Development of 3D in vitro tissue models for the analysis of solar radiation damage of skin

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Development of 3D in vitro tissue models for the analysis of solar radiation damage of skin

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Abstract

The aim of this work was to investigate changes to the molecular composition and conformation of HaCaT cells as a result of simulated solar radiation in a 3D in vitro skin model by Raman spectroscopy. The process to achieve this goal was performed in three main stages: (1) optimisation of the working concentration and volume of two 3D membranes, used as a structural support in the skin model; (2) the construction of the 3D in vitro skin model and; (3) the investigation of the dose-dependent effects of solar radiation on HaCaT cells in the skin model in comparison with the conventional 2D models. The novelty in the 3D culture models is that they have an increased physiological representation of in vivo-like conditions, compared to 2D cell culture models. The introduction of the extracellular matrix enables cells to achieve their natural morphology and polarity, and it improves the mechanical/biochemical signals and cell-microenvironment communication. Moreover, 3D cell cultures offer alternative to animal models, following the regulations against human and animal testing (EU Directive-2010/63/EU and US Public Law 106-545, 2010, 106th Congress). In the first stage, the results revealed that the cell geometry in 3D cultures modifies the uptake and conversion rates of the cytotoxicity assay dye in comparison with 2D models, resulting in an apparent increment in cell viability levels. However, flow cytometry showed no differences in live cells and apoptosis levels between 2D and 3D cultures, although a cell cycle arrest at the S-phase in a cancer cell line cultured in collagen I was observed. The results of this study promotes the use of collagen I and Geltrex in the construction of a 3D in vitro skin model, since the cellular health and viability levels are not affected by these extracellular matrices. The second stage in this
thesis illustrates the methodology to build the skin model. Firstly, human dermal fibroblasts were embedded in collagen I to form the dermis layer. Secondly, after 1 day of incubation, HaCaT cells cultured in a Geltrex layer were seeded on top of the dermis layer to form the epidermis in the in 3D vitro model. The ensemble of these two layers resulted in a simplistic 3D in vitro skin model. In the optimisation of the model, the use of human serum to supplement the media for the cell culture was seen to affect the viability levels of cells in both 2D and 3D models. Thus, the traditional foetal bovine serum was employed in the cell culture. In the final stage, the influence of using 3D matrices in HaCaT cells exposed to simulated solar radiation in comparison with 2D models is reported. The detrimental effects of solar radiation on cell integrity were studied using different techniques. The induced morphological changes were observed through histochemical staining in 2D models as well as the characterisation of the 3D skin model. The viability levels in both culture systems (2D and 3D) were monitored using the colorimetric assay Alamar blue. The viability results suggested that solar radiation had no effects on cell health immediately after irradiation. However, this was associated with the performance of the Alamar blue dye in the 3D membrane. The investigation of the photobiological events occurring at the molecular level in the cell due to the impact of simulated solar radiation was performed by Raman microspectroscopy. The focus was on the cell nuclei, as DNA is the main target of solar radiation. As an immediate effect of simulated solar radiation and cell interaction, Raman spectroscopy suggests induction of single strand breaks, formation of bipyrimidine photoproducts and oxidative damage of bases, whereas as a later-term response, protein damage is observed. Hence, the spectral analysis showed that not only cell cycle is affected when cells are transferred from 2D models to a more complex system as 3D models. Cell responses to external stimuli, drugs or compounds are altered in this transition.
Declaration

I certify that this thesis which I now submit for examination, 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 graduate 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.

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Signed ___________________________  Date  28/03/2020 _________________
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Abbreviations

2D  Two-dimensional
3D  Three-dimensional
UVR Ultraviolet radiation
NIR Near Infrared radiation
CLSM Confocal laser scanning microscopy
AB Alamar blue
ECM Extracellular matrix
EMSC Extended multiplicative signal correction
BM Basement membrane
PBS Phosphate buffered saline
PLSR Partial least squares regression
PCA Principal Component Analysis
HaCaT Human dermal keratinocytes cells
Hela Henrietta Lacks cells
HDF Human dermal fibroblast cells
HS Human serum
FBS Fetal bovine serum
RS Raman spectroscopy
MCTS Multicellular tumour spheroids
IHC Immunohistochemistry
PI Propidium Iodide
DMEM F-12  Dulbecco’s modified Eagle medium: Nutrient mixture F-12

FFPE  Formalin-fixed paraffin-embedding

HEM  Human epidermal melanocytes
# Contents

Abstract .............................................................................................................................. ii

Declaration ......................................................................................................................... iv

Acknowledgments ............................................................................................................... vi

For the giants of my life ..................................................................................................... vi

Abbreviations ..................................................................................................................... vii

Table of Figures .................................................................................................................. xiii

Chapter I ................................................................................................................................. 1

1.1 Introduction .................................................................................................................. 1
1.2 Background .................................................................................................................... 2
1.3 Aims and Objectives .................................................................................................... 8
1.4 Thesis Summary .......................................................................................................... 9
1.5 References .................................................................................................................... 11

Chapter II ............................................................................................................................... 17

2.1 Introduction .................................................................................................................. 17
2.2 Cell culture models ..................................................................................................... 17
   2.2.1 2D cell culture .................................................................................................... 17
   2.2.2 Animal models .................................................................................................. 19
   2.2.3 3D cell culture .................................................................................................. 20
   2.2.4 Commercial skin models .................................................................................. 23
2.3 Summary ....................................................................................................................... 24
2.4 References .................................................................................................................... 26

Chapter III ............................................................................................................................. 30

3.1 Introduction .................................................................................................................. 30
3.2 Extracellular matrix (3D membranes) ....................................................................... 30
   3.2.1 Geltrex ............................................................................................................. 30
   3.2.2 Collagen I, rat-tail .......................................................................................... 31
3.3 Primary cells and Cell lines ......................................................................................... 32
7.1 Abstract ........................................................................................................ 137
7.2 Introduction .................................................................................................... 138
7.3 Experimental section .................................................................................... 140
  7.3.1 Materials .................................................................................................. 140
  7.3.2 HDF and HaCaT cell lines ...................................................................... 140
  7.3.3 Co-culture model preparation ................................................................. 141
  7.3.4 Dosimetry ................................................................................................ 142
  7.3.5 Solar Exposure .......................................................................................... 143
  7.3.6 Light microscopy imaging ...................................................................... 144
  7.3.7 Cell viability measurement with Alamar Blue ........................................ 144
  7.3.8 Raman Spectroscopy ............................................................................. 145
  7.3.9 Data analysis ............................................................................................ 146
7.4 Results and discussion .................................................................................. 149
  7.4.1 Light microscopy imaging ...................................................................... 149
  7.4.2 Cell viability measurement with Alamar Blue ........................................ 150
  7.4.3 Raman analysis ....................................................................................... 151
7.5 Conclusion ..................................................................................................... 165
7.6 References ..................................................................................................... 166
7.7 Supplementary information ......................................................................... 171
Chapter VIII ........................................................................................................ 177

Conclusions .......................................................................................................... 177

8.1 Introduction ..................................................................................................... 177
8.2 The 3D Model ............................................................................................... 178
8.3 The 3D model vs 2D cultures; effects of cell microenvironment .................... 179
8.4 Effects of Simulated Solar Radiation ............................................................ 180
8.5 Raman Microspectroscopic analysis ............................................................ 180
8.6 Future Perspectives ....................................................................................... 181
Appendix 1: Publications .................................................................................... 187

Appendix 2: Conferences and Modules ............................................................. 188
# Table of Figures

**Figure 2.1** Schematic representation of cells cultured in 2D culture. In this format, half of the cell is attached to a plastic surface, while the other half is exposed to media. In this culture model, cells have limited cell-cell communication, they adopt an unnatural morphology (flattened) and therefore it does not mimic in-vivo conditions. .................. 18

**Figure 2.2** Examples of three different 3D cell culture methods. Cells in purple illustrate the possible organization within the different matrix. (Adapted from: https://www.elveflow.com/organs-on-chip/3d-cell-culture-methods-and-applications-a-short-review/) .......................................................... 23

**Figure 3.1** Images acquired with the Confocal Laser Scanning Microscope. In the figure can be observed a) HaCaT, keratinocyte cells, b) Hela cells and c) Human dermal fibroblast cells. The red colour in the images highlight the nucleus of cells. ................................. 32

**Figure 3.2** Schematic representation of the Resazurin reduction to resofurin by viable cells in the AB assay.......................................................... 36

**Figure 3.3** Schematic optic representation of the CLSM.................................................. 38

**Figure 3.4** Non-polar molecule in the absence of an external E (left) and in the presence of a uniform electric field E (right). .......................................................... 40

**Figure 4.1** 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). .......... 57

Figure 4. 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. ................................................................. 59

Figure 4. 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. ............................................................................................................ 60

Figure 4. 4 YOPRO 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. ............................................................................................................. 61

Figure 4. 5 YOPRO 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 ............... 62

Figure 5.1 The components of the skin. This illustration depicts the skin as an elaborated layered structure composed of epidermis and dermis and some structures like hair follicle and nerves. (Adapted from https://www.dermatologysydney.com.au/anatomy-of-the-skin/) .......................................................... 76

Figure 5.2 The epidermis. This illustration depicts the 5 layers of the epidermis and the main type of cells (keratinocytes, melanocytes, Langerhans cells, stem cells and Merkel cells) living within it. (Adapted from: https://slideplayer.com/slide/10688229/) ............... 77

Figure 5.3 Clonogenic assay for HDF primary cells cultured in media with different concentration of HS and FBS .................................................................................. 85

Figure 5.4 Alamar blue response following both a 24 and b 48 h growth on both 2D and 3D culture of HDF cells in HS and FBS supplementation.................................................. 87

Figure 5.5 Schematic representation of the ensemble of the two layers of the skin: epidermis and dermis. The base of the culture was made by HDF embedded in Collagen I matrix. Subsequently, the epidermis layer is generated by culturing keratinocytes on the top of a basement membrane (Geltrex) and laid on the top of dermis. ....................... 88

Figure 5.6 In the upper part of the figure, images of the 3D in vitro tissue model of skin acquired with a microscope at 10X are shown. The visible islands are formed by keratinocytes on the epidermis. The red squares indicate the localization of visible
fibroblast embedded in the collagen matrix. On the bottom is an overview image of the 3D in vitro model of skin in a microwell dish. ................................. 89

**Figure 6. 1** Microscopic examination of the H&E stained control and exposed samples (30, 60, 90, 120 and 180 min) to simulated solar radiation for both times of analysis A) immediately after irradiation and B) 24 h post exposure. ......................................................... 106

**Figure 6. 2** Alamar blue response of HaCaT cells (1×10^5 per well) to simulated solar irradiation for varying exposure times, measured immediately and 24 h post exposure... 108

**Figure 6. 3** Average Raman spectrum of the nuclei of HaCaT cells cultured on a CaF2 disc. The highlighted bands correspond to molecular vibrations originating from nucleic acids within the nucleus of the cells. The red shaded area defines the standard deviation on the mean. ................................................................. 109

**Figure 6. 4** PCA scatter plot (a) and loading of PC2 (b), corresponding to comparison of control versus 180 minutes of exposure. The Raman spectral acquisition was done immediately after irradiation. The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2. No significant differences were found for control vs 30 (p=0.051); 60 (p=0.156); 90 (p=0.986) and 120 (p=0.516), except for control vs 180 (p= 6.00 × 10^{-4}).......................................................... 112

**Figure 6. 5** PCAs scatter plot (a) and loading of PC2 (b), corresponding to comparison of cells exposed for 180 min analysed immediately and 24 hrs post exposure. Red dotted line indicates the zero point in the PC2 loading. The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2.
Statistical differences were observed for each score plot of irradiated cells. Significant statistical differences were found between groups: 30 and 30 (24 hrs) \( p = 3.00 \times 10^{-4} \); 60 vs 60 (24 hrs) \( p = 4.22 \times 10^{-8} \); 90 vs 90 (24 hrs) \( p = 4.62 \times 10^{-6} \); 120 vs 120 (24 hrs) \( p = 7.26 \times 10^{-6} \); and 180 vs 180 (24 hrs) \( p = 3.64 \times 10^{-11} \).

