1.1 Introduction

The story of glycation reaction was started way back in the year 1912 when Louise Camille Maillard first described the glycation reaction after whom the reaction is also known as the Maillard reaction (Maillard, 1912). Glycation is the process whereby sugars bind to the free amino residues of proteins, lipids and DNA macromolecule. Sugars and other reactive carbonyl compounds bind spontaneously to nucleophilic amino groups of amino acids and proteins in a nonenzymatic process. Reducing sugars, such as glucose in basic solutions and lipids by β-oxidation or peroxidation generate formyl (an aldehyde) and ketone groups. Aldehydes and ketones have a highly polarized carbonyl (C=O) group, the oxygen atom of which is electronegative and may react with nucleophiles in proteins and other biomolecules like, DNA and lipids. Under high glucose load (hyperglycemic condition), these biomolecules undergo a nonenzymatic glycation reaction leading to the formation of a complex series of compounds known as the advanced glycation end products (AGEs). This, in turn, results in the deprivation of the functions of the biological macromolecules by altering their structural conformation. Figure 1.1 schematically represents the probable pathway of reaction of biomacromolecules with reducing sugars to form protein AGEs, advanced lipoxidation end products (ALEs) and DNA advanced glycation end products (DNA-AGEs). Glycation and oxidative stress are closely linked and are often referred to as “glycoxidation” process which is believed to be involved in the complications associated with several disorders including diabetes, cardiovascular disease and Alzheimer’s disease, in addition to various forms of cancer (Aldini et al., 2013).

1.2 Glycation of biomacromolecule

1.2.1 DNA glycation

When DNA reacts with sugars in vitro at a physiological temperature, the
Figure 1.1. Schematic representation of probable pathway of macromolecules reacting with reducing sugars to form AGEs/ALEs and DNA-AGEs, respectively (Ahmad et al., 2014)
formation of DNA-bound AGEs is observed (Mustafa et al., 2012; Ahmad et al. 2014). Glycation of DNA has shown to considerably alter the structure of DNA macromolecule and it leads to depurination, strand breaks and mutations such as insertions, deletions and transposition (Ahmad et al., 2011a; Ahmad et al., 2011b).

Therefore, DNA-AGEs could contribute to the loss of genomic integrity, which occurs during aging and may contribute to the age-related complications. More detailed studies on the stability and dynamics of DNA showed that glycation leads to partial unwinding and/or fragmentation of the double helix (Mustafa et al. 2012). Wuenschell et al. (2010) investigated the mutagenic potential of the predominant DNA-glycation adduct carboxy ethyl deoxyguanosine (CEdG) and exhibited that CEdG within the template DNA and the corresponding triphosphate possess different syn/anti conformations during replication which influence base pairing preferences. Oxidative modifications within the DNA lead to reduced gene expression (Nagai et al., 2010). Previous studies have shown that genotoxicity and immunogenicity are incurred in DNA and proteins by carcinogens and reactive oxygen species (ROS) as well (Moinuddin et al., 2014; Shahab et al., 2012b; Shahab et al., 2013a; Shahab et al., 2013b). Moreover, most recently, our research group has also shown that glycation-induced oxidative stress leads to the modification of DNA macromolecule and results in alteration of its structure (Akhter et al., 2013). The structural perturbations in the DNA macromolecules are the consequence of the genotoxic effect of the glycation reaction (Ahmad et al., 2011a). The structural pathway for the formation of DNA bases Amadori products is shown in Figure 1.2 In vitro incubation of DNA with glucose, fructose and glucose-6-phosphate led to UV absorbance and fluorescence changes, indicating that DNA undergoes nonenzymatic browning reaction (Ahmad et al., 2011a; Ahmad et al., 2011b). So far, it has
Figure 1.2. Mechanistic representation of glycation reaction between reducing sugars (ribose, glucose and deoxyribose) and adenine/guanine base (Ahmad et al., 2014).
been shown that among all DNA bases, deoxyguanosine exhibited the highest glycation rate with reactive carbonyl species (RCS) (Li et al., 2008). Therefore, free guanosine, guanine or 2′-deoxyguanosine were used in model incubations with sugar or RCS to identify the structure of possible DNA adducts. N2-carboxyethyl-2′-deoxyguanosine (CEdG A,B) was established as a sensitive marker for DNA modifications by the carcinogenic substance methylglyoxal (MG).

The role of DNA-glycation in vivo has been discussed recently in great detail where they showed the presence of auto-antibodies in type 1 and type 2 diabetes patients against glycated human DNA (Ahmad et al., 2014). Elevated levels of AGEs have been implicated in the pathological complications of diabetes, uremia, Alzheimer’s disease and possibly cancer. CEdG adducts were specifically detected in a human breast tumor and normal adjacent tissue at levels of 3–12 adducts/10^7 dG, suggesting that this lesion may be widely distributed in vivo which is an adduct of DNA glycation only.

