Multicellular tumor spheroids (MCTS) are a powerful biological in vitro model, which closely mimics the 3D structure of primary avascularized tumors. Mass spectrometry (MS) has established itself as a powerful analytical tool, not only to better understand and describe the complex structure of MCTS, but also to monitor their response to cancer therapeutics. The first part of this review focuses on traditional mass spectrometry approaches with an emphasis on elucidating the molecular characteristics of these structures. Then the mass spectrometry imaging (MSI) approaches used to obtain spatially defined information from MCTS is described. Finally the analysis of primary spheroids, such as those present in ovarian cancer, and the great potential that mass spectrometry analysis of these structures has for improved understanding of cancer progression and for personalized in vitro therapeutic testing is discussed.

1. Introduction

Since the establishment of HeLa cells in 1953, cell lines have provided researchers with an almost unlimited supply of cells for experimentation and, since then, a vast number of immortalized cell lines have been established from a range of different cancers. Decades of optimization have led to rapid and reproducible techniques employing cancer cell lines. Cells are typically either grown as an adherent monolayer or in suspension and, despite the profound impacts that these techniques have had on biological research, it has been noted that these simple biological systems do not truly represent the in vivo environment. Monolayer culture conditions fail to capture the tumor microenvironment which involves the complex interaction between heterogeneous cell populations, extracellular matrix (ECM) components, and the influence of a host of secreted factors that are present in the tissue. Additionally, it has been shown that growing cells on ridged 2D structures, such as tissue culture plastics, results in abnormal metabolism and protein expression. This highlights the need for in vitro models which more closely replicate the in vivo situation. Multicellular tumor spheroids (MCTS) are an in vitro model composed of cancer cells which have been allowed to grow as 3D aggregates. These structures replicate gas and nutrient diffusion, gene expression, protein abundance, cell–cell interactions and cell–ECM connections seen in primary tumors. Mass spectrometry approaches hold great potential for elucidating the molecular organization of these structures and how MCTS respond to cancer therapeutics. Together these methods promise to provide insight into the complex molecular mechanisms underpinning cancer progression and response to therapy in a way which is rapid, reproducible, and closely replicates the in vivo conditions.

In the first part of this review we summarize the mass spectrometry approaches used to investigate MCTS as a model focusing on cancer progression and treatment response. There are a number of traditional LC-MS/MS approaches, which have been applied to the MCTS model, as well as other soluble techniques, which aim to provide information about the global abundance of proteins and other molecules. This includes gel and label based techniques such as 2D-PAGE and stable isotopic labeling of amino acids in culture (SILAC), respectively. These techniques have typically focused on identifying proteomic differences between the same cells grown as monolayers or as MCTS.

One short coming of traditional mass spectrometry approaches in this context is that they require homogenization of the sample causing loss of spatially defined information.
formed from homotypic population of cancer cells develop layers of different proliferative capacities making spatial localization of molecules important. In the second part of this review we summarize current methods for investigating spatially defined molecular information and therapeutics response such as mass spectrometry imaging (MSI). The publications summarised are listed in Table 1. Finally we outline a MSI approach established in our lab to investigate peptide abundance in primary ovarian cancer ascites derived spheroids (ADS) and discuss the future application of mass spectrometry to ADS in the pursuit of personalized cancer therapy.

2. MCTS as a Biological Model for Avascularized Tumors

2.1. MCTS Replicate Solid Tumor Structure and Tumorigenicity

MCTS are being used extensively in research and drug development, as they closely replicate avascularized solid tumors. The cell–cell and cell–ECM connections seen in MCTS mimic the in vivo situation and have profound effects on cellular signaling. Consequently, research utilizing this biologically relevant cellular model aims to bridge the gap between research employing monolayer culture and that of in vivo experimentation.

When grown to diameters greater than 500 μm, MCTS from a single cell lines form a core of necrotic cells. They are believed to be a result of the cells’ response to gradients of oxygen, nutrients, pH, CO2, and metabolic waste within the MCTS structure. The cells surrounding the necrotic core seem to have adapted to their challenging conditions, with a majority of cells in this region entering a quiescent state, while most of the cells in the outer regions of the MCTS continues to proliferate. This results in three physiological layers of different proliferative capacities (Figure 1) and this heterogeneous cellular structure profoundly alters growth, survival, and response to treatment as compared to the same cells grown as monolayers.

Furthermore, some cell lines have been shown to be more tumorigenic as MCTS when compared to single cell suspensions in the animal model, supporting their relevance as a biological model. Interestingly, MCTS formed from co-cultures of cancer cells and normal fibroblasts have more tumorigenic properties than homotypic spheroids and both cell types in suspension which correlated with increased spheroid density in cancer–fibroblast co-cultures. This is not surprising considering that signaling between cancer cells and cancer associated fibroblasts are important in cancer progression and it again highlights the importance, and complexity, of heterotypic cell–cell interactions.

2.2. Chemotherapy Response in MCTS

Cells grown as MCTS have been demonstrated to be more resistant to the majority of traditional chemotherapy treatments when compared to the same cells cultured as monolayers. The exact mechanism by which MCTS formation protects cells from chemotherapy has not been fully elucidated. However, inhibited diffusion of the drugs, the maintenance of a quiescent population within the MCTS, expression of pro-survival genes as a result of cell–cell contact, and paracrine/autocrine signaling have been suggested as possible mechanisms. This is supported by studies demonstrating that disruption of cell–cell contacts sensitizes MCTS to chemotherapy treatment. One explanation for higher chemotherapy resistance in MCTS is that the inner quiescent layer is in a non-proliferative G0 cell cycle stage, providing resistance to therapies which target proliferating cells. In melanoma MCTS, cells in G0 were localized to the inner layer of the MCTS with cycling cells on the periphery, which correlated with higher oxygen levels. The cell cycle stages were shown to be similar to a mouse xenograft model with more cell cycle arrest observed distant from blood vessels. Moreover, hypoxia in the central layers of MCTS has been shown to reduce the effectiveness of radiation and chemotherapy as well as being a factor which promotes metastasis and progression. Together, this suggests that the MCTS microenvironment mimics that of a solid tumor giving rise to microenvironment driven phenotypic changes which have vast influences on cancer progression and chemotherapy response.

