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Whole Cell Mass Spectroscopy (WCMS) is a method that has gained popularity in the last decade. WCMS saves time by omitting extensive sample preparation for mass spectrometric analysis of microorganisms. WCMS has been mainly used for profiling and identifying fungi, bacteria and mammalian cells. We utilized WCMS as an assay for differentiating in vitro cellular toxicity. In this study we focused on using HepG2 cells, a liver carcinoma cell line, for WCMS profiling of untreated cells and dosed cells with known toxins. We have optimized the parameters for using two different mass spectrometers and obtained accurate and sensitive profiling of the whole liver cells. To verify the toxic response at the time of WCMS, we tested our cells with the traditional cytotoxicity assays which included lactate dehydrogenase (LDH) or MTT assay(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). This research lays down the groundwork for a technique with great applications in pharmacology, toxicology, and medicinal chemistry.

WHOLE CELL MASS SPECTROSCOPY AS A CYTOTOXICITY SCREENING METHOD

by

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CHAPTER I

INTRODUCTION

Specific Aims

Unexpected liver toxicity is one of the main causes for drug trial failure. In toxicology the preliminary risk assessment of a given compound relies on *in vitro* cytotoxicity assays. The conventional *in vitro* cytotoxicity assays study the effects of a given compound by examining the dose and time dependence to a model cell organism. These cytotoxicity assays present a somewhat limited approach to observe a systemic response, where the cell viability is measured based on the activity of certain proteins, enzymes, or organelles after a specific dosage or time exposure. To understand the specific *in vitro* toxic mechanisms a battery of assays need to be performed. After which, the findings of in vitro analysis do not always correlate with the *in vivo* or the trial-stages of a drug development. The use of whole cell mass spectrometry can accelerate the risk assessment process by providing a quick systemic analysis of a given xenobiotic. While traditional cytotoxic assays present a specific organelle or enzyme response which can provide limited information.

Nanomaterials have potential applications in the pharmaceutical, food, chemical and many other industries[1]. By 2016, the expected market demand of nanomaterials is $ 5.5 billion. Gold nanoparticles are of particular interest due to their unique physical and chemical properties. In the biomedical arena, gold nanoparticles have promising applications as a drug or nucleic acids delivery system. With respect to the chemical and physical properties, nanoparticles fall in a novel category in terms of their behavior. Nanoparticles behave neither like small molecules nor like bulk materials.

Gold nanoparticles are easy to synthesize, and modify to meet a desired objective, yet this accelerates the pace in which a new compound is created. Toxicology risk assessments have yet to catch up with the myriad of nanoparticles and their potential applications. The MTT and the lactate dehydrogenase assays are considered the gold standards in cytotoxicity risk assessment. Gold nanoparticles can interfere with these two assays as they can adsorb key molecules of the assay and/or absorb or scatter specific wavelengths used in either of these assays depending on the gold nanoparticle used. This presents the necessity for a method that does not rely on UV-absorption. Mass spectrometry can bridge this gap with a rapid method that does not depend on a single mechanism or reagent, and can provide a whole cell assessment.

The purpose of this research is to develop a high throughput cytotoxicity screening using Matrix Assisted Laser Desorption/Ionization Time of Flight Mass

Spectrometry (MALDI-TOF-MS). To develop such a method we first have to optimize the instrument conditions, define the limits of detection, and determine the sample preparation method. We used HepG2 cells, an in vitro model of liver carcinoma cells. We hypothesize that untreated HepG2 cells will have a unique mass spectral profile, which will vary after exposing cells to different toxins. We expect to design a revolutionary methodology which can comprehensively examine the toxicological effects of gold nanoparticles and other well established toxins, thus speeding the risk assessment process.

Method optimization to develop a reproducible profile of Whole HepG2 cells in MALDI Mass spectrometer quadrupole time of flight

Obtaining a reproducible mass spectral signal from cells without a digestion, separation, or isolation is a difficult task due to the biochemical complexity of the samples. We will be loading whole cells to the mass spectrometer; which presents high variability within the sample.

To achieve a high throughput method, we aim to minimize the processing steps of cells before the mass spectrometry analysis. We understand that we could not obtain a signal with just direct raw samples for which we have to compromise with some sample preparation.

We hypothesize that we can obtain a reproducible signal for signature mass spectra profile of intact HepG2 cells by optimizing the sample preparation

and acquisition protocols. We expect to develop a standard protocol for mass spectra analysis of untreated HepG2 cells.

- Study 1.1: Instrument optimization for analysis of mammalian cells
- Study 1.2: Effects of sample preparation on cell viability and signal
- Study 1.3: Sample matrix and loading effects on cell signal

Whole cell mass spectrometry as a methodology to test nanomaterial and known toxins cytotoxicity using *in vitro* models

We hypothesize that HepG2 cells will generate a unique mass spectra profile signature depending on the toxin and exposure. We will use well known toxins (aflatoxin B_1, acetaminophen) and verify their cytotoxicity with assays like MTT, Lactate Dehydrogenase, and Tryptan Blue cell viability assessment. We will also examine the cytotoxic effects of gold nanoparticles, whose toxicity is not well understood. We expect to obtain distinct mass spectra for the well-known toxins, an elucidation of a potential nanoparticle toxic dose which would have not been achievable through the traditional toxicology screenings.

- Study 2.1: Evaluate cytotoxic effects of known toxins with traditional lactate dehydrogenase and MTT assays
- Study 2.2: Cross reference a novel compound (gold nanomaterials) effects on HepG2

Intact cell mass spectrometry (ICMS) or whole cell mass spectrometry (WCMS) is an emerging field that arose in the last seventeen years. The

technique was developed to identify microorganisms without the need of trypsination or separation by minimally preparing and directly placing an entire cell sample to be analyzed by a Mass Spectrometer (MS). The use of intact cell mass spectrometry has become a well-established technique for microorganism identification[2].

Other than the use of WCMS, the traditional identification of organism method which uses mass spectrometry relies on generating a mass spectral profile (finger print) after extensive preparation where the cells have gone through isolation and purification techniques like trypsination which generate a wide array of peptides, lipids or oligonucleotides. A selected group of masses measured in the mass spectrometer serve as biomarkers for identifying a given organism. The traditional identification approach has the drawbacks of being time consuming, cost restrictive and give a somewhat fractionated view. Fractionated view in the sense that the sample has to be purified, or separated in some form prior to analysis. The interested party may lose important information of the sample through this purification.

Whole cell mass spectrometry (WC-MS) analysis has been primarily used to identify bacteria [3-7], fungi [8, 9], and a limited extent in vivo and in vitro mammalian cells [2, 10-12]. In a similar manner like the conventional MS identification method, WCMS relies on the generation of a unique array of mass spectral peaks (finger print), which can be correlated to genus and species of the

questioned organism[8]. This new identification technique has ample applications in the diagnostics, taxonomical, pharmaceutical and even biodefense fields[13]. For whole cell analysis, the instrument of choice tends to be the Matrix Assisted Laser Dissociation Induced Mass Spectrometer (MALDI MS)[2, 4, 5, 7-10, 14-25]. MALDI MS is used for several reasons, but the two main drives tend to be the broad mass range, and a relative salt tolerance.

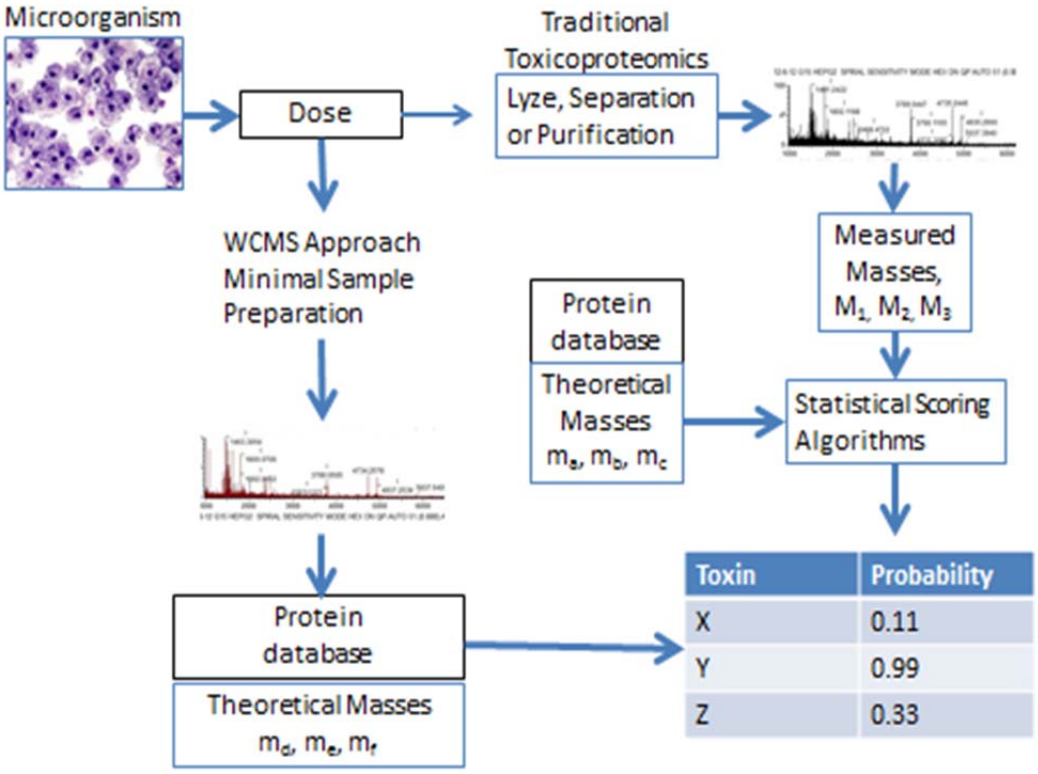


Figure 1. Flow chart of the common approach taken to study cytotoxicity using Mass Spectrometry. Whole cell mass spectrometry aims at minimizing the time and cost consuming steps of separation, lyzing or digestion [26].

In an attempt to build on the microorganism identification WC-MS technique we intended to branch out into the field of toxicology. We hypothesized that evidence of cytotoxicity can be detected using whole cell mass spectra analysis, as the conventional fingerprint technique has been used to determine cytotoxicity in *in vitro* cell samples[26, 27] and identification is attainable using WCMS based on a the conventional technique, we assume we could use this technique for toxicology. Our initial goal was to develop a technique which could ultimately be used for blind screenings as it can be seen in Figure 1. The main difference between the conventional MS toxicity screening and our proposed method lies in the sample preparation before the MS analysis. Our proposed method will not rely on the use of trypsin, chromatography or other digestive/ purifying methods commonly used in the conventional MS analysis Figure 1. The traditional cytotoxicity screening compares masses measured from the fingerprint with theoretical masses and a scoring algorithm will determine the probability that the microorganism has been exposed to a given toxin. If our proposed method would use a similar approach, it could not rely on previous theoretical masses, for which a new database will have to be developed.

A dosed cell will generate a different MS profile spectra than the control cells which have not been dosed. We worked under the assumption that a xenobiotic (foreign chemical) induces a molecular alteration in a given model cell organisms which we could then measure using MALDI-MS. The molecular alteration mass spectra could then be stored in a database and used as a

reference for potential blind screenings. A cytotoxicity assay using WC-MS would be advantageous since it operates with a small number of cells and is cost effective, and high through put.

The purpose of this research is to develop a cytotoxicity assay using Matrix Assisted Laser Desorption/Ionization quadrupole Time of Flight Mass Spectrometry (MALDI-qTOF-MS) for the detection of *in vitro* cytotoxicity. This toxicological Whole Cell Mass Spectrometry method (TWCMS) method was compared with well-established cytotoxicity assays, like the (MTT) and lactate dehydrogenase (LDH) assays. The advantage of using mass spectrometry (MS) lies in the sensitivity, resolution, type of detector and time efficiency which cannot be achieved through the conventional UV -absorption methods. This research intends to provide the groundwork for the development of a methodology with potential uses in toxicology and diagnostics arena.

