2019

3D Growth Substrates in Mammalian Cell Culture in Vitro, their Effects on Cellular Function and Response

Mahmoud Gargotti

Technological University Dublin

Follow this and additional works at: https://arrow.tudublin.ie/sciendoc

Part of the Physical Sciences and Mathematics Commons

Recommended Citation

This Theses, Ph.D is brought to you for free and open access by the Science at ARROW@TU Dublin. It has been accepted for inclusion in Doctoral by an authorized administrator of ARROW@TU Dublin. For more information, please contact arrow.admin@tudublin.ie, aisling.coyne@tudublin.ie, gerard.connolly@tudublin.ie, vera.kilshaw@tudublin.ie.
3D growth substrates in mammalian cell culture \textit{in vitro}, their effects on cellular function and response

BY

Mahmoud Gargotti
PhD Thesis
Supervisor
Dr. Alan Casey

Physics, Clinical and Optometric Sciences/ FOCAS Research Institute
Technological University Dublin

2019
Abstract:

Three-dimensional (3D) cell culture systems have gained increasing interest in drug discovery and tissue engineering due to a number of advantages in providing more physiologically relevant information and potentially more predictive data for *in vivo* tests. Extracellular matrix (ECM) proteins have been developed over in recent years to simulate a natural microenvironment for cells cultured *in vitro*. Conventional cell culture or 2D cell culture form monolayers of cells on a solid surface, which is typically polystyrene or glass, whereas a 3D culture system employ a porous growth matrix on which the cells grow. The transition from cell culture on a flat surface 2D to a 3D model *in vitro* is expected to mimic more realistic culture environments. However, the major limitation of 2D culture is their lack of structural architecture and stroma and not all types of normal epithelial cell are able to adhere and grow on 2D culture.

For this study, two different immortal cell lines were used (HeLa and HaCaT cells) and two commercial 3D substrates (Collagen Rat Tail and Geltrex) were used, in order to evaluate how the transition from 2D to 3D affects viability, cell cycle, live/dead cell, responses to the drug exposure and bio-spectroscopy studies.

The viability of cells were monitored with the aid of the Alamar Blue assay, cellular morphology was monitored with confocal microscopy, cell cycle and cell death studies were performed with flow cytometry and viability of 3D culture in bio-spectroscopy performed with Raman spectroscopy. The viability studies showed apparent differences between the 2D and 3D culture systems, the differences attributed in part to the physical transition from 2D to a 3D environment causing alterations to effective resazurin concentration, uptake and conversion rates. This was verified by flow cytometry, in which no significant differences in viable cell numbers between 2D and 3D systems was
observed. Cell cycle analysis revealed cellular function could be altered by growth on the 3D substrates and the alterations were noted to be dependent on 3D membrane concentration. The use of 3D culture matrices has been widely interpreted to result in “improved viability levels” or “reduced” toxicity or cellular “resistance” compared to cells cultured on traditional 2D systems. Cells were grown on the different substrates (Collagen and CaF$_2$), confirming that the \textit{in vitro} cell culture environment impacts significantly on the cell cycle. The live cell \textit{in vitro} Raman spectroscopic analysis of cells on the 2D CaF$_2$ and 3D Collagen substrates was performed and data were analysed using principal component analysis (PCA). The spectroscopic analysis revealed differences in profiles which reflect the differences in cell cycle for both \textit{in vitro} culture environments. In particular, the Raman spectra of cells grown on CaF$_2$ show indicators of cell stress, which are also associated with cell cycle arrest at the G0/G1 phase. Doxorubicin still induced apoptosis with no difference in the population levels in 3D and 2D culture of apoptotic, necrotic and live cells. Also, cells grown on both substrates were arrested at G0/G1 phase by the Doxorubicin, Raman spectra collected from cells grown in Collagen showed that, Doxorubicin is clearly seen to be present in the nucleolus, nucleus and cytoplasm of the HaCaT cells. Statistical analysis, consisting of principal components analysis (PCA) was used to highlight the Doxorubicin interaction with HaCaT cells grown in 3D cultures. The results of this study show that Cellular health and viability levels were not altered by culture in 3D environments, but their normal cycle could be altered verified by the cell cycle studies performed and these variations must be accounted for in studies employing 3D membranes. The Raman spectra of cells grown on CaF$_2$ show indicators of cell stress, which are also associated with cell cycle arrest at the G0/G1 phase. The bioavailability or effective concentration of the cytotoxicity assay and the chemotherapeutic agent are both affected by the
absorptive nature of the matrix. Despite the differences in the cell cycle in cells grown in 2D and 3D cultures, the efficacies and ultimate effect of the drug on all the cultured cells are the same regardless of culture environment and the variations in the cell cycle for cells grown on different substrates must be accounted for in vitro cellular screening in particular when screening cell cycle dependant toxicants. This study has shown that the use of 3D culture systems has the potential to make a significant impact in the Raman spectroscopy field in particular Collagen is a cost effective substrate replacement for more expensive options.
Declaration

I certify that this thesis which I now submit for examination for the award Doctor of Philosophy (Ph.D), is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Technological University Dublin and has not been submitted in whole or in part for another award in any other third level institution.

The work reported on in this thesis conforms to the principles and requirements of the Technological University Dublin guidelines for ethics in research.

Technological University Dublin has permission to keep, lend or copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

Signature

___________________________________________ Date ____/____/____

Mahmoud Gargotti
Acknowledgements

First of all, I thank Allah, the almighty, for giving me the strength to carry on this thesis and for blessing me with many great people who have been my greatest support in both my personal and professional life. I would like to seize this opportunity to express my deepest regards and gratitude to my supervisor Dr. Alan Casey for his dedication and sincere effort throughout the whole stages of bringing this thesis into light. Dr. Alan has been my role model who I have always admired. His passion and dedication in teaching has a strong effect on his students including me. I was really lucky and blessed to be taught by such a phenomenal teacher who truly believes in the power of education in positively shaping and changing peoples' lives to the best. I would also love to thank Prof. Hugh Byrne for dedicating much of his time to help me with many of the problems I faced while conducting the study that would not have been possible without his constant guidance and constructive feedback.

Last but not least, I would like to express my deepest gratitude to my family for supporting me every stage of my life, their endless love and confidence in me. And finally I would like to thank all colleagues in the Nanolab who provided insight and expertise that greatly assisted the thesis.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M NaOH</td>
<td>1 Moles sodium hydroxide</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensions</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensions</td>
</tr>
<tr>
<td>AB</td>
<td>Alamar Blue</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorption</td>
</tr>
<tr>
<td>CaF₂</td>
<td>Calcium fluoride</td>
</tr>
<tr>
<td>CARS</td>
<td>Coherent anti-stokes Raman scattering</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge coupled device</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinases</td>
</tr>
<tr>
<td>CDM</td>
<td>Cell-derived matrices</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal Laser Scanning</td>
</tr>
<tr>
<td>DH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Modified Eagle Medium: Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrices</td>
</tr>
<tr>
<td>Em</td>
<td>Emission</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward-scattered light</td>
</tr>
<tr>
<td>FT-Raman</td>
<td>Fourier transform Raman</td>
</tr>
<tr>
<td>G₁ phase</td>
<td>First growth phase</td>
</tr>
<tr>
<td>G₂ phase</td>
<td>Second growth phase</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
</tbody>
</table>
IC₅₀  Inhibitory concentration
IR    Infrared
IU    International unit
LBC   Liquid-based cytology
M     Molar
mg    Milligram
min   Minute
mL    Millilitre
mM    Millimolar
M phase Mitotic phase
NCCD  The Nomenclature Committee on Cell Death
nM    Nano-molar
nm    Nano-meter
PAMAM Polyamido amine
PBS   Phosphate buffered solution
PCA   Principal Component Analysis
PED   Poly-ethylene oxide
PEG   Poly-ethylene glycol
PI    Propidium iodide
PMT   Photomultiplier
P-value Probability value
RCCS  Rotary Cell Culture System
RNA   Ribonucleic acid
RNase Ribonuclease
RPM   Round per minuet
RR    Resonance Raman
SD    Standard deviation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERS</td>
<td>Surface enhanced Raman scattering</td>
</tr>
<tr>
<td>S phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>SSC</td>
<td>Side-scattered light</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WP</td>
<td>Work packages</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micro-molar</td>
</tr>
</tbody>
</table>
## Table of Contents

Abstract ........................................................................................................................................... i
Declaration ....................................................................................................................................... iv
Acknowledgements ...................................................................................................................... v
List of abbreviations ...................................................................................................................... vi
Table of Contents .......................................................................................................................... ix
List of Figures .................................................................................................................................. xiv
List of Tables ..................................................................................................................................... xxii

### CHAPTER 1

Introduction ..................................................................................................................................... 1

1.1. Introduction ............................................................................................................................... 2

1.2. 2D culture vs 3D culture ........................................................................................................... 4

1.3. 3D culture and drug discovery ................................................................................................ 12

1.4. Bio-spectroscopy ...................................................................................................................... 14

1.5. Aims and Objectives ................................................................................................................ 17

1.6. Thesis Summary ...................................................................................................................... 18

### CHAPTER 2

Methodology .................................................................................................................................... 21

2.1. Materials .................................................................................................................................. 22
2.2. Cell culture

2.3. 3D substrates preparation

2.4. Confocal Laser Scanning Microscopy (CLSM)

2.5. Alamar Blue Assay (AB)

2.5.1. Cell viability measurement with Alamar Blue (AB)

2.5.2 Assessment of Doxorubicin Cytotoxicity by using Alamar blue

2.6. Flow Cytometry

2.6.1. Cell cycle Analysis

2.6.2. Apoptosis assay

2.7. UV-Visible spectroscopy

2.8. Raman Spectroscopy

2.9. Raman spectroscopy measurements

2.10. Data analysis

2.11. Chapter Summary

CHAPTER 3

Comparative studies of cellular viability levels on 2D and 3D in vitro culture matrices

3.1. Introduction

3.2. Results
3.2.1. Confocal microscopic imagining

3.2.2. Cell viability measurement with Alamar Blue

3.2.3. Apoptosis and necrosis analysis

3.2.4 Cell cycle analysis

3.3. Discussion

3.4. Conclusion

CHAPTER 4

Raman spectroscopy detects biochemical changes due to different cell culture environments in live cells in vitro

4.1. Introduction

4.2. Results

4.2.1 Cell cycle analysis for HeLa and HaCaT cell lines on different substrates

4.2.2. Raman spectroscopic analysis of HeLa and HaCaT cells on CaF$_2$ substrate

4.2.3. Raman spectroscopic analysis of HeLa and HaCaT cells on Collagen substrates

4.2.4. Comparison between CaF$_2$ and Collagen

4.3. Discussion

4.4. Conclusion
CHAPTER 5

Chemotherapeutic efficiency of drugs in vitro: Comparison of Doxorubicin exposure in 3D and 2D culture matrices

5.1. Introduction

5.2. Results

5.3. Discussion

5.4. Conclusions

CHAPTER 6

Monitoring cellular uptake and efficacy of Doxorubicin in 2D and 3D in vitro cell culture platforms using Raman spectroscopy

6.1. Introduction

6.2. Results

6.2.1 Cytotoxicity assays

6.2.2 Cell cycle analysis

6.2.3 Raman spectroscopy

6.2.4 Apoptosis and Necrosis analysis

6.3. Discussion

6.4. Conclusion

CHAPTER 7

Conclusions and final remarks
7.1. Thesis Summary ........................................................................................................122

7.2. Implications of the work on “Bio-spectroscopy” .............................................129

References .......................................................................................................................131

Supplementary Material ...............................................................................................153

Appendix I: Publications ...............................................................................................160

Appendix II: Conferences, Courses and Summer Schools ....................................161
List of Figures

Figure 1.1. Different cell culture systems, cell grown in 2D culture and cell grown in 3D culture (Dhaliwal 2012). (Page 5)

Figure 1.2. Description the advantages of using 3D culture models in vitro to bridge the gap between 2D culture and in vivo, 3Rs principle and using 3D culture to predict drug delivery prior clinical trials (Fitzgerald et al., 2015). (Page 8)

Figure 1.3. Types of 3D cell culture plates. A- Scaffolds 3D culture or from cell-derived matrices. B- Spheroids cell culture, derived from single tumour cell. C- Microfluidic based systems (Fitzgerald et al., 2015). (Page 12)

Figure 1.4. Schematic illustration of Raman Spectroscopy set up. (Page 17)

Figure 2.1. HeLa and HaCaT cell lines. The figure is adapted from the images provided by ATCC and CLS (ATCC-HeLa; CLS-HaCaT). (Page 23)

Figure 2.2. Schematic diagram of the optical pathway and principle in a Confocal Laser Scanning microscope, (Claxton et al., 2006). (Page 25)

Figure 2.3. Reduction of Resozurin sodium salt to strongly fluorescent Resofurin sodium salt in the presence of metabolic active cells (Markaki 2009). (Page 28)

Figure 2.4. Hydrodynamic focusing of the sample through the laser beam (BDBiosciences, 2000). (Page 31)

Figure 2.5. The cell cycle consists of interphase and the mitotic phase. During interphase, the cell grows and the nuclear DNA is duplicated. Interphase is followed by the mitotic phase. During the mitotic phase, the duplicated chromosomes are segregated
and distributed into daughter nuclei. The cytoplasm is usually divided as well, resulting in two daughter cells (The cell cycle). (Page 33)

Figure 2.6. Illustration of the cell death pathway (Jeng et al., 2018). (Page 36)

Figure 2.7. Schematic representation of the energy transition in Raman microspectroscopy with h: Plank’s constant and v0: excitation frequency v: bond vibration frequency. (Page 40)

Figure 3.1. a HeLa cells were seeded on 2D culture for 24 h, nuclei were stained with the nuclear stain NucRed. b HaCaT cells were seeded on 2D culture for 24 h, nuclei were stained with the nuclear stain NucRed. c HeLa cells were seeded on 3D culture (Collagen rat rile) for 24 h and nuclei stained with NucRed. d HaCaT cells were seeded on 3D culture (Collagen rat tile) for 24 h and nuclei stained with NucRed. e HeLa cells were seeded on 3D culture (Geltrex) for 24 h and nuclei stained with NucRed. f HaCaT cells were seeded on 3D culture (Geltrex) for 24 h and nuclei stained with NucRed (scale bar 20 µm). (Page 49)

Figure 3.2. Alamar Blue response following 24, 48 and 72 h growth on both 2D and 3D culture (Collagen) of HeLa and HaCaT cells on both a 6 well plate and b a 24 well plate. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments and relative to a 2D culture control. Statistically significant differences between the 3D culture membrane viability responses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01. (Page 51)

Figure 3.3. Alamar Blue response following 24, 48 and 72 h growth on both 2D and 3D culture (Geltrex) of HeLa and HaCat cells on both a 6 well plate and b a 24 well plate.
Data are expressed as a percentage of three independent experiments ± SD of three individual experiments and relative to a 2D culture control. Statistically significant differences between the 3D culture membrane viability responses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01. (Page 53)

Figure 3.4. YO-PRO-1 and PI stained flow cytometry live, apoptotic and necrotic assay for HeLa (a) and HaCaT (b) cells grown on Collagen (3D) in different concentration and cells grown on plastic (2D) culture. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane live/dead cell analyses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01. (Page 55)

Figure 3.5. YO-PRO-1 and PI stained flow cytometry live, apoptotic and necrotic assay for Hela (a) and HaCaT (b) cells grown on Geltrex® (3D) in different concentration and cells grown on plastic (2D) culture. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane live/dead cell analyses and those of the 2D cultures are denoted by *P < 0.05 and **P < 0.01. (Page 57)

Figure 3.6. Cell cycle analysis of HeLa (a) and HaCaT (b) - cells grown on three different concentrations of Collagen gel (3D) and cells grown on plastic (2D) culture, and percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane cell cycle analyses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01. (Page 59)
Figure 3.7. Cell cycle analysis of HeLa (a) and HaCaT (b) cell grown on two different concentration of Geltrex (3D) and cells grown on plastic (2D) culture, and percentage of cells at G0/G1, S and G2/ M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane cell cycle analyses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01. (Page 61)

Figure 4.1. (a) HaCaT cell cycle analysis, cells were grown on three different substrates (Polystyrene, Collagen and CaF₂), and percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. (b) HeLa cell cycle analysis, cells were grown on three different substrates (Polystyrene, Collagen and CaF₂), and percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D and CaF₂ cultures and the polystyrene cultures are denoted by **P < 0.01 and *** P < 0.001. (Page 72)

Figure 4.2. PCA scatter plot (a) and loading of PC1 (b), corresponding to comparison of cytoplasm, nucleus and nucleolus region of HaCaT cells grown on CaF₂ substrate. The ‘0’ line is indicated with black dashes. PCA scatter plot (c) and loading of PC1 (d) corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HeLa cells grown on CaF₂ substrate. The ‘0’ line is indicated with black dashes. (Page 74)

Figure 4.3. PCA scatter plots (a) and loadings of PC1 (b), corresponding to comparison of cytoplasm, nucleus and nucleolus of HaCaT and HeLa cells grown on CaF₂. The ‘0’ line of PC1 is indicated with black dashes. Band positions corresponding to dominant features of the PC1 are indicated on the loadings. (Page 76)
Figure 4.4. PCA Scatter plot (a) and loading of PC1 (b) corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HaCaT cells grown on 3D culture (Collagen). The ‘0’ line is indicated with black dashes. PCA scatter plot (c) and loading of PC1 (d) corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HeLa cells on 3D culture (Collagen). The ‘0’ line is indicated with black dashes. (Page 80)

Figure 4.5. Scatter plots (a) and first loadings of PCA (b) corresponding to comparison of cytoplasm, nucleus and nucleolus of HaCaT and HeLa cells on 3D culture (Collagen). The ‘0’ line is indicated with black dashes. Band positions corresponding to dominant features of the PC1 are provided on the loadings. (Page 83)

Figure 4.6. Scatter plots of PCA corresponding to comparison of cytoplasm (a), nucleus (b) and nucleolus (c) of HeLa cells grown on CaF\textsubscript{2} and 3D culture Collagen substrate. The black dashes are used to indicate the ‘0’ line. (Page 84)

Figure 4.7. Scatter plots and first loadings of PCA corresponding to comparison of cytoplasm, nucleus and nucleolus of HaCaT cells on CaF\textsubscript{2} and 3D culture Collagen substrate. The ‘0’ line is indicated with black dashes. (Page 87)

Figure 5.1. Confocal image of Hela cells from (a) 2D following 24 h exposure to 2 µM of the Doxorubicin, (b) control Hela cells grown on 3D Collagen Matrix and (c) Hela cells grown on 3D matrix after 24 h exposure to 2 µM DOX. DOX was excited by 488 nm and its emissions recorded at 650 nm (DOX) respectively. (Page 95)
Figure 5.2. Alamar blue cytotoxic response curve following 24, 48 and 72 h exposure of HeLa cells to Doxorubicin on a standard plate. Data is expressed as a percentage of three independent experiments ± SD of the three individual experiments. (Page 97)

Figure 5.3. Alamar Blue cytotoxic response curve following 24, 48 and 72 h exposure of HeLa cells to Doxorubicin on a Collagen coated plate. Data is expressed as a percentage of three independent experiments ± SD of the three individual experiments. (Page 97)

Figure 5.4. Absorption spectrum of cell culture medium, Collagen, medium on Collagen, DOX in medium, DOX in medium on Collagen, recorded in situ in the well plate. (Page 100)

Figure 5.5. Photographic image of visible colour changes observed on a 96 well plate following exposure of DOX containing medium. Top row (a) is 3D exposure of 100 μM DOX in medium which then underwent a serial dilution with medium. The bottom row (b) is a 2D exposure of a serial dilution of 100 μM DOX in medium which then underwent a serial dilution with medium. (Page 100)

Figure 5.6. Dose dependent absorbance of Doxorubicin at 480 nm dispersed in cell culture medium (○) and that of Doxorubicin in cell culture medium after 24h exposure on a Collagen substrate (●). (Page 102)

Figure 6.1. Apoptosis and Necrosis analysis for HaCaT cells grown on Collagen (3D) and cells grown on polystyrene (2D) culture and cells were exposure to 1.25μM DOX and non-exposed cells, cells were stained by YO-PRO-1 and PI. Data is expressed as a percentage of three independent experiments ± SD of the three individual experiments. Statistically significant differences between the 3D culture membrane apoptosis and
necrosis analysis and that of the 2D cultures are denoted by *P < 0.05,**P < 0.01 and ***P < 0.001. (Page 112)

Figure 6.2. Cell cycle analysis of HaCaT cells grown on Collagen (3D) and cells grown on polystyrene (2D) culture and cells were exposure to 1µM DOX, non-exposed cells and the percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane cell cycle analyses and that of the 2D cultures are denoted by *P < 0.05,**P < 0.01 and ***P < 0.001. (Page 114)

Figure 6.3. (a) PCA scatter plot of cytoplasm region, nucleus and nucleolus region of DOX-exposed vs control HaCaT cells grown in Collagen. (b) loading of PC1, corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HaCaT cells grown on Collagen substrate after 24 h exposure to 1 μM of the DOX vs non-exposed cells (control), highlighted regions indicating discriminating DOX features. (Page 116)

Figure S1. Comparison of Raman spectra of two substrates. Mean spectrum of Collagen and CaF2 are indicated with red and black, respectively. The dashed circle highlights the characteristic contribution of water at ~1640 cm⁻¹. (Page 153)

Figure S 2. (a) Absorption of Dox drug on Collagen rat tail (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation. (b) Absorption of MTX drug on Collagen rat tail (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation. (Page 154)

Figure S 3. (a) Absorption of Dox drug on Geltrex (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation. (b) Absorption of MTX drug on Geltrex (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation. (Page 155)
Figure S 4. Confocal images for (a) HeLa cell grown on 2D culture after 24 h exposure to 2 μM of the Doxorubicin, (b) HeLa cell grown on Geltrex after 1 h exposure to 2 μM of the Doxorubicin. (c) HeLa cell grown on Collagen after 1 h exposure to 2 μM of the Doxorubicin. (d) HaCaT cell grown on 2D culture after 1 h exposure to 2 μM of the Doxorubicin. (e) HaCaT cell grown on Geltrex after 1 h exposure to 2 μM of the Doxorubicin. (f) HaCaT cell grown on Collagen after 1 h exposure to 2 μM of the Doxorubicin. DOX was excited by 488 nm and its emissions recorded at 650 nm (scale bar 20 μm). (Page 156)

Figure S 5. Confocal images for (a) HeLa cell grown on 2D culture after 24 h exposure to 5 μM of the Mitoxantrone, (b) HeLa cell grown on Geltrex after 1 h exposure to 5 μM of the Mitoxantrone. (c) HeLa cell grown on Collagen after 1 h exposure to 5 μM of the Mitoxantrone, (d) HaCaT cell grown on 2D culture after 1 h exposure to 5 μM of the Mitoxantrone. (e) HaCaT cell grown on Geltrex after 1 h exposure to 5 μM of the Mitoxantrone. (f) HaCaT cell grown on Collagen after 1 h exposure to 5 μM of the Mitoxantrone. MTX was excited by 607 nm and its emissions recorded at 690 nm (scale bar 10-20 μm). (Page 157)

Figure S 6. Confocal images for (a) HeLa cell grown on Geltrex, mitochondria stained by MitoTracker Red 250 nM, (b) HaCaT cell grown on Geltrex mitochondria stained by MitoTracker Red 250 nM. MitoTracker was excited by 579 nm and emission 599 nm respectively. (Page 158)

Figure S 7. Confocal images for (a) HeLa cell grown on Geltrex, lysosomes were stained by LysoTracker Red 75 nM and LysoTracker red was excited by 577 nm and emission 590 nm respectively, (b) HaCaT cell grown on Geltrex, lysosomes were stained by LysoTracker Green 75 nM and LysoTracker red was excited by ~504 nm and
emission 511 nm respectively. NucRed 2 drops/1 mL and NucRED was excited by 638 nm and emission 686 nm respectively. (Page 159)

List of Tables

**Table 1.** Comparison of 2D and 3D cell culture methods (Kapalańska et al., 2018; Hoarau-Véchot et al., 2018; Edmondson et al., 2014). (Page 6)

Table 2.1. NucRed® and Doxorubicin (absorption / emission). (Page 27)

Table 4.1. Assignments of Raman Bands of cells grown on CaF$_2$ and Collagen substrate (Movasaghi et al., 2007; Notingher et al., 2007; Pobezhimova et al., 2000). (Page 78)

Table 5.1: IC$_{50}$ and Hillslope values for the fitted data values following exposure to DOX under standard culture conditions in both 2D and 3D culture substrates following 24, 48 and 72 h exposure. (Page 89)

Table 6.1. IC$_{50}$ values for the fitted data values following HaCaT cells exposed to DOX under standard conditions in both 2D and 3D (Collagen) substrate following 24 and 72 h exposure. (Page 110)
CHAPTER 1

Introduction
1.1. Introduction

Cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment. Cell culture has been proven to be suitable, convenient and comparable to in vivo studies by the constant development and alteration of many types of substrates. In general, Cells growing in vivo involve a complex, three-dimensional (3D) environment that exhibit various topographical features, spanning several orders of size and the environments organizational structure exposes them to circulating molecules, neighbouring cells, at the nanoscale, cells are in contact with collagen fibrils and other protein polymers that compose the extracellular matrix (ECM) (Pieuchot et al., 2018). With these environmental cues in mind, biologists have developed more physiologically relevant methods for culturing cells and tissues, including new media formulations 3D systems and co-culture techniques. Standard in vitro culture involves the growth of cells on a 2D substrate, while allowing extensive research on cellular systems, response, gene expression and toxicological screening, the growth environment does not resemble those of the in vivo environment. In an effort to improve the relevance of in vitro screening extensive research has developed different models to create what is now termed “3D culture”. 3D culture systems in vitro have become preferential to 2D in certain scenarios due to their ability to simulate/replicate the microenvironment of surrounding tissue that normally occurs in vivo; as such they have found great usage in studies attempting to mimic tumour responses to chemical agents in vivo. The surrounding tissue in vivo has a major effect on drug absorption, metabolism, distribution, and toxicity of the drug thus by the incorporation of said 3D system the relevance in vitro screening and uptake of the chemical under test in vitro can be improved.
Significant efforts have been made to develop the techniques in cell culture to study the physiology and biochemistry of cells when they are growing in vitro and it has been used widely as the model system for drug delivery, toxicity, tissue engineering, immunology, normal and cancer cells.

