Synchrotron based Fourier-transform infrared microspectroscopy as sensitive technique for the detection of early apoptosis in U-87 MG cells

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L. Buriankova[1], Z. Nadova[1], D. Jancura[1], M. Refregiers[2], I. Yousef[2], J. Mikes[3] and P. Miskovsky [1,4*]

[1] Department of Biophysics, Faculty of Science, Safarik University, Kosice, Slovakia

[2] Synchrotron SOLEIL, L'Orme des Merisiers, Saint-Aubin, Gif sur Yvette, France

[3] Institute of Biology and Ecology, Safarik University, Kosice, Slovakia

[4] International Laser Centre, Bratislava, Slovakia

*Corresponding author:

Pavol Miskovsky, Department of Biophysics, Faculty of Science, Safarik University, Jesenna 5, 041 54 Kosice, Slovakia, e-mail: pavol.miskovsky@upjs.sk

Abstract (abstract figure included)

The aim of this study is to show that SR-FTIRM can be successfully applied for the detection of early apoptotic processes in the human glioma cells (U-87 MG). The apoptosis was induced by photodynamic action after irradiation of either photosensitizer hypericin (Hyp) or a complex of Hyp with low-density lipoproteins (Hyp/LDL) incorporated into the cells. The differences between infrared (IR) spectra of non-treated and photodynamically treated U-87 MG cells are mainly manifested in position of Amide I vibrational band, sensitive to changes in protein secondary structure. The conformational transition α-helix to β-sheet results in the shift of Amide I vibration to lower frequency, and can be explained as a consequence of the processes leading to apoptosis. It is also shown that the sensitivity of SR-FTIRM for the detection of early apoptotic signs is comparable, or even higher, than that of classic technique usually used for the detection of early apoptosis, flow-cytometry study of cell staining by Annexin V. Moreover, we have demonstrated by both methods, SR-FTIRM and flow-cytometry, that Hyp/LDL complex loaded U-87 MG cells 24 hours after photodynamic action are mostly late apoptotic/necrotic. In contrast, U-87 MG cells loaded with Hyp alone display apoptosis as prevailing mode of cell death at 4 hours and 24 hours after photodynamic action.

Introduction

Synchrotron based Fourier-transform infrared micro-spectroscopy (SR-FTIRM) has become a valuable technique for examining the chemical make-up of biological molecules by probing their vibrational motions on a microscopic scale [1]. This technique allows *in situ* structure determination of the most important biomolecules (nucleic acids, proteins, lipids) in chosen sub-cellular compartments [1, 2]. The higher spatial resolution and spectral quality, in comparison with "classic" Fourier-transform infrared microscopy, is due to the fact that a synchrotron IR source is 100-1000 times brighter than a conventional thermal source for IR spectroscopy [3]. The higher brightness (flux density) of synchrotron is due to the small effective source and its very narrow emittance. The high brightness of synchrotron source allows the analysis of smaller region with acceptable signal-to-noise ratio and the spatial resolution reaches the diffraction limit ($\lambda/2$) [1, 4, 5]. Thanks to the high spatial resolution of the SR-FTIR technique, the structure of biomolecules inside cells can be probed with a sub-cellular resolution.

The SR-FTIRM has already been successfully applied in several studies of biological systems. The comprehensive review on this subject was provided by Miller and Dumas [1]. The broad spectrum of biological and medical applications of SR-FTIRM includes determination of the protein structure aggregates in brain tissue of patients with Alzheimer´s disease, study of protein folding, investigation of the bone composition during osteoporosis, observation of the changes in the lipid and collagen content and in the structure of myocardium during heart disease and many others [see review 1]. This technique was also successfully used to study cell cycle, differentiation and proliferation of different cell lines and cell death mode [6-12].

With respect to the scope of this work, we would like to emphasize that for the study of programmed cell death (apoptosis) not only SR-FTIRM, but also "classic" IR spectroscopy has been relatively widely used [13-20]. Apoptosis is accompanied by changes in the structure of the three main types of biomolecules (nucleic acids, proteins, lipids). Techniques based on IR spectroscopy are able to analyze simultaneously these structural changes in all three types of biomolecules [21].

