CHAPTER-1

Introduction

Abstract

This chapter gives a general introduction to diabetes mellitus and its classification, epidemiology, symptoms and management of diabetes mellitus. It also gives a brief introduction to gold nanoparticles, especially its biomedical applications and the importance of biological synthesis of gold nanoparticles. In addition, potential novel optical spectroscopic techniques and the objectives of the present study are highlighted.
CHAPTER-1

Introduction

1.1. Diabetes

Diabetes mellitus (DM), a name derived from ancient Greek and Latin terms for ‘excessive urination’ (diabetes) and ‘sweet’ (mellitus), refers to a family of disorders characterized by chronic hyperglycemia. It is a multifactorial, multi systemic endocrine disorder in which the body does not produce (type 1) and/or properly respond (type 2) to insulin, a hormone essential for the entry of glucose from plasma to cells for energy production (American Diabetes Association, 2012). Diabetes mellitus (DM) is a chronic metabolic disease with the highest rates of prevalence and mortality in both developed and developing countries. According to the International Diabetes Federation (IDF), 387 million people in the worldwide are affected by diabetes and that number is likely to increase 592 million by 2035 (IDF, 2014) (Fig. 1.1a and b).

It is estimated that more than 60% of the global population with diabetes is concentrated in Asia (Chan et al., 2009). According to the 2013 estimates by the international diabetes federation (IDF), the highest prevalence in the South East Asia (SEA) region is found in Mauritius (14.8%) followed by India (9.1%). India, Nepal, and Sri Lanka have higher numbers of people with diabetes in rural areas than in urban areas. India, the largest country in the region, has more than 65 million adults with diabetes and has the second highest number of cases in the world after China (Ramachandran et al., 2014).
Fig. 1.1. (a) (IDF) International diabetes federation regions and global projections of the number of people with diabetes, 2014 and 2035 (Adapted from IDF, 2014)
Figure 1.1. (b) Global diabetes prevalence (Adapted from IDF, 2014)
1.2. Classification of diabetes mellitus

Diabetes mellitus can be classified in different ways but one form of classification is as follow (American Diabetes Association, 2004):

- Type I diabetes (Insulin dependent) is due to immune mediated beta-cells destruction, leading to insulin deficiency.
- Idiopathic diabetes is the type 1 diabetes with no known etiologies and is strongly inherited.
- Type II diabetes (Non-Insulin dependent) is due to insulin secretory defect and insulin resistance.
- Gestational diabetes mellitus is any form of intolerance to glucose with onset or first recognition of pregnancy.

However diabetes is mostly classified basically into two major types: type I Diabetes (IDDM) and type II Diabetes (NIDDM).

1.2.1. Type 1 or insulin dependent diabetes mellitus

T1D is a chronic autoimmune disorder that precipitates in genetically susceptible individuals by environmental factors. In Type 1 diabetes mellitus (DM), the body’s own immune system attacks the beta-cells in the islets of Langerhans of the pancreas, destroying or damaging them sufficiently to reduce and eventually eliminate insulin production (Belle et al., 2011). Usually, type 1 diabetes begins in childhood, so it is also termed juvenile diabetes. Type 1 DM patients do not produce enough insulin to sustain life and become dependent on exogenous insulin for survival. The two main forms of clinical type 1 diabetes are type 1a (about 90% of type 1 diabetes) which is thought to be due to immunological destruction of pancreatic β cells
resulting in insulin deficiency; and type 1b (idiopathic, about 10% of type 1 diabetes), in which there is no evidence of autoimmunity (Bastaki, 2005).

Genetic predisposition appears to be a prerequisite for the development of type 1 diabetes. Environmental factors such as viruses, toxins, and diet may be involved in the clinical expression of genetic susceptibility. Once β-cell specific autoimmunity has developed, autoimmune-mediated destruction of β-cell results in the onset of type 1 diabetes.

1.2.2. Type 2 or non-insulin dependent diabetes mellitus

Type 2 diabetes mellitus (T2DM) is characterized by a combination of insulin resistance and relative insulin deficiency that leads to elevated blood glucose levels. In type 2 diabetes mellitus there are certain mechanisms broken that keep regulation between tissue sensitivity to insulin which consequently leads to impaired insulin secretion by the pancreatic beta cells and impaired insulin action through insulin resistance (DeFronzo and Lily, 1987). Type 2 DM results from interaction between genetic, environmental and behavioral risk factors.

1.3. Criteria for diagnosis of diabetes mellitus

A consensus statement published by the World Health Organization (WHO) in 2006 published the current diagnostic criteria for diabetes (WHO, 2006), which are in agreement with those of the American Diabetes Association (ADA). These are

These are:

- a fasting plasma glucose ≥ 126 mg/dL (≥ 7.0 mmol/ L) on two occasions or more
- a 2 hour plasma glucose ≥ 200 mg/dL (≥ 11.1 mmol/L) after 75 g glucose load (oral glucose tolerance test, OGTT)

or

- a random plasma glucose ≥ 200 mg/dL (≥ 11.1 mmol/L)

### 1.4. Symptoms of diabetes

Symptoms are similar in both types of diabetes but they vary in their intensity.

- Excessive thirst
- Frequent urination
- Extreme hunger
- Unexplained weight loss
- Sudden vision changes
- Tingling or numbness in hands or feet
- Feeling very tired much of the time
- Very dry skin
- Sores that are slow to heal
- More infections than usual

### 1.5. Diabetic complications

Hyperglycemia is a pre-requisite for the development of diabetic complications and in chronic diabetes. Diabetic complications are usually classified into two main classes:

- Acute complications
- Chronic complications
1.5.1. Acute complications

The acute metabolic complications of diabetes consist of diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic non-ketotic syndrome (HHNKS).

1.5.2. Chronic complications

Long term elevation in blood glucose levels is associated with chronic complication which affects many tissues and organ systems and is accountable for the majority of morbidity and mortality encountered in diabetes mellitus (Tripathi et al., 2006). The chronic complications of diabetes classified into three categories as given below.