The technique was discovered/developed by Dr. Ross Granville Harrison in 1907 (Freshney 2016). In 1952, the first human cell line establishment by George Otto Gey used cervical cancer tissue taken from the patient, Henrietta Lacks, to create the first an immortal cell line (HeLa) (Lucey et al., 2009). Thus, cell culture for researchers became more important and further cell lines were established in vitro. In general, cells used for culturing can be divided in to three groups: Primary and Continuous cell lines.

Primary cells are isolated directly from donor tissue by either mechanical methods such as dissection and filtration or enzymatic such as trypsin and Collagenase. Many of the cell types do not have the ability to adhere or grow under artificial condition. Primary cells can survive outside of their original environment for some time and have a limited lifetime. Thus, primary cells closely resemble the properties of their original tissue and present the best biological model (Langdon 2004), they have the ability to divided a limited number of times an example is MRC-5 cells, they derived from tissues of human lungs, where they have the ability to double approximately 50 times then die and they are used extensively in studies to determine the viral susceptibility profile (Chen et al., 2017).

Continuous cell lines are able to grow continuously and can originate from both normal and tumorigenic tissue. In vivo proliferation of cells is strictly controlled by a number of proteins which can regulate prognosis of the cell cycle. Cyclin-CDK complexes are regulated by phosphorylation and protein interaction events that tightly
control the timing and extent of CDK activation. Many events must occur for cell lines to become continuous/immortal cell lines, such as mechanical by somatic mutation via hybridization fusion of the host cell with an immortal cell line, by other induced mutation or by viral transformation. Continuous cell lines have been extensively developed resulting in a vast selection of cells lines available through central commercial biological banks allowing cell culture based research to progress with greater relevance to target diseases and organs.

1.2. 2D culture vs 3D culture

The majority of cell culture research has been performed on a two-dimensional (2D) substrate made of polystyrene, such as tissue culture flasks, micro-well plates, and Petri dishes because they offer high cell viability convenience and these traditional cell culture systems have as stated notably improved the understanding the basic of cell culture (Lee et al., 2008). To date, the majority of cell-based assays use traditional two-dimensional (2D) monolayer cells cultured on flat and rigid substrates.

However, when compared to the site of origin of the cell in vivo, most 2D substrates lack the complex and biological information-rich (Duval et al., 2017) environment present in vivo. Cells growing in vivo are surrounded by an extracellular matrix (ECM) (Chitcholtan et al., 2013) which is a support structure of tissue; this ECM gives cells in vivo their mechanical properties and facilitates communication between cells that embedded in the ECM (Gattazz et al., 2014).
The microenvironment that is surrounding a cell can strongly influence the cellular behaviour such as proliferation, differentiation, and metabolism (Jeanes et al., 2011; Lu et al., 2012). Moreover, not all normal epithelial cell types are able to grow and adhere to an artificial substrate (Kim 2005) and in this culture method, cell-cell and cell-extracellular environment interactions are not represented as they would be in the tumour mass (Kapałczyńska et al., 2018).

Figure 1.1. Different cell culture systems, cell grown in 2D culture and cell grown in 3D culture (Dhaliwal 2012).
Table 1.1. Comparison of 2D and 3D cell culture methods (Kapalczyńska et al., 2018; Hoarau-Véchot et al., 2018; Edmondson et al., 2014)

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>2D</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Sheet-like flat and stretched cells in monolayer Shape changed</td>
<td>Natural shape in spheroid/aggregate structures Real shape</td>
</tr>
<tr>
<td>Gene/protein expression</td>
<td>Often display differential gene and protein expression levels compared to <em>in vivo</em> models</td>
<td>Cells often exhibit gene/protein expression profiles more similar to those <em>in vivo</em> tissue origins</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Often proliferate at a faster rate than <em>in vivo</em></td>
<td>May proliferate at a faster/slower rate compared to 2D-cultured cells depending on cell type and/or type of 3D model system</td>
</tr>
<tr>
<td>Access to essential compounds</td>
<td>Unlimited access to oxygen, nutrients, metabolites and signalling molecules (in contrast to <em>in vivo</em>)</td>
<td>Variable access to oxygen, nutrients, metabolites and signalling molecules (same as <em>in vivo</em>)</td>
</tr>
<tr>
<td>Tumoral heterogeneity</td>
<td>Basic</td>
<td>Better approximation via the proliferation gradient, drug penetration and difference in mobility</td>
</tr>
<tr>
<td>Cells interactions</td>
<td>Deprived of cell-cell and cell extracellular environment interactions, no <em>in vivo</em>-like microenvironment and no “niches”</td>
<td>Proper interactions of cell-cell and cell-extracellular environment, environmental “niches” are created</td>
</tr>
<tr>
<td>Multicellular study</td>
<td>Better when studying the immune response</td>
<td>Good in co-culture, but complicated with more than two cell types</td>
</tr>
<tr>
<td>Time of culture formation</td>
<td>Within minutes to a few hours</td>
<td>From a few hours to a few days</td>
</tr>
<tr>
<td>Culture quality</td>
<td>High performance, reproducibility, long-term culture, easy to interpret, simplicity of culture</td>
<td>Worse performance and reproducibility, difficult to interpret, cultures more difficult to carry out</td>
</tr>
<tr>
<td><em>In vivo</em> imitation</td>
<td>Do not mimic the natural structure of the tissue or tumour mass</td>
<td><em>In vivo</em> tissues and organs are in 3D form</td>
</tr>
<tr>
<td>Cost of maintaining a culture</td>
<td>Cheap, commercially available tests and the media</td>
<td>More expensive, more time-consuming, large variety commercially available tests</td>
</tr>
<tr>
<td>Exposure to medium/drugs</td>
<td>Cells in monolayer are equally exposed to nutrients/growth factors/drugs that are distributed in growth medium</td>
<td>Nutrients and growth factors or drugs may not be able to fully penetrate the spheroid, reaching cells near the core</td>
</tr>
<tr>
<td>Drug sensitivity</td>
<td>Cells often succumb to treatment and drugs appear to be very effective</td>
<td>Cells are often more resistant to treatment compared to those in 2D culture system, often being better predictors of <em>in vivo</em> drug responses</td>
</tr>
</tbody>
</table>

Animal model based experiments provide a similar environment to those observed within human cells and organs (Desrochers et al., 2014) and as such have long been
regarded as the “gold standard” of biological testing due to their close resemblance to human exposure. However, these models are expensive, can result in lengthy experimental timeframes (Antoni et al., 2015) and ethical issues (Elliott & Yuan 2011). As a result, the EU have adopted Directive 2010/63/EU took full effect on 1 January 2013, the United States 106th Congress (2000) generated the US public law 106-545 both outlining the protection of animals used for research purposes. These directives are derived from Russell and Burch “The Principals of Human experimental Technique” first published in 1959 where the authors’ were the first to propose adopting the principals of 3Rs, to (Replace, Reduce and Refine the use of animals for scientific purposes). In essence, animals should be replaced with other methods where available or the numbers of animals used in the experiment should be reduced and efforts heightened to simultaneously reduce animal suffering and improve animal welfare.

To bridge the gap between the use of animal models and abide by a 3R based policy, efforts began to improve the relevance of in vitro systems and replace where possible animal exposure with cell culture as seen in fig. 1.2. Of paramount importance was to create other growth environments in vitro that mimic the original in vivo environment as closely as possible. In vitro cell culture needs matrices, also known as scaffolds which can support cell growth, differentiation and organization within their structure (Lee et al., 2008). In toxicity measurement, 3D culture has been found to resemble the results found with in vivo models (Lee et al., 2009), more closely with reduced cost (relative to in vivo studies) and obvious ethical benefits (Ho & Sun 2012). 3D cell culture has the architectural structure to mimic in vivo tissues and possesses the phenotype and functional characteristics of their in vivo tissue counterparts. Therefore, 3D culture provides a more realistic model of biological responses in vitro (Padmalayam & Suto 2012).
Figure 1.2. Description the advantages of using 3D culture models *in vitro* to bridge the gap between 2D culture and *in vivo*, 3Rs principle and using 3D culture to predict drug delivery prior clinical trials (Fitzgerald *et al*., 2015).

There is a large variety of 3D culture systems (Rimann & Graf-Hausner 2012), currently on the market and they are classified in to three categories: a) Scaffolds, b) Spheroids and c) Microfluidic 3D culture systems (fig. 1.3).

a) **Scaffolds**

Scaffolds have been widely used in tissue engineering applications; it is a 3D construct which provides an ECM which can support cells to grow and differentiate, help adhesion of the transplanted cells (Hutmacher 2000) and establish cell-cell and cell-ECM interaction. Cells can migrate between fibres and attach to them (Breslin & O’Driscoll 2013). Scaffolds are produced from natural materials such as Collagen, fibronectin, agarose, laminin and gelatine (Ravi *et al*., 2015) or synthetic polymers like polyethylene oxide (PED) and polyethylene glycol (PEG) (Place *et al*., 2009).
Hydrogels

Hydrogels are 3D matrices or porous scaffolds; they are a water-swollen polymeric network, consisting of cross-linked hydrophilic polymers (Zhu & Marchant 2011). Physically the hydrogels are weak, but it has a biomimetic environment to assist cell differentiation and proliferation (Peck & Wang 2013). Hydrogels have a type of flexibility very similar to normal tissue due to their large water content. Natural hydrogels were gradually replaced by synthetic hydrogels during last two decades because the synthetic hydrogels possess high capacity of water absorption, long service life and high strength (Ahmed 2015). Examples of hydrogels include Matrigel, Myogel, and Collagen I matrices.

Decellularized tissues/organs

These systems have been used extensively as a 3D culture for tissue engineering vascular constructs and may provide the capability to overcome the challenges that were facing current solutions (Keane et al., 2015). By the removal of cellular content and antigen from the tissue by the process of decellularizing tissue and as a result, only the ECM and functional proteins remain, cells grown in this system can show reduced inflammation, foreign body reaction and potential immune rejection (Wong & Griffiths 2014; Fu et al., 2014). In the decellularization process, chemical, enzymatic and physical methods are utilized to remove cells and DNA from the tissue while preserving its structural and regulatory proteins (Gilbert et al., 2006). These cellular tissue or organ scaffolds have been used to culture cells in vitro with great success and shown to aid better understanding in the pathogenicity of disease as well as drug delivery (Dunne et al., 2014; Brown et al., 2006) but they can be expensive.
**Cell-derived matrices**

Cell-derived matrices (CDM) are formed by cells cultured on a biomaterial surface for sufficient time at a high density *in vitro* with the result that the cells become surrounded by a dense ECM extracellular matrix. Then the cells are removed by leaving only ECM that (Decellularized) closely mimics native molecular content and stromal fibre (Kutys et al., 2013) of an *in vivo* environment. CDM have been used in a variety of applications, including coatings for synthetic scaffolds, biomimetic microenvironments for stem cell differentiation, and decellularized tissue engineered heart valves and vascular grafts. While several results are promising, there remains a fair amount of variability in the reported effects CDM have *in vitro* and *in vivo*, likely due to many differences in the cells, methods and applications examined thus far (Fitzpatricka & McDevittb 2015).

**b) Spheroids**

Spheroids are simple 3D culture models which are generated from a wide range of cell types and formed due to their more physiological cell-cell contact geometry (Haycock 2011). They are typically formed from co-culture techniques such as hanging-drop, rotary culture or concave plate methods or single culture (Antoni et al., 2015; Sumi et al., 2017). The spheroid format is particularly useful in cancer research as it enables quick discovery of morphological changes in transformed cells. Cells are embedded in an extracellular matrix (ECM) and left to proliferate and polarize according to the organ of origin (Antoni et al., 2015) and heterogeneous cell aggregates. They are characterized by a necrotic centre as observed in 14- and 19-day cultures (Gebhard et al., 2016) due to the lack of oxygen and nutrient transport (Mehta et al.,
2012) when the diameter reaches greater than 500 µm (Hirschhaeuser et al., 2010; Amaral et al., 2017). Similar to the typical features of tumour growth in vivo (Proskuryakov & Gabai 2010) thereby improving the relevance of cells cultured in this way. However, the use of spheroids culture is limited to certain cell lines, as not all cell types can form 3D spheroid in culture and some cells may accumulate into unpredictable shapes (Mueller-Klieser 2000). The Rotary Cell Culture System (RCCS) is a device designed to grow 3D clusters (spheroid). It is a unique 3D cell culture technology for culturing both suspension and anchorage-dependent cells and it has different features such as high mass transfer of nutrients in media to prevent cell death within the spheroid core. It can also be used with or without scaffolds to generate 3D cell models, with the low shear force in the rotating vessel (low turbulence) allowing the cells to grow in a spheroid it also offers the ability to co-culture multiple cell types in a spheroid 3D culture.

C) Microfluidics

These types of systems have been used for more than thirty years in research labs (Manz et al., 1990), colloquially known as a “lab on a chip“, they are small scale systems (Fig 1.3c) used to culture cells on a bio-chip and facilitating researchers to perform experiments on it (Daw & Finkelstein 2006). 3D culture techniques are used to create the three-dimensional architecture in vitro, where a microfluidic platform can be used to create a similar heterogeneous model culturing living cells in it continuously. The system thereby allows for continuous oxygen introduction, nutrition and waste removal through the culture medium (Larson 2015). Cells cultured on microfluidics are not accurately cultured in 3D unless grown as multi tumour spheroids. Simple
microfluidics systems are now increasingly being fabricated and generated by soft lithography processes to construct patterned environments which are relatively easy to fabricate a suitable with most biological systems (El-Ali et al., 2006).

**Figure 1.3.** Types of 3D cell culture plates. A- Scaffolds 3D culture or from cell-derived matrices. B- Spheroids cell culture, derived from single tumour cell. C- Microfluidic based systems (Fitzgerald et al., 2015).

### 1.3. 3D culture and drug discovery

In drug development, the standard procedure for initial screening is that of monitoring compounds with conventional 2D culture based tests and subsequent animal
model tests, after which just only about 10% of the potential compounds pass the patient trials with less than 1% making it to clinical use (Edmondson et al., 2014). Practices like this result in lengthy and expensive experimental timeframes for industry. Improvement of in vitro screening methods with the use of 3D culture systems offers considerable advantages. 2D cell cultures systems are generally less resistant to the drug treatment compared to tumour cells grown in vivo (Mikhail et al., 2013). Drug testing in 2D culture offers very little information and is often misleading because of different metabolic processes in tumour cells grown on 2D and in 3D culture. Tumour cells also show decreased levels of apoptosis that is induced by radio-chemo therapy when compared with cells grown on 2D culture. Likewise, chemotherapy based on cytotoxicity studies is considerably lower in 3D culture (Ravi et al., 2015).

The advantages of utilizing 3D cell culture in compared to 2D cell culture for evaluating a potential drug includes an increased, cell-cell interaction, ECM-cell interactions, different rates of cellular proliferation, oxygen and nutrient and finally the additional impact of stroma (Lovitt et al., 2014) all combining to help mimic in vivo conditions in vitro. Resistance to a drug in cancer can be divided into two different categories, acquired and de novo. Acquired resistance is defined as a result of the modification on the cells that can occur during exposure of cancer cells to drug and de novo resistance is associated with factors, such as adhesion of cancer cells to ECM that occur prior to drug treatment (Zahreddine & Borden 2013; Meads et al., 2009). Incorporation of a 3D system into drug screening will also mimic this de novo induced drug resistance to potential drugs increasing the relevance of in vitro screening.

Doxorubicin (Adriamycin®) (Dox) is one of the most effective anti-cancer drugs used to treat a multitude of human neoplasms. DOX is an anthracycline antibiotic that is
widely used in chemotherapy. It is a cell-cycle non-specific antitumor drug that exerts its action mainly by inducing apoptosis, autophagy and necrosis (Tacar et al., 2018). One of the mechanisms proposed to explain the effect of doxorubicin on cancerous cells is the intercalating into DNA and impairing topoisomerase II, function which is needed for the proper maintenance of DNA methylation (Lu et al., 2015). This drug is commonly used in drug cytotoxicity in vitro on 2D and 3D culture. Despite of the potential of spheroids for compounds screening and the emergence of technologies for high-throughput drug screening in spheroids, these in vitro models still present some challenges and limitations associated. For instance, there is an urgent need to optimize some of the spheroids production techniques in order to attain these micro-tissues under highly reproducible conditions.

1.4. Bio-spectroscopy

On 28 February 1928, Raman led an experiment with K. S. Krishnan, on the scattering of light, when he discovered what now is called the Raman effect. The advantages of Raman spectroscopy in the analysis of cells, tissue and bio-fluids have been extensively demonstrated in recent years. It allows rapid, non-invasive and high spatial resolution acquisition of structural and biochemical information through the acquisition of point spectra and spectral images. Raman spectroscopy offers a better possibility to study cells in an aqueous environment and thus keep cells alive during the experiments under normal physiological conditions. Raman scattering thus offers an intrinsically higher spatial resolution for mapping or profiling, the limit of resolution being (classically) determined by the wavelength (<1nm for Raman). Raman spectroscopy records the spectral information from cytology samples reflecting its
biochemical composition, including cells from an interrogated liquid-based cytology (LBC) specimen. Raman spectroscopy approaches coupled with multivariate analysis of the acquired data have shown the potential to detect cell abnormalities at molecular levels, which occur prior to the changes in morphology are seen under the light microscope. Raman spectroscopy is a complementary technique whereby incident radiation couples with the vibrating polarisation of the molecule and thus generates or annihilates a vibrational quantum, similarly resulting in a vibrational spectrum (Byrne et al., 2011). The differing underlying mechanisms give rise to complementarity nature of the two techniques. For a vibration to be active in IR spectroscopy a change in the dipole moment is required, whereas for a mode to be Raman active, a change in polarisibility is required. Vibrations of asymmetric, polar bonds thus tend to be strong in IR spectra, whereas Raman is particularly suitable as a probe of symmetric, nonpolar groups. A further implication of the differing physical origins of the techniques is that, whereas IR monitors the absorption of IR radiation, Raman scattering can be employed in the UV, visible or near-IR regions of the spectrum. The application of Raman spectroscopy to biomolecules and even tissues was first demonstrated as early as the 1960s (Lord & Yu 1970; Tobin 1968; Walton et al., 1970), and by the mid-1970s biomedical applications were explored (Yu et al., 1974) Whole cell and tissue studies were carried out on histological samples and one study found that Raman Spectroscopy could discriminate between cancer and normal cells (Lyng et al., 2019; Cicerone & Camp 2017; Rau et al., 2016) and in vivo studies have demonstrated the potential use in diagnostic applications ( Pence & Mahadevan-Jansen 2016; Cordero et al., 2018) and they demonstrated the potential for diagnostic applications. With such diverse potential profile of Raman spectroscopy in the area of bio-related research, there are however some limitations hindering it reaching its full clinical potential. One such limit is that
the substrate choice is standard glass slides to prepare to analyse and screen samples because glass is cheap, optically transparent and readily available. However, it has a Raman signal which can interfere with cellular analysis, and as such bio-spectroscopy has long used Raman clear substrates such as CaF$_2$ slides instead. However, Raman optically clear CaF$_2$ is expensive for large scale Raman spectroscopic experiments. Surface-enhanced Raman spectroscopy (SERS) has become an essential ultrasensitive analytical tool for biomolecular analysis of small molecules, macromolecular proteins, and even cells. SERS enables label-free, direct detection of molecules through their intrinsic Raman fingerprint (Bruzas et al., 2018). The development of three-dimensional (3D) cell culture systems has been effectively applied in several fields including developmental biology, tissue engineering and drug discovery (Ravi et al., 2015). Collagen rat tail as (3D) substrate has been used as Raman substrate to study live cells which is open new perspectives for the application of Raman spectroscopy for prolonged measurements (Bonnier et al., 2010).
1.5. Aims and Objectives

The main aim of this thesis was to develop and characterise a novel 3D growth substrate *in vitro* based on characteristics of known 3D models to mimic natural micro-environment *in vivo*.

The study was designed to investigate the viability of 3D culture compared to 2D culture using normal and cancer cell lines and to clarify how the transition from 2D to 3D culture effects cells *in vitro* by monitoring cellular viability, cytotoxicity responses, cell cycle, live dead cell assay, morphological changes, and bio-spectroscopy.
In this thesis, two commercial products were utilized as three dimensional cell culture models (Collagen and Geltrex), with two different cell lines, human cervical cancer cells (HeLa) and human keratinocyte cells (HaCaT). Different techniques were employed to investigate the viability of 3D culture compared to traditional 2D culture in vitro. Flow cytometry, Raman micro-spectroscopy and confocal microscopy were employed to determine how the transition from 2D to 3D culture effects on the cells in vitro based on viability, cytotoxicity and cell cycle. Following the study of the effects of transitioning from a 2D to a 3D in vitro system, the ECM’s potential to act as a Raman spectroscopic substrate was compared to that of CaF$_2$ a traditional common Raman substrate used in bio-spectroscopy and recommendations for the use of 3D systems in bio-spectroscopic studies were made.

1.6. Thesis Summary

This thesis is designed to demonstrate differences between cell culture substrates and how the transition from 2D culture to 3D culture can affect the outcome of experiments performed in vitro. Two cell lines were employed namely HeLa cervical cancer cells and HaCaT keratinocyte cells in conjunction with commercial 3D culture systems, Geltrex and Collagen rat tail.

