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Mass Spectrometry Imaging of 3D Tissue Models

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A 3D cell culture is an artificially created environment in which cells are permitted to grow/interact with their surroundings in all three dimensions. Derived from 3D cell culture, organoids are generally small-scale constructs of cells that are fabricated in the laboratory to serve as 3D representations of in vivo tissues and organs. Due to regulatory, economic and societal issues concerning the use of animals in scientific research, it seems clear that the use of 3D cell culture and organoids in for example early stage studies of drug efficacy and toxicity will increase. The combination of such 3D tissue models with mass spectrometry imaging provides a label-free methodology for the study of drug absorption/penetration, drug efficacy/toxicity, and drug biotransformation. In this article, some of the successes achieved to date and challenges to be overcome before this methodology is more widely adopted are discussed.

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An organ that has been successfully modeled via 3D cell culture techniques is human skin. A range of commercially available models have become established for toxicological and pharmaceutical studies. These include models of human reconstructed epidermis (HRE), 3D differentiated epidermis cultures derived from human keratinocytes, that is, EpiSkin (Epskin, Lyon, France) and EpiDerm (Mattek, Ashland, USA) and full thickness living skin equivalents (LSE), for example, EpiDermFT (Mattek, Ashland, USA), T-skin (Episkin, Lyon, France) and Labskin (Innovenn(UK) Ltd, York, UK). The full thickness LSEs comprise a differentiated epidermis supported by a dermal component consisting of fibroblasts in a 3D scaffold. A comprehensive review into their use in drug development has been published by Mathes et al.[2]

The combination of 3D tissue models of human skin with MSI potentially provides an elegant label-free methodology for the study of both drug absorption and drug biotransformation in skin. The earliest work in this area was reported by Avery et al.[3] who examined the absorption of the drug imipramine into a commercial 3D tissue model of the epidermis "Straticel." Other studies of a similar type have been reported by Francese et al.[4] and Mitchell et al.[5] In the work of Francese et al.[4] MSI was used to map the distribution of the drug acetretin within a commercial living skin equivalent model with the purpose of investigating the efficiency of curcumin as matrix compared to CHCA. MSI data of Labskin 4 h post treatment samples showed the penetration of acetretin into the epidermal layer. In further development of this work reported by Harvey et al.,[6] the localization of the same drug was analyzed using MALDI-MSI, after the creation of an LSE exhibiting psoriatic-like properties by treatment of the commercial product with the pro-inflammatory cytokine interleukin-22. In this modified model, the distribution of acetretin was studied at 24 h and 48 h post treatment and the data obtained demonstrated that after 48 h, it was possible to observe the drug penetration into the dermal region, whereas after 24 h, it was still localized in the epidermal layer only.[6]

A concern that has been expressed in the use of 3D cell culture models for absorption studies relates to the difference in the absorption properties of such models compared to human skin.[7] It was found in a large-scale validation study carried out in Germany that the permeation of chemicals was overestimated when using 3D models.[7] This seems to be an instance where a discussion of the philosophy of the use of tissue models is appropriate. For acceptance of the use of these models in absorption studies what is required is an acknowledgement that the models are "models," not human skin. In order for the models to be used to predict absorption behavior in human skin, what is therefore required is that their absorption behavior be fully characterized for substrates with a range of physiochemical properties so that conversion/scaling factors can be derived.

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Similar issues surround the use of 3D models of skin for the study of drug biotransformation. This is a complex area where there is not at present even a consensus on the range and distribution of metabolizing enzymes present in human skin itself.[8–10] Nevertheless, given the difficulties in reliably obtaining human skin for metabolism studies (and sufficient skin for a representative study given issues including race, gender, age, and genetic polymorphisms) there has been interest in the use of 3D models in this area. In the United Kingdom, the NC3Rs (National Centre for the Replacement, Refinement, and Reduction of Animals in Research) instigated in 2016 a challenge to researchers "To establish, both qualitatively (which metabolites are produced) and quantitatively (concentration of the metabolites produced), the extent to which skin metabolism determines xenobiotic availability in human skin" (https://crackit.org.uk/challenge-20-metaboderm). Working towards this aim, we became interested in examining, the use of MSI, to localise, where in full thickness human skin and a commercial skin model, the presence of metabolising enzymes could be detected. In order to achieve this, we developed the idea of "substrate-based mass spectrometry imaging" (SBMSI).[11] Here the surface of the skin or model was treated with a known substrate for a specific metabolizing enzyme, left to incubate for 48 h before a section through the skin model was examined by MSI. **Figure 1** shows the results of such an experiment carried out to detect the presence of esterase activity in a full thickness skin model using methyl paraben as a probe.[12] In these data the biotransformed methyl paraben (shown in green) is clearly detected in the epidermal layer, hence indicating esterase activity in this region. Quantitative MSI of the amounts of such metabolites formed could be used to give a measure of the amount of enzyme present in different regions of the skin model and human skin. Derivation of scaling factors would then allow the building of robust in silico models to predict skin metabolism.[13]