### 1.2.2. Protein glycation

The protein glycation is an inevitable, nonenzymatic reaction between reducing sugars and proteins, occurring in all living systems. Although the reaction is slow, it is quite dynamic in nature and starts with the formation of unstable Schiff base, which undergoes a series of reactions leading to the formation of heterogeneous molecules called AGEs (Thornalley et al., 1999). Glucose and glucose-derived dicarbonyls such as glyoxal (GO), MG, glucosone and 3-deoxyglucosone (3-DG) are the major precursors of AGEs. The levels of these precursors determine the formation of different types of AGEs. The predominant AGE modifications include fructosyl-lysine, carboxymethyl lysine (CML), carboxyethyl lysine (CEL) and pentosidine. AGEs are accumulated in the body due to in vivo glycation as well as through intake of exogenous AGEs, which are mainly formed due to overheating of
foods and beverages (Faist and Erbersdobler, 2001). Diabetes promotes the formation of AGEs in vivo, thus, enhancing the overall accumulation. AGEs cause cell damage at various levels, namely (i) alteration of protein structure and function; (ii) protein aggregation, fibril formation and protease resistance (Wei et al., 2012); (iii) aberrant signalling through interaction with the RAGE and (iv) dysfunction of extracellular matrix. AGEs contribute substantially to the progression of diabetic complications, including nephropathy, retinopathy, neuropathy; cardiovascular diseases, cataract, accelerated aging, neurodegenerative diseases and cancer.

Studies of protein glycation have focused on the reaction of aldoses and ketoses, particularly glucose, with lysine residues of proteins. The acyclic form of the monosaccharide reacts reversibly with the lysyl side chain of amino group to form an initial Schiff base adducts. This exists mainly in the cyclic glycosylamine form (Neglia et al., 1983). AGEs are formed slowly throughout life and the concentrations of AGEs found represent a lifelong accumulation of the glycation adducts. This applies to chemically stable AGE residues formed on long-lived proteins. For example, CML, CEL and pentosidine residue accumulation on skin collagen (Shimasaki et al., 2011). Various proteins and enzymes of clinical significance as well as those involved in cellular processes such as glucose metabolism, bioenergetics, cell repair and stress response have been affected by glycation. Plasma proteins especially human serum albumin (HSA), immunoglobulins, apolipoprotein, fibrinogen and transferrin are predominantly glycated and implicated in various disease conditions (Zhang et al., 2008). Hemoglobin undergoes extensive glycation and is an established biomarker for diabetes (Lyons and Basu, 2012). Likewise, early glycation products of albumin could serve as markers for secondary complications of diabetes. Glycation of albumin decreases the antioxidant activity and binding capacity (Rondeau and
Similarly, AGE modified immunoglobulin G presents unique neoepitopes, which elicit autoimmune response in rheumatoid arthritis patients (Ahmad et al., 2012). Increased glycation of apolipoproteins may play a role in the accelerated development of atherosclerosis. Glycation of fibrinogen alters the formation of fibrin network kinetics, which contributes to decreased pore size and lysis rate of fibrin clots (Pieters et al., 2008). Transferrin glycation is associated with increased free radical production, lipid peroxidation and decreased iron-binding capacity (Van Campenhout et al., 2004). It has also been found that glycation affects calcium signaling and calcium-dependent processes. Human paraoxonase 1, a calcium-dependent esterase responsible for metabolism of membrane lipid hydroperoxides, is inactivated by glycation and implicated in coronary heart disease (Mastorikou et al., 2008). Other proteins such as human complement regulatory protein show reduced activity due to glycation, thereby promoting membrane attack complex formation in the target organs of diabetic complications (Qin et al., 2004). Glycation modification results in the diminished enzyme activity of creatine kinase, alanine aminotransferase and cytoplasmic aspartate aminotransferase aiding in the development of diabetic complications (Beranek et al., 2001). Several intracellular enzymes especially those involved in glucose metabolism have been greatly affected by glycation modifications. Key enzymes such as glyceraldehyde 3-phosphate dehydrogenase, bisphosphoglycerate mutase from erythrocytes and pancreatic glucokinase are inactivated by glycation (Zhao et al., 2000). Increased glycation of glycolytic enzymes leads to higher accumulation of carbonyls, which may further modify proteins. Other enzymes which show loss of activity upon in vitro glycation include enolase, nitric oxide synthase, catalase and Cu/Zn superoxide dismutase (SOD1) (Pietkiewicz et al., 2009). In vitro glycation of Na-KATPase at different amino groups has shown differential
catalysis and cation binding, suggesting that glycation not only inhibits enzyme activity but also modulates enzyme kinetics (Garner et al., 1990). Interestingly, glycation-induced loss of functional activity is compensated by increased gene expression. One of the well-studied examples is the decreased functional activity of glutathione system due to carbonyl stress, which is compensated by increased expression of gamma-glutamylcysteine synthetase enzyme (Miyahara et al., 2002). Nevertheless, targeting any of the enzyme or protein involved in the nonenzymatic glycation may prevent their ultimate structure consequently their function as well. The need of the hour is to stop this slow and steady glycation reaction with minimal or no toxicity. The approaches used in the past have shown some deleterious effects on humans undergoing trial in diabetic nephropathy patients against aminoguanidine (AG).