- Microvascular (retinopathy, nephropathy and neuropathy).
- Macrovascular (cardiovascular disease, peripheral arterial disease and stroke).
- Both micro-and macrovascular (diabetic foot ulcers)

1.6. Diabetes and metabolic abnormalities

1.6.1. Diabetes and carbohydrate metabolism

Diabetes mellitus (DM) is a chronic disorder of carbohydrate metabolism caused by abnormal insulin function or insulin deficiency. Defects in carbohydrate machinery and consistent efforts of the physiological systems to correct the imbalance in carbohydrate metabolism pose an over exertion on the endocrine system, which leads to the deterioration of endocrine control. Continuing deterioration of endocrine control exacerbates the metabolic disturbances by altering carbohydrate metabolic enzymes and leads primarily to hyperglycemia (Kuzmanova et al., 2007).
1.6.2. Diabetes and lipid metabolism

Lipid abnormality is a major problem in patients with diabetes. Among individuals with diabetes, 97% had at least one lipid abnormality. Patients with diabetes or metabolic syndrome frequently have higher triglycerides (TGs), lower high-density lipoprotein (HDL) cholesterol, and more cholesterol poor low density lipoprotein (LDL) particles; this combination contributes significantly to their cardiovascular risk. Lipids are also involved in the development of microvascular diabetes complications as well.

1.6.3. Diabetes and protein metabolism

Diabetes mellitus is basically a disorder of carbohydrate metabolism, but with progression of the disease, protein metabolism is also affected. Insulin generally has an anabolic effect on protein metabolism in that it stimulates protein synthesis and retards protein degradation (Sheela and Augustin, 1992). Absolute or relative insulin deficiency leads to negative nitrogen balance with loss of nitrogen from most organs. Increased blood urea levels in DM signify enhanced protein catabolism in liver. The decrease in the total protein concentration in serum diabetes may be ascribed to increased conversion of glycogenic amino acids to CO₂ and H₂O and reduction in protein synthesis secondary to a decreased amount and availability of mRNA.

1.6.4. Diabetes and oxidative stress

DM is associated with oxidative stress, leading to an increased production of reactive oxygen species (ROS), including superoxide radical (O₂), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH) or reduction of antioxidant defense system (Peerapatdit et al., 2006; Sklavos et al., 2010). Oxidative stress (OS) is the imbalance between cellular production of ROS
and the ability of cells to scavenge them. OS has been implicated as a potential contributor to the pathogenesis of several diseases, such as cancer, diabetes and heart disease (Gilgun-Sherk et al., 2002). Abnormally high levels of lipid peroxidation and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and lead to oxidative stress (Subramaniam et al., 2014).

1.6.5. Diabetes and lipid peroxidation

High levels of free radicals can lead to damage of cellular organelles and enzymes increase lipid peroxidation (LPO) and development of complication of diabetes mellitus (Maritim et al., 2003). Lipid peroxidation is a free radical-mediated propagation of oxidative insult to polyunsaturated fatty acids (PUFA) involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants (Korkina et al., 1997). The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination. In the lipid peroxidation initiation step, prooxidants like hydroxyl radical abstract the allylic hydrogen forming the carbon-centered lipid radical (L·`). In the propagation phase, lipid radical (L·`) rapidly reacts with oxygen to form a lipid peroxy radical (LOO·`) which abstracts a hydrogen from another lipid molecule generating a new L· (that continues the chain reaction) and lipid hydroperoxide (LOOH). In the termination reaction, antioxidants like vitamin E donate a hydrogen atom to the LOO· species and form a corresponding vitamin E radical that reacts with another LOO· forming nonradical products. The chain reactions continues until the PUFA substrate is completely consumed. This is known as termination stage (Yin et al., 2011).
1.7. Antioxidants and diabetes mellitus

Oxidative stress induced mainly by hyperglycemia and generation of free radicals, contributes to the development and progression of diabetes and related contributions, it became clear that ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications (Schultz Johansen et al., 2005). Antioxidants are bioactive moieties that originally can be referred to molecules that retard or prevent the utilization of oxygen by human tissues and known to prevent the oxidative system as a whole (Bhardwaj et al., 2014). The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as “free radical scavengers” by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune defense and lower the risk of diabetes.

Endogenous antioxidants in cells can be classified as enzymatic antioxidants and non-enzymatic antioxidants. The major antioxidant enzymes directly involved in the neutralization of ROS and RNS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR).

Generally, non-enzymatic antioxidants are also divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants belonging to endogenous antioxidants, are produced by metabolism in the body, such as lipid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc. While nutrient antioxidants belonging to exogenous antioxidants, are compounds which
cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, (Droge et al., 2002; Willcox et al., 2004) may act as antioxidants in the human body.

1.8. Diabetogenic agents

Many chemicals are used for the induction of DM in the animal models for testing new antihyperglycemic drugs. Alloxan and streptozotocin are the most prominent diabetogenic chemicals in diabetes research. The most disadvantages of alloxan diabetes model is multiorgan damage; hence this diabtogen is not widely employed to the anti-diabetic effect of newer agents.

1.8.1. Streptozotocin

Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromogenes*, is frequently used to induce DM in experimental animals through its toxic effects on pancreatic β-cells. The cytotoxic action of STZ is associated with the generation of ROS causing oxidative damage (Szkudelski et al., 2001). It is generally accepted that STZ brings about insulin-producing β-cell death by directly causing DNA damage, thereby effectively replicating one of the central deleterious effects of oxidative stress, as well as through STZ-induced secondary effects manifested as bona fide oxidative stress. The structure of streptozotocin is given in Fig.1.2.

