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MICRO-RAMAN SPECTROSCOPY IN THE VISIBLE RANGE: A TOOL FOR RAPID INVESTIGATION OF MAMMARY TUMOURS

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Abstract. A surface enhanced Raman effect in the visible range was obtained on areas of surgery instruments either raw or coated with silver thin films. Spectra were collected on *ex vivo* smear samples from animal patients for rapid assessment of tumour margins. An experimental "standard" healthy fat spectrum is proposed.

Key words: micro-Raman spectroscopy, surface enhanced Raman scattering, silver thin films, pulsed laser deposition, spin coating, *ex vivo* investigations, clean surgical margins, veterinary surgery.

1. INTRODUCTION

During the last decades the frequency of tumour diseases in both animals and humans registered a continuous increase. A tremendous intensification of research activity in the field has evolved to identify the aetiology factors and seek for new diagnostic procedures and therapies aimed at reducing mortality and increasing chances to healing.

One very common method to counteract extensive development of cancer tumours is surgery, by means of which malignant tissues are supposedly entirely removed from the body to prevent metastases.

Mammary tumours are common in dogs and cats. About 42% of all tumours in the female dog and 85% in cats affect the mammary gland. Metastases [1–3] occur mainly through the lymphatic system like in human mammary neoplasm [4].

However, treatment options are limited in comparison with human breast cancer. There are several surgical alternatives for mammary tumour removal: lumpectomy, mammectomy, regional mastectomy, unilateral mastectomy, bilateral mastectomy, and radical mastectomy. The appropriate decision is made for each case accounting for the species on one side and the number, size, and location of the tumours on the other side.

It is to underline that there are on the average 5 pairs of mammary glands in female dogs and 4 pairs in female cats positioned on two symmetrical chains extending from axillary to inguinal zones, involving on the average 5 pairs in female dog and 4 pairs in female cat.

A detailed study on tumour recurrence in female dogs that suffered regional mastectomy [5] assumed that in the middle of the mammary chain there is overlap of lymph drainage, from the cranial abdominal mammary gland to the axillary and superficial inguinal lymph nodes simultaneously or only to the axillary lymph node [6, 7] and observed that new tumours developed at a separate site from the initial tumour location in the remaining ipsilateral mammary tissue. The authors' conclusion was that removal of the entire chain of mammary glands would have prevented the need for a second surgical intervention in those female dogs.

The influence of surgical procedure on the survival time, disease free interval and period of new tumours development has been evaluated recently in an ample study [8]. It shows that the simplest surgical technique must be applied to remove the entire lesion and the main lymphatic connections, with clean margins.

Achieving a clean surgical margin represents a technical challenge with important clinical implications. Despite the ongoing debate on healthy tissue conservation the key predictor of local recurrence is the margin status.

Detection of circulating tumour cells in body fluids (*e.g.* lymph, blood) during surgery may substantially enhance the chance to realize a clean margin especially when excision of large surfaces of tissue is required like in dog or cat radical mastectomy.

While in human breast cancer surgery advances have been registered towards *in vivo* discrimination between malignant and healthy tissue using Raman spectroscopy [9–11] veterinary surgery has less taken advantage of these steps forward. Comparative medicine would allow for knowledge and technical interchanges in breast tumour and even intra-cavity tumour surgery based on diagnosing a clean margin through molecular fingerprints that are obtained in Raman spectra as a result of photon inelastic scattering on chemical bonds.

The energy of monochromatic photons incident at a sample surface excites intra-molecular vibration modes whose frequencies are specific to the involved chemical bonds. Since the process develops with energy and momentum conservation a photon of lower energy is emitted (Raman photon) and a transition from one energy level to another occurs resulting in a frequency shift of the emitted

photon. These shifts are seen as individual bands in the Raman spectrum and their assessment would permit accurate analyses.