**Figure 6.6** PLSR against exposure time for Raman spectra of cells analysed immediately after irradiation, (PLSR C) plot of PLSR coefficient for regression against exposure time and (PCA L) PCA loadings of control vs 180 min. The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2 and PLSR co-efficient.

**Figure 6.7** PLSR modeling versus cell viability for Raman spectra of cells analysed 24hrs post exposure, (PLSR C) plot of PLSR coefficient for regression against cell viability and (PCA L) PCA loadings of 180 min vs 180 min (24hrs). The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2 and PLSR co-efficient.

**Figure 7.1** Microscopic examination of the H&E stained co-culture model. The morphology of fibroblast and keratinocytes is similar to that in normal human skin.

**Figure 7.2** Alamar Blue response of the co-culture model to solar radiation for varying exposure times analysed immediately and 24 h post exposure.

**Figure 7.3** PCA scatter plots (a) and first loadings (b) derived from comparison of control and irradiated cells (180 min). ANOVA indicates significant differences for control vs 30
min (P = 0.0018); 120 min (P = 0.0486) and 180 min (P = 1.324 \times 10^{-13})

Figure 7. 4 PCA scatter plots (a) and second loadings (b) derived from comparison of cells
analysed immediately (180 min) and 24 hrs post exposure (180 min). ANOVA indicates
significant differences for control vs 60 min (P = 1.921 \times 10^{-11}); vs 90 min (P = 5.125 \times
10^{-5}), 120 min (P = 6.672 \times 10^{-9}) and 180 min (P = 3.622 \times 10^{-13}), but not for control
vs 30 (P = 0.059).

Figure 7. 5 Partial least squared regression (PLSR) of Raman spectra of cells analysed
immediately after irradiation against exposure time. Exposure time regression co-efficient
(a) and principal component loading (b) of control versus 180 min. The horizontal red
dashed lines represent the zero point of PC1 and PLSR co-efficient. The black vertical
dashed lines highlight regions of conformational and biochemical changes due to the action
of simulated solar radiation in cells.

Figure 7. 6 Partial least squared regression (PLSR) against cell viability for Raman spectra
of cells analysed 24 hrs after irradiation. Regression co-efficient against exposure time (a)
and PCA loading (b) of 180 min immediate versus 180 min 24 hrs post exposure. The
horizontal red dashed lines represent the zero point of PC1 and PLSR co-efficient. The
black vertical dashed lines in the spectra highlight the regions of conformational and
biochemical changes due to the action of simulated solar radiation in cells.
Chapter I

1.1 Introduction

Cell culture is one of the most important tools employed in cellular and molecular biology. The ability to grow cells in an artificial environment has provided appropriate model systems for studying physiology and biochemistry of cells [1][2][3]. On the other hand, animal models are still considered very important in research, due to their physiological, behavioural, or other similar characteristics with humans [4]. However, regulatory developments in both the European Union (EU) and United State of America (US) aim to minimise the use of animal models and redirect research towards models that most appropriately mimic human conditions [5].

Two-dimensional (2D) monolayer cultures have been widely used for cellular research, since 1907 [5]. However, this type of cell culture lacks the structural architecture and stroma of in vivo conditions [1]. The increasing recognition of the inadequacy of these simplistic models has driven increased efforts towards novel in vitro models which accurately represent in vivo cellular conditions. Three-dimensional (3D) cell culture models can serve this purpose. Cells in 3D models are placed within hydrogel matrices allowing the spatial organisation of cells, mimicking in vivo cell counterparts to investigate cell-cell communication and cell-environment interaction [3]. However, the use of 3D matrices can modify the performance or efficacy of chemotherapeutic agents within the matrix [10,11,12]. Artificial skin models allow the study of biological functions at the molecular and cellular level of different skin layers. Although many options are available
commercially, they are delivered fully differentiated, and therefore the effects of external factors such as solar radiation on the differentiation process cannot be studied. Therefore, in this project, a 3D *in vitro* tissue model of skin is developed to better understand the process and risk factors associated with solar damage. Changes to the biochemical composition in the 3D model as a result of the radiation-matter interaction and the effects of 3D matrices in comparison with 2D models were studied and understood by cytotoxicity assays and Raman spectroscopy. Conducting outdoor experiments to use the natural solar radiation can be impractical. Intermittent cloud, variations in solar intensities or seasonal changes can affect the repeatability of the experiments. Therefore simulated solar radiation is used to ensure reproducibility in the experiments. In recent years, Raman spectroscopy has shown potential and popularity among scientists due to its simplicity and versatility. This vibrational spectroscopic technique generates a unique characteristic for a specific chemical structure known as a molecular fingerprint. Besides, this technique has several advantages such as minimum or no sample preparation, low spectral contribution from water [6]. Therefore, in this work, Raman spectroscopy is used as an ideal tool to reveal changes in the biochemistry of cells as a result of the radiation. In addition, to promote Raman spectroscopy as a novel toxicological screening technique oriented towards pharmaceutical and cosmetic industries, its use to identify immediate and later cell responses in epidermal cells irradiated in different culture environments is demonstrated.

**1.2 Background**

One of the most important factors attributed to life on earth is the sunlight. Throughout human history, the sun has been considered a deity among many ancient civilisations (Greek, Roman, Egyptian, Indian, etc.) [9]. In Aztec mythology, the god Huitzilopochtli,
associated with the sun, was both worshiped and feared. Nowadays, scientific research has come to a similar idea, whereby exposure to the ultraviolet radiation (UVR) in sunlight is recognised to have both beneficial and deleterious effects on human health [10].

Plants, animals and humans benefit from the vital energy coming from the sun, using it to synthesise vitamin D3. However, excessive and chronic sun exposure is associated with deleterious effect on health that may lead to skin cancer [11], [12]. This effect is due to the range of frequencies coming from the sun to the Earth. The electromagnetic radiation emitted from sun ranges from 290 to more than 1,000,000 nm. It includes optical radiation such as ultraviolet (UV), visible (light) and infrared (IR) radiation. However, shorter (ionizing radiation) wavelength and longer (microwaves and radiofrequency) wavelength radiation are also emitted. [11]. Although UVR represents approximately 5% of the radiation coming from the sun, it is the most harmful to human well-being. UVR is subdivided into three components with widely differing physical properties and potential for causing biological damage, as shown in Table 1.1

However, not all the subdivisions of the UVR reach the surface of the Earth in the same percentage: UVA (90–95%), UV-B (5-10%) and UV-C (<1%). Fortunately, UV-C, the most damaging and cytotoxic for humans, is mostly absorbed by the ozone layer [14]. Geographic location plays an important role in UV dose exposure. Countries located around the Equator receive higher UV levels, since the sunlight strikes the Earth most directly in these regions.
Table 1. Subdivision of UVR in its three components [11], [13]

<table>
<thead>
<tr>
<th>UVR type</th>
<th>Wavelength range (nm)</th>
<th>Effects on skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-A</td>
<td>315-400</td>
<td>Long-term dermal structure deterioration and clinical signs of photoaging.</td>
</tr>
<tr>
<td>UV-B</td>
<td>290-320</td>
<td>Serious sunburn linked with erythema, edema, ache and blister formation in less than one day of exposure.</td>
</tr>
<tr>
<td>UV-C</td>
<td>100-280</td>
<td>Mostly absorbed in the ozone layer. Used as steriliser due to biocidal properties.</td>
</tr>
</tbody>
</table>

Other factors such as altitude, latitude, cloud or atmosphere particles can also influence the UVR dose due to reflection, scattering or interference with UV photons, the UV dose being weaker as light passes through more atmosphere [15]. The UV part of the solar spectrum is known to be the major causative factor for induced skin pathologies like erythema and inflammation, degenerative aging changes and cancer [14]. This non-ionising radiation is epidemiological and molecularly connected to the three most common types of skin cancer: basal cell carcinoma, squamous cell carcinoma and malignant melanoma [16]. Therefore, it is necessary to increase the amount of studies related to skin and UVR interaction, in an effort to find solutions to some problems related to the present and future of human health.

In scientific research, animal models have played an enormous role in understanding disease progression and drug toxicity assessments. A myriad of research in biology and medicine uses animals for scientific experiments [19]–[22]. The employability of animals in research is due to their anatomical and physiological similarity with humans, promoting
the investigation of a broad spectrum of mechanisms and assess novel therapies in animal models before translating them to humans [22]. New technologies like the manipulation of the genome have even allowed the creation of animals for specific purposes. Tissue-specific methods for gene exclusion are great examples of the advances in mouse genome manipulation. For example, some methods use tetracycline or tamoxifen-induced systems to turn on or off gene transcription, others use fluorescent protein to identify or remove cell lineages in vivo, etc. Murine models are not the only animal models available in research. Scientists have additionally used rats, cats, dogs, rabbits, pigs, sheep, goats, cattle, chickens, zebrafish, amongst others [4]. Even though animal care standards are taken into account in animal experiments, new regulations against human and animal testing (EUDirective-2010/63/EU and US Public Law 106-545, 2010, 106th Congress) require the development of suitable alternative in vitro models to be implemented [4]. Moreover, 85% of early clinical trials for novel drugs in animals fail and, of those reaching the next step, only half are approved for clinical use. One of the reasons why the translation from animals to humans fails could be in part due to a non-appropriate methodology and the fact that animal models do not accurately reproduce the human disease condition [4]. Therefore, even though animal models represent a valuable source of in vivo information for scientists, they fail to accurately represent the same physiology and drug behaviour as in a human. Other alternative such as 3D models, engineering of tissues or computer simulations, may eventually replace the use of animal models in research.

