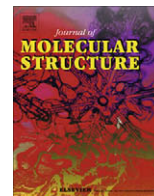




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The hallmarks of breast cancer by Raman spectroscopy

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ABSTRACT

This paper presents new biological results on ex vivo breast tissue based on Raman spectroscopy and demonstrates its power as diagnostic tool with the key advantage in breast cancer research. The results presented here demonstrate the ability of Raman spectroscopy to accurately characterize cancer tissue and distinguish between normal, malignant and benign types. The goal of the paper is to develop the diagnostic ability of Raman spectroscopy in order to find an optical marker of cancer in the breast tissue. Applications of Raman spectroscopy in breast cancer research are in the early stages of development in the world. To the best of our knowledge, this paper is one of the most statistically reliable reports (1100 spectra, 99 patients) on Raman spectroscopy-based diagnosis of breast cancers among the world women population.

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1. Introduction

It has been known for many years that Raman spectroscopy is a valuable research tool, but no one could expect that it will become so fashionable and useful in real world applications. The possibilities are virtually unlimited and carry great potential for almost all aspects of our lives. The main beneficent is telecommunication – Raman fiber amplifiers, Raman fiber lasers, Raman silicon lasers – are only a few examples of applications or potential applications. Nonlinear effects like the stimulated Raman scattering, the nonlinear group velocity dispersion GVD, the four-wave mixing have a key significance in obtaining good work parameters for transmission in optical fibers, such as attenuation, bit error rate (BER), inter-channel crosstalk or capacity. In this contribution we want to demonstrate that Raman spectroscopy has additional powerful tool in medical diagnostics.

Raman spectroscopy is based on the inelastic light scattering and provides information about vibrations of molecules. Raman spectroscopy has been used for chemical analysis for many years, but only recently has the interest exploded to study biological tissue [1–25]. Raman spectroscopy provides important biochemical information, because each molecule has its own pattern of vibrations that can serve as a Raman biomarker. Moreover, these vibra-

tions are generally structure-sensitive reflecting structural changes in distinct environment. We have shown recently that the 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. Although Raman spectroscopy carries great potential for medical diagnostics only a few groups in the world have investigated the feasibility of diagnosing breast cancers. It is obvious that for clinical applications it is necessary to verify and validate the methodology by different groups. It is even more important when we remember that the characteristic set of Raman bands that are visible in the tissue depends on the laser excitation wavelength – different set of bands are enhanced for the excitation with the visible light, different with the near infrared radiation. So that the different spectral regions may provide valuable complementary information.

It is known [29,30] that some substances are produced by the organism in response to the cancer's presence. They are occurring in blood or tissue and 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. The ideal marker would be a “blood test” for cancer in which a positive result would occur only in patients with malignancy. No tumor marker now available has met this ideal. We will show that optical method including Raman spectroscopy may provide such a marker. Possibly it will help to find the hallmarks of cancer.

Many experts in the cancer research anticipate a dramatically different type of cancer science that we have experienced over the past 25 years [30,32]. The most fundamental change will be

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conceptual – simplification, where the complexity of the disease at the clinical level, will become understandable in terms of a small number of basic mechanisms. It is obvious that much of this change will occur at the technical level. We hope that the optical spectroscopy, mainly Raman spectroscopy, will be one of the most important factors on the road to simplification. There are many reasons to expect that Raman spectroscopy will be a valuable diagnostic method. The main advantages of potential employing Raman spectroscopy in medical diagnostics are: immediate in vivo diagnosis, reduction the number of biopsies, combination of biochemical and structural diagnosis. The Raman spectroscopy is non-ionizing method, exhibits chemical and structural specificity and has a potential to remove human interpretation.

The characteristic set of Raman bands depends on the wavelength of the laser beam. Different set of bands are enhanced for the excitation with the visible light, different for the near infrared radiation. So, we have to be very careful when we formulate the quantitative models for the components that are present in tissue, because different components have distinct Raman cross-sections that depend on the wavelength of laser excitation. Thus, it is difficult to estimate the weight of the individual components of the mixture at additional assumption of linear contribution. In spite of these limitations the optical methods provide very significant information – if you compare the Raman spectra at the given excitation wavelength for the normal and the abnormal tissue – you are able to see very essential differences between them. This finding can serve as a biochemical and structural marker of the pathology.

