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Lipid quantification method using FTIR spectroscopy applied on cancer cell extracts ☆

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ABSTRACT

Reprogramming energy metabolism constitutes one of the hallmarks of cancer. Changes in lipid composition of cell membranes also appear early in carcinogenesis. Quantification of various molecules such as lipids evidences the modifications in the metabolism of tumour cells and can serve as potential markers for cancer diagnosis and treatment. Fourier Transform Infrared (FTIR) spectroscopy is a powerful tool used for the detection and characterization of various types of molecules. This technique remains an attractive approach as it is cheap (equipment and reagents), does not require high grade solvents or expensive internal standards, equipment is widely available in standard laboratories and the method is robust and suitable for routine analyses. In this work we established partial least square (PLS) models based on FTIR spectra able to quantify lipids in complex mixtures such as cell extracts. In the first part, we attempted to build PLS models with FTIR spectra of 53 mixtures of 8 well-characterized pure lipids. Second, the PLS models were verified using FTIR spectra of mixtures that did not contribute to the calibration. The third step was the validation of the models on lipid cell extracts. In order to obtain reference values for cell extracts, high performance liquid chromatography was carried out by AVANTI. The lipid distribution were globally similar with both techniques, PLS models and chromatography. Finally, the models were applied to determine the lipid composition of cells exposed to four treatments. We could not evidence significant changes in the lipid composition of cell extracts after treatment, in terms of polar head groups. However, the models established in this study appear reliable and could be applied for high throughput measurements. This article is part of a Special Issue entitled Tools to study lipid functions.

1. Introduction

Lipids comprise diverse group of compounds that play many key roles in cells. Firstly, they provide structural support for cell membranes. Membrane fluidity controls, at least in part, process such as ligand–receptor interaction, endocytosis and antigen presentation. Secondly, they serve as storage for chemical energy, notably with fatty acids. Thirdly, lipids are involved in various signaling pathways. For example, phosphoinositides and ceramides act as precursors for second messengers notably to regulate cell growth, proliferation and motility [1,2]. The biological functions involving lipids (membrane dynamics, energy homeostasis and regulation of the molecular machinery) concern critical aspects of proliferation.

It is now well established that cancer cells adjust their energy metabolism to fuel the chronic and uncontrolled cell growth. In turn, activation of lipid metabolism is an early event in carcinogenesis and a hallmark of many cancers [2–4]. During mitosis, cells have to provide huge amount of lipids in order to build new membranes. Upregulation of de novo lipid synthesis, especially fatty acid synthesis is thus frequently observed in cancers including prostate and breast cancers [3,5]. Lipid composition of cell membranes seems also to be affected very early when normal cells are transformed into malignant cells. Such modifications have recently been described for breast cancer [3].

Quantification of various classes of molecules evidences the modification in the metabolism specific to tumour cells. These modifications are of particular interest as they could serve as potential markers for cancer diagnosis and treatment. This study is focused on lipids. The diversity of lipids in terms of physical and chemical properties complicates their analysis especially in complex mixtures. There is currently no single analytical method available to record the full lipidome of a cell in one experiment [2,3]. In recent years, the

Abbreviations: IR, Infrared; FTIR, Fourier Transform Infrared; hrs, hours; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; CL, cardiolipin; CH, cholesterol; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; PLS, partial least square; iPLS, interval PLS; PLSR, PLS regression; MTT, 3-[4,5-dimethylthiazol-2yl]-diphenyltetrazolium bromide; RMSECV, root mean square error of cross validation; RMSEP, root mean square error of prediction

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lipidomic approach was thus developed thanks to rapid advances in a number of analytical technologies (mass spectrometry, computational method) [1].

A rapid method that could provide a quantitative evaluation of the various lipid classes is still largely missing. Fourier Transform Infrared (FTIR) spectroscopy is a powerful tool used for the detection and characterization of various types of molecules. FTIR spectra are commonly plotted as the absorbance as a function of the wavenumber (1/wavelength). They contain absorption peaks which appear at wavenumbers corresponding to various modes of vibrations of bonded atoms present in the sample molecules. The absorption wavenumber depends on the relative mass and the geometry of the atoms. Resonance between vibrations depends on molecule conformation and further modulates the spectra. IR spectra contain then a wealth of information on both the chemical structure of molecules and on their conformation. Interestingly, polarized FTIR spectroscopy carried out on lipid monolayers or multilayer stacks provide information on the orientation of the different chemical bonds with respect to the membrane plane [6,7].

Two major distinct regions can be distinguished in the IR spectra of lipids (see Fig. 1). The high wavenumber part of the spectrum (3100–2800 cm^{-1}) includes only contribution from C – H stretching vibrations, which mainly originates from the hydrocarbon chains. The low wavenumber region of the spectrum (below 1800 cm^{-1}) is correlated to the polar head groups of the lipids. The tallest peak corresponds usually to the stretching of the ester (ν(C═O)) and is followed by the phosphate contributions around 1240 cm^{-1} ($\nu_{as}(PO_2^-)$) and 1090 cm^{-1} ($\nu_s(PO_2^-)$).[6] Various polar head groups lead to significantly different IR spectra, hence specific spectral signatures which potentially allow lipid identification and quantification.