The particular advantages of Raman spectroscopy in biology and hence in precise diagnosis can be summarised as follows: *i*) sensitivity to many different functional groups, with access to C=C, S-S, CS bonds that are weak in the infrared (IR); *ii*) highly selective fingerprint, since it can discriminate similar compounds; *iii*) non-invasive and non-destructive technique as long as the right excitation photon energy (wavelength) and collection parameters are appropriately chosen, *iv*) no sample preparation is required, and this becomes particularly suitable for *ex vivo* or *in vivo* applications; *v*) compatibility with aqueous solutions gives a high chance to analyses of blood, lymph and other body fluids; *vi*) high spatial resolution permits single cell level analysis; *vii*) sensitivity to molecular orientation through polarization measurements. Measurements can be done *in vitro*, *ex vivo* or *in vivo* as appropriate.

In medical research Raman spectroscopy was reported to have a sensitivity of 100%, a specificity of 100%, and overall accuracy of 93% in identifying carcinomas [11].

A first pilot study for *in vivo* margin assessment during partial mastectomy breast surgery using Raman spectroscopy in women has been done in 2006 to show the feasibility of *in vivo* Raman spectroscopy for intraoperative margin assessment [12]. Raman spectra carefully acquired from proteins, nucleic acids, lipids and carbohydrates in order to develop a classification model for normal and anomalous tissue based on their biochemical composition [13] made available the peak positions and assignments of main Raman vibration modes used to diagnostic cervical cancer in women.

Assignments of the major bands for Raman and IR spectra of the noncancerous and respectively cancerous human breast tissue were recently reported [14] showing good similarity with values assigned in cervical cancer diagnosis research [13] and also with those found in studies on malignant skin tumours examined in vivo using portable Raman instrumentation [15].

Since accumulation times to obtain Raman spectra of high resolution and sufficiently high intensity may be quite long in order to make this technique a true real-time diagnosis tool for surgical margin assessment one solution were to provide a signal enhancement through using surface enhanced Raman scattering (SERS) [16, 17].

Surface enhanced Raman scattering occurs when surface plasmons of metal micro or nanostructures are resonantly excited by incident monochromatic light. Bio-molecules adsorbed or laid onto such substrates provide Raman signals within orders of magnitude (up to 10^8) more intense than in the absence of SERS substrates. That enhancement has effect on both spectral resolution improvement and on drastically shortening of collection times.

In principal any rough metal surface would serve as a SERS substrate but the best known for their surface plasmon activity are copper, silver and gold. Easily oxidation of Cu makes it difficult to be used in surgery but noble metal SERS substrates would be an appropriate choice.

This paper reports the attempts to implement Raman spectroscopy in veterinary oncologic surgery with the aim to provide in the future a real time in vivo technique for assessment of clean margins in both extra- and intra-cavity surgery. The comparative anatomy, physiology, biochemistry and medicine in general would permit the translation of Raman peaks assignments for normal and anomalous cells in humans to canine and feline patients and in turn human surgery could benefit of the findings resulting from SERS accessories employed in *ex vivo* Raman analyses described in this work.

2. EXPERIMENTAL

2.1. MATERIALS AND METHODS

Ex vivo samples of normal (healthy) tissue, breast tumours, skin and pure fat from 10 patients (female cats and female dogs) were investigated through Raman spectroscopy following regional mastectomy (removal of one mammary gland chain, the skin covering the breasts and the corresponding one side lymph nodes). Pure fat was collected also from incisions on the median line in healthy subjects operated for spaying. The tumours were immediately divided into two parts, from which one followed the common way of cytological and histopathology analyses and the second part was sampled for direct Raman exploration. Duration of histopathology analyses extends up to 4 weeks. The time linking excisions and Raman investigations counted 5 to 15 minutes. Thick (3 mm) pieces of tissue were laid on glass microscope slides. Smears were investigated on the scalpel blades used to section the samples of all kinds knowing that anomalous cells if present would be found in smears too.

2.2. PREPARATION AND USE OF SERS SUBSTRATES

The surfaces of the stainless steel blades used in surgery are not optically flat as seen in the atomic force microscopy (AFM) measurements shown in Fig. 1a. Therefore, they have been used as primary SERS substrates.