In recent years optical spectroscopy, including Raman spectroscopy, has become the subject of great interest as a potential in vivo diagnostic tool to provide information about the chemical and morphologic structure of tissue [1–25]. 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]. The most typically used excitation was near infrared with an Nd:YAG laser at 1064 nm, diode lasers (785 nm) or Ti:sapphire laser (800 nm) covering the vibrational spectral region from 600 to 1800 cm^{-1} , with the bands of 1230–1295, 1440–1460 and 1650–1680 cm^{-1} , assigned to the vibrations of the carbohydrate-amide III, proteins and lipids, and carbohydrate-amide I, respectively. We prove that the excitation in the visible region employed in this paper has many advantages over the near infrared region. The Raman resonance enhanced conditions for biological fluorophores allow employing excitation powers as low as 14 mW and short integration times (0.5 s) in comparison with the near IR excitation (typically 100–125 mW and 1 s). These parameters are crucial if we want to avoid haemolysis and photodissociation of the tissue. Moreover, both the Raman spectra and the autofluorescence of the biological sample can be measured at the same time in much broader spectral range of 200–8000 cm^{-1} and the region of 25–200 cm^{-1} can be used to control the morphology and structure through the phonon peaks.

The main goals of bio-Raman spectroscopy at this stage of development are threefold. Firstly, to develop the diagnostic ability of Raman spectroscopy in order to find an optical marker of cancer in the breast tissue. Secondly, to implement the Raman markers in a clinical environment to produce accurate and rapid real time medical diagnoses. Thirdly, to formulate some hypothesis based on Raman spectroscopy results on the molecular mechanisms which drive the transformation of normal human cells into highly malignant derivatives.

2. Method and experimental

Upon removal during the operation, the ex vivo breast tissue is divided by a doctor into two parts, one part is delivered to our lab-

oratory, the second one is provided to the pathology examination. The ex vivo samples neither have been frozen in liquid nitrogen for storage nor fixed in formalin. The fresh tissue samples have been measured immediately after delivering from the hospital. We avoid both the fixation and keeping in nitrogen, because the first process chemically alters the tissue, mainly via cross-links to collagen proteins, the second process modifies the structure resulting in autofluorescence alteration of the tissue. The samples for pathology measurements undergo the standard procedure of thawing at room temperature and keeping moist with different solvents, cutting through the marked locations into 5- μm -thick sections, and staining with eosin.

Raman and fluorescence spectra have been recorded for the ex vivo samples of human breast tissue from 99 patients. For each patients we have measured the normal and abnormal tissue at least at a few spots. (The total numbers of Raman spectra is 1100.) For each patient we have measured Raman spectra for the normal tissue and for the abnormal (malignant or benign lesion) tissue as well as the blood from the circulatory vessels of each patient. In a few cases the complete set of normal, abnormal tissues and blood for a given patient could not be provided from the hospital. The statistics of the patients monitored so far in Laboratory of Laser Molecular Spectroscopy is given below:

- Noninvasive ductal cancer from 3 patients.
- Noninvasive lobular cancer from 1 patient.
- Noninvasive intracystic papillary cancer from 1 patient.
- Intraductal cancer from 2 patients.
- Infiltrating ductal carcinoma (IDC) from 37 patients.
- Mucinous cancer from 1 patient.
- Papillary cancer from 1 patient.
- Infiltrating lobular carcinoma (ILC) from 7 patients, IDC + ILC from 2 patients.
- Infiltrating multifocal carcinoma from 3 patients.
- Fibroadenomas from 22 patients.
- Benign dysplasia from 7 patients.
- Benign dysplasia + fibroadenoma from 1 patient.
- Ductal-lobular hyperplasia from 1 patient.
- Intraductal hyperplasia from 1 patient.
- Cystic mastopathy from 7 patients.
- Cystic mastopathy + fibroadenoma from 1 patient.
- Proliferating breast disease from 1 patient.

This report does not divide the patients into the groups related to age, the type of breast carcinoma and employed medicines. The classification will be the next step of research when the number of the samples for each group is statistically sufficient. In this report we present the data for all the monitored patients.

Raman spectra of the breast tissue were measured with Ramano U1000 (Jobin Yvon) and Spectra Physics 2017-04S argon ion laser operating at 514 nm and output power of 25 mW, and 100 mW, respectively. The laser spot is $d = 500 \mu\text{m}$ in diameter. Light diffusion in the biological tissue results in a sampled diameter of $d \approx 1 \text{ mm}$. As the laser probes an area of typically $\sim 0.25 \text{ mm}^2$, it interacts with a large number of molecules (typically $\sim 10^{11}$). Molecular scale information is obtained through the molecular vibrations, and the synchronized response of the individual molecules in the tissue ensemble following the excitation process. The integration time is 0.5 s at the spectral resolution of 8 cm^{-1} . The experimental condition must be identical for normal and abnormal time to compare the Raman spectra for both cases. 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 pho-

tochemical instability at energies below 100 mW, but for security reasons the output power of the laser was reduced to 25 mW (directly at the sample 14 mW). The spectra have been recorded for the range 200–3600 and 200–8000 cm^{-1} at the laser output 25 and 100 mW.

Additionally, we have measured the resonance Raman spectra of carotenoids in biological matrices of carrot to compare them with the bands of carotenoids observed in the breast tissue. The sample of carrot has been measured without and with gamma irradiation from a cobalt chamber as a function of the irradiation dose. The spectra of the carotenoids embedded in the biological matrix of carrot are very stable and resistant to decomposition or isomerization caused by laser irradiation up to 500 mW. The spectra of the carotenoids embedded in the biological matrix of carrot are also stable and resistant to decomposition caused by gamma irradiation up to 4 kGy.