IR spectrum provides thus a unique signature of the lipid classes and fine details on the structure. It covers a wide range of applications to obtain detailed information about the structure and organization of biomembranes and lipid assemblies, e.g. to study lipid phase and orientation in liposomal and monolayer systems [6,8].

Within the last ten years it has been demonstrated that the IR spectrum of mixtures allows quantifying its constituents [9,10]. In principle, Beer–Lambert law holds true for common analyte and if the concentration of a component is high enough to contribute meaningfully to the IR absorption spectrum, this component may be quantified. However, various wavelengths are generally required to quantify any component in a biological sample and analytical method based on IR spectroscopy must generally be calibrated using reference spectra and multiple wavelength regression. Recently, Dreissig et al. were able to predict the composition of lipid extracts of porcine brain tissue on the basis of a PLS model. This model was calibrated using as reference IR spectra of various amounts of 2 lipid mixtures with an established composition. The results of the prediction were verified by lipid quantification obtained after TLC. Lipid distribution defined by both techniques were similar [11]. In another publication, they achieved the quantitative determination of free fatty acid contents in lipids extracted from poultry feeds by FTIR spectroscopy [12].

FTIR spectroscopy is also used as a fast spectroscopic method for biochemical analysis of cells and tissues. As exploited in a wide range of examples, IR spectroscopy monitors the global chemical composition of a sample and provides a precise signature of all the cell constituents. For instance, it was recently demonstrated to be a useful tool to observe all metabolic modifications induced when cancer cells are exposed to antitumor drugs [13–19]. Interestingly, drugs known to induce similar types of metabolic disturbances present a comparable signature and appear to cluster when spectrum shapes are analyzed [20,21]. In previous works, we could observe some specific spectral changes due to treatments in a spectral region (1800–1700 cm^{-1}) dominated by the absorption of lipids. Given that carcinogenesis affects cell lipid metabolism, anticancer treatment could also have consequences on lipid distribution. Therefore, the question of whether drugs affect or not the lipid composition of cells is of crucial importance.

To investigate this subject, PLS (partial least square) models were established for lipid quantification that would be suitable for cell lipid extracts. This study was divided in four parts. In a first step, we attempted to calibrate PLS models with IR spectra of mixtures of pure lipids. Second, the PLS models were validated with IR spectra of mixtures that were not included in the calibration. The third step consisted of verifying the applicability of these models on cell lipid extracts. In the last part, these models were applied on total lipid extracts originating from cells untreated and treated with four anticancer drugs.

Antitumor drugs belong to two classes characterized by a unique mechanism. The first class, the antimicrotubules (paclitaxel and vincristine), affects in particular microtubules and blocks the formation or dissociation of the spindle apparatus which separates the chromosomes into the daughter cells during cell division [22–24]. The second class includes doxorubicin and daunorubicin; they inhibit class II topoisomerases progression, preventing the DNA double helix from being resealed and therefore stopping cell division [25,26]. In addition to these main targets, a few publications indicate that these drug and especially antimicrotubules could affect lipid metabolism [27–29].

2. Materials and methods

2.1. Lipids and lipid mixture preparation

The following lipids were purchased from Avanti Polar lipids (Instruchemie, The Netherlands) and used without further purification: L-α-phosphatidylcholine (Egg), Cardiolipin (*E. coli*), L-α-phosphatidylinositol (liver, bovine), L-α-phosphatidylserine (brain, porcine), sphingomyelin (brain, porcine) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). They were all received solved in chloroform. Triolein and cholesterol were purchased powdered from Sigma-Aldrich (Bornem, Belgium) and dissolved in chloroform. They were all stored at −20 °C. Phosphatidylcholine (PC), cholesterol (CH) and DOPE were preserved at a concentration of 25 mg/ml, Triolein at 100 mg/ml and cardiolipin (CL), phosphatidylinositol (PI), phosphatidylserine (PS) as well as sphingomyelin (SM) were stored at 10 mg/ml.

Various amounts of pure lipids were combined to train and validate the models based on IR spectra. Eight blends were prepared, each in the absence of one of the lipids. Starting from these mixes, increasing volumes of the missing lipid were added to obtain a variation of this lipid. Table 1 shows the example for PC. A total of 53 mixtures were prepared. The composition of all the mixes is presented in Annex 1.

2.2. Cell culture and treatment

The human prostate cancer PC-3 (ACC 465) cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and was maintained according to the supplier's instructions. The cells were incubated at 37 °C in sealed (airtight) Falcon plastic dishes (Cellstar, Greiner Bio-one, Wemmel, Belgium) in a humidified atmosphere of 5% CO_2. The cells were kept in exponential growth in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2% penicillin/streptomycin (an antibiotic solution). Cell culture medium, FBS and antibiotics were purchased from Lonza (Verviers, Belgium). Cultures were tested twice a month for mycoplasma infections using PlasmoTest from InvivoGen (Toulouse, France).

Cells were grown to sub-confluence i.e. ca. 80% of confluence as defined elsewhere [30] and were incubated with four anticancer drugs used in clinics (daunorubicin, doxorubicin, paclitaxel, vincristine) during 24 h at their IC_{50} concentration. The IC_{50} is defined as the concentration needed to decrease the cell growth of the cancer cells by 50% after 72 h of culturing. For the drugs used in this study, the IC_{50}