After entering β-cells via the GLUT2 glucose transporter, STZ causes DNA damage by DNA alkylation, which in turn rapidly triggers the induction of the DNA repair process. Immediately prior to activation of DNA repair, the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP-1), that regulates the
molecular events responsible for DNA repair, is activated. The level of PARP-1 activation that is correlated with the amount of DNA damage is manifested as an increase in poly (ADP-ribosyl)ation. Thus, intensive poly (ADP-ribosylation) leads to a rapid depletion of cellular NAD$^+$ and ATP levels and the process of cell death becomes activated. Some of the secondary effects of STZ are enhanced dephosphorylation of ATP that provides a substrate for xanthine oxidase which generates more ROS, as well as the liberation of toxic amounts of nitric oxide that participate in further DNA damage. As a result of an overload of primary and secondary STZ actions, the cell is unable to restore homoeostasis and $\beta$-cells undergo destruction by necrosis (Lenzen, et al., 2007).

1.9. Management of diabetes mellitus

Management of diabetes is considered a global problem and successful treatment is yet to be discovered. Diet, life style changes (physical
activity, smoking cessation) and medication play a major role in the management of diabetes mellitus. Lifestyle interventions are not just beneficial before the development of diabetes. Several studies have clearly demonstrated the benefit of a healthful diet, regular exercise, and weight loss in individuals already diagnosed with diabetes (Fowler et al., 2007). Dietary habits are the personal decisions individuals make when choosing their nutrition. Nutrition therapy is generally recommended for primary, secondary, and tertiary prevention. Primary prevention means intervention before the development of diabetes, secondary prevention refers to the time after diagnosis of diabetes, and tertiary prevention can take place when significant numbers of beta-cells remain after diagnosis. Primary prevention is particularly important in type 2 diabetes, because the time of diagnosis and the severity of the disease course can be influenced beneficially by changing daily lifestyle and dietary practices. However, despite this awareness, there is still no universal dietary approach for diabetes prevention and management (Psaltopoulou et al., 2010). Regular exercise/physical activity leads to a number of beneficial physiological changes that favorably improves insulin sensitivity, muscle glucose uptake and utilization, and overall glycemic control (Hayes et al., 2008). If diet and lifestyle changes do not keep blood sugar under normal condition, patients may have to resort to classical drugs (insulin, sulfonylureas, biguanides and thiazolidinediones). Besides the classical drug usage for the treatment of diabetes, several species of plants have been described in scientific and popular literature as having hypoglycemic activity (Verspohi, 2002; De Sousa et al., 2004; Colca et al., 2006). Plants have always been a good source of therapeutic drugs.
1.9.3. Plant based drugs.

Plant-based medicine which uses medicinal plants as the first medicines is a universal phenomenon. Medicinal plants play a key role in the discovery of new therapeutic agents and have attracted much attention as sources of biologically active substances including antioxidants, hypoglycemic and hypolipidemic agents (Tang et al., 2006). Medicinal plants and their active phytochemicals exhibit delay the development of diabetic complications and correct the metabolic abnormalities. According to the search, several plant species have been described as hypoglycemic agents such as Opuntia streptacantha, Trigonella foenum graecum, Momordica charantia, Ficus bengalensis, Polygala senega, Gymnema sylvestre, Allium sativum, Citrullus colocynthis Aloe vera and Morus alba (Naowaboot et al., 2009; Patel, et al., 2012). Among these medicinal plants, Morus alba (Mulberry) is one of the most important plant used in the treatment of diabetes (Naowaboot et al., 2009; Jo et al., 2010; Kwon et al., 2011).

1.9.4. Mulberry

Genus Morus (Mulberry) consists of over 150 species, among these Morus alba L. is dominant. Mulberry trees are growing under varied climatic conditions, ranging from temperate to tropical, all over the world. Mulberry (Morus alba L.) leaves, bark and branches have long been used in Chinese medicine to various diseases. Among different parts of mulberry plant, leaves have been used widely to treat fever, improve eye sight, strengthen joints, reduce high blood pressure, high cholesterol, and neutral fat, prevent thrombus formation and ageing, treat constipation and diabetes, and to promote urination. In addition, mulberry leaves exhibit superior antioxidant, antimicrobial, antihyperglycemic, antiinflammatory, hypolipidemic,
neuroprotective, anti-HIV, anti-hypotensive and anticancer activities (Yang et al., 2012; Jo et al., 2010; Fatch et al., 2013; Kwon et al., 2011). Mulberry leaves contains a lot of nutritional components including 1-deoxynojirimycin (DNJ), soluble dietary fibers, resveratrol, quercetin, rutin, astragalin, Kuwanon G and leachianone and γ-aminobutyric, which is known as a powerful antioxidant, antimicrobial and antidiabetic properties (Naowaboot et al., 2009; Jo et al., 2010; Kwon et al., 2011). Several phytochemicals from plant materials are experimentally proved and widely used as more effective agents against diabetes. Although the phytochemicals from plant materials have potential antidiabetic property, it possess insoluble character leading to lower bioavailability and increased systemic clearance requiring repeated administration or higher dose, which make the drug as a poor candidate for therapeutic use. To overcome these problems, researchers looking for more effective antidiabetic agents preferably from nanoparticulate drug delivery systems, which should be palpable benefits in terms of reduced dosing frequency, increased bioavailability, prevention from degradation specifically against the harsh gastric environment, site specificity and reduced side effects.

1.10. Nanoparticulate drug delivery systems

In recent years, there is growing interest in the potential use of nanomedicines as an alternative treatment for various diseases as these are commonly improves the therapeutic potential of the drugs. Nanomedicine involves applying and further developing nanotechnology to solve the problems in medicine (i.e., to diagnose, treat and prevent diseases at cellular and molecular levels). Nanotechnology is an emerging,
multidisciplinary field that frequently employs techniques and tools from diverse disciplines, including biology, engineering, chemistry and medicine. Nanotechnology is typically known as the study of the control of matter on an atomic and molecular scale, generally structures in the nanometer (10^{-9} m) range, and involves developing materials or devices on that scale. The basic idea behind nanotechnology is that metal, semiconductor and polymeric nanoparticles have novel optical, electronic, magnetic and structural properties that are often not available from individual molecules and bulk solids (Niemeyer, 2001; Nie et al., 2007). In recent years, nanotechnology has been assessed and implemented in different areas of disease management and therapeutics with the hope that it will lead to major advances in diagnosis and treatment (Niemeyer, 2001; Ferrari, 2005; Cuenca et al., 2006; Nishiyama, 2007; Wang, 2008).