2.3. Cancer Biomarkers are Present in MCTS

The biological relevance of the MCTS model has been demonstrated by the appearance of more relevant cancer biomarkers in cells grown as MCTS compared to cells grown as monolayers. One example is the pancreatic cancer cell line PC3, which, in 3D culture conditions, exhibited a shift from E-cadherin to N-cadherin expression and increased CXCR4 and β1 integrin abundance at the cell membrane. Expression of these proteins is similar to that observed in primary pancreatic cancer biopsies.

MCTS have also been shown to more closely resemble gene expression patterns observed in solid tumor. In general, this includes an increase in cell adhesion and cell junction genes with a decreased expression of genes associated with DNA replication and progression of the cell cycle. In the case of head and neck squamous cell carcinoma (HNSCC), increased expression of cytochrome P450 xenobiotic metabolism genes were observed in MCTS compared to monolayers. This has also been reported in hepatocellular carcinomas and more closely replicates the expression observed in cancer tissue. The cytochrome P450 superfamily is thought to be responsible for up to 75% of all drug metabolism processes and the upregulation of these genes in MCTS suggests increased drug resistance mechanisms in these structures compared to the same cells grown as monolayers.

2.4. Conclusion of MCTS as a Biological Model for Avascularized Tumors

MCTS represent a powerful biological model to further analyze and better understand the complex biology and drug response of avascularized tumors. They faithfully replicate the solid tumor in gene expression, protein biomarkers, drug response, gas and nutrient gradients, and the subsequent cellular layers of different proliferative capacities. However, there are still a number of limitations associated with this in vitro model. For example, not all immortalized cells’ lines are able to spontaneously...
| Paper | Author            | Analysis method | Spheroid growth method | Cell origin                | Key findings                                                                                                                                 |
|-------|------------------|-----------------|------------------------|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
|       |                  |                 |                        |                            | **Soluble based mass spectrometry approaches**                                                                                         |
|       |                  |                 |                        |                            | Established that, through the use of purification techniques, a single spheroid can be investigated with LC-MS/MS in a reproducible way. |
|       |                  |                 |                        |                            | tSIRT1 (metabolic), tPIAS1 (signaling), tMRD1 (drug resistance), tZBTB7 (oncogenic transcription factor). Showed that nutrient restriction increases cell viability but also increases effectiveness of chemotherapy. |
|       |                  |                 |                        |                            | Identified differentially regulated proteins in MCTS (↑ Laminin A/C fragment, ↑ P0 ribosomal component, ↑ acidic calponin) |
|       |                  |                 |                        |                            | **Nutrient restriction of glucose or serum results in similar proteomic expression changes in 3D colon cancer cell lines**                   |
|       |                  |                 |                        |                            | Showed that nutrient restriction increases cell viability but also increases effectiveness of chemotherapy.                                |
|       |                  |                 |                        |                            | **Proteomic analysis reveals differences in protein expression in spheroid versus monolayer cultures of low-passage colon carcinoma cells** |
|       |                  |                 |                        |                            | Increase in glycolytic, ECM and cytoskeletal proteins in MCTS compared to monolayers.                                                  |
|       |                  |                 |                        |                            | **Comparative proteomics of ovarian cancer aggregate formation reveals an increased expression of calcium-activated chloride channel regulator 1 (CLCA1)** |
|       |                  |                 |                        |                            | Greatly increased expression of CLCA1 in MCTS (eightfold) in MCTS compared to monolayers. This ion channel is essential in MCTS formation in this context. |
|       |                  |                 |                        |                            | **Proteomic comparison of 3D and 2D glioma models reveals increased HLA-E expression in 3D models is associated with resistance to NK cell mediated cytotoxicity** |
|       |                  |                 |                        |                            | Decreased abundance of proteins involved in proteosomal degradation and antigen presentation in MCTS compared to monolayers. Observed decreased susceptibility to NK cell mediated apoptosis in MCTS. |
|       |                  |                 |                        |                            | **Quantitative proteomic and phosphoproteomic comparison of 2D and 3D colon cancer cell culture models**                                   |
|       |                  |                 |                        |                            | Highly reproducible phosphoproteomic analysis of MCTS. Identified differentially abundant proteins and phosphoproteins involved in proliferation, cell cycle, and DNA repair. |
|       |                  |                 |                        |                            | **Multicellular tumor spheroids combined with mass spectrometric histone analysis to evaluate epigenetic drugs**                        |
|       |                  |                 |                        |                            | Observed significantly different methylation patterns in MCTS compared to monolayers. Treated spheroids with UNC1999, which inhibits methylation of H3D27, and observed decreased H3D27 methylation in the outer layers of the spheroid. Established this as a platform for testing epigenic targeting anti-cancer agents. |
|       |                  |                 |                        |                            | **Proteomic approach toward molecular backgrounds of drug resistance of osteosarcoma cells in spheroid culture system**                 |
|       |                  |                 |                        |                            | Nine out of 11 osteosarcoma cell lines were significantly more resistant as MCTS than as monolayers. This was correlated with cathepsin D, gelsolin and ferritin abundance. |
| Paper | Author | Analysis method | Spheroid growth method | Cell origin | Key findings |
|-------|--------|-----------------|------------------------|-------------|--------------|
| Plasma membrane proteomics of tumor spheres identify CD166 as a novel marker for cancer stem-like cells in head and neck squamous cell carcinoma | Yan et al. (2013) | Membrane fractionation + SDS-PAGE + LC-MS/MS | ULA plates | HNSCC cell lines: CAL27, HN6, HN12, HN13 | Identified differential abundance of proteins involved in differentiation (CD44), cell adhesion (CD166), and numerous signaling pathways (Wnt, integrin, and TGFβ signaling pathways). They focused on CD166 as a protein which was required for spheroid formation and has been associated with malignant progression of a number of cancers. |
| Colorectal cancer derived organotypic spheroids maintain essential tissue characteristics but adapt their metabolism in culture | Rajcevic et al. (2014) | SDS-PAGE combined with LC-MS/MS | Agarose overlay | Primary tumor fragments from patients with colorectal carcinoma | Performed the first global proteomic analysis of “organotypic spheroids” (OS) formed from tumor biopsies. They observed an 86% similarity in protein abundance between OS and the tumors they were derived from demonstrating an in vitro method of tumor aggregate generation which directly replicates the primary tumor it was derived from. |
| 3D neuroblastoma cell culture: proteomic analysis between monolayer and multicellular tumor spheroids | Kumar et al. (2008) | Nuclear and cytoplasmic protein enrichment, 2D electrophoresis and LC-MS/MS | ULA plates | Three neuroblastoma cell lines: SK-N, SK-N-DZ, and IMR-32 | Investigated differential protein abundance between monolayers and MCTS and identified proteins with altered abundance to be heat shock and glycolysis related. |
| Mass spectrometry imaging | Li and Hummon (2011) | MALDI + LC-MS/MS | Agarose overlay | HCT116 colon cancer cell line | Established the work flow for MALDI imaging of MCTS. Found increased expression of various survival proteins. |
| Imaging mass spectrometry of 3D cell culture systems | Liu and Hummon (2016) | MALDI MSI | Agarose overlay | HCT116 colon cancer cell line | Were able to semiquantitatively track the distribution of the platinum based drug, oxaliplatin, and its metabolite in MCTS. |
| Chemical imaging of platinum based drugs and their metabolites | LaBonia et al. (2016) | MALDI MSI | Agarose overlay | HCT116 colon cancer cell line | Were able to track the penetration of irinotecan into MCTS delivered via a 3D printed fluidic device which better replicated the dynamic drug dosing which occurs in vivo. |
| Drug penetration and metabolism in 3D cell cultures treated in a 3D printed fluidic device: assessment of irinotecan via MALDI imaging mass spectrometry | Hiraide et al. (2016) | MALDI MSI | Cell matrix type 1 (gelatin based scaffold) | Primary colon cancer derived spheroids, and the colon cancer cell lines; HCT116 and DLD1 | Identified high abundance of the phospholipid Pt(18.0/20.4) on the periphery of primary cell derived MCTS. This was not present in cell line derived spheroid showing the shortcomings of the cell line MCTS model. |
| Accumulation of arachidonic acid-containing phosphatidylinositol at the outer edge of colorectal cancer | Li and Hummon (2016) | MALDI MSI | Agarose overlay | TFK-1 bile duct carcinoma cell line | Were able to quantitatively track the distribution of platinum and palladium based drugs into cancer spheroids. |
| Quantitative bioimaging of platinum group elements in tumor spheroids | Neihoff et al. (2016) | LA-ICP-MS | Spinner Flask | HCT116 and DLD1 | Traced the penetration of liposomal doxorubicin into MCTS. Observed similar penetration between free drug and liposomal drug however there was slower metabolism of the liposomal drug. Strong precedence for using the MCTS model to investigate uptake of liposome encased compounds. |
| Analyzing liposomal drug delivery systems in 3D cell culture models using MALDI imaging mass spectrometry | Lukowski et al. (2017) | MALDI MSI | Agarose overlay | Colon cancer cell line: HCT116 | |
Figure 1. Graphical representation of a multicellular tumour spheroid formed from homotypic dissociated cells. The structure has proliferating cells on the outer layer (blue) a quiescent inner ring (purple) and a necrotic core (black). These layers are a result of limited diffusion of oxygen and nutrients in conjunction with increased waste and CO₂ in the centre of the structure. (Layers are drawn for illustration purposes only and are not drawn to scale)

