CHAPTER – 6

Optical redox ratio using endogenous fluorescence to assess the metabolic changes associated with treatment response of bioconjugated gold nanoparticles in streptozotocin-induced diabetic rats

6.1. Introduction

Diabetes mellitus (DM) is a major endocrine disease, involving metabolic disorders characterized by hyperglycemia with disturbances of carbohydrate, protein and lipid metabolism. It is also characterized by impairment in carbohydrate utilization by cells and the abnormal regulation of blood glucose resulting from insufficient or defective insulin secretion, resistance to insulin action or both. The International Diabetes Federation estimates that more than 387 million people were affected worldwide and this number is expected to increase 592 million by the year 2035 (IDF, 2014). Chronic hyperglycemia increases oxidative stress in a variety of tissues by excess generation of ROS through various pathways, such as increased advanced glycation products, altered redox equilibrium, mitochondrial respiration chain deficiencies and NAD(P) oxidase (Rahimi et al., 2005). As a result, ROS generation and oxidative stress in hepatic cells trigger the development of diabetes and its complications (Rahimi et al., 2005). Mitochondria are a major source of ROS and free radicals even in the presence of antioxidant defense mechanisms. Mitochondrial metabolic coenzymes NADH and FAD are the primary electron carriers which can be used in multiple metabolism processes including glycolysis and oxidative phosphorylation (Skala et al., 2007; Quinn et al., 2013). These enzymatic cofactors serve an important role in mitochondria as electron carriers linking the tricarboxylic acid (TCA) to the electron transport
chain. These metabolic coenzymes NADH and FAD are becoming increasingly recognized as key metabolic indicators of the state of cellular metabolism associated with health and disease (Skala et al., 2007; Ostrander et al., 2010; Shah et al., 2014; Shaiju et al., 2015). Therefore, direct monitoring of these two coenzymes can be used to study the metabolic activity of cells and tissues. These metabolic coenzymes NADH and FAD are autofluorescent and can be monitored by non-invasive optical techniques without any exogenous labels. Further, the optical redox ratio can be determined by calculating the ratio of the measured fluorescence emission intensities of FAD and NADH (Ostrander et al., 2010; Quinn et al., 2013; Gohulkumar et al., 2014; Shaiju et al., 2015). This optical redox ratio provides relative changes in the oxidation-reduction state in the cell without the use of exogenous stains or dyes, and can thus be measured in vivo in both human and animal studies. Moreover, the optical redox ratio is a prominent indicator of oxidative stress and has potential to non-invasively detect treatment response sooner than current methods (Maleki et al., 2012).

Recently, gold nanoparticles have been extensively used as medicinal agents for the therapeutic applications of several diseases (BarathManiKanth et al., 2010; Fratoddi et al., 2014; Patra et al., 2015). Many studies have demonstrated that green synthesis mediated AuNPs provide new opportunities for the treatment of diabetes (BarathManiKanth et al., 2010; Venkatachalam, et al., 2013). The present study has demonstrate the green chemistry approach for the synthesis of gold nanoparticles using Morus alba leaf extract (MLE) where MLE act as both reducing as well as stabilizing agent/capping agent.

Mulberry (Morus alba) leaf has been used in the traditional medicine for anti-diabetes, anti-hyperlipidemias and the prevention of coronary artery disease. Due to the presence of several phytochemicals (proteins, phenolics,
rutin, isoquercitin, quercetin, astragalin and kaemferol), mulberry leaf extract possess pharmacological importance and have been reported to significantly reduce blood glucose in diabetic induced animals and exert hypoglycemic and hypolipidemic effects in the treatment of diabetes (Kwon et al., 2013; Yang et al., 2013). Moreover, Mulberry (Morus alba) leaf is rich in immune sugars 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). Hence the present study was designed to evaluate the antidiabetic effect of mulberry mediated gold nanoparticles (MAuNPs) in comparison with mulberry leaf extract (MLE) for monitoring endogenous fluorophores emissions and to quantify the metabolic changes in the mitochondrial redox state to liver tissues of STZ-induced diabetic rats using a fluorescence spectroscopic technique. Furthermore, the differentiation between the control and the diabetic treated groups and the validation of the data set using the spectral data were performed using PC-LDA.

6.2. Review of Literature

Zheng et al., (2003) have used optimal autofluorescence excitation and emission wavelengths for diagnosis of bladder cancer. Significant changes in fluorescence intensity of excitation-emission matrices (EEMs) were observed between the normal and tumor bladder tissues and the results showed that tissue autofluorescence of bladder tumor was characterized by an increase in the emission of tryptophan and porphyrin and a decrease in the emission of collagen, NADH and FAD compared to the normal tissues. The results demonstrated that autofluorescence spectroscopy can distinguish malignant from normal bladder tissue and that excitation wavelengths of 280 and 330 nm
are the most significant for differentiation between normal and malignant bladder mucosa with a high degree of diagnostic accuracy.

