Journal of Molecular Liquids 141 (2008) 145–148

Contents lists available at ScienceDirect

Journal of Molecular Liquids

journal homepage: www.elsevier.com/locate/molliq

Breast cancer diagnostics by Raman spectroscopy

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ARTICLE INFO

Article history:
Available online 3 April 2008

Keywords:
Breast cancer
Raman spectroscopy
PCA

ABSTRACT

This paper presents new results for the normal, malignant and benign tissues by Raman spectroscopy. To the best of our knowledge, this is one of the most statistically reliable research (321 spectra from 44 patients) on Raman spectroscopy-based diagnosis of breast cancers among the world's women population. The paper demonstrates that Raman spectroscopy is a powerful medical diagnostic tool with the key advantage in breast cancer research.

1. Introduction

The real world applications of Raman spectroscopy are virtually unlimited and carry great potential in all aspects of our lives. The main beneficent is telecommunication and medical diagnostics. In this contribution we want to demonstrate the power of Raman spectroscopy in medical diagnostics.

Among the different spectral techniques, Raman spectroscopy is usually the first choice for observing and investigating biological tissue [1–25]. The Raman spectroscopy is based on the inelastic light scattering and provides the information about vibrations of molecules. Thus, Raman spectroscopy provides important biochemical information, because each molecule has its own pattern of vibrations that can serve as a Raman biomarker. Moreover, these vibrations are generally structure-sensitive reflecting structural changes in a distinct environment and the sensitivity to molecular environment of the biological tissue can be employed as an optical marker of a normal healthy tissue and a malignant tissue. Additionally, Raman spectroscopy is very sensitive to the structure and phase transitions [26–28] which can be very useful in monitoring morphological features, for example, the morphological changes associated with nuclear enlargement, a qualitative indicator of cancer (the nucleus-to-cytoplasm ratio) used by pathologists.

It is known [29–31] that some substances are produced by the organism in response to the cancer's presence. They are called tumor markers. The ideal marker should be easily and reproducibly measured, should exhibit the correlation with the stage of the malignancy and respond to clinical treatment. No tumor marker now available has met this ideal. We believe that optical method including Raman spectroscopy will provide such a marker. Possibly it will help to find the hallmarks of cancer.

There are many reasons to expect that Raman spectroscopy will be a valuable diagnostic method. The main advantages of employing Raman spectroscopy are: immediate in vivo diagnosis, reduction in the number of biopsies, and combination of biochemical and structural diagnosis. Moreover, the Raman spectroscopy is a non-ionizing method, it exhibits chemical and structural specificity and has a potential to remove human interpretation. The effort to develop an optical fiber needle probe for medical Raman applications as well as implementation of Raman spectroscopy in real time for medical diagnosis has been undertaken [6,7].

2. Method and experimental

We have measured the ex vivo breast tissue samples removed during the operation. The ex vivo breast tissue has been divided by a doctor into two parts, one of them has been measured in our laboratory, the second one has undertaken the pathology examination. The fresh tissue samples have been measured immediately after delivering from the hospital. We avoid both the fixation in formalin and keeping in nitrogen, because the first process alters the tissue chemically, mainly via cross-links to collagen proteins, the second process modifies the structure resulting in autofluorescence alteration of the tissue.

Raman and fluorescence spectra have been recorded for the ex vivo samples of human breast tissue. We have recorded 321 spectra from 44 patients, including 125 spectra of normal tissue and 196 spectra of abnormal tissue. Raman spectra of the normal tissue, the malignant or benign tissue as well as the blood from the circulatory vessels for each

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doi:10.1016/j.molliq.2008.02.015

patient have been measured. The malignant tissue samples were represented by the following types of cancer: infiltrating ductal cancer, infiltrating lobular cancer, mucosinum cancer, intracystic papillary cancer. The benign lesion tissue samples were represented by fibroadenoma, benign dysplasia and ductal lobular hyperplasia.

