

## RAMAN SPECTROSCOPY INVESTIGATIONS OF THE CD1 NU/NU MOUSE SKIN PRE-CARCINOMA DAMAGES

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### Abstract

Cancer is one of the most studied pathology and early detection could be helpful for treatment or other types of interventions that may stop the fatal evolution. The preclinical studies can be applied on experimental models, especially on rodents. Cancer can be induced experimentally most often by the application of chemical carcinogens, tumor cells inoculation or xenografts.

FT-Raman spectroscopy in conjunction with the surface enhanced Raman scattering (SERS) has been applied for preliminary investigation on skin tissue from mice models in order to evaluate the possibility to record analytical spectral fingerprint in tissue diagnostic. FT-SERS spectra from the UV and chemically damaged tissues showed dominant characteristic bands from collagen and nucleic acids, whereas the bands attributed to proteins are observed with less extent.

### Rezumat

Cancerul este una din patologiile cele mai studiate, iar detectarea timpurie ar putea fi de folos în aplicarea unui tratament, sau a altor tipuri de intervenții ce pot opri evoluția fatală. Studiile preclinice se pot aplica pe modele experimentale, mai ales pe rozătoare. Inducerea cancerului se poate realiza cel mai adesea prin aplicarea carcinogenilor chimici, inoculare de celule tumorale sau xenogrefe.

Spectroscopia vibrațională Raman cu transformata Fourier (FT-Raman) combinată cu tehnica de amplificare a semnalului, SERS (*surface enhanced Raman scattering*), s-a aplicat pentru investigații preliminare ale țesutului cutanat prelevat de la modele de șoarece, pentru a evalua posibilitatea înregistrării amprente spectrale analitice în diagnosticarea tisulară. Spectrele FT-SERS înregistrate în cazul țesuturilor afectate de expunerea la UV sau chimic, au prezentat benzi dominante caracteristice pentru colagen și acizii nucleici, în timp ce benzile atribuite proteinelor au fost mai slab reprezentate.

**Keywords:** FT-Raman, SERS (*surface enhanced Raman scattering*), skin pathology, mice

### Introduction

Tumor models require important and non-invasive techniques to control the evolution of the pathology for early diagnosis and treatment surveillance [10]. Early diagnostic in cancer represents an aim that increases the possibilities for treatment and interventions in order to reduce or exclude the fatal pathologic evolutions [1, 3]. Spectroscopy could be an opportunity in this direction because of its ability to measure skin content and it can be applied for the evaluation of pathology evolution [6, 7, 12]. As an important protection barrier of the body, the skin consists of the following layers: *epidermis*, *dermis* and *subcutis* [2, 7]. The main dangerous types of skin pathologies are the cancers. Three examples are the most important: basal and squamous cell cancer, sarcoma of skin and malignant (cutaneous) melanoma [9, 15]. Because of the increased incidence of the skin cancers, the research activities for treatment and prophylaxis are important [1, 15]. All these aspects are increased in our days in direct correlation with environment degradation and protection reduction of (including skin). The most common diagnostic technique consists in histological investigations of the biopsies [1, 11]. FT-Raman spectroscopy has been recently successfully applied in an increasing number of cancer diagnostic applications because of its unique ability to identify and differentiate molecular structures involved in tissue pathology [6, 7, 8]. For a normal screening, a small portion of skin is sufficient for a proper measurement [6, 7, 13]. Previous studies confirmed that the biopsies can be preserved in 4-10% formalin solutions without influencing the Raman signal [6]. The FT-Raman and surface enhanced Raman scattering (SERS) measurements could represent a confirmation method associated with a histological evaluation on biopsies or directly applicable on whole body in experimental models to avoid the biopsy procedure by application of some modern techniques and carriers. This last application requires special anesthesia and breathing conditions. Using appropriate formulation and methods it is applicable for the detection of target tumor tissues from *in vivo* models. For routine applications in medicine, this technique has to be improved regarding the acquisition time, the signal-to-noise ratio and reproducibility. It has been recently demonstrated that SERS technique can be successfully applied in tissue measurements using colloidal nanoparticles. The SERS signal from tissue is strong, sensitive and selective for the molecular tissue components from the close vicinity of the nanoparticles [6, 7, 13]. Moreover, silver and gold nanoparticles are very useful in bio-diagnostic, especially in cancer target measurements [6, 13, 17]. Skin cancer experimental models can be obtained using, in most of the cases, mice such as CD-1 Nu/Nu type (hairless mice) [11, 15]. Some specific

chemicals and UV exposure determine the cancer evolution in the characteristic stages: initiation, promotion and final progression [3, 11, 15]. The most common chemicals are 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) as initiators and promoters [3, 15]. The initiation stage is associated with the chemical application and doesn't produce visible tumors. The visible tumor appears after a long term application (several weeks) of tumor promoter (TPA) [12, 15, 16]. An important role in skin carcinogenesis is played by ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamines synthesis that is induced by TPA, the tumor promoting agent [4, 10, 16].