Manjunath et al., (2004) carried out laser-induced fluorescence spectroscopy in the normal and malignant oral tissues at different excitation wavelengths (275, 325 & 425 nm). The results show that a fluorescence spectral difference was more prominent at 325 nm excitation wavelength. They formulated several discrimination methodologies based on PCA and evaluated the intensity differences between different emission peaks (resultant peaks of curve analysis). The results suggest that intensity ratio of bound NAD(P)H to collagen seems to be more suitable for the discrimination between the normal and malignant oral tissues.

Kirkpatrick et al., (2005) have used endogenous fluorescence spectroscopy to measure metabolic changes in response to treatment with N-4-(hydroxyphenyl) retinamide (4HPR) in ovarian and bladder cancer cell lines. Fluorescence signals consistent with nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and tryptophan were measured to monitor cellular activity through redox status and protein content. The results revealed that redox signal changes depended primarily on changes consistent with NADH fluorescence, whereas the FAD fluorescence remained relatively constant. Further, the results suggest that fluorescence redox values along with changes in tryptophan fluorescence may be used as an endpoint marker for chemopreventive drugs.

Skala et al., (2007) have investigated the potential for in vivo precancer diagnosis with metabolic imaging via multiphoton fluorescence lifetime imaging microscopy (FLIM) of the endogenous metabolic cofactor, reduced NADH. The results demonstrated that the protein-bound NADH lifetime decreased with low grade pre-cancers (mild to moderate dysplasia) and high
grade pre-cancers (severe dysplasia and carcinoma in situ (CIS)) when compared to the normal tissues. Further, they observed that the protein-bound NADH relative to free NADH decreased with low grade pre-cancers in vivo. The results suggest that multiphoton FLIM is a powerful tool for the non-invasive characterization and detection of epithelial pre-cancers in vivo.

Pu et al., (2010) have measured the fluorescence spectra of normal human prostate cancerous tissues at 340 nm excitation wavelength. The result shows that there is a reduced contribution from the emission of collagen and increased contribution from nicotinamide adenine dinucleotide (NADH) in cancerous tissues when compared with the normal tissue. The results suggest that NADH and collagen may present a potential native biomarker for prostate cancer detection.

Shaiju et al., (2011) have carried out autofluorescence spectroscopic screening of normal volunteers with and without lifestyle oral habits and patients with oral sub mucous fibrosis (OSF). The spectra from different sites of habitu’es, non-habitu’es and OSF patients were analyzed using the intensity ratio, redox ratio and linear discriminant analysis (LDA). The spectral disparities among these groups are well demonstrated in the emission regions of collagen and flavin adenine dinucleotide. The results revealed that LDA gives better efficiency of classification than the intensity ratio technique. The results further confirm that the clinical application of autofluorescence spectroscopy along with LDA, yields spontaneous screening among individuals, facilitating better patient management for clinicians and better quality of life for patients.

Maleki et al., (2012) have used cryo fluorescence imaging to measure the metabolic changes in the kidneys of diabetic mice. The results show that the mitochondrial redox ratio (NADH/FAD) was decreased in diabetic mice compared with control mice. The results further suggest that redox ratio may
used as a quantitative marker of oxidative stress during the diabetic progression.

Lin et al., (2012) have investigated the spectral characteristics of the normal and cancerous nasopharyngeal tissues by laser-induced autofluorescence spectroscopy and recorded the autofluorescence excitation emission matrix by *in vitro*. To quantify the changes of collagen and NAD(P)H in cancerous nasopharyngeal tissues peak ratio algorithm (I455 ± 10 nm/I380 ± 10 nm) was used. The results suggest that the excitation wavelength at 340 nm was an excellent diagnostic excitation wavelength for cancerous nasopharyngeal tissues.

Shaiju et al., (2013) have reported the efficacy of autofluorescence spectroscopy to serve as a single entity to discriminate tumor tissues from normal ones by analyzing the variations in both endogenous fluorophores and chromophores involved. The emissions from prominent fluorophores collagen, flavin adenine dinucleotide, phospholipids and porphyrin were analyzed at 320 and 410 nm excitations. The results conclude that potential of fluorescence spectroscopy as a single entity to evaluate the prominent fluorophores as well as the hemoglobin concentration within normal and tumor brain tissues.

Ghanian et al., (2014) have used cryo fluorescence imaging to investigate the effects of low (10 mg/kg) and high (25 mg/kg) doses of 3-iodothyronamine (T1AM) on the metabolism in the kidney and heart of mice. The results revealed that a significant increase in redox ratio was observed in the high dose-treated tissues which further indicate that reduced oxidative stress compared to the low dose-treated tissues or the control tissues. These results demonstrated that while T1AM at a high dose has a protective effect and
may exert its effect through alternative pathways at different doses and at tissue specific levels.

Shah et al., (2014) have investigated the potential of autofluorescence spectrum with metabolic cofactors reduced NADH and FAD for chemotherapeutic response in human head and neck squamous cell carcinoma (HNSCC). The optical redox ratio and fluorescence lifetimes of NADH and FAD resolved a response after 24 hours of treatment with targeted therapies and chemotherapies in HNSCC cells. The results indicated that optical metabolic imaging showed promise to identify effective drug candidates during drug development. Additionally, applying optical metabolic imaging to measure treatment response early has potential to impact quality of life for HNSCC patients.