Raman spectra of the breast tissue were measured with Ramanor U1000 (Jobin Yvon) and argon ion laser (Spectra Physics 2017-04S) operating at 514nm and the output power of 25mW, and 100mW, respectively. The laser spot is $d = 500\mu m$ in diameter. Light diffusion in the biological tissue results in a sample diameter of $d \approx 1mm$. The integration time is 0.5s at the spectral resolution of $8cm^{-1}$. The spectra have been recorded for the range $200–3600cm^{-1}$. The reproducibility of data for the breast tissue has been examined carefully to avoid photoisomerization or photodecomposition caused by irradiation with the laser beam. The power density of the laser beam was kept as low as possible in Raman measurements to avoid or minimize degradation of the sample. We have never seen photochemical instability at energies below 100mW, but for security reasons the output power of the laser was reduced to 25mW corresponding to 14mW directly at the sample.

The Raman spectra were analyzed also using the principal component analysis (PCA) and MATLAB least-squares fitting algorithm [32].

3. Results

In Fig. 1 we compare the Raman spectra for the normal and the malignant tissues (infiltrating ductal cancer) recorded for the same patient at identical experimental conditions. One can easily see the significant differences in the Raman spectra of the normal tissue and the malignant tissue. The normal tissue exhibits characteristic Raman bands: C–C

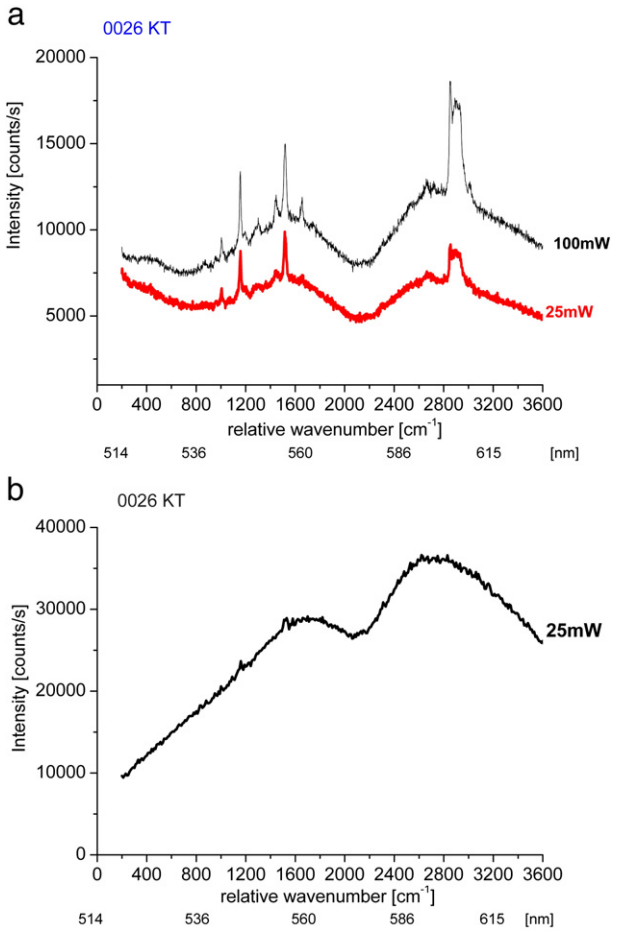

Fig. 1. Raman spectra of the normal (a) and the malignant (b) (infiltrating ductal cancer) tissues for the same patient.

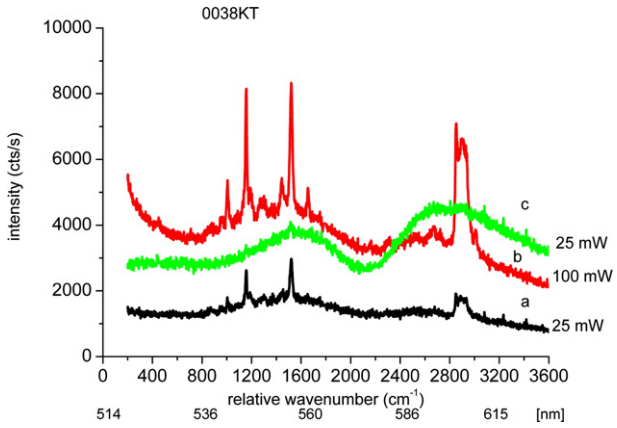

Fig. 2. Raman spectra of the normal (a, b) and the benign (c) tissues for the same patient.

