CHAPTER - 6

Endogenous porphyrin as an important biomarker in assessing tumor proliferation following nanoencapsulated silibinin treatment in DMBA-induced hamster buccal pouch carcinogenesis

6.1. Introduction

Oral cancer is the sixth most common cancer worldwide that results from the oral habits of smoking, reverse smoking and betel nut chewing, accounting for at least 40% of all malignancies. Oral malignancy progresses in a multistep process that begins with premalignant alterations of the oral mucosa. Oral squamous cell carcinoma (OSCC) constitutes more than 90% of oral cancer (Min et al., 2012). Despite significant advances in various forms of treatment, the 5-year survival rate for early stage malignancies is approximately 80% but can be as low as 19% for cases with metastasis (Neville and Day, 2013). One approach to this problem would be to improve the ability of oral health care professionals to detect relevant potentially malignant lesions or cancerous lesions at their earliest or most incipient stage. Therefore, early detection and the clarification of the detailed molecular mechanism of OSCC are urgently needed.

In recent years there has been much interest in the use of optical spectroscopy as a tool to augment the current protocols for cancer diagnosis, (Liu, 2011) as it has the capability to probe the metabolic changes of tissue that accompany disease progression. By investigating the metabolic processes, the diseases can be diagnosed. The capability to obtain in vivo information about specific biochemical/morphological changes that take place during the development or regression of neoplasia can provide a rich source of diagnostic information, and can further support the understanding of biological processes involved. Optical spectroscopy has shown that tissue
fluorescence spectra contain such diagnostic information about the metabolic activity of epithelial cells. Autofluorescence spectroscopic techniques are safe, non-invasive, highly sensitive, short testing time, less sample requirement consumption and cost effective compared with other spectroscopic techniques and hence affordable to financially backward patients for the identification of premalignant and malignant oral lesions from benign lesions and normal oral mucosa (de Veld et al., 2004; Mallia et al., 2010; Yuvaraj et al., 2014; Nazeer et al., 2014). The tissues contain a number of key fingerprint native endogenous fluorophore molecules such as tryptophan, collagen, elastin, reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and endogenous porphyrins which are related to metabolic processes. The normal and pathological tissues have different content of endogenous fluorophores and would emit different fluorescence spectra; which can distinguish pathological tissue from normal one. More recently, fluorescence guidance has taken advantage of evaluating intrinsic metabolic and structural changes that occur within tumors by exploiting the heme biosynthetic pathway (Roberts et al., 2011). Porphyrins are synthetic precursors to the prosthetic group heme in hemoglobin and protoporphyrin IX (PpIX) is an intermediate in the biosynthesis of heme and can be amassed since the conversion process into heme is slow (Miah, 2009). Wavelengths in the range 400-410 nm is most suitable for tissue excitation as it provides the greatest amount of information related to endogenous porphyrin and allowed more accurate diagnosis of oral tumors. In particular, endogenous porphyrin (PpIX) has characteristic fluorescence properties and its preferential accumulation in certain cell types may be further considered as one of the spectral markers for the detection of oral cancer (Smits et al., 2005). Use of this spectral biomarker as potential disease marker may lead to the development of simple clinical tools for early detection of numerous diseases
as well as for monitoring disease progression and cell proliferation (Miah, 2001). Hence, endogenous porphyrin autofluorescence measurement can be used to monitor the treatment response of anticancer drugs.

