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Effects of Cells Density and Positioning on Optimized FTIR Biospectroscopy

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Researchers have found a big interest in biological application of Fourier Transform Infrared (FTIR) spectroscopy. Evaluating many diseases, staging them and studying the chemical structures of different formed compounds in diseases are some of the research applications of FTIR. Cancer is also one of these diseases. Researchers are trying to set up FTIR methods to detect and diagnose cancer cells and follow up the treatment steps using FTIR. In this regard, cancer cells and tissues are under investigation. In order to study cancer cells in lab, it is important to find out the proper cell density on the disk, at the first step. In this regard, the effect of different densities and positioning of cancer cells on FTIR supporting disk are studied in the present project. At the first step calibration of the instrument is checked using bovine serum albumin (BSA). Cancer cells were collected from culture dishes and washed with normal saline, twice. Different concentrations of cells (10000-320000 cells/uL) were located on ZnSe disks and dried prior to spectroscopy. The samples were scanned in the mid-infrared range of 4000-400 cm-1, with the resolution of 2 cm-1. Each spectrum was collected by 100 sample scans. Microscopic images of the disk were also taken to find out the distribution of the cells on the disk. The results of this study showed that the right amount of cell number and positioning on the disk is a very important parameter in bio-spectroscopic quality for biological purposes.

Keywords : A549, A2780,Cell, Density, FTIR, HepG2, Spectroscopy, Spectrum.

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1. Introduction

Fourier Transform Infrared (FTIR) spectroscopy detects the vibrations of the atoms of molecules, following the infrared (IR) radiation. IR makes vibrations in each specific atom and each atom makes vibrations specific to only that atom (Stuart, 2004) Therefore, each molecule, with its unique structure, will have a unique infrared absorption and spectrum. That is how IR spectra are known as the fingerprint of any molecule.

In addition to structural analysis of other chemical compounds, FTIR spectroscopy has had a wide application in biological research (Kumar Eri, Chanti Naik, Padma, Ramana, Madhu, & Gopinath, 2014).

Cancer grading of frozen samples of lymphoma patients (Andrus & Strickland, 1998), characterizing mineralized structure of bones (A.L. & Mendelsohn, 2005), multiple sclerosis lesions (LeVine & Wetzel, 1998), detection of dysplasia and early cancer of oral tissue (. T.C.BakkerSchut, 2000), as well as squamous cell carcinoma (Conti, Giorgini, Pieramici, Rubini, & Tosi, 2005), morphologic studies on colorectal carcinomas (Lasch & Naumann, 1998), lesion detection in esophagus of patients (Stone, Kendall, Shephard, Crow, & Barr, 2002), spatial and chemical studies on the components of atherosclerosis in arteries (Bonnier, et al., 2006), chemical composition of lymph nodes in breast cancer patients (Smith, Kendall, Sammon, Christie-Brown, & Stone, 2003) are few examples of wide biological use of FTIR.

Likewise, biological samples of cells and tissues have been a subject of infrared spectroscopy for structural analysis and diagnostic purposes (J. R. Mourant, Yamada, Carpenter, Dominique, & Freyer, 2003). However, to get validate spectra of cancer cells, it is important to set up the basics of correctly applying the cells. It is important to know if cell density and the volume of applied cell suspension actually disturb FTIR spectra or not. Therefore, the aim of the present study was to study the importance of cell density in the FTIR spectra of cancer cells.

2. Methods and Materials

2.1. Materials

The cells, human hapatocarcinoma HepG2 cell line (IBRC C10096) and human lung adenocarcinoma A549 cell line (IBRC C10681) were purchased from the Iranian biological research center. Human ovary carcinoma A2780 cell line (C461) was prepared from National Cell Bank of Iran. All the materials used in this project were purchased form Gibco®, life technologies®, unless otherwise specified.

2.2. Methods

2.2.1. Cell Culture Protocol

The cells were thawed and passaged three times, before the test. The cells were kept in humid CO_2 incubator at 37°C. At the time of the experiment, the cells were trypsinized and suspended in media. Then the cells were centrifuged at 700g, 4°C, for 10 min, and washed with normal saline, twice. The cells were then counted based on trypan blue assay and appropriate cell concentrations (10000, 20000, 40000, 80000, 160000 or 320000 cells/uL) were made in normal saline. Suitable volume of the cell suspension (10 µL or 100 µL) was placed on a ZnSe disk. The suspension was then dried out in a vacuumed chamber at 400 mmHg for 5 min. The disk was later placed in the FTIR chamber to obtain the spectrum.