3. Results and discussion

We have studied normal, malignant and benign lesions in human breast ex vivo tissue samples obtained from the breast surgeries and studied with Raman spectrometer.

Fig. 1 presents the Raman spectra for the normal and the malignant tissue (infiltrating ductal cancer) of the same patient. One can easily see the significant differences in the Raman spectra of the normal tissue and the malignant tissue. The normal tissue has the characteristic set of Raman bands: C–C (1150 cm^{-1}) and C=C (1520 cm^{-1}) stretching vibration of carotenoids and at 2850 cm^{-1}

2940 cm^{-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 Raman bands of carotenoids and lipids.

In most studied cases the normal tissue has lower autofluorescence than the malignant tissue, although for certain types of cancer they are comparably low. Fig. 2 presents the comparison between the Raman spectra for the normal and the malignant tissue of the same patient for another type of cancer – infiltrating lobular cancer. It is worth emphasizing that although the fluorescence is low and nearly identical in both types of tissue, the characteristic Raman bands of carotenoids and lipids can be seen only in the normal tissue.

Fig. 3 compares the Raman spectra for the normal tissue and the benign lesion tissue of the same patient. We can see that although both tissues have nearly identical low autofluorescence, only the normal tissue shows the Raman peaks.

These findings provoke a question:

1. Does the lack of characteristic Raman peaks in the malignant tissue indicate that there are less carotenoids and fat in the malignant tissue or that their peaks are simply hidden in the high background autofluorescence of the tissue?

From the diagnostic point of view this question is not quite essential. To find a marker it is enough to say – the peaks are vis-

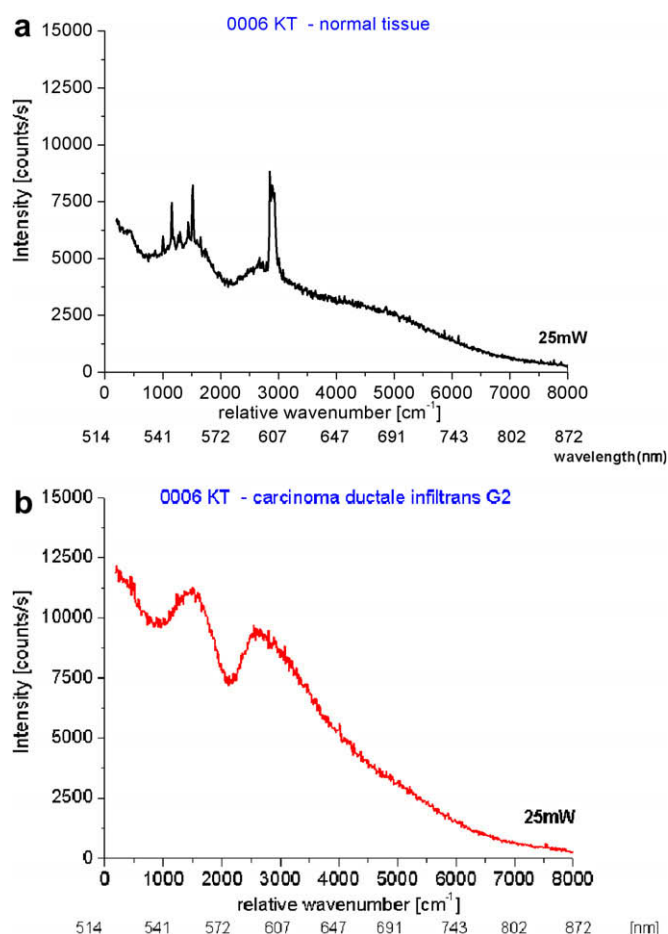


Fig. 1. Raman spectra of the normal tissue (a), and the malignant (infiltrating ductal cancer) tissue (b) of the same patient.