The field of whole cell mass spectroscopy has had the challenge of obtaining reproducibility among different labs and instruments [15, 28]. The sample preparation also varies among labs and instruments. Many of the whole cell[1] mass spectroscopy studies have used bacteria as their model organism [29-32], adherent mammalian cells present a greater challenge due to their biochemical complexity and their clumping nature. To obtain reproducible spectra, it is

[1] The term Intact cell mass spectroscopy is used interchangeably with whole cell mass spectroscopy to refer to a similar technique.

necessary to have a meticulous protocol and rigid sample preparation [4]. Factors that influence the reproducibility in the sample preparation are: type and concentration of MALDI matrices, the number of cells used, and the washing steps [31]. Prior to optimizing the sample preparation steps we need to determine the optimal instrument conditions.

Study 1.1: Instrument optimization for analysis of mammalian cells

This research has had the opportunity to work with two MALDI-MS-TOF instruments. The preliminary work was done using a linear setup, while the bulk of the work was done with an instrument that had an orthogonal time of flight tube and a quadrupole in front of the analyzer. The tandem quadrupole –time of flight analyzer (qTOF) with the orthogonal setup provide advantages which the linear mass spectrometer cannot achieve. A qTOF setup has higher sensitivity and can reach a resolution of around 10000 full width half maximum (FWHM). The mass accuracy of the instrument is also very high reaching 5 to 10 ppm. Additionally qTOF allows to read a mass range up to 20000 m/z [33] p.180. However, despite the high efficiency provided by the orthogonal qTOF, the duty cycle is less than in the linear setup therefore losing some of the ions produced at the source [33] p.140. We tested different instrument variables in the MALDI HD Synapt G2 qTOF among which were sensitivity, resolution, laser intensity, and the quadrupole settings. The first three were fairly straight forward, and can be adjusted according to the sample necessity. Though, the quadrupole setting was a bit trickier to setup.

The quadrupole in the instrument we used for assay development is in front of the TOF analyzer. This quadrupole placement was designed to improve the signal to noise ratio, which can present us with a challenge since the transmission of ions is not as broad range as in a linear setup of the 4700 MALDI Mass Spectrometer. To allow a high transmission and a lower loss of ions to the time of flight analyzer, we did not apply the DC resolving voltage to the quadrupole. The DC voltage is suppressed by using the MS acquisition mode in the software [34].

To optimize the largest range of transmission we experimented with the quadrupole manual profile using necrotic cells, a PEG calibrant, and a control healthy sample. We aimed to examine which setting would provide the best signal and broadest ion transmission. We systematically modified the quadrupole manual profile's ramp and dwell time in order to have a broader range of transmission and avoid biomarker peak suppression. Based on previous work on mammalian whole cell mass spectroscopy our interest mass range was in 1500 -20,000 Da. This mass range hints that our biomarker peaks are peptides or fragmented oligonucleotides and not whole proteins.

Study 1.2: Effects of sample preparation on mass spectra signal

The sample preparation is another key element in obtaining a reproducible mass spectra signal. We aimed to minimize the number of steps before loading the sample to the MALDI-plate to obtain a high through put analysis. We

recognized that placing raw samples brought too much noise in the spectrum due to the biochemical complexity nature of cell sample. Therefore we had to include washing steps before loading our samples to the MALDI mass spectrometer. The purpose of the washing steps is to remove any leftover cell culture medium, trypsin, fetal bovine serum and salts which increase the signal to noise ratio. The washing-steps recommended in the literature are rinsing with 1x Phosphate Buffer Solution (PBSx1)[11], water[35], 150 mM ammonium acetate[16] or a combination of these rinses.

To the best of our knowledge, HepG2 intact cell or whole cell mass spectroscopy has not been reported in the literature, reinforcing the need to evaluate the washing steps, since no washing results in a noisy spectrum, while washing 3 times with PBSx1 followed by 3 washes with ammonium acetate results in 100 % cell death. Initially, we tried to minimize cell death by the washing steps, since we believe it would be impractical when you are trying to develop a cytotoxicity mass spectral assay. Though as our worked progressed, we realized that having viable cells at the point of the mass spectral analysis is unattainable, just by the very nature of the acid matrix that destroys the integrity cell. For this reason the author would recommend the disuse of the label *Intact cell mass Spectroscopy* and favor the use of whole cell mass spectroscopy, as both are used interchangeably in the literature.

The cell viability aspect is of extreme importance; since a whole cell toxicity mass spectral assay would is not able to determine cell viability, which is important in field of toxicology and cell culture in general. Rather, whole cell mass spectral cytotoxicity assay would be used to determine a systemic molecular modification brought upon by exposure to a xenobiotic.

Cell number

For any assay, method or technique it is important to know what is the working range. What are the upper and lower limits of detection? In this case the number of cells/μL of matrix which we can measure reasonably.

Washing Steps

To test for the effect on mass spectral signal by the cell washing steps, we harvested HepG2 p. 4 them using trypsin, and compared using a final confluence of cells ranging from 580-1160 cells/μL. To minimally rinse our cells from impurities we tried washing our samples in the following manner:

a) Rinse with only PBSx1 (0°C)
b) Once with PBSx1 (0°C) followed by one wash with 150 mM (0°C)
c) Three washes with PBSx1 (0°C) followed by three washes with 150 mM (0°C)
d) Once with PBSx1 (0°C) followed by three washes with 150 mM (0°C)
e) Once with PBSx1 (0°C) followed by three washes with DI H_2O (0°C)

Study 1.3: Sample matrix and loading effects on cell signal

Matrix Selection

We have to determine which MALDI matrix is optimal to obtain a consistent spectrum from healthy HepG2 cells. We had narrowed it down to sinapinic acid and α-cyano cinnamic hydroxyl acid, the main difference between these two lie in the mass range for which the matrix is best suited. If we want to keep the spectrum below 10,000 Da range, we work with the α-cyano matrix, while sinapinic acid can be used if we desire to examine larger masses. The α-cyano matrix has been found to be more reproducible from spectra to spectra [35].

Loading Effects

Extensive work previously been done addressing the manner in which the cells are loaded onto the MALDI plate and the effect this has on the reproducibility of the signal. The loading method has varied among different research groups with techniques ranging from the mix-volume method [11], dried-droplet deposition, sandwich method [2] or the use of slightly more sophisticated instrumentation like a nebulizers [35] to load to the plate. Each variation of the method is intended to optimize matrix/sample crystallization which would enable ionization of the large molecules for the mass spec analysis.

Due to the extensive research in the MALDI loading technique we did not dove into exploring what the optimal sample loading technique on the MALDI

plate. We did quickly dabble with the sandwich and the dry droplet techniques. Neither of these two techniques produced any positive significant differences among them, for which we stuck to the mixed method technique for our protocol.

Overriding the cell detachment or trypsination

One of the goals of this project was to develop a high through put assay. The whole cell assay sample preparation was not as time consuming like SDS PAGE or other separation techniques; yet, it was still taking us 6 hours of sample preparation. The extensive manipulation along the way of preparing the sample made the procedure highly prone to human or methodological errors. To minimize the error exposure and time requirement, we experimented with an ambitious protocol modification overriding the cell detachment and centrifugation steps of the initial sample preparation. The comparison of the initial and modified sample preparation protocols can be observed in Figure 2 and Figure 3 respectively. We aimed to minimize human manipulation of the samples and reduce the time required to prepare the samples.

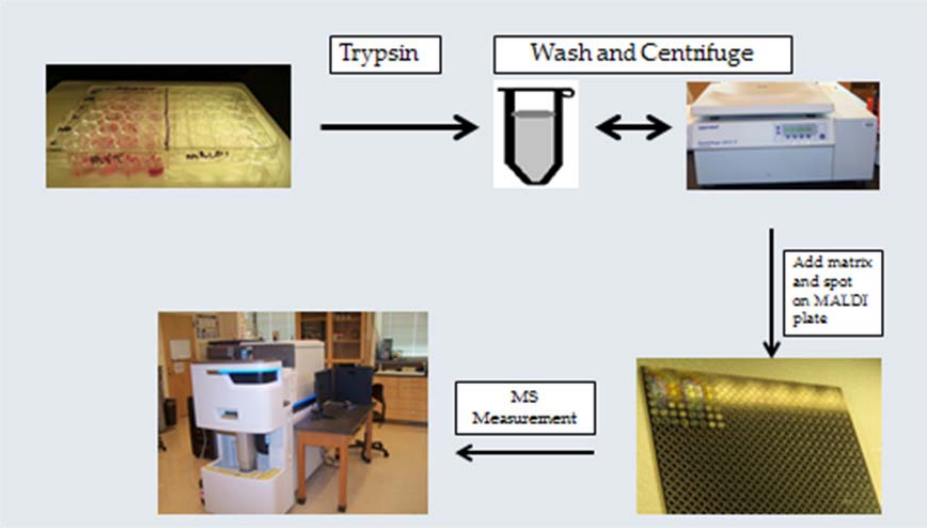


Figure 2. Flow chart of the initial sample preparation for TWCMS. This initial method required harvesting the cells from the well plate, and several steps of washing, centrifuging, decanting and re-suspending.

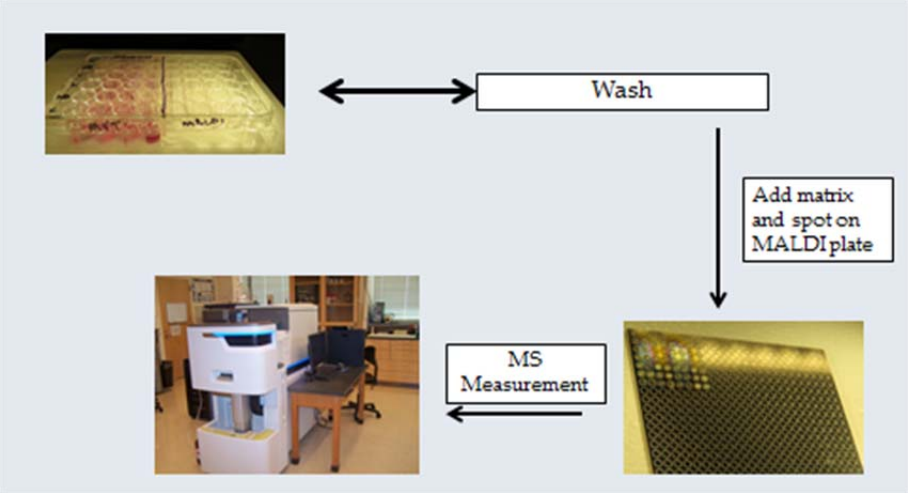


Figure 3. Improved sample preparation method,which skipped the detaching and centrifugation steps.

Aim 2: Whole cell mass spectrometry as a methodology to test nanomaterial and known toxins cytotoxicity using in vitro models

The liver is an important organ in the activation, metabolism and elimination of any given compound entering the human body. The detoxification or intoxication is a result of the xenobiotic interaction with the iso-enzyme family of cytochrome P 450 [36, 37]. We chose to study liver toxicity since unexpected hepatotoxicity frequently halts drug development during first trials [26].

For this study we chose to work mainly with HepG2 cells, a cancerous immortalized cell line originally from hepatocellular carcinoma disease of a fifteen-year-old patient. We were using cancerous cells as a basic model for the development of the methodology; once the methodology is developed and shown to be robust we will apply it towards noncancerous cells. Though, the use of cancerous cells can highlight potential cancer treating compounds [38].

Our first aim was to establish the protocol for the whole cell mass spectral analysis. Once we determined what the steps were, we continued to see if we could detect cytotoxicity using this MS technique. To do so, we needed reference compounds which would induce toxicity on our cell model to later be analyzed using the MALDI qTOF.