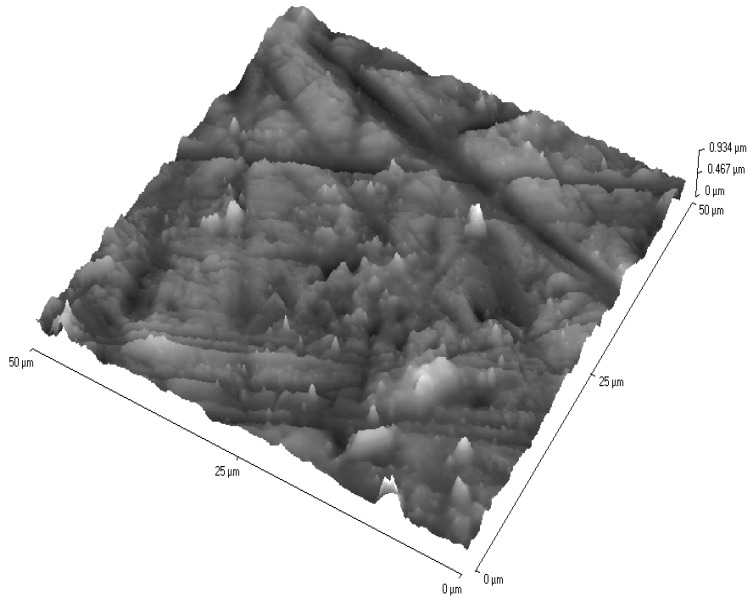
To maximize the surface plasmon effect on the Raman signal amplification silver coating of raw scalpel blades resulted in more appropriate SERS substrates [18].

Coating occurred through two techniques: pulsed laser deposition (PLD) and spin coating. PLD consists in depositing films on various substrates through pulsed laser ablation of a bulk target [18].

The process was carried out in vacuum 10^{-6} Torr using a PLD 2000 workstation (PVD Products Ltd.) with a COMPEX Pro 201 excimer laser (λ = 248 nm). Stainless still scalpel blades where used as substrates held at room temperature.

Spin coating of similar blades was run using a silver paste soluble in polar solvents such as water and ethanol [19]. For this work the paste was diluted in ethanol and then a few droplets were set on the stainless steel blade surface. Spinning rotation was gradually increased from 100 rpm to 500 rpm for 5 min. The as spin-coated blades were then dried in vacuum 90 minutes at 160^{o}C and finally left to cool down to ambient temperature. Adhesion of either way deposited silver coatings was checked through the simplest possible test, *i.e.* the "scotch test". None of the Ag films showed tendency to exfoliation.

AFM investigations of both PLD and spin-coated Ag films proved that the roughness of the surfaces is comparable. Therefore, the lot of following experiments was developed trough spin-coating of the scalpel blades view the significantly lower cost and shorter duration of this process in comparison with PLD.



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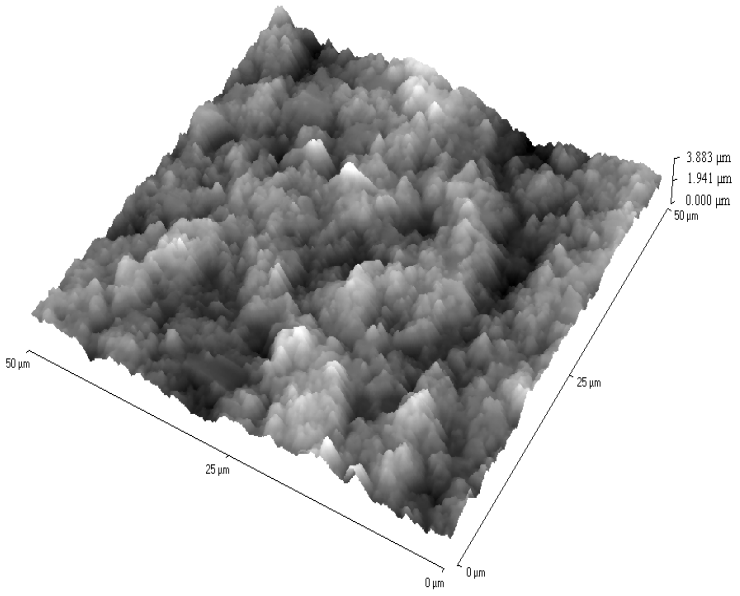

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Fig. 1 – AFM measurements performed over a 50 μm^2 area on a) raw stainless steel surgical blades (RMS roughness is 0.11 μm); b) silver coated surgical blades (RMS roughness is 0.5 μm).