Results of numerous scientific research studies done in nanotechnology and nanomedicine are inspiring the scientific community to discover new, innovative, non-invasive tools at the nano-scale level for such purposes because of its unique properties such as the small size, controlled release of drugs and reduced toxic side-effects. Nanomedicine refers to the research and development of technologies, devices and drug delivery systems for prevention, diagnosis and treatment of disease at the nano-scale. Delivering therapeutic drug to the target site is a major problem in treatment of many diseases. A conventional application of drugs is characterized by limited effectiveness, poor biodistribution and lack of selectivity (Nevozhay et al., 2007). These limitations and draw-backs can be overcome by controlling drug delivery. In controlled drug delivery systems (DDS) the drug is transported to the place of action, thus, its influence on vital tissues and
undesirable side effects can be minimized. In addition, DDS protects the drug from rapid degradation or clearance and enhances drug concentration in target tissues; therefore, lower doses of drug are required (Nevozhay et al., 2007). This modern form of therapy is especially important when there is a discrepancy between a dose or concentration of a drug and its therapeutic results or toxic effects. Cell-specific targeting can be achieved by attaching drugs to individually designed carriers. Recent developments in nanotechnology have shown that nanoparticles (structures smaller than 200 nm in at least one dimension) have a great potential as drug carriers. Due to their small sizes, the nanostructures exhibit unique physicochemical and biological properties (e.g., an enhanced reactive area as well as an ability to cross cell and tissue barriers) that make them a favorable material for biomedical applications.

The whole system leads to a special function related to treating, preventing or diagnosing diseases sometimes called smart-drugs or theranostics (Wang et al., 2008). The primary goals for research of nanobio-technologies in drug delivery include:

- More specific drug targeting and delivery,
- Reduction in toxicity while maintaining therapeutic effects,
- Greater safety and biocompatibility and
- Faster development of new safe medicines.

The main issues in the search for appropriate carriers as drug delivery systems pertain to the following topics that are basic prerequisites for design of new materials. They comprise knowledge on (i) drug incorporation and
release, (ii) formulation stability and shelf life, (iii) biocompatibility, (iv) biodistribution and targeting and (v) functionality. Recently, researchers have introduced numerous metal nanoparticle-based innovative inventions for potential theranostic applications in the field of biomedical research (Thanh et al., 2010; Arvizo et al., 2012; Mukherjee et al., 2015).

1.11. Noble metal nanoparticles

Noble metals (gold, silver, platinum and palladium) have been widely used for the synthesis of stable colloids which are useful in the areas of optoelectronics (Tamura and Fujihara, 2003), catalysis (Jia, et al., 2009), photothermal therapy, SERS detection and biological labeling (Philip, 2010). In addition, noble metal nanoparticles inspired the researchers due to their remarkable role in detection and treatment of dreadful diseases such as diabetes, cancer, human immunodeficiency virus (HIV), tuberculosis (TB), and Parkinson disease geared towards biological and biomedical applications. Among several noble metal nanoparticles, gold nanoparticles (AuNPs) have been considered as important area of research due to their unique and intense plasmon resonance in the visible range and their potential applications in biomedical field (Dreaden et al., 2012; Fratoddi et al., 2014).

1.12. Gold nanoparticles

Gold nanoparticles (AuNPs) are widely used in many fields as preferred materials for their unique optical and physical properties, such as surface plasmon oscillations for labeling, catalysis, imaging, and sensing. Recently, much advancement were made in biomedical applications with better biocompatibility in disease diagnosis and therapeutics (Fratoddi et al., 2014; Mukherjee et al., 2015). The use of gold in medicine is not new, because
gold has been used in medicine from ancient era for many therapies. The medical therapy, which involved in the use of gold is commonly known as “Chrysotherapy”. The earliest medical use of gold can be traced back to the Chinese in 2500 BC. They were the first to prepare and use red colloidal gold as the, “drug of longevity”. Red colloidal gold is still in use today in India in the form of Ayurvedic medicine for rejuvenation and revitalization during old age under the name of Swarna Bhasma (“Swarna” meaning gold, “Bhasma” meaning ash). During the 5th millennium B.C., the extraction of gold started near Varna (Bulgaria) and it is believed that “soluble” gold appeared around the 5th or 4th century B.C. in Egypt and China. It was referred with different names such as soluble gold and drinkable gold, before the term “colloid” (from the French word, colle) was coined by Graham in 1861 (Graham, 1861). “Aurum potabile” or “drinkable gold” was used to cure diseases like arthritis and heart problems, venereal diseases, dysentery, epilepsy and tumors and also for the diagnosis of syphilis, a method which remained in use until the 20th century. By the end of 16th century, colloidal gold was routinely used to make ruby glass and for coloring ceramics, methods that are still in use now. The most famous examples of the use of colloidal gold in ruby glass are the Lycurgus Cup that was manufactured in the 5th to 4th century B.C. and the “Purple of Cassius”, that has been known since the 17th century (Savage, 1973). The Lycurgus Cup (Fig. 1.3) appears ruby red in transmitted light and turns green in reflected light, due to the presence of gold colloids.
Fig. 1.3 The Lycurgus Cup 1958, 1202.1 in reflected (a) and transmitted (b) light. Scene showing Lycurgus being enmeshed by Ambrosia, now transformed into a vine-shoot.