The main IR spectral features such as modifications of the protein structure determined by shift in the positions of Amide I and Amide II bands, fragmentation of DNA revealed by the alterations of vibrational modes of DNA, and changes of stretching vibrations in CH_2 and CH_3 groups of lipid molecules have been shown to be a consequence of apoptosis. Changes in the position and intensity of Amide I peak are in a good correlation with the change in the percentage of apoptotic population of Jurkat cells [13]. In leukemia cells under apoptosis, it was determined that the protein structure shifts from predominantly β-sheet to unordered coil structure, and that quantification of apoptotic cells correlates very well with the determination of apoptosis by two standard methods (flow-cytometry and DNA ladder formation) [14, 15]. Further, it has been shown that significant changes in the IR spectra of apoptotic cells, mainly attributed to conformational changes of DNA, are associated with decreasing amounts of detectable DNA [16]. It was also observed that the apoptotic index is inversely proportional with the spectral area 900-1200 cm^{-1} assigned to the nucleic acids (NAs) [18]. There is evidence that apoptosis induces a conformational disorder of lipid acyl chains as indicated by a marker band in the IR spectrum of an apoptotic cell corresponding to the exposure of phosphatidylserine on the outer leaflet of the membrane [19]. All these findings strongly show that IR spectroscopy, especially SR-FTIRM can provide unique insights into properties of apoptotic processes. However, it should be pointed out that the above mentioned results are often contradictory. This can be attributed to the fact that in different studies, different types of cell lines are used, and the IR spectra are often obtained from cells at different stages of apoptosis. Furthermore, the mode of the apoptosis induction (drugs, radiation, photodynamic action) can influence the IR spectra of apoptotic cells.

In present work, we induced apoptosis by photodynamic action caused by illumination of either Hyp or Hyp/LDL complex localized in U-87 MG cells. Hyp (Fig. 1) is a natural photosensitizing pigment occurring in plants of the genus *Hypericum* [for reviews see 22-24]. Previously, it has been clearly demonstrated that under suitable concentration and illumination conditions Hyp is a very effective and potent inducer of apoptosis in many cell lines, including glioma cells such as U-87 MG cells [24-27].

The reason for the use of Hyp/LDL complex in addition to Hyp alone in this study stems from the following. Upon administration into the blood stream Hyp associates with serum proteins, mainly with LDL. Because of the enhanced expression of specific LDL receptors in many types of transformed cells (cancerous cells) in contrast to non-transformed cells, LDL could play a key role in the targeted delivery of hydrophobic and amphiphilic photosensitizers to tumor cells in photodynamic therapy (PDT) [28]. Our previous studies have shown binding of both monomer and aggregate forms of Hyp to LDL [29-31]. It was also demonstrated that over-expression of LDL receptors results in an increased Hyp accumulation within U-87 MG cells, and that a different delivery strategy leads to a different type of cellular response to Hyp photoactivation [32].

The aim of this study is to show that SR-FTIRM spectroscopy, can be successfully used for the detection of the early apoptotic processes in the cells after photodynamic action. In particular, we show that this technique is capable to distinguish between very subtle differences in cellular response induced by photoactivation of Hyp, delivered to cells alone or complexed with LDL. In the SR-FTIRM spectra, the Amide I band seems to be the most sensitive marker to these subtle differences. The correlation of SR-FTIRM results with the outcome of standard flow cytometry method for the detection of apoptosis is also discussed.

Materials and Methods

Chemicals: Hypericin (Hyp), Dulbecco´s modified Eagle´s medium (DMEM), Fetal Bovine Serum (FBS) and penicillin/streptomycin were purchased from Gibco invitrogen (France), low-density lipoproteins (LDL) from Merck (Darmstadt, Germany) and Ultroser® G from Pall Life Sciences (France)

Cell cultivation: Human glioma cells (U-87MG) were cultured as monolayers and grown in Dulbecco´s modified Eagle´s medium (DMEM) containing L-glutamine (862mg L^{-1}) and glucose 4500 mgl^{-1} supplemented with 10% Fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% sodium-pyruvate (control cells). In order to induce an increased expression of LDL receptors in U-87MG cells, the 10% FBS was replaced by 2% Ultroser®G-serum without lipids in cell media 24 hours before experiment (cells with

elevated LDL receptors). The cells were kept at 5% CO_2 atmosphere. To prevent an accidental photoactivation of Hyp, all experiments were done in dark conditions.