Currently, conventional therapeutic approaches such as chemotherapy, radiation, combinational chemotherapy and surgical treatments are widely accepted to treat or eradicate tumor(s). Unfortunately, these therapies pose high risks. Chemotherapy-associated side effects and secondary neoplasm are some of the most common long-term complications of chemotherapeutic treatment (Singh and Singh, 2012). To overcome these challenges, the use of nanoparticles (NPs) as a vehicle for delivering anticancer therapeutics or as therapeutic agents themselves, has been widely explored in recent years. The nanoparticle drug system has significant advantages, including increasing the solubility of drugs in the body to enhance its absorption, high surface area to volume ratio which affect the pharmacokinetics, improving in vivo bioavailability and biodistribution of the associated drug molecule are main features of nanotechnology based drug delivery systems (Danhier et al., 2010; Frank et al., 2014). Therefore, nanoparticles could act as an effective delivery vehicle and are developed to overcome major obstacles associated with natural phytochemicals bioavailability like poor solubility, stability, absorption and rapid systemic metabolism and its anticancer effect. Moreover, it can significantly enhance the bioavailability and therapeutic efficacy of drugs with reduced side effects. Among many natural anti-cancer agents, silibinin is a favorable phytochemical that has demonstrated remarkable therapeutic potential for various cancers (Deep and Agarwal, 2010). Silibinin is a plant derived flavonoid present in the plant *Silybum marianum* (milk thistle), which is responsible for multiple pharmacological activities including antioxidant, antiinflammatory, antiproliferative and anticarcinogenic (Wellington and Jarvis, 2001; Sangeetha et al., 2010;
Sangeetha et al., 2012). Hence, the present study is designed to evaluate the antitumor efficacy of silibinin-loaded nanoparticles (SILNPs) in comparison with free silibinin for monitoring the changes in the endogenous porphyrin emission against DMBA-induced oral carcinogenesis. In addition, multivariate statistical techniques, including principal components and linear discriminant analysis (PC-LDA) are utilized to develop diagnostic algorithms for differentiation between the control and the experimental groups. The receiver operating characteristic (ROC) curve is also conducted to further evaluate the performance of PC-LDA algorithms to monitor the therapeutic response of SILNPs using autofluorescence spectroscopy for oral cancer diagnosis.

6.2. Review of Literature

Palmer et al., (2002) have observed that the largest differences between normal and dysplastic tissues obtained in the excitation wavelength of 410 nm. Dysplastic and malignant samples had increased fluorescence above 600 nm, when compared with normal samples indicating that dysplastic lesions exhibited a fluorescent characteristic which is not present in normal mucosa. Their results demonstrated sensitivity, specificity and accuracy of fluorescence to differentiate histologically normal tissue from dysplastic and neoplastic 90%, 91% and 91%, respectively.

Ebihara et al., (2003) have used the hamster cheek pouch model for differentiation between the healthy tissues and the different stages of oral premalignancy and malignancy by laser induced fluorescence after tissue exposure to 5-Aminolevulinic acid (ALA) at 405 nm excitation. They observed that fluorescence intensity was significantly lower in healthy tissue than in pathological tissues. The results further revealed that tissue exposure to ALA to differentiate between healthy tissue and the different stages of oral
premalignancy and malignancy and the effectiveness for diagnostic purposes of ALA application at 90 minutes.

Chen et al., (2005) have used time-resolved autofluorescence spectroscopy for effectively distinguishing normal oral mucosa (NOM) from oral premalignant lesions including verrucous hyperplasia (VH), epithelial hyperplasia (EH) and epithelial dysplasia (ED). They obtained accuracy rate of 93% for ED, of 75% for VH and EH, and of 100% for NOM samples by leave-one-out method and the FDA algorithm. In addition, all oral premalignant lesions (including VH, EH and ED) could be distinguished from NOM samples by FDA algorithm. The results revealed that time-resolved autofluorescence spectroscopy at 633 nm emission under 410 nm excitation, based on two-component lifetime calculation and Fisher's discriminant analysis (FDA) was a very sensitive technique for in vivo diagnosis of oral premalignant lesions.

Mallia et al., (2008) have developed a spectral ratio reference standard (SRRS) to discriminate different grades of oral cancer. They recorded laser-induced autofluorescence (LIAF) emission spectra from oral mucosa in the 420-720 nm spectral range on a miniature fiberoptic spectrometer from 14 anatomical sites of 35 healthy volunteers and 91 sites of 44 patients, with excitation at 404 nm from a diode laser. They used three spectral ratio reference standard (SRRS) (intensity ratio F_{500}/F_{635}, F_{500}/F_{705} and F_{500}/F_{685}) scatter plots to differentiate the normal mucosa from hyperplasia, hyperplasia from dysplasia and dysplasia from squamous cell carcinoma (SCC). The results suggest that the F_{500}/F_{685} ratio was more suited to understand tissue progression from normal to premalignant and malignant with 100% sensitivity and specificity.