Preliminary experimental results concerning the application of the Raman and SERS spectroscopic measurements of tissue from CD1 Nu/Nu mouse skin carcinoma model are reported in this paper.

## **Materials and Methods**

### ***Chemicals***

DMBA (Fluka) and TPA (Sigma-Aldrich), and the solvents from Chimpar Bucharest were used as received.

For UV exposure an ET-UV lamp was used. The main solvent for tumor agents was acetone. For obtaining the silver (Ag) colloidal nanoparticles, AgNO<sub>3</sub>, sodium citrate and tridistilled water were used.

### ***Animals***

Female CD-1 Nu/Nu mice (8 weeks old) were purchased from Charles River (Germany). The UMFT (University of Medicine and Pharmacy Timisoara, Romania) Bioethical Committee aproved the protocol and institutional guidelines were followed in the handling and care of the animals. For the present study 2 animals from a group of 10 mice that were treated for skin carcinoma development were chosen. The animals were accommodated in the UMFT Biobase and they were maintained in optimal conditions. Standard food was used. The animals had free access to food and water. The day/night cycle was 12/12 hours and the humidity was over 55%.

### ***Tumor induction protocol***

The DMBA was applied 2 times/week, during the first 2 weeks and after that TPA was used 2 times/week. The substances were dissolved in acetone, for DMBA 200 nmol/100μL acetone and for TPA 5 nmol/100μL acetone as in the protocols presented in literature [4,8]. The actual measurement was performed after 10 weeks of photochemical application. After DMBA application the mice were exposed to UV-lamp 2 times/week, 5 min/day, (from week 3 to 6) to accelerate the pathologic process.

Biopsy preparation: the skin was excised from the most damaged skin area. Thin excised tissue samples were cut in 20  $\mu\text{m}$  sections for FT-Raman analysis, with the total dissection of skin layer. The same procedure was applied for the tested organs. The samples were kept in a 10% formalin solution mixed with the colloidal silver solution 1/1, v/v.

#### ***Raman spectral acquisition***

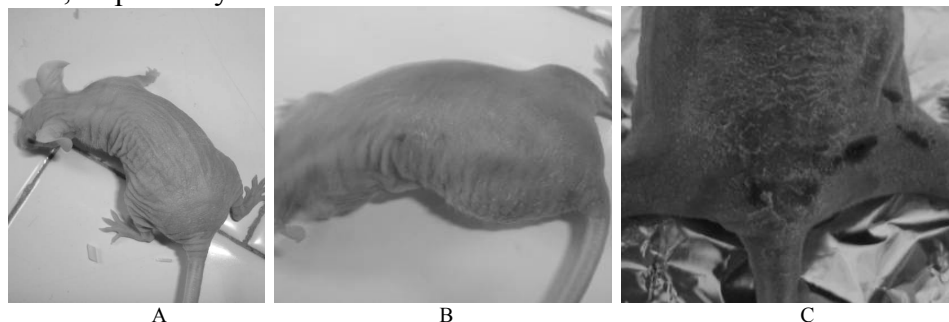
FT-Raman/FT-SERS spectra have been recorded using an Equinox 55 Bruker spectrometer with an integrated FRA 106 S Raman module fiber optic coupled with a Ramanscope II micro-Raman setup. A Nd:YAG laser operating at 1064 nm line with an output laser power of 350 mW was employed for excitation and a Ge detector operating at liquid nitrogen temperature, for detection. The spectral resolution was 4  $\text{cm}^{-1}$  and different numbers of scans ranging from 50 to 350 were accumulated for optimal data acquisition for each sample.

#### ***Silver colloidal solution preparation***

The silver colloidal solution was prepared by dissolving in triple distilled water the  $\text{AgNO}_3$  and heating until boiling. An aliquot of sodium citrate was added to the boiling solution and the final silver colloidal suspension was concentrated by centrifugation at 5500 rpm for 35 minutes.

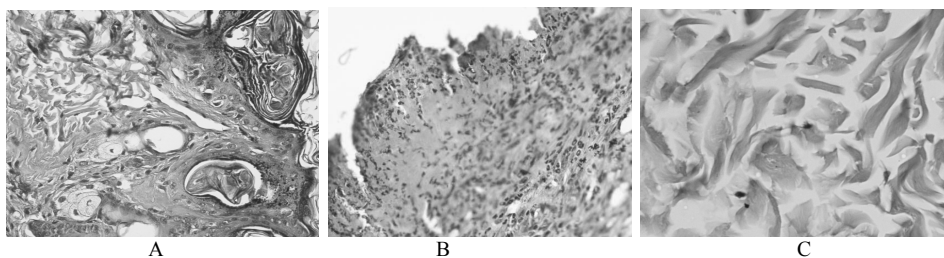
### **Results and Discussion**

The general aspects regarding the technique implementation and the general histological evaluation of the skin, liver and lungs were the first steps of the experiment. Different degrees of toxicity were also observed in this phase of the experiment as the samples were collected from exposed organs such as liver. The principal aspects of damage and pathologic skin after the photochemical exposure are presented in figure 1, whereas figures 2 and 3 show the microscopic details of the dermo-epidermal tissue and lung or liver status, respectively.