A new technique discovered in the 20th century by Wilhelm Roux allowed the study of animal cell behaviour in vitro [23]. This technique was pre-established when Wilhelm Roux, a clinical doctor, used warm saline to maintain chicken embryos for some days. Even though it was not strictly considered a cell or tissue culture, it helped to developing the
tissue culture principle. A few years later, in 1907 Ross Granville Harrison achieved and published the first official cell culture technique. Harrison used nerve cells to study the origin and growth of fibres [5]. This accomplishment has led to define cell culture as the removal of animal cells and its propagation and cultivation *in vitro* in an artificial environment that is suitable for its growth [23]. For more than a century, cell culture has contributed to understanding the fundamental biophysical and biomolecular mechanisms of cells assembled into tissues and organs. Therefore, it is possible to study cell behaviour such as cell differentiation, migration, growth, etc. Different fields in science use cell culture, for example biomedics, tissue engineering, regenerative medicine, biophysics, industrial practices, to mention but a few [24]. A typical 2D cell culture entails cells adhered to a flat surface, usually a Petri dish of glass or polystyrene to offer support for cells. When cells grow in 2D, they have access to a similar amount of nutrients and growth factors existing in the medium, thus, a homogeneous growth and proliferation is observed on the surface of the petri dish or cell culture flask [25].

Although 2D cell cultures are well-accepted and have significantly advanced our understanding of cell behaviour, [26], [27] it is recognised that 2D methods do not reproduce the natural structure and stroma of tissues or tumours. In fact, tissue architecture, mechanical and biochemical cues and cell-cell communication are lost in this simple cell culture model [26][24]. In a tumour, cell and cell-extracellular environment interaction is observed. This communication is responsible for cell differentiation, proliferation, vitality, expression of genes and proteins, drug metabolism and other cellular functions. These previous features are absent in 2D cell culture. Therefore, there is a need to consider alternative models that better mimic the real conditions and architecture found in tissue or tumours [26][28].
In a tissue, live cells interact with each other and their extracellular matrix (ECM). This communication is possible due to biochemical and mechanical cues, establishing a 3D network preserving the specificity and homeostasis of the tissue. 3D cell culture tries to better mimic the physiology in which cells can interact in a 3D network and represent specificity of native tissues better than conventional 2D models [26]. The capability of 3D models to mimic specific aspects of cells in vivo allows studying morphogenesis, cellular differentiation, genotypic and phenotypic response to compounds in drug and toxicity screening assays [29]. There are different methods to develop 3D cell culture models that are built according to specific research interest. These methods are divided into 3 categories: a) suspension cultures on non-adherent plates, b) cultures in concentrated medium or in gel-like substances and c) cultures on scaffold. The perfect method of 3D cell culture has not yet been determined and the construction of each model is based on the tissue architecture needed in the experiment [30].

This project will mainly focus in the use of gel-like substances to develop an in vitro 3D model of skin and the effects of using these matrices in comparison with 2D models. The epidermis, the outermost layer of the skin is highly organised in four different layers, the stratum corneum, granular layer, spinous layer and basale layer. The majority of epithelial cells that make up the epidermis are keratinocytes [16]. Keratinocytes and other cell types interact with neighbouring cells and their ECM, including the basement membrane to which they are attached. The ECM generates diverse functions due to the multiple endogenous factors present, which can potentially meliorate cell proliferation, migration and the development of many cell types [31] [32]. It is essential for the organisation and cell function in a tissue; therefore, 3D culture methods that involve gel-like substances to reproduce the ECM possibly better mimic in vivo skin situations [30].
In order to visualise the bilayer of the skin, the cells embedded in the 3D *in vitro* tissue model and identify the morphological changes occurring after irradiation, histochemical staining (H&E) techniques are appropriate methods to achieve this goal. As such, H&E is a standard process which can elucidate the impact at a cellular level. The basic dye haematoxylin has an affinity for negatively charged molecules such as DNA and RNA, thus the cell nuclei can be revealed [33]. The acidic dye eosin binds to molecules, which are positively charged inside the cytoplasm. Both stains provide a general overview of the cell integrity [33].

Raman spectroscopy is an ideal tool to understand and monitor the biochemical changes occurring in the artificial skin, due to the effects of simulated solar radiation. Raman spectroscopy is a non-invasive technique that records distinctive optical signals via molecular vibrations in tissue samples [35]. The Raman phenomenon is based on inelastic scattering of photons incident on a material. When light is scattered due to the interaction with a sample, most of the incident light is scattered elastically and it is named Rayleigh scattering. However, a small fraction (1 in 1x10^6 of photons) of the light is scattered inelastically, and the loss of energy, known as Raman shifts, corresponds to transitions between rotational or vibrational energy levels of chemical bonds [36]. Thus, Raman spectroscopy can be used to identify functional groups of a material, monitoring changes in cellular composition as a function of malignancy or classification of cells [37].

**1.3 Aims and Objectives**

The primary aim of the project is to develop a 3D *in vitro* tissue model of skin, following EU directives to minimise animal testing and to better understand the processes and risk factors associated with UV/solar damage in comparison with the traditional 2D cell culture.
In parallel, the project will demonstrate the applicability of in situ Raman microscopic spectroscopy for the study of biochemical and physiological processes in skin. Raman spectroscopy is a frequently used method for analysis of biological samples and often proposed as a potential diagnostic tool; however, it remains under-utilised as a potential technique in the bio-industrial environment and the characterisation of chemical modifications at a microscopic scale. This project is undertaken to demonstrate that appropriate experimental methods and data handling methods, the technique is certainly adaptable and transferable to such research applications.

1.4 Thesis Summary

Chapter I provides the context within which this study is conducted, highlighting three important areas of study: 1) the effects of solar radiation damage of skin, 2) the development of a new cell culture method, which better mimics real tissue conditions and follows EU directives to minimise animal testing and 3) the suitability of Raman spectroscopy for the study of physiological processes in skin. In addition, the aims and objectives of this thesis are described.

Chapter II describes the materials, methods and the background to the experimental techniques employed for the development of a 3D in vitro model of skin. For instance, the principle of Raman spectroscopy (RS), the theory regarding confocal laser scanning microscopy (CLSM) and two cellular assays: Alamar Blue (AB) and Clonogenic assay.

Chapter III provides a brief historical background of cell culture and discuss the disadvantages of using animal models and the importance of moving from conventional 2D cell cultures to a more realistic 3D models. Moreover, additional information concerning
the limitations of pursuing studies of UV radiation effects on commercially available skin models is presented.

**Chapter IV** has been adapted from the published journal article entitled ‘Comparative studies of cellular viability levels on 2D and 3D in vitro culture matrices’, Analytical Methods, 2015, 7, 10000-10017, and presents a comparison between 2D and 3D cell culture by using two commercial membranes in different concentrations and volumes. This chapter shows that transfer from 2D to 3D culture does not necessarily affect the viability of the cells. Moreover, this work served as a foundation study for the development of 3D in vitro model of skin by studying collagen I, rat-tail and Geltrex membranes as the ECM in 3D cell culture.

**Chapter V** presents the background to understanding the basic anatomy and physiology of the skin. In addition to the methodology employed to develop a 3D in-vitro model of skin, the comparison of two sera media supplements via two cell-based assays is also discussed in this chapter.

**Chapter VI** consists of the published journal article entitled ‘Monitoring the biochemical changes occurring to human keratinocytes exposed to solar radiation by Raman spectroscopy’, *J. Biophotonics*, 2020; e202000337. This work presents an analysis of the biochemical and morphological changes occurring to HaCaT cells exposed to simulated solar radiation in a traditionally 2D model. Changes in the cell viability and morphology are monitored using the Alamar Blue viability assay and haematoxylin and eosin staining, whereas Raman spectroscopy reveal molecular alterations in the genome as a result of the irradiation.

**Chapter VII** reproduces the submitted manuscript entitled ‘Biochemical impact of solar radiation exposure on human keratinocytes monitored by Raman spectroscopy; effects of
cell culture environment’, *J. Biophotonics*, 2021. Herein is illustrated the construction of a simplistic 3D in vitro skin model exposed to simulated solar radiation, compared to 2D culture. Raman spectroscopy revealed the modifications in the cell nuclei at the molecular level due to the radiation and the effects of culturing cells in a 3D format.

Chapter VIII summarises the overall conclusion of this thesis. Starting from the convenience to translate from the traditional 2D cell culture to a more elaborated 3D model. The methodologies and techniques employed in this work and the suitability of Raman spectroscopy as a non-invasive tool to investigate the molecular changes occurring in keratinocytes cultured in different microenvironments (2D and 3D models) exposed to external agents as simulated solar radiation.

### 1.5 References


Available:


A. Modenese, L. Korpinen, and F. Gobba, “Solar Radiation Exposure and Outdoor


10.1002/bies.950170711.


Chapter II

Tissue cell culture

2.1 Introduction

In this chapter, a brief historical background of cell culture is provided. The disadvantages of using animal models and the importance of moving from conventional 2D cell cultures to more realistic 3D models are discussed. Finally, additional information concerning the limitations of pursuing studies of UV radiation effects on commercially available skin models are presented.

2.2 Cell culture models

2.2.1 2D cell culture

For more than a century, cell culture has contributed to understanding the fundamental biophysical and biomolecular mechanisms of cells, assembled into tissues and organs. Therefore, it has been possible to study the characteristics of cell behaviour, such as cell differentiation, migration, growth, etc. A wide range of different fields in science have come to rely on 2D cell culture, for example biomedicine, tissue engineering, regenerative medicine, biophysics, industrial practices, to mention but a few.[1]

A typical 2D cell culture entails adhesion of cells to a flat surface, usually a flask, to offer support for cells (figure 2.1) [2]. When cells grow in 2D, they have access to a similar amount of nutrients and growth factors existing in the medium, and therefore, a homogeneous growth and proliferation is observed on the surface of the Petri dish or cell
culture flask [2]. In 2D cell culture, cells undergo a process termed contact inhibition, which reduces, and/or stops cell division once a monolayer of cells has been achieved across the surface of culture flasks. Standard operating procedures (SOP) suggest that cells can be used for experiments, passaged or frozen down when cells have reached approximately 80% confluency [3].

Figure 2. 1 Schematic representation of cells cultured in 2D culture. In this format, half of the cell is attached to a plastic surface, while the other half is exposed to media. In this culture model, cells have limited cell-cell communication, they adopt an unnatural morphology (flattened) and therefore it does not mimic in-vivo conditions.

Although such 2D cultures have provided important information in microscopic visualisation of cells and a suitable set up for biochemical, immunological, and pharmacological applications, [4] this simple cell arrangement does not accurately depict or simulate realistic conditions and does not reflect the essential physiology of real tissues [5]. Notably, cell geometry and complex processes such as cell-cell communication or mechanical/biochemical signalling are modified or altered in 2D models [6]. Thus, culturing in 2D models could generate misleading results [2]. For instance, in the field of drug discovery, the processes of drug testing are first performed in vitro in 2D cell culture, followed by animal models and finally clinical trials. Only 10% of the compounds successfully reach clinical development [21]. This poor outcome is partially associated with
the nonrepresentative nature of 2D cell culture, in which the cell-drug response can be altered since cells in monolayers express different phenotype than cells in vivo [2]. Therefore, it is recognised that improved models for in vivo conditions are required to improve research analysis, and thus, animal models became popular in industry and academic areas.