form MCTS and those which do require extensive optimization and validation which limits the use of this model. Additionally, these structures fail to replicate a number of aspects of the solid tumor such as heterogeneous cell populations, paracrine and endocrine signaling, and immune cell interactions all of which play a role in cancer progression. To address this, currently more complex in vitro systems are being evaluated, including methods to encourage MCTS formation by co-culturing with other cell types, such as fibroblasts, and replication of the complex signaling which occurs in vivo. These methods are an exciting avenue for the future development of the MCTS model.

3. Generating and Maintaining MCTS in Culture

MCTS were first documented in the 1970’s and have experienced a renaissance in the last 5 years, likely due to advancements in culturing techniques. MCTS is an umbrella term describing 3D structures composed of cancer cells alone or co-cultured with other cell types with or without matrix or other scaffolds. The simplest, and most widely used, means of generating MCTS consist of suspensions of homotypic cancer cells which are encouraged to aggregate by a range of methods. The best characterized include culturing on non-adhesive surfaces, hanging drop, and spinner flask (reviewed in ). Establishing MCTS in non-adhesive tissue culture plates is the most common method of growing these structures. These techniques are rapid and inexpensive and can potentially be employed in high throughput drug testing. To standardize MCTS size with this method, cells can be seeded into 96 well round bottom plates, but cultures usually still contain a mix of attached cells and MCTS. The hanging drop method involves growing MCTS in drops of media on the underside of tissue culture plastic held by surface tension. This technique generates consistent MCTS but is labor intensive and unsuitable for long term culturing. Finally, the spinner flask method utilizes suspension culture, which is continuously stirred to encourage MCTS formation. This method is useful for large scale production of MCTS. However, the sheer force used to generate MCTS can result in phenotypic changes and heterogeneous distribution of cell numbers within the MCTS.