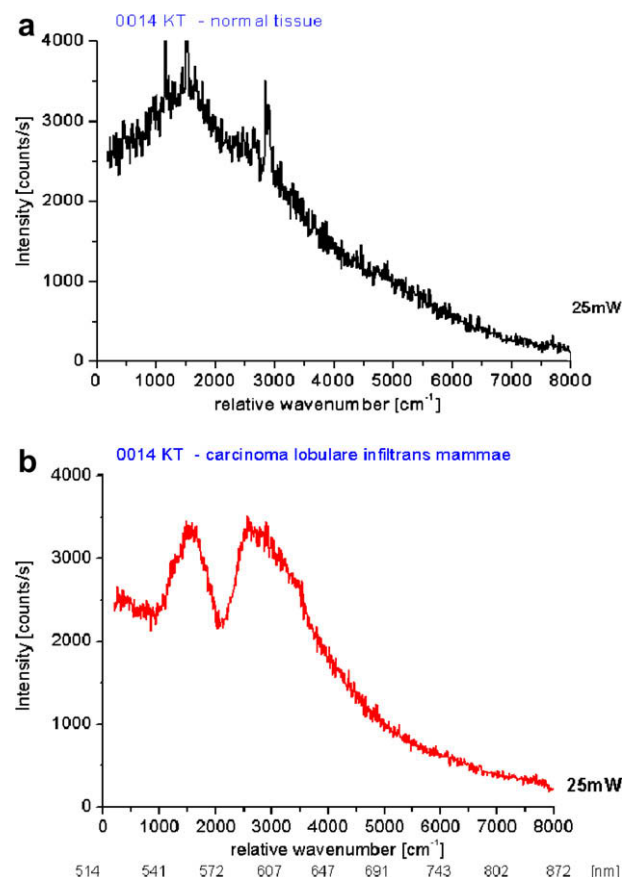


Fig. 2. Raman spectra of the normal tissue (a), and the malignant tissue (infiltrating lobular cancer) (b) of the same patient.

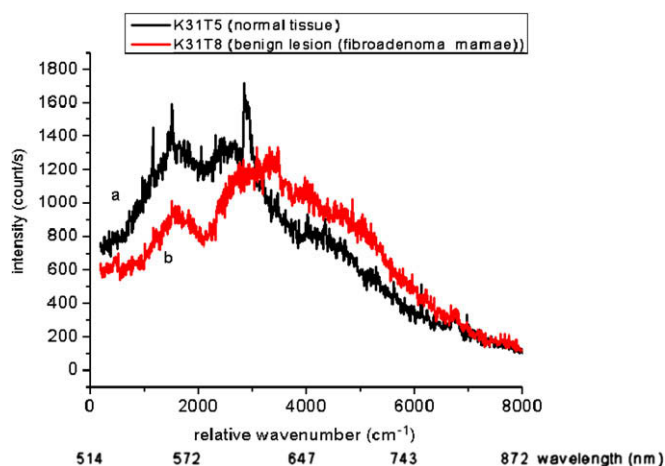


Fig. 3. Comparison between the Raman spectra of normal (a) and benign lesion (fibroadenoma mammae) (b) of the same patient.

ible 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. 4 may help to find the answer to the above mentioned question. The most typical situation found in our research is presented in Fig. 1 with the autofluorescence of the malignant tissue much higher than that of the normal one. However, we have recorded also the cases that are presented in Fig. 4. It represents the situation where both types of tissues of the same patient – normal and malignant – have high and relatively similar autofluorescence. In fact, the autofluorescence of the malignant tissue with respect to the normal tissue at around 560 nm is even lower. However, one can see that in spite of the lower autofluorescence of the malignant tissue with respect to the normal tissue at around 560 nm, again only the normal tissue shows the characteristic Raman peaks of carotenoids and lipids.

The results presented in Fig. 4 simply suggest that the lack of characteristic peaks in the malignant tissue is related to disappearance of the carotenoids and lipids bands in the malignant tissue. Another method to remove fluorescence is employing low temperature Raman spectroscopy [31]. Which effectively quench autofluorescence of the tissue. Raman measurements.

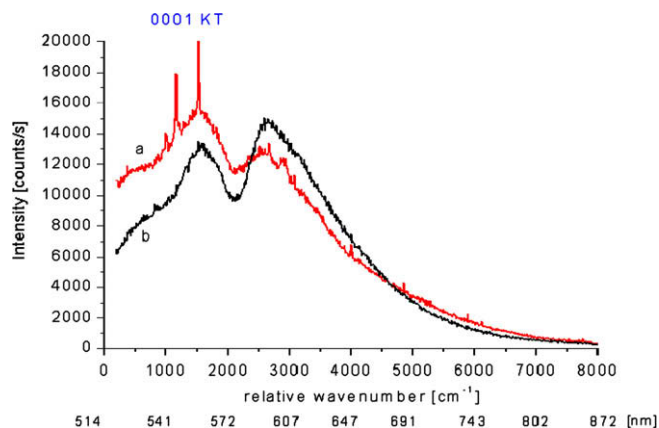


Fig. 4. Raman spectra of the normal tissue (a) and the malignant (multifocal infiltrating carcinoma) tissue (b) of the same patient.

Fig. 5 shows Raman spectra for the normal and abnormal (fibroadenoma mammae) tissue at 293–285 K (Fig. 5 inserts) and at 250–77 K. One can see that at low temperatures the autofluorescence of both tissues is reduced significantly. However, the characteristic set of peaks is clearly visible in the normal tissue like for the ambient temperature of 293 K. It indicates, that the peaks of carotenoids and lipids simply disappear in the abnormal tissue.