Bellini et al., (2008) used fluorescence spectroscopy to evaluate the accumulation of porphyrin (PpIX) in mice bearing renal cell carcinoma. They
found significant differences between the normal and tumor-bearing kidneys in autofluorescence shape occurred in the 600-700 nm spectral region. The results suggest that the excitation wavelength at 405 nm was an excellent diagnostic excitation wavelength for renal carcinoma tissues.

Tristao et al., (2010) have used fluorescence spectroscopy to evaluate the levels of erythrocytes porphyrin of healthy and 5/6 nephrectomy (NX) treated wistar animals. They observed the emission spectra with excitation at 405 nm. They found significant differences between the normal and NX treated rats, autofluorescence shape occurred in the 600-700 nm spectral region.

Mallia et al., (2010) have utilized laser-induced autofluorescence (LIAF) and diffuse reflectance (DR) for clinical study to improve early oral cancer diagnosis and tissue grading. The spectra were recorded LIAF and DR emission from oral mucosa on a fiber-optic spectrometer by illumination with a 404 nm diode laser and tungsten halogen lamp in 36 healthy volunteers and 40 lesions of 20 patients. The results suggested that LIAF/DR technique, in conjunction with curve-fitting, as a promising classification tool for differentiate dysplasia and SCC in clinical trial and proved its potential for early detection of oral cavity cancer and tissue grading.

Ebenezar et al., (2012) have evaluated the diagnostic potential of fluorescence excitation spectroscopy (FES) technique for the detection and characterization of normal and cancerous oral lesions in vivo. Fluorescence excitation (FE) spectra from oral mucosa were recorded between 340 and 600 nm excitation wavelengths for an emission wavelength at 635 nm using a fiberoptic probe spectrofluorometer. The results revealed that the FES technique was able to distinguish normal from WDSCC tissues with a specificity of 100%, with corresponding sensitivity of 100%. Results of the
pilot study demonstrated that the FE spectral changes due to porphyrin have a good diagnostic potential; therefore, porphyrin can be used as a native tumor marker.

Khosroshahi and Rahmani, (2012) have applied Laser-induced fluorescence spectroscopic technique for the evaluation and detection of normal and malignant bone cells at an excitation of 405 nm. The results revealed that the fluorescence intensity and the area under the spectra of malignant bone cells was less than that of normal. Further, the area ratio and shape factor also changed as normal cells centered at about 486 and 575 nm and for malignant cells about 482 and 586 nm respectively. The results suggest that LIAF spectroscopic technique provides significant potential for the discrimination between normal and malignant bone cells.

Rajasekaran et al., (2013) have studied the native fluorescence characteristics of human urine samples by excitation-emission matrices (EEMs) and fluorescence emission spectral measurement at 405 nm excitation wavelength. The fluorescence spectra of urine samples of cancer patients exhibited considerable spectral differences in both EEMs and emission spectra with respect to normal subjects. Using the fluorescence intensity values of the emission spectra different ratios were calculated and were used as input variables for a multiple linear discriminant analysis across different groups. The discrimination revealed that fluorescence emission spectra excited at 405 nm discriminates the cancer patients from normal subjects with a sensitivity and specificity of 95% and 94.4%, respectively, with an overall accuracy of 94%. Based on the fluorescence emission characteristics of urine and statistical analysis, they concluded that the fluorophores nicotinamide adenine dinucleotide (NADH) and flavins may be considered as metabolomic markers of cancer.
Sulfikkarali and Krishnakumar, (2013) have investigated the chemopreventive effects of naringenin-loaded nanoparticles (NARNPs) in comparison with free NAR against 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis by the laser-induced autofluorescence (LIAF) spectroscopic technique. LIAF emission spectra from the hamster buccal mucosa of the control and experimental groups animals were recorded in the 350-700 nm spectral range on a miniature fiber optic spectrometer from different anatomical sites of each group, with excitation at 404 nm from a diode laser. LIAF emission spectra showed significant difference between the control and tumor tissues. On a comparative basis, the results revealed that the treatment of nanoparticulate naringenin was found to be more effective than free naringenin in completely preventing the formation of tumor and also improved the status of endogenous porphyrins to a normal range in DMBA-induced hamster buccal pouch carcinogenesis. The results suggest that LIAF spectroscopy could be a very valuable tool for rapid and sensitive detection of endogenous fluorophore changes in response to chemopreventive agents.