Sample´s preparation for SR-FTIRM experiments: U-87MG cell line was cultured on low-e slides at the concentration of 10^5 cells per ml in different cultivation media (10 % FBS, 2% Ultroser®G). Thereafter cells were incubated with Hyp and/or with Hyp/LDL complex for 3 hours. Cells with non-elevated LDL receptors (10%FBS) were cultured in DMEM in presence of 500 nM Hyp. Cells with elevated LDL receptors (2% Ultroser®G) were cultured in DMEM in presence of Hyp/LDL=30:1 complex ratio, which corresponds to 500 nM of total concentration of Hyp. Samples were then irradiated with 4J/cm^2 at 600 nm wavelength. At several times after irradiation (4 and 24 hours) cells were washed with PBS (pH=7.2) and then fixed with 3.8% formalin for 20 minutes. After fixation with formalin, cells were washed with water and air dried.

SR-FTIRM experiments: The experiments were performed at Synchrotron SOLEIL (St. Aubin, Gif Sur Yvette, France) at the SMIS infrared beamline. The experimental set-up was previously described in detail by P. Dumas, and M. J. Tobin [2]. Briefly, the synchrotron light is collected from a bending magnet in the electron storage ring. The IR beam is extracted by the combination of plane water cooled and ellipsoidal mirror. The IR beam is re-collimated and directed toward the IR microscope. The collimated beam of synchrotron IR light enters the FTIR spectrometer before the IR microscope. The sample area is spectroscopically sampled and the image is built up by raster-scanning specimen through the focused beam. The single element detector is used for this configuration. The microscope is equipped with reflecting Schwarshild-type objective to avoid absorption and chromatic aberration over the large mid-IR-spectral range.

The IR spectra were recorded in reflection mode by the IR Continuum XL microscope coupled to a ThermoNicolet 5700 micro-spectrometer. Each individual cell was selected visually and the IR spectra were taken at the nucleus location of the cell with an aperture of 8×8 μm^2. Each spectrum was acquired after 100 accumulations with 4 cm^{-1} spectral resolution. Data acquisition and processing were performed using Omnic software (Version 7.3, Thermo Electron Corporation). Each final IR spectrum is the average of at least of 11 individual cell spectra.

Flow-cytometry experiments: The flow-cytometry experiments were performed as reference experiments. The samples were prepared under identical incubation conditions as in SR-FTIRM experiments. Evaluation of apoptotic cells was performed using the Alexa Fluor®488 Annexin V (Molecular Probes)/ propidium iodine (PI) staining. Cells with photoactivated Hyp were harvested by low-speed centrifugation and washed with PBS. Cells were resuspended in 100 µl of Annexin-V binding buffer and incubated with 5 µl of Alexa Fluor®488 Annexin-V for 15 min at room temperature. After the incubation, the cells were diluted with 400 µl of Annexin-V binding buffer and stained with PI (final concentration 1 µg/ml). Control cells stained with Alexa Fluor®488 Annexin V or PI alone were used to compensate for the flow cytometric analysis. The measurements were performed on a BD FACSCalibur flow-cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW/488 nm argon laser. The externalization of phosphatidylserine (PS) into outer leaflet of the plasma membrane as a sign of apoptosis was detected by a green fluorescence of bounded Annexin V to PS in the FL-1 channel. The dead cells were identified by a red fluorescence of PI marker (bound to DNA in nucleus) in the FL-3 channel.

Results and discussion

IR spectra of untreated U-87 MG cells: An average IR spectrum obtained from several U-87 MG cell is shown on Figure 2B. The broad band centered at ~3300 cm^{-1} comes from O-H stretching and N-H vibrations (proteins, carbohydrates and water). These vibrations are not usually used for the investigation of cell's processes like apoptosis, differentiation, or cell death. Asymmetric stretching of CH_3 groups of lipids is localized at 2962 cm^{-1} and symmetric one has maximum at 2877 cm^{-1}. Asymmetric and symmetric modes of CH_2 stretching vibrations appear at 2927 and 2853 cm^{-1}, respectively. Proteins are characterized by two dominant peaks: Amide I at 1650 cm^{-1} and Amide II at 1633 cm^{-1}. The Amide I band is sensitive to the secondary structure of proteins and corresponds to the vibration of C=O group of the peptide bond with a small contribution of the C-N stretching vibration [33]. The Amide II band arises predominantly from the coupled N-H in-plane bending and C-N stretching vibrations in peptide bond and is not usually used in the conformational analysis because it overlaps with amino acid side chain vibrations.