2.2.2 Animal models

In scientific research, animal models have played an enormous role in understanding human anatomy and physiology since they were first employed in the 6th century BC in Greece [7]. The employability of animals in research is due to their anatomical similarity with humans, promoting the investigation of a broad spectrum of mechanisms and assessment of novel therapies in animal models before translating them to humans [8]. A wide variety of animals have been employed in research, as they have short lifespan, ease of handling and high reproductive rate [9]. Small animals include rodents such as mice, rats and rabbits; whereas large animals include dogs, goats, sheep, pigs and horses [10]. Studies with animals have greatly contributed to the development of vaccines, antibiotics, disease progression and drug assessment [8], [11]–[14]. New technologies, like the manipulation of the genome, have even allowed the creation of animal models for specific purposes [7]. These animals have been modified by recombinant DNA, which involves gene deletions, replacements or additions [15]. Despite the benefits of animal models, they fail to mimic the human disease condition [10], [16]. In a toxicity study, the drug TGN1412 successfully tested in different animals for the treatment of immunological diseases (sclerosis and rheumatoid) caused organic failure in human patients [17]. Other studies have showed that the immune system response in murine models varitates significantly in comparison to
humans in both innate and adaptive immunity [18]. In cancer research, animal models are limited in reproducing the complex processes observed in human carcinogenesis, physiology and progression [18]. In dermatological studies, animal models have provided a great comprehension of skin pathophysiological mechanisms and allowed testing therapeutic approaches at preclinical level [16]. However, the acquired information is limited by structural, physiological, and molecular differences between human and animals. Beside the skin thickness, animals possess a more permeable skin than humans. This difference is attributed to the haired and nude skin ratio between the two species [16]. Moreover, even though animal care standards are taken into account in animal experiments, a high priority following European regulations against animal testing for cosmetic ingredients (2009/1223/EU) and the REACH guideline for chemicals (2006/1907/EC), require the development of suitable alternative in vitro models to be implemented [7], [16]. Therefore, even though animal models represent a valuable source of in vivo information for scientists, they fail to accurately represent the same physiology and drug behaviour as in a human. Alternative methods such as 3D models, engineering of tissues or computer simulations, may eventually replace the use of animal models in research.

2.2.3 3D cell culture

Understanding the basic anatomy and physiology of the human body gives humankind greater chances to extend life expectancy as well as improve the quality of life. Therefore, it is important to understand how tissues form and function. However, the majority of studies on cells have been in 2D cell culture, which fails to replicate the in vivo cellular microenvironment [1]. A novel methodology, which represents more accurately the microenvironment of cells in real tissue, is that of 3D cell culture [2]. 3D culture methods
generate an artificial environment in which cells grow and communicate with their surroundings in all three dimensions [17]. This feature contributes to improving biological mechanisms such as cell number monitoring, viability, morphology, proliferation, differentiation, response to stimuli, cell-cell communication, etc [17]. Polarity is another important cell feature. Usually, epithelial cells are polarised with apical and basolateral surfaces which play an important role in tissue organisation. This feature is lost in 2D models, since cells are attached to a plastic surface. However, in a 3D model, apical basolateral polarity is maintained [18].

There are different types of 3D cell cultures, which are chosen according to the research interest (Figure 2.2). Most of them employ a matrix-based substrate, such as gels or scaffolds, but others use suspension methods. In multicellular tumour spheroids (MCTS), cancer cells agglomerate and grow in suspension or embedded in gels [20]. As the names implies, MCTS are based on a structure that replicates the physical and biochemical features of a tumour, and can be subdivided into four types of structures: round, mass, grape-like and stellate spheroids [20]. However, one of the disadvantages for this type of cell culture is that it uses animal-derived or human-derived matrices which often contain unwanted growth factors and viruses, leading to a possible disease transmission [20]. Synthetic scaffolds have also been employed to reconstruct the ECM for cells. Usually, the materials used for the fabrication of these synthetic scaffolds are polymers, titanium or ceramic-based. One of the advantages of using synthetic materials is that their mechanical properties can be modified according to the cell culture required and their chemical composition is well defined. In this method, cells grow on the matrix surface or in the pores [21]. One drawback when working with scaffolds is their biodegradation feature, since this natural decomposition of the material could affect cell activity in unknown conditions [21],
Hydrogels are another material to grow cells in a 3D environment. Hydrogels can be defined as reticulated structures of cross-linked polymer chains forming 3D matrices or porous scaffolds which origin could be natural or synthetic [5], [23]. Additionally, hydrogels possess important features that make them suitable for 3D cell culture, such as high water content, variation in stiffness, porosity or elasticity, they can be coupled with adhesion and growth/differentiation factors and they can be produced by combining synthetic and natural materials [21]. Natural hydrogels are animal-derived proteins. The materials used to produce natural hydrogels are collagen, albumin, fibronectin, laminin, agarose and alginate [5], [21]. For instance, Collagen I, Myogel and Matrigel are hydrogels that have been employed in 3D cell culture to replicate the microenvironment for cells [5], [24], [25]. Collagen-based hydrogels are one of the most used 3D cell culture systems. This popularity arises since collagen is the most abundant protein in mammals, [26] collagen type I being the most abundant in tissues [27].
Figure 2.2 Examples of three different 3D cell culture methods. Cells in purple illustrate the possible organization within the different matrix. (Adapted from: https://www.elveflow.com/organs-on-chip/3d-cell-culture-methods-and-applications-a-short-review/)

2.2.4 Commercial skin models

A huge variety of products used in daily life have emerged from cosmetic or pharmaceutical companies and cleaning services. These products are directly in contact with the skin, and therefore, they should be previously evaluated and tested before sale. The use of animals as tools to test cosmetic products is strictly forbidden, as stated in the Seventh Amendment to the Cosmetics Directive of European Union Commission [28]. Alternative systems in which these products can be accurately tested is urgently needed [28]. The development of 3D models of skin as alternatives to animal ones is gaining popularity in academic and industry research [29]. These 3D models better mimic the structural architecture and stroma of real skin tissue than 2D models [5]. Skin equivalent models commercially available include EpiDerm™, obtainable from MatTek corporation, in a ready to use format. It uses human keratinocytes from neonatal foreskin, cultured to
generate a stratified epidermis grown on polycarbonate filters [29]. However, in the study of Tyfali et al., [29] it was shown that the EpiDerm\textsuperscript{TM} system presented inconsistencies in the continuous lipidic matrix formation (in the stratum corneum), resulting in higher permeability of the model, compared with real human skin. Other reconstructed skin models are EpiSkin\textsuperscript{®} and SkinEthic\textsuperscript{®}. These models present similar features as real skin like morphology, biochemical markers and lipid composition. Studies of phototoxicity and topically applied chemicals have been successfully performed in these models. However, as in the case of EpiDerm\textsuperscript{TM}, their barrier function is less developed compared to real skin. [28], [30] Importantly, the commercially available models are delivered fully differentiated, complicating the study of the effects of UV radiation on the differentiation process. This project therefore proposes to further develop 3D skin models in which processes such as the effects of UV radiation can be studied, and in parallel, will demonstrate the suitability of \textit{in situ} Raman microscopic spectroscopy for the study of physiological processes in skin. In chapter 5 is presented the optimization of a 3D in vitro model of skin.

\textbf{2.3 Summary}

Since ancient times, humans have tried to understand the basic anatomy, function and physiology of tissues. Different methods such as 2D models and animal models have contributed to achieve this task. However, 2D models fail in generating the natural environment of cells, where they can migrate and proliferate in a 3D microenvironment. Animal models, which provide this microenvironment, fail to reproduce human disease conditions. However, a novel 3D cell culture method has gaining popularity in scientific and industry area since it is able to reproduce \textit{in vivo} like conditions and human cells can be employed in the model. This 3D cell system has been applied in cancer research, drug
discovery, neuroscience and others. However, as an emerging culture method needs improvement and an established method is still required.

This project will develop and study an artificial skin model following the EU directives to minimise animal testing. The proposed 3D model will mainly focus in the use of gel-like substances to develop an *in vitro* 3D model of skin. Geltrex, which is a ready to use basement membrane obtained from murine Engelbreth-Holm-Swarm tumor, will be use as a base to seed keratinocytes to replicate the epidermis, whereas, collagen I, which is one of the main components of the dermis, was chosen to embed human dermal fibroblasts (HDF) and reproduce an artificial dermis layer. As a first step in the 3D model of skin development, the dermis will be produce to set the base for the skin model, after one day incubation, the epidermis will be laid on the top of the dermis and raised to air-liquid interphase to develop the different layers of the epidermis. Following this procedure, and unlike the commercially available models, this epidermis development process will allow to study the UV/solar radiation effects on the differentiation process of the epidermis. Complete information regarding the artificial skin development is provided in chapter 5.
2.4 References


Chapter III

Methodology

3.1 Introduction

In this chapter are described the materials, methods and the background to the experimental techniques employed for the development of a simplistic 3D *in vitro* model of skin. All the techniques performed in this study were optimised to obtain accurate and reproducible data. Specific uses and details concerning the techniques are described in the corresponding chapters.

3.2 Extracellular matrix (3D membranes)

In this study, an artificial skin model is developed by providing cells a suitable 3D culture system that better mimics *in vivo* conditions. Two cell culture membranes, namely Geltrex and collagen I rat-tail, were employed to replicate the extracellular matrix (ECM) in the 3D *in vitro* tissue model of skin.

3.2.1 Geltrex

Geltrex is a commercially available ECM protein, which is extracted and purified from murine Engelbreth-Holm-Swarm tumour. Geltrex is mainly composed of laminin, collagen IV, entactin and heparin sulphate proteoglycan [1]. This rich mixture of proteins and
glycoproteins provides physical support for cells and allows their proliferation, adhesion, migration and differentiation [2]. Some studies [2], [3] involve the use of Geltrex to replicate a 3D microenvironment for cells, since it accurately simulates the ECM. Within the 3D environments, important proteins like collagen, laminin and elastin exist that provide support and architecture for cells [4], [5]. In cancer research, Geltrex has been employed to culture cancer and epithelial cells to investigate cancer cell dynamics and cellular changes in response to the microenvironment [6].

One of the targets in this project is to obtain a simplistic 3D in vitro tissue model of skin. Geltrex was employed to replicate the basement membrane found in the epidermis, providing support for keratinocytes in the 3D in vitro model of skin. Geltrex is a ready to use substrate, no thawing; diluting or premature gelling is required, which facilitate cell culture. This basement membrane (BM) matrix, used in this work, was obtained from Biosciences (Dublin, Ireland) and kept at 4°C, according to the manufacturer’s instructions.

### 3.2.2 Collagen I, rat-tail

Collagen I is a fibrous protein, which has been widely used in 3D cell culture to replicate a structural and biochemical support for cells [7], [8]. Within the collagen I matrix, cells can grow, differentiate, attach and migrate, thus, allowing the study cell functions [7]. In 3D cell culture, collagen I, usually derived from rat-tail, is the leading class of ECM protein used, [9] and it has been employed to culture variety of cell types, including keratinocytes [10] dermal fibroblast, [9] endothelial cells, [11] etc.

In this study, collagen I, rat-tail, was obtained from Gibco Biosciences (Dublin, Ireland) and used to develop the dermal layer of skin. In order to obtain a collagen-based substrate to replicate the extracellular matrix found in the dermis, it is required to mix collagen
(3mg/ml), sterile 10X phosphate buffered saline (PBS), sterile distilled water (dH2O) and sterile 1 M sodium hydroxide (1 M NaOH).