In the view of the presented results two questions arise. First, what mechanisms lead to the disappearance of the carotenoids and lipids bands in the malignant tissue. Second, what the origin of the tissue autofluorescence is and why the autofluorescence of the abnormal tissue is usually higher than that of the normal tissue for the same patient. The mechanisms seems to be related and answering the question about the autofluorescence of the tissue may provide important hints which way we should follow in our further investigations.

First, it is important to understand what the origin of the tissue autofluorescence is. The autofluorescence peak with maximum at 560 nm may be partially related the tissue blood with hemoglobin as a predominant fluorophore (αQ_1 band, 540), and (βQ_1 band, 560 nm). To estimate the contribution of blood to the tissue autofluorescence we have measured the Raman spectra of the whole blood (as well as the separated components such as the blood cells and the blood plasma) from the circulatory blood vessels for each patient. Fig. 6 presents the comparison between the Raman spectra of the fresh ex vivo normal tissue, the malignant tissue and the whole blood from the circulatory blood vessels of the same patient.

One can see that the fluorescence from the blood is much lower than the autofluorescence of the malignant tissue and lower than the autofluorescence of the normal tissue. Moreover, Fig. 7 shows that the blood has its own pattern of characteristic peaks dominated by the bands of proteins (1440–1460 and 1650–1680 cm^{-1}) and hemoglobin (1370 cm^{-1}) in contrast to the normal breast tissue that is dominated by the bands of carotenoids (C–C (1150 cm^{-1}) and C=C (1520 cm^{-1})) stretching vibration as well as the bands of lipids (at 2850–2940 cm^{-1} for the C–H symmetric and asymmetric vibrations of the hydrocarbon chain). It indicates that the whole blood in the tissue does not dominate the autofluorescence of the samples and cannot be responsible for the differences in autofluorescence of the normal and abnormal tissue.

To learn more about the origin of the tissue autofluorescence (other than from the whole blood) we have concentrated on the fluorescence of the main components of the tissue. As it is well known the main components of the tissue: collagen, epithelial cell cytoplasm, cell nucleus, calcium oxalate dehydrate, calcium hydroxyapatite, cholesterol do not have emission in the visible region, except of flavins and carotenoids. The other components of the biological tissue such as tryptophan, pyridoxine, elastin, collagen, NADH exhibit emission at shorter wavelengths. However, majority of the biological tissue components can be precursors of fluorescent products. As our results presented so far provide a strong indication that the Raman bands of carotenoids show a specificity to distinguish between the normal and abnormal tissue we have concentrated on the fluorescence from carotenoids. We have measured the resonance Raman spectra of carotenoids in biological matrices of carrot to compare them with the bands of carotenoids observed in the breast tissue. As it is well known [33] the unique selectivity of the resonance Raman spectroscopy provides a powerful method of probing the structure of carotenoids when they are bound to sub-cellular structures. It is possible because the other pigments as well as molecules such as peptides, lipids and carbohydrates have no absorption in the 450–550 nm range and give no direct contribution the resonance Raman spectrum.

Fig. 8 shows the Raman and fluorescence spectra for carotenoids in carrot biological matrix that we have recorded in our laboratory.