Gohulkumar et al., (2014) have investigated the metabolic changes in chemopreventive response of prepared silibinin loaded nanoparticles (SILNPs) relative to the efficacy of free silibinin (SIL) against 7,12-dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch (HBP) carcinogenesis using fluorescence spectroscopy. The tumor tissues are characterized by a decrease in the emission of collagen, NADH and FAD compared to the control tissues. Further, the results revealed that a significant decrease in the optical redox ratio was observed in DMBA-induced tumor tissues, which indicates increased metabolic activity compared to the control tissues. Moreover, an oral administration of SIL and its nanoparticulates restored the status of endogenous fluorophore emission and led to a higher redox ratio in the buccal mucosa of DMBA-painted animals. The results of this study further suggest that the fluorescence spectroscopic technique in conjunction with PC–LDA has a potential for rapid and sensitive detection of
specific metabolic alteration and changes in the endogenous fluorophores in response to anti-cancer drug treatments.

Shaiju et al., (2015) have used minimally invasive fluorescence spectroscopic technique to study the effect of carbon tetra chloride (CCL₄) on the different stages of liver diseases such as fibrosis and cirrhosis in a rodent model. The results revealed that a drastic increase in redox ratio was observed for fibrosis and cirrhosis, compared with the normal control. Further, the redox ratio has decreased considerably for the reversal and reaching a level similar to that of normal control. The results further demonstrated that fluorescence spectroscopy along with PC–LDA algorithm has the potential for rapid and accurate minimally invasive diagnosis and detection of structural changes due to liver injury resulting from various intoxicants.

From the above literature survey, it was clear that fluorescence spectroscopy can provide valuable information regarding the collagen, NADH, FAD and cellular metabolism present in the tissues. Hence, the present chapter has been designed to evaluate the antidiabetic efficacy of the MAuNPs in comparison with MLE for monitoring endogenous fluorophores emission and to quantify the metabolic changes in the redox state against STZ-induced diabetic liver tissues by using fluorescence spectroscopic technique. Principal component and linear discriminate analysis (PC-LDA) has been carried out to classify the autofluorescence emission spectra acquired from the control and the experimental group of rats. Receiver operating characteristic (ROC) testing was also conducted to further evaluate the performance of PC-LDA algorithms on autofluorescence spectroscopy for the diagnosis of diabetes.

6.3. Fluorescence

The phenomenon, fluorescence of substances, had been observed for hundreds of years and was explained by the British scientist Sir George G.
Stokes in 1852 that fluorescence can be induced in certain substances by illuminated with ultraviolet light. He formulated Stokes’s law, which states that the wavelength of the fluorescent light is always greater than that of the exciting radiation; therefore fluorescence is the phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at longer wavelengths. Thus, fluorescence is the emission that results from the return to the lower orbital of the paired electron. Such transitions are quantum mechanically allowed and the emissive rates are typically of nearly \(10^8\) Sec\(^{-1}\). These high emissive rates result in fluorescence lifetimes of nearly 10 nano seconds.

The lifetime is the average period of time when a fluorescence biomolecule, fluorophore remains in the excited state. For atoms excited by a high temperature energy source, this emission is commonly termed as optical emission. For molecules, it is termed fluorescence if the transition occurs between states of the same spin. There are certain factors that control the occurrence of fluorescence. It is seen that whenever the interaction between excited molecules and surrounding is strong, radiation less decay will occur. But if the interaction between them is ineffective at achieving large energy, transfer needs to take it to the lower electronic state. The radioative decay thus dominates and the molecule fluoresces. The various energy levels involved in the absorption and emission of light by a molecule is classically presented by a Jablonski energy diagram, named in honor of the Polish physicist Professor Alexander-Jablonski. A typical Jablonski diagram illustrates the singlet ground (S (0)) state, as well as the first (S (1)) and second (S (2)) excited singlet states as a stack of horizontal lines as shown in Fig. 6.1. The thicker lines represent electronic energy levels, while the thinner lines denote the various vibrational energy states (rotational energy states are ignored). Transitions between the states are illustrated as straight or wavy arrows, depending upon whether the
transition is associated with absorption or emission of a photon (straight arrow) or results from a molecular internal conversion or non-radiative relaxation process (wavy arrows). Vertical upward arrows are utilized to indicate the instantaneous nature of excitation processes, while the wavy arrows are reserved for those events that occur on a much longer timescale.

Fig.6.1 Jablonski diagram

Absorption of light occurs very quickly (approximately a femto second, the time necessary for the photon to travel a single wavelength) in discrete amounts termed quanta and corresponds to excitation of the fluorophore from the ground state to an excited state. Likewise, emission of a photon through fluorescence or phosphorescence is also measured in terms of quanta. The excess vibrational energy is converted into heat, which is absorbed by neighboring solvent molecules upon colliding with the excited state.
fluorophore. An excited molecule exists in the lowest excited singlet state (S (1)) for periods on the order of nanoseconds (the longest time period in the fluorescence process by several orders of magnitude) before finally relaxing to the ground state. If relaxation from this long-lived state is accompanied by emission of a photon, the process is formally known as fluorescence. The closely spaced vibrational energy levels of the ground state, when coupled with normal thermal motion, produce a wide range of photon energies during emission. As a result, fluorescence is normally observed as emission intensity over a band of wavelengths rather than a sharp line.