Chapter 1 provides the introduction and background to highlight the fundamentals of cell culture, different cell culture and substrates, doxorubicin, bio-spectroscopy and the aims – objectives of the thesis.

Chapter 2 describes the methodology that has been used throughout the study in more detail. The principles of 3D cell culture and the use of multivariate analysis techniques
to extract information from the huge and complex data sets are discussed. Moreover, the theory behind the bio-imaging techniques, cytotoxicity assays and flow cytometry assays which were employed as a complementary technique to 3D culture assessment by Raman spectroscopy will be explained in detail.

**Chapter 3** presents a comparison between 2D and 3D culture by using two commercial products of 3D culture in different concentrations and volumes of 3D culture. The chapter will provide information how cellular health and viability levels are not altered by culture in 3D environments, but their normal cycle can be altered as indicated in the cell cycle studies performed.

Chapter 3 has been adapted from the published journal article entitled ‘Comparative studies of cellular viability levels on 2D and 3D in vitro culture matrices’, Cytotechnology, 2017, DOI: 10.1007/s10616-017-0139-7.

**Chapter 4** mainly focuses on how the cell culture environment can considerably influence the cell cycle profile, and therefore the diversity of biochemical profiles of individual cells within a mixed cell population, depending on substrate and also cell type. Moreover, these differences manifest as spectroscopic markers of cellular stress which are particularly prominent in cells on CaF$_2$ substrates, widely used for Raman analysis.

Chapter 4 has been adapted from the published journal article entitled ‘Raman spectroscopy detects biochemical changes due to different cell culture environments in live cells in vitro’, ABC, 2018, DOI: 10.1007/s00216-018-1371-5.

**Chapter 5** mainly focuses on the use 3D culture in cytotoxicity assay compared with 2D culture as well as localisation of Doxorubicin in the cells (2D and 3D culture). The
Chapter will discuss that the bioavailability or effective concentration of the cytotoxicity assay and the chemotherapeutic agent are both affected by the absorptive nature of the matrix as is clearly evident by the variations in spectral properties and cellular responses.

Chapter 5 has been adapted from the published journal article entitled ‘chemotherapeutic efficiency of drugs in vitro: Comparison of Doxorubicin exposure in 3D and 2D culture matrices’, Toxicol In Vitro, 2016, DOI: 10.1016/j.tiv.2016.02.022.

Chapter 6 shows that DOX induced apoptosis and cells grown in different substrates were arrested at G0/G1 phase. Whereas, there is no different between 3D and 2D cultures in apoptotic, necrotic and live cells. However, 3D cultures are a better representation of the action of DOX. Raman spectroscopy is not only able to detect DOX inside cells and profile its specific subcellular localisation, but, it is also capable of demonstrating the local biochemical changes elicited by the drug and the sub cellular region responses.

Chapter 7 summarises the main findings of the thesis to build a more holistic approach to demonstrate differences between 2D and 3D culture, implication of this work on biospectroscopy studies and recommendations for the area based on finding of this thesis.

In the chapters which are adapted from the papers, the format of the original has been retained, but changes in figure and section numbering have been made, as required for the thesis format. Supplementary material related to the corresponding work is also added at the end of the thesis.
CHAPTER 2

Methodology
In the previous chapter, the background and aims of the study are discussed and the importance of 3D culture for in vitro studies, as well as their mechanism of action in terms of toxicology. This chapter is designed to provide more background information about the techniques and assays which were employed throughout the study.

2.1. Materials

Cell culture media, all supplements, foetal bovine serum, L-glutamine, ampicillin, streptomycin, trypsin Doxorubicin (DOX) and PropidiumIodide (PI) were purchased from Sigma Aldrich Ltd (Arklow, Co. Wicklow, Ireland). Geltrex_ hESC-qualified Ready-To-Use Reduced Growth Factor Basement Membrane Matrix. Catalogue Number A1569601 and LOT Number 1851583—Collagen I Rat-Tail (Gibco), YO-PRO-1 stain (Gibco™) and Alamar Blue™ (AB) and the NucRed_ Live 647 ReadyProbes_ were purchased from Biosciences (Dublin, Ireland).

2.2. Cell culture

HeLa cells (human cervical cancer; ATCC CCL-2 (fig. 2.1); purchased from ATCC (Manassas, VA, USA)) and HaCaT cells (human dermal keratinocyte (fig. 2.1); purchased from the Leibnitz Institute DSMZ—German Collection of Microorganisms and Cell Cultures), were both adapted to culture in DMEM/F12 supplemented with 10% foetal bovine serum, 1% L-glutamine, penicillin and streptomycin (50 mg/mL), under standard conditions of 5% CO₂ at 37°C and humidity of 95%. Cells were cultured until they reached approximately 80% confluency.
Figure 2.1. HeLa and HaCaT cell lines. The figure is adapted from the images provided by ATCC and CLS (ATCC-HeLa; CLS-HaCaT).

2.3. 3D substrates preparation

Collagen I Rat Tail (Gibco) was used for a preparation of the Collagen gel; 3 mg/mL sterile solution was mixed with sterile 1 M sodium hydroxide (1 M NaOH), Phosphate Buffered Saline x10 (PBS10x) and sterile distilled water. Three different Collagen based substrates were produced and tested by varying the concentration of the Collagen content in the gel to 2.5, 2 and 1.5 mg/mL, respectively. Each of these concentrations was used to produce Collagen substrates incubated for 45 min -1 h at 37°C to allow the gel to form. All preparation steps were performed on ice to ensure premature gelation did not occur.

Geltrex is similar to Matrigel, in that both are derived from the Engelbreth-Holm-Swarm tumour and as such are of very similar structures. Geltrex was chosen due to its consistent protein concentration from lot-to-lot, extensive supplier production functional testing on each lot and the system comes ready to use, which means no thawing, diluting, or premature gelling facilitating a higher throughput of experiments. Geltrex is
a ready to use substrate system, and, as such, minimal substrate preparation was needed. Briefly, the Geltrex stock was placed on ice to avoid premature gelation and used in different volumes; 250, 200, 150 and 100 µl per well in 24 well plates and 1.5, 1 and 0.5 mL per well in 6 well plates, to form substrates of differing thickness. The Geltrex coated plates were then incubated for 1 h until basement membranes were formed.

2.4. Confocal Laser Scanning Microscopy (CLSM)

The basic concept of confocal microscope was originally developed by Marvin Minsky in the mid-1950s (Claxton et al., 2006). The light emitted by the laser system passes through light source pinhole aperture, a dichromatic mirror, onto the sample and the emitted fluorescence is collected only from the focal point (Nwaneshiudu et al., 2012) (Fig. 2.2).
Figure 2.2. A. Zeiss LSM 510 Confocal Laser Scanning Microscope, Schematic diagram of the optical pathway and principle in a Confocal Laser Scanning microscope, (Claxton et al., 2006).

To assess whether any significant morphological differences were present in the tested lines when grown on the ECM, live cell microscopy was performed with the aid of a Zeiss LSM 510 Confocal Laser Scanning Microscope (CLSM). The nucleus, being the most dominant feature of a cell, was stained for image clarity but also to ensure that no alterations to the nuclear region occurred. HeLa and HaCaT cells were seeded in Matek 35 mm glass bottomed culture vessels at a density of $1 \times 10^5$ in a volume of 200 µl of 10% FBS DMEM/F12. The cells were then incubated for 1 h to encourage the cells to attach to the glass bottom culture dishes, after which 2 mL 10% FBS DMEM/F12 was added. For the 3D culture, cells were seeded exactly in the same
fashion, except that the glass bottom was pre-coated with the desired substrate. For Collagen, substrates were prepared as previously described, at Collagen concentrations of 2.5, 2 and 1.5 mg/ml (100 µl/dish), respectively, Geltrex, 150 µl/dish and 100 µl/dish and 2D substrate. After 24 h incubation with 5% CO₂ at 37°C, cells were removed and stained with NucRed® Live 647 ReadyProbes® Reagent, as per the manufacturer’s instructions. Briefly, after 24 h incubation, cells were washed with 2 mL PBS and two drops (100 µl) of the as purchased stain were added per 1 mL of medium. Cells were then incubated for 20 min and washed with PBS prior to imaging. Cells were then imaged live in PBS and the NucRed® Live 647 was excited with a 633 nm Helium Neon laser and the emission detected at 660–675 nm (table 2.1).

For Doxorubicin (DOX) exposure, all images were taken on a Zeiss 510 LZSM confocal inverted microscope with an external argon ion laser (488 nm). Briefly, confluent cells were trypsinized and, at a concentration of 1 × 10⁵ cells/ml, 100 µl of cell suspension was placed onto 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA, USA). Glass dishes were incubated for 1 h. Following incubation, 1 mL of cell culture media was added to cell suspensions. Dishes were re-incubated and allowed to attach for 24 h. This protocol was altered to image the HeLa cells grown and exposed to Dox on the Collagen substrates. For this, the glass bottomed culture dishes were coated with 100 µl of Collagen and allowed to gel as per the gel formation outlined previously. HeLa cells were then seeded and allowed to attach for a period of 24 h. After the 24 h attachment, both the glass and Collagen substrate culture dishes were dosed with 2 µM of the Doxorubicin under standard exposure conditions. DOX was excited by 488 nm and its emission recorded at 650 nm (table 2.1).
Table 2.1. NucRed® and Doxorubicin (absorption / emission).

<table>
<thead>
<tr>
<th>Doxorubicin and Probe</th>
<th>Abs *(nm)</th>
<th>Em*(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>488 nm</td>
<td>650 nm</td>
</tr>
<tr>
<td>NucRed®</td>
<td>638 nm</td>
<td>686 nm</td>
</tr>
</tbody>
</table>

2.5. Alamar Blue Assay (AB)

The Alamar blue AB assay measures the innate metabolic activity of cells. The oxidised indigo blue, non-fluorescing form of this chromogenic indicator dye is reduced by cellular dehydrogenases to a reduced pink fluorescent form, which can be easily monitored by spectroscopic means. The (AB) assay monitors the proliferation of human and animal cell lines, bacteria, and fungi (Kuda & Yano 2003; O’Brien et al., 2000; Pettit et al., 2005; Al-Nasiry et al., 2007; Mosmann 1983). The AB assay has been widely used in studies of cell viability and cytotoxicity (Vega-Avila & Pugsley 2011; Rampersad 2012; White et al., 1996). Alamar blue is a water-soluble dye which is a sensitive oxidation-reduction indicator employed for in vitro quantification of the cell viability. Resazurin is the non-toxic and active compound of Alamar Blue®, and it is cell permeable, blue in colour and virtually non-fluorescent. Live and healthy cells convert resazurin to resorufin, which produces very bright red fluorescence that can be measured spectroscopy to measure viability and cytotoxicity (Fig. 2.3). For viability experiments both HeLa and HaCaT cells were placed in different size plates and Collagen and Geltrex substrates were used to grown cells in 3D culture. After 24 h incubation medium was removed and cells were washed with warmed PBS, an AB solution (5% [v/v]) prepared in medium (without FBS or supplements) and was
subsequently added to each well in plates according to manufactures instruction and incubated for 3 h. AB conversion was measured by fluorescent on a spectrometer (Spectra Max - M3) at 540 nm excitation and 595 nm emission.

Figure 2.3. Reduction of Resozurin sodium salt to strongly fluorescent Resofurin sodium salt in the presence of metabolic active cells (Markaki 2009).

2.5.1. Cell viability measurement with Alamar Blue (AB)

For AB viability experiments, both HeLa and HaCaT cells were seeded at a density of $2 \times 10^4$ cells per well (1 mL) in 24 well plates and $1 \times 10^5$ cells per well (1 mL) in 6 well plates, respectively. Collagen substrates were used at constant volumes of 200 µl per well in 24 well plates and 500 µl per well in 6 well plates. All plates were divided into four parts of the differing concentrations of gel, 2.5, 2, 1.5 mg/ml and finally without Collagen (2D) as a control. Geltrex, plates were divided into parts according to their volume, with uncoated 2D controls, 250, 200, 150 and 100 µl in 24 well plates and 1.5, 1, 0.5 mL in 6 well plates (The experiments were performed in triplicates and each plate contained a 2D control). After 24, 48 and 72 h incubation, the medium was
removed and cells were washed with pre-warmed PBS. An AB solution (5% [v/v]) was prepared in medium (without FBS or supplements) and was subsequently added to each well according to the manufacturer’s instructions, and incubated for 3 h. AB conversion was measured by a plate reading spectrometer (Spectra Max—M3) by monitoring fluorescence as a measure of AB dye conversion, using 540 nm excitation and 595 nm emission.

### 2.5.2. Assessment of Doxorubicin Cytotoxicity by using Alamar blue (AB)

For the AB assay, HeLa cells were seeded in 96 well microtiter plates (Nunc, Denmark), plates were coated with Collagen 2.5 mg/mL (50 µl) per well and finally without Collagen (2D) as a control at a density of $1 \times 10^5$, $5 \times 10^4$, $4 \times 10^4$ and $3 \times 10^4$ cells/ml for 24, 48 and 72 h exposures respectively and HaCaT cells were seeded at a density of $1 \times 10^6$ cells/ml for 24 and 72 h exposures respectively. At least three independent experiments were conducted with six replicate wells employed per concentration per plate in each independent experiment. After 24 h of cell attachment, plates were washed with 100 µl/well PBS and treated with increasing concentrations of DOX, incubated for the required exposure time and then the viability was assessed with the aid of the Alamar blue assay as previously reported (Casey et al., 2007; Davoren et al., 2006). Briefly, the control medium and/or the test exposure were removed, the cells were rinsed with PBS and start AB measurements as described previously (2.5.1). The mean fluorescent units for the six replicate cultures were calculated for each exposure treatment and the mean blank value was subtracted from these.
2.6. Flow Cytometry

Flow cytometry is a sophisticated instrument measuring multiple physical characteristics of a single cell such as size and granularity simultaneously as the cell flows in suspension through a measuring device. Flow Cytometry involves the use of a beam of laser light projected through a liquid stream that contains cells, or other particles, which when struck by the focused light give out signals which are picked up by detectors. These signals are then converted for computer storage and data analysis, and can provide information about various cellular properties. It has the ability to measure the properties of individual cells, and measures fluorescence and optical characteristics of an individual cell and cells suspension inside. The cells are drawn like a stream made by surrounding sheath called as a coaxial, which creates a laminar flow that force a single cell to pass through an interrogation point (Macey, 2010; Wilkerson, 2012). To excite fluorescent compounds in the sample of cells, most flow cytometers use a laser as the source of light (Adan et al., 2016) (Fig. 2.4.b). When a particle deflects the incident laser light, light scattering occurs and depending on the physical properties of cells in can be scattered in different directions forward-scattered (FSC) is proportional to cell size, side-scattered (SSC) is proportional to cell granularity (Suthanthiraraj & Graves 2018).
Figure 2.4. Hydrodynamic focusing of the sample through the laser beam (BD Biosciences, 2000).

2.6.1. Cell cycle Analysis

The cell cycle refers to the orderly sequence of events that takes place between one cell division and the next cell division that is regulated or controlled within a time period. The cell cycle, it is the progression of the cell through a full cycle of division, and it is required for cell growth. In general the cell cycle is divided into four identifiable component parts (Fig. 2.5) and these are the gap 1 phase G1, synthesis phase S, gap 2 phase G2 and mitotic phase M. Cells can enter the first gap phase (G1) from the quiescent state G0 or, if they are proliferating, after completing cytokinesis. The progression through G1 is mitogen dependent up to the restriction point (Fan et al., 2018). DNA synthesis takes place in S phase, generating exactly two identical sister chromosomes. G2 phase is a period of rapid cell growth and protein synthesis during which cells get ready for mitosis. The next phase of the cell cycle is the mitotic phase (M phase), it is starts with the nuclear division, corresponding to the separation of daughter chromosomes and usually ends with division of cytoplasm (Osés-Ruiz &
Talbot 2017; Fan et al., 2018). Progression through the mammalian cell cycle requires the accurate orchestration of a sequence of events. Among the countless elements taking part in this process, the sequential activation of heterodimeric CDK–cyclin complexes (cyclins and their counterpart cyclin-dependent kinases (CDKs)) has been described as the key regulatory events. The kinase activity of CDKs is tightly regulated by the binding to cyclins, the activating subunits which are expressed in an oscillatory way, the binding to negative regulators (CDK inhibitors, CKI) and phosphorylation/dephosphorylation events (Manchado et al., 2012; Diaz-Moralli et al., 2013). Cell cycle checkpoints are surveillance mechanisms that monitor the order, integrity, and fidelity of the major events of the cell cycle. There is four important checkpoints during the cell cycle, the first one G1 checkpoint between G1/S, second checkpoint between S/G2, third checkpoint between G2/M and fourth checkpoint in mitotic phase between metaphase and anaphase. These include growth to the appropriate cell size, the replication and integrity of the chromosomes, and their accurate segregation at mitosis (Barnum & O’Connell 2016).
The cell cycle consists of interphase and the mitotic phase. During interphase, the cell grows and the nuclear DNA is duplicated. Interphase is followed by the mitotic phase. During the mitotic phase, the duplicated chromosomes are segregated and distributed into daughter nuclei. The cytoplasm is usually divided as well, resulting in two daughter cells (The cell cycle).

Figure 2.5. The cell cycle consists of interphase and the mitotic phase. During interphase, the cell grows and the nuclear DNA is duplicated. Interphase is followed by the mitotic phase. During the mitotic phase, the duplicated chromosomes are segregated and distributed into daughter nuclei. The cytoplasm is usually divided as well, resulting in two daughter cells (The cell cycle).

Briefly to monitor the cell cycle, cells were seeded in T-25 cm² flasks at a density of 1.5 × 10⁶ (5 mL of medium) per flask. For Collagen, flasks were divided into four groups, two flasks with 2.5 mg/mL Collagen, two flasks with 2 mg/ml Collagen, two flasks with 1.5 mg/ml Collagen and two flasks without Collagen (2D). For Geltrex, flasks were divided into three groups, two flasks with 3.75 mL Geltrex, two flasks with 1.87 mL Geltrex and two flasks without Geltrex (2D). For CaF₂, cells were seeded at a density of 1.5 × 10⁶, 200 µl of cell suspension were deposited per slide, on three slides.
and kept in polystyrene petri dishes with 3 mL of growth medium. Flasks petri dishes were incubated in a 5% CO₂ at 37°C for 24 h. After 24 h incubation, cells were washed twice with prewarmed PBS and were collected by trypsinization, after which the trypsin was removed by centrifugation (1200 RPM for eight min), after which cells were fixed in ice cold, 70% ethanol and prepared for analysis immediately or stored in the fridge for a maximum of 2 days. Briefly, for analysis, cells were washed twice with PBS, to remove any residue fixative and resuspended in 2 mL PBS. 100 µg/ml Ribonuclease was added to ensure that only the DNA content was stained. After five min incubation with RNase at room temperature, DNA content was then stained with Propidium Iodide (PI) at a staining concentration of 50 µg/mL. The sample was again incubated at room temperature for 20 min, after which it was immediately analysed. A minimum of 10,000 single cell events per sample were analysed. All samples were analysed with the aid of a BD Accuri™ C6 Flow Cytometre and the experiments were performed in triplicate. 3D cultures were performed identically with the addition of the cells grown on the membrane.

For DOX exposure, cells were again seeded in polystyrene flasks T-25 cm², pre-coated with 1 mL of 2.5 mg/mL Collagen, at a density of 1.5 × 10⁶ /mL, 5 mL of cell suspension per flask and in polystyrene flasks T-25 cm² without Collagen (2D). Flasks were prepared in two groups and cells were incubated in 5% CO₂ at 37°C for 24 h. After 24 h incubation, for the first group, cells grown in 3D and 2D were washed twice with pre-warmed PBS and were collected by trypsinisation, the trypsin was removed by centrifugation (1200 RPM for eight min), after which cells were fixed in ice cold, 70% ethanol and prepared for analysis immediately or stored in the fridge for a maximum of 2 days. For the second group, medium was replaced with other medium containing 1
µM DOX. After 24 h incubation, samples were prepared as described previously in cell cycle analysis.

2.6.2. Apoptosis assay

Over the past decade, the Nomenclature Committee on Cell Death (NCCD) has formulated guidelines for the definition and interpretation of cell death from morphological, biochemical, and functional perspectives (Gulluzzo et al., 2018). Cell death mode includes including intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, NETotic cell death, lysosome-dependent cell death, autophagy-dependent cell death, immunogenic cell death, cellular senescence, and mitotic catastrophe; these are associated with different morphological features and cases. Apoptosis is accompanied by rounding-up of the cell, reduction of cellular volume (pyknosis), retraction of pseudopodes, chromatin condensation, nuclear fragmentation (karyorrhexis), classically little or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing (but maintenance of its integrity until the final stages of the process). Necrosis is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Gulluzzo et al., 2018; Kroemer et al., 2009) (fig. 2.6).
Figure 2.6. Illustration of the cell death pathway (Jeng et al., 2018).

To monitor apoptosis populations, cells were seeded in T-25 cm² flasks at a density of $1.5 \times 10^6$ (5 mL of medium) per flask. For Collagen, flasks were divided into four groups, two flasks with 2.5 mg/mL Collagen, two flasks with 2 mg/mL Collagen, two flasks with 1.5 mg/mL Collagen and two flasks without Collagen (2D). For Geltrex, flasks were divided into three groups, two flasks with 3.75 mL Geltrex, two flasks with 1.87 mL Geltrex and two flasks without Geltrex (2D). Flasks were incubated in a 5% CO₂ at 37°C for 24 h. Following incubation, the cells were washed twice with prewarmed PBS and were collected by trypsinization, after which the trypsin was removed by centrifugation. The cells were then washed twice with pre-warmed PBS and
stained with the YO-PRO-1/Propidium iodide (PI) dyes, whereby 1 µl of YO-PRO-1 dye (100 µM) and 1 µl of PI (1 mg/ mL) were used to stain cells at per 1.5 × 10^6 cell/ml. After staining of a cell population, apoptotic cells show a green fluorescence, whereas dead cells show green and red fluorescence. After incubation on ice for 30 min, the cells were analysed by flow cytometry within 30 min, using 488 nm excitation and reading the fluorescence at both 530 and >575 nm in order to visualize three groups: live cells, apoptotic cells and necrotic cells. All samples were analysed with the aid of a BD AccuriTM C6 Flow Cytometre and the experiments were performed in triplicate.

For DOX exposure, cells were seeded in polystyrene flasks T-25 cm2, pre-coated with 1 mL of 2.5mg/mL Collagen, at a density of 1.5 × 10^6 /mL, 5 mL of cell suspension per flask and in polystyrene flasks T-25 cm^2 without Collagen (2D), and flasks were prepared in two groups. Cells were incubated in 5% CO₂ at 37°C for 24 h. After 24 h incubation, for the first group, cells grown in 3D and 2D were washed twice with pre-warmed PBS and were collected by trypsinisation, after which the trypsin was removed by centrifugation (1200 RPM for eight min). For the second group, cells grown in 3D and 2D culture medium were replaced with other medium containing 1.25 µM DOX. After 24 h incubation samples were prepared for as described above in live/dead assay.

2.7. UV-Visible spectroscopy

UV-vis spectroscopy is an absorption spectroscopy technique; it relies on a sample containing species that absorb light in the ultraviolet to visible range. Since its
development in the 1950’s the UV-Visible Spectrophotometer (Molecular spectroscopy) has evolved into an accurate and reliable analytical tool and it has become one of the most utilised instruments in today’s scientific laboratory. Ultraviolet-visible (UV-Vis) spectra are derived when the interaction between incident radiation and the electron cloud in a chromophore results in an electronic transition involving the promotion of one or more of the outer shell or the bonding electrons from a ground state into a state of higher energy. The UV and visible spectral bands of substances generally are broad and do not possess a high degree of specificity for compound identification. Nevertheless, they are suitable for quantitative assays and, for many substances, are useful as an additional means of identification. Such types of instrument comprise the following components in their constructions a) Light Source, b) Monochromator, c) Cell Compartment, d) Detector and e) Signal Processing System. UV/Vis characterised by, fast sample analysis, suitable for a wide variety of analysis, much simpler than chromatographic techniques, user-friendly interface and little maintenance required.