The poison is in the dose, everything is toxic. This is the fundamental axiom of toxicology where any xenobiotic (foreign chemical) can have detrimental effects depending on the concentration, dosage or exposure. Different kinds of

chemicals will produce different kinds of toxic responses. There are xenobiotics that can have more than one type of toxic response. The fundamental question on our research is if we can detect or notice a difference between the mass spectral profiles of a control cell versus cells that have been exposed to a chemical at a toxic dose.

To determine whether or not we are administering a toxic dose to our cell organisms we used cell viability assays. To determine cell viability we primarily used the MTT assay; we also used the LDH and tryptan blue cell viability assay. LDH seemed appealing since the measurements can go in parallel with the MS and the MTT measurements, and both procedures decant the medium as an initial step. Though the LDH was disfavored due to time demand and it has been reported that nanoparticles can interfere with the LDH stability. The LDH assay, an enzymatic reaction monitored through UV, might not be suitable for a toxicology analysis of compounds from the emerging field nanoscience [39].

Study 2.1: Evaluate cytotoxic effects of reference toxins with MTT and LDH assays

We aimed to use whole cells in our TWCMS analysis, reducing the processing time compared to other MS based cytotoxicity screenings. We expected to obtain unique toxicological mass spectral profile for each type of cellular response to any given xenobiotic. An approach inspired by the method which uses mass spectrometry for microorganism identification. Mass spectrometry microorganism identification relies on specific proteins, peptides,

oligonucleotides or lipids peaks to generate a unique mass spectra profile. A handful of these peaks are used as biomarkers and stored in a database.

The aim of doing whole cell MS analysis is to minimize the preparation time, and still obtain specific protein biomarker peaks without the need of the arduous sample preparation. Using the digestion approach, research has shown unique toxicological cellular responses represented through biomarker peaks from multiple drugs on HepG2 cell lines [26]. In the same manner, as intact cell mass spectroscopy microorganisms identification arose from models which used digestions or separations, we aim to obtain toxicity biomarkers peaks from HepG2 with intact cell mass spectroscopy. Whole cell or intact cell MS analysis is the technique on which we will base our toxicity method.

The use of intact cells or whole cell analysis as a toxicological tool has only been approached by Dong et. all 2011, who claimed they were able to differentiate apoptotic cells from healthy cells. [11, 40]. Dong et. al. 2011 had a high level of reproducibility and claimed to be the most rapid approach towards the detection of apoptosis in mammalian cells. The aforementioned study was correlate the advent chemically induced apoptosis (programed cell death) with the expression of unique MALDI TOF MS peaks [11]. This paper was ground breaking since early development of the WCMS field, researchers claimed that the detection of virulence or toxicity would not be possible using WCMS analysis[15].

Despite the innovative approach of Dong's group, we would disagree that what they observed was specifically the event of apoptosis. Dong et all claimed they were unable to distinguish between necrosis and healthy cells, where they induced necrosis to HeLa cells by physical disruption through freezing and sonication. The group was able to distinguish apoptosis, after incubating the cells for twenty four hours with apoptposcamptothecin, etoposide, and andrographolide. These three chemicals are known to induce apoptosis and were confirmed of doing so by flow cytometry. Our disagreement stems in that Dong was measuring the systemic molecular alteration after exposure to the chemical rather than having a technique which measures apoptotic versus healthy cells. This group did not use chemical to cause necrosis, and were unable to distinguish between the healthy cells, while for each of the apoptotic inducing chemicals had a slightly different mass spec profile. Therefore the difference that they were measuring was either the ultimate toxicant, or the modified molecular target, and not a symptom of apoptosis.

The idea of the molecular alteration has been presented frequently throughout this document as we aimed to show that exposure to different chemicals will have a response which we can detect through different mass spectra profiles. We aim to use the MALDI- qTOF-MS to detect healthy HepG2 cells from cells going through different toxic cellular responses produced by the exposure to different chemicals. This work will potentially lay the ground for the development of an MS toxicity-response database, which can be used for a quick

toxicity screening of any given compound being developed. Though, for the development of such a database it is necessary to have an agreement of a rigorous protocol for sample preparation and analysis among different labs and for each cell line. This might be unpractical or even unfeasible, nevertheless this technique could be used as an accompanying method for toxicologists who are trying to assess molecular responses on a given organism.

MTT assay

The MTT assay is considered an *in vitro* toxicity gold standard for the screening of a compounds effect on the cell viability. The MTT is colorimetric assay that was developed by Tim Mossman in 1983 [41]. The assay is based on the UV absorbance of the formazan crystals at wavelengths of around 570 nm. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reacts only with living cells, since only living mitochondria has the necessary mithocondrial reductase which cleaves tetrazolium ring into an insoluble formazan crystal (Figure 4). [41]

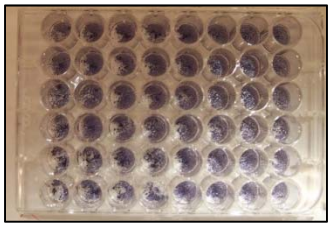


Figure 4. Example of a plate with HepG2 cells which have been incubated with MTT solution.

To evaluate the working range of the MTT we performed serial dilutions of cell into the wells in a 48-well plate. The upper limit of cells can be adjusted accordingly with the volume or concentration of the MTT solution. The minimal amount of cells we needed for a consistent reading was 10,000 cells per well (Figure 5). When dealing with an expensive cell line or very low number of cells, the MTT assay can be restrictive to evaluate cytotoxicity. As it will be further presented, the MS assay we are developing works with very few cells highlighting one of the advantages this alternative MS assay.

The MTT is a reliable assay for measuring *in vitro* cell viability, though this assay is challenged by the other factors besides the low cell number previously mentioned. One limitation is that the assay correlates cell viability with mitochondrial reductase activity. Where a given compound can solely inactivate or enhance the mitochondrial activity and the plate reading will be interpreted as cell viability or increase [42]. A second limitation lies in the physical/optical properties of the compound been screened, as they can interfere with the plate reader since they can absorb at wavelengths also ranging from 500-600 nm. [43, 44].

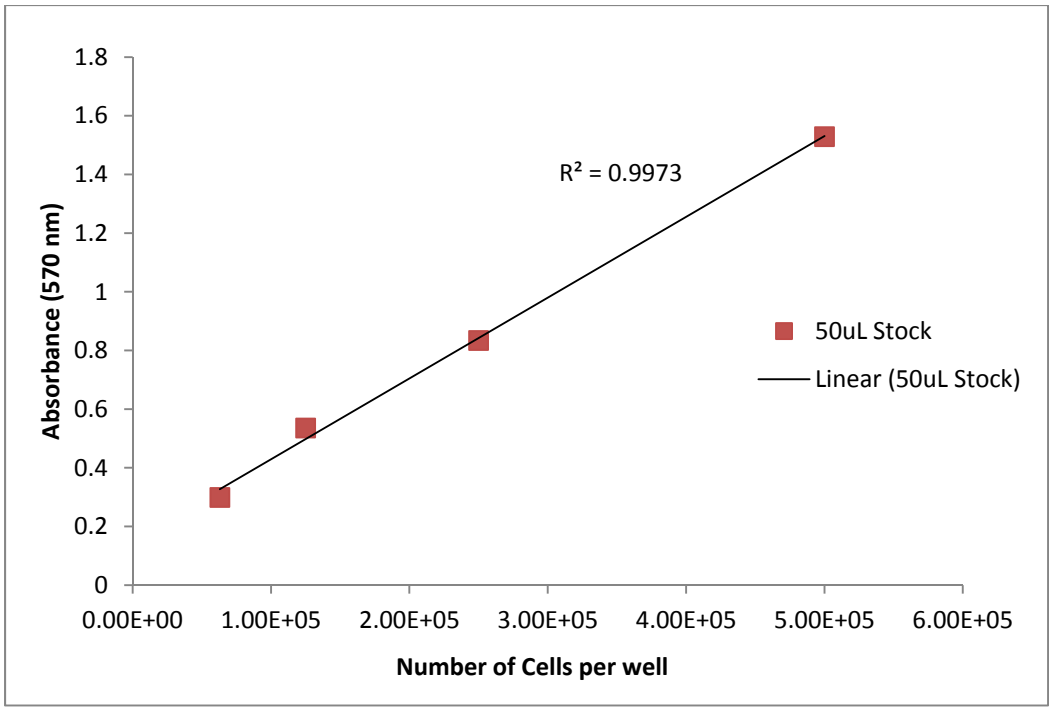


Figure 5. Calibration of the MTT assay in 48 well plate.

LDH assay

The lactate dehydrogenase (**LDH**) assay is based on the change in absorbance of the LDH catalyzed reduction of NADH to β-nicotinamide adenine dinucleotide (NAD^+). Most damaged eukaryotic cells release extracellular LDH [42]. Therefore the LDH activity can be used as a cytotoxic indicator, this activity can be measured through the change in absorbance of NADH at 340 nm [39]. Some nanoparticles can interfere with the LDH stability, for which the LDH might not be suitable for a toxicology analysis of compounds from this emerging field [39].

To perform the LDH assay it is necessary to have the pyruvate and NADH as reagents. The assay is a kinetic assay which studies the reduction to β-

nicotinamide adenine dinucleotide, where lactate dehydrogenase is the catalyst of this reduction. Using a UV spectrophotometer the reduction rate can be quantified. The reduction-rate ratio between of the dose and control cells are compared giving the percentage of LDH that was released.

$$\mathrm{NADH} + \mathrm{Pyruvate} \xrightarrow{\mathrm{LDH}} \mathrm{NAD}^{+} + \mathrm{Lactate}$$

Study 2.2: Cross reference a novel compound (gold nanomaterials) effects on HepG2

To examine the method developed we experimented using a compound which has commonly been referred to as nontoxic due to its inert nature, this compound is gold nanoparticle. We hypothesized that we would be able to detect signs of toxicity at lower doses than what traditional methods like MTT and LDH are able to detect. We used gold nanoparticles with an average size of 12 nm, enabling physical endocytosis[44] and potentially triggering toxic pathways. Some nanomaterials like carbon nanotubes have shown to interfere with this assay [43], reinforcing the necessity for an alternative rapid method for screening nanomaterials.

Gold Nanoparticles

Nanoparticles (NP) have promising uses in therapy and diagnosis, cosmetic and food industry. NP can be used as delivery agents for drugs or nucleic acids inside the cells. Gold nanoparticles (AuNP) have a lot of potential applications since they are easy to make, chemically stable, and have unique optical properties [44]. Despite the promising uses of nanoparticles, the toxicological effects on humans and the environment remains relatively unknown [44, 45].

An important element in the development of this new MALDI-TOF-MS toxicity method lies in the optical properties of gold nanoparticles. Gold nanoparticles can scatter light in the visible spectrum at varying wavelengths depending on their physical characteristics [46] they can also interfere with fluorescence, or light absorbance based assays such as the LDH and the MTT. Additionally, gold nanoparticles can adsorb molecules from the cell medium involved in the redox reactions of the traditional cytotoxic assays [44]

CHAPTER II

CHEMICALS AND METHODS

Cell Culture

HepG2 cell line was obtained from ATCC. The cells were thawed from a liquid nitrogen container, and suspended in 12 mL of Dulbelcos Eagle Medium (37°C) with 10 % Fetal Bovine Serum and Penicillin Strip. The cell suspension was then transferred to 75 cm^2 incubation flask. The cell flask was incubated at 37°C and 5% CO_2. The medium was changed twice per week. When cells reached a confluence of <90% they were subcultured into 3 incubation flasks or well plates corresponding to the experiment on hand.

Subculturing the cells

At 95 % confluence cells were subcultured, into further flasks or well plates. The flasks were decanted from medium washed using PBSx1 at 37°C.

MALDI solutions

TFA (0.1%)

Decanted 10 µL of triflouroacetic acid (Fluka 91703) into 10 mL volumetric flask (Pyrex A), diluted to 10 mL with DI H_2O.