### 3.3 Primary cells and Cell lines

In biological research, cell culture typically involves culturing either one of two different types of cells: primary cells and immortal cells. Primary cells are isolated directly from different donor tissues and are non-immortalised cells (limited number of passages). On the other hand, immortalised cells can be continually passaged over extended periods of time and in some cases, as for HaCaT cells, they usually are derived from the same donor.[12] Both types of cells are employed in studying physiological, pathophysiological and differentiation processes of cells.[13]

![Figure 3. Images acquired with the Confocal Laser Scanning Microscope.](image)

In this project, HaCaT cells (figure 3.1, a) (Human dermal keratinocyte: purchased from the Leibnitz Institute DSMZ- German Collection of Microorganisms and Cell Cultures) and HeLa cells (figure 3.1, b) (human cervical cancer: ATCC CCL-2: purchased from ATCC)
(Manassas, VA, USA)) were used to compare cellular viability on 2D and 3D in vitro matrices. Additionally, the HaCaT cell line was cultured on Geltrex to mimic the outermost layer of skin, called the epidermis, while HDF primary cells (figure 3.1, c) (Human Dermal Fibroblast: purchased from SIGMA-ALDRICH) were cultured within collagen I rat-tail to replicate the dermis of skin.

3.4 Cell culture serum

In 1956, an American physician and pathologist Harry Eagle developed a basic medium useful for in vitro cell cultivation. Nowadays, DMEM or Dulbecco’s modified Eagle’s medium, which is a variation of the first medium produced by Eagle, is widely used in cell culture. The DMEM is mainly composed of a complement of amino acids, vitamins, organic salts and glucose contributing to support cells growth. In addition to cell culture medium, a serum is required for most types of cells in order to promote optimal cell growth and proliferation. The serum supplements the medium with buffer capacity, transportation proteins, antioxidants, cytokines, and growth factors [14]. Foetal bovine serum and Human serum are the most common supplement in culture medium [15].

3.4.1 Human serum vs. Foetal bovine serum

In order to build the 3D in vitro model of skin, it is relevant for this study to provide a culture system resembling the human nature of skin as closely as possible. Human serum (HS) and foetal bovine serum (FBS) were tested as a supplement in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) growth medium to evaluate the effects of HS and FBS in culturing HDF cells to replicate the dermis layer of skin.
FBS is a common supplement in cell, tissue and organ culture [16], [17]. FBS contains important growth factors, cytokines and proteins such as bovine serum albumin (BSA), alpha-1-antiproteinase, plasminogen, lactoperoxidase, kniogen (LMW II), alpha-2-HS-glycoprotein, hemiferrin, prothrombin, apolipoprotein A-I, integrin beta-1, IGFBP2, IGF II, TGF-beta1 contributing to cell growth and proliferation. Despite the widespread use of FBS in cell culture, [18] some issues should be taken into consideration when working with FBS; for example, its composition varies from batch-to-batch, as does the possibility of disease transmission due to prions, bacteria and viruses [18]. Some studies in skin tissue culture reported immune response by antibody detection against FBS proteins in burn patients receiving keratinocytes graft cultured using FBS. On the other hand, HS represents another choice to supplement medium in cell culture. Among its protein content can be found albumin, complement factor H, angiotensinogen, prostate-specific antigen [17]. There are clear differences in protein content between HS and FBS, which could also affect cell culture. Heger et al. [19] reported that HS and FBS differently influence the behaviour of cells in culture, which may have an impact on experimental results, especially in 3D cultures. Mazlyam et al. [16] reported that HS supplementation provides good culture-expanded fibroblast that proliferate rapidly, maintaining normal cell cycle.

In this study, cell viability of HDF in HS and FBS supplementation was compared to optimise the 3D in vitro model of skin. Cell survival and viability levels were compared by using the clonogenic assay (Section 2.5.2) in 2D models and the alamar blue assay (Section 2.5.1) for 2D and 3D model correspondingly.
3.5 Cellular Assays

A cellular assay is a method to test the cytotoxicity of a physical, chemical or biological agent [11]. In the present work, the Alamar blue and the clonogenic assays were used to test the cell viability of Hela, HaCaT and HDF cells in different experiments to develop the layers of skin.

3.5.1 Alamar blue

The Alamar Blue assay (AB) is a very popular method, used over the past 50 years to assess cell viability and cytotoxicity of fungi, bacteria, human and animal cells [20][21]. Due to its non-toxic properties, sensitivity and since it is a cost-effective method, the AB assay has become one of the most referenced methods to assess metabolic function and cellular health in research [22]. Additionally, the bioassay offers other advantages, since it can be employed simultaneously with other experiments such as mRNA, immunophenotyping, apoptosis assays and cytogenetics [22]. A variety of studies can be monitored by using AB assay such as: apoptosis, cell cycle function and control, test compound toxicology in medicine and in environmental risk assessments and antimicrobial susceptibility testing [23]–[25].

AB is an oxidation-reduction indicator assay. In the AB assay, Resazurin (oxidised form) is a blue, non-fluorescent, and non-toxic active ingredient, which can permeate through cell membranes. It undergoes a colour change in response to the chemical reduction of growth medium, resulting from metabolic active cells. Inside of the cell, there are different coenzymes which have specific intrinsic reduction potentials, such as NADPH (E_0=320 mV), FADH (E_0=220), FMNH (E_0=210 mV), FMNH (E_0=210 mV) and NADH (E_0=320 mV). These coenzymes contribute to cellular respiration metabolic reactions. In the AB
assay, the Resazurin, having a reduction potential of +380 mV, has a great affinity to uptake electrons, resulting in reduction by the coenzymes to resofurin. This reduced form of the indicator is pink in colour and highly fluorescent (figure 3.2). Therefore, using the AB assay is possible to measure spectrophotometrically cellular proliferation by exposing cells to 530-560 nm light and reading the fluorescent signal produced at 590 nm, as a measure of the metabolic activity of cells [22][26].

Figure 3. 2 Schematic representation of the Resazurin reduction to resofurin by viable cells in the AB assay.

3.5.2 Clonogenic Assay

The clonogenic, or colony formation, assay is an in vitro experiment that tests the capacity of a single cell to form a colony [27]. Puck and Marcus first described the clonogenic or colony formation assay in 1956 [28]. The clonogenic assay consists of placing cells in a defined growth environment, affected by external stress, and testing the capacity of the cell to produce a colony (>50 cells), as a measure of the impact of the stress. This technique is useful to monitor long-term toxic effects on cells, such as survival after irradiation, and it
can be suitable for any cell growing in culture [29]. The colonies can be visually counted. However, even though it is a very useful technique in research, it is time consuming, since the incubation time for colony formation can take several days (1-3 weeks) [30] [31].

In this present work, the clonogenic assay was used to assess the colony formation of HDF in two different sera: Human (HS) and Foetal bovine serum (FBS) in the culture medium. More detailed information concerning the comparison between HS and FBS serum is provided in chapter 4.

### 3.6 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a very useful technique for acquiring high-resolution (3D) images of cells or other complex morphological structures, approximately 200 nm of resolution depending on the numerical aperture of objective and the wavelength of illumination [32]. This powerful mode of microscopy has become very popular in medicine and biology since it allows visualisation of cell organelles in a non-invasive manner and it provides the capacity to acquire well-defined optical sections from which 3D images can be reconstructed [32]–[34]. In general, a CLSM system is equipped with a laser source, a scanning microscopic device and an optical detection system [34]. The process to acquire images starts when the laser beam from the source passes through a pinhole where it becomes a divergent beam of expanded diameter, which passes through an excitation (interference) filter of appropriate wavelength, to remove any background light. The beam is directed to a dichromatic mirror to reflect the light onto an objective lens, which focus the light on the desired focal plane of the sample. When the laser light reaches the sample, it will produce fluorescence from endogenous and/or exogenous fluorophores, which is red shifted to longer wavelength than that of the source laser. Some of the fluorescence is
collected by the objective lens, transmitted by the dichroic mirror, and delivered to the detector. Although most of the light collected with the CLSM system originates from the focal plane, some can originate from shallower or deeper in the sample, and be scattered into the direction of collection. This can cause an undesirable blurring of an image. In confocal mode, the out of focus light can discarded by refocussing the collected light through a pinhole, before the detector, producing a sharper image. This is achieved since the focus inside the sample and the pinhole aperture are positioned at optically conjugated points, thus blocking out-of-focus light. Therefore a high-resolution and 3D image can be reconstructed from the sample (figure 3.3.) [34], [35].

Figure 3. 3 Schematic optic representation of the CLSM.
In this work, live cell microscopy was performed with a Zeiss LSM 510 Confocal Laser Scanning Microscope (CLSM) equipped with a x60 oil immersion objective and argon (488 nm) and He (543 nm) lasers.

### 3.7 Raman spectroscopy

Raman spectroscopy (RS) is a label-free optical technique widely used in different fields of science such as physics, biology, and chemistry [36]–[38]. It can acquire molecular information of the analysed sample based on the intrinsic vibrational and rotational modes in molecules and their interaction with monochromatic light. Thus, RS can be used for chemical structure analysis, chemical fingerprinting and chemical imaging.

#### 3.7.1 Classical approach to Raman phenomenon

According to the classical theory, when an atom or non-polar molecule is subject to an external electric field (\( \mathbf{E} \)), the latter modifies the original charge distribution in the molecule. The positive charge nuclei will be attracted to the negative pole and electrons toward positive pole (figure. 3.4), creating an induced dipole moment, which is parallel to the electric field (\( \mathbf{E} \)) [31].
Non-polar molecule in the absence of an external $E$ (left) and in the presence of a uniform electric field $E$ (right).

In the Raman phenomenon, a molecule interacts with the incoming radiation ($E$) (laser source), which produces a change in the polarisability ($\alpha$) of the molecule. The polarisability-radiation interaction induces a dipole moment ($\mu$) in the molecule, which oscillates with the frequency of the incident radiation, resulting in the emission of photons of different frequencies.

The induced dipole moment can be written as:

$$\mu_{\text{ind}} = \alpha_{\rho\sigma} E_0 \cos (w_0 t)$$

where $\alpha_{\rho\sigma}$ is the polarisability tensor, which is a measure of the ease with which the molecule can be polarised along each direction. In equation 2, the small displacements of the electron cloud in the normal coordinates of the molecule are represented mathematically, expanded in a Taylor series.

$$\alpha_{\rho\sigma}(Q) = (\alpha_{\rho\sigma})_0 + \sum_k \left( \frac{\partial \alpha_{\rho\sigma}}{\partial Q_k} \right)_0 Q_k + \cdots$$
The polarisability tensor at the equilibrium position is represented by $\alpha_{\rho\sigma}$ and $Q_k$ is the $k^{th}$ normal mode coordinate of the molecule associated with the vibrational frequency $\omega_k$. The normal modes can be written as:

$$Q_k = Q_{k0} \cos (\omega_k t)$$

By combining eq.3 and 2 and replacing the result in eq.1 the linear induced dipole moment obtained is:

$$\mu_\rho^0 = \alpha_{\rho\sigma_0} E_{\rho_0} \cos(w_0 t) + \sum_k \left( \frac{\partial \alpha_{\rho\sigma}}{\partial Q_k} \right)_0 \frac{Q_{k0} E_{\rho_0}}{2} \left[ \cos (t(w_0 - w_k)) + \cos(t(w_0 + w_k)) \right]$$

The first term represents an oscillating dipole that radiates at a frequency $w_0$, corresponding to Rayleigh scattering, the second term contains the frequency $w_0 - w_k$ corresponding to Raman Stokes and $w_0 + w_k$ to Raman anti-Stokes [32].