Shaiju et al., (2014) have demonstrated the potential of fluorescence spectroscopy as a tool for the discrimination between adjacent normal and tumor tissues of intra axial tumors such as astrocytoma and glioma and extra axial tumors such as meningioma and schwannoma. Spectra excited at 410 nm along with PC–LDA analysis are found to be the best combination for the effective classification of adjacent normal and tumor tissues.

Yuvaraj et al., (2014) have used the autofluorescence spectroscopy for discriminating the saliva of normal subjects and oral cancer patients based on spectroscopic characterization of porphyrin at 405 nm excitation. In the case of OSCC, they observed distinct fluorescence emission peaks at 625 nm and
687 nm and their intensities were 3.4 and 2 times higher than that of normal saliva respectively. This pilot study revealed that salivary porphyrin provides discrimination of OSCC from the normal.

Francisco et al., (2014) have evaluated the efficacy of fluorescence spectroscopy for discrimination of normal oral mucosa, oral cancer and potentially malignant disorders. They used 115 individuals, of whom 55 patients presented oral squamous cell carcinoma, 30 volunteers showing normal oral mucosa and 30 patients had potentially malignant disorders. They classified spectra and compared with histopathology for evaluating the efficiency in diagnostic discrimination employing fluorescence. They further classified the spectra by decision tree algorithm (C4.5). They observed high variance spectral data, the specificity and sensitivity of 93.8% and 88.5%, respectively obtained at 406 nm excitation. The results suggest the potential use of fluorescence spectroscopy as an important tool for oral cancer diagnosis and potentially malignant disorders.

From the above literature survey, it is clear that fluorescence spectroscopy can provide valuable information regarding the endogeneous fluorophores present in the tissues. Hence, the present chapter has been designed to investigate the chemopreventive effects of prepared silibinin-loaded nanoparticles SILNPs relative to efficacy of free silibinin (SIL) in modifying the changes in the endogenous fluorophores during DMBA-induced oral carcinogenesis by autofluorescence spectroscopy.

6.3. Instrumentation setup

The autofluorescence measurements were performed using the spectrofluorometer (Fluorolog-III; Jobin Yvon Inc., USA), with a fiberoptic accessory for in vivo applications measured from Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. The
fiber-optical probe was placed perpendicular to the surface of the hamster buccal pouch in the control and the experimental groups. The fiber optic probe of numerical aperture, 0.22 and 1 cm outer diameter was used for the measurements. 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 buccal mucosa site. 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. A correction factor was uniformly applied to all spectra to compensate for the changes made by the test tube. After spectral acquisition from control and experimental animals in each group, the test tube was cleaned and disinfected with 2% glutaraldehyde solution. The excitation light was a 450 W Xenon lamp. The signal was then amplified and displayed on the computer monitor. The excitation wavelength of 410 nm was selected using Datamax™ software (Datamax, Round Rock, Texas, USA) and the in-built double-grating monochromator. Emission spectra in the range of 450 to 750 nm were recorded at 410 nm excitation.