Chemicals

1:1 (ACN: 0.1 % TFA)

Mixed 5 mL of acetonitrile (Fisher Biotech Lot. no. 024930 with 5 mL of TFA (0.1%

Sinapinic acid matrix

Weighed 0.010 g of sinapinic acid (Fluka 85429), diluted it in 1 mL of 1:1 ACN:0.1 %TFA. The solution was filtered through a 0.22 µm filter.

CHCA matrix

Weighed 0.0122 g of α-cyano-4-hydroxycinnamic acid (Fluka 70990), diluted it in 1 mL of 1:1 ACN:0.1 %TFA. The solution was filtered through a 0.22 µm filter.

150 mM ammonium acetate

Weighed 0.1137 g of ammonium acetate (Fluka 09688), diluted it in 10 mL of DI H_2O.

PEG MALDI calibration standard

This mixture was formulated and provided by Triad Mass Spec Facility. Each stock was diluted to 10mg/mL (in 1:1 Water:ACN), 10 µL of 1000 PEG, 10 µL of 2000 PEG, 20 µL of 3000 PEG, 40 µL of 4000 PEG, 60 µL of 6000 PEG, 60 µL of 8000 PEG, plus 30 µL of 2 mg/mL NaI (in 1:1 Water:ACN).

MALDI Protocol

The goal is to have between 500-1000 cells / µL of Matrix on the target MALDI plate. Preferably the sample should not have cell agglomeration, and wash the cells accordingly to remove the salts from the medium.

Sample washing

1. Harvest cells using trypsin EDTA 0.05 %
2. Centrifuge harvested cells at 1000 RPM (4 °C, 5-10 min). From this step on you should keep the sample and solutions in ice bath or 0°C.
3. Count cells using Hepatocytometer
4. Transfer one million cells to start the washing process.
5. Wash cells and re-suspend with 1xPBS, centrifuge 1000 RPM, repeat twice
6. Wash cells and re-suspend with 150 mM ammonium acetate, centrifuge 1000 RPM, repeat twice
7. Decant solution and re-suspend in the matrix, serial dilution Performing another cell count is advisable since cells might have been discarded in the washing process.
8. Load 0.5-1 µL to the MALDI plate, preferably with α-CHCA matrix.

MTT assay

The supernatant medium of the wells was decanted and replaced with medium containing the MTT solution and minimal fetal bovine serum. We incubated for two to four hours after which we decanted the medium containing the MTT solution. The purple formazan can be seen at the bottom of the well plates Figure 4. The purple formazan is diluted with a strong organic solvent or detergent. The UV absorbance readings with a Gen5 plate reader at 570 nm. The control well absorbance value serves as a reference for the dosed samples.

CHAPTER III

AIM 1: METHOD OPTIMIZATION TO DEVELOP A REPRODUCIBLE PROFILE OF WHOLE HEPG2 CELLS IN MALDI MASS SPECTROMETER QUADRUPOLE TIME OF FLIGHT

Results of Study 1.1

The manual setting on the quadrupole profile has the following input variables:

Table 1. Represents the input table in the tune page software to setup the manual quadrupole profile options found in the instrument software of the MALDI-qTOF synapt G2. These numbers are the ones that gave us the broadest ion transmission.

Mass	Dwell Time (% Scan Time)	Ramp Time (% Scan Time)
1. 1000	5	15
2. 4000	55	25
3. 8000		

As we can see in Figure 6, the ions will shift depending on the quadrupole profile. The three spectra were taken from the same spot/sample of a HepG2 sample. When setting up the quad profile the mass entry is of primordial

importance. The auto quad profile tends to favor the masses below 3000 Da. We aimed at having a transmission between 1500 and 20000 Da, therefore the auto quadrupole profile would not be favorable. We tried to promote masses above 3000 Da by making the minimal mass be 3000 Da (top spectra). We did get a better transmission of the higher mass ions though the ion suppression is too drastic for the masses below 3000 Da. Maximizing the second mass (bottom spectra) on the input table had similar ion transmission and suppression as in the auto quad profile.

To further evaluate the ion transmission and suppression of the quadrupole configuration, we experimented using a polyethylene glycol (PEG) standard. An example of these experimentations for ion transmission can be seen in Figure 7. The PEG MALDI calibration mix was a formulation of multiple standards with masses ranging from 1000 up to 8000 Da. Figure 7, shows the suppression of ions greater than 4500 by having the maximum mass be 4500 Da.

As we systematically analyzed the different input parameters of the manual profile we found the broadest ion transmission for mass range of 1000-15000 Da with the configuration presented in Table 1. The maximum mass that can be entered is 8000 Da, and this configuration was found favorable both in the cell and PEG analysis.

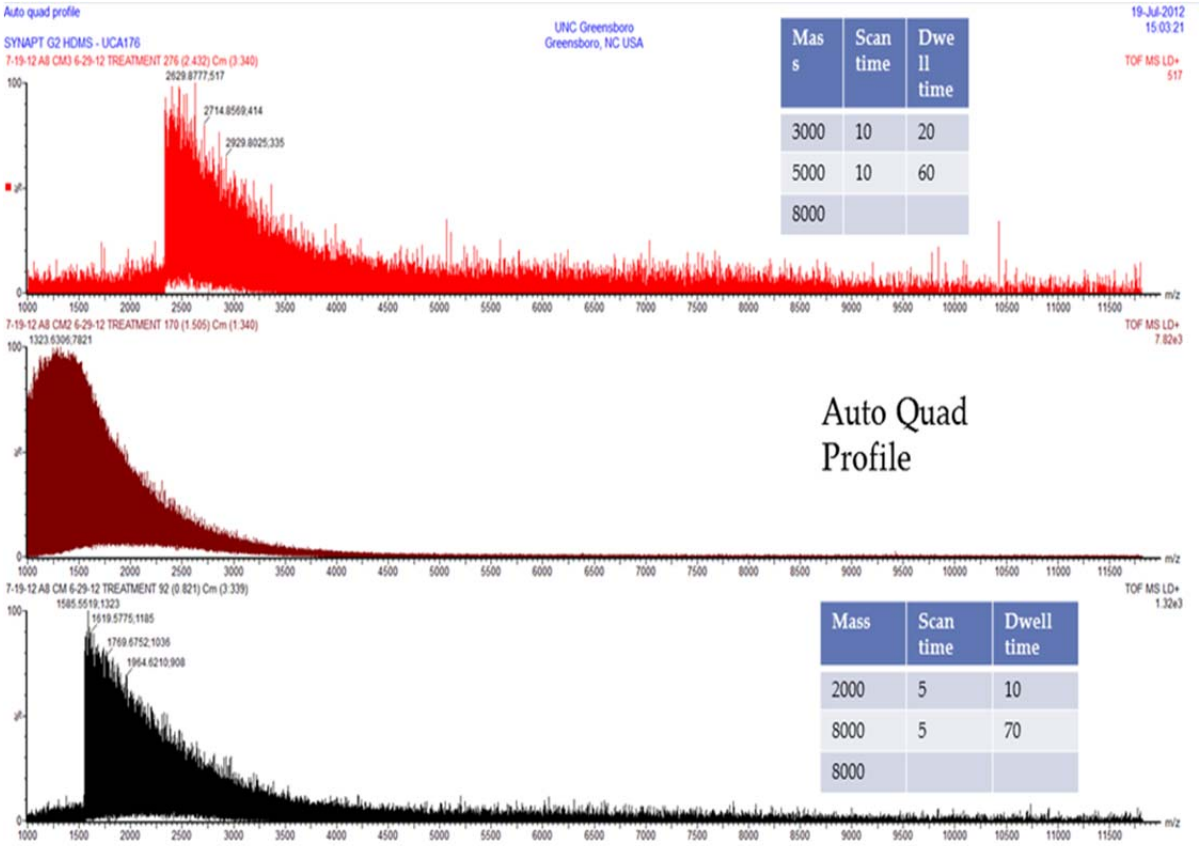


Figure 6. HepG2 MS spectra. The three spectra show the effect of the quad profile on ion transmission and suppression on the same MALDI spot.

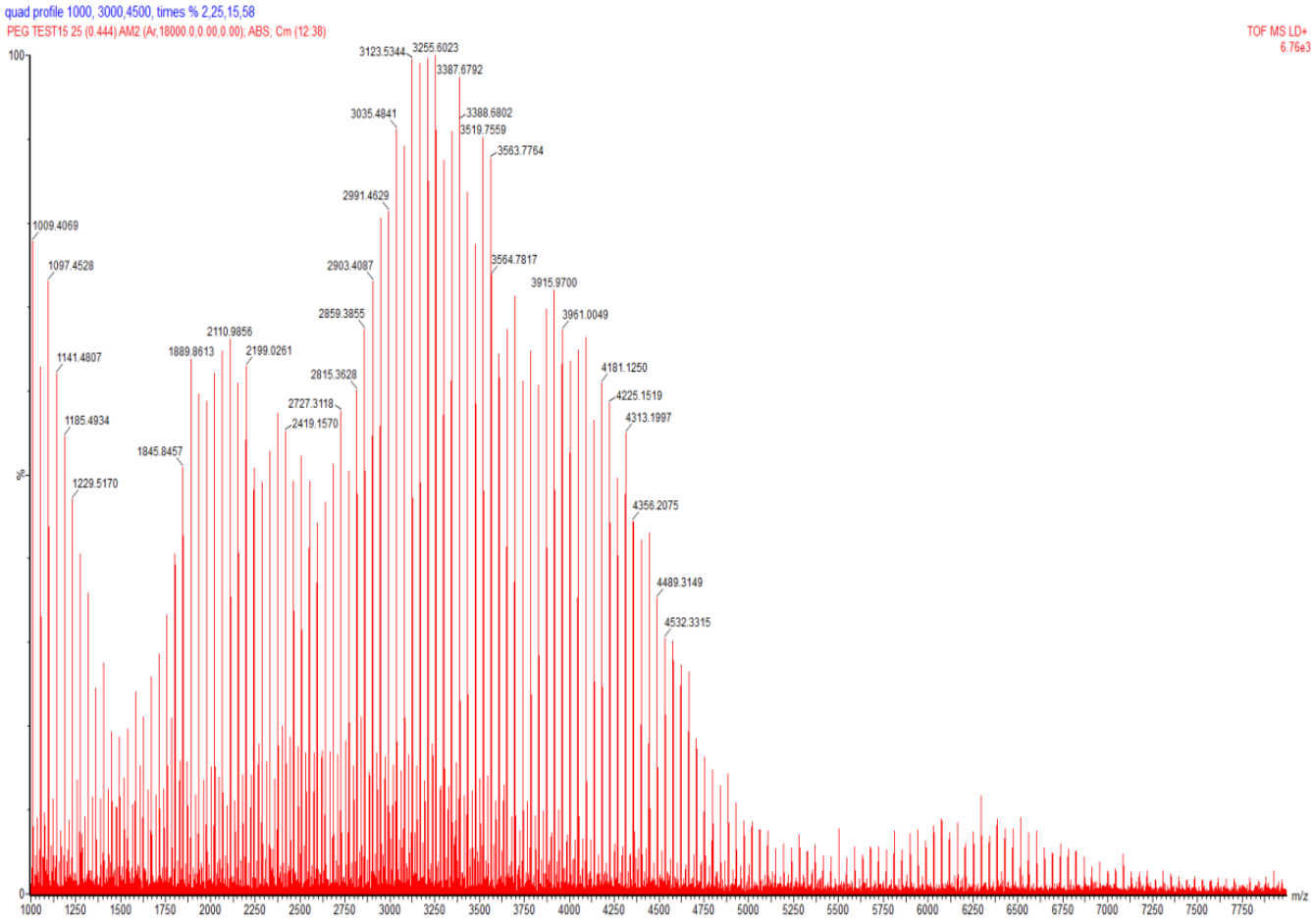


Figure 7. PEG calibrant collected with the quadrupole profile described in Table 1.