6.3.1. Autofluorescence

Tissue autofluorescence originates from native tissues. Under UV and blue light irradiation, all biological tissues emit fluorescence from various endogenous fluorophores in tissue with a broad distribution in the visible wavelength region. This fluorescence is referred to as autofluorescence, or endogenous fluorescence. The spectrum is also influenced by the optical properties of tissue. Strong absorbers such as hemoglobin may decrease the overall intensity of the fluorescence spectrum, without changing its shape by absorbing the excitation light (Gillenwater et al., 1998). It can also absorb fluorescence light at certain wavelength which in turn changes the appearance of the recorded fluorescence spectrum by generating dips in the spectrum and the illusive presence of the false peaks. Naturally, the shape of the spectrum also depends on the excitation wavelength, since this will determine what energy transitions in the fluorophores are possible. Most often, excitation in the UV or blue wavelength region is used for laser-induced fluorescence (LIF) studies of tissues. The autofluorescence spectra are very complex and the contributions from the different fluorophores cannot usually be separated but can be recognized by their emission peaks.
Since the biological sources of this fluorescence are endogenous to the tissue, this type of tissue fluorescence is called “autofluorescence”. The molecules responsible for this are called fluorophores and include such biological substances as connective matrix (collagen, elastin), cellular metabolic coenzymes (reduced nicotinamide adenine dinucleotide [NADH]), flavin adenine dinucleotide ([FAD] and flavin mononucleotide [FMN]), aromatic amino acids (tryptophan, tyrosine, phenylalanine), byproducts of the heme biosynthetic pathway (porphyrins) and lipopigments (lipofuscin, ceroids). Each group of fluorophores is characterized by distinct excitation and emission wavelength ranges. It is common for a single excitation wavelength to excite many fluorophores and conversely, for the emission signals of many fluorophores to overlap, since the absorption and emission bandwidths of these molecules can be broad.

The oxidized forms NAD and FAD are electron acceptors in the metabolism of the cell. After accepting an electron the carriers are reduced into the forms NADH and FAD, respectively. Later these reduced forms transfer the electrons to oxygen in the mitochondria, and they once again become oxidized. It is the reduced form NADH and the oxidized form FAD that are fluorescent in the visible region. Depending on the state of the cell the balance between the concentrations of oxidized and reduced forms will change and lead to differences in the fluorescence spectrum, which therefore can give information about the metabolic function of the cell and tissue. Collagen and elastin are proteins that are found in supportive tissue and are important fluorophores in the extracellular matrix (Monici, 2005).

Fluorescence spectroscopy has gained increasing importance in recent years, especially in molecular and cellular biology and in many associated areas of biochemical analysis, because it offers many advantages such as
extreme sensitivity, a high degree of selectivity, gives a wide spectrum of information in very short time and exceptional flexibility in measuring a range of solid and liquid sample formats.

Light-induced fluorescence spectroscopy of biological tissues is based on the physical phenomenon that when with a light beam in appropriate spectral region one irradiates some biological sample it could re-emit the light with a spectrum, related to its biochemical content. These molecules, which are in the tissue and re-emit the light, are called fluorophores and the process itself is called fluorescence. Light sources that can be used include incoherent light sources such as Xe or Hg lamps, light-emitting diodes or monochromatic laser light. When light penetrate into the tissue, it could cause reflection from the tissue layers and non-homogeneities in the tissue; could cause absorption, as well as fluorescence (Fig. 6.2). When fluorescence is observed in situ the resultant spectrum is superposition of several overlapping contributions of various fluorophores, concentrations and special distribution vary depend on the stage of tissue pathology. It is typical to observe changes in intensity, or appearance/disappearance of fluorescent maxima with progression towards neoplasia. These spectral changes could indicate tissue pathological condition and stage of the lesion growth. The principle of autofluorescence spectroscopy is based on the fact that different diseased tissues contain different morphohistological characteristics and intrinsic fluorophores that give rise to different fluorescence emission spectra when the tissues are excited at a suitable wavelength (Kortum et al., 1996). If only endogenous fluorophores, naturally existing in the sample, are used to obtain fluorescent signal from the tissue one could observe autofluorescence. Several endogenous fluorophores are often involved in the transformations that occur in the neoplastic process and are therefore interesting for quantitative research.
These include: tryptophan, tyrosine, collagen, elastin, NADH, FAD and porphyrin.