There are also a number of more complex 3D culturing methods developed in order to more closely replicate the in vivo environment for any particular biological situation. These include co-culturing with other cell types, culturing in matrices, such as hydrogels, and in microfluidic devices (reviewed in ).

3.1. Primary Cell Derived Three Dimensional Cell Culture

In addition to being formed from dissociated cells in culture, 3D aggregates also appear as part of ovarian cancer malignant progression and will be defined here as “AD5”. Ovarian cancer is characterized by dissemination of tumor cells into the abdominal cavity causing the formation of malignant cancer-cell rich fluid called “ascites.” Within the ascites ovarian cancer cells exist as both single cells and spherical aggregates. These structures are thought to be of importance in inherent and acquired chemo resistance, which is a defining characteristic of ovarian cancer, and represents a reservoir of cancer cells which drive metastases.

4. Analysis Techniques for Investigating MCTS

Despite many advances in culturing methods, there remains the barrier of analyzing these 3D structures in a way which maintains their biologically important structures. A range of techniques have been developed to investigate the morphology, topography, growth, cellular organization, gene expression, cell cycle patterns, and invasive and metastatic potential of MCTS (reviewed in ). This includes a range of microscopy based techniques, flow cytometry, traditional proteomic, genomic, metabolomic, and transcriptomic techniques as well as the application of mathematical modeling.

However, in this complex 3D context, many of these techniques are inadequate. For example, colorimetric dyes and lysate based techniques, such as flow cytometry and western blots, lose the spatial information during sample preparation. Additionally, fluorophore based techniques, such as microscopy and histology, require foreknowledge of molecules of interest limiting the potential of these techniques to discover novel molecular aspects of these structures.

To overcome the issues related to loss of spatial information, the MCTS can be separated into solubilized layers via serial trypsinization. This technique was first applied and validated in the 1980’s to characterize the different cell populations in MCTS formed from mouse mammary tumor cell line. Briefly, it involves washing the MCTS with trypsin, removing the dissociated cells forming the outer layer, and repeating until the entire spheroid is separated. Sutherland et al. (1980) demonstrated that this approach separated the MCTS consistently and went on to characterize an inner layer of cells as...
Figure 2. Workflow for LC–MS/MS and MALDI MSI analysis of MCTS. **LC–MS/MS**: Cells are harvested and lysed before specific fractionation techniques are applied. This is followed by processing including tryptic digest for investigation of peptides. The sample is loaded into the LC–MS/MS and spectra are acquired before data analysis to identify molecules and perform relative quantification of abundance. **MALDI MSI**: Spheroids are embedded and sectioned onto conductive slides. Matrix and calibrants are applied to the sections before spectra are acquired in a MALDI-TOF instrument. Spectra are coregistered with images of the sections to acquire spatially localised molecular information.

quiescent while the outer layers were proliferative, however to a lower extent than the same cells grown as monolayers.\(^{[31]}\)

This technique is compatible with a range of analysis techniques, including mass spectrometry, and is discussed further in Section 4.2.1.

### 4.1. Traditional Mass Spectrometry Techniques for Analyzing MCTS

One way to address some of the short comings of the techniques discussed above is to investigate the molecular underpinnings of these structures using mass spectrometry (Figure 2). Mass spectrometry analyses have the potential to identify vast numbers of proteins, post translational modifications (PTMs), lipids, metabolites, and other analytes with relative quantification. No foreknowledge of the molecule of interest is required, making these techniques ideal for discovery approaches. These techniques have been used in cancer biology to identify biomarkers for cancer detection,\(^{[54–57]}\) to predict chemotherapy response,\(^{[38–62]}\) and to investigate the biology of cancer progression.\(^{[63]}\)

In the case of MCTS, mass spectrometry has been employed to identify molecules which are involved in MCTS formation or confer MCTS specific characteristics as well as tracing penetration of therapeutics into these structures.

One of the barriers to effective elucidation of the proteome using mass spectrometry is the great complexity of the sample. This complexity can result in higher abundance analytes suppressing the signals from less abundant, but no less biologically important, ones.\(^{[64]}\)

To overcome this, a range of fractionation techniques are employed in traditional mass spectrometry work flows in order to reduce the complexity of the sample.

Liquid chromatography (LC) techniques have become the standard approach for fractionation of analytes before mass spectrometry analysis. The most commonly used LC technique is reverse phase liquid chromatography (RP-LC).\(^{[65]}\)

One limitation of RP-LC is that highly polar analytes are difficult to separate as they are not well retained on the column. This can be overcome through the use of hydrophilic interaction liquid chromatography (HILIC)\(^{[66]}\) and this technique has been employed to separate carbohydrates,\(^{[67]}\) peptides,\(^{[66]}\) and polar pharmaceuticals.\(^{[68]}\)

It is yet to be widely applied for the analysis of MCTS, however holds potential and might result in a complementary data set. Together, these LC techniques provide effective fractionation of a range of complex samples allowing for effective identification and relative quantification in MS instruments.

Recently, label free proteomic analysis of MCTS was achieved for single MCTS formed from the colon cancer cell line: HCT116.\(^{[69]}\)

Initially, they established that a single MCTS (approximately 1 mm in diameter) contains an average of 39 ± 4 μg of protein. Through careful protein concentration, via acetone precipitation, and sample preparation the authors were able to minimize any protein losses resulting in the identification of more than 1350 proteins from a number of duplicate runs with high reproducibility and relative quantification. Through the application of Pearson’s correlation they were able to show that variations in identified proteins between different single MCTS were purely due to random variations in spectra\(^{[69]}\) supporting the high level of reproducibility in LC-MS/MS analysis of MCTS and the possible application with high throughput platforms.