1.2.3. Lipoproteins glycation

In diabetes, hyperglycemia induces modification of plasma and tissue proteins by nonenzymatic glycation, a gradual process that culminates with the formation of irreversible AGE. AGE accumulates particularly at sites of atherosclerotic lesions, but the mechanisms whereby AGE contributes to diabetes-induced accelerated atherogenesis are not fully understood. Low-density lipoproteins (LDLs), either oxidized or glycated, are present in the plasma and in the affected vasculature of diabetic patients (Virella et al., 2003). Although there are several studies on the effect of glycated proteins on vascular cells (Younis et al., 2008), only few data exist on the effect of irreversibly glycated LDL (AGE-LDL) on endothelial cells. AGE interact with specific cellular receptors, the best characterized of which is the receptor for AGE (RAGE). Interestingly, elevated levels of AGE-LDL are present in the sera of euglycemic or normolipemic patients with atherosclerosis. AGE-
LDL is present in the vessel wall atheroma and exerts proatherogenic effects (Sima, 2002; Hodgkinson et al., 2008).

It has been found that recognition of glycated LDL by LDL receptor is impaired, while the uptake by monocytemacrophages are enhanced, which contributes to hyperlipidemia and accelerated foam-cell formation, respectively (Toma et al., 2009).

It has been hypothesized that in diabetes, hyperglycemia and the ensuing increased formation of AGE-LDL may directly affect the vascular endothelium by activating RAGE, which in turn may induce a series of changes leading to a prooxidant and pro-inflammatory state, finally generating endothelial dysfunction characteristic for micro-and macroangiopathy. It has also been reported that AGE-LDL induce in human endothelial cell (HEC) an increased expression of RAGE, p22phox, p67phox and NOX4, leading to an increase in NADPHox activity, and an augmented expression of monocyte chemoattractant protein-1. High glucose augments the effect of both nLDL and AGE-LDL on HEC (Toma et al., 2009).

Moreover, the glycation of lipoproteins in diabetes was first reported by Schleicber et al. (1981). It has been reported that in healthy nondiabetic people the serum concentration of glycated apolipoprotein B (apo B), although lower than in diabetic people was on average 5 mg/dl, representing ≈4% of the total apo B (the major component of the protein moiety of LDL) (Tames et al., 1992). It has been reviewed that the possibility of lipoproteins glycation, including very-low-density lipoprotein (VLDL), LDL and also HDL could contribute to atherosclerosis. It has been consistently observed both in tissue culture and during in vivo turnover studies that glycated LDL is not cleared by the physiological LDL receptor (Wang et al., 1998). It thus has a slower catabolic rate than nonglycated LDL. Glycation of LDL apo B involves epitopes close to its receptor-binding site, suggesting that a
conformational change in the binding site influences recognition by LDL receptors. Glycated LDL is thus more likely to be cleared by scavenger receptors on macrophages and endothelial cells, to which its binding is not compromised by glycation, and subclasses of scavenger receptors specific for AGEs have been described (Schmidt et al., 1999). Furthermore, glycation and oxidation are by no means mutually exclusive naturally occurring modifications of LDL, because glycation itself generates free radicals (glycoxidation) (Jenkins et al., 2004). Glycooxidated LDL is present in the atheromatous plaque (Imanaga et al., 2000). It has, however, been shown in vitro that prior oxidation is not essential for glycation of LDL to occur (Li et al., 1996).

1.3. Advanced glycation end products: source and target

AGEs are formed endogenously when the carbonyl groups of reducing sugars nonenzymatically react with the free amino groups on proteins, lipids and DNA. AGEs are generated in vivo as a normal consequence of metabolism, but their formation is accelerated under conditions of hyperglycemia, hyperlipidemia and increased oxidative stress. Although glucose is relatively slow in reacting with proteins, highly reactive dicarbonyl compounds (generated as a result of glucose autooxidation, lipid peroxidation and the interruption of glycolysis by reactive oxygen species) are capable of rapid AGE formation. Dicarbonyls such as GO, MG and 3-DG interact with intracellular proteins to form AGEs, and can also diffuse out of the cell and react with extracellular proteins. Excessive AGE accumulation results in significant cellular dysfunction by inhibiting communication between cells, altering protein structure and interfering with lipid accumulation within the arterial wall (Barlovic et al., 2010).