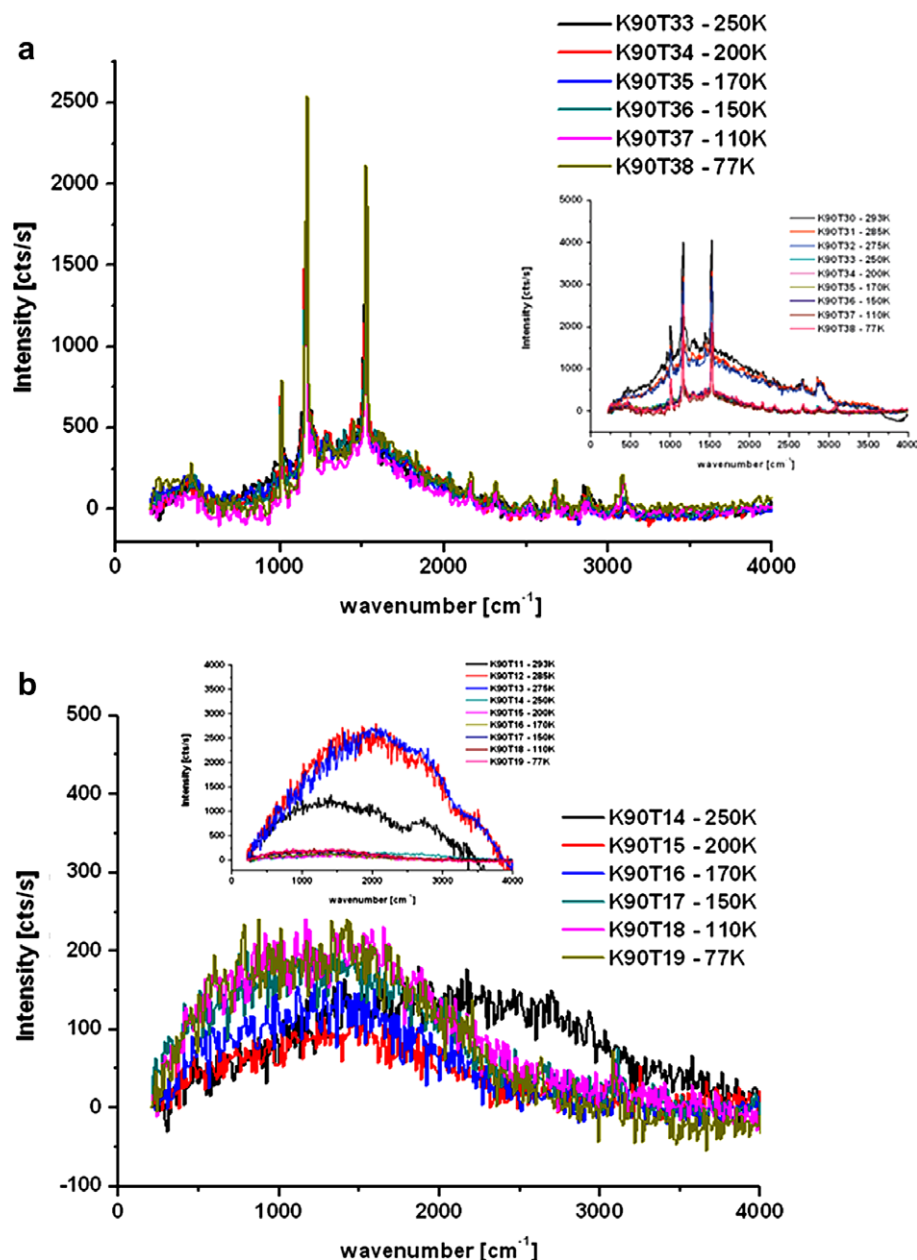


Fig. 5. Low temperature Raman spectra of the normal tissue (a) as a function of temperature (the inset shows spectra in the temperature range of 293–250 K) and (b) the fibroadenoma mammary tissue as a function of temperature (the inset shows spectra in the temperature range of 293–250 K).

One can see that the sample without irradiation shows much weaker fluorescence than the sample irradiated with gamma irradiation from a cobalt chamber. The maximum of the fluorescence peak at around 560–570 nm for the carotenoids in the biological matrix of carrot is exactly at the same wavelength as that for the malignant breast tissue. Moreover, the intensity of fluorescence of the carotenoids is similar to that of the normal breast tissue (Fig. 1a). When the carrot sample is irradiated with gamma irradiation, the fluorescence increases significantly and its magnitude at 560 nm becomes similar to that of the malignant breast tissue (Fig. 1b). The most obvious explanation of the fluorescence increase in the carrot sample irradiated with gamma irradiation is generation of various kinds of radicals that contribute to such a spectacular increase in emission at around 560 nm. It is well known that the highly unsaturated chain confers the instability of β -carotene that leads to reactions such as isomerisation or radical generation. It has been shown recently [34] that irradiation

with 10 kGy 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 picture that emerges from the analysis of the results presented so far provides strong indication that the disappearance of the Raman peaks of carotenoids and lipids as well as and the autofluorescence increase in the malignant tissue may be related to free radical oxidation of variety of biological material including pigments such as carotenoids and lipid products. In the view of the suggestion that the free radical oxidation of pigments is responsible for the autofluorescence of the tissue we can go one step further and attract attention to the other phenomenon which is apparently beyond the scope of this study, namely 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

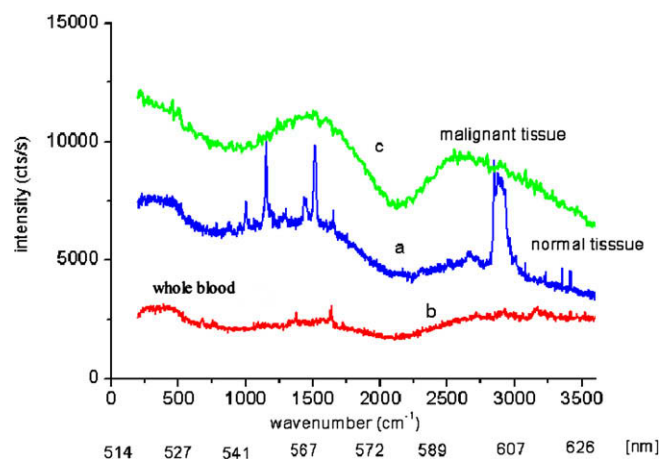


Fig. 6. Comparison between the Raman spectra of the fresh ex vivo normal (a), blood cells (b), and malignant tissue (c) of the same patient at 25 mW.