When the light emitted by a sample is measured and the excitation light is at a constant wavelength, an emission spectrum is collected. Excitation of the tissue autofluorescence may be done with the excitation light delivery fibre or fibre bundle is placed in direct contact with the tissue.
Differences in tissue components and structure modify the fluorescence emission, including its intensity and behaviour, so that normal and diabetic induced liver tissues may be discriminated after fluorescence analysis. This technique has been used to detect diseased tissues in a variety of organ systems, such as the cervix of uterus, colorectum, lungs and head and neck (Lin et al., 2012; Quinn et al., 2013; Sulfikkarali and Krishnakumar, 2013; Nazeer et al., 2014; Shah et al., 2014). These studies suggest the possibility of using autofluorescence spectroscopy in disease diagnosis.

**6.4. Experimental design**

In the experiment, a total of 24 rats (6 normal rats and 18 diabetic surviving rats) were used. The experimental rats were divided into four groups of six each.

**Group 1:** Normal control (NC) rats

**Group 2:** Diabetic control (DC) rats

**Group 3:** Diabetic rats were received MLE (100 mg kg^{-1} bw) (D+MLE) in aqueous solution daily using an intragastric tube for 45 days.

**Group 4:** Diabetic rats were received MAuNPs (2.5 mg kg^{-1} bw) (D+AuMNPs) in aqueous solution daily using an intraperitoneal injection for 45 days.

The dose of MLE and MAuNPs used in this study was chosen based on previous studies (El-Sayyad et al., 2011; BarathManiKanth et al., 2010). At the end of the experimental period, the liver tissues were excised and the tissues were further processed for experiments. One portion of the tissue specimens
from each group were frozen in liquid nitrogen and then stored at -80° C until fluorescence spectroscopic analysis.

**6.5. Instrumentation setup**

The autofluorescence emission spectra were obtained from different anatomical sites of the control and the experimental diabetic liver tissues using a hand-held optical fiber probe attached to Fluorolog-III spectrofluorometer (Jobin Yvon Inc., USA) measured from Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. The fiber-optical probe was located perpendicular to the surface with a spot size of 2 X 6 mm. The fiber-optic probe of numerical aperture of 0.22 and 1 cm outer diameter was used (Fig. 6.3a). The bifurcated Y-type fiber optic probe originating from the spectrometer end merges to become a single fiber bundle as it comes in contact with the tissue (Fig. 6.3b). The excitation light was a 450 W Xenon lamp. The desired excitation wavelength was selected and is transmitted to the site through one arm of the Y-type cable and the received fluorescence signal was directed back to the spectrometer through the other arm. The excitation wavelength of 320 nm was selected using Datamax™ software (Datamax, Round Rock, Texas, USA) and the in-built double-grating monochromator. Emission spectra in the range of 350 to 550 nm in 1nm increments were recorded for 320 nm excitation wavelength.

**6.6. Data preprocessing and analysis**

All spectra were baseline corrected and the data in 350-550 nm range were extracted using datamax™ Software. Data values of each spectrum were normalized with respect to the intensity of the peak at around 450 nm. The normalization data was further analyzed using a curve fitting program (Origin, Version 8.0) with Gaussian spectral functions. The area under the curve and peak intensity of each group were extracted and subjected to one-way analysis of variance (ANOVA) using software package SPSS-17.0.
Fig. 6.3 (a) Spectrofluorometer with the fiber-optic probe and (b) Fiber-optic probe bundle for in vivo analysis
6.7. Redox ratio evaluation

To accurately determine the redox ratio, the detected NADH and FAD autofluorescence intensity was considered as the measurement of cellular metabolism (Ostrander et al., 2010). The reduction and oxidation ratio was computed using the relation (Kirkpatrick et al., 2005; Sivabalan et al., 2010)

\[
\text{Redox ratio} = \frac{\text{FAD intensity}}{\text{FAD intensity} + \text{NADH intensity}}
\]

Where, FAD intensity and NADH intensity are the emission intensity at 520 ± 10 and 450 ± 10 nm, respectively.

6.8. Results
6.8.1. Fluorescence spectral features

The present study was carried out to investigate the effect of MAuNPs and MLE on streptozotocin induced metabolic changes in liver tissues of diabetic rats using fluorescence spectroscopy. The typical averaged fluorescence emission spectra of normal control (NC), diabetic control (DC), D+MLE and D+MAuNPs treated rat liver tissues in the range of 350 to 550 nm at 320 nm excitation are shown in Fig. 6.4. The entire emission spectra were baseline corrected and normalized with respect to the peak at 450 nm. At 320 nm excitation, two broad peaks were mainly observed around 380 and 460 nm with a shoulder was observed around 520 nm. These three peaks have been reported as due to emission from endogenous fluorophores such as collagen, NADH and FAD, respectively. Significant differences in the peak intensity were determined between control and diabetic group of rats. This difference indicates the change of fluorophores composition during the progression of diabetes. Diabetic control liver tissues had increased in the collagen peak at around 380 nm compared with those for the normal control liver tissues. Nevertheless, decreased fluorescence intensity was observed upon an
Fig. 6.4. Average autofluorescence emission spectra from normalized to the intensity at around 450 nm for NC, DC, D+MLE and D+MAuNPs of liver tissues.
administration of MAuNPs and MLE treatment to diabetic group of rats. Interestingly, decreased fluorescence intensity was observed upon an administration of MAuNPs treatment when compared with MLE treated diabetic liver tissues. Further, curve-fitting analysis was performed to estimate the area under these endogenous fluorophores in all the spectra and is given in Fig. 6.5. The average area of the collagen peaks for the control and the experimental groups are plotted in Fig. 6.6. As seen from Fig. 6.6, the significant increase in the area of the peak around 380 nm was observed, which indicates an increase in the amount of collagen in diabetic control liver tissues. Moreover, administration of MLE and MAuNPs to diabetic liver tissues showed a decrease in the area of this peak, contrast with the diabetic control liver tissues. However, much decreased area of the peak at around 380 nm was observed in the administration of MAuNPs compared with treatment with MLE. As we have normalized with the entire emission spectra with respect to the NADH peak at around 460 nm, the evaluation of peak intensity and area were not considered for the analysis. However, significant peak shift was observed between the control and the experimental groups because normalization has no influence on the peak shift. In addition, the small shoulder peak around 520 nm is observed in the spectra of diabetic control liver tissues, whereas in the case of control and treatment groups, this peak appears very weak.