The middle intense peak at 1240 cm^{-1} is assigned to the asymmetric phosphate stretching vibrations (υ_{as} PO_2^-) and the band at around 1080 cm^{-1} results from symmetric phosphates stretching vibrations (υ_s PO_2^-) of phosphodiester groups of nucleic acids [34].

IR spectra of U-87 MG cells after photodynamic action induced by Hyp and Hyp/LDL: Figures 3 and 4 represent the proteins contribution region of IR spectra collected from U-87 MG cells treated with either Hyp alone or with Hyp/LDL complex (Hyp/LDL=30:1) measured 4 hours (Fig.3) and 24 hours (Fig.4) after illumination. The most significant changes appear in the region of Amide I vibrations. The position of the maximum of Amide I band in the cells treated with Hyp alone (at 4 hrs) is shifted from 1650 cm^{-1} to 1624 cm^{-1} (Fig. 3, Table 1) and to 1640 cm^{-1} (at 24 hrs) (Fig.4, Table 1). Similar shifts of the Amide I band to the final position at 1630 cm^{-1} (at 4 hrs) (Fig. 3, Table 1) and 1644 cm^{-1} (at 24 hrs) (Fig. 4, Table1) are observed for cells treated with the Hyp/LDL complex. For a better resolution of Amide I band and to determine the exact position of the maxima of each vibration contributing to the Amide I band, the second-derivative IR spectra are shown in Figures 3 and 4. It is evident that the Amide I band in untreated U-87 MG (control) cells has only one peak centered at 1652 cm^{-1}. This finding indicates the dominancy of α-helix secondary structure of the proteins present in untreated cells. In contrast, the second-derivative IR spectra in the Amide I region of the Hyp and Hyp/LDL treated cells (at 4 hrs after illumination) are characterized by the appearance of a new band localized at 1622 cm^{-1}. This is a consequence of the conformational transition of the secondary structure of proteins from α-helix to β-sheet structure. The final position of Amide I band in SR-FTIRM spectra at 1640 cm^{-1} and at 1644 cm^{-1} is observed 24 hour after illumination for cells treated with Hyp alone and for Hyp/LDL treated cells, respectively. The second-derivative spectrum has only one peak centered at 1643 cm^{-1} and at 1647 cm^{-1}, respectively. This position corresponds to the unfolding of proteins to unstructured coil. Thus, the overall secondary structure of proteins in U-87 MG cells after photodynamic action induced by the irradiation of either Hyp alone or Hyp/LDL complex demonstrates a shift from α-helix to β-sheet (4 hrs after illumination) and from β-sheet to disordered conformation (24 hrs). Similar structural changes in the proteins during apoptosis were also observed by others. Liu et al. demonstrated structural shifts from β-sheet in the normal cells to unordered coil in the

apoptotic cells [14, 15]. Zhou et al. observed an increase of the β-sheet structure occurrence in membrane proteins of HL60 apoptotic cells [17]. Pozzi et. al. suggested a correlation between the value of the shift of β-sheet wavenumber and the percentage of apoptotic cells [13]. Taking all together, one can conclude that the analysis of the Amide I position provides useful tool for the detection of early apoptotic signs.

Flow-cytometry (Annexin V+PI staining) study of U-87 MG cells after treatment with Hyp or Hyp/LDL: To make a correlation between the detection of apoptosis by SR-FTIRM and flow-cytometry experiments, we performed experiments where apoptotic cells quantification was assessed by detecting of Annexin V staining (externalization of the PS molecules in plasma membrane during apoptosis).

Cell survival (staining by PI) and occurrence of apoptotic signs were tested 4 and 24 hours after Hyp photoactivation in U-87 MG cells (4 J/cm^{-2}) (Fig.5 and Table 2). Annexin V/PI double-negative cells are defined as live cells (Fig. 5, lower left quadrant), Annexin V-positive/PI-negative cells are defined as early apoptotic cells (Fig. 5 lower right quadrant), and Annexin V/PI double-positive cells are defined as late apoptotic/ necrotic cells (Fig. 5 upper right quadrant). PI only positive cells are considered dead (Fig. 5 upper left quadrant). Fig. 5A shows the typical distribution of control cells (with Hyp alone and without irradiation). Fig. 5B shows the distribution of cells incubated with Hyp alone measured 4 and 24 hours after irradiation. There is a slight shift of the cell population toward apoptotic region (lower right quadrant) 4 hours after irradiation. The amount of apoptotic cells is dramatically increased 24 hours after Hyp photoactivation (Fig. 5 and Table 2). Fig. 5C shows the distribution of cells incubated with Hyp/LDL complex. In this case, almost half of cells population is still alive even after 24 hours after the photodynamic action. The rest of the cells undergo late apoptosis or necrosis (Fig. 5 upper right quadrant).