2.3. RAMAN MEASUREMENTS

All Raman spectra were acquired using a LABRAM HR 800 (Horiba Jobin Yvon) micro-Raman spectrometer, in the backscattering geometry with a $\lambda = 632$ nm HeNe laser for excitation source.

To date have been reported *in vivo* Raman experiments on human subjects using 830 nm and 785 nm excitation wavelengths that limit the spectral range to 1800 cm^{-1}, while amide II proteins (CH stretching Raman mode) and N-H stretching (DNA, proteins) that are found only in cancerous cells [14] exhibit Raman lines above 2200 cm^{-1}.

The $\lambda = 632$ nm has been chosen on several considerations: *i*) it would provide a reasonable signal intensity, because $I_{\text{Raman}} \sim(1/\lambda)^4$; *ii*) it is less energetic than the other visible wavelengths (*e.g.* 514 nm, 488 nm) and thus the danger to get the samples surfaces modified is entirely removed even when acquiring the spectra would take longer then a few minutes; *iii*) its penetration in the biologic tissue goes beyond the subcutaneous fat layer; *iv*) laser power at the sample surfaces is about one order of magnitude smaller than for IR (*e.g.* 785 nm) irradiation.

A 1800 gr/mm diffraction grating was used and the spectral range extended from 100 cm^{-1} to 4000 cm^{-1} to cover all vibrations of biological interest, *i.e.* S-S, C-S, C-C, OH, NH, and H_2O. Depending on the substrates used (raw or Ag coated)

the laser power at the sample surface was set to 10 mW (raw) or 5 mW (silver coated) for a 0.5 mm laser spot. The integration times were 6 seconds on Ag and 30 seconds on stainless steel substrates. The resolution was 1 cm^{-1}.

3. RESULTS AND DISCUSSION

About 60 Raman spectra/patient were obtained for each of the 10 investigated patients on thick tissue samples laid on microscope glass slides (skin and tumour slices) and on smears collected on both raw stainless steel scalpel blades and Ag-coated blades, either used for initial incisions (subcutaneous fat) or for sampling the breast tumours.

Spectra taken on thick tissue samples (not shown here for low relevance matters) show interference on a moderate background of fluorescence centred at about 1200 cm^{-1}.

However, the range between 2800 cm^{-1} and ~3000 cm^{-1} corresponding to bands assigned to saturated bonds of lipids, fatty acids, polypeptide proteins and aromatic and aliphatic amino acids [14] is well displayed.

Spectra taken on smears laid on raw stainless steel surfaces are fluorescence free and demonstrate a slight SERS effect owing to the roughness produced by the industrial processing of the blades. Those spectra were collected in a quite long time, 25 min over the whole frequency range, in order to achieve an acceptable intensity for a 10 mW power at the sample's surface. In this case a "real time" *in vivo* margin assessment would be out of question although the resolution is excellent and no fluorescence background is created.

A true surface enhanced Raman effect is demonstrated through the spectra acquired from smears laid on silver coated scalpel blades.

The RMS roughness value of the Ag coated surfaces was 0.5 μm in comparison with 0.11 μm of uncoated ones (Fig. 1) as measured by AFM. That has led to a 10^5 amplification factor of spectra intensities along with drastically reducing the collection time from 25 min to 3 min. The laser power at the surface of the samples had to be reduced to 5 mW using attenuation filters and the acquisition times reduced as mentioned above in order to be able to record the spectra without saturation of the CCD of the micro-Raman instrument. This demonstrates the feasibility of the technique using a portable Raman instrument in a future stage.

In Fig. 2 are shown micro-images of smear samples from healthy fat subcutaneous tissue (Fig. 2a), healthy skin (Fig. 2b) and mammary tumour (Fig. 2c) of a female dog subjected to unilateral radical mastectomy and in Fig. 2d – image of subcutaneous fat from healthy female cat (spayed).

Figure 3a,b displays the corresponding SERS spectra.