1.12.1. Optical property of gold nanoparticles

For last few decades, metallic nanoparticles have fascinated researchers due to their colorful colloidal solutions. Mie was the first to explain the red color of gold nanoparticle in 1908 by solving Maxwell's equation for an electromagnetic light wave interacting with small metallic spheres. The color exhibited by metallic nanoparticles is due to the coherent excitation of all the “free” electrons within the conduction band, leading to an in-phase oscillation and is known is surface plasmon resonance (SPR). Thus,
the color of metallic nanoparticles may change with their size due to surface plasmon resonance.

Unique optical property of nanomaterials may also be due to quantum size effect, which arises primarily because of confinement of electrons within particles of dimension smaller than the bulk electron delocalization length. This effect is more pronounced for semiconductor nanoparticles, where the band gap increases with a decreasing size. The same quantum size effect is also shown by metal nanoparticles, when the particle size is >2 nm.

1.12.2. Surface plasmon resonance in gold nanoparticles

Gold nanoparticles show a strong absorption band in the visible region when the frequency of the electromagnetic field is resonant with the coherent electron motion, which is called surface plasmon resonance absorption (Bohren, et al., 1983). Free electrons in the metal (d electrons in silver and gold) travel through the material. The mean free path in gold and silver is ~50 nm. In particles smaller than ~50 nm, no scattering is expected from the bulk. This means interactions with the surface dominate. When the wavelength of light is much larger than the nanoparticle size it sets up standing resonance conditions as represented in Fig. 1.4. Light in resonance with the surface plasmon oscillation causes the free-electrons in the metal to oscillate. As the wave front of the light passes, the electron density in the particle is polarized to one surface and oscillates in resonance with the light’s frequency causing a standing oscillation. The resonance condition is determined from absorption and scattering spectroscopy and is found to depend on the shape, size, and dielectric constants of both the metal and the surrounding material. This is referred to as the surface plasmon resonance, since it is located at the surface.
**Fig. 1.4. Origin of surface plasmon resonance due to coherent interaction of the electrons in the conduction band with electromagnetic field**

As the shape or size of the nanoparticles changes, the surface geometry changes, causing a shift in the electric field density on the surface. This causes a change in the oscillation frequency of the electrons, generating different cross-sections for the optical properties including absorption and scattering.

**1.12.3. Application of surface plasmon resonance in biomedical research**

In the recent years, surface plasmon resonance (SPR) has become one of the major methods for studying and determination of biologically active materials exhibiting affinity interactions. SPR biosensors are increasingly used in biochemistry and bioanalytical chemistry to determine antibody-antigen interactions, to investigate DNA hybridization, to diagnose bacteria- and virus-induced diseases, to identify hormones, steroids, and immunoglobulins, to investigate blood plasma coagulation. Using SPR
biosensors, it is possible to analyze the mixtures of substances with a very similar chemical structure because SPR allows identifying only those analytes that specifically interact with biologically active substance immobilized on the surface of SPR biosensor. Therefore, at present SPR is one of the most promising methods for determining the interactions between ligand and receptor, antigen and antibody, thus being increasingly used in diagnostics and biomedical research (Kausaite, et al., 2007).

Due to the strong surface plasmon absorption, gold nanoparticles offer great potential in photothermal therapy applications. It has been found that the strong absorbed radiation is converted efficiently into heat on a picosecond time domain due to electron-phonon and phonon-phonon processes. Thus, upon the laser irradiation at the surface plasmon absorption band, the nanoparticles absorb photon energy and then immediately transfer into heat energy. If the nanoparticles are incorporated or incubated with biomolecules, cells or tissues, this heat energy will cause the sharp increase on the local temperature around the nanoparticles and thus cause the damage of the surrounding materials. This photothermal destruction can be used for disease or cancer therapy.

1.13. Biomedical applications of gold nanoparticles

Gold nanoparticles (AuNPs) have recently emerged as an attractive candidate for delivering various therapeutic agents such as drugs, peptides, proteins, and nucleic acids into their target (Dreaden et al., 2012; Fratoddi et al., 2014). AuNPs have received considerable attention during the past decade due to their biological potential applications with in clinical diagnosis, biosensing and drug delivery.
1.13.1. Clinical diagnosis of gold nanoparticles

AuNPs show easily tuned physical properties, including unique optical properties, robustness, and high surface areas, making them ideal candidates for developing biomarker platforms. Modulation of these physicochemical properties can be easily achieved by adequate synthetic strategies and give gold nanoparticles advantages over conventional detection methods currently used in clinical diagnostics. Gold nanoparticles have been primarily used for labeling and bioimaging applications (Sperling et al. 2008). The gold nanoparticles are directed and enriched at the region of interest, providing contrast for observation and visualization. The interaction of gold nanoparticles with light can be used for the visualization of particles using optical microscopy, fluorescence microscopy, photothermal, and photoacoustic imaging. In addition, the interaction of gold nanoparticles with both electron waves and X-rays can also be used for visualization, e.g., using TEM. Other noninvasive diagnostic tools such as magnetic resonance imaging (MRI) and X-ray computed tomography (X-ray CT) have also utilized gold nanoparticles as contrasting agent due to the ease of surface modification and higher X-ray absorption coefficient, respectively (Jain et al., 2012).

1.13.2. Biosensing applications of gold nanoparticles

The myriad shapes and surfaces of AuNPs available today, their relatively easy conjugation to molecules of interest, and their low toxicity are favorable features for biological applications. Gold nanoparticles have been widely used to construct biosensors because of their excellent ability to immobilize biomolecules. AuNPs based biosensors can be classified into optical biosensors, electrochemical biosensors and piezo-electric biosensors. Gold nanoparticles can be used as passive labels or as active sensors (Sperling
et al., 2008). Many kinds of biosensors, such as enzyme sensor, immunosensor, and DNA sensor, have been prepared based on the application of gold nanoparticles (Huo, 2007). In biosensors, AuNPs are the transducers, producing a measurable signal on biological recognition of the primary event occurring in close proximity, most often on their surfaces. AuNPs-based tests for various biomarkers are already commercially available (e.g., Nanosphere, Merck, and BB International), offering highly sensitive and specific detection of proteins and nucleic acids associated with infections, and with heart, kidney, and genetic diseases. The development of DNA-labeled gold nanoparticles has opened up a new field of bionanotechnology. Gold nanoparticle probes heavily functionalized with thiolated oligonucleotides have emerged as an attractive alternative to molecular probes for nucleic acid detection. A two-color-change method for detection and simultaneous validation of single-nucleotide polymorphisms in DNA target using Ag/Au core–shell and pure gold nanoparticle probes was proposed (Cao et al. 2005). Finally, gold nanoparticles can be used for developing sensitive electrochemical detection methods. These methods are coupled with enzyme detection. If molecules in a narrow gap between electrodes are labeled with gold nanoparticles, there is a decrease in resistance that can be measured.