AGEs are well reported to bind to the RAGEs which activates nuclear factor kB (NF-kB), triggering oxidative stress, thrombogenesis, vascular
inflammation and pathological angiogenesis (Yamagishi et al., 2007), thereby contributing to many of the long-term complications of diabetes. More recently, AGEs have been implicated in the pathogenesis of type 2 diabetes by contributing to the development of insulin resistance and low-grade inflammation known to precede the condition (Tahara et al., 2012). Apart from endogenous AGE formation, AGEs and their precursors are also absorbed by the body from exogenous sources such as cigarette smoke and through consumption of strongly heated processed foods. Browning of food during cooking is used to enhance the quality, flavor, color and aroma of the diet. This process (known as the Maillard reaction) generates large quantities of AGEs. Factors that enhance AGE formation in foods include high lipid and protein content, low water content during cooking, elevated pH and the application of high temperature over a short time period. More AGEs are generated in foods exposed to dry heat (grilling, frying, roasting, baking and barbecuing) than foods cooked at lower temperatures for longer time periods in the presence of higher water content (boiling, steaming, poaching, stewing or slow cooking) (Uribarri et al., 2005). Kinetic studies have demonstrated that ≈10–30% of dietary AGEs consumed are intestinally absorbed (Faist and Erbersdobler, 2001), with only one-third of ingested AGEs excreted in urine and feces. Plasma AGE concentration appears to be directly influenced by dietary AGE intake and the body’s capacity for AGE elimination (Delgado-Andrade et al., 2012). Low-AGE diets in animal studies have been shown to reverse insulin resistance and chronic inflammation, inhibit the progression of atherosclerosis and prevent experimental diabetic nephropathy and neuropathy (Uribarri et al., 2007), but whether these results can be translated to humans is uncertain. Cross-sectional and case–control studies involving humans with impaired renal function or diabetes have demonstrated associations between elevated AGE intakes and serum biomarkers of
oxidative stress, endothelial dysfunction, inflammation, hyperlipidemia and hyperglycemia (Chao et al., 2010). AGEs have also recently been implicated in the dysfunction and death of pancreatic beta cells, leading to the hypothesis that excessive AGE formation and oxidative stress possibly have a role in the development of type 1 and type 2 diabetes (Coughlan et al., 2011). Low-AGE diets have been suggested as a possible future therapeutic option for healthy individuals at risk for the development of type 1 or type 2 diabetes (Vlassara and Striker, 2011). Through reduced consumption of highly processed heat treated foods, dietary AGE restriction may represent a relatively simple, non invasive therapy for the effective treatment of many of the metabolic disturbances attributed to excessive AGE levels. This competent review might help to better understand to stop the glycation menace either by dietary AGEs restriction as reviewed here or by using novel therapeutic drugs and novel bioconjugation approach discussed elsewhere.

1.4. Interrelations of ROS, RCS and AGEs

The nonenzymatic glycation of DNA or protein is the process which links chronic hyperglycemia to a series of pathophysiological alterations and considered important in the development of diabetes and the associated diseases (Ahmad et al., 2014). AGEs are direct pathogenic and accumulate in the plasma, serum and tissues of patients in different diseases, e.g. diabetes, end stage renal disease, cardiovascular, aging and arthritis (Peppa et al., 2003).

The excess free radical production under hyperglycemic conditions originates from mitochondrial respiration, cytochrome p450, xanthine oxidase, PKC dependent activation of NADH/NADPH oxidase and RAGE-triggered cellular oxidant stress (Bandeira et al., 2013). Under hyperglycemic conditions several pathways gets activated, namely (i) autooxidation of glucose which leads to the actual glycation reaction thus forming ROS
CHAPTER 1

species, (ii) the generation of sorbitol pathway, (iii) activation of PKC pathway by diacyl glycerol generation (DAG), (iv) activation of glucosamine and (v) mitochondrial pathway. All the above pathways ultimately lead to the formation of ROS and result in deleterious effects on protein, DNA and LDL macromolecule.

Figure 1.3 shows the free radical formation and the check points where this slow and steady can be stopped. Furthermore, ROS, within certain boundaries, is essential to maintain homeostasis. Most recently, new sources of ROS generation have been reported (Brieger et al., 2012). These ROS species are also generated in the early and the advanced glycation processes and these species have been shown to exhibit cytotoxicity (Figure 1.4.). During the rearrangement process of the glycation reaction there is also the generation of free radicals like ·OH and O2· (Ahmad et al., 2011b).