6.4. Experimental design

The local institutional animal’s ethics committee (IAEC), (Register number 160/1999/CPCSEA), Annamalai University, Annamalainagar, India, approved the experimental design. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian council of Medical Research, India. A total number of 36 animals were divided into six groups and each group contained six animals. Group I hamsters served as the control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Group II, III and IV hamsters were treated with 0.5% solution of DMBA in liquid paraffin using a No. 4 sable brush, three
times a week for 14 weeks on their left buccal pouches (Shklar, 1999). Group II animals received no other treatment. Groups III and IV hamsters were orally given silibinin at a dose (50 mg/kg body weight/day) and SILNPs (dose equivalent to 50 mg/kg body weight/day of SIL), dissolved in 1 ml of 5% dimethyl sulfoxide (DMSO) and dissolved in distilled water, respectively, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the end of the experiment. Groups V and VI hamsters received the same dose of SIL (50 mg/kg body weight/day) and SILNPs alone (dose equivalent to 50 mg/kg body weight/day of SIL) as in group III and IV throughout the experiment period. The dose of silibinin used in this study was chosen based on a dose response study undertaken by us that demonstrated maximum chemopreventive efficacy at this dose which has been reported in previous studies (Kaur et al., 2010; Sangeetha et al., 2012). The experiment was terminated at the end of 16 weeks and hamsters were sacrificed by cervical dislocation after an overnight fast. The buccal pouches 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. The remaining tissue samples were immediately fixed in 10% neutral buffered-formalin and embedded in paraffin. In group V (SIL alone) and group VI (SILNPs alone), the epithelium was normal, intact and continuous. Therefore, groups (I-IV) were chosen for further fluorescence spectroscopic analysis.

6.5. Data Processing and Analysis

All spectra were baseline corrected using DatamaxTM software. Normalizing a fluorescence spectrum removes absolute intensity information; algorithms developed from normalized fluorescence spectra rely on differences in spectral line shapes for diagnosis. Data values of each
spectrum were normalized with respect to maximum intensity wavelength in the 500 ± 10 nm for 410 nm excitation. One way analysis of variance (ANOVA) test was carried out checking the variations in the prominent fluorescence peak intensity between the control and the experimental groups using the statistical software package SPSS-17.0.

6.6. Results

6.6.1. Spectral features of 410 nm excited spectra

Fig. 6.1 shows the average autofluorescence spectra from the control and the experimental groups were recorded in the emission range of 440-750 nm at 410 nm excitation wavelength. The broad autofluorescence peak at 500 nm seen in the autofluorescence spectra is characteristic of all normal and diseased epithelial tissues (Mallia et al., 2010). This peak has been reported as due to emission from endogenous fluorophores such as reduced nicotinamide adenine dinucleotide hydrogenase (NADH) and flavin adenine nucleotide (FAD) (Mallia et al., 2010). However, the endogenous fluorescence peak at 500 nm is reduced in DMBA-induced tumor tissues relative to the control tissues and no further peak was observed at longer wavelengths. Significant differences between the control and the experimental tissue in autofluorescence peak were observed in the 600-700 nm spectral regions. In particular, the emission from endogenous phorphyrins of protoporphyrin IX demonstrably characterized the autofluorescence spectroscopy of cancerous tissues. As can be seen from Fig. 6.1, fluorescence spectrum consists of two main peaks centered about 635 and 670 nm and a broad peak with a shoulder around 700 nm is observed in the DMBA-induced tumor tissue when compared with the control tissues. In fact, a most intense fluorescence peak was observed at 635 nm in DMBA-alone treated tissues. This may be a result of the increased fluorescence
Fig. 6.1 Average autofluorescence emission spectra from the control, DMBA-alone, DMBA + SIL and DMBA + SILNPs
of endogenous porphyrins accumulated in DMBA-induced tumors. In addition, oral administration of SIL and SILNPs to DMBA treated tissues showed a decrease in endogenous porphyrins fluorescence intensity compared with the DMBA-alone treated tissues. However, much lower fluorescence intensity at 630 nm and no more peaks was observed at longer wavelength in the SILNPs administered group of DMBA treated animals tissues.