In a recent study, Lewis et al.[14] have utilized MSI to study wound healing in a full thickness 3D skin model. The aims of this experiment were to develop a project for the testing wound-healing products. Sections of skin wounded by incision were examined by MSI after 4 days. It was observed that the wound in the skin had healed and migration of epidermal cells into the wound bed could be observed. Using a multivariate statistics approach signals associated with the wound bed were highlighted and then identified by a combination of accurate mass measurement and MS/MS. All of the 14 metabolites highlighted as having elevated levels within the wound bed were associated with biochemical pathways associated with wound healing including reepithelialization, reduction of inflammation, and cell proliferation.

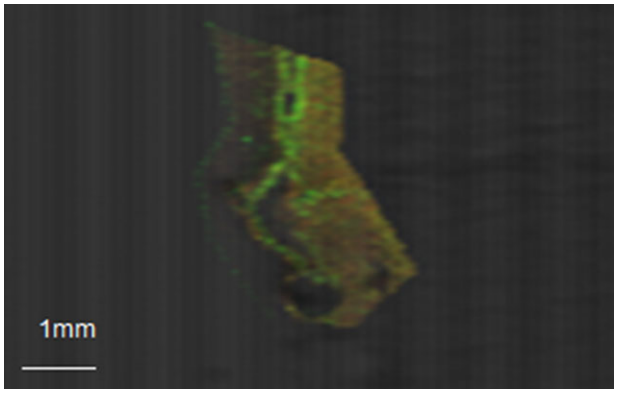

Figure 1. Substrate-based mass spectrometry imaging (SBMSI): MSI used to detect the presence of esterases. A commercially available full thickness 3D skin model was treated with the esterase probe methyl paraben. After 48 h, sections were taken and examined by MSI; the image shows the localization of methyl paraben (red) and its esterase-generated phase 1 metabolite *p*-hydroxybenzoic acid (green) confirming the presence of esterase activity in the model.[11]

An area where use of 3D models might be expected to increase is in the study of chemical toxicity. Most countries in the world require comprehensive toxicity testing of newly created chemicals, which may be used as/in industrial chemicals, pesticides, food additives, biotechnology products, or pharmaceuticals. Legislation requires the conduction of specific tests depending on whether the chemicals are, for example, mono-constituent or multi-constituent substances, mixtures of chemicals, pesticide formulations, or cosmetic products. There are several legislative organizations in the world, including the Organization for Economic Co-operation and Development (OECD), which provides regulatory frameworks to assess the safety of any chemicals, which have to be agreed by government, industry, and independent laboratories (http://www.oecd.org/). One aspect of the legislation studies the effect of chemicals on human health through assessing toxicokinetics, topical toxicity, and systemic toxicity. There is clearly a role for 3D tissue models in this area and indeed an increased use of 3D tissue models of human skin in toxicology was stimulated by European legislation, Directive 76/768 EEC which detailed with the upcoming prohibition of the use of animal models for the toxicity testing of cosmetics and cosmetic ingredients. It has to be considered in risk management strategies carried out by industry that societal pressure could lead to similar legislation being drafted in other areas of chemical/pharmaceutical safety and hence research now into the use of 3D models for toxicokinetic studies in these other areas seems essential. Indeed 3D models would seem to be ideal platforms for investigations into adverse outcome pathways (AOP) and associated molecular initiating events (AOP) widely proposed as the route to large-scale toxicity screening of chemicals.[15]

MSI has also emerged as a powerful analytical tool to visualize specific species and drug therapeutics in 3D cell culture models of cancer: tumor spheroids. Spheroids have become essential tools for in vitro research in this area due to their ability to replicate the tumor microenvironment.[16] These spheroid models mimic the complexity and structure of in vivo tumors that is not possible by traditional 2D cell culture.[17] Additionally, spheroids are a cost-effective way of modeling the intricate processes of tumor environments, with added benefits of higher throughput results and avoiding ethical issues associated with animal models.[18] For the analysis of drug penetration, spheroids are of a particular interest due to the formation of a pathophysiological gradient within its structure.[19] Spheroids consist of three layers, a necrotic core encapsulated by a quiescent layer and an outer proliferating region. The structure of spheroids mimics the physiological barrier for drugs to penetrate making it a highly representative model of a solid tumor.[19] By understanding the molecular composition and structure of spheroids,

the distribution of drugs and the responses from treatment can be further understood. Therefore, an MSI approach to monitor drug penetration is a highly desirable method for pharmaceutical development.