Many Raman features of the human breast tissue involve characteristic C-H bands of fatty acids [14] attributed to different CH, CH_2, and CH_3 groups whose frequencies range from about 1400 to 3000 cm^{-1}.

In the present work dog and cat fat from healthy spayed females (Fig. 2a,d) were investigated *ex vivo*. The corresponding SERS spectra were compared with each other (Fig. 3b) and also with the Raman lines reported *in vitro* in reference [14] for human subjects.

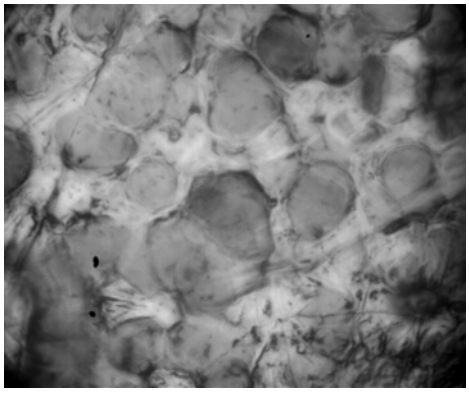
The H-C=C vibrations produce a band at 3009 cm^{-1}, corresponding to fatty acids and is weak in proteins. In human research that band was thought to play an important role [14] knowing that proteins are related to malignant tumour tissue. However, in figure 3a this line shows in both malignant tissue (spectrum c) as confirmed by histopathology and in normal tissue (skin b) as well as in subcutaneous fat (spectrum a).

Since fat is present in nearly all tissues (breasts, abdomen, thorax) it looks quite unlikely to use this line for benign *versus* malignant assessments.

On the contrary, the line at 1558 cm^{-1} reported for carotenoids [14] and not found in malignant breast tissue (Fig. 3a,b) could make a start to establish a marker.

In all SERS spectra investigated in this work a peak appears at ~2740 cm^{-1} whose assessment has been not found in references [13] and [14]. This peak (like many other ones) is common to both malignant and benign samples and is also seen in Fig. 3 of reference [14]. Since this Raman line is properly seen in the spectrum of pure fat it may be related to CH vibrations in fatty molecules and not to proteins.

The 3311 cm^{-1} OH vibration mode has been found in all malignant samples (histopathology confirmation) and it is seen in the representative spectrum in Fig. 3a. Neither fat spectra nor healthy tissue related spectra show that feature.



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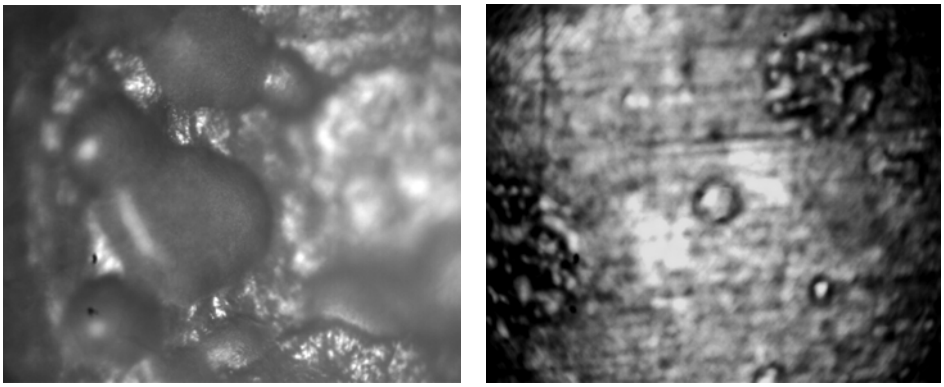
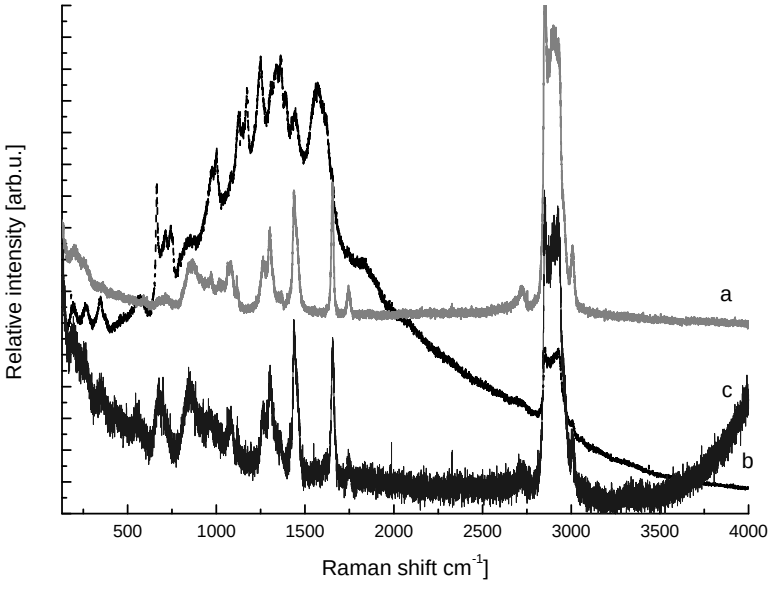