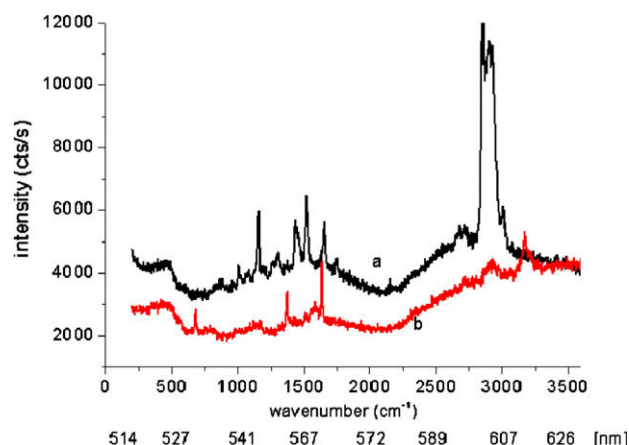


Fig. 7. Comparison between the Raman spectra of the fresh ex vivo normal (a), and blood cells (b) of the same patient at 100 mW.

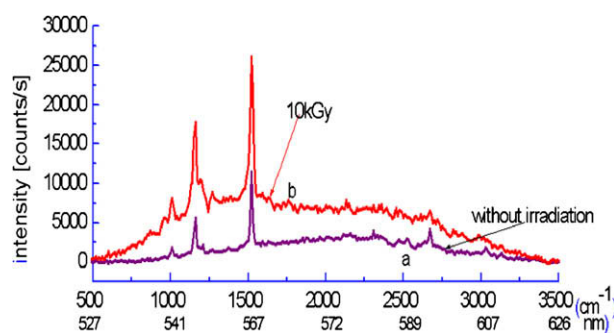


Fig. 8. Resonance Raman spectrum of carrot, (a) without gamma irradiation, (b) gamma irradiated with 10 kGy.

[35]. 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 many years, the biochemistry and formation are still a matter of debate and controversy. The age-related pigments are classically known as “lipofuscin”. 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. However, it has been shown that only lipids and carotenoids are precursors for the fluorescent products that have emission around 560 nm [35] which cover the region of the malignant breast tissue autofluorescence. The disappearance of the lipid bands in the tumor tissue (the band at 2850–2940 cm^{-1}) may be related to lipid peroxidation by reactive oxygen (superoxide anion radical $\text{O}_2^{\cdot-}$) or their iron complexes that lead to lipid hydroperoxides and cyclic peroxides, which are further decomposed, or degraded into various saturated and unsaturated carbonyl compounds, mainly, aldehydes. Unsaturated aldehydes are often cytotoxic or genotoxic [35].

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. This mechanism seems to be particularly effective in the malignant breast tissue. The Raman and autofluorescence results for the breast tissue presented in the paper provide some contribution to the hypothesis that the tumorigenesis in humans is a multi-step process related to the free radical oxidation with accumulation of age-related pigments fluorophores. The free radical theory of aging, proposed by Harman [36] is now one of the leading theories explaining the biochemical basis of age-related pigments formation. Our Raman results presented here provide strong support that the same theory may explain many of the processes that drive the progressive transformation of normal human cells into highly malignant derivatives.

4. Analysis of the Raman spectra for the breast tissue samples by the PCA statistical methods

When we have a large amount of samples (1100 spectra from 99 patients) and each of them contains a lot of information in each spectrum we need any method that will help to extract the most important features or components. The most frequently used method in this respect is the principal component analysis (PCA), with each Raman spectrum represented as a vector of intensity values corresponding to each wavelength. The Raman spectra were analyzed using the PCA method and MATHLAB least-squares fitting algorithm using PLS Toolbox Version 4.0 for use with MATLAB [37]. Fig. 9 shows the PCA score plot (model – SNV, mean center, first derivative) for all the recorded Raman spectra and all the samples. One can see that the samples belong to one of three characteristic groups. The samples in the left and the right circles are separated along PC1. In the left circles there are almost exclusively the malignant and benign breast tissues. In the right circle there are almost exclusively the normal tissues.

Generally, the PCA analysis does not provide the answer what the physical meaning of the PC1 component is. However, in this case it is easy to recognize it looking at the loading plot in Fig. 10. This is the loading plot as a function of the wavenumber (the model with the SNV, mean center, first derivative procedures has been applied). We can see that the loading plot shows 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.

Comparing the Raman spectra with 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 from Fig. 10.