Carbonyl stress is an imbalance of RCS production and carbonyl scavenging mechanisms, which originate from a multitude of mechanistically related pathways, such as glycation, autooxidation of sugars, amino acid metabolism, lipid peroxidation and UV damage (Sergei et al., 2008). The formation of RCS during glycation reaction is shown in Figure 1.4 Generation of reactive intermediate products is an important step in the glycation. These compounds are known as α-dicarbonyls and include 3-DG, glyoxa and MG. 3-DG rapidly reacts with protein amino groups to form AGE such as imidazolone, pyrraline and CML (Jono et al., 2004). MG may be produced by nonenzymatic pathways from spontaneous decomposition of triose phosphates, autooxidation of carbohydrates and glucose degradation (Nagaraj et al., 2002). In addition to reaction with arginine residues to form imidazolone adducts, MG reacts with lysine residues in protein to form CEL and the imidazolium cross-link, MG-lysine dimer. On the other hand, product
Figure 1.3. Schematic representation of the formation of ROS and RNS under hyperglycemic condition: probable check points “×” to control the reaction (Ahmad et al., 2014).
Figure 1.4. Schematic representation of the formation of early and advanced glycation end products. Probable check points “×” to control the reaction (Ahmad et al., 2014).
-ion physiological conditions can yield number of AGEs such as CML, pentosidine, glyoxal-lysine dimer and other nonfluorescent AGEs (Nagai et al., 2012). α-Oxoaldehydes are metabolized and inactivated by enzymatic conversion to the corresponding aldonic acids, catalyzed by the glutathione-dependent glyoxalase system (Birkenmeier et al., 2010).

1.5. Antigenicity of glycated LDL

Among the proteins, lipoproteins are modified as a consequence of oxidation and glycation. Endothelial cells (EC), monocytes/macrophages, smooth muscle cells (SMC) and lymphocytes are all competent to enhance the rate of oxidation and glycation of LDL.

The pro-inflammatory properties of glycated LDL seems to be considerably enhanced as a consequence of their immunogenicity. The immunogenic effect of glycated LDL was first reported by Steinbrecher et al. (1984) based on the immunization of laboratory animals with several types of modified LDL. Steinbrecher (1987) as well as Palinski et al. (1995) characterized its immunogenic epitopes. Furthermore, human auto-antibodies to oxidized LDL (oxLDL) were the first to be characterized and purified (Yla-Herttuala et al., 1994; Virella et al., 2000). Oxidation and glycation of lipoproteins may alter their structure sufficiently to deliver them immunogenic. Curtiss and Witztum (1983) also performed studies to explore the possible immunogenic properties of glycated lipoproteins. They injected guinea pigs with homologous LDL that had been subjected to \textit{in vitro} glycation in the occurrence and absence of cyanoborohydride, producing heavily (60% of lysine residues glycated) and mildly (6% of lysine residues glycated) modified particles, respectively. The modified particles were highly immunogens, stimulating the production of antibodies that did not interact with unmodified LDL, but that did interact with other reductively glycated proteins. Glucitol-lysine, the product of cyanoborohydride reduction of Schiff
base, was an important fraction of the epitope. *In vivo*, however, glucitol-
lysine does not exist, and the initial stable product of glycation that is
fructose-lysine (FL), was not recognized by the antibody raised against
reductively glycated LDL. When the *in vitro* glycation was performed without
cyanoborohydride, resulting in the formation of FL as would occur *in vivo*,
the more mildly modified LDL was a much less potent immunogen. The
presence of antibodies to reductively glycated LDL leads to accelerated
clearance of these particles from guinea pig plasma, but non-reductively
glycated LDL had no effect on its rate of clearance (Witztum *et al*., 1983). It
appears that the difference in antibody response and subsequent clearance
rates may be in part the result of different degrees of LDL modification and in
element the result of epitope differences. However, the existence of even low
levels of antibodies to the less modified, FL-containing glycated LDL may
have pathophysiologic consequence, because it suggests the presence of
circulating immune complexes, which are believed to be potently atherogenic.

1.6. Diabetes mellitus

The worldwide frequency of diabetes mellitus has risen dramatically over the
past two decades, from an estimated 30 million cases in 1985 to 177 million
in 2000. Based on current trends, more than 360 million individuals will have
diabetes by the year 2030 (Inzucchi *et al*., 2015). Estimates about 194 million
individual worldwide to have diabetes in 2003 and is expected to increase to
about 333 million by 2025 (International Diabetes Federation, Diabetes Atlas,
2012). As might be expected, the countries with the largest populations have
the highest number of persons with diabetes. Only Bangladesh and Nigeria
are exceptions of the world’s 10 most populous countries are not amongst the
10 countries with the highest diabetes numbers for 2010. There are marked
differences between developing and developed countries. For developing
countries, adult diabetes numbers are likely to increase by 69% from 2010 to