($1150cm^{-1}$) and C=C ($1520cm^{-1}$) stretching vibration of carotenoids and at $2850–2940cm^{-1}$ for the C–H symmetric and asymmetric vibrations of lipids (fat) which are not visible in the malignant tumor tissue. Moreover, the comparison between Fig. 1a and b demonstrates spectacular changes in the fluorescence spectra. One can see that the fluorescence is much higher in the malignant tissue than in the normal tissue. It appears that changes in autofluorescence are at least as characteristic of the state of the tissue as the disappearance of the bands of carotenoids and lipids.

In most studied cases the normal tissue has lower autofluorescence than the malignant tissue, but for certain types of cancer they are comparable. It is worth emphasizing that even when the autofluorescence is comparable in the normal and the malignant tissues, the characteristic peaks can be observed only in the normal tissue.

Fig. 2 compares the Raman spectra for the normal and the benign lesions. We can see that both tissues have autofluorescence much lower than that of the malignant tissue (Fig. 1), but only the normal tissue exhibits the characteristic set of Raman peaks. This finding provokes a question if the lack of characteristic Raman peaks in the malignant tissue indicates that the carotenoids and lipids disappear to any reasons in the malignant tissue or that they are simply hidden in the high background autofluorescence of the tissue. From the diagnostic point this question is not quite essential. To find a marker it is enough to say—the peaks are visible in the normal tissue whereas they are not observed in the malignant one and make effort to find a qualitative way of estimation. However, answering this question is important for cancer science, because it may help to understand molecular mechanisms which drive the transformation of normal human cells into malignant derivatives.

Fig. 3 may help to find the answer to the above mentioned question. It represents the case where both types of tissues have

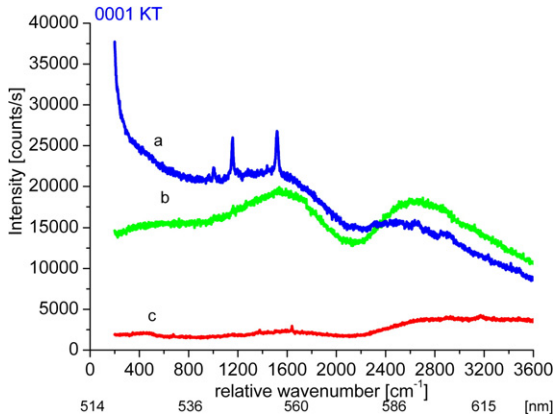

Fig. 3. Raman spectra of the normal (a) and the malignant (b) (multifocal infiltrating carcinoma) tissues and blood (c) for the same patient.

nearly identical and relatively high autofluorescence. One can see that inspite of lower autofluorescence of the malignant tissue with respect to the normal tissue at around 560nm, only the normal tissue shows the Raman peaks.

The results presented in Fig. 3 provide evidence that the lack of characteristic peaks in the malignant tissue simply demonstrates that there are less carotenoids and fat in the malignant tissue. Additionally they are partially hidden in the high background of the malignant tissue autofluorescence.

It is important to understand what the reason of the tissue autofluorescence is. The autofluorescence peak with maximum at 560nm may be partially related to the tissue blood with heamoglobin as a predominant fluorophore (β Q_1 band, 540), and (α Q_1 band, 560nm). To estimate the contribution of blood to the tissue autofluorescence we have measured the Raman spectra of the blood as well as the separated components such as the blood cells and the blood plasma from the circulatory blood vessels for each patient. Figs. 3 and 4 present the comparison between the Raman spectra of the fresh ex vivo normal tissue, the malignant tissue and the blood cells from the circulatory blood vessels of the same patient. One can see that the fluorescence from the blood components is much lower than the autofluorescence of the malignant tissue.