2.2.2. FTIR Spectroscopy

The cells suspension, at desirable concentration is placed on the central part of a ZnSe disk, in a vacuumed chamber. The cells are then rapidly dried in the vacuumed chamber for 5 min at 400 mmHg. Later, the disk is placed in the sample holder in the FTIR chamber (WQF-510, Rayleigh Optics, China). The sample was scanned in the mid-infrared range of 4000-400 cm^{-1} with the resolution of 2 cm^{-1}. Each spectrum was collected by 100 sample scans.

2.2.3. Microscopic Images

Microscopic images were taken using a camera focused on the optic lenses of an inverted microscope.

2.2.4. Analysis

Spectra analysis was performed using Essential FTIR® software version 3.00.019.

3. Results and Discussion

A lot of molecular species can be identified, detected or quantified within a biological sample using FTIR (Holman & McKinney, 2003) (Krafft & Sergo, 2006). Recently, FTIR spectroscopy of biological samples has become the interest of lots of

investigators. As cancer is also one of the major concerns of biologists, physicians and researchers, FTIR application in cancer studies is also improving. The aim of the current study was to study the importance of cancer cells density on the resulting FTIR spectra.

At the first step, the instrument had to be calibrated, to check if the effect of water vapor in the atmosphere can be diminished and if the instrument can detect major peaks of a known sample, which is BSA in this case. In order to omit this background effect, azote gas was injected to the sample chamber of the FTIR instrument and spectrum of normal saline as a background was obtained (Figure 1). Then the spectrum of normal saline, as a sample, was obtained. As it is observed in Figure 2, since the background spectrum was obtained from normal saline, the IR spectrum of normal saline, as a sample did not have any IR peaks. Normal saline is the buffer that is used for cancer cells preservation right before spectroscopy. FTIR spectroscopy could successfully zero the effect of background (Figure 1).

At the next step, the validity of the spectra was tested with BSA. Figure 2 shows the principle peaks of FTIR of BSA. The principle peaks include the peaks near 1650 cm^{-1} that indicates the amide I band and results from the C=O stretching vibrations of the peptide bond. Also the peaks near 1540 cm^{-1}(N-H bending vibration/C-N stretching vibration) and 1240 cm^{-1} (C-N stretching vibration/N-H bending vibration) which are characteristics of the amide II band, and amide III band, respectively. The peak near 3300 cm^{-1} is considered to be N-H bending vibration and the peak near 1400 cm^{-1} results from the protein side-chain COO- (Protein Secondary Structural Analysis by FTIR, 2015).

Since the spectra obtained from the FTIR instrument were valid and reproducible, the next step was to study the effect of cell density on the obtained spectra. In this regard, two tests were performed; 1- 10 µL of HepG2 cell suspension was placed on ZnSe disk in two states; either localized at the center of the disk or widely distributed on the disk. Furthermore, 100 µL of the cell suspension was also placed on the disk for

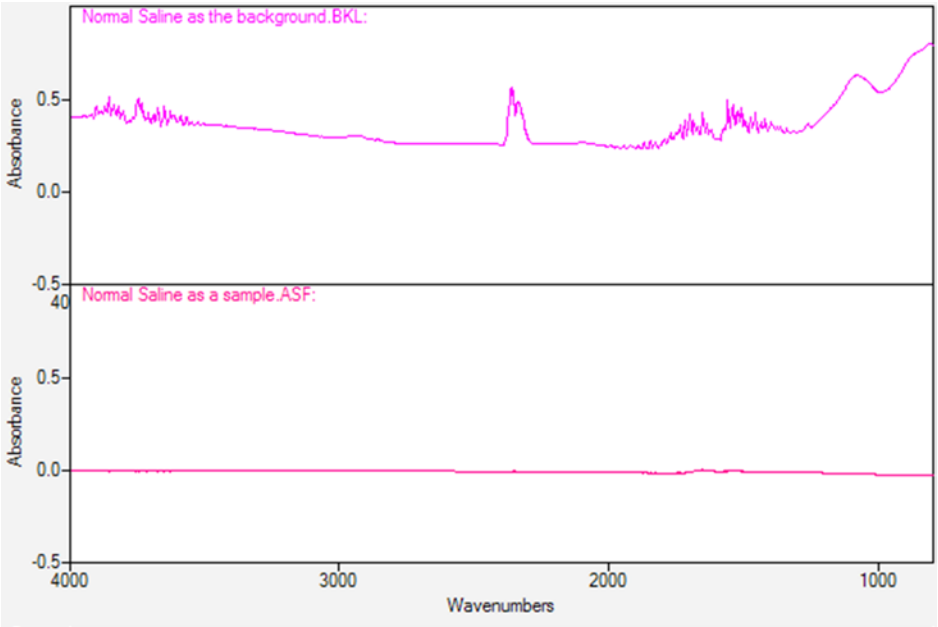

Figure 1. Checking the ability of the FTIR spectrometer to correctly zero the effect of background.Normal Saline is placed on ZnSe disk, dried and then the spectrum is collected as a background (top). When the spectrum of normal saline is collected as a sample, no significant infrared peak is obtained (bottom).