1.13.3. Drug delivery applications of gold nanoparticles

Gold nanoparticles provide an excellent platform for drug delivery system (DDS) design due to the functional versatility of their monolayers. Drug delivery applications of AuNPs has peaked interests over the past decade owing to its intrinsic tunable optical properties that can be used directly or indirectly for the treatment of disease. Gold nanoparticles are frequently used as carriers as they are inert and nontoxic. A second
advantage is that gold particles are easy to synthesize. With their unique chemical and physical properties, gold nanocarriers provided a new group of target-specific deliveries of therapeutic agents and have emerge as promising carrier for delivery of various molecules with therapeutic properties. The therapeutic agent could be small drug molecules or large biomolecules, such as DNA, RNA, and proteins. They are able to penetrate the cell to facilitate cellular internalization and connective tissue permeation, thus enabling the drugs to be delivered efficiently to the targeted cell without clogging capillaries. The latest achievements in the applications of gold nanoparticles as drug delivery tools for the therapy of human diseases. The idea of multifunctional gold nanoparticles for drug delivery is presented in Fig. 1.5.

![Diagram of AuNPs application](image)

**Fig. 1.5 Diverse application of AuNPs in human therapy (Ghosh et al., 2008)**
1.13. Synthesis of nanoparticles

Generally nanotechnology based synthetic methods are most commonly developed on the basis of two rational designs which are “top-down” and the “bottom-up” approach. In the top-down approach, nanoparticles are produced by size reduction from a suitable starting material. Size reduction is achieved by various physical and chemical treatments. A major drawback of the top-down approach is the imperfection of the surface structure. Such defects in the surface structure can have a significant impact on physical properties and surface chemistry of the metallic nanoparticles due to the high aspect ratio (Thakkar et al., 2010). Bottom-up, or self assembly, refers to the construction of a structure atom-by-atom, molecule-by-molecule, or cluster by-cluster. In this approach, initially the nanostructured building blocks (i.e. nanoparticles) are formed and subsequently, assembled into the final material using chemical or biological procedures for synthesis. A distinct advantage of the bottom-up approach is the enhanced possibility of obtaining metallic nanoparticles with comparatively lesser defects and more homogeneous chemical compositions.

1.13.1. Synthesis of gold metal nanoparticles

Over the past several years research on developing efficient methods for the large-scale synthesis of gold nanoparticles (AuNPs) are being intensely pursued. Several methods are used for synthesis of metallic nanoparticles (NPs) such as physical, chemical, enzymatic and biological (Mittal et al., 2013). Schematic representation of different methods to produce gold nanoparticles is presented in Fig. 1.6. Physical methods are including plasma arcing, ball milling, thermal evaporate, spray pyrolysis, ultra
Fig. 1.6. Schematic representation of different methods to produce gold nanoparticles (Adapted from Mittal, 2013)
thin films, pulsed laser desorption, lithographic techniques, sputter deposition, layer by layer growth, molecular beam epistaxis and diffusion flame synthesis of nanoparticles. Similarly, the chemical methods are used to synthesized NPs by electro deposition, sol-gel process, chemical solution deposition, chemical vapour deposition, soft chemical method, Langmuir Boldgett method, catalytic route, hydrolysis, co-precipitation method and wet chemical method. However, altogether these methods are energy and capital intensive; employ toxic chemicals and non-polar solvents in the synthesis procedure and later on synthetic additives or capping agents, thus precluding their applications in clinical and biomedical fields. Physical and chemical methods have been using high radiation and highly concentrated reductants and stabilizing agents that are harmful to environmental and to human health (Mittal et al., 2013). Therefore, the need for the development of clean, reliable, bio-compatible, benign and eco-friendly process to synthesize nanoparticle leads to turning researchers towards ‘green’chemistry and bioprocesses. Biosynthesis of gold nanoparticles thus became an eco-friendly alternative to the currently available physical and chemical methods.

1.13.2. Biological methods for synthesis of gold nanoparticles

Synthesis of gold nanoparticles by physical methods requires sophisticated equipments and is thus quite expensive. Gold nanoparticles synthesized by chemical methods often require toxic reducing and stabilizing agents. These toxic substances adsorbed on the surfaces of the gold nanoparticles limit its applications in biomedical fields. Therefore, there is an overwhelming need to develop environmentally benign processes for the synthesis of gold nanoparticles without using any toxic chemicals. Biosynthesis of gold nanoparticles thus became an eco-friendly alternative to
the currently available physical and chemical methods. Researchers have
used biological methods for the synthesis of nanoparticles, by adopting
simple protocols, involved in the process of the reduction of metal ions by
using biological extracts as a source of reductant either extracellularly or
intracellularly (Kumar et al., 2011; Mittal et al., 2013). In recent years,
several researchers have accomplished the biosynthesis of metal
nanoparticles using microbes as well as extracts of plants. Microbial synthesis
is of course readily scalable, environmentally benign and compatible with the
use of the product for medical applications, but production of
microorganisms is often more expensive than the production of plant
extracts. Moreover, plants (especially plant extracts) are able to reduce metal
ions faster than fungi or bacteria. Furthermore, in order to use an easy and
safe green method in scale-up and industrial production of well-dispersed
metal nanoparticles, plant extracts are certainly better than plant biomass or
living plants (Mittal et al., 2013). Many biomolecules in plants such as
proteins/enzymes, amino acids, polysaccharides, alkaloids, alcoholic
compounds, and vitamins could be involved in bioreduction, formation and
stabilization of metal nanoparticles. Reduction potential of ions and reducing
capacity of plants which depend on the presence of polyphenols, enzymes,
and other chelating agents present in plants have critical effects on the
amounts of nanoparticle production.