We have discussed [20–23] the origin of the tissue autofluorescence. The picture that emerges from the analysis provides some indication that the disappearance of the bands of carotenoids and lipids as well as the pronounced increase of autofluorescence of the malignant tissue may be related to free radical oxidation of pigments such as carotenoids and lipids. In the view of this suggestion we can go one step further and attract attention to the other phenomenon which is apparently beyond the scope of this study—the age-related progressive accumulation of pigments. The age-related progressive accumulation of pigments is a consistently recognized phenomenon in men and animals and is regarded as a hallmark of aging [31]. The pigments may occupy up to 40% of the cytoplasmic volume in post-mitotic cells of old animals. Although intensive studies including morphological, pathological and biochemical research of these enigmatic substances have been carried out for more than a century, the biochemistry and formation are still a matter of debate and controversy. The age-related pigments (APF- age-pigment like fluorophores) are classically known as "lipofucsin". They can be produced from a variety of biological materials including carotenoids, lipids, proteins, carbohydrates, ascorbic acid, possibly nucleic acids. A majority of them are precursors of fluorescent products. It has been shown recently [33] that irradiation with 10kGy dose resulted in a general increase of quinone radical content in all of the investigated samples and significant decrease of carotenoids, as revealed by the electron paramagnetic resonance spectroscopy. The disappearance of the lipid bands in the tumor tissue (the band at 2850–2940cm^{-1}) may be related to lipid peroxidation by reactive oxygen (superoxide anion radical O^{-2}) or their iron complexes that leads to lipid hydroperoxides and cyclic peroxides, which are further decomposed, or degradated into various saturated and unsaturated carbonyl compounds, mainly, aldehydes. Unsaturated aldehydes are often cytoxic or genotoxic [31].

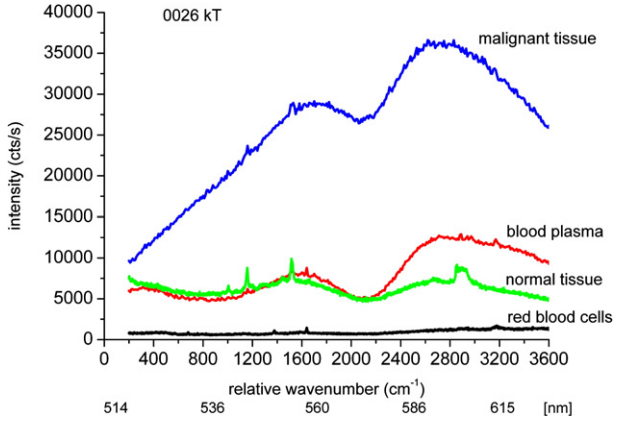

Fig. 4. Comparison between the Raman spectra of the fresh ex vivo normal tissue, blood malignant tissue, blood cells, blood plasma of the same patient at 25 mW.

To summarize this part of the discussion, our results combined with the above mentioned results from literature strongly suggest that the characteristic Raman bands of carotenoids and lipids observed in the normal breast tissue samples disappear followed by increasing autofluorescence of their radical products in the malignant breast tissue. The Raman and autofluorescence breast tissue results presented in the paper provide some contribution to the hypothesis that the tumorigenesis in humans is a multistep process like aging with the accumulation of age-related pigments fluorophores. The free radical theory of aging, proposed by Harman [34] in 1956 is now one of the leading theories explaining the biochemical basis of age-related pigments formation. Our Raman results presented here provide some hints that the same theory may explain many of the processes that drive the progressive transformation of normal human cells into highly malignant derivatives.