6.8.2. Optical Redox Ratio

NADH is primarily fluorescent in its reduced biochemical state, whereas FAD is only fluorescent in its oxidized form. Therefore, NADH and FAD are important metabolic coenzymes which will provide information on the metabolic changes in the mitochondrial redox state associated with disease progression. Relative change in such oxidation and reduction rate is termed as
Fig. 6.5. The curve-fitting analysis for the average autofluorescence spectra showing constituents bands from NC, DC, D+MLE and D+MAuNPs of liver tissues.

Fig. 6.6. Area under the emission peak of collagen around 380 nm evaluated by Gaussian curve fitting. Data are shown as mean + SD.
redox ratio. The redox index or the ratio between flavoproteins and NADH has been proved to be a good indicator of the cell functioning since it is close to oxidation-reduction equilibrium. The redox ratio is more precise indicator of the oxidation-reduction state than the measurements of the averaged NADH and FAD fluorescence intensity alone. The most common method of measuring this redox state of the tissues is the computation of the ratio of fluorescence intensity of FAD and NADH, as this ratio is considered to be sensitive to cellular metabolism as well as vascular oxygen supply. Distinct differences in the optical redox ratio value were observed from control and different pathological conditions of diabetic group of rats and is shown in a box plot in Fig. 6.7. From this result, it is clear that there is a considerable decrease in the redox ratio for diabetic control liver tissues from that of the normal control liver tissues. A decrease in the redox ratio usually indicates increased cellular metabolic activity, as is typically observed in diseased tissues. Conversely, the redox ratio value was significantly higher in the MLE and MAuNPs-treated diabetic liver tissues compared to diabetic control liver tissues. Interestingly, the treatment of rats with D+MAuNPs resulted in an increased redox ratio value compared to those from any other diabetic groups, indicating a decrease in metabolic activity.

6.8. Multivariate analysis

The detailed spectra differences in the whole range were analyzed by PC-LDA. PCA allows for the reduction in the dimensionality of spectral datasets, thereby facilitating the identification of any clustering patterns that may exist. To evaluate the effective diagnostic algorithms for tissue classification based on spectra, the PC-LDA discriminant analysis was carried out with leave one out cross validation technique. An assessment of the LDA model’s ability to predict each tissue can be gained by examining Fig. 6.8. This
Fig. 6.7. Optical redox ratio [Auto fluorescence intensity of FAD/ (NADH+FAD)] for NC, DC, D+MLE and D+MAuNPs of liver tissues.
Fig. 6.8. Canonical discriminant of the function 1 and function 2 discrimination values for each lesion are plotted. The center of each cluster is marked by an star in the appropriate color.
plot of two canonical functions clearly distinguished the tissues as a group in the plot. When the canonical component scores are plotted, they reveal relationships existing between the samples such as natural clustering of the data. Fig. 6.8 exhibits the two-dimensional scatter plot of the two canonical functions (Function 1 versus Function 2), which shows that NC and DC group separated compared with two separate groups. Further, NC and D+MAuNPs group exhibit variations resulting in distant segregation across function 1, whereas the DC and D+MLE group each with some overlap are separated mainly by function 2. To prevent over-training, the leave one out cross validation procedures were used. In canonical discriminant analysis, the group centers (group averages) of the functions are well differentiated for the normal control and the experimental groups. The results of the classification efficiency analysis are shown in Table 6.1. From the results, overall classification efficiency is obtained 83%.

The first principal component (PC1) by definition encompasses the largest variation in the multidimensional space of the original variables; PC2 is orthogonal and includes the largest remaining variation, etc. In the present study, we utilized the two principal components (PC1 and PC2) for the data reduction of PCA and it appears to give a reasonable separation of the diabetic groups from the control (Fig. 6.9). Separation in PC space between control and experimental groups were interpreted in terms of differences occurred in their fluorescence spectra (i.e. endogenous fluorophore features). Misclassifications occurred mostly between NC and D+MAuNPs groups and such overlap was understandable because of the intrinsically similar components between the two kinds of tissue sample.

