated with the respective antigens (10 μg/ml).
Fig. 33. Direct Binding of serum antibodies from diabetes patients to native poly-L-lysine (■), glycated poly-L-lysine (■) and ROS-glycated poly-L-lysine (■) and NaBH$_4$ reduced glycated poly-L-lysine ( ). Serum samples from normal human subjects served as control. The microtitre plates were coated with the respective antigens (10 µg/ml).
Fig. 34. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 1) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 35. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 2) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 36. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 3) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 37. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 4) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 38. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 5) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 39. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 6) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 40. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 7) inhibition by native poly-L-lysine (○), glyated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 41. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 8) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 42. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 9) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 43. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 10) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 44. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 11) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 45. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 12) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 46. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 13) inhibition by native poly-L-lysine (○), glycated poly-L-lysine ( ●), ROS-glycated poly-L-lysine ( ▲) and NaBH₄ reduced glycated poly-L-lysine ( ■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 47. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 14) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 48. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 15) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 49. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 16) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 50. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 17) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 51. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 18) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 52. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 19) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 53. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 20) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 54. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 21) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 55. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients. Serum antibodies (sample 22) inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
TABLE 9

Antibodies against native poly-L-lysine and modified forms of poly-L-lysine in the sera of diabetes patients

<table>
<thead>
<tr>
<th>Sera No.</th>
<th>Native poly-L-lysine</th>
<th>Glycated poly-L-lysine</th>
<th>ROS-glycated poly-L-lysine</th>
<th>NaBH₄ reduced glycated poly-L-lysine</th>
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</table>

Mean = SD  

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<td>54.8 ± 5.1</td>
<td>51.6 ± 5.7</td>
<td>32.2 ± 5.1</td>
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</table>

Statistical data shows significant binding of modified forms of poly-L-lysine than native poly-L-lysine (p<0.001) with antibodies in the diabetes sera.
In the case of diabetic patients having secondary complications, the observed inhibition at 20 μg/ml, with native, glycated, ROS-glycated and NaBH₄ reduced glycated forms of poly-L-lysine was in the range of 12.8% to 18.5%, 55% to 68%, 51% to 63% and 30.4% to 49.2% respectively (Figs. 56-66). The data (Table 10) reveal significant inhibition of antibodies by the modified forms of poly-L-lysine as compared to native poly-L-lysine (p<0.001).

**Purification of diabetic patients IgG**

Sera 3, 7 and 9 of diabetes patients with secondary complications showing maximum recognition of modified forms of poly-L-lysine, were used for the purification of IgG. IgG was also isolated from normal human subject. IgG purified by affinity chromatography on Protein A-Sepharose column eluted as a symmetrical single peak (Fig. 67). Purified IgG migrated as a single homogenous band on SDS-PAGE under non-reducing conditions (Fig. 67 inset).

**Anti-glycated poly-L-lysine and anti-ROS-glycated poly-L-lysine antibodies binding to IgG from diabetes patients**

Diabetes induced glycation in the IgG isolated from various diabetic patients was probed by our experimentally induced antibodies against Amadori-rich glycated poly-L-lysine. Immune complexes were formed between the IgGs purified from the sera of diabetes patients and the induced anti-glycated poly-L-lysine antibodies. The immune complexes thus formed were used in competition inhibition ELISA on the plates coated with glycated poly-L-lysine. The induced anti-glycated poly-L-lysine antibodies were inhibited to the extent of 46 to 55.6 percent at a maximum concentration of 20 μg/ml of the IgG from the diabetes patients (Fig. 68).

However, when immune complexes were formed between the IgGs isolated from the diabetes patients and experimentally induced anti-ROS-glycated poly-L-lysine antibodies, the observed inhibition in the activity of our induced antibodies ranged from 42 to 51 percent in the competitive binding assay on a plate coated with ROS-glycated poly-L-lysine (Fig. 69).
Fig. 56. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 57. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 58. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 59. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum 4 inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 60. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Inhibitor Concentration, pg/ml

Fig. 61. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 62. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum 7 inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Inhibitor Concentration, pg/ml

Fig. 63. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 64. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum 9 inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 65. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
Fig. 66. Detection of autoantibodies against native and modified poly-L-lysine in the diabetes patients having secondary complications (DC). DC serum inhibition by native poly-L-lysine (○), glycated poly-L-lysine (●), ROS-glycated poly-L-lysine (▲) and NaBH₄ reduced glycated poly-L-lysine (■). The microtitre plate was coated with glycated poly-L-lysine (10 µg/ml).
TABLE 10

Antibodies against native poly-L-lysine and modified forms of poly-L-lysine in the sera of diabetes patients having secondary complications

<table>
<thead>
<tr>
<th>Sera No.</th>
<th>Native poly-L-lysine</th>
<th>Glycated poly-L-lysine</th>
<th>ROS-glycated poly-L-lysine</th>
<th>NaBH₄ reduced glycated poly-L-lysine</th>
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<td>54.2</td>
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<td>61.0</td>
<td>59.0</td>
<td>39.8</td>
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</table>

Mean ± SD 16.1 ± 1.8 62.5 ± 4.8 57.5 ± 3.7 41.5 ± 6.2

Statistical data shows significant binding of modified forms of poly-L-lysine than native poly-L-lysine (p<0.001) with antibodies in diabetic sera with secondary complications.
Fig. 67. Elution profile of an isolated IgG from diabetes patients with secondary complications on protein-A Sepharose affinity column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Fig. 68. Inhibition of anti-glycated poly-L-lysine IgG binding to glycated poly-L-lysine by IgG isolated from sera of normal healthy subject (○) and 3 diabetes patients with secondary complications (■, ▲, ●). The microtitre plate was coated with glycated poly-L-lysine (10 μg/ml).
Fig. 69. Inhibition of anti-ROS-glycated poly-L-lysine IgG binding to ROS-glycated poly-L-lysine by IgG isolated from sera of normal healthy subject (○) and 3 diabetes patients with secondary complications (■, ▲, ●). The microtitre plate was coated with ROS-glycated poly-L-lysine (10 µg/ml).
For control experimentation IgG isolated from normal human subject produced only 15 to 18 percent inhibition in the activities of anti-glycated poly-L-lysine and anti-ROS-glycated poly-L-lysine antibodies.

The percent inhibition data of the IgGs isolated from the diabetes patients and normal human subject with anti-glycated poly-L-lysine and anti-ROS-glycated poly-L-lysine antibodies has been presented in Table 11.
TABLE 11

Binding of anti-glycated poly-L-lysine and anti-ROS-glycated poly-L-lysine antibodies to IgG from the sera of normal subject and diabetes patients

<table>
<thead>
<tr>
<th>Serum Isolated IgG</th>
<th>Maximum % inhibition at 20 µg/ml</th>
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<th>ROS-glycated poly-L-lysine</th>
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<td>Normal healthy subject</td>
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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^ε-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). •OH generated by Fenton reaction between Fe^{2+} and Amadori product-derived endogenous H_2O_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 •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).
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_{2}O_{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 neoepitopes 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 \('\text{OH} \) 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 NaBH\(_4\) 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/analtered) 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.
References


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