Absorbance Spectroscopy

To monitor gel uptake and retention of the DOX, cellular spectroscopic studies were performed on Collagen gels. The gels were prepared in an identical manner as that in the cellular studies and dosed with media containing the required concentration as in the cellular exposure protocol. The DOX content was monitored, in situ in the well plates, with the aid of a Molecular Devices Spectr amax M3 spectrometer by means of absorbance measurements at 480 nm.
2.8. Raman Spectroscopy

Is a non-invasive analytical tool which is increasingly being explored for its potential in clinical applications including chemotherapeutic development.

Raman is an optical spectroscopic technique, based on the transitions between vibrational levels of electronic states. In more detail, the Raman effect is a inelastic scattering technique involving the coupling of the incident photons or electromagnetic radiation with the molecular vibrations. When the frequency of the scattered light is less than the incident one because the molecule absorbs energy from the incident photon and elevates itself from a lower energy state to an excited vibrational state, this is called Stokes Raman scattering. Conversely, anti-Stokes scattering occurs when the scattered photons are higher in energy than the incident photons, as a result of the annihilation of molecular vibration (Fig. 2.7) (Tu & Chang 2012).
Figure 2.7. Schematic representation of the energy transition in Raman microscopy with $h$: Plank’s constant and $v_0$: excitation frequency $\nu$: bond vibration frequency.

At room temperature, most molecules will be in the lowest energy state, the Raman spectrum is commonly recorded as the Stokes line intensity as a function of wavenumber or Raman shift. Since the vibrations are characteristic of the molecular structure, the Raman spectrum provides a spectroscopic “fingerprint” of the material (Byrne et al., 2014). A Raman spectrophotometer typically consists of 4 major parts; the light source (laser), optics (filters and focussing objective), wavelength selector (grating) and the detector (Photodiode, CCD, PMT). The set-up for a typical Raman spectrometer is shown in Figure 2.8. The laser illuminates a sample through a microscope objective, and then the collected Raman-shifted light is directed to a diffraction grating to disperse the Raman scattered beam into specific frequencies which
are subsequently focused on an array of detectors, such as a high sensitivity CCD. The spectrometer is equipped with a notch or edge filter to eliminate the elastically scattered photons (Rayleigh photons) (Downes & Elfick 2010; Notingher 2007).

In addition to the basic Raman effect or spontaneous Raman, various phenomena were discovered over the last few years, such as a) Resonance Raman (RR), occurs when the laser frequency is close to an electronic transition in the material, b) surface enhanced Raman scattering (SERS), which uses a nanoscale noble metal surface to enhance the signal, c) coherent anti-Stokes Raman scattering (CARS), involving two coherent lasers, and d) Fourier Transform Raman (FT-Raman) using the Michelson interferometer. (Cîntă Pînzaru et al., 2004; Gala & Chauhan 2015).

Due to its non-invasive analytical capability, Raman spectroscopy has seen an increase in applications in various fields, from environmental sciences, pharmaceutical development and drug discovery, to research and diagnostics (Paudel et al., 2015).

Briefly to prepare samples for Raman analysis, HeLa and HaCaT cells were harvested by trypsin detachment and seeded in Matek 35 mm glass bottom culture vessels pre-coated with 100 µl of Collagen 2.5 mg/mL at a final concentration at a density of 1 × 10^5 cells per-dish in a volume of 200 µl of 10% FBS supplemented DMEM/F12. For CaF_2, cells were seeded at a density of 1 × 10^5, 200 µl of cell suspension were deposited per slide and kept in polystyrene Petri dishes with 3 mL of growth medium. After 24 h incubation in 5% CO_2 humidified incubator at 37°C, the medium was removed, cells were washed with pre-warmed PBS and medium was replaced with Phenol Red free medium supplemented with 10% FBS for spectroscopic measurements.
For DOX exposure, HaCaT cells were harvested by trypsin detachment and seeded in Matek 35 mm glass bottom culture vessels pre-coated with 100 µl of 2.5 mg/ml Collagen at a density of 20,000 cells per-dish in a volume of 200 µl of 10% FBS supplemented DMEM/F12. The cells were then incubated for 1 h to allow the cells to attach to the glass bottom culture dishes pre-coated with Collagen (3D), after which 3 mL 10% FBS DMEM/F12 was added. After 24 h incubation in a 5% CO₂ humidified incubator at 37°C, the medium was removed, cell were washed with pre-warmed PBS and medium was replaced with medium containing 1µM DOX. After 24 h incubation, the medium was removed and the cells were washed with pre-warmed PBS and replaced with another 3 mL medium free Phenol Red 10% FBS DMEM.

2.9. Raman spectroscopy measurements

A Horiba Jobin-Yvon LabRAM HR800 spectrometer equipped with a 785 nm, 300 mW diode laser as source (70 mW at the sample), a 16-bit dynamic range Peltier cooled CCD detector, and a x100 immersion objective (LUMPlanF1, Olympus, N.A. 1.00) was employed throughout the study, producing a spot on the sample of ~1 µm diameter. The 300 lines/mm grating and 100 µm confocal hole was used to acquire spectra from the two different substrates and both cell lines (HeLa, HaCaT) in the fingerprint region, from 400 cm⁻¹ to 1800 cm⁻¹, and accumulated twice for 30 seconds to improve the signal to noise ratio. The system was pre-calibrated to the 520.7 cm⁻¹ spectral line of silicon. Point spectra were acquired from subcellular regions on the different substrates, Collagen and CaF₂. 20 point spectra, each from the cytoplasm, nucleus and nucleolus of live cells were acquired (60 spectra per cell line) and 20 cells were analysed per substrate.
2.10. Data analysis

At least three independent experiments were conducted for each endpoint. Test results for each endpoint where appropriate, were expressed as a percentage of the 2D control ± standard deviation (S.D.). Control values were set at 100%. IC₅₀ of drugs and the differences between samples and the control were evaluated using the statistical analysis package Prism 7 (Graphpad) and Minitab14. Statistically significant differences were set at p ≤ 0.05. Normality of data was confirmed with Q–Q percentile plots and Kolmogorov–Smirnov tests. Equality of variances was evaluated using Levene tests. One-way analysis of variances (ANOVA) followed by Dunnett’s multiple comparison tests was carried out for normally distributed samples with homogeneous variances. Non-parametric tests, namely Kruskal–Wallis followed by Mann–Whitney-U-tests were applied to samples without normal distribution and/or inhomogeneous variances. Raman spectral pre-processing and analysis were performed using Matlab 2017 (Mathworks, USA), using algorithms developed in house and the scripts are adapted from the CLIRSPEC Open Access Scripts (Henderson 2015). Prior to analysis, spectra were smoothed (Savitsky-Golay filter, 5th order, 7 points) and any obvious outliers were removed from the dataset. The reference spectrum constituting background signal was subtracted and spectra were vector normalised to improve spectral quality and minimise the possible background contributions into the spectra. After pre-processing, principal components analysis (PCA) was employed as an unsupervised multivariate approach to analyse the Raman data. PCA is based on determination of a number of principal components which correspond to the variables in the data sets to reduce dimensionality. In each data set, data is separated into the groups based on variances (principal components) in data sets. A number of principal components are determined according to their efficiency to separate the data sets from
each other; the 1st principal component shows the highest variance in the data set and the other principal components follow it as 2nd most, 3rd most and 4th most variance and so on. The order of the PCs denotes their importance to the dataset. PC1 describes the highest amount of variation, PC2 the second greatest and so on. In all analyses, the first two principal components accounted for > 63 % of the variance of the datasets, and higher order PCs did not add further clarity to the analysis. This statistical method was preferred for this study to highlight the variability existing in the spectral data set recorded during the different experiments. Another advantage of this method is the observation of loadings which represent the variance for each variable (wavenumber) for a given PC. Analysing the loadings of a PC can give information about the source of the variability inside a data set, derived from variations in the molecular components contributing to the spectra.

2.11. Chapter Summary

In this chapter, all experiments conditions from sample preparation to all 3D cultures spectroscopic and microscopic methods used were optimised in order to get the best data from cellular and drug investigations and to have accurate and validate scientific results.
CHAPTER 3

Comparative studies of cellular viability levels on 2D and 3D

in vitro culture matrices

The following chapter has been adapted from the published journal article entitled “Comparative studies of cellular viability levels on 2D and 3D in vitro culture matrices”, Cytotechnology (2018) 70: 261.

Author list: Mahmoud Gargotti, Ulises Lopez Gonzalez, Hugh J. Byrne, Alan Casey
3.1. Introduction

Traditionally, 2D monolayer cultures have been favoured as *in vitro* models for cellular research, due to the ease and convenience of set up with little loss of cellular viability. Typically, 2D substrates used *in vitro* are made from polystyrene or glass, and support cell growth to form a flat, two-dimensional cellular layer (Freshney 2016). Although such 2D cultures have significantly contributed to the understanding of basic cellular biology, they have limitations (Lee *et al.*, 2008). 2D based growth substrates lack the structural architecture and stroma (Drife 1986) present *in vivo* and not all types of epithelial cells can adhere and grow well on the artificial substrates (Kim 2005), limiting the uses of standard *in vitro* techniques. *In vivo* animal models are faced with a considerable higher level of ethical issues, stringent regulation control and these models are (Annabi *et al.*, 2014). Expensive and can result in lengthy experimental timeframes (Antoni *et al.*, 2015). Critically, the use of *in vitro* alternatives to animal models is increasingly encouraged by both EU and US regulatory bodies (EU Directive-2010/63/EU and US Public Law 106-545, 2010, 106th Congress) (European Union 2010; United States, 2000). To bridge the gap between *in vitro* and *in vivo* models and to improve the relevance of *in vitro* models, 3D culture models are being increasingly developed. 3D cell culture has the architectural structure to mimic the *in vivo* extra cellular matrix (ECM) and aims to produce cultures which possess the phenotype and functional characteristics of their *in vivo* counterparts, resulting in a more realistic biological response *in vitro* (Padmalayam & Suto 2012). In cancer research, 3D cultures have found favour as they are thought to mimic events occurring *in vivo* during progression and formation of cancer (Kim 2005). Currently there is a large variety of 3D culture systems on the market (Rimann & Graf- Hausner 2012), ranging from scaffolds, including, animal derived (Matrigel®, Collagen) or plantderived (QGel®
Matrix, 3-D Life Biomimetic, Puramatrix), scaffold-free, including low adhesion plates, micropatterned surfaces, hanging drop, suspension using methyl cellulose, rolling vessel or magnetic levitation (Riss 2014). Scaffold based systems are a 3D construct which provides an ECM that supports cell growth and differentiation (Hutmacher 2000). In scaffolds, cells can migrate between fibres and attach to them (Breslin & O’Driscoll 2013). Scaffolds are typically produced from natural materials such as Collagen, fibronectin, agarose, laminin and gelatin (Ravi et al., 2015) or synthetic polymers like poly (ethylene oxide) (PED) and poly (ethylene glycol) (PEG) (Place et al., 2009). Hydrogels are 3D matrices or porous scaffolds consisting of hydrophilic polymers (Annabi et al. 2014). Physically, the hydrogels are weak, but they provide a biomimetic environment to assist cell differentiation and proliferation (Peck & Wang 2013). Examples of hydrogels are Matrigel, Myogel and Collagen I matrices (Worthington et al., 2015). Decellularised tissue membranes are prepared by decellularising tissue by a combination of physical, chemical and enzymatic reactions, whereupon cells can be grown successfully for tissue engineering applications (Gilbert et al., 2006). Cell-derived matrices (CDM) are formed by cells cultured on a biomaterial surface at high density in vitro for sufficient time so that the cells produce their own ECM, whereupon the cells are removed, leaving only ECM that closely mimics native molecular content and stromal fibre (Kutys et al., 2013). Basement membrane extract and Collagen are the most common types of ECM used (Antoni et al., 2015), and two commercial examples of this type of membrane are used in this study, namely Rat Tail derived Collagen I, and Geltrex. These two membranes have been employed as substrates for 3D cell culture and the cell viability and function have been monitored, and compared to conventional 2D cultures, to determine which basement supports growth with least impact on cell function. To further monitor the effect of these
membranes and their potential for more relevant in vitro screening, a normal and cancer cell lines were chosen for growth on both basement membranes, and for consistency with previous studies (Bonnier et al., 2015; Casey et al., 2016).

3.2. Results

3.2.1. Confocal microscopic imagining

Images of live HeLa and HaCaT cells grown on both extracellular matrices (Collagen and Geltrex) and 2D cultures were recorded by CLSM. Nuclear staining was performed with NucRed® Live 647 ReadyProbes® reagent, as described in the Materials and Methods section. Due to the increased physical depth of the culture vessel caused by the presence of the ECM, two different objective lenses were used: cells grown on Collagen were imaged with a × 20 lens (Fig. 3.1.c, d) whereas, for cells grown on 2D and the Geltrex ECM, a × 63 oil immersion lens (Fig. 3.1.a, b, e, f) was employed. In all cases, minimal or no differences were observed in the cells examined, the nuclear membrane was unaltered and the 3D membrane was clearly visible in all images obtained.
Figure 3.1. a HeLa cells were seeded on 2D culture for 24 h, nuclei were stained with the nuclear stain NucRed. b HaCaT cells were seeded on 2D culture for 24 h, nuclei were stained with the nuclear stain NucRed. c HeLa cells were seeded on 3D culture (Collagen rat tile) for 24 h and nuclei stained with NucRed. d HaCaT cells were seeded on 3D culture (Collagen rat tile) for 24 h and nuclei stained with NucRed. e HeLa cells were seeded on 3D culture (Geltrex) for 24 h and nuclei stained with NucRed. f HaCaT cells were seeded on 3D culture (Geltrex) for 24 h and nuclei stained with NucRed (scale bar 20 µm).

3.2.2. Cell viability measurement with Alamar Blue

Cells were cultured, gels prepared and cells seeded as outlined in the Materials and Methods section. Following 24, 48 and 72 h incubation, cellular viability levels were monitored with the AB assay. The AB assay measures the innate metabolic activity of cells (Bonnier et al., 2015). The oxidised indigo blue, nonfluorescing form of this
chromogenic indicator dye is reduced by cellular dehydrogenases to a pink fluorescent form, which can be easily monitored spectrophotometrically. The HeLa and HaCaT cells, when cultured on Collagen gel (Fig. 3.2.a, b) in both the 6 well plate and 24 well plates and in the first 24 h exhibit higher fluorescence intensity than those cells grown in traditional 2D culture indicative of an increase in cellular viability on the 3D culture membrane. After 48 and 72 h exposure, fluorescence intensity was reduced compared to those cells growing in traditional 2D culture, as seen by a drop in calculated viability levels when compared to that of the 2D control. HeLa cells were noted to be significantly influenced; viability levels were approximately decreased by 50% compared to cells grown on 2D culture after 48 and 72 h incubation in 24 well plates.
Figure 3.2. Alamar Blue response following 24, 48 and 72 h growth on both 2D and 3D culture (Collagen) of HeLa and HaCaT cells on both a 6 well plate and b a 24 well plate. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments and relative to a 2D culture control. Statistically significant differences between the 3D culture membrane viability responses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01.
In contrast, for cells that were cultured on Collagen membrane of 2.5 mg/mL concentration ECM (Fig. 3.2.a), the average viability level had dropped by 20% when compared to the conventional 2D control in all incubation periods in 6 well plates. When cultured on Geltrex® (Fig. 3.3.a, b), both HeLa and HaCaT cells showed an increased conversion of the AB dye after 24, 48 and 72 h incubation. This increased fluorescence has been typically interpreted as a higher level of cellular viability (Antoni et al., 2015; Cartmell et al., 2003).
**Figure 3.3.** Alamar Blue response following 24, 48 and 72 h growth on both 2D and 3D culture (Geltrex) of HeLa and HaCat cells on both a 6 well plate and b a 24 well plate. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments and relative to a 2D culture control. Statistically significant differences between the 3D culture membrane viability responses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01.
To verify whether the results of the AB assay were indeed due to increased cellular viability in the 3D matrices compared to 2D, live cell flow cytometry studies were performed. A live, apoptotic, necrotic cell triplex assay was performed by using YO-PRO-1 and PI in combination to quantify the amount of live/apoptotic and necrotic cells after 24 h incubation on both tested 3D ECMs. For flow analysis, they were then harvested by enzymatic removal and stained with both YO-PRO-1 and PI. Cell doublets were excluded from the analysis by agitating the samples immediately prior to the analysis and area scaling with the BD Accuri software. As can be seen (Fig. 3.4.a, b), cells cultured on the Collagen ECM displayed slight differences in the levels of live, apoptotic and necrotic cells when compared to 2D substrates. Specifically, the cells grown on all concentrations of Collagen ECM displayed nominally lower viability levels than the 2D controls.
**Figure 3.4.** YO-PRO-1 and PI stained flow cytometry live, apoptotic and necrotic assay for HeLa (a) and HaCaT (b) cells grown on Collagen (3D) in different concentration and cells grown on plastic (2D) culture. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane live/dead cell analyses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01.
A very similar trend was also observed for the HaCaT cells cultured on Geltrex. The viability values of 2D controls were 95%, and the viability values of 3D cultures were 92 and 95% (Fig. 3.5.a, b). These results indicate that in both tested ECMs on both cell lines, the AB variations noted were not due to a difference in viability but a difference in dye uptake or conversion mechanisms, as previously stated.
Figure 3.5. YO-PRO-1 and PI stained flow cytometry live, apoptotic and necrotic assay for Hela (a) and HaCaT (b) cells grown on Geltrex® (3D) in different concentration and cells grown on plastic (2D) culture. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane live/dead cell analyses and those of the 2D cultures are denoted by *P < 0.05 and **P < 0.01.
3.2.4 Cell cycle analysis

In order to determine whether there were any differences between the cyclic behaviour of the cells cultured on the 3D substrates, cell cycle studies were performed on both cell lines in 2D and 3D cultures. Cells were grown as previously on various different working concentration or volumes of the ECM under study and incubated for 24 h on the ECM prior to analysis. For analysis, cells were then harvested by enzymatic removal, fixed and stained as detailed earlier and DNA content in the cells was monitored by a BD Accuri™ C6 Flow Cytometer. As before, cell doublets were excluded from the analysis by agitating the samples immediately prior to the analysis and area scaling with the BD Accuri software. The cells grown in Collagen did show variations when compared to those grown in traditional 2D culture, after 24 h of incubation. In the HeLa cells, there were significant increases to the number of cells in the G0/G1 and S-phase, with a corresponding reduction of cells in the G2/M phase, indicating that the cells may have been arrested in the G0/G1 or S phase as a result of culture on the Collagen substrate. In contrast these differences were not observed in the HaCaT cells with only marginal differences in the cell cycle checkpoint populations indicating they were not arrested to the same degree as the HeLa cells (Fig. 3.6).
**Figure 3.6.** Cell cycle analysis of HeLa (a) and HaCaT (b) - cells grown on three different concentrations of Collagen gel (3D) and cells grown on plastic (2D) culture, and percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane cell cycle analyses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01.
In contrast to the observations on the Collagen substrate, Geltrex, which is used at a fixed working concentration and at different volumes, resulted in less alteration to the cell cycle than Collagen. Only the HeLa (Fig. 3.7) cell line displayed variation in cell phases when compared with 2D culture, for which slight increases in the G2/M phase with a corresponding decrease in the G0/G1 were observed.
Figure 3.7. Cell cycle analysis of HeLa (a) and HaCaT (b) cell grown on two different concentration of Geltrex (3D) and cells grown on plastic (2D) culture, and percentage of cells at G0/G1, S and G2/ M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D culture membrane cell cycle analyses and that of the 2D cultures are denoted by *P < 0.05 and **P < 0.01.
3.3. Discussion

When the cells were viewed under the CLSM, the scaffold structures were clearly visible in both the Collagen and Geltrex (Fig. 3.1.c, f). As can be seen in Fig. 3.1, some minor morphological differences were apparent between the HeLa or HaCaT cells grown in conventional 2D (Fig. 3.1.a, b) when compared to those on the Collagen based or Geltrex 3D membrane (Fig. 3.1.c, f). However, nuclear staining confirmed that the nuclear integrity of both tested cells were not significantly altered by culture on either the Collagen or the Geltrex substrate and it is postulated that the morphological differences observed are attributed to the growth on a soft porous membrane in comparison to that of the 2D glass substrate. The in vitro viability of both cell lines was assessed in both 3D environments and all membrane variations were compared to a traditional 2D culture system used as control. Significant (P ≥ 0.05) differences were noted between the viabilities of the two cell lines on the 3D membranes and 2D substrates. These differences presented themselves as an apparent increase in the viability levels of both cell lines on the 3D matrices, but this is in fact due to an increase in the conversion rate of the resazurin to resorufin in the AB assay, due to the transition from a 2D to a 3D system (Bonnier et al., 2015). Indeed, similar effects were observed for an exposure to the chemotherapeutic agent Doxorubicin (Casey et al., 2016). The effect was notably different in the Collagen based 3D matrix at a concentration of 2.5 mg/ml (6 well plates) for both the cell lines, but it is postulated that this may have been due to the increased physical density of the higher concentration of Collagen of restricting nutrient levels to the cells or more likely the increased density of the membrane hindering the conversion of the dye by binding to the fibrous mesh of the ECM. It was also noted that, while increases in AB conversion were also observed when both cell lines were cultured on the Geltrex® ECM, this effect was of a much
smaller magnitude than the variations observed in the Collagen ECM, suggesting that the effect, while not eliminated, can be minimised by employing a different ECM. In previous studies comparing the viability of cells grown in conventional 2D cultures to that of cells grown on Collagen gel matrices, the apparent increased viability observed using the Alamar Blue cytotoxicity assay was attributed to differences in the diffusion and conversion rates of the test dye due to the alteration of the geometry and morphology of the test system (Bonnier et al., 2015). However, when the culture period was extend past 24 h, significant (P ≥ 0.05) variations in the AB assay responses to those of a 2D control were observed, as a drop in cellular viability. The current study again indicates that, rather than affecting a significant change in the cell metabolism, the 3D matrix (Collagen or Geltrex) composition and concentration alters the exposure conditions of the cells to the dye (AB), but notably that the effect can be reduced by ECM type, concentration and exposure period, and the observed effects should be taken into account when comparing cellular exposures in 2D and 3D matrices. The apoptosis results were in contrast to the AB studies and verified the postulation that there were no differences in cellular viability in 2D and 3D systems after 24 h exposure (Fig. 3.4). The cells cultured on the highest working concentration of the Collagen ECM (2.5 mg/ml) showed the highest level of cellular viability of 96%, which, although not significantly different to that of the 2D control (94%), gives support to the notion that the highly concentrated fibrous membrane of the 2.5 mg/ml concentration Collagen ECM restricted the diffusion of the AB in the test environment, resulting in a lower conversion rate in the AB studies. In contrast to the HeLa cells, no variations were noted in cell viability levels in the HaCaT cultures as a function of Collagen concentration, cultures yielding viability levels of 97% in the 2D and an average of 98% in all the 3D concentrations tested. This gives further support to the notion that cultures
grown on 3D do not have an increased viability as indicated by the AB conversion rates, but that the different cell growth environments can themselves influence the conversion rates of the cytotoxicity assay (Bonnier et al., 2015), resulting in an apparent increased viability in 3D matrices compared to 2D cultures. Identical studies were then performed with the Geltrex based 3D cultures, in which, again, no variations were noted between viability levels of 2D and 3D cultures. In the HeLa cell line, no differences in viability were noted between different volumes of Geltrex employed to form the membrane, as was the case with the Collagen based membranes, 2D cultures yielding 94% viability and the 3D yielding 92 and 93% viability levels, differences which fall outside statistical significance, again providing supportive evidence that observed viability levels were only a result of the transition from 2D to 3D. In vivo, the proliferation of cells is strictly controlled by numbers of proteins which can regulate prognosis of the cell cycle. However, the onset of carcinoma and indeed the immortalisation process of cells can alter the normal control of the cell cycle (Stacey et al., 2009). There are three important checkpoints during cell cycle, the first, G1 checkpoint between the G1/S phase, the second, G2 checkpoint between the G2/M phase and the spindle checkpoint in the mitotic phase between metaphase and anaphase (Han et al., 1995; Gorbsky 2001; Seluanov et al., 2009). Interestingly, statistically significant (P ≥ 0.05) differences were noted in the cell cycle assay, which were seen to be dependent on the working concentration of the Collagen concentration, cell population numbers in the G0/G1 phase decreasing and S-Phase population numbers increasing with decreasing Collagen working concentration, indicating that the presence of the Collagen substrate most likely altered the cycle of the HeLa cells (Fig. 3.6) by arresting cells in the G0/G1 phase. This effect, while also apparent for the HaCaT (Fig. 3.6) cells, was notably of lesser extent, the greatest variation being observed at the lowest working concentration.
of Collagen, indicating that the normal HaCaT line was not as susceptible to alteration in cell cycle by Collagen as the HeLa line. In contrast to the Collagen, only the HeLa (Fig. 3.7) cell line displayed variations in cell phases when compared to that of the 2D culture, slight increases in the G2/M phase with a corresponding decrease in the G0/G1 being observed. No variations in the HaCaT (Fig. 3.7) line were observed, both 2D and 3D cultures showing little or no variation in cell populations at each checkpoint, indicating that the HeLa and HaCaT cell cycle were largely or completely unaltered by the transition from 2D to that of 3D Geltrex culture. The observed cell cycle interruptions are thought to be the cause of the decreasing cellular viability levels determined with the AB assay for the longer term exposure on the Collagen membrane. The effect causes a reduced proliferation rate of the cells on the Collagen, resulting in a reduction in the number of cells present on the 3D matrix for the 48 and 72 h exposures when compared to that of the 2D control, resulting in a lower assay conversion rate on the membranes.