1.13.3. Biological synthesis of metallic nanoparticles by plants

The use of plant systems has been considered as a green route and a
reliable method for the biosynthesis of nanoparticles owing to its
environmental friendly loom. In producing nanoparticles using plant extracts,
the extract is simply mixed with a solution of the metal salt at room
temperature. The reaction is complete within minutes. The nature of the plant extract, its concentration, the concentration of the metal salt, the pH, temperature and contact time are known to affect the rate of production of the nanoparticles, their quantity and other characteristics (Dwivedi and Gopal, 2010). The ability of various plant systems to produce Au nanoparticles with different morphologies has been demonstrated by different research groups in which the influences of reaction parameters to the formation of nanoparticles (Kumar et al., 2011; Mittal et al., 2013). Nanoparticles produced by plants are more stable and the rate of synthesis is faster than in the case of microorganisms. Moreover, the nanoparticles are more various in shape and size in comparison with those produced by other organisms. Many researchers have reported the biosynthesis of metal nanoparticles by plant leaf extracts and their potential applications (Kumar et al., 2011; Mittal et al., 2013; Mukherjee et al., 2015).

1.14. Plausible mechanism

The exact mechanism for the synthesis of nanoparticles using plant extract still remains unclear. However, it was already well established that plant extract helps for the formation and stabilization of nanoparticles. Many investigators have already established the role of plant extract as reducing agent as well as stabilizing agent during the formation of metal nanoparticles especially gold and silver nanoparticles. According to published literature, presence of polyphenolic/alcoholic compounds, aldehydes/ketones and proteins present in the plant extract might be responsible for the reduction of HAuCl₄ to gold nanoparticles and stabilization of the nanoparticles (Mittal et al., 2013; Mukherjee et al., 2015). It is well established that both low (12-22 kDa) and high molecular weight proteins (~150 kDa) play a major role for the
reduction of metal ions to metallic nanoparticles. However, it depends upon the nature of plant as well as the source of leaf. The overall mechanism for the formation and stabilization of AuNPs is schematically presented in Fig. 1.7.

Newman and Blanchard, (2006) demonstrated that the involvement of proteins supports the formation and stabilization of AuNPs. They demonstrated that reduction of HAuCl₄ occurs due to transfer of electrons from the amine to the metal ion, resulting in the formation of Au⁰ (gold nanoparticles) as follows.

\[
\text{HAuCl}_4 + 3\text{NR}_3 \rightarrow \text{Au}^0 + 3\text{NR}_3 + \text{H}^+ + 4\text{Cl}^-\]

Therefore, in the present work, a single-step green chemistry approach for the synthesis of gold nanoparticles using mulberry (Morus alba L.) leaf extract, where mulberry leaves act as both reducing as well as stabilizing agent/capping agent. Mulberry (Morus alba) leaf extract (MLE) has been chosen for the synthesis of AuNPs because of several reasons, namely (i) it is a traditionally important medicinal plant (ii) contains several phytochemicals like, rutin, isoquercitin, quercetin, astragalin and kaemferol (iii) rich in immunosugars such as glucose analogue 1-Deoxynojirimycin (DNJ), which might be beneficial for suppressing abnormal high blood glucose levels and exert hypoglycemic and hypolipidemic effects in the treatment of type 2 diabetes (Naowaboot et al., 2009; Kwon et al., 2011; Yang et al., 2013). Therefore, mulberry mediated gold nanoparticles (MAuNPs) are mostly biocompatible and could act as an effective delivery system for improving its therapeutic activity against diabetes and its complications. Hence, the mulberry mediated gold nanoparticles (MAuNPs) has been successfully developed in the present study.
Fig. 1.7. Mechanism for the formation and stabilization of AuNPs

1.15. Optical spectroscopic techniques used in the present study

In recent years, there has been much interest in the use of optical spectroscopy as a tool to augment the current protocols for disease diagnosis, as it has the capability to probe the morphological/biochemical changes of tissues that accompany disease progression. Optical techniques may potentially obviate many of the limitations of conventional biochemical methods by providing non-invasive, minimal time required for the study, reproducibility, high-resolution morphological and biochemical analyses of suspect lesions in real time. A variety of optical diagnostic techniques have been proposed for distinction between normal and diseased tissues, such as autofluorescence spectroscopy (AF), diffuse reflectance spectroscopy (DRS), elastic scattering spectroscopy (ES), Fourier transform infrared (FT-IR) spectroscopy and Raman spectroscopy (RS) (Kortum et al., 1996; Bozkurt et al., 2012; Singh et al., 2014). These spectroscopic techniques each have
separate physical bases and all have the potential to become an adjuvant method to conventional biochemical detection methods. Essentially, all spectroscopy techniques have the same mode of action, based on the interaction of light with matter and dependent on the fact that the optical spectrum displays biochemical constitutes of tissue under examination by measuring the signals of fluorescence, absorption and scattering (Bigio and Bown, 2004; Kortum et al., 1996). Qualitative and quantitative analysis of the biochemical changes can be performed by studying spectral features and measuring their intensities over the spectral range providing important information about disease diagnosis and disease stages (Bozkurt et al., 2012; Singh et al., 2014).

