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Raman microspectroscopy discrimination of single human keratinocytes exposed at low dose of pesticide

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ABSTRACT

Raman spectroscopy is a useful technique for early diagnosis of cellular damage related to the exposure at toxic chemicals, because biochemical changes related to action mechanism of chemicals can be detected in Raman spectra. In this investigation Raman microspectroscopy has been used, in correlation with the principal component analysis method, to detect biochemical changes occurring in cultured human cells as a consequence of exposure at a commercial pesticide. Cultured human keratinocyte cells have been exposed at increasing concentrations of pesticide for 24 h. Viability tests indicated that the cells vitality is almost completely preserved when the concentration of active ingredient of pesticide is very low (5×10^{-8} M, about two orders of magnitude lower than the cytotoxic concentration at 24 h exposure). Nonetheless, the analysis of Raman spectra allows to state that a biochemical change occurs: it involves mainly the protein linkages between aminoacids (amide I bonds) and, at a minor amount, lipids. On the whole, principal components analysis is able to classify into two separate clusters the control and exposed human cells.

1. Introduction

Pesticides are chemical products extensively used in agriculture, industrial and household activities mainly for insecticidal and anti-parasitic purpose. A commercial pesticide is usually composed of active ingredients and formulants. Active ingredient is called the agent having the properties to prevent, destroy, repel or mitigate a pest. Among active ingredients, Deltamethrin is a pyrethroid chemical widely used for synthesizing pesticide products which are very effective in damaging the central nervous system of pests due to its effects on voltage sensitive sodium channels [1]. Instead, formulants are any other chemical in the formulation of the pesticide, which does not possess any specific function against the pests. Formulant agents are added to the active ingredients both to better solubilize it and to increase efficiency, by optimizing the assumption by the target. Therefore, they are relatively non-toxic and they have few side effects. Nonetheless, it is interesting to investigate if and how such formulant agents modify the toxicity of pesticide in humans. In particular, investigation of cultured cells exposed to a commercial pesticide is important to evaluate the eventual biochemical modifications at cellular level caused by the pesticide, especially when low dose exposure occurs.

Raman microspectroscopy has become a well-established technique to investigate biochemical changes induced by exposure to chemicals at single cell level. It is a vibrational spectroscopy technique in which monochromatic radiation from a laser is focused onto a sample in order to induce vibrational modes in the functional groups of the sample and create inelastically scattered photons with energies and intensities characteristic of such functional groups. In fact, the main cellular components, as protein, nucleic acids, lipids and carbohydrates, scatter light inelastically: therefore, eventual changes of such components upon stress conditions can affect the Raman spectrum. Several investigation have shown that Raman microspectroscopy can reveal differences between spectra of normal and chemically stressed cells [2–10].

Sometimes Raman spectra of control and stressed cells have been analyzed by means of multivariate statistical techniques to obtain data reduction and evaluation of differences between them [2,7,8]. In fact, rather than relying on the use of individual spectra to distinguish groups of cells, multivariate techniques employ information from the whole data set of all spectra to classify them. Principal components analysis (PCA) is a data reduction technique that summarizes the variation between the spectra by reducing it into a small number of principal components (PCs) scores, while retaining the majority of information contained within the spectra [11]. These components describe consistent differences between

Abbreviations: Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; p, proteins; c, carbohydrates; l, lipids; n.a, nucleic acids; str, stretching; wagg, wagging; twist, twisting; sciss, scissoring.

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the spectra, allowing separation of the spectra into groups based on similarities or disparities in the spectral characteristics.

In our previous work, we have verified the possibility of Raman technique to detect molecular changes in single human cells exposed to pure Deltamethrin solution at a concentration value (10^{-6} M) at least two orders of magnitude lower than that established as the cytotoxic value (between 10^{-4} M and 2.5×10^{-4} M) for 24 h exposure [12]. In the present work we extend the results obtained for the Deltamethrin to a Deltamethrin-based commercial pesticide (“Decaflow”) containing formulant agents as well, in order to evaluate the capability of Raman spectroscopy to detect cellular changes after low dose exposure of such chemical. Our results show that the cytotoxic concentration for 24 h exposure of Deltamethrin as active ingredient of Decaflow is lower than that of pure Deltamethrin of about one order of magnitude. In addition, biochemical modifications occur in cells exposed to Decaflow at a very low Deltamethrin dose (5×10^{-8} M for 24 h) and characterized by viability value similar to that of control cells. Such modifications mainly involve amide I bond of the protein components. Although the differences between Raman spectra of control and exposed cells are very small, PCA technique is able to distinguish control from exposed cells.

2. Materials and methods

2.1. Cell cultures

Normal human keratinocyte (HUKE) cells were grown in Epilife medium, supplied with Keratinocyte Medium Supplement, at 37 °C, 5% CO_2. The cells were exposed for 24 h to different Decaflow solutions containing increasing concentrations of Deltamethrin from 5×10^{-8} M to 2.5×10^{-4} M. This latter value has been chosen because it corresponds to Deltamethrin concentration in the Decaflow solution suggested (by manufacturer company) for commercial use. The Trypan blue and MTT methods were used as viability tests to evaluate the cytotoxic concentration of the chemical.