The results of the FACS analysis are summarized in Table 2. In cells incubated with Hyp alone, the number of early apoptotic cells increases in a time dependant manner from 14 % at 4 hours after illumination to 61 % at 24 hours after illumination. The cells treated by Hyp/LDL complex showed both apoptosis as well as necrosis signs. 24 hours after illumination the percentage of cells in the late apoptosis/necrosis was even higher than early apoptotic cells (36 % vs. 6 %). The determination of the late apoptosis/necrosis 24

hour after illumination of U-87 MG cells in the presence of Hyp/LDL complex is an a good correlation with the SR-FTIRM experiments where the existence of unordered coil structure of proteins was observed. Thus, it can be suggested that the process of necrosis is accompanied by conformational transition from ordered to unordered structure of proteins. Although flow-cytometry measurements were not able to detect apoptosis in U-87 MG cells treated with Hyp/LDL complex 4 hours after illumination (only 3 % of cell population demonstrated signs of apoptosis), IR spectra are sufficiently sensitive to reveal conformational transition from α-helix to β-sheet in protein structure.

Conclusion

The modification of the protein secondary structure in U-87 MG cells loaded with either Hyp or Hyp/LDL complex after photodynamic treatment could be explained by on-going apoptosis confirmed by flow-cytometry experiments. In the IR spectrum of the cells 4 hours after irradiation, the overall secondary structure of proteins has been significantly shifted from α-helix to β-sheet (1640 - 1622 cm^{-1} shift). The shift of the Amide I band in the IR spectrum strongly indicates events of the early apoptosis. The unordered coil structure of proteins has been observed in the cells 24 h after irradiation. This finding likely corresponds to the final stages of apoptosis, in which the cells loses their structure and proteins are degraded. The flow-cytometry results have further supported this possibility as the majority of cells was at late apoptosis/necrosis stage at 24 hours after irradiation. Taking all together, our results demonstrate that SR-FTIRM is a powerful technique for the detection of the early stage of apoptosis (the apoptotic signs are detected even earlier than by flow-cytometry), and in combination with other apoptotic assays can provide unambiguously a determination of the apoptosis induced by various factors (e.g. oxidative stress induced by photodynamic action, drugs, radiation).

Acknowledgement: This work was supported by the Slovak Research and Development Agency under the contracts APVV-0449-07 and lpp-0337-06. We would like to thanks Synchrotron SOLEIL for the access to the SMIS beamline and the SMIS staff for technical help and advices.

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Tables:

	Amide (cm^{-1})	**Amide II (cm^{-1})**
Control	1650	1533
Hyp, 4 hrs after irrad.	1624	1511
Hyp, 24 hrs after irrad.	1640	1526
Hyp/LDL, 4 hrs after irrad.	1630	1514
Hyp/LDL, 24 hrs after irrad.	1644	1517

Table 1 Table of vibrational position of Amide I and Amide II groups

	ANNEXIN V$^-$ / PI$^-$ (LIVE CELLS)	**ANNEXIN V$^+$/ PI$^-$** (EARLY APOPTOTIS)	**ANNEXIN V/ PI$^+$** (LATE APOTOSIS/NECROSIS)
Control	82 %	3 %	13 %
Hyp, 4 hrs after irrad	68 %	14 %	16 %
Hyp, 24 hrs after irrad	3 %	61 %	34 %
Hyp/LDL, 4 hrs after irrad	43 %	3 %	54 %
Hyp/LDL, 24 hrs after irrad	58 %	6 %	36 %

Table 2 Response of U-87MG cells to the PDT treatment in the different period after irradiation (4h, 24h)

Figures:

Fig. abstract . Visible image of human glioma cell U-87 MG cell and corresponding Infrared spectrum collected from the point marked in the VIS image of cell

Fig.1 Chemical structure of hypericin

Fig. 2 (A) Area of selection of scanned nucleuses of human glioma cells U-87MG**,** (B) average IR spectrum from marked nucleuses of human glioma cell (U-87MG)

Fig. 3 (A) Normalized average SR-FTIR spectra of Amide region (4 hours after irradiation): (1) untreated (control) cells (average of 14 spectra), (2) cells treated with Hyp alone (average of 35 spectra), (3) cells treated with Hyp/LDL complex (ratio =30:1) (average of 22 spectra), 2^{nd} derivatives of Amide I region: (B) control cells, (C) cells treated with Hyp alone, (D) cells treated with Hyp/LDL complex.