Results of Washing Steps

The number of cells will determine firstly whether there is a signal from the mass spec. Yet, we found that rinsing with Ammonium Acetate improves the signal to noise ratio of the spectra. In some cases the peak with the highest relative abundance will shift as seen in Figure 8. Where a spectra on top was washed Once with PBSx1 (4°C) followed by three washes with DI H_2O (4°C), and the one on the bottom was washed with PBSx1 (4°C) followed by three washes with 150 mM (4°C). We can observe that the highest intensity peaks are around 2331 when washed with H_2O though the highest intensity was at 4337 Da when washed with ammonium acetate. The signal to noise ratio significantly improves when the cells are rinsed with ammonium acetate for salt removal. Figure 9 highlights the importance of the ammonium acetate wash as we can see a significant amount of noise with samples only washed once with 150 mM ammonium acetate after an initial PBS wash.

Though you can obtain a reproducible spectrum by washing the cell samples with H_2O and PBS, ammonium acetate provided better noise reduction. Despite our systematic analysis, the washing is not the limiting factor to get a signal, which has allowed a variety in the protocols for sample-preparation throughout the literature.

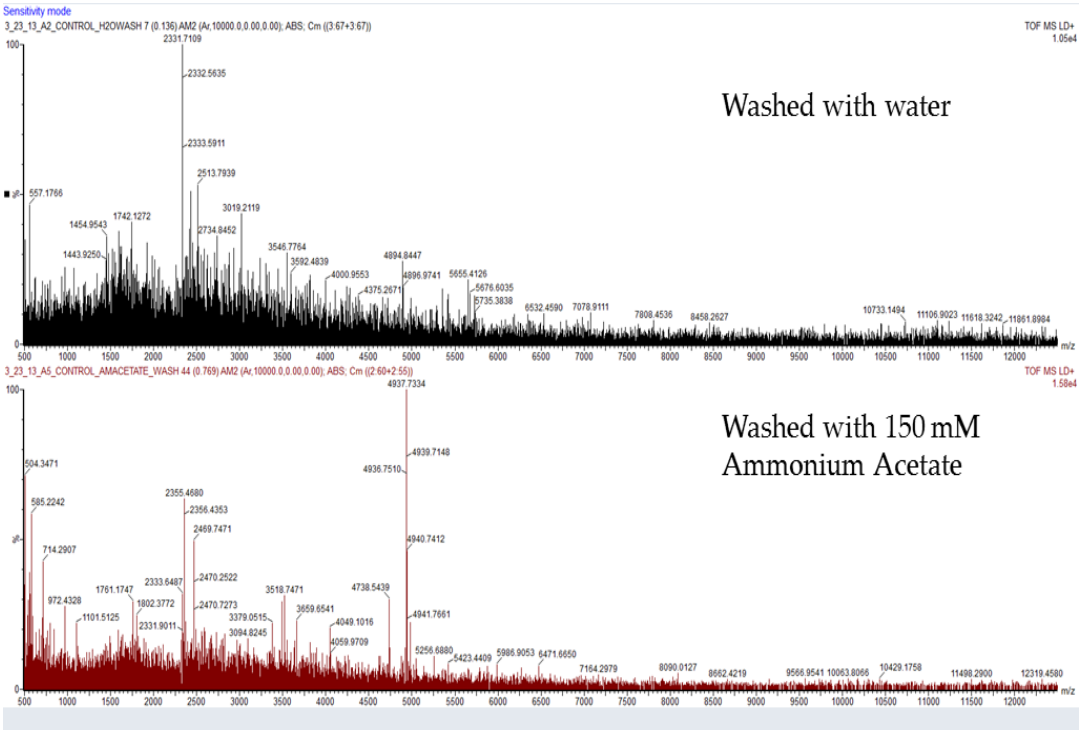


Figure 8. Comparison of the washing effect on the sample. The top spec was washed with DI H2O after a preliminary wash with PBSx1. Bottom spec was washed with 150 mM ammonium acetate after a preliminary wash with PBSx1.

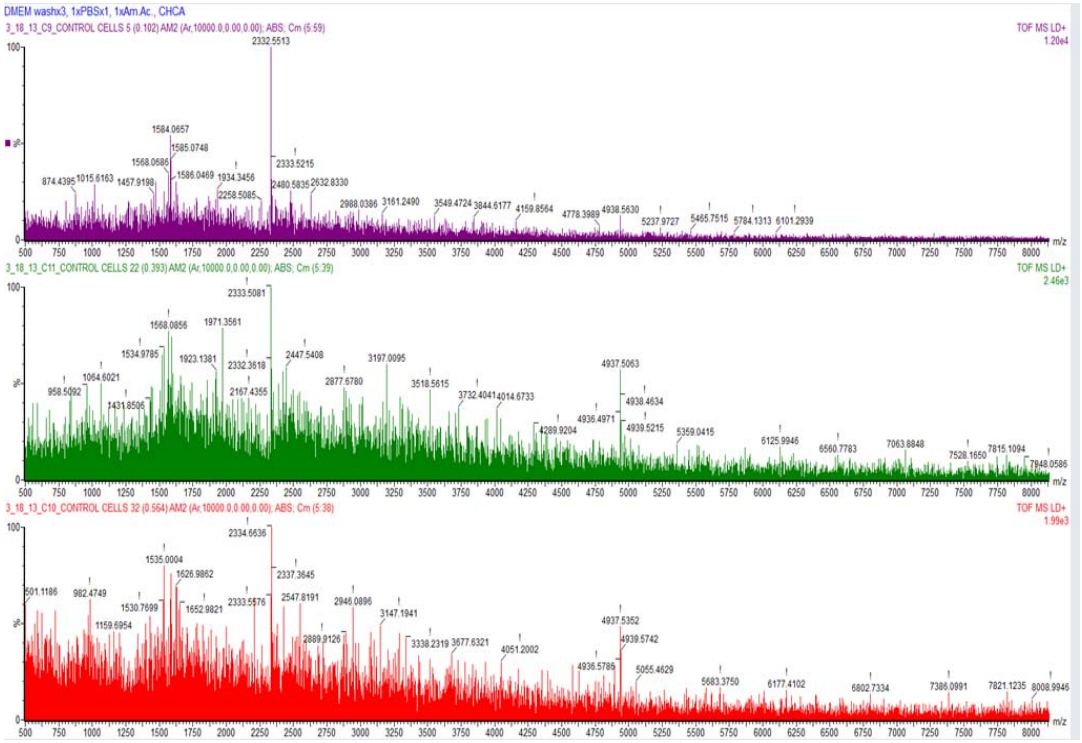


Figure 9. WCMS of HepG2 cells. Samples were washed once with PBSx1 followed by once with 150 mM Ammonium Acetate.

Experimental Setup for Cell Number

To test the limit of detection we made serial dilutions from with either SA or α-CHCA MALDI matrix cell stock. The initial cell stock had $3x10^2$ cells/μL, and was diluted down to $5x10^2$ cells/μL.

Results of Cell Number experimentation

We found that the working cell number range for whole cell mass spectral analysis was $5.8x10^2$ cells/μL up to $1.2x10^3$ cells/μL as it can be seeing in Figure 10. Figure 11 shows the spectra of three different spots of HepG2, the confluence was of $5x10^2$ cells/μL and suspended in α-CHCA MALDI matrix. Figure 11 shows the high reproducibility of this technique. To the best of the knowledge of the author, this is the first whole cell mass spec profile of HepG2 cells, and the first whole cell analysis performed on a MALDI-qTOF with an orthogonal TOF tube.

Obtaining the profile of the HepG2 was our eureka moment, we were very pleased with the results. Though, the sample preparation to obtain such a profile was still consuming close to six hours of bench work to finally analyze the samples. Part of our research objective was to develop a high through put assessment, which motivated us to make the protocol modification in our sample preparation.

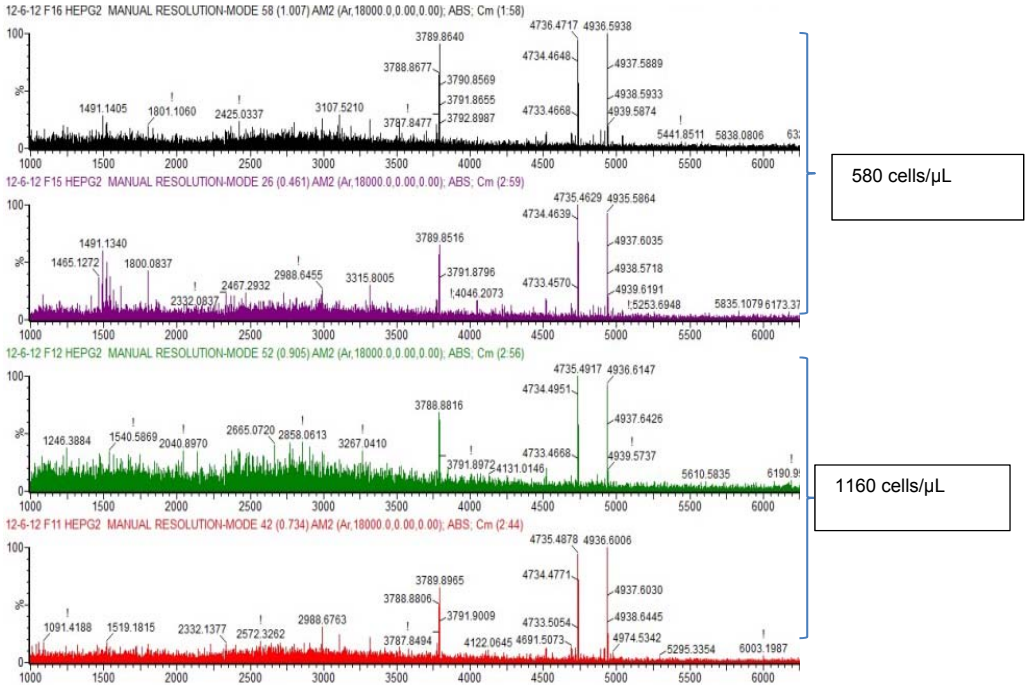


Figure 10. HepG2 WCMS in α-CHCA matrix.

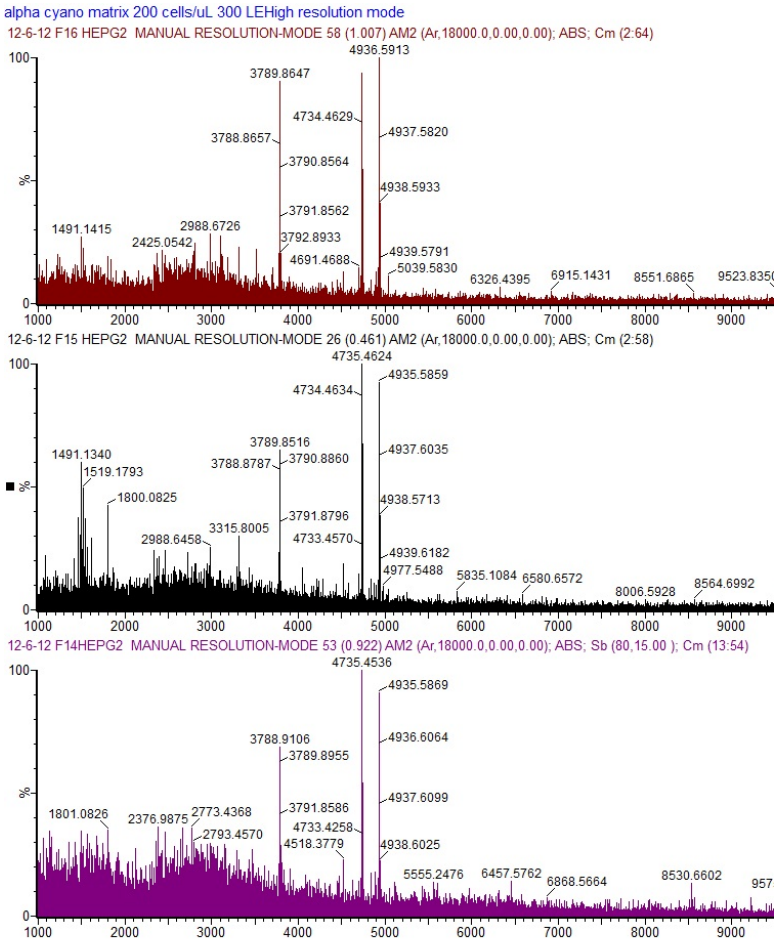


Figure 11. The mass spectra profile of whole cell of HepG2 in the MALDI qTOF

Experimental Setup 1.3

We will ran experiments in parallel with untreated HepG2 cell lines to examine which matrix and sample deposition results produces the most consistent profile.