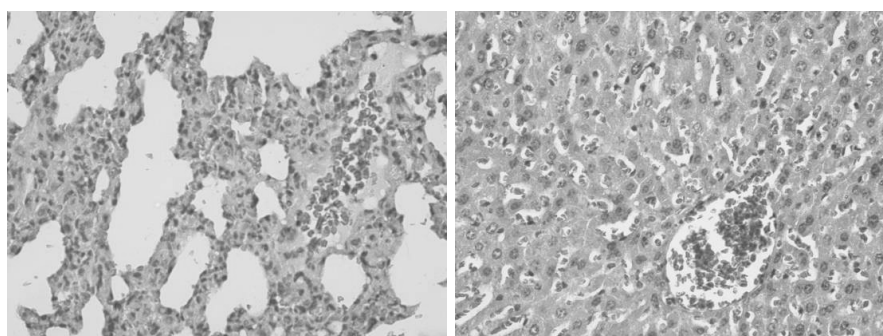


**Figure 1.**

Pathological skin evolution on hairless mouse (CD1 Nu/Nu) in 10 weeks.  
A. week 1; B. week 3; C, week 10.

**Figure 2.**

- A. Skin: dermo-epidermal tissue with reduced damage (HE x 400).  
 B. Skin: epidermal ulceration, inflammatory acute dermal process (HE x 400)  
 C. a high collagen density dermal tissue (HE x 400)

**Figure 3.**

Lung stasis (left) and liver stasis detail in pericentrolobular area (right). HE x 400.

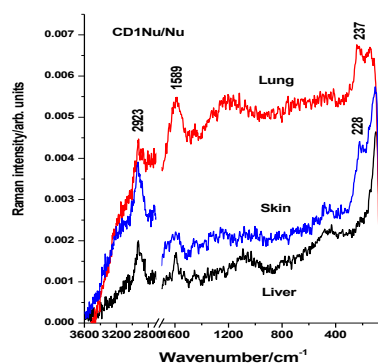
The liver and lung suffered drastic damages after the application of chemical compound, but these aspects were not fatal during the experiment.

#### *Raman spectroscopy*

It is well known that the Raman scattering of tissue is extremely weak. Generally, the quantum yield of Raman spectroscopy is 6–10 orders of magnitude lower than other methods. Because of the danger of denaturation or even pyrolysis, the low signal intensity cannot be compensated by raising the laser power. Therefore all components of the experimental protocol have to be optimized carefully, especially for application in medicine.

For Raman spectroscopy of tissue we used the histological verified biopsy samples as shown in the Figures 2 and 3.

The first attempt to measure directly the tissue samples is shown in figure 4.



**Figure 4.**

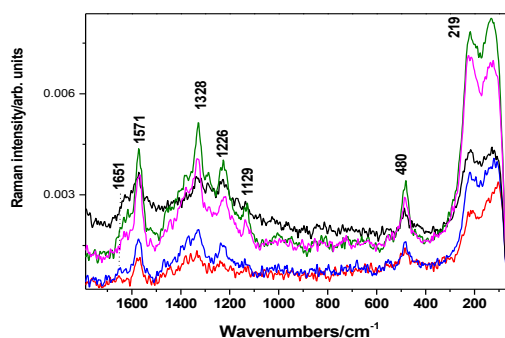
FT-SERS spectra collected from the CD1Nu/Nu hairless mouse immediately after dissection and tissue inoculation with colloidal nanoparticles. Excitation: 1064 nm, 300 mW.

Although slight spectral differences could be observed in the 1589  $\text{cm}^{-1}$  band shape as well as in the 400-1300  $\text{cm}^{-1}$  spectral range, this experimental approach does not allow a reliable molecular characterization of the tissue specimens. It must be pointed out the evidence of the Ag clusters chemisorptions proved by the vibrational mode in the 228-237  $\text{cm}^{-1}$  region. It is obvious that the 1064 nm line used for the Raman scattering excitation is not favorable because of the high local heating of the samples and results in a strong fluorescence background and a low signal-to-noise ratio. At present, the measuring time of 15 minutes for weak scattering tissues with the freshly incubated nanoparticles probe is not practical.