Fig. 4 (A) Normalized average SR-FTIR spectra of Amide region (24 hours after irradiation): (1) untreated (control) cells (average of 14 spectra), (2) cells treated with Hyp alone (average of 47 spectra), (3) cells treated with Hyp/LDL complex (ratio =30:1) (average of 11 spectra), 2^{nd} derivatives of Amide I region: (B) control cells, (C) cells treated with Hyp alone, (D) cells treated with Hyp/LDL complex.

Fig. 5 AnnexinV (FL1-H channel) and propidium iodide (FL3-H channel) staining of U-87 MG cells (A.) control cells, (B.) cells treated with Hyp alone (4 hours after irradiation), (C.) cells treated with Hyp alone (24 hours after irradiation), (D) cells treated with Hyp/LDL complex, (ratio 30:1) (4 hours after irradiation), (E) cells treated with Hyp/LDL complex, (ratio 30:1) (24 hours after irradiation)

Fig. abstract:

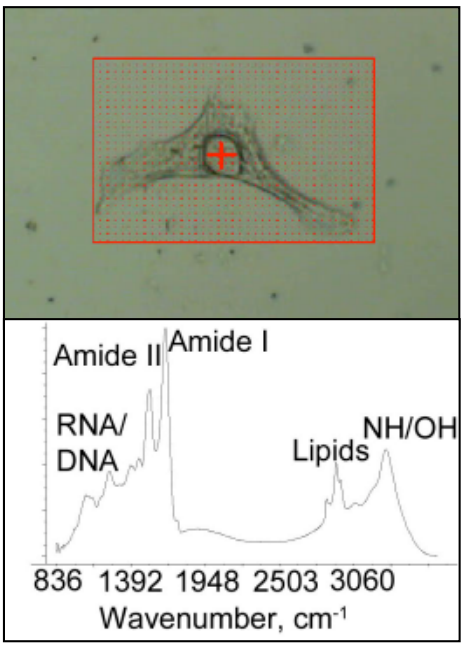


Fig.1:

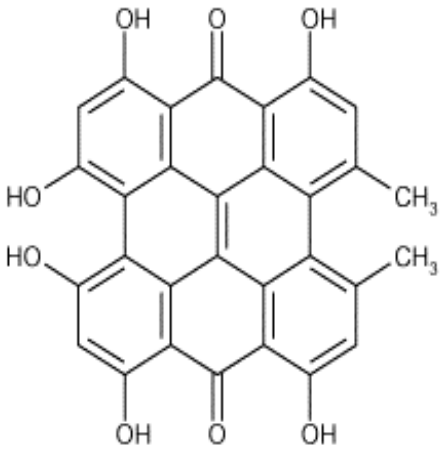
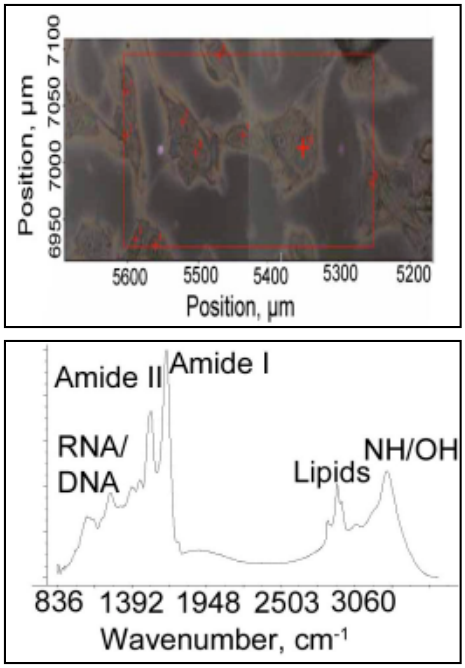


Fig. 2:

(A)


Position, µm
6950 7000 7050 7100
5600 5500 5400 5300 5200
Position, µm
Amide II
Amide I
RNA/
DNA
Lipids
NH/OH
836 1392 1948 2503 3060
Wavenumber, cm-1