Results of Study 1.3

For the linear MALDI TOF we found better results using the SA matrix finding some reoccurring peaks above the 10000 Da. For the MALDI qTOF we found that the α-cyano matrix to give us the best results in trying to obtain a reproducible spectrum.

Experimental Setup of overriding the cell detachment or trypsination

To test if we could obtain reproducible spectra from whole cell analysis we worked with cells that were cultured in well plates. After decanting the growth media from the well, instead of removing the cells from the well plate using warm trypsin for eight minutes, we performed the washing steps directly with the cells still attached. To finally add the volume of matrix necessary for to obtain the working cell confluence range previously described.

Results of overriding the cell detachment or trypsination

To our satisfaction, performing the washes directly from the well plate allowed for reproducible whole cell spectra profile. The spectra generated from the direct well plate analysis produced similar peaks to the ones generated after extensive washing procedure as it can be seeing in Figure 12.

This improvement in the technique can have great repercussions in the field of whole cell mass spectroscopy, and facilitate its transition to be used as a tool for toxicity screenings. This protocol modification has the advantages of saving time and minimizing error susceptibility to error by the reducing the steps.

The disadvantage that this modification brings an increase in cost as more matrix may be necessary to achieve right cell confluence.

The trypsination and centrifugation steps take up to two hours per sample to prepare. This can be significantly increased if dealing with multiple samples. While the washing steps directly from the plate reduces the time significantly as the washing is done without the need of centrifugation, therefore saving ten minutes per washing step. Additionally, it is more time efficient dealing with a multi-well plate than with capped centrifuge tubes.

Directly washing in the well plates allows the cells will remain adhered to the plate until the addition of the matrix. Since the cells remain attach to the plate surface, the washing solutions can have a greater contact with the cells to remove impurities. Where the in the previous procedure the centrifugation step clumps the cells together and the researcher had to manually re-suspend to guaranty an appropriate washing. During the re-suspension step cells were frequently lost by adhering to the pipette tip or decanting of the previous washing solution.

It is worth noting that the direct plate analysis technique presented indications of storability, as the spectrum presented in Figure 12 was loaded the day after the washing steps. Storability can be achieved by allowing the matrix to crystalize in the well plate and storing it away from the light, to later be dissolved with minimal matrix and loaded to the well plate.

Figure 12 represents the combined spectra of multiple spot analysis of a samples prepared under similar condition and final cell confluence. As we can observe the spectra obtain without the use of centrifugation has less noise than the one that went through trypsination and centrifugation. The greater noise found in the bottom spectrum can be a result of human error or the exposure to the highly biochemically reactive trypsin.

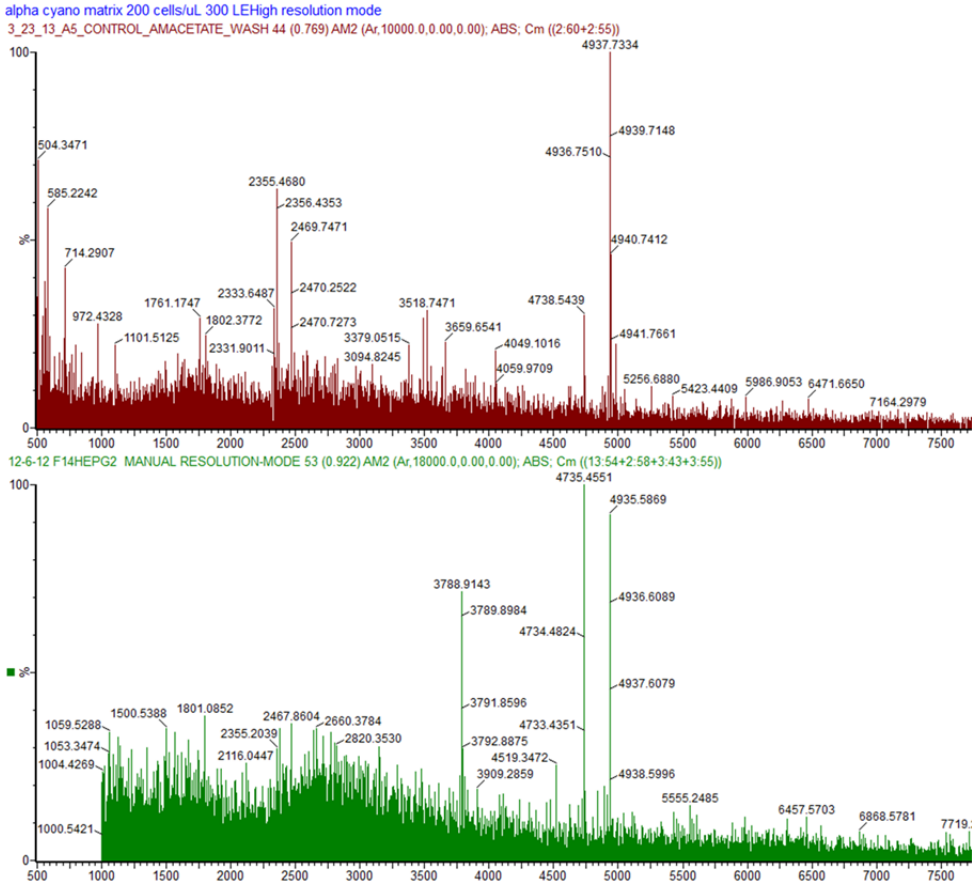


Figure 12. The two mass spectra profile represent different techniques of sample preparation. The top spectrum did not have the cells go through trypsination, while the bottom spectrum did use trypsin to detach the cells for further washing.

CHAPTER IV

AIM 2: WHOLE CELL MASS SPECTROMETRY AS A METHODOLOGY TO TEST NANOMATERIAL AND KNOWN TOXINS CYTOTOXICITY USING *IN VITRO* MODELS

Experimental Setup of Study 2.1

We address the gold nanomaterial in the study 2.2. For our reference toxins we have chosen to work with aflatoxin B_1 (AFB_1), acetaminophen, and hydrogen peroxide. Other compounds were examined, though the toxicity screenings did not show an reduction in cell viability.

Results

After a dose dependent MTT assay we selected to use 400 μM of AFB_1 to be screened in the MS analysis as it can be seen in Figure 14. Our MTT analysis showed robust results from the AFB_1 dosage to compare with the Mass Spectrometry, and LDH. We were able to optimize the conditions for working with HepG2 cell line and the MTT. HepG2 showed resistance to a several compounds during our preliminary screenings (data not shown). For example, hydrogen peroxide did not show toxicity up to the 5 mM concentration Figure 13, higher than what was reported in the literature [47-49]. Acetaminophen showed a

reduction in 78 % cell viability at 30 mM (Figure 15), though for MS analysis we increased the dosage to 60 mM. We were unable to obtain conclusive result from the MTT for the analysis of hydrogen peroxide for which we went with the LDH assay.

In our whole cell analysis we were able to detect a difference in the mass profile between healthy cells and ones dosed with AFB_1 and hydrogen peroxide as it can be seen in Figure 17. Though for acetaminophen we were unable to detect a difference in the mass spectra between healthy and dosed cells.

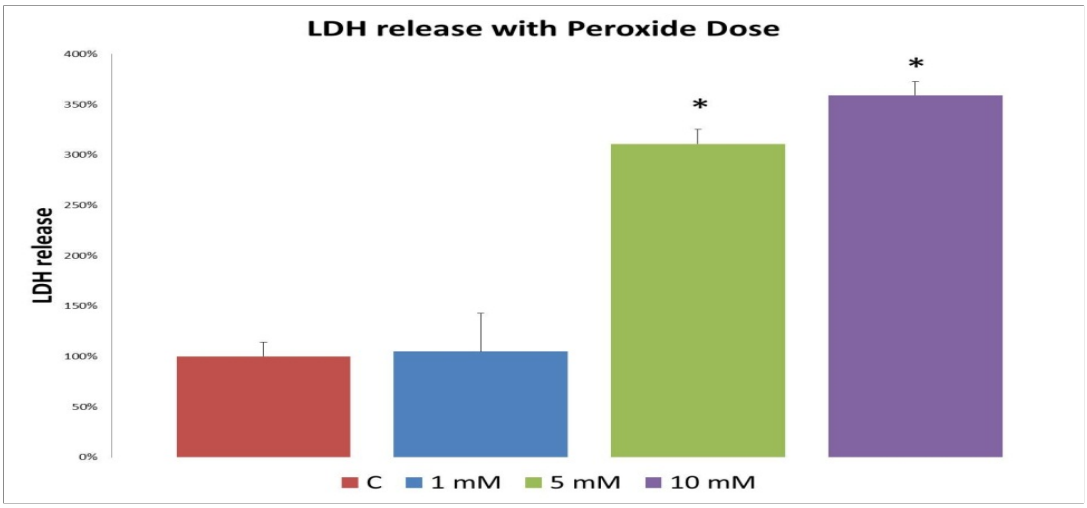


Figure 13. % LDH leakage of HepG2 cell lines in supernatant medium. ANOVA analysis gave a P<0.01, *= T-test with a P<0.01. Error bars Standard Errors. Measurement done in duplicates, with n=4.

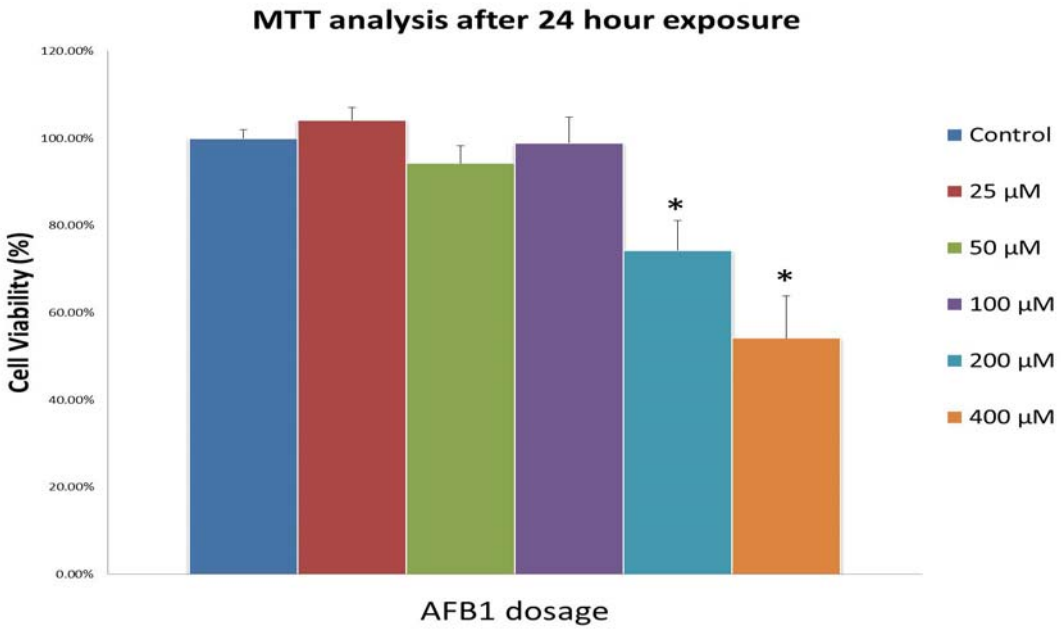


Figure 14. Varying dosage of AFB_1 after 24 h. ANOVA analysis gave a P<0.01, *= T-test with a P<0.01. Error bars Standard Errors. Measurement done in duplicates, with n=8. We decided to go with 400 uM as an appropriate dosage for the MS measurements.