3.4. Conclusion

In summary, this study presents a comparison between 2D and 3D culture by using two commercial products of 3D culture in different concentrations and volumes of 3D culture. Thus, the study shows that transfer from 2D to 3D culture does not necessarily affect the viability of the cells. Moreover, differences in fluorescent detection of the AB assay are primarily due to an increased cell surface area exposed to the surrounding environment which leads to an increase in uptake and conversion rates of dye and not to changes in cellular viability levels. Viability levels were verified via flow cytometry and no differences in live cell and apoptosis levels between cells grown on 2D culture and cells grown in 3D culture were noted. However, when the culture length was increased these increases in AB conversion were reduced, ultimately displaying a reduced
viability on 3D when compared to a 2D. It was subsequently shown that transfer from 2D to 3D culture can influence cell cycle by inducing an interruption at the S-phase of the cell cycle interruptions result in a decreased cellular numbers due to a lower proliferation rate of cells on the Collagen membrane and should be accounted for in experimental planning. The results of this study strongly support the use of 3D culture in cytotoxicity assays to improve the relevance of drug or toxin screening protocols is a viable option, as there is no loss in cellular viability. They may indeed provide a more comparable culture environment to that of in vivo exposures, but appropriate controls and experimental validations must be incorporated into the protocols at every assessed time point. Numerous chemotherapeutic compounds work by processes of DNA intercalation and inhibition of macromolecular biosynthesis (Parker 2009), and as such, are most effective at set cell checkpoints. If the cell culture environment employed arrests the cell at a particular checkpoint, as is observed in this study, the efficacy of a drug could potentially be enhanced or delayed. Indeed, in previous study (Casey et al., 2016) variations in Doxorubicin toxicity at short term cellular exposures were observed resulting from a transition from 2D to 3D Collagen membrane. Such responses may have been due to the alteration of cell cycle, altering the mechanism of action of the Doxorubicin. Therefore, in choosing a membrane for screening drug toxicity, consideration must be given to the membrane effect on cellular systems. If basic functions like cell cycle can be influenced by experimental protocols this may in turn reduce or indeed improve the efficacy of tested drugs, depending on their mode of action. A viable option, as there is no loss in cellular viability, and may indeed provide a more comparable culture environment to that of in vivo exposures by appropriate controls and experimental validations must be incorporated into the protocols at every assessed time point.
CHAPTER 4

Raman spectroscopy detects biochemical changes due to different cell culture environments in live cells in vitro

The following chapter has been adapted from the published journal article entitled “Raman spectroscopy detects biochemical changes due to different cell culture environments in live cells in vitro”, Analytical and Bioanalytical Chemistry, 2018, 410(5–6):1-14.

Author list: Mahmoud Gargotti, Esen Efeoglu, Hugh J. Byrne, Alan Casey
4.1. Introduction

The transition from 2D to 3D culture models in vitro has been a significant development in the understanding of differences in tumour formation mechanisms in vitro (Freshney 2016; Antoni et al., 2015; Padmalayam & Suto 2012; Breslin & O’Driscoll 2013). 3D cell culture has the architectural structure to mimic the in vivo environment (Kim 2005; Breslin & O’Driscoll 2013) via an extra-cellular matrix (ECM) and aims to produce cultures which possess the phenotype and functional characteristics of their in vivo counterparts, resulting in a more realistic biological response in vitro (Padmalayam & Suto 2012). Over the last decade, new models such as Collagen gels and more complex multicomponent systems such as Matrigel have emerged, providing the cell a suitable ECM (Prestwich 2008; Weaver et al., 1995).

It has been demonstrated that the different culture environments do not significantly affect the viability and health of the cells, and any apparent differences in viability levels between 2D and 3D culture have been demonstrated to be a result of the differing physical characteristics of the 2D and 3D environments which cause an increase in conversion rates of quantifying viability dyes (Casey et al., 2016, Bonnier et al., 2015). However, while not necessarily affecting the viability of the cells, it has subsequently been shown that, compared to 2D cultures, the 3D environment can influence cell cycle as a result of cell cycle arrest in the G0/G1 or S phase and retardation of the S-G2/M transition, as indicated by flow cytometric analysis (Gargotti et al., 2017).

Raman spectroscopy can be employed to further explore this phenomenon at a biochemical level. The technique has been shown to be a powerful analytical technique for the analysis of biological materials (Notingher 2007; Ember et al., 2017; Butler et al., 2016), and it has a high molecular specificity. The advantages of Raman
spectroscopy in the analysis of cells, tissue and bio-fluids have been extensively demonstrated in recent years (Ellis et al., 2013; Clemens et al., 2014; Liu et al., 2014). It allows rapid, non-invasive and high spatial resolution acquisition of structural and biochemical information through the acquisition of point spectra and/or spectral images (Butler et al., 2016; Lieber et al., 2008; Swain & Stevens 2007; Notingher & Hench 2006). As a label free technique, Raman spectroscopy presents several advantages in the study of live cells compared to high content molecular analysis with optical imaging, in which cellular processes are visualised through specific labels. In comparison to infrared spectroscopic analysis, Raman offers submicron spatial resolution and has the advantage that water has a relatively weak contribution, which is an extremely strong infrared absorber. Due to these advantages, Raman spectroscopy offers a better possibility to study cells in an aqueous environment and thus keep cells alive during the experiments under normal physiological conditions (Draux et al., 2009). The technique has thus been widely used for the analysis of cell-drug and cell nanoparticle interaction at a sub-cellular level (Farhane et al., 2018a; Efeoglu et al., 2017). Currently, in bio-spectroscopy, one of the most widely used substrates is CaF$_2$ windows, as the material permits the transmission of visible, near and mid IR light with low losses and UV grade CaF$_2$ does not have any characteristic Raman bands in the cellular spectral fingerprint region (400-1800 cm$^{-1}$) which allows the analysis of biological samples (Gee et al., 1966). Although CaF$_2$ has many advantages, such as low background signal and high optical transmission, this substrate is expensive and significantly increases sample analysis costs for clinical applications (Baker et al., 2018). Standard glass and quartz have stronger backgrounds and are more frequently used during stimulated and coherent anti-stokes Raman setups, in which the Raman signal is relatively strong or turned to a specific wavenumber (Gee et al., 1966). Some studies have previously investigated
metal-coated Raman substrates for biological analysis (Cui et al., 2016; Kerr et al., 2015). However, the release of metal ions in the biological environment can have adverse effects on live cultured cells, hindering the lifetime of the sample (Kerr et al., 2015).

This work aims to investigate how the substrate induced changes in cell cycle are manifest in the spectroscopic profiles of the cell cultures. To do this, and for consistency with previous studies (Bonnier et al., 2015; Casey et al., 2016; Gargotti et al., 2017), normal and cancer cell lines were chosen for growth on both a commercially available 3D basement membranes (Collagen) and 2D systems (Polystyrene, CaF$_2$). The cell cycle for each cell line on each substrate was profiled using flow cytometric analysis, and the spectral signatures of the cytoplasm, nucleus and nucleolus of cells grown on standard glass slides coated with Collagen were compared to those of cells on the CaF$_2$ substrate, with the aid of principal components analysis (PCA).

4.2. Results

4.2.1 Cell cycle analysis for HeLa and HaCaT cell lines on different substrates

The cell cycle is constituted by the G1 phase (cell growth), followed by S phase (DNA synthesis), and then by G2 phase (cell growth), whence the mitotic phase is reached, at which the cell undergoes mitosis and cytokinesis, leading to the formation of two daughter cells, in the G0 phase, which then progress to the G1 phase, etc. In order to determine whether there were any differences between the cell cycle profiles of the cells cultured on the 3D in comparison to the 2D substrates, cell cycle analysis studies were performed on both cell lines on polystyrene, CaF$_2$ and Collagen, revealing significant cell line dependent differences in profiles of cell grown on different
substrate/environments (Fig. 4.1). For the case of HaCaT cells, minimum differences are apparent between the cell cycle profiles of cells grown on polystyrene, compared to the 3D Collagen environment (Fig. 4.1.a). The cell cycle profile of HeLa cells, however, shows significant differences between the two (Fig. 4.1.b), indicating relatively fewer cells in the S and more cells in the G2/M phases in the conventional 2D growth environment, as previously been reported by Gargotti et al..

On CaF$_2$ substrates, favoured for Raman spectroscopic analysis, the cell cycle profile is markedly different for both cell lines. For the HaCaT cells, there were significant increases to the number of cells grown on CaF$_2$ in the G0/G1 phase, with a corresponding reduction of cells in the G2/M phase, indicating that the cells may have been arrested in the G0/G1 phase as a result of culture on the CaF$_2$ substrate compared with those grown on 3D culture (Fig. 4.1.a). Although elevated with respect to those on Collagen, the cell populations of HeLa cells on CaF$_2$ are not as significantly different to that of the HaCaT, the population levels in the S and G2/M phases are similar within the standard deviations of the measurements.
Figure 4.1. (a) HaCaT cell cycle analysis, cells were grown on three different substrates (Polystyrene, Collagen and CaF₂), and percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. (b) HeLa cell cycle analysis, cells were grown on three different substrates (Polystyrene, Collagen and CaF₂), and percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D and CaF₂ cultures and the polystyrene cultures are denoted by **P < 0.01 and *** P < 0.001.
4.2.2. Raman spectroscopic analysis of HeLa and HaCaT cells on CaF$_2$ substrate

The ability to acquire Raman spectra from cells in the presence of the saline without interference to the spectra has been demonstrated by (Bonnier et al. 2010), resulting in a series of band assignments for cellular components at molecular level (Movasaghi et al., 2007; Notingher et al., 2006; Pobezhimova et al., 2000) (see table 4.1). After initially culturing the HaCaT cells (normal keratinocyte) for 24 h on CaF$_2$ and 3D culture (Collagen), the medium was replaced with phenol free DMEM, which is used to improve the visibility of the cells and subcellular features under white light illumination, before taking any point spectra of live cells. 20-point spectra were acquired from each cellular region of 20 different cells, as previously described by Bonnier et al. (2010). Fig. 4.2 shows scatter plots of PCA (Fig. 4.2.a) and the loading of the first PC (Fig. 4.2.b) corresponding to cytoplasm, nucleus and nucleolus of HaCaT cells grown on CaF$_2$. Different cellular regions are indicated respectively by, blue for cytoplasm, red for nucleus and green for nucleolus. The spectra corresponding to the nucleus and nucleolus (nuclear areas) are clearly differentiated from cytoplasm of HaCaT cells grown on CaF$_2$ according to PC1 (Explained variance 41%) based on their biochemical features. In the first PCA loading obtained from comparison of the combined nuclear region and cytoplasm of HaCaT cells grown in CaF$_2$, the spectra relating to the cytoplasm of HaCaT cells scored negatively (PC1< 0), while nucleus and nucleolus spectra scored positively (PC1 ≥0) (Fig. 2 b). To facilitate the analysis of the loadings, table 4.1 details common assignments of cellular spectral features derived from literature (Movasaghi et al., 2007; Notingher et al., 2006; Pobezhimova et al., 2000). In the loading, positive features are dominated by nucleic acid features (785 and 810 cm$^{-1}$) corresponding to biochemical composition of nucleus and nucleolus. On the other hand, negative features of the first loading show lipid and protein features such as
those at 715, 1438 and 1600-1700 cm\(^{-1}\) (Fig. 4.2 b, table 4.1). Similar differentiating profiles have previously been observed, for example for lung cancer cell lines (Farhane \textit{et al.}, 2015a)

\textbf{Figure 4.2.} PCA scatter plot (a) and loading of PC1 (b), corresponding to comparison of cytoplasm, nucleus and nucleolus region of HaCaT cells grown on CaF\(_2\) substrate. The ‘0’ line is indicated with black dashes. PCA scatter plot (c) and loading of PC1 (d) corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HeLa cells grown on CaF\(_2\) substrate. The ‘0’ line is indicated with black dashes.
HeLa cells were cultured under the exact same conditions as the HaCaT cells and the same data acquisition was applied. Briefly, the HeLa cells were grown on CaF$_2$ and 3D culture (Collagen) and spectra were acquired from cytoplasm, nucleus and nucleolus of individual cells. Fig. 4.2 shows the scatter plots (Fig. 4.2.c) and the first loading of PCA (Fig. 4.2.d), corresponding to cytoplasm, nucleus and nucleolus of HeLa cells grown on CaF$_2$. The spectra corresponding to the nucleolus are clearly differentiated from the cytoplasm of HeLa cells grown on CaF$_2$ according to PC1, although, in comparison with the observations for HaCaT cells, the spectra for the nucleus are less well differentiated, and straddle the positive and negative sides of PC1, which represents the most significant variance (33%) among the data. The loading of PC1 of HeLa cells grown in CaF$_2$, is nevertheless dominated on the positive side by nucleic acid features associated with the nuclear region and negative features of protein features associated with the cytoplasm (Fig. 4.2.d). The differentiating loadings of PC1 in Fig. 4.2 are remarkably similar for the two cell lines and are consistent with those observed in numerous studies of a range of cell lines, conducted under similar experimental conditions (Bonnier et al., 2010; Farhan et al., 2015a; Efeglu et al., 2016).

A comparison between HeLa and HaCaT cells grown on CaF$_2$ and 3D culture (Collagen) was performed. Fig. 4.3 shows the scatter plots and the loading of the first PCs corresponding to a pairwise comparison of cytoplasm, nucleus and nucleolus of HeLa and HaCaT cell lines after 24 h growth on the CaF$_2$ substrate. The scatter plots of PCA and data sets relating to the different cell lines are colour coded, blue for HaCaT cells and red for HeLa cells. Although a complete differentiation is not observed for any of the subcellular regions, the data is partially discriminated for each. The spectra relating to HaCaT cells predominantly scored negatively according to PC1 (PC1<0), while those of HeLa cells scored positively (PC1≥0). The different cell lines are best
differentiated by their nucleolar regions, consistent with the studies of different lung cell lines, by Farhane et al. (2015a).

**Figure 4.3.** PCA scatter plots (a) and loadings of PC1 (b), corresponding to comparison of cytoplasm, nucleus and nucleolus of HaCaT and HeLa cells grown on CaF$_2$. The ‘0’ line of PC1 is indicated with black dashes. Band positions corresponding to dominant features of the PC1 are indicated on the loadings.
Fig. 4.3a (cytoplasm) shows the scatter plots of PCA corresponding to pairwise comparison of the cytoplasm of HeLa and HaCaT cell lines according to PC1, which represents the most significant variance (34%) among the data, originating from biochemical differences in the cytoplasm of the different cell lines. Fig. 4.3b (cytoplasm) shows the loading of the first PC, corresponding to cytoplasm of HeLa and HaCaT cells. The predominant features which differentiate the HeLa and HaCaT cells are the positive features associated with phospholipids, nucleic acid, lipids, protein, Collagen, DNA and RNA (715-9, 873 cm\(^{-1}\), 1265 cm\(^{-1}\), 1299, 1438 cm\(^{-1}\) and 1655-80 cm\(^{-1}\)). On the other hand, the dominant negative features include those associated with DNA/RNA and amino acids (785 cm\(^{-1}\), 810 cm\(^{-1}\) and 1575 cm\(^{-1}\)) (table 1). Fig. 4.3a (nucleus) compares the spectra corresponding to the nucleus of HeLa and HaCaT cell lines according to PC1, which represents the most significant variance (42%) among the data, originating from biochemical differences in nucleus of different cell lines. Fig. 4.3.b (nucleus) shows the loading of the first PC, which differentiates the nucleus of HeLa and HaCaT cells. The predominant features which differentiate the HeLa and HaCaT cells are identified as the positive features characteristic of amino acid, methoxy groups, phospholipids, protein and lipids (1003 cm\(^{-1}\), 1029 cm\(^{-1}\), 1204 cm\(^{-1}\), 1445 cm\(^{-1}\), 1615 cm\(^{-1}\) and 1655-80 cm\(^{-1}\)) (table 4.1). Fig. 4.3.a (nucleolus) shows the spectra corresponding to the nucleolus of HeLa and HaCaT cell lines according to PC1, which represent the most significant variance (44%) among the data. Fig. 4.3.b (nucleolus) shows the first loading of the PCA, corresponding to the nucleoli of HeLa and HaCaT cells. The positive features of the loading are dominated by HeLa cell related bands, while negative features relate to the HaCaT cells. The predominant features which are used to differentiate the HeLa and HaCaT cells are observed as the negative features
associated with amino acid, nucleic acid and DNA/RNA (729 cm\(^{-1}\), 785 cm\(^{-1}\), 830 cm\(^{-1}\), 852 cm\(^{-1}\) and 1094 cm\(^{-1}\)) (table 4.1).

**Table 4.1. Assignments of Raman Bands of cells grown on CaF\(_2\) and Collagen substrate** (Movasaghi *et al.*, 2007; Notingher *et al.*, 2007; Pobezhimova *et al.*, 2000).

<table>
<thead>
<tr>
<th>Band</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>715-9 cm(^{-1})</td>
<td>C-N (membrane phospholipids head)/adenine, CN(_2)(CH(_3))(_3) (lipids), choline group</td>
</tr>
<tr>
<td>729 cm(^{-1})</td>
<td>Adenine</td>
</tr>
<tr>
<td>785 cm(^{-1})</td>
<td>U, T, C (ring breathing modes in the DNA/RNA bases), backbone O-P-O (RNA)</td>
</tr>
<tr>
<td>810 cm(^{-1})</td>
<td>phosphodiester (Z-marker) DNA</td>
</tr>
<tr>
<td>830 cm(^{-1})</td>
<td>proline, hydroxyproline, tyrosine</td>
</tr>
<tr>
<td>850 cm(^{-1})</td>
<td>most probably due to single bond stretching vibrations for the amino acids and valine and polysaccharides</td>
</tr>
<tr>
<td>852 cm(^{-1})</td>
<td>proline, hydroxyproline, tyrosine</td>
</tr>
<tr>
<td>873 cm(^{-1})</td>
<td>symmetric breathing of tryptophan (protein assignment)</td>
</tr>
<tr>
<td>918 cm(^{-1})</td>
<td>proline, hydroxyproline</td>
</tr>
<tr>
<td>938 cm(^{-1})</td>
<td>proline, hydroxyproline</td>
</tr>
<tr>
<td>980 cm(^{-1})</td>
<td>C-C stretching β-sheet (proteins)</td>
</tr>
<tr>
<td>1003 cm(^{-1})</td>
<td>phenylalanine, C-C skeletal</td>
</tr>
<tr>
<td>1029 cm(^{-1})</td>
<td>O-CH(_3) stretching of methoxy groups</td>
</tr>
<tr>
<td>1081 cm(^{-1})</td>
<td>ν(_1)CO(_3)^2, ν(_3)PO(_4)^3, ν (C-C) skeletal of acyl backbone in lipid or collagen</td>
</tr>
<tr>
<td>1094 cm(^{-1})</td>
<td>DNA</td>
</tr>
<tr>
<td>1167 cm(^{-1})</td>
<td>lipid</td>
</tr>
<tr>
<td>1184 cm(^{-1})</td>
<td>Guanine, Cytosine</td>
</tr>
<tr>
<td>1249 cm(^{-1})</td>
<td>Amide III, Guanine and Cytosine</td>
</tr>
<tr>
<td>1265 cm(^{-1})</td>
<td>Amide III of collagen, Amide III (collagen assignment), Amide III, ν(CN), δ(NH) amide III, α-helix, collagen (protein assignment)</td>
</tr>
<tr>
<td>1299 cm(^{-1})</td>
<td>Lipid</td>
</tr>
<tr>
<td>1315 cm(^{-1})</td>
<td>Guanine (B, Z-marker)</td>
</tr>
<tr>
<td>1335 cm(^{-1})</td>
<td>nucleic acid, collagen</td>
</tr>
<tr>
<td>1438 cm(^{-1})</td>
<td>CH(_3), CH(_2) deformation</td>
</tr>
<tr>
<td>1445 cm(^{-1})</td>
<td>δ(CH(_3)), δ(CH(_3)), CH(_2)CH(_3) bending</td>
</tr>
<tr>
<td>1448-9 cm(^{-1})</td>
<td>CH(_2)CH(_3) deformation</td>
</tr>
<tr>
<td>1575 cm(^{-1})</td>
<td>ring breathing modes in the DNA bases, G, A (ring breathing modes of the DNA/RNA bases)</td>
</tr>
<tr>
<td>1587 cm(^{-1})</td>
<td>phenylalanine, hydroxyproline</td>
</tr>
<tr>
<td>1615 cm(^{-1})</td>
<td>tyrosine, tryptophan, C=C (protein)</td>
</tr>
<tr>
<td>1655-80 cm(^{-1})</td>
<td>T, G, C (ring breathing modes of the DNA/RNA bases)-amide I (protein)</td>
</tr>
<tr>
<td>1660 cm(^{-1})</td>
<td>Amide I</td>
</tr>
</tbody>
</table>
4.2.3. Raman spectroscopic analysis of HeLa and HaCaT cells on Collagen substrates

Fig. 4.4 shows the scatter plots (a) and the first loadings of PCA (b) corresponding to cytoplasm, nucleus and nucleolus of HaCaT cells grown on 3D culture (Collagen). The spectra corresponding to the nucleus and nucleolus (nuclear areas) are clearly differentiated from those of the cytoplasm of HaCaT cells grown on 3D culture (Collagen), according to PC1 which represents the most significant variance (34%) among the data originating from differences in biochemical composition between the nuclear and cytoplasmic areas. According to the loading of PC1 of HaCaT cells grown in 3D culture (Collagen), the spectra relating to the cytoplasm of HaCaT cells scored negatively (PC1 < 0), while nuclear and nucleolus spectra scored positively (PC1 ≥ 0) (Fig. 4.4. b).
Figure 4.4. PCA Scatter plot (a) and loading of PC1 (b) corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HaCaT cells grown on 3D culture (Collagen). The ‘0’ line is indicated with black dashes. PCA scatter plot (c) and loading of PC1 (d) corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HeLa cells on 3D culture (Collagen). The ‘0’ line is indicated with black dashes.