(B)

Fig.3:

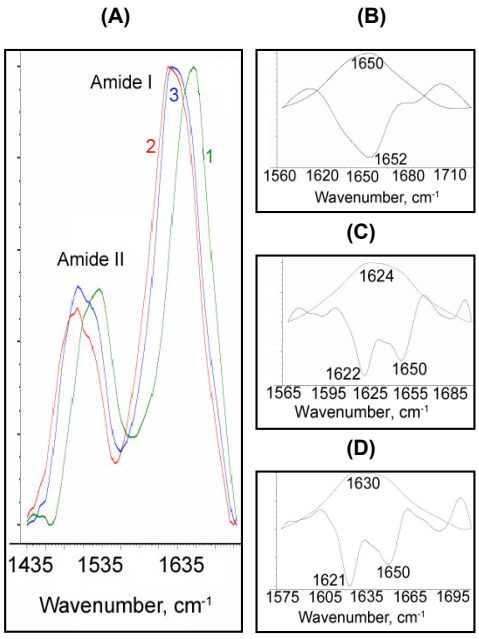

(A)
(B)
Amide I
3
2
1
Amide II
1435 1535 1635
Wavenumber, cm-1
1650
1652
1560 1620 1650 1680 1710
Wavenumber, cm-1
(C)
1624
1622
1650
1565 1595 1625 1655 1685
Wavenumber, cm-1
(D)
1630
1621
1650
1575 1605 1635 1665 1695
Wavenumber, cm-1

Fig. 4:

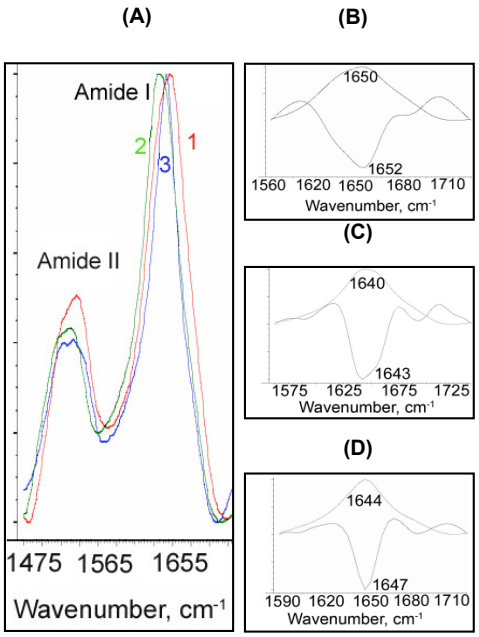
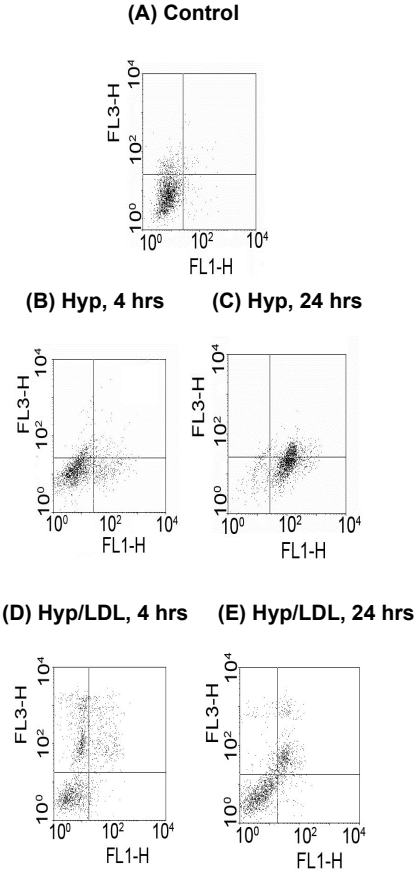

(A)
(B)
Amide I
Amide II
1
2
3
1475 1565 1655
Wavenumber, cm-1
1650
1652
1560 1620 1650 1680 1710
Wavenumber, cm-1
(C)
1640
1643
1575 1625 1675 1725
Wavenumber, cm-1
(D)
1644
1647
1590 1620 1650 1680 1710
Wavenumber, cm-1

Fig. 5


(A) Control
FL3-H
FL1-H
(B) Hyp, 4 hrs
(C) Hyp, 24 hrs
(D) Hyp/LDL, 4 hrs
(E) Hyp/LDL, 24 hrs