In addition to being formed from dissociated cells in culture, spherical aggregates can also be grown directly from a tissue biopsy fragment\(^{[70]}\) which has the advantage of maintaining more tissue-like characteristics such as ECM, capillaries, and tumor cell heterogeneity.\(^{[70]}\)

For example, Rajcevic et al. (2014) found that, when colorectal cancer biopsy fragments were grown as 3D aggregates, they formed a distinct organization with an outer layer of epithelial cells and an inner core of mesenchymal cells,
and a small number of infiltrating immune cells, showing the complex heterogeneity in tumors and how they can be replicated using these in vitro techniques. They went on to perform the first global proteomic analysis of these “organotypic spheroids” (OS) using SDS-PAGE coupled with LC-MS/MS and comparing them to cancer tissue directly obtained from a biopsy. They were able to identify 1315 proteins with 35 upregulated and 70 downregulated in OS compared to cancer tissue. They highlighted an 86% similarity in proteins identified in OS and tumor biopsies with the proteins that were found to be differentially abundant being mostly ECM and cytoskeletal proteins, which is to be expected as the spatial organization of this cellular structure is changed ex vivo. Despite the small differences in proteomes in these organotypic spheroids compared to tumor biopsies, this method holds promise for further improving upon the biological relevance of these in vitro techniques.

4.1.1. 2D Gel Based Fractionation Combined with MS Analysis

2D electrophoresis techniques, such as 2D-PAGE, are fractionation methods commonly employed in proteomic mass spectrometry workflows. It typically involves separating proteins based on their isoelectric point in the first dimension and by molecular weight in the second dimension and is an effective method for separating proteins out of complex samples. Once a protein spot is separated it can be readily identified using mass spectrometry. However, one protein spot can contain a number of proteins, with the most abundant being the most likely to be identified.

An example of the use of 2D-PAGE coupled with LC-MS/MS compared HepG2 liver cancer cells lines grown as monolayers or as MCTS in collagen based scaffolds. This approach identified more than 823 ± 45 spots in monolayer culture and 762 ± 65 in MCTS with 73 of these spots shown to be significantly differentially abundant between the two groups. This was followed up with in-gel tryptic digestion and LC-MS/MS identifying a number of proteins involved in cytoskeletal organization and indicative of a shift toward anaerobic glycolysis, which has been widely observed in MCTS. The microenvironmental stresses on cancer, replicated through MCTS culture, are vital in the shift to glycolytic metabolism which mediates angiogenesis, tumor progression, and metastasis.

Through immunoblotting for stabilized HIF-1α, they identified increased levels of hypoxia in MCTS and established that, when grown as MCTS, these cells could replicate a number of aspects of an avascularized tumor. The role of hypoxia in driving tumor progression, and reducing the effectiveness of anti-cancer therapy, has been described in a number of contexts. In addition to altered metabolism and hypoxia response, an increased abundance of ECM and cytoskeletal proteins were identified in MCTS, which both play a role in cancer progression, particularly in migration and metastasis.

A similar approach was also applied to three neuroblastoma cell lines grown in low attachment plates. Through combined nuclear and cytoplasmic enrichment followed by 2D electrophoresis, they identified 18 spots differentially abundant in MCTS compared to monolayer cells. These proteins were identified via in-gel tryptic digestion followed by LC-MS/MS and were characterized as heat shock and glycolysis related. The altered metabolism and expression of heat shock proteins (HSPs), seen only in the MCTS, replicates protein expression in solid tumors further establishing MCTS as a strong biological model. The increased abundance of stress response proteins, such as HSPs, have been associated with apoptosis resistance, angiogenesis, and chemo resistance and are expected to arise in response to the stressful conditions in the inner layers of the MCTS.

2D electrophoresis, with MALDI MS mass fingerprint protein identification, was used to investigate the proteomic differences in primary colon cancer cell lines grown as MCTS or as monolayers. Through the generation, and early passage analysis, of colorectal cancer cell lines they aimed to reduce the impact that long term culturing has on the phenotype of cancer cell lines. Four proteins spots showed significantly altered abundance in MCTS compared to monolayer culture when analyzed via 2D electrophoresis. These were then identified by MALDI MS mass fingerprinting as acidic calponin, laminin A/C, 15-hydroxy prostaglandin dehydrogenase, and acidic ribosomal protein P0 which are associated with the cytoskeleton, prostaglandin metabolism, and ribosomal function, respectively. Laminin A/C was identified by MALDI MS, however in the 2D electrophoresis it was approximately 30 kDa smaller than the expected size of the full length protein. They concluded that this represented a truncated version of the proteins which was more abundant in MCTS compared to monolayers. This difference was more pronounced in more compact MCTS and coincided with high levels of apoptosis. The abundance of this protein in 2D cultures under hypoxia and/or serum deprivation was investigated to determine whether these particular stresses are responsible for the cleavage of this protein in MCTS. Hypoxia alone were not sufficient to alter the abundance of this protein fragment in 2D cultures, however serum deprivation alone, or combined hypoxia and serum deprivation, resulted in an increased abundance of this truncated protein. It was demonstrated that the truncated laminin A/C protein fragment is a product of caspase-6 activation through the use of a caspase-6 inhibitor. This supports early findings of caspase-6 activation and protein cleavage within the MCTS. Interestingly, apoptosis and consequently caspase-6 activation, was present in localized areas throughout the spheroid. This is in contrast to the well characterized morphology of MCTS specifically the necrotic core, suggesting that these properties may not be universal.