Fig. 4.4 shows the PCA scatter plot (c) and the loading of PC1 (d) corresponding to cytoplasm, nucleus and nucleolus of HeLa cells grown on 3D culture (Collagen).
Different cellular regions are colour coded as before, blue for cytoplasm, red for nucleus and green for nucleolus, respectively. In contrast to the spectra obtained from CaF$_2$, the spectra corresponding to the nucleus and nucleolus (nuclear areas) are clearly differentiated from those of the cytoplasm of HeLa cells grown on 3D culture (Collagen), according to PC1 (explained variance 42%). According to the loading of PC1 obtained from comparison of nuclear region and cytoplasm of HeLa cells grown in 3D culture (Collagen), the spectra relating to the cytoplasm of HeLa cells scored negatively (PC1 < 0), while nuclear and nucleolus spectra scored positively (PC1 ≥ 0) (Fig. 4.4.d). Notably, although a better discrimination between the subcellular regions of HaCaT cells was observed, the differentiating profiles of PC1 obtained from the analysis of cellular compartments of HeLa cells on CaF$_2$ and Collagen surfaces showed a high degree of consistency in terms of Raman spectral bands in negative and positive features of the loadings.

Fig. 4.5 shows the scatter plots and the loading of the first PC corresponding to cytoplasm, nucleus and nucleolus of HeLa and HaCaT cells lines after 24 h growth in 3D culture (Collagen). The scatter plots of PCA and data sets relating to substrates are colour coded as, blue for HaCaT cells and red for HeLa cells. Fig. 4.5.a (cytoplasm) shows the spectra corresponding to the cytoplasm of HeLa and HaCaT cell lines according to PC1, which represent the most significant variance (37%) among the data, originating from biochemical differences of the cytoplasm of the different cell lines. The spectra relating to the cytoplasm of HaCaT scored negatively according to PC1 (PC1 < 0), while the cytoplasm of HeLa spectra scored positively (PC1 ≥ 0). Fig. 4.5b (cytoplasm) shows the first loading of the PCA, corresponding to pairwise comparison of cytoplasm of HeLa and HaCaT cells. The positive features of loading 1 can be attributed to HeLa cells, while negative features represent HaCaT cells. The most
dominant features which differentiate the HeLa and HaCaT cells are the positive features of lipids/phospholipids, nucleic acids, DNA/RNA, amino acids (717-9 cm\(^{-1}\), 785 cm\(^{-1}\), 810 cm\(^{-1}\), 850 cm\(^{-1}\), 918 cm\(^{-1}\), 938 cm\(^{-1}\), 1003 cm\(^{-1}\), 1081 cm\(^{-1}\), 1265 cm\(^{-1}\), 1299 cm\(^{-1}\), 1438 cm\(^{-1}\) and 1655-80 cm\(^{-1}\)) (table 4.1). Fig. 4.5.a (nucleus) shows the spectra corresponding to the nucleus of HeLa and HaCaT cell lines according to PC1, which represents the most significant variance (31 %) among the data, originating from biochemical differences in the nucleus of different cell lines. The spectra relating to the nucleus of HaCaT scored negatively according to PC1 (PC1<0), while those of the nucleus of HeLa spectra score positively (PC1≥0). Fig. 4.5.b (nucleus) shows the first loading of the PCA, corresponding to nucleus of HeLa and HaCaT cells. The positive features of the loading of PC1 represent the differentiating biochemical features of HeLa cells, while negative features relate to the HaCaT cells. The most dominant features which differentiate the HeLa and HaCaT cells are the positive features of phospholipids/lipids, nucleic acids, DNA/RNA, amino acids and proteins (717-9 cm\(^{-1}\), 785 cm\(^{-1}\), 850 cm\(^{-1}\), 938 cm\(^{-1}\), 980 cm\(^{-1}\), 1003 cm\(^{-1}\), 1029 cm\(^{-1}\), 1094 cm\(^{-1}\), 1249 cm\(^{-1}\), 1335 cm\(^{-1}\), 1448-9 cm\(^{-1}\) and 1656-80 cm\(^{-1}\)) (table 4.1). On the other hand, the dominant negative features include those of lipids, guanine, cytosine, adenine and dipeptides (1167 cm\(^{-1}\), 1184 cm\(^{-1}\) and 1587 cm\(^{-1}\)).

Fig. 4.5.a (nucleolus) shows the spectra corresponding to the nucleolus of HeLa and HaCaT cell lines according to PC1, which represent the most significant variance (35 %) among the data, originating from biochemical differences in nucleolus. The spectra relating to the nucleolus of HaCaT scored negatively (PC1<0), while nucleolus of HeLa spectra scored positively according to PC1 (PC1≥0). Fig. 4.5.b (nucleolus) shows the first loading of the PCA, corresponding to nucleolus of HeLa and HaCaT cells. The positive features of the loading of PC1 can be related to HeLa cells, while negative
features relate to the HaCaT cells. The most prominent loadings which differentiate the HeLa and HaCaT cells are the positive features of phospholipids/lipids, nucleic acid, DNA/RNA, proteins, Guanine and protein (717-9 cm\(^{-1}\), 785 cm\(^{-1}\), 980 cm\(^{-1}\), 1094 cm\(^{-1}\), 1315 cm\(^{-1}\), 1335 cm\(^{-1}\), 1448-9 cm\(^{-1}\), 1575 cm\(^{-1}\) and 1660 cm\(^{-1}\)) (table 4.1).

![Figure 4.5](image)

**Figure 4.5.** Scatter plots (a) and first loadings of PCA (b) corresponding to comparison of cytoplasm, nucleus and nucleolus of HaCaT and HeLa cells on 3D culture (Collagen). The ‘0’ line is indicated with black dashes. Band positions corresponding to dominant features of the PC1 are provided on the loadings.
4.2.4. Comparison between CaF$_2$ and Collagen

A comparison between the spectra of cells grown on CaF$_2$ and 3D culture (Collagen) was performed (see supplementary materials); scatter plots and the loadings of the first PC corresponding to cytoplasm, nucleus and nucleolus of HeLa and HaCaT cells are provided in Fig. 4.6 and 4.7, respectively.

Fig. 4.6 is a representation of the scatter plots obtained from the pairwise comparison of different cellular regions of HeLa cells grown on CaF$_2$ and Collagen substrates. Notably, for all cellular regions, HeLa cells show little or no discrimination between 3D culture (Collagen) and CaF$_2$ in the scatter plots of PCA (Fig. 4.6.a, b and c).

**Figure 4.6.** Scatter plots of PCA corresponding to comparison of cytoplasm (a), nucleus (b) and nucleolus (c) of HeLa cells grown on CaF$_2$ and 3D culture Collagen substrate. The black dashes are used to indicate the ‘0’ line.
Fig. 4.7 is a representation of the plots obtained from pairwise comparison of cytoplasm, nucleus and nucleolus of HaCaT cells grown on different substrates. In contrast to the results from HeLa cells, clear discrimination is observed between 3D culture (Collagen) and CaF$_2$ in the scatter plots of PCA for each subcellular region (Fig. 4.7). The scatter plots of PCA and data sets relating to the different cell lines are colour coded, blue for HaCaT cells grown on CaF$_2$ and red for HaCaT cells grown in Collagen. The spectra relating to cells on CaF$_2$ predominantly scored negatively according to PC1 (PC1<0), while those cells in Collagen scored positively (PC1≥0). Fig. 4.7.a (cytoplasm) shows the spectra corresponding to the cytoplasm of HaCaT cells on CaF$_2$ and HaCaT cells in Collagen according to PC1, which represent the most significant variance (51 %) among the data, originating from biochemical differences in the cytoplasm of the cells grown on different substrates. Fig. 4.7.b (cytoplasm) shows the loading of the first PC, corresponding to cytoplasm of HaCaT cells grown on CaF$_2$ and HaCaT cells grown in Collagen. The dominant negative features include those of phospholipids/lipids, nucleic acid, DNA/RNA and amino acid (717 cm$^{-1}$, 785 cm$^{-1}$, 1003 cm$^{-1}$ and 1094 cm$^{-1}$) (table 5.1). Fig. 4.7.a (nucleus) compares the spectra corresponding to the nucleus of HaCaT cells grown on CaF$_2$ and HaCaT cells grown in Collagen according to PC1, which represents the most significant variance (52 %) among the data, originating from biochemical differences in nucleus of different substrates. Fig. 4.7.b (nucleus) shows the loading of the first PC, which differentiates the nucleus of HaCaT cells grown on CaF$_2$ and HaCaT cells grown in Collagen. The predominant features, which differentiate the HaCaT cells grown on CaF$_2$ and HaCaT cells grown in Collagen, are identified as lipids in the positive loadings (1438 cm$^{-1}$), while the dominant negative features include those of phospholipids/lipids, nucleic acid, DNA/RNA and amino acids (717-9 cm$^{-1}$, 785 cm$^{-1}$, 1003 cm$^{-1}$ and 1094 cm$^{-1}$) (table
4.1. Fig. 4.7a (nucleolus) shows the spectra corresponding to the nucleolus of HaCaT cells grown on CaF$_2$ and on Collagen according to PC1, which represents the most significant variance (63 %) among the data. Fig. 4.7b (nucleolus) shows the first loading of the PCA, corresponding to the nucleoli of HaCaT cell grown on CaF$_2$ and HaCaT cells grown in Collagen. The negative features of the loading are dominated by HaCaT cells grown on CaF$_2$ related bands, while positive features relate to the HaCaT cells grown in Collagen. The predominant differentiating features are observed as the positive features of lipids at 1438 cm$^{-1}$ and the negative features of phospholipids/lipids, nucleic acid, DNA/RNA, amino acids (717-9 cm$^{-1}$, 785 cm$^{-1}$, 1003 cm$^{-1}$, 1094 cm$^{-1}$ and 1575 cm$^{-1}$) (table 4.1).
Figure 4.7. Scatter plots and first loadings of PCA corresponding to comparison of cytoplasm, nucleus and nucleolus of HaCaT cells on CaF₂ and 3D culture Collagen substrate. The ‘0’ line is indicated with black dashes.
4.3. Discussion

3D culture better mimics the extracellular conditions encountered by cells in their native environment and can facilitate the acquisition of accurate information on cell differentiation, migration and cell homeostasis (Kim et al., 2004; Kim 2005). 3D cell culture has the architectural structure to mimic the in vivo extra cellular matrix (ECM) and aims to produce cultures which possess the phenotype and functional characteristics of their in vivo counterparts, resulting in a more realistic biological response in vitro (Padmalayam & Suto 2012). New models such as Collagen and more complex multicomponent systems such as Matrigel have been developed to provide an ECM to grow the cells in vitro (Petersen et al., 1992; Weaver et al., 1995). However, a number of studies have shown that there can be modifications to the cell composition, metabolism and also phenotype when the cells are grown in ECM compared to 2D culture (Lupanova et al., 2010; Wu et al., 2009).

The translation from 2D culture to 3D culture has been seen to influence cell cycle by inducing an interruption at the S-phase of cell cycle, as evidenced by flow cytometric analysis of cell cycle phase transitions using a Propidium Iodide stain for the DNA content (Gargotti et al., 2017). Notably, in comparison to cells grown in a conventional 2D environment (Polystyrene), significant differences in the cell cycle profile of both cell lines was observed on the CaF$_2$ substrate, manifest as significantly higher cell numbers in the G0/G1 phase, and correspondingly lower numbers in the S and G2M phases, particularly for the case of HaCaT cells. A different trend is observed for the Collagen substrates, although to a lesser extent, and therefore there is a greater similarity of the profiles of the conventional polystyrene Petri dish and 3D Collagen environment, particularly for the HaCaT cell line. Comparing the cell cycle profiles of
the two cell lines, the differences between the 2D CaF$_2$ and Collagen environment are more pronounced for the HaCaT cell line.

In this study, we used two different types of cell lines which represent normal and cancer cells models, and are among the most commonly used human cell lines in scientific research. The PCA of data collected in this work demonstrates a clear separation and they do not show high variances. In all cases, the cellular samples are mixed populations, and microscopically, the different phases of the cell cycle are not discernible. The Raman spectroscopic profiling of the subcellular regions is of random cell selections within the population. In the case where the subcellular regions of the cells are not mutually differentiated, as is the case for example for the comparison of cell lines on either CaF$_2$ or Collagen substrates (Fig. 4.3 and 4.5), or HeLa cells on CaF$_2$ compared to Collagen (Fig. 4.6), pairwise PCA simply identifies the variance within the combined dataset, and the PC loading represents that variance. Although some differences in cell cycle profile are evident in Fig. 1a, b, as discussed in “Cell cycle analysis for HeLa and HaCaT cell lines on different substrates” section, these differences are not apparent in the comparison of the spectral profiles of the subcellular regions, indicating that it is difficult to directly correlate such differences in cell cycle profile with the spectroscopic profiles.

Spectroscopically, HaCaT cells grown on the CaF$_2$ and Collagen environments are best differentiated in all subcellular regions, as shown in Fig. 4.7. Notably, comparing the cell cycle profiles for the two different substrates in Fig. 4.1a, it can be seen that the most significant differences are the relative populations in the G0/G1, and in the G2/M phases. The loadings of the differentiating PCs in Fig. 4.7 indicate distinctive nucleic acid contributions for all subcellular regions, particularly that at 785 cm$^{-1}$, assigned to ring breathing modes of the DNA/RNA bases, as well as backbone O-P-O stretching of
RNA. Notably, in all cases, these present as positive bands for cells grown on CaF$_2$, and a similar differentiating profile is observed for the nucleoli of HaCaT cells compared to HeLa, on CaF$_2$. This feature, and the doublet of features at 785 cm$^{-1}$ and 810 cm$^{-1}$ observed in the differentiating loading for cytoplasm of HaCaT vs HeLa cells grown on CaF$_2$ (Fig. 4.3), have previously been observed in studies of oxidative stress in nanoparticle exposed cell cultures, and may be related to non-coding RNA formation as a result of reactive oxygen species generation (Efeoglu et al., 2017). This band has also been seen to be prominent in the study of the subcellular interactions of the chemotherapeutic agent, Doxorubicin, particularly in the cell nucleus and nucleolus (Farhane et al., 20017). The spectroscopic differentiation reveals therefore signatures of cellular stress, which are not evident in the 3D cell culture environment.

CaF$_2$ is a microcrystalline material, which in itself renders it as an ideal substrate for either infrared or Raman spectroscopy, as any intrinsic vibrational modes are low frequency, below the fingerprint regions of biological interest (<500 cm$^{-1}$) (Crystran). It is generally considered biocompatible, and therefore optimal for Raman microscopic profiling of cells (Kerr et al., 2015). However, the cell cycle profiles of Fig. 4.1a and 4.1.b clearly show that the cell populations are significantly perturbed by the crystalline substrate. In contrast, the cell cycle profiles of the conventional 2D polystyrene Petri dish and 3D Collagen substrates are comparable, at least for HaCaT cells. For HeLa cells, in contrast, there is a significant difference between the cell cycle profiles on the conventional 2D and 3D substrates (Gargotti et al., 2017).
4.4. Conclusion

The study demonstrates that the cell culture environment can considerably influence the cell cycle profile, and therefore the diversity of biochemical profiles of individual cells within a mixed cell population, depending on substrate and also cell type. Using Raman spectroscopy, these differences manifest as spectroscopic markers of cellular stress which are particularly prominent in cells on CaF$_2$ substrates, widely used for Raman analysis. The study therefore flags a potentially important consideration for applications of Raman spectroscopy for cellular analysis, particularly of external factors such as anti-cancer agents whose mode of action are understood to be cell cycle specific (Denbigh et al., 2017).

In contrast, the ECM mimicking material, Collagen is seen to be an ideal substrate for Raman spectroscopic measurements it absorbs the cell culture medium, facilitating a prolonged viability of cultured cells, even on glass substrates, and when compared to CaF$_2$, it is minimally disruptive to the cell cycle profile, as measured against cells grown on conventional polystyrene Petri dish substrates.

As a substrate for Raman microscopy, due to its low density, Collagen has minimal contribution to the spectral background. The underlying glass substrate has similarly negligible contribution and, in immersion, the background is that of the medium, dominated by water, facilitating spectral pre-processing. Preparation time is low and the Collagen option is therefore significantly lower cost when compared to standard Raman substrates strengthening its potential.
CHAPTER 5

Chemotherapeutic efficiency of drugs in vitro: Comparison of Doxorubicin exposure in 3D and 2D culture matrices

The following chapter has been adapted from the published journal article entitled “Chemotherapeutic efficiency of drugs in vitro: Comparison of Doxorubicin exposure in 3D and 2D culture matrices”, Toxicology in vitro, 33, (2016), 99-104.

Author list: Alan Casey, Mahmoud Gargotti, Frank Bonnier, Hugh J. Byrne
5.1. Introduction

In recent years, directives from both the EU Directive 2010/63/EU, United States 106th Congress (2000) and the US Public Law 106–545 have driven strategies to minimise the use of in vivo animal models in evaluating toxicity of materials. The strategies have been likened to the notion of a three R (Replace, Reduce and Refine) strategy, put forward by Russell and Burch (1959) in relation to testing methods. As such, significant efforts have been directed towards the development of more “realistic” in vitro testing methods which better represent in vivo conditions. One such method which has received considerable interest is the use of 3D culture systems (Breslin and O'Driscoll, 2013; Brown, 2013; Chen et al., 2015; Elliott and Yuan, 2011; Kimlin et al., 2011). The use of Collagen gels or other commercially developed systems are reported to provide the cells with a microenvironment or matrix which is analogous to the extracellular matrix (ECM) experienced by cells in vivo and is widely considered to be a better representation of the cancerous cell phenotype when compared to those grown in conventional 2D culture (Petersen et al., 1992; Prestwich, 2008; Weaver et al., 1995). The effects on cells grown in such systems compared to 2D systems is widely documented and it has been reported that cells can exhibit different behaviours when cultured in 3D when compared to 2D, such as toxicological response, phenotype, metabolism and composition (Sowa et al., 2010; Wang et al., 1998; Fourre et al., 2008; Harisi et al., 2007). Despite being widely used, research is still on going into the effect of the additional ECM on cellular function and responses. For example, it has been shown that antibodies against B1-integrins exhibit different behaviours when tested in 2D compared to 3D models (Wang et al., 1998); induced Doxorubicin-resistance by the extracellular matrix in human osteosarcoma and HT1080 cells has been demonstrated (Fourre et al., 2008; Harisi et al., 2007); and reduced radiation induced toxicity when
cells are grown in a 3D environment has also been reported (Sowa et al., 2010). However, in an earlier study comparing the viability of cells grown in conventional 2D cultures to that of cells grown on Collagen gel matrices, the apparent increased viability observed using the Alamar Blue cytotoxicity assay was attributed to differences in the diffusion and conversion rates of the test dye due to the alteration of the geometry and morphology of the test system (Bonnier et al., 2015). The study indicates that, rather than affecting a significant change in the cell metabolism, the 3D matrix alters the exposure conditions of the cells to the dye and this should be taken into account when comparing cellular exposures in 2D and 3D matrices. The study is extended here to include exposure of the cell cultures in 2D and 3D environments to the commercially available chemotherapeutic agent Doxorubicin (DOX), chosen as a model compound, also clearly traceable due to its strong colour and fluorescence (Farhane et al., 2015a). Cellular viability after exposure was evaluated with the Alamar Blue (AB) in vitro cytotoxicity assay. The results further support earlier findings that the cell substrate and cell geometry can influence the outcome of the cell viability assay and highlight the need for adaption of existing basic protocols and indeed careful consideration in the interpretation of the results yielded.

5.2. Results

Fig. 5.1 shows the confocal microscopy images of HeLa cell cultures grown on 2D (a) and 3D matrices (c) exposed to 2 μM DOX medium for 24 h, as well as the unexposed controls on the 3D matrices (b). As can be seen in Fig. 5.1, no significant morphological differences were apparent between the HeLa cells grown in conventional 2D (Fig. 5.1a) when compared to that of the Collagen based 3D (Fig. 5.1b), indicating

94
that cellular growth characteristics of the HeLa cells were not significantly altered by growth on the Collagen substrate. Furthermore, it was verified, by monitoring the emission of the DOX by laser excitation, that the intracellular localisation site of the DOX remained unchanged in both the 2D (Fig. 5.1a) and 3D (Fig. 5.1c) systems and that the DOX (red) was localised primarily within the cell nucleus.

Figure 5.1. Confocal image of Hela cells from (a) 2D following 24 h exposure to 2 μM of the Doxorubicin, (b) control Hela cells grown on 3D Collagen Matrix and (c) Hela cells grown on 3D matrix after 24 h exposure to 2 μM DOX. DOX was excited by 488 nm and its emissions recorded at 650 nm (DOX) respectively.

Fig. 5.2 shows the dose dependent cytotoxic response of HeLa cells to DOX exposure, as measured using the AB assay, at time points of 24, 48 and 72 h. The continuous lines show a fit of the cytotoxic responses to Eq. (1):

\[
y = \min + \frac{\max - \min}{1 + \left(\frac{x}{IC_{50}}\right)^{\text{Hillslope}}} \tag{5.1}
\]

Where IC_{50} is the mean inhibitory concentration. In the case of the dose dependent curves in Fig. 5.2 and 5.3 the elicited responses at the highest doses do not reach the
maximum (0% viability). According to the description of “Operational models of pharmacological agonism” by Black and Leff (Black & Leff 1983), the action is one of a partial agonist, and IC\textsubscript{50} represents the concentration at which half the maximum observed affect is elicited. A clear dose dependence is observable, and, as illustrated in Table 5.1, the IC\textsubscript{50} value decreases with increasing exposure time, indicative of a progressive toxic response, as, for example, experimentally demonstrated and numerically simulated using a phenomenological rate equation approach by Maher \textit{et al.} (2014), for the \textit{in vitro} response to Poly (amido amide) (PAMAM) dendrimer exposure. Fig. 5.3 shows the equivalent cytotoxic response for HeLa cells on the Collagen matrices. IC\textsubscript{50} values determined according to the fit of Eq. (5.1) are also tabulated in Table 5.1, for all time points. After 24 h exposure, according to the AB assay, DOX is significantly less toxic to the HeLa cells in the 3D cell culture matrix than in the 2D culture. Notably, however, this difference is not in evidence for longer exposure times. It should also be noted that the dose dependent response at 24 h on the Collagen matrices has become a sharp, step like response. This response was reproducible over 6 independent measurements each using 8 replicate wells per concentration of DOX.
Figure 5.2. Alamar blue cytotoxic response curve following 24, 48 and 72 h exposure of HeLa cells to Doxorubicin on a standard plate. Data is expressed as a percentage of three independent experiments ±SD of the three individual experiments.

Figure 5.3. Alamar Blue cytotoxic response curve following 24, 48 and 72 h exposure of HeLa cells to Doxorubicin on a Collagen coated plate. Data is expressed as a percentage of three independent experiments ± SD of the three individual experiments.
Table 5.1. IC\textsubscript{50} and Hillslope values for the fitted data values following exposure to DOX under standard culture conditions in both 2D and 3D culture substrates following 24, 48 and 72 h exposure.