In this study, Raman spectroscopy is performed to biochemically characterise the 3D model of skin, and any changes to it, due to external factors (radiation). Initially, RS will be employed to characterise biochemically the physiology of cells grown in 2D and 3D culture environments.
3.8 References


Chapter IV

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

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In addition to assisting in drafting the manuscript, Ulises Lopez Gonzalez specifically contributed the cell viability measurements using Alamar Blue for Hela and HaCaT cells for 48 and 72 hrs of incubation time and the corresponding data analysis.

The referencing format of the original publication has been retained.
4.1. Abstract

In this study, the cellular viability and function of immortalized human cervical and dermal cells are monitored and compared in conventional 2D and two commercial 3D membranes, Collagen and Geltrex, of varying working concentration and volume. Viability was monitored with the aid of the Alamar Blue assay, cellular morphology was monitored with confocal microscopy, and cell cycle studies 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 dependant on exposure time, but also due to the effect of the membrane itself on cellular function. 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 hr culture. The results showed the observed effect was different after shorter exposure periods, was also dependent on working 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. 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. The results of this study show that 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 and such variations must be accounted for in studies employing 3D membranes for in vitro cellular screening.
4.2 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 2005). 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 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 and 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 and Graf- Hausner 2012), ranging from scaffolds,
including, animal derived (Matrigel®, Collagen) or plant derived (QGel® Matrix, 3-D Life Biomimetic, Puramatrix), scaffold-free, including low adhesion plates, micropatterened 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 and 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 and 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, 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).

4.3 Materials and methods

4.3.1 Materials

Cell culture media, all supplements, foetal bovine serum, L-glutamine, ampicillin, streptomycin, trypsin and Propidium Iodide (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), YOPRO 1 stain (Gibco™) and Alamar Blue™ (AB) and the NucRed® Live 647 ReadyProbe® were purchased from Biosciences (Dublin, Ireland).

4.3.2 Cell culture

HeLa cells (human cervical cancer; ATCC CCL-2; purchased from ATCC (Manassas, VA, USA)) and HaCaT cells (human dermal keratinocyte; purchased from the Leibnitz Institute DSMZ—German Collection of Microorganisms and Cell Cultures), were both adapted to culture in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 10% foetal bovine serum, 1% L-glutamine and penicillin and streptomycin (Mukherjee et al. 2011; Casey et al. 2016; Cody et al. 2013; Herzog et al. 2007), under standard conditions of 5% CO₂ at 37°C and humidity of 95%. Cells were cultured until they reached approximately 80% confluence. Cells were harvested by trypsin
detachment and seeded at a density of $1 \times 10^5$ cells per well (1 ml) in 6 well plates and $2 \times 10^4$ cells per well (1 ml) in 24 well plates. All experiments were performed in triplicate and incubated for 24, 48 and 72 h prior to measuring cell viability.

4.3.3 Collagen substrate 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.

4.3.4 Geltrex® substrate preparation

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 through put 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.
4.3.5 Confocal laser scanning microscopy

To assess whether any significant morphological differences were present in the tested lines when grown on the ECM, live cell microscopy was performed with 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% CO$_2$ 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 of the 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.

4.3.6 Cell viability measurement with Alamar Blue

The Alamar Blue (AB) assay quantitatively monitors the proliferation of human and animal cells, bacteria and fungi (Kuda and Yano 2003; O’Brien et al. 2000; Pettit et al. 2005; Al-Nasiry et al. 2007; Mosmann 1983). It has been widely used in studies of cell viability and
cytotoxicity (Vega-Avila and Pugsley 2011; Rampersad 2012; White et al. 1996). 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.

4.3.7 Flow cytometry

Cells were seeded in T-25 cm$^2$ 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; all samples were analysed with the aid of a BD Accuri™ C6 Flow Cytometre.
4.3.8 Cell cycle analysis

Cells were grown in 3D and 2D at the same initial seeding concentration of $1.5 \times 10^6$ cells per flask and again 5 ml medium volume in T-25 cm$^2$ flasks. 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.

4.3.9 Apoptosis and necrosis analysis

Cells were seeded on both 2D and 3D substrates, as was done for the cell cycle analysis. 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 YOPRO1/Propidium iodide (PI) dyes (Biosciences Ltd, Dublin, Ireland), whereby 1 µl of YOPRO1 dye (100 µM) and 1 µl of PI (1 mg/ ml) were used to stain cells at per $1.5 \times 10^6$ 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.

4.3.10 Statistical analysis
At least three independent experiments were conducted for each endpoint. Test results for each endpoint were expressed as percentage of the 2D control ± standard deviation (SD). Control values were set as 100%. Differences between samples and the control were evaluated using the statistical analysis package Prism 7 (Graphpad). Statistically significant differences were set at \( P \leq 0.05 \). 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 were 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.

4.4 Results
4.4.1 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 \( \times 20 \) lens (Figure 4.1c, d) whereas, for cells grown on 2D and the Geltrex ECM, a \( \times 63 \) oil immersion lens (Figure 4.1a, 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 4. 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. 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).

4.4.2 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 (Figure 4.2.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. In contrast, for cells that were cultured on collagen membrane of 2.5 mg/ ml concentration ECM (Figure 4.2.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® (Figure 4.3.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 4. 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.
Figure 4. 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.
4.4.3 Apoptosis and necrosis analysis

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 YOPRO 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 YOPRO 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.

Figure 4. YOPRO 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.
As can be seen (Figure 4.4.(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.

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% (Figure 4.5.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 4. 5** YOPRO 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 **$P < 0.01$.
4.4.4 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.

**Figure 4. 6** Cell cycle analysis of HeLa (left) and HaCaT (right) - 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.

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 (Figure 4.6).

**Figure 4.** Cell cycle analysis of HeLa (left) and HaCaT (right) 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.

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 (Figure 4.7) 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.

4.5 Discussion

When the cells were viewed under the CLSM, the scaffold structures were clearly visible in both the Collagen and Geltrex (Figure 4.1.c, f). As can be seen in Figure 4.1., some minor morphological differences were apparent between the HeLa or HaCaT cells grown in conventional 2D (Figure 4.1.a, b) when compared to those on the Collagen based or Geltrex 3D membrane (Figure 4.1.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 ($P \geq 0.05$) 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 ($P \geq 0.05$) 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.
4.4). 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 (Figure 4.6) by arresting cells in the G0/G1 phase. This effect, while also apparent for the HaCaT (Figure 4.6) 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 (Figure 4.7) 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 (Figure 4.7) 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.

### 4.6 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.

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Chapter V

Development of a 3D *in vitro* tissue model of skin

5.1 Introduction

In this chapter, the background to understanding the basic anatomy and physiology of the skin is presented. In addition to the methodology employed to develop a 3D *in-vitro* model of skin, the comparison of two sera media supplements (HS and FBS) via two cell-based assays is also discussed.

5.2 Basic Anatomy and Physiology of the Skin

The skin, which is often referred to as the integumentary system, is considered by many to be the largest organ in the human body [1]. It has a surface area of 1.5 – 2 m² and comprises about 15 to 20 % of total body tissue weight. The integumentary system is more than just an organ, however; it is the body’s first line of defence [1]. It acts as a mechanical barrier against external physical, chemical and biologic deleterious substances. Ultra-violet (UV) radiation, extreme temperatures or harmful microorganisms are some examples of these noxious substances [1], [2].

The skin has many different functions in the body, including thermoregulation, excretion, absorption, metabolic regulation, evaporation management and aesthetics. However, it is also an organ of sensuality and psychological well-being [1]. An important fact about the
skin is that it is personalised; it shows modifications according to age, gender, ethnicity and anatomic location [1].

The skin consists of two anatomically distinct layers: the outermost layer, called the epidermis and the underlying dermis, both of which have a distinctive complex structure and purpose. The ancillary structures such as hair follicles, nails, sebaceous and sweat glands are also considered part of the integumentary system (Figure 5.1) [3].

![Skin Anatomy](https://www.dermatologysydney.com.au/anatomy-of-the-skin/)

**Figure 5. 1 The components of the skin. This illustration depicts the skin as an elaborated layered structure composed of epidermis and dermis and some structures like hair follicle and nerves. (Adapted from https://www.dermatologysydney.com.au/anatomy-of-the-skin/)**

### 5.2.1 The epidermis

The epidermis is the outermost layer of the skin and its principal function is to serve as a protective barrier between the environment and the body (figure 5.2.) [1], [2], [4]. This
stratified and keratinising squamous epithelium is constantly renewing by a process called desquamation [1], [2], [4]. In this self-renewal system, the new cells located in the basal layer of the epidermis (stratum basale or stratum germinativum) push the old cells up to the stratum spinosum, then to the stratum granulosum and finally to the stratum corneum [1], [2], [4]. The layers of the epidermis (stratum basale, spinosum, and granulosum) are mainly composed of keratinocytes (90-95 %), but the last layer, the stratum corneum, is basically constituted by corneocytes (enucleated keratinocytes) to which a set of lipids are covalently bound to generate an effective biological barrier [1], [2], [4] [5].

Figure 5. 2. The epidermis. This illustration depicts the 5 layers of the epidermis and the main type of cells (keratinocytes, melanocytes, Langerhans cells, stem cells and Merkel cells) living within it. (Adapted from: https://slideplayer.com/slide/10688229/)

There are, however, other cell types in the skin epidermis that are also important, although they exist in a much lower quantity. The melanocyte is an oval or fusiform, dendritic cell
that inhabits the basal layer of epidermis. The ratio of melanocytes to keratinocytes is 1:10 in the epidermal basal layer, being the amount of melanocytes in the skin, irrespective of race. The main function of melanocytes is to produce melanin which is stored above the keratinocyte nucleus [6]. This natural pigment provides the human epidermis with a dark colour and, additionally, the melanin provides protection against UV radiation [5].

Langerhans cells (LCs) are another type of dendritic cell, which possess only one nucleus. These phagocytic cells are seeded from common macrophage precursors in the epidermis. The function of LCs is still not well understood, although they are known to be involved in immune process [7]. Merkel cells (MCs), found in touch-sensitive areas of the skin, are fundamental components of the integument. They are localised at the dermo-epidermal junction. This strategic location allows them to have a connection with the dermal sensory nerve endings, and thus they play an important role in mediating different aspects of touch responses [7]. Toker cells (TCs) are focused in the basal layer or are arranged into glandular structures growing up to the spinosum layer. Among their features is their oval-shaped nuclei and the presence of one or two nuclei [8].