The samples for Raman microspectroscopy consisted of cultured cells on poly-lysine coated glass coverslips. After 24 h exposition, control and treated cells were washed three times in phosphate-buffered saline (PBS) and then fixed in paraformaldehyde 3.7%, in order to stop autolysis and prevent morphological and chemical degradation when they are moved out of the incubator (as needed for data acquisition). Then, the samples were stored in Petri dishes filled with PBS solution until Raman spectra were performed. Before such measurements, cells were rinsed in deionized water, in order to remove residual PBS from the surface of the cells.

2.2. Raman instrumentation

Raman spectra were recorded at room temperature by means of a Raman confocal micro-spectrometer apparatus (Labram from Jobin-Yvon Horiba) using the 488 nm line of an Ar ion laser as excitation source. The laser beam was focused by an Olympus optical microscope with a 100× oil immersion objective (1.4 numerical aperture): a diffraction limited spot less than 1 μm diameter resulted at the focal plane. The laser intensity on the sample was properly fixed at about 10^5 W/cm^2, in order to obtain a good signal/noise ratio, but avoiding thermal damage of the sample. This choice was also supported by other literature papers [13,14] reporting about Raman spectra of HeLa cells measured with the 488 nm line and laser light intensity values comparable to that used in this experiment. In fact, no thermal damage was observed in the investigated cells. As a matter of fact, the absorbed fraction of the incident light by HUKE cells measured at 488 nm is quite weak (about 9%). The glass coverslip containing the cells was transferred onto a microscope slide provided of a well, which was filled with PBS solution. The face of the coverslip where the cells were adherent was placed into the PBS, so that the laser beam through the glass coverslip probed fixed single cells kept in PBS solution. It was checked the reproducibility of data, in order to ensure that this configuration did not lead to degradation of the samples, related to photochemical reactions and/or sample heating, within the acquisition time for each measurement. The immersion of cellular samples inside PBS solution also permits the dissipation of the laser energy without destroying the cells (as frequently occurs when air objectives are used). Each Raman spectrum was measured in the spectral range from 900 to 1800 cm^{-1}, with an acquisition time of 60 s. Scattered light from the sample was collected by the same 100× oil immersion objective (in backscattering geometry) and passed through a notch filter to suppress the elastically scattered laser light. Then, Raman scattered light crossed a squared confocal hole (600 μm diagonal) and the 200 μm entrance slit of a spectrometer equipped with a 600 grooves/mm grating. Signal was detected by means of a CCD, cooled at 223 K. A separate CCD camera was used to record white light microscope images of the cells being probed. The spectral resolution was about 5.5 cm^{-1}/pixel. Photon counting technique was used for signal recording.

2.3. Spectral processing and data analysis

Raman spectra were recorded from 15 different single cells, randomly chosen on each of the glass coverslip containing control and exposed cells. A typical Raman spectrum of a control cell is shown in Fig. 1a (continuous line), where the background spectrum (due to the optics, coverslip, paraformaldehyde and PBS solution) is also reported (dashed line): this latter spectrum was measured in a point of the coverslip where no cell was present. In fact, it has been verified (by comparing the background Raman spectrum with the spectrum of the glass coverslip) that the influence of PBS and paraformaldehyde on cell Raman spectra is negligible. For each cell spectrum, this background was firstly multiplied for a correction factor, so that the broad band due to the coverslip (centered at about 920 cm^{-1}) could nearly overlap the corresponding band of the cell spectrum. This background so corrected was subtracted from the same cell spectrum. In this way, a spectrum similar to that shown in Fig. 1b is obtained. Moreover, the cellular fluorescence was also subtracted from each cellular spectrum by using a second order polynomial function, so that the Raman spectrum of each single cell could be obtained, as shown in Fig. 1c. Further, the Raman spectrum of each cell was vector normalized, in order to minimize the effect of variation either of sampling cellular volume or laser power on the spectral intensity of each single cell. After such pre-treatment procedure, the spectra from each cell type (control and exposed ones) were averaged over the 15 measured cells, in order to obtain a mean Raman spectrum specific of each cell type.

In addition to the spectral averaging method, the analysis of vector normalized spectra from single cells was also carried out by means of PCA method. This is a multivariate statistical technique applied to reduce the information contained in a spectroscopic data set (including many spectra each one consisting of many variables, corresponding to the spectral intensity values) to a few relevant variables, known as principal components (PCs). In fact, such method consists in expressing each spectrum of the data set (which can be written as a matrix of the intensity values) as a linear combination of PCs, which are computed from the covariance of the data set. The PCs maintain all the variance of the original data, but the first PC contains the most of the variance