<table>
<thead>
<tr>
<th>Time Point (Hr)</th>
<th>2D IC\textsubscript{50} (µM)</th>
<th>3D IC\textsubscript{50} (µM)</th>
<th>Hillslope 2D</th>
<th>Hillslope 3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.2 ± 0.3</td>
<td>3.6 ± 1.33</td>
<td>1.2 ± 0.44</td>
<td>11 ± 11</td>
</tr>
<tr>
<td>48</td>
<td>0.3 ± 0.22</td>
<td>0.40 ± 0.08</td>
<td>0.67 ± 0.35</td>
<td>1.0 ± 02.2</td>
</tr>
<tr>
<td>72</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.0202</td>
<td>1.11 ± 0.11</td>
<td>3.1 ± 1.33</td>
</tr>
</tbody>
</table>

The lower toxic response of the HeLa cells to DOX exposure after 24 h has been previously interpreted as an induced resistance of the cells in the 3D culture environment (Breslin & O’Driscoll 2013; Brown 2013; Chen et al., 2015; Elliott & Yuan, 2011; Kimlin et al., 2011). However, it has recently been demonstrated that the different cell growth environments can themselves influence the conversion rates of the cytotoxicity assay (Bonnier et al., 2015), resulting in an apparent increased viability in 3D matrices compared to 2D cultures. In order to establish whether similar factors affect the uptake and interaction of the DOX solution within the cells, a spectroscopic approach was employed.

Fig. 5.4 compares the absorbance spectrum, as recorded in situ in the well plates, of the Collagen gel, the cell culture medium, the cell culture medium on the Collagen, 100 µM DOX in cell culture medium, and 100 µM DOX in cell culture medium on the
Collagen. The spectra are uncorrected for any background due to reflection from the well plate, which gives rise to the flat baseline of absorbance 0.05. The cell culture medium exhibits an absorption maximum at ~570 nm, characteristic of phenol red, and a weaker maximum at ~410 nm, which derives from the FBS (Casey et al., 2008). The DOX in medium is dominated by the combination of the phenol red peak and the absorption of the DOX itself, with maxima at ~500 nm and 480 nm. The Collagen, as expected, is transparent in the visible, only showing an onset of absorption < 400 nm. Notably, the Collagen spectrum also shows a flat baseline until this point, indicating negligible loss of light due to scattering. In contrast, the medium on Collagen shows an increased baseline of absorbance ~0.1 in the transparency region, the distinctive phenol red peak at ~570 nm is absent, and a broad absorption feature appears centred at ~450 nm. The spectrum of DOX in medium on Collagen similar displays a strong scattering background in the transparency region, no evidence of the phenol red feature, and, taking into account the increased scattering baseline, a reduced absorbance of the DOX at 500 nm.
**Figure 5.4.** Absorption spectrum of cell culture medium, Collagen, medium on Collagen, DOX in medium, DOX in medium on Collagen, recorded in situ in the well plate.

**Figure 5.5.** Photographic image of visible colour changes observed on a 96 well plate following exposure of DOX containing medium. Top row (a) is 3D exposure of 100 μM DOX in medium which then underwent a serial dilution with medium. The bottom row (b) is a 2D exposure of a serial dilution of 100 μM DOX in medium which then underwent a serial dilution with medium.
The spectral changes are well visualised in the photographic image of (Fig. 5.5) which shows a 3D exposure of a serial dilution of 100 μM DOX in medium (a). The bottom row (b) shows a 2D exposure of a serial dilution, again of 100 μM DOX in medium which was then serially diluted with fresh medium. The results indicate not only an interaction of the DOX with the Collagen gel, but also the cell culture medium. The increased scattering background of the spectrum of the medium on Collagen indicates the precipitation of medium constituents on the gel, potentially resulting in a depletion of the medium itself. The reduced DOX absorbance also indicates an interaction with the gel, and/or the precipitated constituents, potentially resulting in reduced bioavailability of the drug. Fig. 5.6 shows the dose dependent absorbance at 480 nm of DOX dispersed in the cell culture medium, with that of the supernatant extracted from a Collagen gel substrate, in the absence of cells, after an exposure time of 24 h. The zero concentration absorbance corresponds to that of the cell culture medium, in the case of the DOX supernatant, without the contribution of the scattering from the Collagen gel. Of importance to the effective dose administered to the cells, the rate of increase of DOX absorbance with concentration is considerably lower for the Collagen supernatant (slope 0.0028) than the DOX medium (slope 0.0044), indicating that the effective concentration of DOX in the supernatant, which is bioavailable to the cells, is substantially lower than that of the actual dose. From the linear fits to the dose dependent absorbance, the reduction can be calculated to be a factor of ~1.6.
Figure 5.6. Dose dependent absorbance of Doxorubicin at 480 nm dispersed in cell culture medium (○) and that of Doxorubicin in cell culture medium after 24h exposure on a Collagen substrate (●).

As demonstrated previously, the effective concentration of the AB assay is also effectively reduced by absorption by the porous Collagen gel (Bonnier et al., 2015). As a further potentially confounding factor, although the gel is rinsed thoroughly between DOX exposure and the application of the AB assay, the previous exposure to the DOX may “clog” the matrix and further influence the availability of the AB to the cells. The absorbance (at 570 nm) of the supernatant AB solution on the Collagen gel was monitored, before and after dose dependent exposure to DOX followed by thorough rinsing. Compared to the absorbance of the initial AB solution, which showed an average of 0.356, the absorbance of the AB supernatant of an unexposed Collagen gel was found to be reduced to 0.20 after 3 h, a factor of 1.78. The result is consistent with that previously reported for AB on Collagen gels (Bonnier et al., 2015). In gels which
were exposed to a varying range of DOX, from 6.25 μM to 100 μM, and subsequently thoroughly rinsed, as described in the Section 5.2, the absorbance of the supernatant was found to be the same, and therefore independent of DOX exposure.

5.3. Discussion

The in vitro cytotoxicity assessment of the effects of the chemotherapeutic agent DOX in cells cultures in a 2D compared to a 3D environment indicate a significant difference between the cultures and potentially an increased resistance (Fourre et al., 2008; Harisi et al., 2007) of the cells to the drug in the 3D environment. However, a similar study of the same cell line (Bonnier et al., 2015), cultured in the same two environments, demonstrated that, although the cytotoxicity study gave similar indications of reduced toxic response of the two cultures, no differences in cell population viability were apparent in a flow cytometric screening measurement. The phenomenon was explained by a combination of the absorption and therefore reduced bioavailability of the assay dye by the porous Collagen matrix, and the different effective surface areas of the cell exposed to the dye solution and therefore different uptake rates. The current study extends the previous study of cytotoxicity assays alone (Bonnier et al., 2015) to the case of the sequential exposure to the chemotherapeutic agent subsequently washed and followed by the cytotoxic assay. The change from the 2D to the 3D cell culture environment significantly affects the profile of the dose dependent toxicity response, at all time-points, and notably at the 24 h time-point. The dose dependent response of toxicity assays has been comprehensively described in the treatment by Black and Leff (Black & Leff, 1983) and, more recently, the phenomenological rate equation model of Maher et al. (2014) demonstrated that the
dose and time dependent responses can be reproduced by consideration of a complex cascade of events from the uptake of the external agent, to the registration of the cellular response by the cytotoxicological assay. The response profile is sensitive to the uptake rate, cellular reactions and the assay responses. The results clearly indicate that the Collagen matrix affects the effective concentration of the exposure solution, reducing it by a factor of ~1.6 which in part would contribute to the observed increase in viability previously perceived as a drug resistance (Fourre et al., 2008; Harisi et al., 2007) of cells to Doxorubicin when grown in a 3D environment. Correspondingly the effective concentration of the cytotoxic assay solution is in itself reduced by a factor of 1.78 (Bonnier et al., 2015) coupled with a different effective surface area of the cell exposed to the dye solution and therefore different uptake rate; result in the reduced cytotoxic response recorded. To elicit the same degree of cell death (50%), at 24 h exposure, the actual concentration required is increased from 1.2 ± 0.3 μM to 3.6 ± 1.3 μM, which can be approximately accounted for by a combination of the two factors (1.6/1.78 = 2.85).

The reductions of the effective concentrations of both the DOX and AB may account for the unusual cytotoxicity profile observed for 24 h exposure, in Fig. 5.4 Notably, however, the absorption of the assay by the matrix appears to be independent of the previous DOX exposure protocol, indicating that the respective influences of the gel on the two exposure measuring protocols they are independent of each other and can be assessed as such.

5.4. Conclusions

Although cytotoxic assessment of the effects of chemotherapeutic agents on cells in vitro may indicate a lower toxic response of cells grown on 3D matrices compared to
those cultured on conventional 2D plates, interpretation of the results as a difference in cell metabolism, potentially due to increased drug resistance due to the presence of the environment which better mimics the *in vivo* extra cellular matrix should be made with caution. In the case of cells grown on Collagen gels, the cell culture matrix shows clear interaction with the cell culture medium, itself potentially resulting in medium depletion. The bioavailability or effective concentration of the cytotoxicity assay and the chemotherapeutic agent are both affected by the absorptive nature of the matrix as is clearly evident by the variations in spectral properties and cellular responses. The study re-emphasises the need to adapt the cytotoxicity protocols commonly employed for 2D cell cultures for translation to cells grown on or in 3-D matrices.
Chapter 6

Monitoring cellular uptake and efficacy of Doxorubicin in 2D and 3D *in vitro* cell culture platforms using Raman spectroscopy

Under review 2019 Cytotechnology
6.1. Introduction

Legislative pressures, such as the EU Directive-2010/63/EU on the replacement, reduction and refinement of animal experimentation, have prioritised the development of rapid, cost effective in vitro techniques for toxicological and pharmaceutical screening applications, amongst others (European Union 2010; United States, 2000). However, cells grown in traditional 2D culture have a different physiological environment when compared to those grown in 3D culture (Mirbagheri et al., 2019), and have also been reported to exhibit, for example, different drug sensitivity levels (Imamura et al., 2015; Adcock et al., 2015). In vitro 3D culture conditions better mimic those of in vivo cell growth conditions, and such models have several advantages compared to traditional 2D cultures, such as more cell-extracellular-matrix contact area; better physical properties, and increased cell survival (Mirbagheri et al., 2019). The geometrical structure of 3D culture plays an essential part in understanding the fate of cells when they undergo proliferation, differentiation, or apoptosis (Adcock et al., 2015). Moreover, tumour cells in vivo have a complex microenvironment, consisting of normal cells and tumour stroma, including an extracellular matrix (ECM), basement membrane, inflammatory cells, fibroblasts and blood vessels, all of which contribute to tumour development (Wang et al., 2017; Petrova et al., 2018). Recently, studies have demonstrated the importance of stroma in mammary gland carcinogenesis, as well as how stroma is involved in the malignant progression of epithelial tumours (Werb & Lu 2015). Thus, 3D in vitro models can also provide more significant information on cell interactions that may lead to the development of new therapeutics (Ho & Sun 2012).

Notably, however, it has been demonstrated that the different in vitro culture environments do not significantly affect the viability and health of the cells, and any apparent differences in measured viability levels between 2D and 3D culture have been
demonstrated to be a result of the differing physical characteristics of the 2D and 3D environments, which can impact on the bioavailability and uptake rates of cytotoxicity assays and chemotherapeutic agents, giving potentially misleading results (Casey et al., 2016; Bonnier et al., 2015). The growth environment can, however, influence the cell cycle, as seen for the comparison of human dermal (HaCaT) and cervical (HeLa) cell lines on 2D (Petri dish) and 3D (Collagen) substrates (Gargotti et al., 2017). It is important, therefore, to evaluate the effects, if any, which the cell growth environment has on the efficacy of, for example cell cycle dependent chemotherapeutic agents.

Doxorubicin (DOX) is one of the most effective chemotherapeutic drugs used to treat a multitude of human neoplasms. It is a sequence selective DNA intercalating agent targeting topoisomerase II, causing DNA damage and the formation of reactive oxygen species (ROS) which are primarily responsible for its cytotoxic effects (Chaikomon et al., 2018). DOX as a drug is cell cycle-nonspecific, but a maximal effect during the G0/G1-phase of the cell cycle has been reported (Lüpertz et al., 2010; Denard et al., 2012), resulting in damage repair, cell cycle arrest or apoptosis, depending upon the extent of DNA damage (Davalli et al., 2018; Yang et al., 2016).

Raman spectroscopy can be employed to explore the phenomenon of cell cycle at a biochemical level (Pavillona et al., 2018), and has more recently been employed to probe the biochemical changes due to 3D vs 2D cell culture environments in live cells in vitro (Gargotti et al., 2018). Raman spectroscopy has also been used, not only to detect and monitor drugs inside cells at a subcellular level, in a 2D environment, but also to fingerprint the cellular response to this exposure and the adverse drug effect (Owen et al., 2006; Szafraniec et al. 2016; Farhane et al., 2015; Farhane et al., 2017; Farhane et al., 2018a; Farhane et al., 2018b). In the case of DOX, it has been shown that the drug is in fact taken up rapidly in the nucleolus of the cell, interacting with the
RNA, before saturating, and accumulating in the nucleus, and later in the cytoplasm (Farhane et al., 2017), demonstrating that the label free spectroscopic technique can add much to the current understanding of biological processes and drug interactions.

The aim of this work is to investigate whether differences in the cell cycle, as a result of different cell culture environments, impact on the efficacy and/or mode of action of DOX. To this end, cell cycle apoptosis/necrosis analysis of HaCaT cells grown on 2D and 3D cultures (Collagen) were quantified with flow cytometry using with YO-PRO-1 dye as green fluorescent apoptosis marker and Propidium iodide I dye as red fluorescent dead cells marker in a live-dead assay. The cytotoxicity of DOX was monitored on HaCaT cells grown in 2D and 3D culture to evaluate the viability levels and the half maximal inhibitory concentration (IC$_{50}$) of the drug determined from the Alamar Blue assay. IC$_{50}$ is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. Raman spectroscopy is employed to confirm the subcellular distribution of the drug in the nucleolus, nucleus and cytoplasm of the cells, to fingerprint the DOX mechanism of interaction and the physiological responses in the normal cell lines grown in 3D versus 2D cultures.

6.2. Results

6.2.1 Cytotoxicity assays

For the case of immortalised human cervical (HeLa) cells, it has previously been demonstrated that the different culture environments do not significantly affect the viability and health of the cells, and any apparent differences in viability levels between 2D and 3D culture have been demonstrated to be a result of the differing physical
characteristics of the 2D and 3D environments which cause an increase in conversion rates of quantifying viability dyes (Casey et al. 2016, Bonnier et al. 2015).

In this study, the cytotoxic response of HaCaT cells grown on 3D cell culture (Collagen) and 2D cell culture exposed to DOX was measured using the AB assay, at time points of 24 and 72 h, in order to determine the IC₅₀ value. The IC₅₀ is the concentration of the drug required to kill 50% of the cells in the experiment. As illustrated by Table (6.1), HaCaT cells exhibit no difference in the IC₅₀ values, within experimental error, as measured using the AB cytotoxicity assay, determined to be 3.41 ± 0.5 μM for the 3D and reduced to 3.15 ± 1.39 μM for 2D cell culture respectively after 24 h incubation. For longer exposure times of 72 h, the IC₅₀ values were seen to reduce to 0.89 ± 0.12 μM on 2D culture and 0.96 ± 0.24 μM on Collagen, also similar within experimental error. For subsequent analyses, a dose of 1 μM DOX for 24 h exposure was chosen for cells in both cell culture environments.

Table 6.1. IC₅₀ values for the fitted data values following HaCaT cells exposed to DOX under standard conditions in both 2D and 3D (Collagen) substrate following 24 and 72 h exposure.

<table>
<thead>
<tr>
<th>HaCaT cell</th>
<th>IC₅₀ - 24 h</th>
<th>IC₅₀ - 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX 2D</td>
<td>3.15 ± 1.39</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>DOX collagen</td>
<td>3.41 ± 0.5</td>
<td>0.96 ± 0.24</td>
</tr>
</tbody>
</table>

6.2.2. Cell cycle analysis

Cell cycle analysis was performed on HaCaT cells exposed to 1 μM DOX cultured for 24 h in the 2D and 3D (Collagen) environments, to determine whether any effects or differences in cell cycle behaviour were apparent for cells treated with the drug and cultured on different substrates. For analysis, cells were harvested by enzymatic
removal, fixed and stained as detailed in the Materials and Methods section and DNA content in the cells was monitored by a BD Accuri™ C6 Flow Cytometry after propidium iodide staining. Cell doublets were excluded from the analysis by agitating the samples immediately prior to the analysis and area scaling with the BD Accuri™ software.

Figure 6.1 shows the cell cycle of non-exposed and exposed HaCaT cells grown in 2D and 3D environments. Non-exposed cells grown on collagen did show slight variations when compared to those grown in traditional 2D culture, after 24 h of incubation. HaCaT cells grown in Collagen show a partial arrest in S-phase, with a corresponding reduction of cells in the G2/M phase compared to cells grown on 2D. However, after 24 h exposure to DOX, significant increases in the number of cells in the G0/G1 and S-phase were observed, with a corresponding reduction of cells in the G2/M phase, indicating that the effect of DOX was a strong arrest in G0/G1 and S phase in the cells grown in both 2D and 3D substrates, although cells grown in collagen still show a partial arrest in S phase after DOX exposure.
Figure 6.1. Cell cycle analysis of HaCaT cells grown on Collagen (3D) and cells grown on polystyrene (2D) culture and cells were exposure to 1µM DOX, non-exposed cells and the percentage of cells at G0/G1, S, and G2/M phases of cell cycle. Data are expressed as a percentage of three independent experiments ± SD of three individual experiments. Statistically significant differences between the 3D substrate cell cycle analyses and that of the 2D cultures are denoted by *P < 0.05,**P < 0.01 and *** P < 0.001.

6.2.3. Raman spectroscopy

Figure 6. 2a shows the scatter plots of PCA of the data sets relating to unexposed (positive) and exposed (negative) HaCaT cells grown in Collagen, indicated with open and closed circles, respectively and coded with colours according to the different cellular regions. Cytoplasm, nucleus and nucleolus are indicated with blue, red and black, respectively. The same colour coding system is used for exposed cells, but with the use of closed circles. The spectra corresponding to the cytoplasm, nucleus and nucleolus are clearly differentiated for both exposed and unexposed cells according to PC1, which represents the most significant variance (51%), (67%) and (52%), respectively among the data sets, originating from biochemical differences between
unexposed and exposed HaCaT cells. For all cellular regions, exposed cells are clearly differentiated from unexposed cells, indicating that the spectral differences are larger than the intrinsic variability of each region.

Figure 6.2b shows the loading of PC1 of control cells (positive) versus exposed cells (negative) in the cytoplasm, nucleus and nucleolus of HaCaT cells grown in Collagen (3D culture) after 24 h incubation. In both nucleolus and nucleus of HaCaT cells grown in Collagen, DOX features at 440 and 465 cm\(^{-1}\), respectively related to C-C-O and C-O vibrations, features at 1085, 1215 and 1245 cm\(^{-1}\) related to C-O-H, C-O and C-H, features at 1430 and 1450 cm\(^{-1}\) derived from guanine and cytosine are clearly noted in the spectra of the nuclear area which are absent in control cells and are related to DOX-DNA and RNA intercalation, inducing damage and/or conformational changes (Farhane et al., 2015; Farhane et al., 2018a; Rabbani et al., 2005). Lipids are predominantly represented by peaks at 700-720 cm\(^{-1}\), while other prominent features include those at 1003 cm\(^{-1}\) (phenylalanine (Phe)), 1339 cm\(^{-1}\) (Phe, tyrosine(Tyr), nucleic acid) and 1600-1700 cm\(^{-1}\) (tyrosine and phenylalanine ring vibration, C=C and Amide 1). The band at 1600-1700 cm\(^{-1}\) is evident in all regions and can be attributed to a change in cellular proteins, compared with non-exposed HaCaT cells grown on Collagen. In the cytoplasmic area, a slight increase in the DOX features at 440, 465 and 1085 cm\(^{-1}\) (Fig. 6.2b) is evident.

The Raman profiles clearly show the intracellular distribution of DOX in the nucleoli, nucleus and cytoplasm of the cells, after 1\(\mu\)M exposure for 24 h, as observed by Farhane et al., although the features observed are considerably weaker than those observed for the cells grown on CaF\(_2\) (Farhane et al., 2018a).
Figure 6.2. (a) PCA scatter plot of cytoplasm region, nucleus and nucleolus region of DOX-exposed vs control HaCaT cells grown in Collagen. (b) loading of PC1, corresponding to comparison of cytoplasm region, nucleus and nucleolus region of HaCaT cells grown on collagen substrate after 24 h exposure to 1 μM of the DOX vs non-exposed cells (control), highlighted regions indicating discriminating DOX features.
6.2.4. Apoptosis and Necrosis analysis

Live/dead cell studies, using the YO-PRO-1, Propidium iodide I dye combination, were employed to measure the proportion of apoptotic cells in 3D matrices compared with 2D cell culture before and after exposure to 1 µM DOX after 24 h incubation, in order to establish whether the cell culture environment impacted on the \textit{in vitro} toxicity of the drug. Briefly, after exposure, cells were harvested by enzymatic removal and stained with both YO-PRO-1 and PI. Cell doublets were excluded from the analysis by agitating the samples immediately prior to the analysis and area scaling with the BD Accuri software.

As can be seen in Figure 6.3, non-exposed HaCaT cells cultured on the Collagen substrate displayed differences in the levels of live, apoptotic and necrotic cells when compared to 2D substrates. ~98% of non-exposed HaCaT cells cultured on the Collagen were live, ~88% of non-exposed HaCaT cells cultured on the 2D were live, and ~7% of non-exposed HaCaT cells cultured on the 2D were in the apoptotic and necrotic stage.

DOX exposure was seen to induce a mixture of apoptosis and necrosis in HaCaT cells grown in Collagen (3D) and 2D cell cultures. As shown in Figure 6.3, ~20% of HaCaT cells in each of the different cell culture substrates were live, compared to non-exposed HaCaT cells grown in 2D and 3D culture (Fig. 6.3), ~60% of HaCaT cells grown both in Collagen and 2D culture were in the apoptotic stage, while ~20% were found to be necrotic.

Notably, although some differences were observed in the cell cycle analysis of Figure 6.1, no difference is discernible in the effect of DOX on HaCaT cells grown in different culture environments, and, in fact, the differences in live/dead profile between
the cell culture environments, observed for non-exposed cells, is now dominated by the
effect of DOX.

Figure 6.3. Apoptosis and Necrosis analysis for HaCaT cells grown on Collagen (3D) and cells
grown on polystyrene (2D) culture and cells were exposure to 1.25µM DOX and non-exposed
cells, cells were stained by YO-PRO-1and Propidium iodide I dye. Data is expressed as a
percentage of three independent experiments ± SD of the three individual experiments.
Statistically significant differences between the 3D substrate apoptosis and necrosis analysis
and that of the 2D cultures are denoted by *P < 0.05,**P < 0.01 and *** P < 0.001.

6.3. Discussion

It has been argued that the high attrition rate of anticancer drugs during clinical
development is because their anticancer activity tends to be overestimated on a 2D-
culture-based screening platform (Imamura et al., 2015). In contrast, 3D culture has
been shown to better mimic the in vivo tumour microenvironment than a 2D culture
(Weigelt et al., 2014; Hirschhaeuser et al., 2010). However, it has been demonstrated
that the different culture environments do not significantly affect the viability and health
of the cells, and any apparent differences in viability levels between 2D and 3D culture
have been demonstrated to be a result of the differing physical characteristics of the 2D
and 3D environments which cause an increase in conversion rates of quantifying
viability dyes (Casey et al., 2016, Bonnier et al., 2015). Nevertheless, the cell cycle profile of cells grown in 2D environments has been demonstrated to be significantly different from that of cells grown in a 3D environment (Gargotti et al., 2017). The differences in cell cycle profile are evident in the Raman spectroscopic profiles at a subcellular level (Gargotti et al., 2018). Analysis of the cytotoxicity the chemotherapeutic agent DOX in cells grown in 2D and 3D environments indicated that differences in the toxicity profiles were observed, interpreted as a difference in cell metabolism, potentially due to increased drug resistance due to the presence of the environment which better mimics the in vivo extracellular matrix. However, the reduced efficacy of the drug to cells grown in the 3D environment can be accounted for by a sequential reduction of the effective concentration of the test compound and assay, due to absorption within the Collagen gel, and the increased effective surface area of the cell exposed to the drug and assay in the 3D environment inducing a higher uptake of both drug and assay, thereby influencing the toxic impact of the drug and conversion rate of resazurin, (Casey et al., 2016).