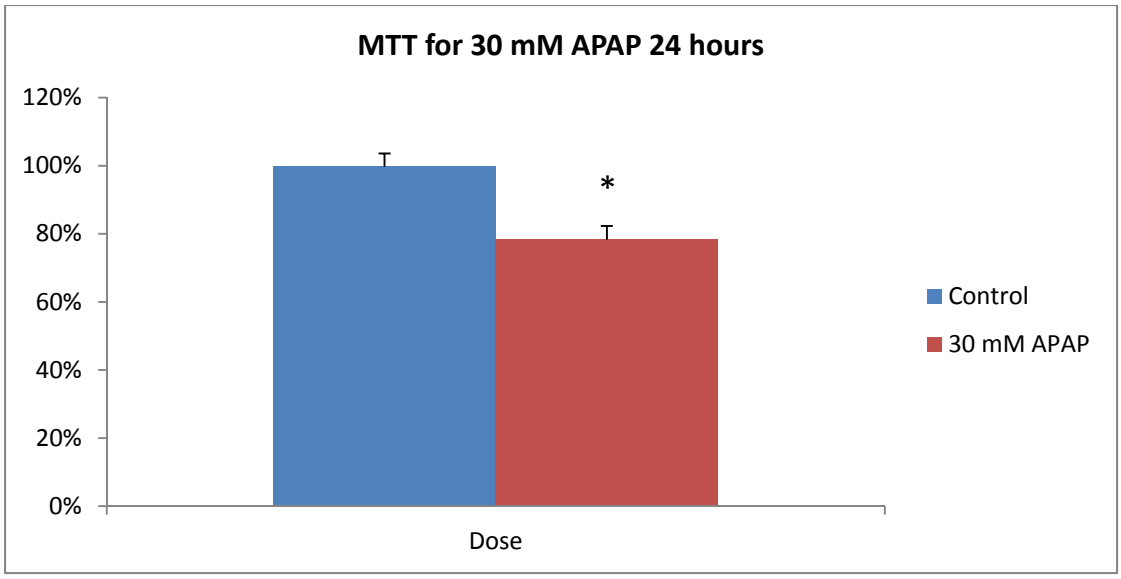


Figure 15. MTT analysis of Acetaminophen, for MS screening we doubled the dose to 60 mM. ANOVA analysis gave a P<0.01, *= T-test with a P<0.01. Error bars Standard Errors. Measurement done in duplicates, with n=10.

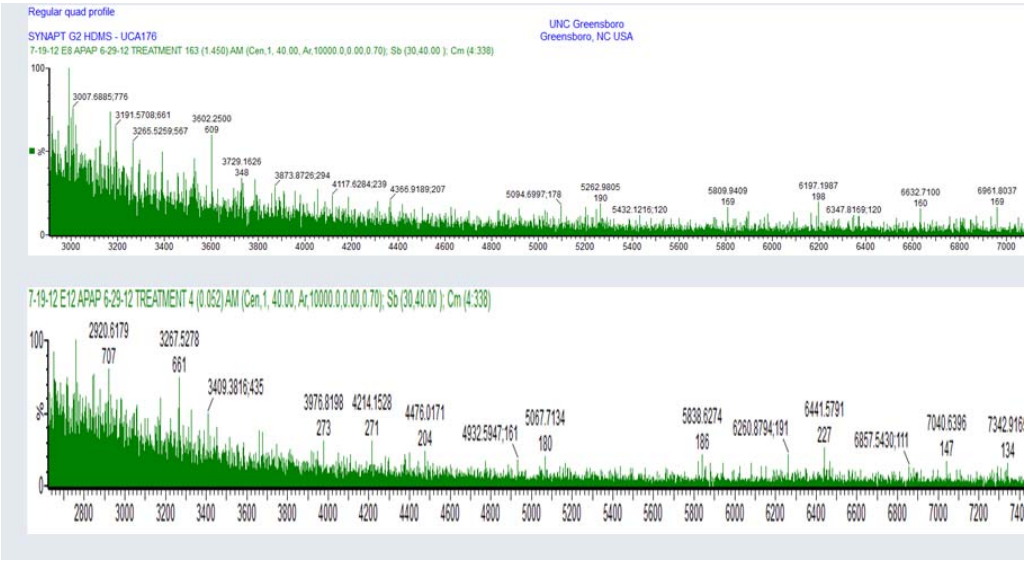


Figure 16. MS analysis of HepG2 dosage after 24 h incubation with 60 mM acetaminophen.

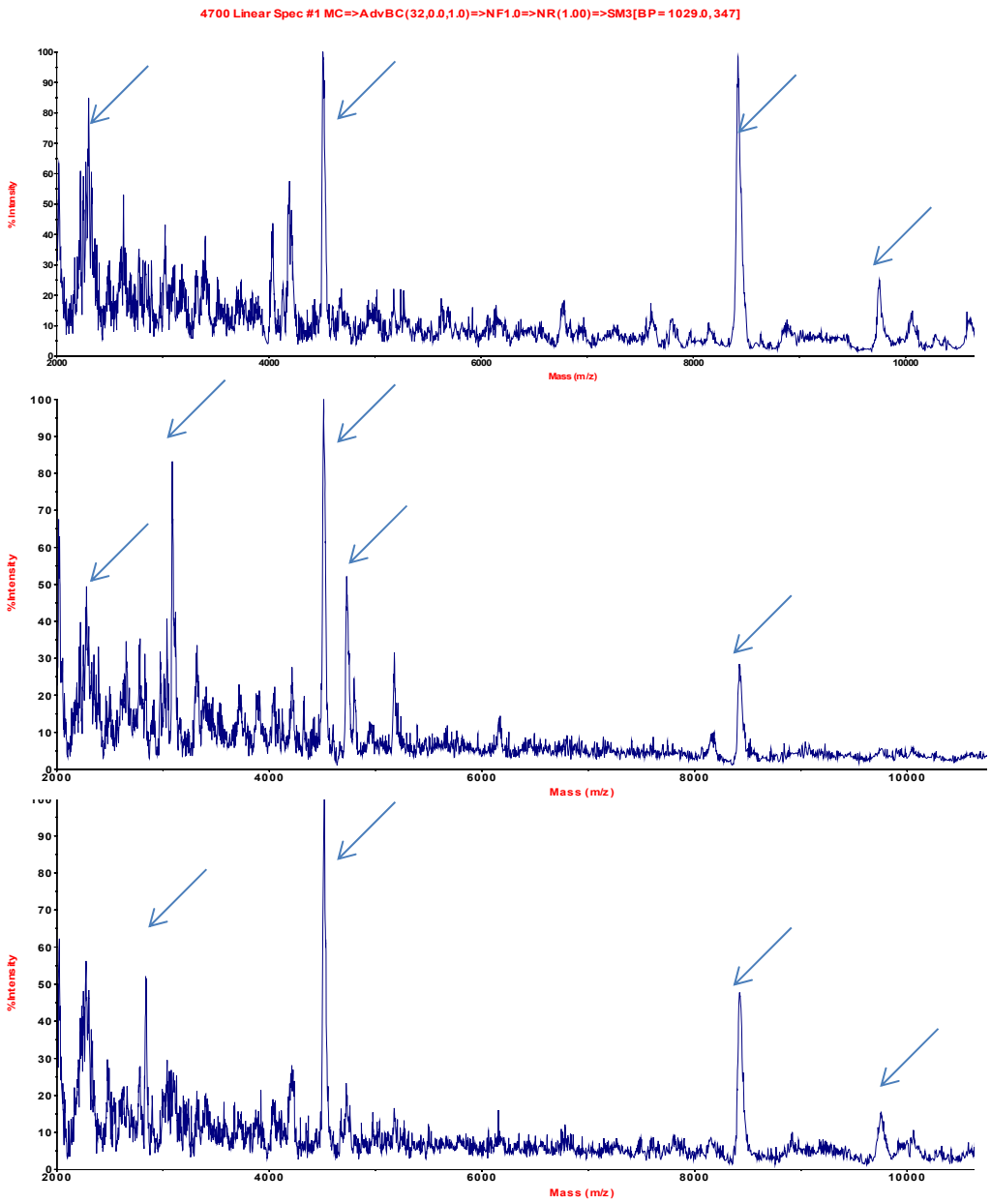


Figure 17. shows a response to different toxins though at a very low signal. This sample was collected with 4700 MALDI TOF from Applied Biosystems. Top Spectra are control HepG2 cells with no dose. Middle spectra-is the AFB1 dose, and bottom spectra-is a Hydrogen Peroxide.

Results of 2.2

We were able to detect a difference in spectra with TWCMS as it can be seen in Figure 18 from the dosage of the Gold nanoparticles. Though, as we suspected the traditional cell viability assay were inconclusive for HepG2 (Figure 19) in their results as the cell did not have a dose response to GNP. The author believes this is a result of the optical properties of GNP. We experimented with a different cell line, and we also did not obtain a dose response (Figure 21) as it would be expected with most xenobiotics. These results highlight the importance of alternate toxicity screening methods to evaluate the toxicity of new compounds like nanomaterials.

From the MS results we were unable to distinguish between 75 μM and 150 μM dosage, though we were able to differentiate from the control sample. The MTT analysis after 24 and 48 hour incubation did not give us a dose dependent response as we would expect. This intrigued us, leading us to test a different cell line model. We tested the dose dependence MTT assay with A10 cell lines, and they also did not present a dose dependent response to the GNP.