Conventional methodologies including fluorescent microscopy[20] and magnetic resonance imaging[21] have previously been used to measure drug distribution in 3D tissues. These methods, however, require the addition of labels or probes which can impact the efficacy of drug distribution, and possibly alter the biological composition of 3D samples. MSI has the ability to directly map a variety of molecular species, drugs, and metabolites within 3D culture models without the use of any labels or probes.[22] Spheroids and drug treatment are therefore not compromised when analyzed by MSI.

Li and Hummon were the first to examine the molecular composition within spheroids using MSI.[23] MALDI-MSI was used to determine the spatial distribution of predominant species in 3D colon cancer cells, HCT116. Specific species including cytochrome C and histone H4 were identified and validated by protein sequencing and LC-MS/MS. This approach successfully located and identified specific peptides without prior knowledge or labeling, proving it to be a valuable methodology to analyze the true genetic makeup within spheroids. An alternative study also utilized the MALDI-MSI approach to examine lipid metabolism of 3D primary colon cancer cells.[24] Identification of phospholipids in the surface region of spheroids gave a further understanding of the importance of colon cancer progression. These studies revealed the benefits of spatial distribution of specific species in spheroids to distinguish possible biomarkers and potential targets for cancer therapeutics.

The ability of MALDI-MSI has expanded to the analysis of drug distribution and molecular responses in spheroids. Liu et al. demonstrated time-dependent distribution of a chemotherapeutic, irinotecan, and its metabolites by MALDI-MSI on colon spheroids, validated by LC-MS/MS.[25] A more recent study used this technique in combination with iTRAQ MS/MS for the identification of proteomic changes in response to a combinational chemotherapeutic drug, FOLFIRI.[26] These projects have demonstrated MSI to be an excellent methodology for exploring pharmaceutical distribution within spheroids. Subsequently, further studies have utilized this approach to determine the spatial distribution of other therapeutics. Liu and Hummon quantitatively tracked the penetration and metabolism of platinum-based drugs in colon cancer spheroids by MALDI-MSI in combination with UPLC-MRM.[27] Results from this study show great potential as a pre-clinical methodology for the analysis of metal-based drugs. The Lukowski group has applied the MSI-spheroid approach for analyzing the efficacy of liposomal drug delivery.[28] The study, however, showed slower metabolic rates compared to free-drug delivery and suggested expanding the approach to actively quantify the amount of drug in spheroids for future work.

This multiplex nature of MSI creates an advantage for analyzing the efficacy of chemotherapeutics for treatment of different cancers. Further studies, however, could be achieved to evaluate alternative cancers and representative therapeutics due to current research primarily focused on colon cancer cell lines. Additional benefits of this approach also allow for high throughput analysis of representative biological models that also impacts the usage of animal models in such experiments. MSI could also be further expanded to actively quantify drug concentrations within spheroids; however, methods including LC-MS can still be utilized for this.[27,28] Nonetheless, MSI of spheroids offers a valuable approach for pre-clinical screening of therapeutics for the pharmaceutical industry.

MSI has therefore emerged as an attractive tool for the analysis of drug distribution within 3D cell cultures models, such as skin and spheroids. MSI techniques have the ability to localize drugs and metabolites within the structure of the models and in addition biological changes in response to treatment/exposure can be observed.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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[1] A. Gonneaud, A. Claude, F. Boudreau, F. Boisvert, *Proteomics* **2017**, *17*, 1700023.
[2] S. H. Mathes, H. Ruffner, U. Graf-Hausner, *Adv. Drug Deliv. Rev.* **2014**, *69–70*, 81.
[3] J. L. Avery, A. McEwen, B. Flinders, S. Francese, M. R. Clench, *Xenobiotica* **2011**, *41*, 735.
[4] S. Francese, R. Bradshaw, B. Flinders, C. Mitchell, S. Bleay, L. Cicero, M. R. Clench, *Anal. Chem.* **2013**, *85*, 5240.
[5] C. A. Mitchell, M. Donaldson, S. Francese, M. R. Clench, *Methods* **2016**, *104*, 93.
[6] A. Harvey, L. M. Cole, R. Day, M. Bartlett, J. Warwick, R. Bojar, D. Smith, N. Cross, M. R. Clench, *Proteomics* **2016**, *16*, 1718.
[7] M. Schäfer-Korting, U. Bock, W. Diembeck, H-. Dösing, A. Gamer, E. Haltner-Ukomadu, C. Hoffmann, M. Kaca, H. Kamp, S. Kersen, M. Kietzmann, H. C. Korting, H-. Krachter, C-. Lehr, M. Liebsch, A. Mehling, C. Möller-Goymann, F. Netzlaff, F. Niedorf, M. K. Robbelke, U. Schöfer, E. Schmidt, S. Schreiber, H. Spielmann, A. Vuia, M. Weimer, *ATLA Altern. Lab. Anim.* **2008**, *36*, 161.
[8] N. Manevski, P. Swart, K. K. Balavenkatraman, B. Bertschi, G. Camenisch, O. Kretz, H. Schiller, M. Walles, B. Ling, R. Wettstein, D. J. Schaefer, P. Itin, J. Ashton-Chess, F. Pognan, A. Wolf, K. Litherland, *Drug Metabol. Disposit.: Biol. Fate Chem.* **2015**, *43*, 126.
[9] Z. Z. van Eijl S., J. Cupitt, M. Gierula, C. Gotz, E. Fritsche, R. J. Edwards, *PLoS One* **2012**, 7.
[10] F. Oesch, E. Fabian, K. Guth, R. Landsiedel, *Arch. Toxicol.* **2014**, *88*, 2135.