It has been shown that transfer from 2D to 3D culture can influence cell cycle by inducing an interruption at the S-phase resulting in a decreased number of cells due to a lower proliferation rate on the Collagen substrate (Gargotti et al. 2017). HaCaT cells grown on different substrates treated with DOX showed that cells were arrested in G0/G1 phases (Fig. 6.1). The effect of 3D culture (Collagen) on cells has been previously demonstrated; cells were arrested in S-phase of cell cycle, so by the nature of the experiment the cells here in first 24 h before exposure in 3D (Collagen) the cell cultures may have had a higher population in the S phase than that of the 2D culture regardless they have similar behaviour after 24 h exposed to DOX. DOX is known to induce apoptosis in normal cell types and tumour cells via different mechanisms (Wang
et al., 2004) but both apoptosis and cell cycle arrest after DNA damage is involved (Levine 1997) and the results presented in this study suggest that this hold true for both 2D and 3D culture. The results clearly show that HaCaT cells grown in collagen and 2D culture had the similar pattern in apoptosis, necrosis and live cells (Fig. 6.3) and the presence of the substrate did not alter the efficacy of the DOX.

Raman spectroscopy can track the kinetics of the uptake and accumulation of the chemotherapeutic drug such as DOX at a subcellular level in vitro (Farhane et al., 2018a; Farhane et al., 2017), and also can differentiate the biochemical responses associated with the subcellular regions of nucleolus, nucleus and cytoplasm, both in terms of the mechanisms of action, and the subsequent cellular metabolic responses. Raman spectroscopic analysis of drug-cell interactions has focused both on changes in the cellular Raman spectra upon drug application, and also during intracellular tracking of the drug and its metabolites (Farhane et al., 2015; El-Mashtoly et al., 2014; Farhane et al., 2018a; Farhane et al., 2017; Dana et al., 2015). In 2D cell cultures on CaF₂ substrates, Farhane et al. have previously shown that the uptake of DOX occurs in the nucleolus first, the nucleus and finally cytoplasm. However, Gargotti et al., have shown that cells grown on CaF₂ are highly stressed, bringing into question the validity of the results of Farhane et al.. In this study, after 24 h exposure, DOX is clearly seen to be present in the nucleolus, nucleus and cytoplasm of the HaCaT cells. The DOX features are most prominent in the nucleolus, then the nucleus and the cytoplasm is consistent with the kinetics of uptake of the drug observed by Farhane et al., although the diminished relative contribution is consistent with the dilutionary effect of the matrix, which absorbs some of the exposure doses, as reported by Casey et al.,. In HaCaT cells grown in Collagen (Fig. 6.2), obvious decreases of DNA and RNA features for example at 728, 785-795, 813, 1270 and 1300 cm⁻¹ were observed in the spectra of the nucleus.
and nucleolus of treated cells compared to non-treated cells, these changes are due to DOX-DNA interactions inducing DNA synthesis interruption (Gewirtz 1999) and changes in DNA conformation (decrease of DNA B form) (Nawaz et al., 2013) inducing early cell apoptosis (Lin et al., 2012; Brauchle et al., 2014). The results of the Raman spectroscopic investigation therefore clearly demonstrate signatures of the interaction of the DOX with the HaCaT cells grown in 3D culture.

6.4. Conclusion

In conclusion, this work presents a comparison between 2D and 3D culture by using normal cells treated with DOX. This study demonstrates that, despite the differences in the cell cycle of cells grown in 2D and 3D cultures, the efficacies and ultimate effect of the drug on all the cultured cells are the same regardless of the culture environment. Using a 3D culture system such as Collagen in Raman spectroscopy studies, we can clearly observe the drug (DOX) in the cells cultured the 3D environment and despite the associated cytotoxicity of DOX being less, its sites of localisation remains un-altered (it is still in the nucleolus), and the ultimate effect the drug remains the same. Moreover, despite the effect of 3D culture on cell cycle, the impact of the drug (DOX) was clear on the cell cycle. This study clearly demonstrates the potential benefits of using 3D culture (Collagen) as a non-expensive substrate that can offer advantages over standard 2D substrates used in Raman spectroscopy.
Chapter 7
Conclusions and final remarks
The majority of anticancer drugs that enter clinical trials exhibit little or no therapeutic benefit and fail to obtain regulatory approval (DiMasi et al., 2010). This high failure rate consumes billions of dollars annually and contributes to the high cost of those few drugs that are eventually approved. It has been argued that the high attrition rate of anticancer drugs during clinical development is because their anticancer activity tends to be overestimated on a 2D-culture-based screening platform (Imamura et al., 2015), and the most of the published data regarding known cell-based processes are derived from experiments performed in two-dimensional (2D) conditions where cells are grown on rigid materials such as polystyrene and glass. These traditional cell monolayer cultures, grown under simplified and conditions that do not adequately reflect the essential physiology of real tissues. Despite their simplicity, 2D cultures remain very attractive for laboratory purposes because of their simplicity and low cost. This study would suggest that there is a direct correlation between the cell cycle and the type of cell culture substrate. If basic functions like cell cycle can be influenced by experimental protocols this may in turn reduce or indeed improve the efficacy of tested drugs, depending on their mode of action.

The use of 3D culture matrices have widely been interpreted to result in a “reduced” toxicity or cellular “resistance” to many chemotherapeutic agents. But as shown throughout this study, there is no difference between 2D and 3D viability levels and the observed “reduced” toxicity or “resistance” to the tested drug was simply an effect of concentration reduction within the 3D culture by dispersion into the 3D membrane. For this reason, future studies involving testing of drug responses in vitro which incorporate 3D culture, may indeed provide a more comparable culture environment to that of in vivo exposures, but this reduction in effective concentration should be accounted for. The variations in the cell cycle for cells grown on different
substrates must be accounted for in vitro cellular screening in particular when screening cell cycle dependant toxicants. These have great potential to make a significant impact in the Raman spectroscopy field in particular in the study of live cells, Collagen a cost effective 3D system facilitates cell growth, offers an improved micro-environment in vitro with nutrient retention to prolong the life of the cell allowing spectroscopic measurements to be taken and is Raman feature clear substrate which may one day allow the full potential of Raman spectroscopy in the clinical area to be achieved.

The main aim of this study was to establish the use of novel 3D growth substrates in vitro assessment protocols, viability, cytotoxicity and Raman spectroscopy. This study was designed to investigate the potential of 3D culture compared to 2D culture using both normal and cancer cell lines to clarify how the transition from 2D to 3D culture effects the cells in vitro by determining the viability of cells, morphological changes, cytotoxicity responses and how these changes manifested themselves in Raman spectroscopy.

7.1. Thesis Summery

In chapter III, 2D and 3D culture systems were directly compared, for this two commercial products of 3D culture namely Rat Tail derived Collagen I and Geltrex were used in different concentrations and volumes to form 3D culture scaffolds. The cell viability and function were monitored and compared to conventional 2D cultures, to determine which basement supports growth with least impact on cellular function. To further monitor the effect of these membranes and their potential for more relevant in vitro screening, a normal and cancer cell lines were chosen for growth on both basement membranes, and for consistency with previous studies (Bonnier et al., 2015; Casey et
Viability was monitored with the aid of the Alamar Blue assay, cellular morphology was monitored with confocal microscopy, and cell cycle and cell death mechanism studies were performed with flow cytometry.

The viability studies showed apparent differences between the 2D and 3D culture systems, the differences attributed in part to the physical transition from 2D to 3D environment causing alterations to effective resazurin concentration, uptake and conversion rates, which was dependent on exposure time, but also due to the effect of the membrane itself on cellular function as shown in cells grown in Geltrex, viability were reduced and no cell cycle interruption in cells grown in compared with Collagen. These effects were verified by flow cytometry, in which no significant differences in viable cell numbers between 2D and 3D systems were observed after 24 h culture. The results showed the observed effect which was different after shorter exposure periods, was also dependent on working volumes and concentration of the 3D system and could be mediated by altering the culture vessel size. Cell cycle analysis revealed cellular function could be altered by growth on the 3D substrates and the alterations were noted to be dependent on 3D membrane concentration. It was subsequently shown that transfer from 2D to 3D culture can influence cell cycle by inducing an interruption at the S-phase of the cell cycle interruptions which may result in decreased cellular numbers due to a lower proliferation rate of cells on the Collagen membrane and should be accounted for in experimental planning. The results of this study strongly support the use of 3D culture in cytotoxicity assays to improve the relevance of drug or toxin screening protocols, as there is no loss in cellular viability. They may indeed provide a more comparable culture environment to that of in vivo exposures, but appropriate controls and experimental validations must be incorporated into the protocols at every assessed time point. If the cell culture environment employed arrests the cell at a
particular checkpoint, as is observed in this study, the efficacy of a drug could potentially be enhanced or delayed. Indeed, in previous study (Casey et al., 2016) variations in Doxorubicin toxicity at short term cellular exposures were observed resulting from a transition from 2D to 3D Collagen membrane. Such responses may have been due to the alteration of cell cycle, altering the mechanism of action of the Doxorubicin. Therefore, in choosing a membrane for screening drug toxicity, consideration must be given to the membrane effect on cellular systems. If basic functions like cell cycle can be influenced by experimental protocols this may in turn reduce or indeed improve the efficacy of tested drugs, depending on their mode of action. A viable option, as there is no loss in cellular viability, and may indeed provide a more comparable culture environment to that of in vivo exposures by appropriate controls and experimental validations must be incorporated into the protocols at every assessed time point.

In chapter IV, monitored how the substrate-induced changes in the cell cycle are manifested in the spectroscopic profiles of the cell cultures. To do this, and for consistency with previous studies (Casey et al., 2016; Garotti et al., 2017), the human cervical cancer cell line (HeLa) and human keratinocyte cell line (HaCaT), were again chosen for growth on both a commercially available 3D basement membranes (Collagen) and 2D systems (polystyrene, CaF₂). The cell cycle for each cell line on each substrate was profiled using flow cytometric analysis, and the spectral signatures of the cytoplasm, nucleus and nucleolus of cells grown on standard glass slides coated with Collagen were compared to those of cells on the CaF₂ substrate, with the aid of principal component analysis (PCA). HeLa immortalised human cervical cells and HaCaT dermal cells were cultured on three different substrates, conventional polystyrene cell culture dishes, CaF₂ slides as a commonly used Raman substrate, and
glass slides coated with Collagen rat tail, as a mimic of the extra-cellular matrix (ECM) environment. A cell cycle study, based on percentage DNA content, as determined using propidium iodide staining and monitored by flow cytometry, was performed on cells of both types, grown on the different substrates, confirming that the in vitro cell culture environment impacts significantly on the cell cycle. Live cell in vitro Raman spectroscopic analysis of cells on the 2D CaF$_2$ and 3D Collagen substrates was performed and data was analysed using principal component analysis (PCA).

The spectroscopic analysis revealed differences in profiles which reflect the differences in cell cycle for both in vitro culture environments. In particular, the Raman spectra of cells grown on CaF$_2$ show indicators of cell stress, which are also associated with cell cycle arrest at the G0/G1 phase. The study therefore flags a potentially important consideration for applications of Raman spectroscopy for cellular analysis, particularly of external factors such as anti-cancer agents whose mode of action is understood to be cell cycle specific (Denbigh et al., 2017). In contrast, the ECM mimicking material, Collagen is seen to be an ideal substrate for Raman spectroscopic measurements; it absorbs the cell culture medium, facilitating a prolonged viability of cultured cells, even on glass substrates, and when compared to CaF$_2$, it is minimally disruptive to the cell cycle profile, as measured against cells grown on conventional polystyrene Petri dish substrates. As a substrate for Raman spectroscopy, due to its low density, Collagen has minimal contribution to the spectral background. The underlying glass substrate has similarly negligible contribution and, in immersion, the background is that of the medium, dominated by water, facilitating spectral pre-processing. Preparation time is low and the Collagen option is therefore significantly lower cost when compared to standard Raman substrates strengthening its potential. This work contributes to the field of Raman spectroscopic analysis by providing a fresh look at the
significance of the effect of in vitro culture environment to cell cycle and the sensitivity of Raman spectroscopy to such differences in cell metabolism.

In chapter V, the work was extended comparing the viability of cells grown in conventional 2D cultures to that of cells grown on Collagen gel matrices but this time with the addition of a known chemotherapeutic drug. When cells are grown in 3D systems the apparent increased viability observed using the Alamar Blue cytotoxicity assay was attributed to differences in the diffusion and conversion rates of the test dye due to the alteration of the geometry and morphology of the test system (Bonnier et al., 2015). The study indicates that, rather than affecting a significant change in the cell metabolism, the 3D matrix alters the exposure conditions of the cells to the dye and this should be taken into account when comparing cellular exposures in 2D and 3D matrices. The study is extended here to include exposure of the cell cultures in 2D and 3D environments to the commercially available chemotherapeutic agent Doxorubicin (DOX), chosen as a model compound, which was clearly traceable due to its intense colour and fluorescence (Farhane et al., 2015). Cells grown in a 3D in vitro matrix environment have been reported to exhibit significantly different properties to those in a conventional 2D culture environment. However, comparison of 2D and 3D cell culture models have recently been noted to result in differing responses of cytotoxic assays, without any associated change in viability. The effect was attributed to differing conversion rates and effective concentrations of the resazurin assay in 2D and 3D environments, rather than differences in cellular metabolism. In chapter V, the efficacy of a chemotherapeutic agent, (DOX), was monitored and compared in conventional 2D and 3D Collagen gel exposures of immortalized human cervical cells (HeLa). Viability was monitored with the aid of the Alamar Blue assay and drug internalisation was
verified using confocal microscopy. Drug uptake and retention within the Collagen matrix was monitored by absorption spectroscopy.

The viability studies showed apparent differences between the 2D and 3D culture systems, the differences attributed in part to the physical transition from 2D to a 3D environment causing alterations to dye resazurin uptake and conversion rates. The use of 3D culture matrices has widely been interpreted to result in “reduced” toxicity or cellular “resistance” to the chemotherapeutic agent. The results of this study show that the reduced efficiency of the drug to cells grown in the 3D environment can be accounted for by a sequential reduction of the effective concentration of the test compound and assay. This is due to absorption within the Collagen gel inducing a higher uptake of both drug and assay thereby influencing the toxic impact of the drug and conversion rate of resazurin. The increased effective surface area of the cell exposed to the drug and assay in the 3D environment. The chapter re-emphasises the need to adapt the cytotoxicity protocols commonly employed for 2D cell cultures for translation to cells grown on or in 3-D matrices.

In chapter VI the data presented shows that despite the differences in cell cycle on the different substrates, the efficacies of DOX are the same in cells grown in different substrate as shown in the apoptosis/necrosis analysis. In this study, a 3D culture system (Collagen), is employed as a model in vitro using a human keratinocyte cell line (HaCaT), in order to demonstrate the efficacy of a chemotherapeutic agent, Doxorubicin on the 3D system. Cell cycle arrest, apoptosis and DNA content were quantified with flow cytometry using YO-PRO-1 and propidium iodide dye in a live-dead assay,
viability was monitored with the aid of the Alamar Blue assay and Raman microspectroscopy profiling was employed to confirm the drug internalisation, localisation.

The results of this study show that, in order to establish whether the cell culture environment impacted on the *in vitro* toxicity of the drug. When cells are grown in Collagen substrates the Doxorubicin still induced apoptosis with no difference between 3D and 2D culture in the levels of apoptotic, necrotic and live cells. Cell cycle analysis was performed on HaCaT cells exposed to DOX cultured in the 3D (Collagen) and 2D substrates, to determine whether any affects or differences in cell cycle behaviour were apparent for cells treated with the drug and cultured on different substrates. Also cells grown on both substrates were arrested at G0/G1 phase by the Doxorubicin and cells grown in Collagen still show partial arrest in S phase as a result of cells grown in Collagen 24h before exposed to DOX. Raman spectra collected from cells grown in Collagen showed that, Doxorubicin is clearly seen to be present in the nucleolus, nucleus and cytoplasm of the HaCaT cells. Statistical analysis, consisting of principal components analysis (PCA) was used to highlight the Doxorubicin interaction with HaCaT cells grown in 3D cultures. However, despite the differences in the cell cycle in cells grown in 2D and 3D cultures, the efficacies and ultimate effect of the drug on all the cultured cells are the same regardless of culture environment. Using a 3D culture system such as Collagen in bio-spectroscopy studies, we can clearly observe the drug (DOX) in the cells cultured the 3D environment and despite the associated cytotoxicity of DOX being less, its sites of localisation remains un-altered (it is still in the nucleolus), and the ultimate effect the drug remains the same. Moreover, despite the effect of 3D culture on cell cycle, the impact of the drug (DOX) was clear on the cell cycle.
In summary despite the differences in the cell cycle between cells grown in 2D and 3D cultures, the efficacies and ultimate effect of the drug on all the cultured cells are the same regardless of culture environment and the 3D substrate (Collagen) was noted to be the best choice for bio-spectroscopy analysis.

7.2. Implications of the work on “Raman spectroscopy”

Raman spectroscopy is a non-invasive, label-free technique which excites vibrations of molecular bonds. Raman micro spectroscopy has been used not only to detect and monitor drugs inside cells at a subcellular level but also to fingerprint the cellular response to this exposure and the adverse drug effect (Owen et al. 2006). In Raman spectroscopy, one of the most widely used substrates is CaF₂ windows, primarily as the material permits the transmission of visible, near and mid IR light with minimal losses coupled with the fact that UV grade CaF₂ does not have any characteristic Raman bands in the cellular spectral fingerprint region (400–1800 cm⁻¹) which allows the analysis of biological samples (Gee et al., 1966) without substrate interference. Glass and polystyrene have background signals interfere with sample signals, but these substrates used as coted substrate such as in 3D substrate. However, using a 3D substrate such as Collagen is an ideal substrate for Raman spectroscopic measurement, Collagen due to it is low density, has minimal contribution to the spectral background, the preparation time is low and the Collagen as a substrate for bio-spectroscopy option is significantly lower cost when compared to standard CaF₂ Raman grade optical substrates. The data collected from Raman spectra of cells grown on CaF₂ have been noted to show indicators of cell stress, which are also associated with cell cycle arrest at the G0/G1 phase (Gargotti et al., 2018). CaF₂ as a substrate, is expensive.
and significantly increases sample analysis costs limiting clinical applications (Baker et al., 2018), whereas the relative low cost of when compared to CaF$_2$ makes Collagen the best option in Raman spectroscopy studies.
References


Brown, R A (2013). In the beginning there were soft collagen-cell gels: towards better 3D connective tissue models?, *Cell Res.* 319 (16), 2460–2469.


Cordero, E; Latka, I; Matthäus, C; Schie, I W & Popp, J (2018). In-vivo Raman spectroscopy: from basics to applications. *J. of Biomedical Optics*, https://doi.org/10.1117/1.JBO.23.7.071210


Gala, U & Chauhan, H (2015). Principles and Applications of Raman Spectroscopy in


Supplementary Material

Raman spectra recorded on different substrates

A good substrate for biological applications of Raman spectroscopy should have characteristics such as an adherent surface to support the culture of cells, and the background signal should be low to avoid masking the Raman signals from the sample. The polystyrene of conventional Petri dishes itself has a strong Raman signatures, many of the bands of which overlap with features of interest for analysis of cellular spectra, and thus Raman compatible substrates such as CaF$_2$ are favoured (Kerr et al. 2015). Fig. 4.S1 shows the mean Raman spectra obtained from 3D culture (Collagen on glass) and CaF$_2$ substrates. The data recorded from different substrates show that the 3D culture and CaF$_2$ yield similar spectra. No specific features from the Collagen or the underlying glass can be seen and the ~1640 cm$^{-1}$ band in the CaF$_2$ and the Collagen spectrum is that of the water (Bonnier et al. 2011).

![Graph showing Raman spectra of Collagen and CaF$_2$ substrates](image)

**Figure S 1.** Comparison of Raman spectra of two substrates. Mean spectrum of Collagen and CaF$_2$ are indicated with red and black, respectively. The dashed circle highlights the characteristic contribution of water at ~1640 cm$^{-1}$.
Figure S 2. (a) Absorption of Dox drug on Collagen rat tail (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation. (b) Absorption of MTX drug on Collagen rat tail (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation.
Figure S 3. (a) Absorption of Dox drug on Geltrex (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation. (b) Absorption of MTX drug on Geltrex (3D culture) after 1, 3, 6, 24, 48 and 72 h incubation.
Figure S 4. Confocal images for (a) HeLa cell grown on 2D culture after 24 h exposure to 2 μM of the Doxorubicin, (b) HeLa cell grown on Geltrex after 1 h exposure to 2 μM of the Doxorubicin. (c) HeLa cell grown on Collagen after 1 h exposure to 2 μM of the Doxorubicin. (d) HaCaT cell grown on 2D culture after 1 h exposure to 2 μM of the Doxorubicin. (e) HaCaT cell grown on Geltrex after 1 h exposure to 2 μM of the Doxorubicin. (f) HaCaT cell grown on Collagen after 1 h exposure to 2 μM of the Doxorubicin. DOX was excited by 488 nm and its emissions recorded at 650 nm (scale bar 20 μm).
Figure S 5. Confocal images for (a) HeLa cell grown on 2D culture after 24 h exposure to 5 μM of the Mitoxantrone, (b) HeLa cell grown on Geltrex after 1 h exposure to 5 μM of the Mitoxantrone. (c) HeLa cell grown on Collagen after 1 h exposure to 5 μM of the Mitoxantrone. (d) HaCaT cell grown on 2D culture after 1 h exposure to 5 μM of the Mitoxantrone. (e) HaCaT cell grown on Geltrex after 1 h exposure to 5 μM of the Mitoxantrone. (f) HaCaT cell grown on Collagen after 1 h exposure to 5 μM of the Mitoxantrone. MTX was excited by 607 nm and its emissions recorded at 690 nm (scale bar 10-20 μm).
Figure S 6. Confocal images for (a) HeLa cell grown on Geltrex, mitochondria stained by MitoTracker Red 250 nM, (b) HaCaT cell grown on Geltrex mitochondria stained by MitoTracker Red 250 nM. MitoTracker was excited by 579 nm and emission 599 nm respectively.
Figure S 7. Confocal images for (a) HeLa cell grown on Geltrex, lysosomes were stained by LysoTracker Red 75 nM and LysoTracker red was excited by 577 nm and emission 590 nm respectively, (b) HaCaT cell grown on Geltrex, lysosomes were stained by LysoTracker Green 75 nM and LysoTracker red was excited by ~504 nm and emission 511 nm respectively. NucRed 2drops/1 mL and NucRED was excited by 638nm and emission 686 nm respectively.
Appendix I: Publications


“Comparative studies of cellular viability levels on 2D and 3D in vitro culture matrices” (published) Cytotechnology, doi: 10.1007/s10616-017-0139-7.


“Monitoring cellular uptake and efficacy of Doxorubicin in 2D and 3D in vitro cell culture platforms using Raman spectroscopy” (paper submitted to Analyst journal).
Appendix II: Conferences, Courses and Summer Schools

26\text{th} October 2017  
Flow Cytometry Seminar and workshop day

12\text{th} – 15\text{th} July 2016  
CLIRSPEC summer school.

8\text{th} - 10\text{th} June 2016  
Microscopy Society of Ireland 40\text{th} Annual meeting.

29\text{th} Feb - 2\text{nd} March 2016  
Raman based application for clinical diagnostics (Raman 4 Clinics).