Further, the scatter plot of the pairwise comparison of each spectrum belonging to NC vs DC, DC vs D+MLE, DC vs D+MAUNPs and D+MLE vs
Table 6.1 Classification efficiency between NC, DC, D+MLE and D+MAuNPs of liver tissues.

<table>
<thead>
<tr>
<th>Groups (no. of spectrum = 15)</th>
<th>NC</th>
<th>DC</th>
<th>D+MLE</th>
<th>D+MAuNPs</th>
<th>Classification Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>93.3</td>
</tr>
<tr>
<td>DC</td>
<td>0</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>86.6</td>
</tr>
<tr>
<td>D+MLE</td>
<td>0</td>
<td>2</td>
<td>11</td>
<td>2</td>
<td>73.3</td>
</tr>
<tr>
<td>D+MAuNPs</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>12</td>
<td>80.0</td>
</tr>
</tbody>
</table>
Fig. 6.9. Plot of the first principal component (PC1) vs the second principal component (PC2) for NC, DC, D+MLE and D+MAuNPs of liver tissues.
D+MAuNPs treated groups by using PC-LDA model are shown in Fig. 6.10. A separating line on the scatter plots was loaded by the PC-LDA model for the diagnostic significance of different tissue groups. Table 6.2 summarizes the classification results of control and diabetic liver tissues. The diagnostic sensitivities of 95.0 %, 63.5 %, 83.3 % and 80.6 %; specificities of 89.0 %, 71.3 %, 79.4 % and 76.8 % and accuracies of 98.6 %, 72.2 %, 81.8 % and 78.4 % respectively, were achieved for differentiation of NC vs DC, DC vs D+MLE, DC vs D+MAuNPs and D+MLE vs D+MAuNPs treated tissues.

Further to evaluate the performance of the diagnostic model developed by PC-LDA algorithms, the receiver operating characteristic curve (ROC) is generated in Fig. 6.11. ROC is plotted by taking the true positive rate (sensitivity) as the vertical coordinate and the false positive rate (1-specificity) as the horizontal axis under a series of different threshold values. It can reflect the performance of a binary classifier. The area under the curve (AUC) is a common global measure for quantifying the diagnostic accuracy of a laboratory test. The larger AUC value for the classifier has the greater forecast accuracy. In the current study, the area under the ROC curve for NC vs DC, DC vs D + MLE and DC vs D+MAuNPs, treated tissues were 0.96, 0.88, 0.92 and 0.86 respectively.

6.9. Discussion

It is a well-known fact that metabolic and biochemical properties of tissues vary significantly during the different progression of the disease. The present study has demonstrated a quantitative method for measuring autofluorescence-based redox ratio that is sensitive to monitor the metabolic changes in mitochondrial redox state and have begun elucidating the metabolic pathways that contribute to these endogenous fluorophores changes during the progression of diabetes and its complications. In addition, autofluorescence-based redox ratio are becoming of interest for the early
Fig. 6.10. Pairwise discriminant plot based on linear discriminant analysis for NC vs DC, DC vs D+MLE, DC vs D+MAuNPs and D+MLE vs D+MAuNPs at 320 nm excitation.
Table 6.2. Classification efficiency classified using PC-LDA for the differentiation of the normal control and the experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC vs DC</td>
<td>95.0</td>
<td>89.0</td>
<td>98.6</td>
</tr>
<tr>
<td>DC vs D+MLE</td>
<td>63.5</td>
<td>71.3</td>
<td>72.2</td>
</tr>
<tr>
<td>DC vs D+MAuNPs</td>
<td>83.3</td>
<td>79.4</td>
<td>81.8</td>
</tr>
<tr>
<td>D+MLE vs D+MAuNPs</td>
<td>80.6</td>
<td>76.8</td>
<td>78.4</td>
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</tbody>
</table>
Fig. 6.11. The receiver operator characteristic (ROC) curves showing the diagnostic performance of discriminant scores of NC vs DC, DC vs D+MLE, DC vs D+MAuNPs and D+MLE vs D+MAuNPs treated groups.
detection of liver disorders and oxidative damage in chronic hyperglycemia. This method can also be used in conjunction with multivariate data analysis technique based algorithm to interpret the vast spectral information for the exact discrimination of liver tissues having various progressions of diabetes and type of damage. In the present study, excitation around 320 nm is the ideal wavelength to obtain autofluorescence emission from endogenous fluorophores present in liver tissues including collagen, NADH and FAD, respectively.