2030, compared to 20% for developed countries, whereas total adult populations are expected to increase by 36% and 2% respectively (Shaw et al., 2010). In the incidence of both type 1 and type 2 diabetes mellitus there is considerable geographic variation. Finland has the highest incidence of type 1 diabetes mellitus, whereas in Japan, China, its prevalence is least. Northern Europe and the United States have an intermediary rate. Much of the increased risk of type 1 diabetes mellitus is believed to reflect the frequency of high risk human leukocyte antigen (HLA) alleles among ethnic groups in diverse geographic locations. The prevalence of type 2 diabetes mellitus and its harbinger, impaired glucose tolerance (IGT), is highest in certain pacific islands, intermediate in countries such as India and the United States, and relatively low in Russia. This variability is due to genetic, behavioural and environmental factors. India is 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 raise about 80 million by 2030. The International Diabetes Federation (IDF) estimated the total number of diabetes subjects to be around 36 million in India in 2003 and this is further set to rise to 73.4 million by the year 2025. Another report published recently, estimated it to be 50.8 million by the year 2010 and will shoot up to 87.0 million by the end of year 2030 (Shaw et al., 2010). Diabetes is the foremost cause of mortality. A recent estimate suggested that diabetes is the fifth leading cause of death worldwide and is liable for almost 3 million deaths annually, which is a 1.7-5.2% of deaths worldwide.

Diabetes mellitus refers to a group of common metabolic disorders that allocate the phenotype of hyperglycemia. Several types of diabetes mellitus exist and are caused by a composite relation of genetics and environmental factors, viral infection and autoimmune disease have been implicated (Paik et
Mechanisms by which induced oxidative stress is implicated in the diabetic complications are partly known, including activation of transcription factors, AGEs and protein kinase C. Depending on the etiology of the diabetes mellitus, factors contributing to hyperglycemia include decreased glucose utilization, reduced insulin secretion, and increased glucose production. The metabolic dysregulation associated with diabetes mellitus causes secondary pathophysiologic changes in multiple organ systems that impose a remarkable burden on the individual with diabetes. 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 kidneys, all the excess glucose cannot be absorbed. This excess glucose secreted in urine along with water and electrolytes as well as ions required by cells to regulate the electric charge and flow of water molecules across the cell membrane. This causes polydipsia, polyuria, and weight loss as classical symptoms of the diabetes. These symptoms together with a random plasma glucose concentration ≥200 mg/dL (11.1 mmol/L) 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, 2008).

Diabetes mellitus is classified on the basis of the pathogenic process that leads to hyperglycaemia (American Diabetes Association, 2007). The two broad classification of diabetes mellitus are designated as type 1 and type 2. Both types of diabetes are preceded by a phase of abnormal glucose homeostasis as the pathogenic processes progresses. Type 1 diabetes is the result of near-total or complete insulin deficiency. Type 2 is a heterogeneous group of disorders characterized by variable degrees of impaired insulin, secretion insulin resistance and increased glucose production. Distinct metabolic and genetic defects in insulin secretion give rise to the common
phenotype of hyperglycaemia in type 2 diabetes mellitus and have important potential therapeutic implications now that pharmacologic agents are accessible to target precise metabolic derangements. Type 2 diabetes mellitus is preceded by a period of abnormal glucose homeostasis classified as impaired fasting glucose (IFG) or IGT. The term insulin-dependent diabetes mellitus (IDDM) and noninsulin-dependent diabetes mellitus (NIDDM) are obsolete. Since many individuals with type 2 diabetes eventually require insulin treatment for control of glycaemia. Other etiologies for diabetes mellitus include precise genetic defects in insulin secretion or action, metabolic abnormalities that impair insulin secretion, mitochondrial abnormalities and a host of conditions that impair glucose tolerance.

1. Type 1 diabetes (usually leading to absolute insulin deficiency, β-cell destruction)
   a. Immune Mediated.
   b. Idiopathic.

2. Type 2 diabetes (insulin resistance with may range from predominantly insulin resistance with relative insulin deficiency to a predominantly insulin secretory).

3. Other specific types of diabetes
   a. Genetic defects of β-cell function
   b. Genetic defects in insulin action
   c. Infections
   d. Diseases of the exocrine pancreas
   e. Endocrinopathies
   f. Uncommon forms of immune-mediated diabetes
   g. Drug- or chemical-induced
   h. Other genetic syndromes sometimes associated with diabetes