In Fig. 6.2, the average fluorescence emission spectra were normalized further with respect to the peak at ~ 500 nm, and hence this peak intensity is not considered for the analysis. Considering the peak around 500 nm, a blue shift of about 7 nm is observed in the case of DMBA-induced tumor tissue compared to the control tissues. This observed spectral shift of the peak positions in the spectra of cancer tissues may be due to different microenvironments of these endogenous fluorophores in the tissues. DMBA-induced tumor tissues showed enhanced peak intensity at wavelengths of about 635, 670 and 700 nm, corresponding to the fluorescence spectrum of PpIX, that were not observed in the emission spectra of the control tissues (Fig. 6.2). As in the case of DMBA + SIL and DMBA + SILNPs-treated tissues, the intensity of these peaks decreased compared to DMBA treated tissues. Nevertheless, the 635 nm peak is less prominent in DMBA+SIL-treated tissue and the 670 nm peak appears broadened (Fig. 6.2). On the other hand, in DMBA+SILNPs tissues the peak intensity emission around 670 and 700 nm was absent and the intensity of the 635 nm emission was further reduced. The normalized autofluorescent intensities at 635, 670 and 700 nm between the control and the experimental animals in each group are given in Fig. 6.3.
Fig. 6.2 Average autofluorescence spectra from hamster buccal mucosa normalized to the intensity at ~ 500 nm for the control, DMBA-alone, DMBA + SIL and DMBA + SILNPs
Fig. 6.3 Comparison of peak intensities at 635, 670 and 700 nm to the ~ 500 nm normalized autofluorescence spectra for the control, DMBA-alone, DMBA + SIL and DMBA + SILNPs. Data shown are in the form mean ±SD. Significance (* = p < 0.05, and ** = p < 0.005) of differences resulted on pair wise ANOVA between control vs DMBA-alone, DMBA-alone vs DMBA + SIL and DMBA + SIL vs DMBA + SILNPs.
6.6.2. Fluorescence intensity ratio analysis

Scatter plot of normalized autofluorescence intensity ratio, 500/635 from 410 nm excitation spectra are given in Fig. 6.4. Discrimination lines were drawn between the control, DMBA, DMBA + SIL and DMBA + SILNPs at values that correspond to the average ratio values of 5.25, 0.33, 1.22 and 2.81 for respective groups. The cut off value chosen from the scatter plot of the fluorescence intensity ratio gives good discrimination. The ratio shows a decreasing trend with increasing tumor volume (Chapter 4, Table 4.1) with the lowest values for DMBA-alone and the highest for the control tissue.

Fig. 6.4 Discrimination scatter plots for spectral intensity ratio (normalized fluorescence intensity [NFI] 500/635) for the control and the experimental groups
6.6.3. Multivariate analysis

A discriminant function between the control and the experimental group is given in Fig. 6.5. The first two discriminant functions (function 1 and function 2) are plotted along x-axis and y-axis. Group averages are represented by centroids. The distances between these centroids are found to be nearly equal for all the groups. As shown in Fig. 6.5, clusters corresponding to the control and DMBA+SILNPs treated tissues are closer, while clusters of DMBA and DMBA+SIL treated tissues are placed far apart from each other. Classification efficiency of standard models was evaluated by LOOCV data. Fifteen out of 18 spectra were correctly classified as the control. Seventeen out of 20 spectra were correctly classified as DMBA-alone treated tissue, 13 out of 17 and 14 out of 18 spectra were correctly classified as DMBA+SIL and DMBA+SILNPs treated tissues respectively. A summary of classifications for all groups is shown in Table 6.1.

The pairwise discriminant scores for the control, DMBA, DMBA+SIL and DMBA+SILNPs are shown in Fig. 6.6. Discrimination line drawn at 0 gives a good separation. The diagnostic sensitivities of 100%, 83.33%, 89.47% and 83.33% and specificities of 100%, 90.0%, 95.0% and 82.35%, respectively, were achieved for classification of Control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues. The number of positive and negative predictive values was obtained using this position of pairwise discriminant scores. These values were applied for binary calculations to obtain the performance level of PC-LDA. The positive predictive value (PPV) and negative predictive value (NPV) of the prospective data set for the diagnosis of Control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues are 100%, 88.23%, 89.47% and 83.33% and 100%, 85.71%, 90.0% and 82.35% respectively.
Fig. 6.5 Statistical clustering by canonical discrimination analysis. The function 1 and function 2 discriminance values for each case are plotted.
Fig. 6.6 Pairwise discriminant plot based on linear discriminant analysis for control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues at 410 nm excitation.
The accuracies of 100%, 86.84%, 94.59%, 82.08% for discriminating Control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues are shown in Table 6.2. In the statistical results, a high level of accuracy was obtained in distinguishing normal from diseased samples. The Matthew’s correlation coefficient (MCC), which reflects the quality of classifications is also calculated for Control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues as 1.0, 0.78, 0.89 and 0.72, respectively.