4.1.2. Label Based MS Approaches

Minute differences in sample preparation, sample injection, and between each MS run can have profound effects on the MS results. This is particularly true when attempting to quantify differential protein abundance between samples. This results in a large number of technical replicate runs being required before any quantitative difference can be confidently identified. To overcome this, mass tags, such as SILAC, iTRAQ, and dimethyl labeling, can be incorporated into the samples, typically labeling proteins or peptides, during tissue culture or after cell lysis. The addition of these mass tags to one of the comparative groups allows multiple samples to be mixed and prepared in an identical manner while facilitating their separation within the mass spectrometer.
Through the use of such labeling techniques, multiple samples can be investigated simultaneously facilitating accurate relative quantification, reducing bias, and increasing reproducibility.\(^{[64]}\)

SILAC combined with LC-MS/MS, was applied by Musrap et al. (2015) to compare the proteome of the ovarian cancer cell line, OV90, grown adherently or as MCTS.\(^{[76]}\) Through the use of this method 1533 proteins were identified with 13 more abundant in MCTS and 6 less abundant when compared to adherent cells. From this they focused on calcium-activated chloride channel regulator 1 (CLCA1) for further analysis. This protein was found to be eightfold more abundant in MCTS compared to adherently grown cells and has been implicated in a number of biological process including cell–cell adhesion and apoptosis.\(^{[85]}\) Chloride channels have previously been implicated in ovarian cancer–stroma interactions facilitating metastasis\(^{[86]}\) and they observed that siRNA knockdown of CLCA1 or chemical inhibition of chloride channels inhibited aggregation of MCTS in these cell lines. From these findings they suggest that MCTS formation, and some aspects of cancer progression, may be dependent on chloride channels. However, further research is required to understand the importance of these results.

Dimethyl labeling combined with LC-MS/MS was employed by He et al. (2014) to investigate global protein abundance in U251 cells, a human glioma cell line, cultured as monolayers or MCTS.\(^{[74]}\) They were able to identify 363 differentially abundant proteins between monolayers and MCTS with the majority of these involved in metabolic processes, particularly glycolysis, which supports previous findings regarding the altered metabolism in 3D culture.\(^{[87]}\) Additionally, they highlight changes in the abundance of proteins involved in antigen presentation and proteasome function, which plays a vital role in antigen presentation,\(^{[88]}\) in MCTS. This includes human leukocyte antigen E (HLA-E), a well-known ligand of CD94/NKG2A that inhibits natural killer cell function.\(^{[89]}\) Its expression was further investigated with western blot, supported by RT-PCR and FACS, and found HLA-E to be significantly more abundant in MCTS compared to monolayers which was reflected in greater resistance of MCTS to NK mediated cytotoxicity in vitro and in animal models.

**4.1.4. Conclusion of Traditional Mass Spectrometry Techniques for Analyzing MCTS**

Traditional mass spectrometry techniques have proven to be a valuable tool for characterizing the molecular underpinnings of MCTS. In particular, they have identified many molecular differences conferred by growing cells in 3D conditions which, compared to monolayer culture, more closely replicates conditions found within solid tumors. Specifically, mass spectrometry techniques have revealed altered abundance of proteins involved in metabolism, stress responses, survival, cell–cell and cell–ECM interactions, decreased proliferation, and immune evasion. Additionally, a number of proteins involved in MCTS formation itself have been identified. For example, the importance of chloride channels in MCTS formation in ovarian cancer.\(^{[76]}\) Although mass spectrometry has been able to elucidate the altered abundance of proteins in these various MCTS contexts, further experiments are required to determine the biological relevance of these results. Mass spectrometry approaches toward analyzing MCTS do, however, provide a powerful foundation for future research into the molecular underpinnings of cancer progression.

**4.2. Mass Spectrometry Techniques to Obtain Spatially Defined Information from MCTS**

Cellular heterogeneity and cell–cell contacts play a large role in cancer progression and drug resistance.\(^{[91]}\) With the advent of in vitro models which replicate this heterogeneity, such as MCTS, comes the need for analysis techniques which provide spatially defined molecular information in a manner which maintains the biologically relevant features of the model structure. This is seen in MCTS which are heterogeneously organized 3D structures and, in the case of MCTS formed from dispersed cultured cells, form three physiological layers as a result of oxygen, nutrient, and waste gradients (Figure 1).\(^{[11]}\) The different conditions in different layers of the MCTS have significant influences on the molecular and phenotypic nature of the cells\(^{[27]}\); however, the mass spectrometry approaches described above have been limited to whole MCTS analysis. This may result in a loss of information, for example, high abundance proteins from the proliferative or necrotic regions may mask lower abundance proteins from the less translationally active quiescent region. This is of particular importance when investigating the impact of therapeutic agents on MCTS as therapeutic penetration and spatial accumulation of the therapeutic is of great importance.\(^{[19]}\)

**4.2.1. Serial Trypsinization Followed by Mass Spectrometry**

As discussed above (Section 4), serial trypsinization is a method which can provide spatially defined molecular information by
separating the MCTS into solubilized layers. This is ideal for LC-MS/MS analysis of different layers of the MCTS which can provide spatially defined information about peptide and PTM abundance as well as penetration and accumulation of therapeutic agents.

Serial trypsinization combined with LC-MS/MS has recently been applied to investigate the effect of the epigenetic drug UNC1999, a small molecule inhibitor of EZH1 and EZH2,[92] in a colon cancer MCTS model.[93] EZH1 and 2 are the catalytic subunit of the polycomb repressive complex 2[94] which controls the di- and trimethylation of histone H3 lysine 27 (H3K27).[95] Hypermethylation of this histone has been associated with cancer progression and identified as a possible therapeutic target.[96] Previously, the MCTS model has been shown to better replicate the epigenetic profile of primary cancer cells[97] making this a strong platform for testing of epigenetic drugs. Serial trypsinization followed by LC-MS/MS revealed vast differences in methylation patterns between monolayers and MCTS. Interestingly, there were also significant epigenetic differences between the different layers of the MCTS highlighting the biological heterogeneity of the structure. They followed this with an investigation into the influence of UNC1999 and found significant epigenetic changes, including a decrease in H3K27 trimethylation in the outer two layers of the treated MCTS. They did not observe this in the core component of the MCTS however, and attributed this to incomplete penetration of the drug. Phenotypically, this decreased H3K27 trimethylation in the treated MCTS resulted in decreased proliferation and deterioration of the MCTS structure into sequential layers. However, it requires extensive optimization for each cell line. Although the above study has employed a number of techniques to confirm that the MCTS shrinks by consistent volumes with each trypsinization, this reproducibility has not been confirmed in terms of spatially defined molecular information.