4. Gestational Diabetes Mellitus
1.6.1. Type 1 and type 2 diabetes mellitus

Complete insulin deficiency caused by autoimmune-mediated destruction of pancreatic β-cells characterizes type 1 diabetes. This condition is also called “juvenile diabetes” or “insulin-dependent diabetes”. The main reason of the β-cell loss is a T-cell mediated immune attack (Rother, 2007). It is thought to be caused by a combination of viral infection and environmental factors, superimposed on a genetic susceptibility. It accounts for less than 10% of those with diabetes in the United States, but the prevalence may be rising. The disorder may be further sub classified into type 1A if autoimmune markers are found, usually at the time of diagnosis (A Report on Diabetes care, 1997) Type 1B diabetes is an absolute insulin deficiency in which no autoimmune markers can be recognized. Type 1B diabetes may be more ordinary in people of Asian heritage (Abiru et al., 2002). Type 1 diabetes is a multifactoral autoimmune disease thought to arise from a complex interaction between both environmental insult(s) and genetic susceptibility. Several autoantibody markers have been detected in autoimmune diabetes including insulin autoantibodies (IAA), islet cell antibodies (ICA), glutamic acid decarboxylase-65 (GAD-65) autoantibodies and antibodies to tyrosine phosphatases IA-2 and IA-2β (Lan et al., 1996; Lu et al., 1996). Many triggers have been proposed for the development of type 1 diabetes in genetically susceptible individuals. Viruses such as coxackie virus, enteroviruses, and rubella have been proposed as culprits but have not been definitively exposed to induce type 1 diabetes (Lammi et al., 2005). Food additives or toxins, such as nitrosamines, have also been proposed as a cause of diabetes (Helgason et al., 1981). Some investigators have also implicated cow’s milk as an initiating factor in the development of autoimmunity in type 1 diabetes (Oute et al., 1999). Whatever initiating mechanism is, the autoimmune destruction of β-cells leads to a progressive decline in the body’s...
insulin secretory capacity. Eventually, this decline manifests itself in hyperglycemia after a large carbohydrate load, a glucose tolerance test or such as a meal. When less than 80% of β-cells has been damaged, patients develop the first clinical symptoms of diabetes (Powers, 2008).

Type 2 diabetes is a heterogeneous group of conditions that constitute less than 90% of diabetes. Like type 1 diabetes, type 2 diabetes also involves both genetic susceptibility and environmental factors, although the genetic component may be greater than in type 1 diabetes. It is caused by a combination of insulin resistance and comparative insulin deficiency with increased hepatic glucose production. It is important to note that individuals experience predominantly insulin resistance and insulin deficiency. Insulin resistance is generally thought to precede insulin deficiency. Obesity is linked with induced insulin resistance and may be the reason that, type 2 diabetes is more common in obese individuals. The precise mechanism by which obesity leads to insulin resistance is not completely described but may be related to several biochemical factors, such as free fatty acids, tumour necrosis factor-α and leptin, other substances. In addition, many genetic polymorphisms may play a part in insulin resistance, possibly through post-insulin receptor signal transduction mechanisms (Powers, 2008). Overweight and obesity are strongly associated with development of type 2 diabetes and may be responsible for the majority of the growing diabetes pandemic (Wannamethee and Shaper, 1999). Furthermore, weight loss is strongly associated in prospective studies with decreased progression from impaired glucose tolerance (IGT) to type 2 diabetes (Knowler et al., 2002). Insulin resistance alone, however, does not cause diabetes. Most obese people do not develop type 2 diabetes, despite increased insulin resistance (Polansky, 2000). For type 2 diabetes to emerge, there must also be relative insulin deficiency. Before type 2 diabetes develops, the pancreatic β-cells induce their production.
of insulin to compensate for induced insulin resistance. It has been proposed, that there is measurable β-cell hypertrophy present in obese subjects who do not have diabetes. For unclear reasons, β-cell secretory capacity gradually declines in some individuals, leading to the development of type 2 diabetes. As β-cell insulin secretory capacity declines, type 2 diabetes begins to develop. Initially, hyperglycemia is only observed after large meals, as in type 2 diabetes. As β-cell function declines further, however, hyperglycemia becomes more severe. Studies have suggested that approx 40% of β-cell mass lost in individuals who have glucose intolerance, and less than 60% may be lost when clinical type 2 diabetes develops (Butler et al., 2003). Relative insulin deficiency and hepatic insulin resistance also lead to induced hepatic gluconeogenesis, which further worsens hyperglycemia. Eventually, the degree of hyperglycemia worsens and becomes virtually universal if left untreated (Powers, 2008). Still the cause of β-cell failure in type 2 diabetes is unknown. In addition to a genetic predisposition, studies have also revealed higher rates of apoptosis and decreased β-cell mass in patients with type 2 diabetes (Butler et al., 2003). There are also induced amounts of amyloid deposits in the islets of patients with type 2 diabetes (Khan et al., 1999).

1.7. Atherosclerosis

Of the 50 million deaths that occur in the world, 40 million occur in developing countries. Already a extensive proportion of these deaths are due to cardiovascular diseases. It is projected that by the year 2025 well over 80–90% of all the cardiovascular diseases in the world will be occurring in low income and middle income countries. This increase in cardiovascular disease is due to a number of causes which include the following: (1) urbanization with increasing levels of obesity; (2) conquest of deaths in childhood and infancy from nutritional deficiencies and infection; (3) increasing use of tobacco worldwide and (4) increasing longevity of the population so that a
higher proportion of individuals reach the age when they are subject to chronic diseases.