1.15.1 **Fluorescence spectroscopy technique**

Light is composed of energy packages known as photons. Tissues on being exposed to light may cause absorption / reflection / scattering of photons. Tissues are known to contain light reactive biomolecules known as fluoropores. These fluoropores absorb energy from light and emit fluorescent light of lower energy and longer wave length. Study of this emitted light from tissue will provide valuable insight of biological status of the tissue. The concentration and distribution of fluorophores vary as the tissue undergoes malignant transformation. This becomes clearly evident in the spectroscopic pattern produced. In general, pathologic tissues usually emit a lower fluorescence because of modified distribution of the native biofluorophores or have a lack of emission because of molecular or environment changes, such as collagen matrix breakdown in tissue remodeling, increased hemoglobin absorption in high vascularized areas, epithelial thickening in malignant
progression, and decreased flavin adenine dinucleotide concentration in metabolic functions.

Autofluorescence spectroscopy has the potential to provide real-time, non-destructive, and quantitative means for characterizing tissue pathology. Fluorescence techniques are being increasingly employed to investigate both morphological and biochemical changes in different tissue types (Pu et al., 2010; Shaiju et al., 2011; Gohulkumar et al., 2014). Fluorescence spectroscopy is well suited for the diagnosis of diseased tissues because of its sensitivity to minute variations in the amount and the local environment of the native fluorophores present in the tissues (Kortum et al., 1996). There are three main types of fluorophores used for tissue diagnostic studies: exogenous fluorophores, endogenous fluorophores, and fluorophores synthesized in the tissue from a precursor molecule that is given externally. Endogenous fluorophores give rise to autofluorescence phenomenon. Examples of endogenous fluorophores include collagen, elastin, nicotinamide adenine dinucleotide (NADH), tryptophan, porphyrins, and flavin adenine dinucleotide (FAD). Collagen and elastin are mainly responsible for spectral changes associated with structural changes within the tissues and cells. Other fluorophores like FAD, NADH, tryptophan, and porphyrins are mainly responsible for spectral changes associated with changes in cellular metabolism and functional processes. A number of fluorophores, e.g., NADH and flavins producing autofluorescence in the visible regime have proved extremely useful for bioimaging. Flavins are the derivatives of riboflavin, the most common of them being flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN with emission maxima at 520 nm, exhibits much brighter fluorescence efficiency than FAD. Intracellular riboflavin, flavin
coenzymes, and flavoproteins show slightly shifted fluorescence (540-560 nm) compared to flavins (Kortum et al., 1996). Autofluorescence phenomenon has been used for diagnosis of various organs (Pu et al., 2010; Shaiju et al., 2011; Gohulkumar et al., 2014). These studies proposed promising results for using fluorescence spectroscopy as an important possible tool for diagnosis of various diseases

1.15.2. Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared (FT-IR) spectroscopy has been applied as a new and powerful bio-spectroscopic technique to study biological samples and uniqueness as a non-destructive method in identifying vibrational structure of various materials. This rapid, specific and non-invasive technique enables to investigate the state of chemical bonds and the relative concentrations of lipids, proteins, carbohydrates and phosphorylated molecules. In the past decade, the rapid developments in the field of infrared spectroscopy, have demonstrated its potential utility for non-invasive disease diagnosis. It is based upon the absorption of infrared light by vibrational transitions in covalent bonds, where the intensities provide quantitative information and the frequencies relate to the nature of these bonds, their structure and their molecular environment. The spectra allow measuring complex molecular vibrational modes. Various bimolecular components of the cell give a characteristic IR spectrum, which is rich in structural and functional aspects (Bozkurt et al., 2012). FT-IR spectroscopy has largely been employed to study membrane lipids. Spectra of lipids provide a unique signature of the lipid class and fine details of the structure (chain unsaturation and length). The structure and organization of biomembrane can also be studied. It allows the detection and characterization of lipid phase
transition and the measurement of lipid orientation in monolayer/multilayer systems. FTIR spectroscopy is also a method of choice for the experimental determination of protein secondary structure (Bozkurt et al., 2012). Amide vibrations are the largest bands in protein spectra and are based on the amide bond present in proteins. Nine characteristic amide bands have been identified but only three of them are usually used to investigate the secondary structure of protein. The exact frequencies of amide I (C=O stretching, 1700-1600 cm\(^{-1}\)) and amide II (N-H bending, 1600-1500 cm\(^{-1}\)) absorption is influenced by the strength of hydrogen bonds involving amide C=O and N-H groups and the geometry of the polypeptide chain. Each type of secondary structures is associated with specific frequencies at which amide I and II occur. The other advantage of this technique is that it can detect even small alterations in the molecular parameters associated either with the administration of antidiabetic drugs or with the development of pathologies, which are not easily detected by morphological methods.

1.16. Objectives of the present study

The present study mainly focused on the following objectives to explore the spectroscopic detection and evaluation of the antidiabetic efficacy of mulberry mediated gold nanoparticles (MAuNPs) in comparison with mulberry leaf extract (MLE) alone against STZ-induced diabetic rats.

- Preparation of MAuNPs using bio-reduction method.

- Characterization of MAuNPs using UV-Visible spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM), atomic force microscopy (AFM), X-ray diffraction (XRD), and Fourier transform infrared (FT-IR) spectroscopy.
- Determination of antioxidant (DPPH and hydroxy radical assay) and anti-microbial effect of MAuNPs against human pathogens.

- Investigation of biodistribution pattern of MAuNPs in selected organs in wistar rats using inductively coupled plasma optical emission spectrometer (ICP-OES).

- Determination of in vivo toxicity studies of MAuNPs in selected organs in wistar rats through intraperitoneal injection over a period of 7 days.

- Determination of histopathological changes and various biochemical parameters (plasma glucose, plasma insulin, enzymatic and non-enzymatic antioxidants, hepatic and renal functional markers (AST, ALT, ALP and urea, creatinine) and lipid peroxidation (TBARS) in STZ-induced diabetic rats.

- Study of the metabolic changes in the various endogenous fluorophores like collagen, NADH, and FAD during STZ-induced diabetic liver tissues by autofluorescence (AF) spectroscopy.

- Investigation of the alterations in the biochemical, functional and structural changes at the molecular level during STZ-induced diabetic liver tissues by FT-IR spectroscopy.