4.2.2. MALDI Mass Spectrometry Imaging

An alternative approach to analyzing these structures in a spatially relevant manner is through the use of MSI, typically MALDI MSI. The technique uses a matrix which co-crystallizes with the sample before directing a laser at a discrete region which facilitates ionization and sublimation. This produces a mass spectrometry analysis at each laser point which, when combined across a section, can provide spatially defined identification and relative quantification of analytes. This technique has been employed to investigate a vast range of analytes such as peptides,[98] and drugs[99] in MCTS. MALDI MSI also has the potential to investigate a number of PTMs, such as glycosylation[100] and has been widely utilized to investigate PTM profiles across tissue sections. While serial trypsinization followed by LC-MS/MS has been utilized to investigate PTMs in MCTS,[101] this is yet to be performed using MALDI MSI.

In addition to providing spatially defined molecular information, MALDI MSI also has the advantages of being able to analyze multiple samples during one run (such as with a tissue microarray [TMA]) and to investigate hundreds of molecules including those from different classes (e.g., peptides and PTMs) in a single experiment.[102]

However, compared to other mass spectrometry approaches, MALDI MSI has lower sensitivity resulting from interference from the matrix and the small amount of material analyzed at each laser point.[103] While fractionation techniques can be used in conjunction with MALDI, this is not the case with MALDI MSI performed on tissue sections, which further reduces the sensitivity of the technique.[103] The complexity of the samples in MALDI MSI also impedes identification of biological analytes. Typical identification techniques include peptide mass fingerprinting[104] and sequencing via tandem MS,[105] both of which require isolation of the analyte. As a result, complementary mass spectrometry analyses, such as LC-MS/MS, are often required for accurate analyte identification. However, the advent of more sensitive MALDI MSI techniques, such as FT-MS, hold potential for direct identification of biological analytes on tissue.[106]

Although MALDI MSI investigating peptides has been applied to the MCTS model, it is more commonly used to trace the penetration of pharmaceutical compounds within the structure as their chemical structure and molecular weight are known and are generally distinct from intrinsic analytes.

The first MALDI MSI investigation of MCTS was performed by the Hummon group in 2011[108] who investigated protein abundance in MCTS, derived from the colon cancer cell line HCT116, using MALDI MSI. By embedding the MCTS in gelatin followed by crossectioning, they were able to perform MALDI MSI on these structures and demonstrated visualization of a number of proteins through consecutive sections. Some of these were localized to specific regions of the MCTS structure, specifically the necrotic core and proliferative outer layer. They performed protein identification by solubilizing MCTS, performing 1D gel electrophoresis and analyzing specific bands with LC-MS/MS before confirming protein identifications by comparing the mass identified by LC-MS/MS with the m/z values seen in the MALDI MSI. Through this process they were able to identify cytochrome C and histone H4 and map their distribution.

In addition to proteins and drugs, MALDI MSI is also able to analyze other biological molecules such as lipids[107] and glycans.[108] Hiraide et al. (2016) used MALDI MSI to characterize the lipid profile of primary tissue derived colon cancer MCTS[107] and identified high expression of a lipid mass on the periphery of the MCTS. Laser capture microdissection (LCM) of the outer regions of the MCTS followed by LC-MS/MS was used to identify this mass as phosphatidylinositol (18:0/20:4). This phospholipid can be converted to phosphatidylinositol 3,4,5-trisphosphate which triggers the Akt pathways and consequently activates various tumor promoting processes.[109] Interestingly, this phospholipid was not observed in MCTS formed from cultured cell lines which they suggest is due to heterogeneity of cancer cells within the primary tumor which would not be present in the highly homogenous cell lines.[110]

MALDI MSI was also utilized by Liu and Hummon (2016) to investigate the penetration of the platinum based chemotherapy, oxaliplatin. They performed an on tissue chemical derivatization using diethylthiocarbamate to overcome platinum’s low ionization efficiency in MALDI MSI.[111] They observed an
accumulation of platinum in the periphery and the core of the MCTS, as has been observed previously by LA-ICP-MS.\cite{112} However they were also able to identify an inactive methionine bound metabolite localized to the core of the MCTS showing that MALDI can provide additional information about drug penetration into MCTS with appropriate sample preparation. They suggest that the localization of the inactive metabolite to the necrotic core is a product of increased detoxification in this area or an expulsion of this product from the quiescent layer. This quiescent layer is consistently devoid of platinum based chemotherapeutics,\cite{99,111–113} despite complete penetration of the drugs, suggesting that these cells possess detoxification or drug efflux mechanisms.

Recently Lukowski et al. (2017)\cite{114} have employed MALDI MSI to investigate the penetration of doxorubicin encased in a liposomal envelope in the first experiment of its kind. Liposomes are phospholipid structures which have low toxicity, can enhance drug delivery, and reduce clearance of the drug from the body.\cite{115} Comparing penetration of free and liposome encased doxorubicin into the MCTS formed from the colon cancer cell line HCT116, they did not observe a significant difference in penetration efficiency. However, an increased accumulation of the free drug at the outer layers of the MCTS was observed at an earlier stage, suggesting that there is a delayed release of the drug from the liposome.