At one time, atherosclerosis was thought to be a degenerative disease that was an inevitable consequence of aging. Research in the last two decades has shown that atherosclerosis is neither a inevitable nor degenerative disease. On the dissimilar, atherosclerosis seems to be a chronic inflammatory condition that is converted to an acute clinical event by the induction of plaque rupture, which in turn leads to thrombosis. Figure 1.4 depicts a model of the sequence of changes in the artery wall that lead to a clinical event. The underlying hypothesis presented here is that components of the fatty streak, the earliest lesion, which itself is not clinically significant, are also responsible for the latter events that lead to clinically significant disease. The first is lipoprotein transport into the artery wall. The receptor-mediated endocytosis does not require concentration-dependent process. The seminal findings by Brown and Goldstein (1986) that atherosclerosis is induced in multiple species by mutations that involve a single gene, the LDL receptor, provide strong evidence that elevations in LDL levels are sufficient to induce all the components of the atherosclerotic reaction.

Widely spread clinical manifestations of atherosclerosis such as coronary heart disease (CHD), renovascular hypertension, cerebrovascular stroke, and violation of the lower limbs vascular permeability, are the result of formation of advanced atherosclerotic lesions in a vascular wall. A trigger mechanism for the progression of atherosclerotic lesions is an intracellular lipid deposition and subsequent foam cell formation with excessive production of connective tissue matrix components and, possibly, cellular proliferation and inflammatory reactions (Orekhov et al., 1991). Atherosclerosis can be usually described as an excessive fibro fatty, proliferative several cell types, such as smooth muscle cells, monocyte

Figure 1.5. Progression of atherosclerosis to late complications (Koenig and Khusevinova, 2007)
derived macrophages, platelets and lymphocytes (Libby et al., 2005). During
the last three decades, the autoimmune hypothesis of atherosclerosis was
developed and the evidence for an important role for auto-antibodies against
modified low density lipoprotein (LDL) and LDL-containing circulating
immune complexes (LDL-CIC) in atherogenesis has been accumulated.
Immunological factors appear to contribute to the progression of
atherosclerosis as many other factors including alterations in plasma lipid and
lipoprotein levels, clotting factors, arterial smooth muscle cell metabolism,
platelet function, and blood pressure regulation. In a number of recent studies
it has been suggested that the presence of LDL-CIC in the blood promotes the
onset and progression or development of atherosclerotic lesions in the vessel
wall. It has been demonstrated that modified LDL, especially LDL-CIC act as
the primary agents responsible for excessive cholesterol accumulation in
vascular cells (Lopes-Virella et al., 1997). The atherogenic properties of LDL
containing immune complexes suggest them as a candidate marker for
atherosclerosis.

1.8. Immune alterations in diabetes/atherosclerosis
The main causes of morbidity and mortality in diabetes are micro and
macrovascular complications, including nephropathy, atherosclerosis and
retinopathy. As the definition of atherosclerosis as a chronic inflammatory
disease became widely accepted, it became important to define the triggers of
vascular inflammation. Glycated lipids and lipoproteins emerged as major
pathogenic factors in atherosclerosis. Modified forms of LDL (mLDL) are
pro-inflammatory by themselves, but, in addition, mLDLs including AGE-
product-modified LDL (AGE-LDL) induce autoimmune responses in humans.
The autoimmune response involves T cells in the arterial wall and synthesis of
IgG antibodies. The IgG auto-antibodies that react with mLDLs generate
immune complexes (IC) both intra and extravascularly, and those IC activate

the harmonize system as well as phagocytic cells via the ligation of Fcg receptors. *In vitro* studies proved that the pro-inflammatory activity of IC containing AGE-LDL (AGE-LDL-IC) is several-fold higher than that of the glycated/modified LDL molecules. Clinical studies support the pathogenic role of AGE-LDL-IC in the progression of macro vascular disease patients with diabetes. In type 1 diabetes, high levels of AGE-LDL-IC were associated with internal carotid intima-media thickening and coronary calcification. In type 2 diabetes, elevated levels of AGE-LDL in IC predicted the occurrence of myocardial infarction. There is also evidence that AGE-LDL-IC is involved in the pathogenesis of diabetic retinopathy and nephropathy. The pathogenic role of mLDL-IC is not unique to diabetic patients, because those IC are also detected in non-diabetic individuals. But AGE-LDL-IC are likely to reach elevated concentrations and have a more prominent pathogenic role in diabetes due to augmented antigenic load secondary to high oxidative stress and to induced autoimmune responses in type 1 diabetes.

1.9. **Objectives of present study**

This study was initially aimed to glycate commercially available calf thymus DNA (CT-DNA) and LDL using reactive carbonyl species like D-ribose with the following objectives:

- To glycate DNA/ LDL by using D-ribose as a reducing sugar.

- To study and characterize the structural changes induced in DNA/LDL by various physico-chemical techniques.

- To generate polyclonal antibodies against native and D-ribose modified DNA/LDL in experimental animals.
To examine the binding of native and glycated DNA/LDL in diabetic/atherosclerotic sera.