Another important part of the skin is the basement membrane. This thin barrier is the boundary between the two skin compartments and it plays an important role in maintaining tissue architecture and other functions within the skin.

5.2.1.1 Basement Membrane

The basement membrane (BM) is a 50-100 nm layer of extracellular matrix protein complex, structured to separate the epithelium, endothelium, nerve and muscle from adjacent connective tissue stroma [9]. In skin, the BM is located in between the epidermis and dermis, a strategic location which allows it to serve as a structural support to cells and
as a semipermeable selective barrier [9]. Some of the functions performed by the BM are cell traffic control and diffusion of bioactive molecules in both directions (epidermis and dermis). This membrane also stores a variety of cytokines and growth factors that can be released when they are needed [9].

In the cutaneous basement membrane, keratinocytes and fibroblast play an important role, supplying a variety of proteins such as hemidesmosomal plaque proteins, collagen (IV, V and VII), laminin (1, 5 and 6) and nidogen/entactin, [8] each protein enhancing the strength of the matrix and performing different activities. Collagen IV is a non-fibrillar collagen that constitutes 50 % of all BM. Laminin is a non-collagenous protein, which interconnects with collagen IV to provide structure and stability to the matrix. Nidogen or entactin make up 2 %-3 % of the BM and, together with perlecain, stabilise the network built with collagen IV. [10], [11]

In this work, a basement membrane called Geltrex [12] is used in the 3D in vitro tissue model of skin to replicate the basement membrane found in real skin. Details concerning the components of Geltrex are given in chapter 3, section 3.2.1.

5.2.1 Dermis

The Corium, better known as the dermis, is the layer of the skin that underlies the epidermis. It is mainly composed of extracellular matrix (ECM) proteins. The ECM in the dermis is primarily made up of collagen fibres [13]. There are at least 16 different types of collagen, although collagen type I, II and III are the most abundant in the human body (80-90%) [13]. On skin, collagen type IV and I are the major components of dermis layer. Some of the differences among these collagen varieties are that Collagen I, II and III are organised to form long thin fibrils, while Collagen IV is packed to form a two-dimensional
reticulum. However, all collagen types accomplish the same purpose, which is helping tissues resist stretching [14].

The dermis can be divided into two parts: the papillary and reticular layer. Both of them are made up of the same components (collagen fibres), but in a distinct distribution and organisation [12]. The reticular layer consists of large and well organised collagen fibres, while the papillary layer contains thinner collagen fibres and different organisational structures. Other features like blood vessels, hair follicles, oil and sweat glands are in the reticular layer [15]. The dermis has various functions; it serves as a substrate for water and nutrient diffusion and provides mechanical strength to the epidermis [16]. It is composed mostly of collagen I and elastin fibres, although the distributions of these two fibres are not equal. In the reticular dermis, collagen fibres are thicker, ordered and more numerous than in the papillary dermis and basement membrane. Both dermal layers contain different cell types, including neurons, fibroblasts, and leukocytes [15].

The ubiquitous fibroblasts are spindle shaped cells with an oval and flat nucleus. They are found in the dermis, contributing to the extracellular matrix with different components such as collagen, fibronectin and elastin [14]. Additionally, fibroblasts synthesise different growth factors such as keratinocyte growth factor, vascular endothelial growth factor, insulin growth factor, cytokines and interleukin 6, that are important for wound healing processes [14]. Moreover, fibroblasts, together with keratinocytes, perform an important function in cutaneous repair processes [17]. Skin cells interact with each other by dynamic reciprocity. This type of communication allows them to restore normal tissue homeostasis after injury [18]. Mast cells are a type of white blood cell that are located around the dermal arterioles and venules. They are found frequently making contact with nerve fibres. Thus,
mast cells can receive stimulatory signals and release a variety of vasoactive and pro-inflammatory mediators, resulting in an effective defence mechanism in the dermis [15], [17], [19].

In this project, the different layers of the skin are simulated by combining Collagen I matrix with HDF to replicate the dermis and HaCat cells cultured on Geltrex membrane to form the epidermis. The ensemble and description of the 3D in vitro model of skin is detailed in the following sections.

5.3 Materials and Methods

5.3.1 Materials

Cell culture medium, foetal bovine serum (FBS), type AB male human serum (HS) and trypsin were purchased from Sigma Aldrich Ltd (Arklow, Co. Wicklow, Ireland). Carbol fuchsin solution was purchased from VWR international Ltd (Poole, England). Geltrex® hESC-qualified Ready-To-Use Reduced Growth Factor Basement Membrane Matrix. Catalogue Number A1569601 and LOT Number 1851583—Collagen I Rat-Tail (Gibco), and Alamar Blue™ (AB) were purchased from Biosciences (Dublin, Ireland).

5.3.2 Cell culture

HDF cells (human dermal fibroblast (adult); 106-05A); purchased from Sigma Aldrich Ltd (Arklow, Co. Wicklow, Ireland), and HaCaT cells (human dermal keratinocyte; purchased from the Leibnitz Institute DSMZ—German Collection of Microorganisms and Cell Cultures), were both cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 10% foetal bovine serum. Cell culture plates were under standard conditions of 5 % CO₂ at 37°C and humidity of 95 %. Different cell
densities were employed in each experiment. Specific details concerning cell densities are given in the corresponding experiment.

5.3.3 Clonogenic assay

The clonogenic assay was performed to test the HDF cell viability on 2D model cultured in medium supplemented with HS and FBS. The culture medium preparation was done by combining DMEM F/12 supplemented with different concentrations of both HS and FBS. Cells were cultured until they reached approximately 80% confluency. Prior to trypsin detachment, cells were washed with phosphate buffered saline and incubated with trypsin/EDTA solution for 5 min. Once cells were detached, they were washed and seeded at a density of 300 cells per microwell (3ml) dish. Six well plates were employed, but only 5 wells per plate were used to culture cells in media with different concentration of HS and FBS. The first well had a concentration of 100% HS, the second 75% HS and 25% FBS, the third 50% HS and 50% FBS, the fourth 25% HS and 75% FBS and the fifth 100% FBS. Cells were cultured for a period of 9 days until visible colonies were formed under standard conditions of 5% CO₂ at 37 °C and humidity of 95%. The colonies formed were rinsed with PBS, fixed and stained with 20% carbol fuchsin solution. After that, colonies were rinsed with deionised water and allowed to dry to perform colony count. The experiment was performed in triplicate and incubated for 9 days prior to colony formation count.

5.3.4 Cell viability measurement with Alamar Blue

The AB assay was used to measure the cell viability of HDF primary cells in both 2D and 3D substrates cultured in DMEM F-12 medium supplemented with 10% HS and 10% FBS. HDF were seeded at 1x10⁵ cells per well (2 ml). In 3D models, Collagen substrates were
used at constant volume of 400 µl per well in 12 well plates. The three plates were divided into 2 parts, half of the plate was for 2D culture while the other half for 3D culture. After 24 and 48 h of incubation, the medium was removed and the plates were washed with Phosphate Buffered Saline (PBS). An AB solution (5% [v/v]) was prepared in unsupplemented medium to be added to each well. The plates were subsequently incubated for 3h. In order to measure the cell viability, the plates were placed in the spectrometer (Spectra Max-M3) to read the fluorescence as a measure of AB dye conversion.

5.3.5 3D in vitro model development

5.3.5.1 Dermis development and Collagen substrate preparation

In this work, Collagen I Rat Tail (Gibco) was used to replicate the ECM found in the dermis. In the substrate preparation, 3 mg/ml sterile solution of Collagen gel was mixed with 1 M sterile sodium hydroxide (1 M NaOH), Phosphate Buffered Saline x10 (PBS10x) and sterile distilled water. The substrate was produced with a concentration of the Collagen in the gel of 2.5 mg/ml. The Collagen substrate was incubated for 45 min -1 h at 37°C in 12 well plates to allow gel formation. All preparation steps were performed on ice to avoid premature gelation. Once the gel was formed a density of $1 \times 10^6$ HDF cells were embedded within the gel and incubated for 1 day to form the dermis.

5.3.5.2 Epidermis development and Geltrex® substrate preparation

In order to reproduce a system which mimics epidermis conditions, it was necessary to employ HaCaT cells seeded on a Geltrex matrix. Geltrex is a ready to use substrate system. Therefore, minimal substrate preparation was needed. As an important step, Geltrex stock was placed on ice to avoid premature gelation. The volume of Geltrex used on the top of
the dermis layer was 200 µl per well in 12 well plates. After that, the culture system was incubated for 1 h to allow membrane formation. Finally, a density of 1x10^5 cells was seeded on the top of the newly formed membrane to replicate the epidermis of the skin. The 3D in vitro model was incubated for 13 days until a 3D in vitro model of skin was developed.

### 5.4 Results and discussion

This section presents the development of a 3D in vitro human model of skin. Usually, FBS supplement is used to provide growth factors during cell culture. In this study, human cells (HaCaT and HDF) are used to generate the model of skin, thus, it appears logical to use HS supplement instead of an animal-derived serum (FBS) to develop an artificial human skin. However, when HDF were cultured in 10% HS supplement in DMEM F-12 media, surprisingly cell viability was affected and no growth was observed on the 75 mm flask. According to Mazlyzam et al. [19] HS represents another choice to culture HDF resulting in larger numbers of fibroblast produced in shorter time than using FBS. Nevertheless, the opposite result was observed in this study when HDF were cultured in HS. As a result, the clonogenic assay was used to observe the colony formation in the conventional 2D model. Different concentrations of HS and FBS supplement mixtures were used to test HDF proliferation and colony formation. Additionally, the Alamar blue assay was employed as an alternative method to check HDF cell viability in HS and FBS in both the 2D and 3D models.
5.4.1 Clonogenic Assay

The clonogenic assay allowed an evaluation of the capacity of HDF primary cells to produce a colony between control cells cultured in DMEM F-12 media supplemented with 10% FBS and cells that have undergone exposure to the same medium with different concentrations of HS and FBS.

Figure 5.3 Clonogenic assay for HDF primary cells cultured in media with different concentration of HS and FBS.

After 9 days of incubation, colonies were formed and counted. The results for each endpoint were expressed as percentage of the control (+/-) standard deviation. The control value of the maximum colony formation was set as 100%. Maximum colony formation was observed for 100% FBS, and clearly, from Figure 5.3, it can be observed that HDF cultured in a concentration were the medium was supplemented with 100% HS results in less than 20% colony formation in comparison with control. It is also noticeable that decreasing HS percentage in the media increases the chances of colony formation.
Human serum H6914 is produced from whole blood that is allowed to clot. It contains growth factors from the white blood cells and platelets present on the serum. However, the use of HS (H6914) in HDF monolayer was not favourable, since cells were not able to proliferate in this serum.

The clonogenic assay is not appropriate to study colony formation of HDF in the Collagen I (3D model) matrix, as the HDF are embedded at different depths in the matrix and, while a conventional microscope can be used to visualise cells on the surface of the flask, some cells would not be visible to be counted when embedded in the Collagen substrate, leading to misleading results. Consequently, Alamar blue was used as an additional assay to study the HDF cell viability in 2D and 3D models.