FTIR spectroscopy.

2- the spectra of cell suspensions with DNA around 1200 to 1400 are missing or are wide and indistinguishable when some

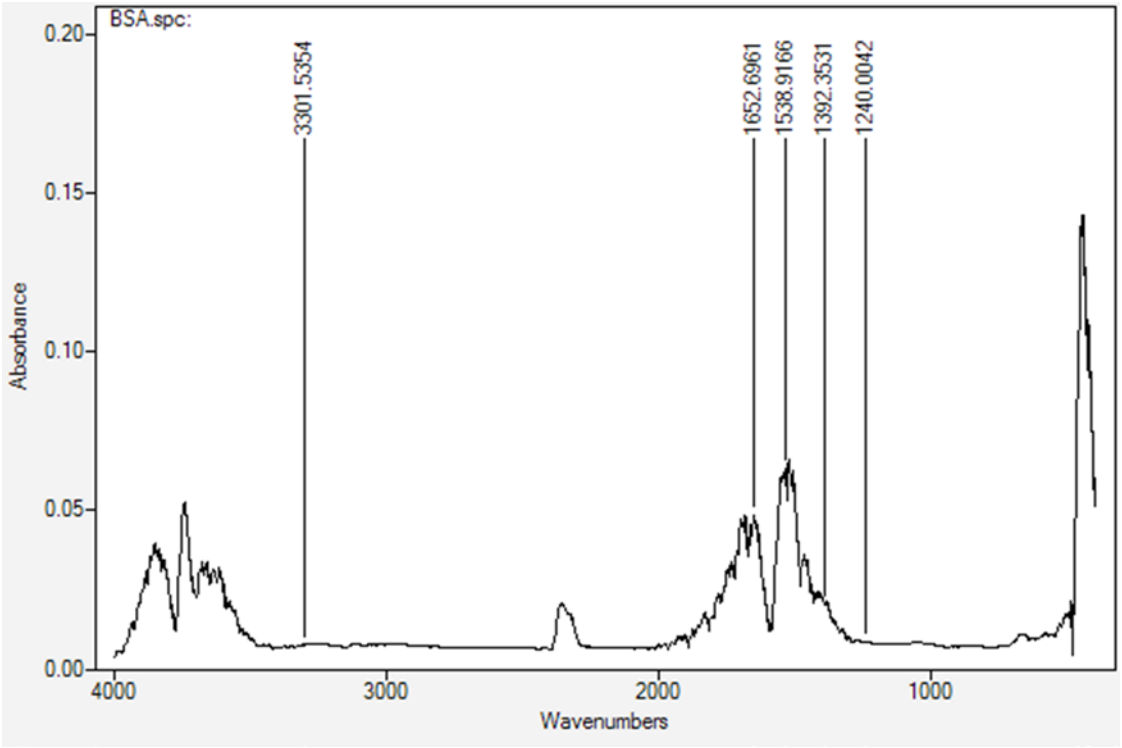

Figure 2. FTIR spectrum of bovine serum albumin (BSA).

different concentrations were obtained.

Figure 3 compares the effect of cells distribution on the disk. At all three states, the principle peaks of amides exist. However, 100 uL of the sample, results in a wide peak instead of 2 typical neighbor peaks of amide I and II. On the other hand, widely spread volume of cell suspension is tough to make a more monolayer cells compared to the cells centrally localized on the disk. However, these data did not show considerable differences between these two states of cell distribution on the disk. In general, the results of this section show a very clear difference on the appearance of different peaks in the amide, nucleic acid ad lipids regions. It looks like the best result presenting all regions with sharp and accountable peaks is when right amount of cells are placed all over the spectroscopy area (Figure 3, top). Some peaks like around carbohydrate and phosphate backbone of spectroscopy area on the disk is lost (Figure 3, middle), and different groups related peaks are missing or gets unclear and wide when too much tight layer of cells are placed on the disk such as the spectrum presented in Figure 3, bottom.