The previously reported results [6, 7] in Raman skin investigation using an FT-Raman equipment and fiber optic probe in detriment of lower spectral resolution, displayed better signal-to-noise ratio and showed a relative intensity change for the pathologic skin and for the most affected area comparing to the normal one. All the lesions, pigmentations, collagenosis, influenced directly the Raman measurement. For example, a comparison between a normal skin, a psoriatic skin and other pathologic aspects including Kaposi sarcoma, showed that the pigmented skin presented significant changes in the 1200-1600  $\text{cm}^{-1}$  spectral range, including the band position, width and relative intensity of the amide III,  $\delta\text{CH}_2$ , amide II, and in the high wavenumber range, over 2800  $\text{cm}^{-1}$  for the  $\text{CH}_2$ ,  $\text{CH}_3$  and OH stretching modes [7]. The main changes in spectral observations can be attributed to collagen influence. A lot of pathologic processes of the skin could determine collagen fiber densification, which increased immediately the Raman bands assigned to this molecular compound. This process is present in the case of skin carcinoma, actinic

keratosis, preliminary phases of cutaneous melanoma, etc [6, 7]. Secondary, the bands for lipids were evaluated [7] and water signs of skin inflammation, that could also be influenced by therapeutic treatment with anti-inflammatory compounds.

FT-SERS spectra collected from different skin lesion regions after 24 hours of colloidal skin incubation are displayed in figure 5. It appears that the Ag nanoparticles have been efficiently adsorbed into the tissue *epidermis* and *dermis* since the signal collected displayed sharper bands with a good reproducibility. The strong band located at  $219\text{ cm}^{-1}$  is characteristic for the Ag-molecule bond when the species adsorbed on the nanoparticles present oxygen within a functional group involved in adsorption. Such SERS feature is characteristic for the proteins and nucleic acids. The amide I and III from proteins fingerprint at  $1651$  and  $1226\text{ cm}^{-1}$  are clearly observed with a medium intensity. The strong band at  $1328\text{ cm}^{-1}$  with a complex shape suggests either the nucleic acids or collagen contribution.



**Figure 5.**

FT-SERS spectra collected from different points of the skin lesions after 24 hours following the nanoparticles incubation. Excitation: 1064 nm, 300 mW.

Such fingerprint bands could be further considered important regarding the collagen aspect that is changed in different pathologies. Overall, the FT-SERS spectra suggest the possibility to monitor the skin damage, since the band shape and relative intensity are sensitive from point to point.

During the experiment, we observed the apparition of skin damages and a dry skin syndrome correlated with skin lesions (Figures 1-A, B, C). These aspects are correlated with data in the literature that present the experimental possibility to obtain a dry skin syndrome and the preliminary phases of skin carcinoma [4, 14]. The cancer initiator and

promoter can develop a systemic toxicity observed especially on the liver because of its systemic penetration [2, 5]. These data indicate the need of a good toxicity monitoring during the experiment.

The first changes of the skin are only general damages and lead to the idea that the apparition of the specific tumor is influenced by the period of exposure. The most significant damages are dry skin, lesions and irritations that could develop important signs of pathology (Figure 1). All the pathologic aspects could be confirmed by a histological hematoxylin eosin staining more specific for the skin (Figure 2). The chemical compounds applied on the skin are known for their capacity of tumor induction [4, 5]. The same type of evaluation was applied for the liver and lungs for the correlation with spectral data (Figure 3). The status of these organs didn't indicate a fatal stage and previous studies suggested a reduced rate of lethality [2]. Our work developed the idea that this type of measurements can be a diagnostic method in skin analysis and appreciation of the cutaneous evolution after the treatment application. Every influence on skin composition leads to changes in peaks aspect. NIR-FT-Raman evaluation of pathologies such as psoriasis and Kaposi sarcoma revealed similar changes as presented in the literature [7]. The method is non-invasive, it is easy to apply and the data on the whole body can be correlated with the analysis on surgical detached skin after mixing with the nanosolution. It could be useful to determine very fast the stage of the disease and even if a skin biopsy is performed, the animal can be kept alive. This spectral technique is adaptable on the whole body of the animal and on the detached biopsies, generally after the combination of colloidal nanoparticles with the biopsies. FT-Raman combined with SERS technique (FT-SERS) measurements are more specifically in skin diagnosis on a biopsy.

### Conclusions

The damage of the skin surface is important for establishing a preliminary for diagnosis and early stage treatments. The common techniques in tissues evaluation, such as histological analysis, could be completed by novel vibrational spectral techniques that can be appreciated as sensitive indicators of the pathological stage.

In the present study we demonstrated the possibility to obtain high quality FT-SERS spectra from the skin tissue with photochemically induced cancer using FT-Raman and SERS technique and silver colloidal nanoparticles. The preliminary results suggest that the FT-SERS technique could be successfully applied to skin cancer diagnostic or therapy monitoring.

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