6.6.4. Receiver operating characteristic (ROC) curve analysis

The performance of PC-LDA for the control and the experimental tissues, receiver operating characteristic (ROC) curves was generated at different threshold levels as shown in Fig. 6.7. The integration AUC yielded classification for Control vs DMBA, DMBA vs DMBA+SIL and DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues as 1.0, 0.98, 0.99 and 0.98, respectively, for 410 nm excitation spectra.

6.7. Discussion

One of the most critical points in cancer treatment is early stage diagnosis and the follow up of treatment efficacy. Most types of cancer can be treated effectively if they are detected at an early stage, so that the patient achieves full recovery. The most effective and efficient treatment is linked to early detection. The development of optical techniques for quantifying tissue fluorescence is crucial for several aspects of cancer treatment, including the detection of specific tumor biomarkers, monitoring of tumor biochemical abnormalities and drug-induced antiproliferative effect (Bellini et al., 2008; Sulfiikkarali and Krishnakumar, 2013). Based on these, many groups have showed interest to probe the possibility of using autofluorescence spectroscopy as one of the methods in early diagnosis and treatment response of cancer (Spliethoff et al., 2014; Gurushankar et al., 2014). Various
Fig. 6.7  The ROC curves showing the diagnostic performance of discriminant scores of function for discerning control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues
stages of tumor growth are correlated with changes in the concentration and metabolic activity of endogenous fluorophores, which reflected on the autofluorescence spectra and can be used for further diagnostic purposes. Among various endogenous fluorophores, endogenous porphyrins are a good fluorescent metabolic indicator in tissues, like others well-known fluorescent metabolic indicators such as reduced NADH and FAD (Olivo et al., 2011; Gurushankar et al., 2014). As a result of tumor specific alterations, porphyrin may rapidly get accumulated in tumors compared with the surrounding normal tissue and it is the most commonly used target in autofluorescence-detecting oncology. Hence, the endogenous porphyrin can be used as a reliable spectral marker and very useful tool for monitoring the anti-proliferative effect of cancer.