Collagen is the most widespread fibrous protein in the body, with several important functions, and any marked change in its structure and composition is manifested in a variety of pathological conditions. Therefore, the quantitative analysis of collagen can serve as a valuable indicator of diabetes complication. The average collagen intensities of diabetic control and diabetic induced treatment group animals were compared with the normal control animals to understand the progression of diabetes. In this study, it was observed that the intensity of the collagen peak around 380 nm was increased in the diabetic control liver tissues when compared with normal control liver tissues. Besides the spectral intensity, one more spectral parameter, the area under the curve (AUC) of the collagen peak was also used to support the spectral intensity measurements (Fig. 6.6). Excessive deposition of collagen could occur during an imbalance in its metabolism. In a diabetic body, collagen is continuously exposed to high glucose in vascular and extravascular fluids. High glucose levels in diabetes can easily induce the glycation of various structural and functional proteins, including plasma proteins and collagen. A significant increase in the collagen fluorescence in diabetic liver tissues may be due to the increased generation of free radicals, lipid peroxidation, and glycosylation (Kaviarasan et al., 2007) In addition, a close association between increased oxidative stress and hyperglycemia has been postulated to
contribute significantly to the accelerated accumulation of advanced glycation end products (AGEs) and the cross-linking of collagen in diabetes mellitus. Thus, collagen is a prominent target of nonenzymatic glycation. Hence, the present results suggest that the monitoring of collagen can serve as ideal markers for non-invasive assessment of diabetes and its complications.

Extensive evidence has shown that chronic hyperglycemia leads to a series of biochemical events resulting in a production of high levels of ROS and eventual oxidative stress. Mitochondria play an important role in the maintenance of cellular redox status, acting as a redox sink and limiting NADPH oxidase activity. However, when the proton potential threshold is surpassed, mitochondria are also a significant source of ROS, which may further stimulate NADPH oxidases, creating a vicious cycle of ROS production (Thimm and Szibor, 2015). The concentration of intracellular NADH and FAD are good biomarkers of mitochondrial function and mitochondrial anomalies. These two metabolic coenzymes serve a major role in mitochondria as electron carriers linking the tricarboxylic acid (TCA) cycle to the electron transport chain. Relative changes in the ratio of these two metabolic coenzymes were used to estimate the redox state of a cell. A significant difference in the optical redox ratio value was observed between the control and experimental group of diabetes. From this result, it was found that there is a considerable decrease in the redox ratio for diabetic control liver tissues from that of normal control liver. A decrease in the redox ratio usually indicates increased metabolic activity because redox ratio has an inversely proportional to the metabolic state of a cell and likely reflecting levels of oxidative phosphorylation. Reduced oxidative phosphorylation capacity is unlikely to be the general cause of all forms of insulin resistance but might accelerate its progression and subsequent organ dysfunction via increased production of reactive oxygen species. Thus, the lower redox ratio that we observe in the diabetic control
liver tissues is consistent with reduced oxidative phosphorylation when compared with normal control liver. Moreover, the decreased redox ratio is closely associated with increased oxidative stress and high degree of neovascularization. Interestingly, treatment with MAuNPs and MLE to diabetic liver tissues statistically increased the optical redox ratio compared to that of diabetic control liver tissues. The antidiabetic potential of MLE and its nanoparticles may also be attributed to its antioxidant properties. The antioxidant potential of MAuNPs might be probably due to the presence of the presence of different flavonoid molecules such as rutin, isoquercitrin, quercetin 3-(6-acetylglucoside), astragalin and kaempferol 3-(6-acetylglucoside), and chlorogenic acid. Published literature demonstrates the presence of these flavonoid molecules have potential antioxidant and antidiabetic efficacy (Kwon et al., 2013; Yang, et al., 2013). These flavonoid molecules may conjugate with the as synthesized MAuNPs and may occupy the surface of the nanoparticles to form in situ drug delivery systems. Moreover, MAuNPs offer many active sites for free radical scavenging because of their large surface to volume ratio and also in situ nanoparticulate drug delivery systems are often interacting with the diseased tissue at higher concentration than normal drug (MLE). Thus, a combination of all these factors may explain the superior antidiabetic effect and also the changes in the redox state compared to leaf extract alone.

Considering the complexity of biological tissue, multivariate statistical analysis (PC-LDA), which incorporates the entire fluorescence spectra data for analysis, is a more robust and rigorous to differentiate spectra with different progression of diabetic tissue. In the present study, a high level of sensitivity and specificity was achieved for classifying and differentiating between the control and diabetic groups. Moreover, ROC analysis showed that PCA,
followed by LDA, could differentiate between control and experimental tissue groups with AUC results of 0.86 to 0.96 in this study.

6.11. Conclusion

In conclusion, the present study has demonstrated that optical redox ratio can be used as a quantitative marker of oxidative stress in diabetes mellitus. These fluorescence based redox ratio measurements represent valid and reliable, non-invasive approaches to detect changes in metabolic pathways during the various progression of diabetes mellitus. The present results show that endogenous collagen emission increases in diabetic control liver tissues which might be due to the increased generation of free radicals, lipid peroxidation, and glycosylation. Further, there was a significant decrease in the optical redox ratio is observed, which indicates an increased metabolic activity when compared to the control liver tissues. Upon administration of MAuNPs and MLE, a gradual reversal of the disease to normal conditions was observed as indicated by the increase in collagen levels and redox ratio. Overall, the treatment of MAuNPs was found to be more effective than MLE in completely preventing the hyperglycemia mediated oxidative stress in STZ induced diabetic rats. The present study further suggest that fluorescence spectroscopy has a potential for the rapid and sensitive detection of specific metabolic alterations without disturbing cell or tissue function and potentially monitor the efficacy of therapeutic regimens.