In order to test the importance of cell density, the spectra of various concentrations of cells were obtained at the next step. Figure 4 shows the microscopic images of the various concentrations of A549 cells dried on ZnSe disk. The images show that increasing the cells' concentration causes multilayer gathering of cells, which somehow blocks the penetration of microscope light at higher densities. The FTIR spectroscopy of such cells is shown in Figure 5A and 5B for A549 and A2780 cell lines, respectively. The results show that in both cell lines, the principle peaks of amide I and II exist. However, at higher cell densities, intensity of the peaks reduces.

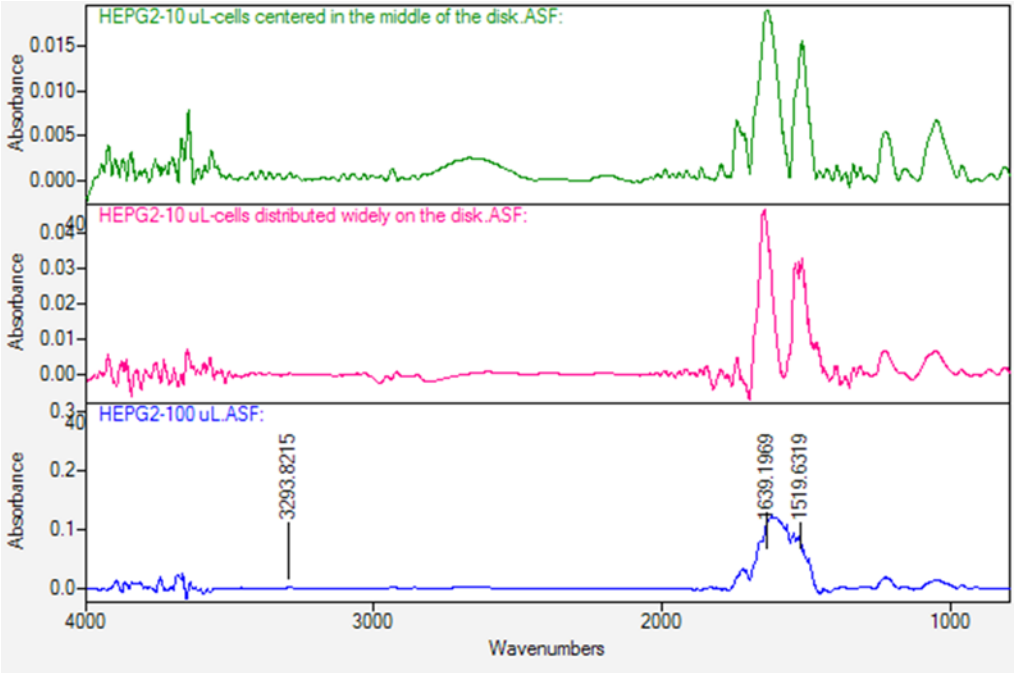

Figure 3. FTIR spectra of HepG2 cells in 3 different positioning states. 10 µL cell suspension was placed on the ZnSe disks, distributed widely on the disk (top), distributed at the center of the disk (middle), or 100 µL of the cell suspension covering the whole disk surface (bottom).

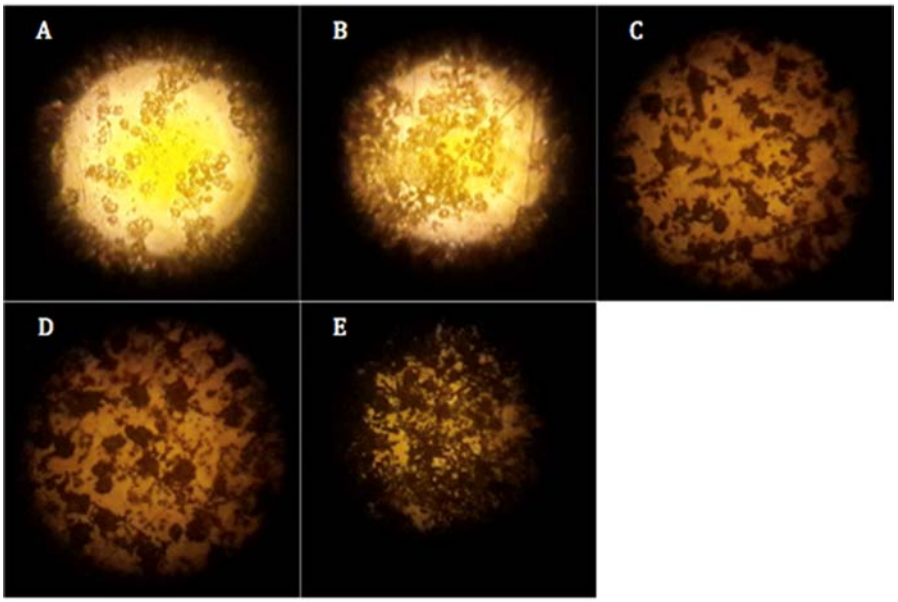

Figure 4. Microscopic images of A549 cell line distribution, at different concentrations, on ZnSe disk after being dried in a vacuumed chamber. A) 10000 cells/µL, B) 20000 cells/µL, C) 40000 cells/µL, D) 80000 cells/µL and E) 160000 cells/µL.