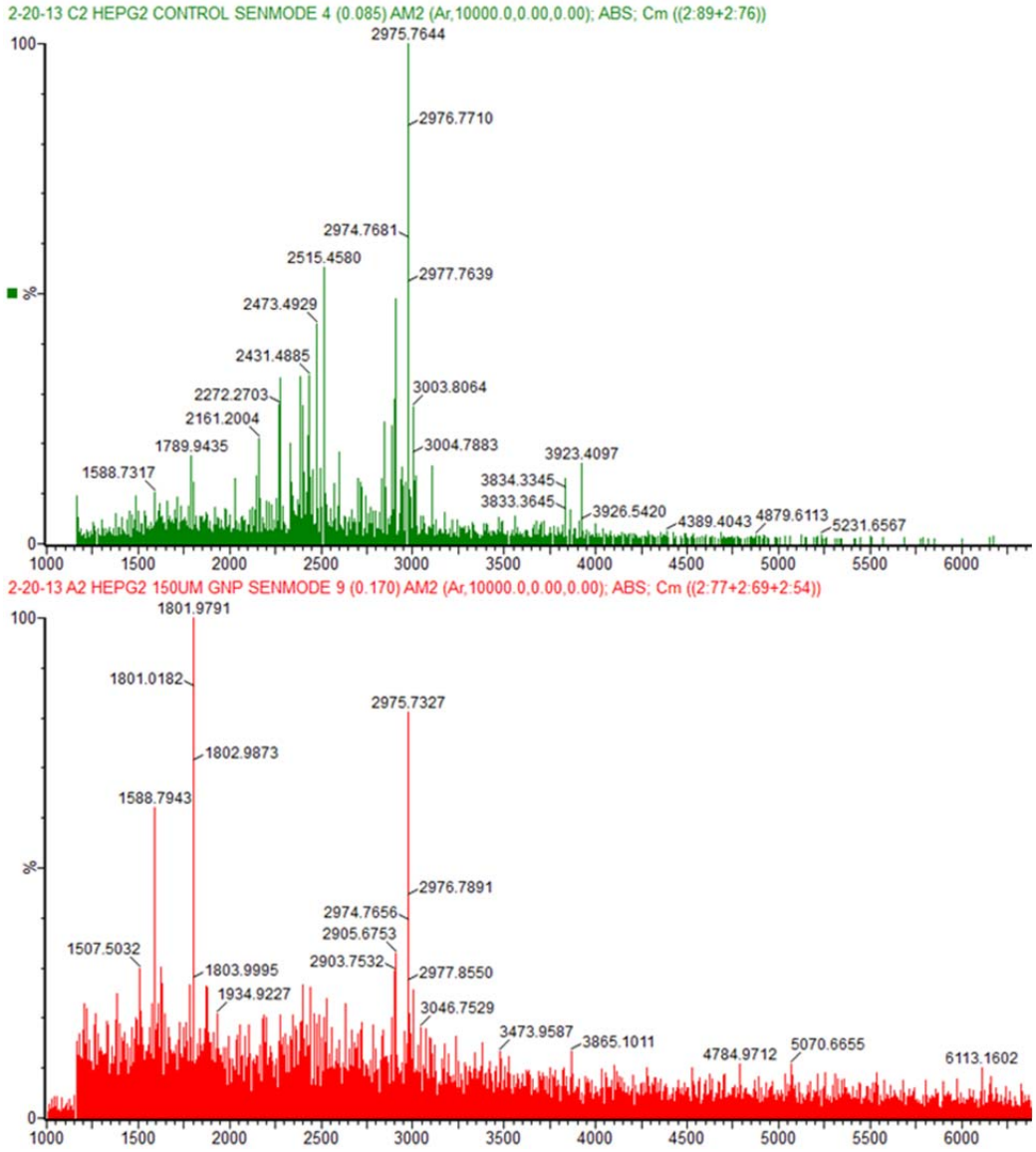
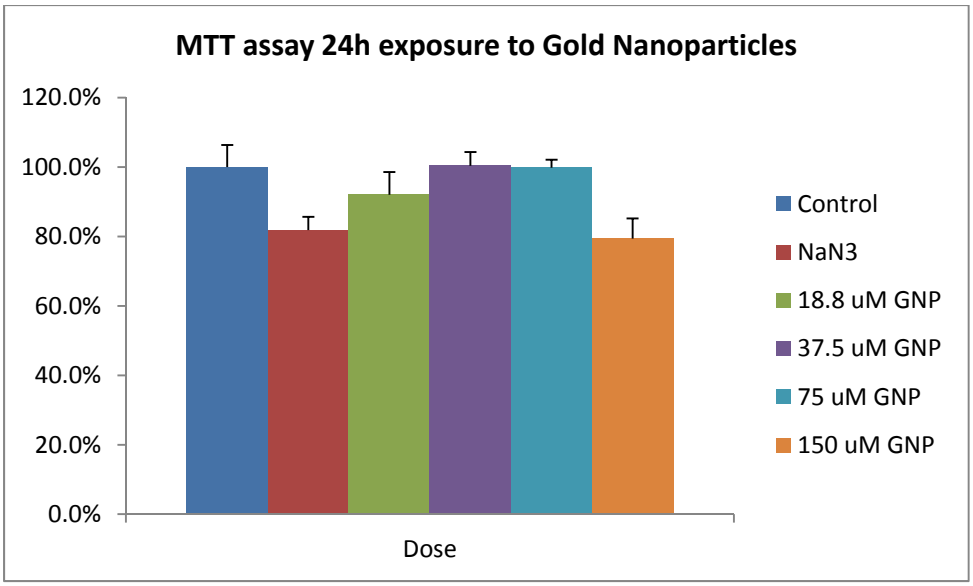


Figure 18. HepG2 TWCMS control and dosed with GNP



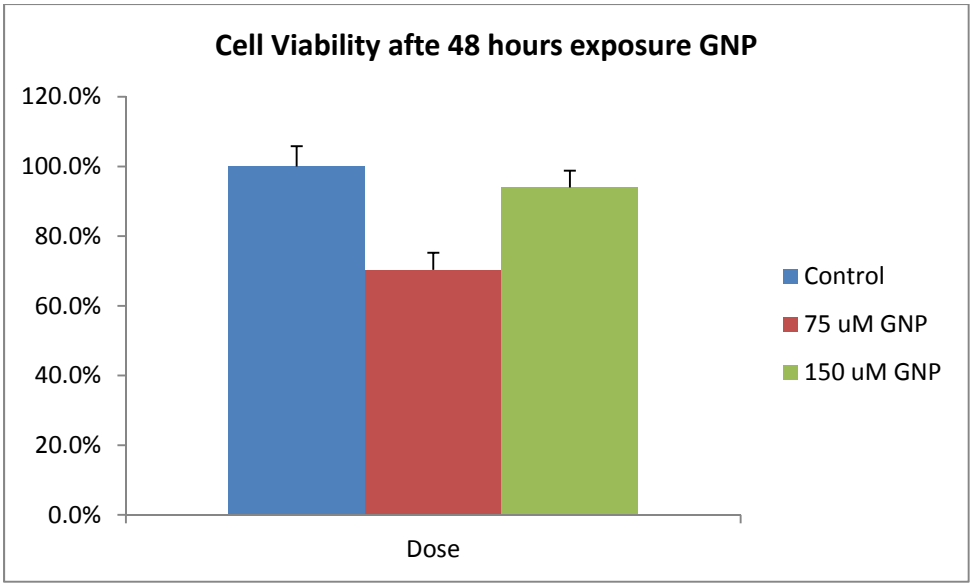


Figure 19. HepG2 MTT analysis to GNP dosage. These two results represent the HepG2 MTT assay after 24 and 48h dosage with Gold nanoparticles. These results do not present the expected dose response.

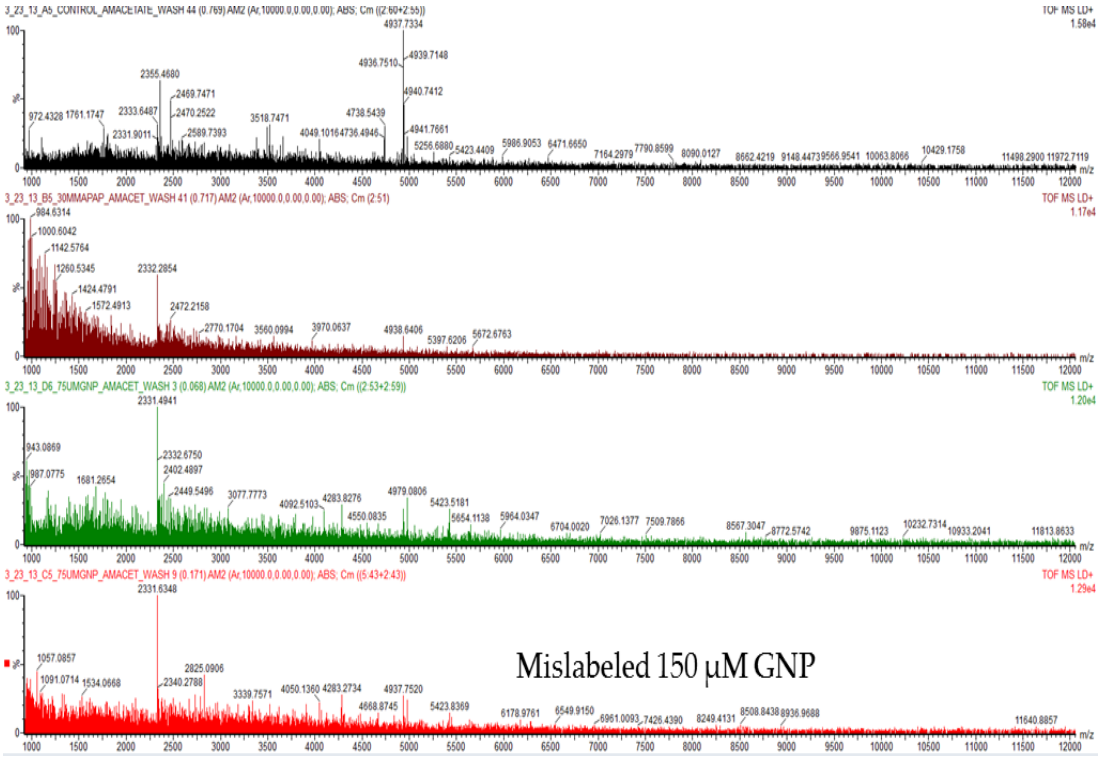


Figure 20. Comparison of the doses. From top to bottom, control, 30 mM acetaminophen, 75 uM GNP, and 150 uM GNP.

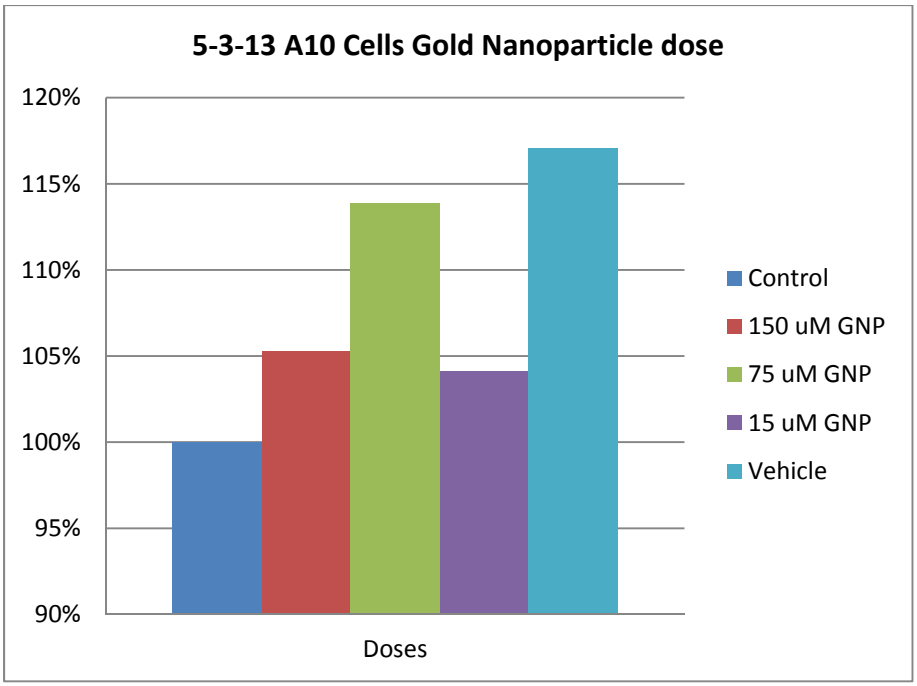


Figure 21. MTT analysis on A10 cells after GNP dosage.

CHAPTER V

DISSCUSSION AND FUTURE DIRECTIONS

Throughout our work we were able to develop the protocol for evaluating whole cells for HepG2 using MALDI qTOF MS. An improvement on previous whole cell analysis, since orthogonal time of flight analyzer have yet to presented on previous work, nor HepG2 analysis had been presented. A major improvement on whole analysis was performed by overriding the centrifugation and harvesting steps presented in most whole cell analysis. This improvement will accelerate the high through put capabilities of this type of analysis.

A key element for whole cell analysis has been that the cell integrity has to be disrupted in order to get a reproducible signal in the mass spectrometer. Our attempts to analyze viable cells were fruitless. We were only able to obtain an MS profile after freezing or prolonged time with acidic MALDI matrix. For this reason the author discourages the use of the terminology intact cell mass spectroscopy for this type of assays.

We were able to detect a difference in mass spectral profile with GNP when we were unable to obtain conclusive results from traditional cell viability assays. This capability of detecting difference among GNP dosage highlights the importance of the method for the emerging field of nanoscience.

To continue on our work, the standardized protocol should be examined with wider array of compounds and be executed in parallel with proteomic work to determine what the peaks are representing.

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