To analyze the data we have employed the statistical methods. The most frequently used method in this respect is principal component analysis, with each Raman spectrum represented as a vector of intensity values corresponding to each wavelength [35].

The PCA score plot (model—SNV, mean center, first derivative) (not shown here) indicates that the studied samples belong to one of the three characteristic groups. The samples are separated along the first principal component PC1 (66.44%). For the negatives scores on PC1 there are almost exclusively the malignant and benign tissues. For the positive scores on PC1 there are almost exclusively the normal tissues. The meaning of the PC1 component has been found from the loading plot. The loading plot (not shown here) demonstrates the most pronounced changes around the characteristic Raman peaks: C–C and C=C stretching bands of carotenoids and C–H symmetric and asymmetric bands of lipids (fats) we have already presented in Figs. 1–4.

Comparing the histopathological description provided by the hospital we have evaluated the sensitivity and the specificity of the studied samples taking into account the first principal component PC1—the characteristic Raman peaks. The sensitivity has been calculated as the proportion of the samples with a positive test result (lack of the Raman peaks) to the total number of the samples that have the target disorder (malignant tissue or benign tissue). We have found the sensitivity of 72% for the malignant tissue and of 62% for the benign tissue. The specificity has been calculated as the proportion of the samples with a negative test result (appearance of the Raman characteristic peaks) to the total number of the samples that does not have the target disorder (normal tissue). We have found the specificity for the normal tissue of 83%.

4. Conclusions

The Raman results for the normal, malignant and benign breast tissues presented here can be summarized as the follows:

1. At the laser excitation of 514nm the normal breast tissue has a characteristic set of Raman bands: C–C and C=C stretching bands of carotenoids and C–H symmetric and asymmetric bands of lipids (fats) which are not visible in the malignant tissue and in the benign tumor tissue.
2. The normal tissue has lower autofluorescence than the malignant tissue, the benign tumor tissue has autofluorescence similar to that of the normal tissue.
3. The Raman and autofluorescence methods provide good sensitivity and specificity for discrimination between the normal and abnormal (malignant or benign) tissues. Combining the sensitivity and specificity with respect to the PCA1 and PC2 components may help to

discriminate between the malignant and benign tissues. We have found the sensitivity of 72% for the malignant tissue and of 62% for the benign tissue and the specificity for the normal tissue of 83% with respect to the first PCA1 component—the characteristic Raman peaks.

4. The results presented here support earlier investigations from the other laboratories [6,18] that the Raman spectra for the normal tissue are dominated by lipids, whereas they reveal significant discrepancy related to the role of carotenoids as a possible Raman markers. According to the micro-spectroscopic model of human breast tissue proposed recently by Shafer-Pelitier et. al.[8] carotenoids show no or minor specificity and cannot be treated as a marker for discrimination of normal, benign and malignant breast tissues. In contrast to the results presented by Shafer-Pelitier et. al.[8] our results demonstrate that Raman bands of carotenoids combined with the autofluorescence of the tissue exhibit key features to diagnose malignancy.
5. The free radical theory of aging, proposed by Harman in 1956 is now one of the leading theories explaining the biochemical basis of age-related pigments formation. Our Raman results presented here provide some hints that the same theory may explain many of the processes that drive the progressive transformation of normal human cells into highly malignant derivatives.
6. The next step in employing Raman spectroscopy in breast tissue diagnostics is related to the progress in obtaining a good quality Raman signal with the optical fibers coupled with a biopsy needle and incorporated into the Raman spectrometer for breast tissue measurements in vivo. The results presented in the paper provide the Raman markers – the characteristic Raman peaks and the autofluorescence of the sample – that can be useful for the preclinical and clinical in vivo breast tissue studies with Raman spectrometer coupled to the probe via the optical fiber designed for medical applications.

Acknowledgements

We gratefully acknowledge the support of this work through the grant Nr3 T11E 04729 in 2005–2008 and the Dz. St 2007. The support from Marie Curie Chair MEXC-CT-2006-042630 is also acknowledged.

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