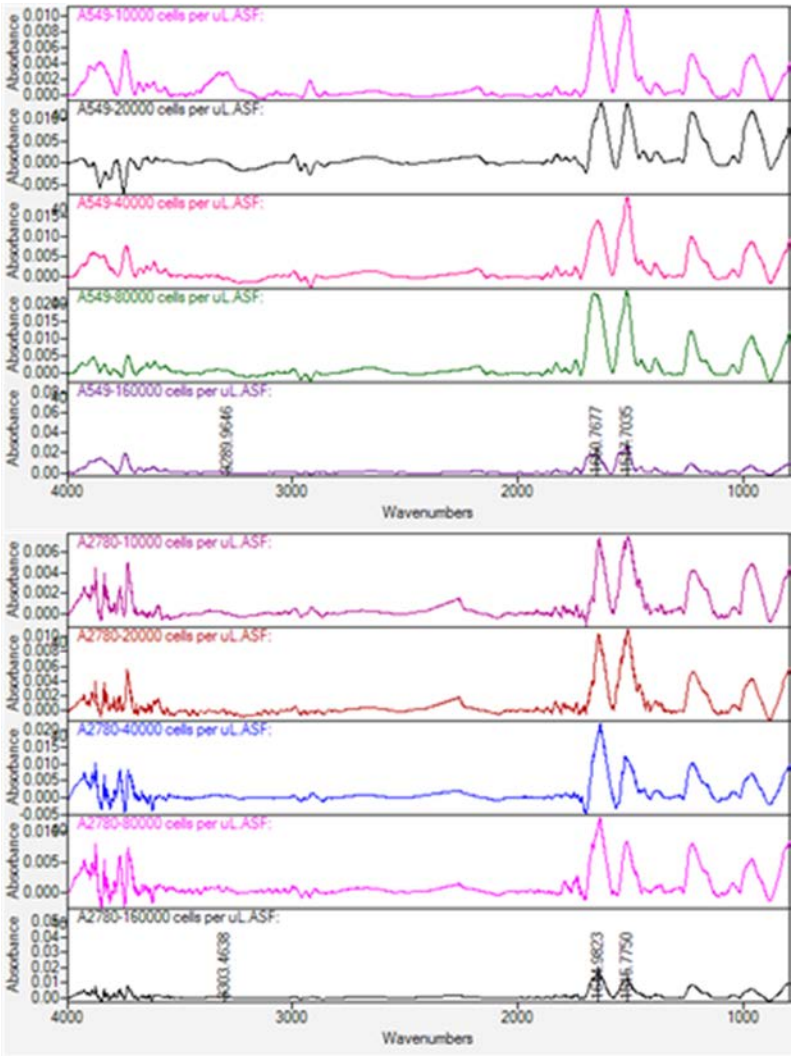

Figure 5. FTIR spectra of [A)] A549 and [B)] A2780 cell lines at different cell concentrations.

The results of this section show that that although sample cell number does not have significant effect on the peak tips and their wavelength location, but do have effect on peaks appearance and their relative high and area under the curve (AUC). Peaks locations are important for the measurement of chemical groups' modifications within the cellular biomolecules, however, peaks heights and AUCs are important to measure the relative concentrations of different biomolecules and chemical groups within the cellular sample. So, sample cell concentration might not be that important when the purpose of the bio-spectroscopy study is to measure the chemical characteristics of different groups in the cells or tissues, but is indeed very much critical when it comes to the measurement of chemical groups appearance or disappearance and relative changes due to interactive parameters like a drug or disease or abnormality.

4. Conclusion

In the current study, the importance of cell density and position on the disk was investigated. Different volumes of cell suspension or different cell densities were placed on ZnSe disk, dried and the FTIR spectra of the samples were collected. We found out that cell density doesn't affect the location of the peaks but it affects the intensity and AUC of the peaks.

Furthermore, placing the cells at the most central part of the disk comes of with the most unique spectra with distinguishable peaks of protein, DNA and lipid regions.

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