To further the pursuit of revealing spatially defined molecular information in MCTS, we have performed MALDI MSI on ADS, grown from primary ovarian cancer cells in what is, to the best of our knowledge, the first ever analysis of its kind performed on ADS. Ovarian cancer cells were isolated from the ascites (patient 1 (serous ovarian carcinoma, stage 3C)), and maintained as monolayers before being grown as ADS per Li et al. (2011)\cite{116} except polyHEMA coated plates were used in place of agarose during ADS formation. These structures reached a diameter of approximately 600 μm after 9 d in culture after which they were formalin fixed and paraffin embedded (FFPE). In brief, ADS were isolated via centrifugation and washed in PBS before adding matrigel to encase the structures. Following incubation at 37 °C for 4 h, a 1 h formalin fixation was performed. This was followed by a shortened processing, to maintain ADS integrity, before they were embedded in paraffin and sectioned at 6 μm thickness onto indium tin oxide (ITO) (Bruker Daltonics, Bremen, Germany) conductive glass slides for MALDI MSI analysis. These sections were then prepared for MALDI imaging as previously described.\cite{116} Briefly, following deparaffinization and citric acid antigen retrieval (10 mM citric acid, pH = 6), ADS sections were digested with trypsin gold (Promega, Madison, WI), followed by incubation in a humidified chamber at 37 °C for 2 h. Internal calibrants\cite{117} and α-cyano-4-hydroxycinnamic acid matrix (CHCA, Bruker Daltonics) was then overlayed onto the ADS section using an ImagePrep station (Figure 2).

Mass spectra were acquired using an UltraflexXtreme MALDI-TOF/TOF instrument (Bruker Daltonics) with FlexControl (version 3.4) and FlexImaging software (version 4.0) in positive reflectron mode. The data was acquired at 50 μm lateral resolution with a laser frequency of 2 kHz over an m/z range of 800–4500 Da (Figure 2). Following data acquisition, CHCA matrix was removed using 70% ethanol before the ADS sections were H&E stained and scanned using a Nanozoomer (Hamamatsu, Shimadzu) (Figure 3). The spatial distribution of peptides was visualized using SCiLS lab software (version 2016a, GmbH, Bremen, Germany) in the form of ion intensity maps.

Using this approach, we were able to identify more than 200 m/z values that were unique to the ADS structure. Of these, we identified 15 m/z values whose intensity correlated with discriminative regions of the structure (proliferative, quiescent, and necrotic layers). We predict that this represents region specific protein abundance (Figure 3).

These preliminary experiments show that MALDI MSI can be effectively applied to ADS. However, this is with the caveat that these cells were maintained as monolayers before being encouraged to grow as 3D structures, which is expected to not accurately replicate pathological ADS. Into the future we aim to apply these techniques to a broader patient cohort, to investigate drug penetration in this context and to utilize this platform to investigate the molecular underpinnings of cancer progression and chemoresponse.

The techniques described above allow for spatially defined elucidation of analytes present in solubilized fractions of the MCTS or across slide mounted sections. Despite the limitations inherent with each of these techniques they have provided relevant information about protein expression, phospholipids, metabolism, and drug distribution in a spatially defined manner. Finally we have presented, for the first time, a MALDI MSI approach to investigate peptide abundance in aggregates formed from ascites derived primary ovarian cancer cells.

5. Outlook for Mass Spectrometry of Ovarian Cancer ADS

MCTS are typically used as a model of avascularized tumors, however, ovarian cancer is characterized by the presence of...
6. Conclusion

Despite their discovery more than 40 years ago, MCTS models have only recently emerged as a powerful platform for in vitro research and drug testing. They have repeatedly been shown to better replicate and represent the avascularized tumors compared to the same cells grown as monolayers in regard to gene expression, antigen expression, proliferation, hypoxia, nutrient, waste, and gas diffusion gradients.\(^{[8,9,36,37,124–127]}\)

The application of mass spectrometry analysis techniques to this biological model promises to provide new insight into cancer progression and treatment response. This includes identification of altered metabolism, cell–cell interactions and cell–ECM connections as well as antigen expression.\(^{[9,10,71,74,76,90,128]}\)

Although additional techniques are required to complement proteomics analysis of these structures, it represents a powerful foundation from which novel cancer research can be developed.

Recently, MSI has been used to investigate spatially defined peptide expression and drug penetration in MCTSs.\(^{[99,111,113,129,130]}\)

The ability to image therapeutics and molecules in a spatially defined way provides a window into solid tumors and their response to therapy. This has the potential to lead to a better understanding of cancer progression, pharmacokinetics, and the role of heterogeneous cell population in response to treatment.

In the context of ovarian cancer, being able to investigate the molecular underpinnings of a patient’s individual disease in a rapid and informative manner through mass spectrometry analysis of ADS represents an avenue toward personalized cancer therapy. This platform has potential to identify effective treatments and/or molecular markers, which predict response to treatment. Personalized therapy is the way of the future for cancer treatment\(^{[131]}\) and in particular in ovarian cancer, which is characterized by high recurrence rates and drug resistance, highlighting the need for better methods to inform appropriate treatment to improve patients outcome.

The development of novel and more sophisticated mass spectrometry techniques, including MSI, and their applications and implementation in clinical laboratories, will contribute significantly to making personal medicine a reality.

Abbreviations

CLCA1, Calcium-activated chloride channel regulator 1; ECM, Extracellular matrix; H3D27, Histone 3 lysine 27; HNSCC, Head and neck squamous cell carcinoma; LCD, Laser capture microdissection; MALDI, Matrix assisted laser desorption/ionization; MCTS, Multicellular tumor spheroids; MSI, Mass spectrometry imaging; OS, Organotypic spheroids; PIP3, Phosphatidylinositol 3,4,5-trisphosphate; PRC2, Polychrome repressive complex 2

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Conflict of Interest

The authors have declared no conflict of interest.

Keywords

cancer, drug resistance, mass spectrometry-LC-MS/MS, mass spectrometry-MALDI-TOF, multicellular tumor spheroids, ovarian cancer

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