In the present study, the significant changes is observed between the control and the experimental animals in normalized autofluorescence emission intensity in the 440-750 nm spectral region at 410 nm excitation. Normalized datasets can provide a comparative quantification of a specific fluorophores with better classification efficiency. The results of the present study, demonstrated that the enhanced autofluorescence endogenous emission observed at 635, 670 and 700 nm in DMBA-alone treated tissues and these increased intensities are considered to be dependent on the accumulation of endogenous porphyrins (PpIX) (Fig. 6.3). It is normally supposed that an enhanced accumulation of porphyrins in cancerous tissues can be a consequence of enhanced proliferation of cells, increased activity, breakdown of RBC’s and increased neovascularization in DMBA-alone treated tumor tissue (Bellini et al., 2008; Valdes et al., 2011). In contrast, oral administration of SIL and SILNPs to DMBA painted animals shows significantly decreased intensity at around 630, 670 and 700 nm. However, much lower fluorescence intensity at 630 nm observed in the oral
administration of SILNPs to DMBA painted animals indicate that there is a controlled revival of porphyrin relative to that of the DMBA+ SIL treated tissues. Wyld et al., (1998) studied the relationship between the different cell cycle phases and the rates of PpIX generation in synchronized and unsynchronized bladder cancer cell. In this study, cells in certain phases of the cell cycle produce greater amounts of PpIX (Pogue et al., 2010). Many researchers have reported that, actively proliferating cells produce more PpIX than quiescent cells which results in increased cellular proliferation combined with architectural changes in the stroma and angiogenesis during oral carcinogenesis. Moreover, the PpIX levels in the DMBA+SIL and DMBA+SILNPs treated tissues were 72% and 95% lower than that of DMBA-alone treated tumor tissues. This observation strongly supports the belief that cellular proliferation changes occurred during the tumor transformation process, modify the endogenous porphyrin emission properties between the control and the experimental tissues. Taken together, the present results suggest that there is a strong correlation between cellular proliferation and endogenous porphyrin emission in DMBA-alone treated tumor tissue. Hence, endogeneous porphyrin (PpIX) emission is considered to provide a powerful and rapid method for evaluating antiproliferative effect during preclinical therapeutic investigations in hamster buccal mucosa (results as briefly discussed in chapter 4). Furthermore, the high antitumor activity of the nanoparticulate silibinin might circulate in blood vessels for prolonged periods, increasing the availability at tumor target sites through leaky blood vessels (EPR effect) and maintain effective therapeutic concentrations for a longer period of time (Sulfiikkarali and Krishnakumar, 2013). In addition, the death of cancer cells might lower transport resistance around blood vessels thereby facilitating the movement of nanoparticles deeper into the tumor tissue and its microenvironment (Iyer et al., 2006; Acharya and Sahoo, 2011).
In canonical discriminant analysis, the group centroids of the functions are well separated for the control and the experimental tissues. Using pairwise discriminant analysis, the overall diagnostic sensitivity of 83.33% to 100% and specificities of 82.35% to 100%, respectively, was achieved for classification of Control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues. The accuracies for discriminating of Control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues are in the range 82.08% to 100%. In the statistical results, a high level of accuracy is obtained in distinguishing between the control and the experimental tissue. Moreover, the positive predictive values of 100%, 88.23%, 89.47% and 83.33% and negative predictive values of 100%, 85.71%, 90.0% and 82.35%, respectively are using pairwise groups. The Matthew's correlation coefficient (MCC), which reflects the quality of classifications, is also calculated for Control vs DMBA, DMBA vs DMBA+SIL, DMBA vs DMBA+SILNPs and DMBA+SIL vs DMBA+SILNPs treated tissues as 1.0, 0.87, 0.96 and 0.72, respectively. The ROC curves in Fig. 6.7 illustrate good classification efficiency (AUC > 0.6) using PC-LDA. ROC results further confirm that PC-LDA is the most robust diagnostic methods. These results show the advantage of ROC-curve analysis in determining diagnostic accuracies in therapeutic response under SILNPS treatment. The basic motivation of this statistical study is to show statistical significance of the results. Gurushankar et al., (2014) have obtained a sensitivity of 100%, for the discriminantion of DMBA-induced tumor tissue from control hamster buccal mucosa and obtained sensitivities of 76.0% and 93.0% and specificities of 73.3% and 86.0% to differentiate between native hesperetin and its nanoparticulate treatment from tumor tissues using pairwise discriminant analysis method. In the present study, a
better classification efficacy was achieved with close agreement with previous studies.

6.8. Conclusion

In conclusion, the present study reveals that the autofluorescence spectroscopic characterization of DMBA-induced oral carcinogenesis, as well as the therapeutic prognosis of response to silibinin and its nanoparticulates treatment. Concurrently, progress has been made in the identification of biomarkers that might be used to assess risk of progression or responsiveness to therapeutics. In the current study, the emission spectra of tissues from the control and the experimental animals were studied under the excitation wavelength of 410 nm with emission ranging from 440-750 nm to explore the possibility of porphyrin as a potential tumor marker. The observed increase of endogeneous porphyrin emission peaks at 635 nm, 670 and 700 nm, might be due to increased cell proliferation, which has been observed in DMBA-induced tumor tissues. However, oral administration of SIL and SILNPs to DMBA treated tissues shows significantly decreased endogenous porphyrin. Moreover, this study indicates the potential efficacy of SILNPs that is more effective than SIL in controlled revival of porphyrin relative to that of the DMBA+ SIL treated tissues. The results suggest that porphyrin may be considered as one of the metabolic markers for the detection of oral cancer. In addition, the PC-LDA modeling on fluorescence spectroscopy provides good tissue classification between the control and the experimental groups. The results of the present study further suggest that endogenous porphyrin fluorescence could be considered as specific spectral biomarkers points to the utility for monitoring anti-proliferative effect of cancer.