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 according to the histopathological description (malignant tissue or

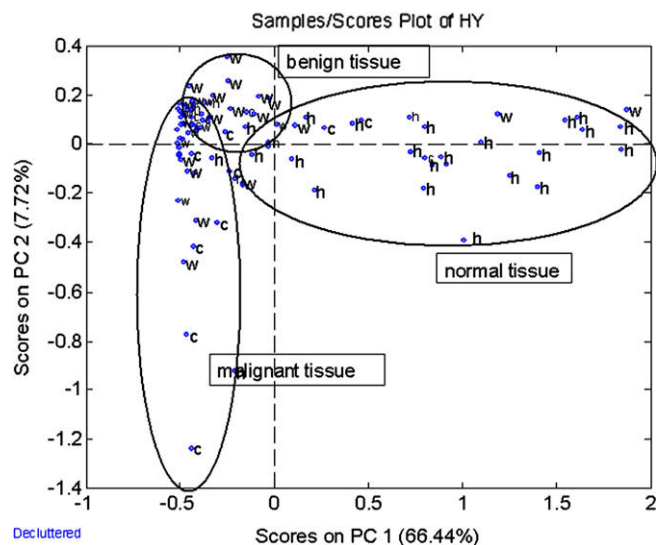


Fig. 9. PCA score plot for 44 patients (model- mean center, SNV, 1-st derivative) for the Raman spectra. Samples description : h- normal tissue, c- malignant tissue, wbenign tissue.

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 do not have the target disorder according to the histopathological description (normal tissue). We have found the specificity for the normal tissue of 83%.

5. Conclusions

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

1. At the laser excitation of 514 nm 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. Statistically, the normal tissue has autofluorescence lower than the malignant tissue.

3. The benign tumor tissue has autofluorescence similar to that of the normal tissue.
4. The results clearly illustrate good discrimination of the normal and the pathological breast tissue as well as the ability of Raman spectroscopy to accurately diagnose the breast cancer. Moreover, the results demonstrate how the diagnostic scheme can be adjusted to obtain the desired degree of sensitivity and specificity. We have obtained the sensitivity of 72% for the malignant tissue and of 62% for the benign tissue as well as the specificity for the normal tissue of 83% with respect to the first PC1 component – the characteristic Raman peaks. Thus, the Raman spectroscopy provide very good sensitivity and specificity for discrimination between the normal and abnormal (malignant or benign) tissues.
5. We have found the sensitivity of 55% for the malignant tissue and of 32% for the benign tissue with respect to the second principal component PC2 – the autofluorescence of the sample. It indicates that 68% of the benign tissue has the autofluorescence comparable to the normal tissue in contrast to the malignant tissues with only 45% of the samples.
6. Combining the sensitivity and specificity related to the PCA1 and PCA2 components may help to discriminate between the malignant and benign tissue.
7. 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 possible Raman markers. According to the micro-spectroscopic model of human breast tissue proposed recently by Shafer-Peltier et al. [8] carotenoids show no or minor specificity and cannot be treated as a marker for discrimination of normal, benign and malignant breast tissue. In contrast to the results presented by Shafer-Peltier et. al. [8] our results demonstrate that Raman bands of carotenoids combined with the bands of lipids and the autofluorescence of the tissue exhibit key features to diagnose malignancy.
8. The results presented in the paper correspond very well to the recent screening cancer studies for 1112 women collected between 1981 and 2006 [38]. According to their report cancer turns up five times more often in women with extremely dense breasts than in those with the fatty breast tissue. Breast density comes from the presence of more connective, duct-lining and milk-gland tissue other than fat.

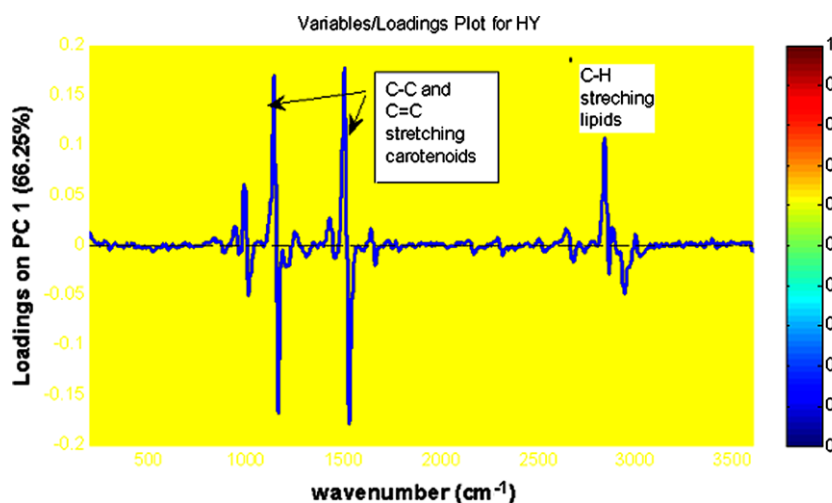


Fig. 10. PCA loading plot for 44 patients (model- mean center, SNV, 1-st derivative) for the Raman spectra.

9. The Raman and autofluorescence breast tissue results presented in the paper provide some contribution to the hypothesis that the tumorigenesis in humans is a multi-step process like aging with accumulation of age-related pigment fluorophores. The free radical theory of aging, proposed by Harman [36] 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.
10. We believe that in a very near future a good quality Raman signal will be obtained with the optical fibers coupled with a biopsy needle and incorporated into 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.

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