c d

Fig. 2 – Micro-images of smears investigated through SERS on Ag-coated scalpel blades: a) subcutaneous fat (healthy dog spayed); b) healthy skin (dog); c) malignant tumour (dog) as assessed by histopathology analysis; d) subcutaneous fat (healthy cat spayed).

The range of peaks reported for *in vitro* Raman measurements on human breast tissue [14] is entirely found in the spectra in Fig. 3a, collected *ex vivo* on smears from dog patients and corresponding to images in Fig. 2a, b, c.

From Fig. 3b it is observed that "healthy fat" SERS spectra are identical for two different species.



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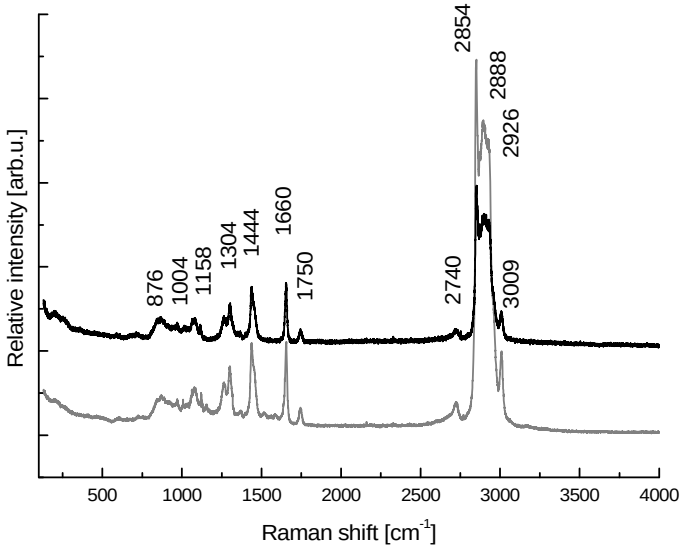

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Fig. 3 – a) SERS spectra taken on smears laid on Ag-coated scalpel blades (corresponding respectively to the samples shown in Fig. 2a, b, c; b) SERS spectra of pure fat (black-dog; gray-cat) corresponding to images a and c in Fig. 2.

4. CONCLUSIONS

Ex vivo investigation of mammary tumours in female dogs was carried out through Raman spectroscopy with visible light ($\lambda = 632$ nm) as an excitation source.

Use of scalpel blades coated with silver produced a spectacular enhancement of the signal (10^5) proving the SERS effect and led to a significant reduction of the acquisition times (from 25 min to 3 min). This result will impact on transferring SERS on surgical instruments and on using portable Raman spectrometers in veterinary oncologic surgery.

Raman spectra of pure fat from healthy female cats and female dogs show identical Raman lines at frequencies previously assigned to CH_x vibrations from *in vitro* studies on human samples.

The experimentally obtained fat spectrum from *ex vivo* samples could be employed as a "standard" spectrum at least in veterinary surgery.

Future work is planned to investigate the roles of the peaks at 1558 cm^{-1} and 3311 cm^{-1} in benign *versus* malignant assessments in the quest of clean surgical margins.

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