[11] N. Couto, N. Jillian, R. Bojar, et al., Presented at 65th ASMS Conf. on Mass Spectrometry and Allied Topics, Indianapolis, IN, June 4–8, **2017**.
[12] A. Suzanne, G. Helene, K. Nancy, P. Marie-Helene, N. Patrick, M. Jacques, *Drug Metabol. Pharmacokinet.* **2010**, *25*, 568.
[13] J. C. Madden, S. Webb, S. J. Enoch, H. E. Colley, C. Murdoch, R. Shipley, P. Sharma, C. Yang, M. T. D. Cronin, *Comput. Toxicol.* **2017**, *3*, 44.
[14] E. E. L. Lewis, L. Freeman-Parry, R. A. Bojar, M. R. Clench, *Int. J. Cosmetic Sci.* **2018**, https://doi.org/10.1111/ics.12446.
[15] National Research Council, *Toxicity Testing in the 21st Century: A Vision and a Strategy*, The National Academies Press, Washington, DC **2007**.
[16] K. Klimkiewicz, K. Weglarczyk, G. Collet, M. Paprocka, A. Guichard, M. Sarna, A. Jozkowicz, J. Dulak, T. Sarna, C. Grillon, C. Kieda, *Cancer Lett.* **2017**, *396*, 10.
[17] R. Edmondson, J. J. Broglie, A. F. Adcock, L. Yang, *Assay Drug Dev. Technol.* **2014**, *12*:207.
[18] K. Nam, A. S. T. Smith, S. Lone, S. Kwon, D. Kim, *J. Lab. Autom.* **2015**, *20*, 201.
[19] G. Mehta, A. Y. Hsiao, M. Ingram, G. D. Luker, S. Takayama, *J. Control Release* **2012**, *164*, 192.
[20] R. Edmondson, J. J. Broglie, A. F. Adcock, L. Yang, *ASSAY Drug Dev. Technol.* **2014**, *12*, 27.
[21] E. S. O'Neill, A. Kaur, D. P. Bishop, D. Shishmarev, P. W. Kuchel, S. M. Grieve, G. A. Figtree, A. K. Renfrew, P. D. Bonnitcha, E. J. New, *Inorg. Chem.* **2017**, *56*, 9860.
[22] X. Liu, A. B. Hummon, *Anal. Chem.* **2015**, *87*, 9508.
[23] H. Li, A. B. Hummon, *Analyt. Chem.* **2011**, *83*, 8794.
[24] T. Hiraide, K. Ikegami, T. Sakaguchi, Y. Morita, T. Hayasaka, N. Masaki, M. Waki, E. Sugiyama, S. Shinriki, M. Takeda, Y. Shibasaki, S. Miyazaki, H. Kikuchi, H. Okuyama, M. Inoue, M. Setou, H. Konno, *Sci. Rep.* **2016**, 6.
[25] X. Liu, E. M. Weaver, A. B. Hummon, *Anal. Chem.* **2013**, *85*, 6295.
[26] G. J. Labonia, K. R. Ludwig, C. B. Mousseau, A. B. Hummon, *Anal. Chem.* **2018**, *90*, 1423.
[27] X. Liu, A. B. Hummon, *Sci. Rep.* **2016**, 6.
[28] J. K. Lukowski, E. M. Weaver, A. B. Hummon, *Anal. Chem.* **2017**, *89*, 8453.