5.4.2 Cell viability measured with Alamar blue

The in vitro cellular viability of HDF cultured in media and supplemented with HS and FBS was examined with the AB assay. Some differences were noted between the viability levels of HDF embedded in the two sera. However, some considerations must be taken when comparing cell viability of 2D and 3D (Collagen I matrix) models using AB assay, as stated in chapter III, section 3.6. In a 2D model, cells are directly exposed to the test dye (AB), whereas different geometry in 3D is presented when cells are embedded in collagen I substrate. Thus, Collagen could alter the exposure conditions of the cells to the dye. [17] Therefore, in this experiment the cell viability is not compared between the two cell culture models but between the sera (HS and FBS) in each model (2D and 3D) separately.

For instance, after 24 h incubation, HDF cultured in 2D and 3D models with FBS supplementation present the highest fluorescence intensity in the experiment, as shown in Figure 4.6. This result suggests an apparent increase in viability levels when using FBS as a
supplement in both 2D and 3D models. The same result was noted after 48 h incubation. Cell viability for HDF cultured with FBS supplementation in 2D and 3D culture showed higher viability than those cultured with HS. These results are in agreement with those obtained previously in the 5.4.1 Clonogenic assay section where the highest percentage of colonies formation happened in the presence of FBS. HS is not an appropriate supplement in the medium to culture a 3D in vitro model of skin since cell viability and proliferation is affected during the culture.

Additional information regarding HDF behaviour in HS could be acquired by using live cell flow cytometry. However, to perform such a study, a density of 1X10^6 cells is required, which was not achievable in HS cultures. Since the main objective is to develop a 3D in vitro model of skin, it was necessary to continue with the process and it was decided that FBS as a supplement for the DMEM-F-12 medium would be used to culture the artificial skin model.

**Figure 5.4** Alamar blue response following both (a) 24 and (b) 48 h growth on both 2D and 3D culture of HDF cells in HS and FBS supplementation.
5.4.3 Development of a 3D in vitro tissue model of skin

The construction of the 3D in vitro model of skin was done in two stages: firstly, dermis layer generation and secondly, ensemble of epidermis. A dermal substitute was generated by embedding HDF into a 3D collagen I matrix. After one-day incubation, the Geltrex matrix was laid on the top of the dermis substitute and incubated for one hour and subsequently HaCaT cells were cultured on top of it to resemble the epidermis layer (figure 5.5).

![Figure 5.5 Schematic representation of the ensemble of the two layers of the skin: epidermis and dermis. The base of the culture was made by HDF embedded in Collagen I matrix. Subsequently, the epidermis layer is generated by culturing keratinocytes on the top of a basement membrane (Geltrex) and laid on the top of dermis.](image)

The incubation period after the ensemble of the two layers was 13 days. During the cell growth, pictures of the epidermis formation were acquired by using a microscope at 10X. In figure 5.6, keratinocytes can be observed forming small islands resembling spheres. Cells do not form a monolayer, but rather keratinocytes grow on the top of each other, forming two to three layers.
In this project, a simplistic 3D \textit{in vitro} tissue model of skin is under construction. Although theoretically two layers of skin (epidermis and dermis) have been developed, it is required to include more experiments that validate the model. For example, Hematoxylin and Eosin (H&E) technique could provide visual information regarding the layers and cells on the skin. The addition of melanocytes could improve the model. The inclusion of more cells in the skin model could create more realistic conditions, hence, the effect of melanocytes on epidermal morphogenesis and UV/solar radiation damage of skin could be studied.

\textbf{Figure 5.6} In the upper part of the figure, images of the 3D \textit{in vitro} tissue model of skin acquired with a microscope at 10X are shown. The visible islands are formed by keratinocytes on the epidermis. The red squares indicate the localization of visible fibroblast embedded in the collagen matrix. On the bottom is an overview image of the 3D \textit{in vitro} model of skin in a microwell dish.
5.5 Conclusions

According to the results obtained in the clonogenic assay and Alamar blue assay, it was determined that FBS is more appropriate than HS to culture the 3D in vitro model of skin, since HDF can not proliferate in HS.

Moreover, a 3D in vitro model of skin was developed and it could bridge the gap between in vitro and in vivo studies. However, more optimisation is required, such as melanocytes implementation and H&E techniques to identify and visualise cells and different layers of the model. This optimisation could lead to a more realistic human skin model and it could provide important information on the effects of solar radiation-tissue interaction, which is another important object of study in this project.
5.6 References


Chapter VI

Monitoring the biochemical changes occurring to human keratinocytes exposed to solar radiation by Raman spectroscopy

The following chapter has been adapted from the published journal article entitled ‘Monitoring the biochemical changes occurring to human keratinocytes exposed to solar radiation by Raman spectroscopy’, J. Biophotonics, 2020; e202000337.

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Ulises Lopez Gonzalez performed all experiments, analysis and drafting of the manuscript. Alan Casey advised on the experimental protocol. Hugh J. Byrne contributed to data analysis and drafting of the manuscript.

The referencing format of the original publication has been retained.
6.1 Abstract

Solar radiation exposure is recognised to be a significant contributor to the development of skin cancer. Monitoring the simultaneous and consecutive mechanisms of interaction could provide a greater understanding of the process of photocarcinogenesis. This work presents an analysis of the biochemical and morphological changes occurring to immortalised human epithelial keratinocyte (HaCaT) cell cultures exposed to simulated solar radiation (SSR). Cell viability was monitored with the aid of the Alamar Blue assay (AB), morphological examination was done with Hematoxylin and Eosin staining (H&E) and changes to the biochemical constituents (nucleic acids and proteins) as a result of the radiation insult were demonstrated through a combination of Raman microspectroscopy and multivariate analysis of spectral patterns. The spectral results suggest that SSR induces changes to the conformational structure of DNA as an immediate result of the radiation, whereas alteration in the protein signature is mostly seen as a later response.

Abbreviations: SSR, simulated solar radiation; AB, Alamar blue; H&E, Hematoxylin and Eosin staining; RS, Raman spectroscopy; PCA, Principal Component Analysis; PLSR, Partial Least Squares Regression

6.2 Introduction

Solar radiation is a fundamental factor for life on earth, but also the most effective carcinogenic agent [1]. The composition of the solar spectrum ranges from radio waves through infrared (IR) further divided into IR-A ($\lambda = 780 – 1400 \text{ nm}$), IR-B ($\lambda = 1400 – 3000 \text{ nm}$), and IR-C ($\lambda = 1 \text{ mm} – 3000 \text{ nm}$), to visible light ($\lambda = 400 – 780 \text{ nm}$) and ultraviolet (UVR) composed of UV-C (wavelength; $\lambda = 100 – 280 \text{ nm}$), UV-B ($\lambda = 280 – 315 \text{ nm}$),
and UV-A ($\lambda = 315 – 400$ nm). Significant attention is focused on the UV region of the solar spectrum, [3–6] since it is the initiator of multiple biochemical events in skin cells, such as generation of free radicals, alteration in the structure of DNA and proteins, chronic depression of key physiological processes and physiological stress, resulting in reduction of cell growth and cell division [6,7]. Additionally, IR radiation can induce premature skin aging through stimuli of vessel formation, inflammatory cells recruitment and oxidative DNA damage, [8] whereas visible light has been implicated in erythema, pigmentation and radical production [9]. Excessive and recurrent exposure to solar radiation can greatly generate adverse health effects, including skin cancer, cutaneous malignant melanoma, non melanoma basal cell carcinoma and squamous cell carcinoma [10,11]. The metabolic cell response to solar radiation insult can occur acutely and in a delayed manner [4]. Inflammatory response, originated from a cascade of cytokines, vasoactive and neuroactive mediators and DNA single and double strand break are highly associated to direct effects of UVR on skin, [4,10] whereas generation of reactive oxygen species (ROS), protein modifications and oxidation, and energy depletion are some of the later responses to solar radiation [11,12]. Histological techniques such as routine Hematoxylin and Eosin staining (H&E) [13], immunohistochemistry (ICH) [14], and the more sophisticated in situ hybridization techniques [15] are commonly used in medical diagnosis of skin neoplasms. These techniques can highlight morphological changes to the cellular architecture, subcellular spatial distribution and show a wide range of normal and abnormal cell and tissue components [11–14,16,17]. However, such techniques do not, however, monitor or provide insight into biochemical changes at a tissue/cellular level, or differentiate the initial, direct photochemical changes and the subsequent metabolic perturbations induced by sunlight. In this context, Raman spectroscopy offers an alternative to study the
interaction mechanisms between solar radiation and the molecular species and structures within the tissue and individual cell. [3,11] This technique has been employed successfully for diagnosis of metabolic disorders, cell death mutagenesis, carcinogenesis processes, among others [11, 18–21]. As a label free, optical microspectroscopic technique, it offers several advantages such as high spatial resolution (~0.5 µm to 1.5 µm) which allows spectral measurements at the subcellular level (e.g. nuclei, cytoplasm) [19, 22]. Experiments in aqueous media can also be performed since water has a relatively weak contribution, compared to IR absorption spectroscopy, enabling studies with cells alive in normal physiological conditions [19, 23]. The present work aims to highlight the potential of Raman spectroscopy coupled with multivariate analysis for the detection and analysis of direct photobiological effects and the later impact of the solar radiation on the cell metabolism. As an initial exploration of the techniques, the responses of in vitro keratinocyte cell cultures are monitored, immediately after exposure to solar irradiation, and 24hrs post irradiation. Multiple environmental factors such as weather conditions, time of day, geographical location, to name but a few, can influence the natural solar irradiance [2]. Therefore, in this study, an artificial source of full spectrum, simulated solar irradiation was used to maximise reproducibility, stability and reliability of the data.

6.3 Materials and methods

6.3.1 Materials

Cell culture media, foetal bovine serum, L-glutamine and trypsin were purchased from Sigma Aldrich Ltd (Arklow, Co. Wicklow, Ireland). Alamar Blue® (AB) was purchased from Biosciences (Dublin, Ireland). Cell culture media (phenol red- free) were purchased from Thermo Fisher Scientific.
6.3.2 Cell culture

HaCaT, immortalised human dermal keratinocyte cells, were purchased from the Leibnitz Institute DSMZ, German Collection of Microorganisms and Cell Cultures. Cells were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F- 12 (DMEM/F12) supplemented with 10% foetal bovine serum, and 1% L-glutamine in a humidified atmosphere containing 5% CO2 at 37°C [24]. Cells were cultured until they reached approximately 80-90% confluency. Cells were detached by trypsin and seeded at a concentration of 1 X 10^5 cells per well in 6 well plates for the AB assays, onto sterilised, circular calcium fluoride CaF_2 windows (Crystan, UK) immersed in Petri dishes (30 mm diameter, Stardust, USA) for Raman spectroscopic measurements and on glass cover slips immersed in Petri dishes for fixed cell, light microscopy imaging [25]. All samples were incubated for 24 h prior to irradiation, to allow cell attachment.

6.3.3 Dosimetry

A Q-sun solar simulator (Q-panel, Cleveland, USA) was employed to irradiate the samples to explore the effects of solar damage to cells. This simulator provides irradiance over the entire solar spectrum, including the UVA and UVB regions [26]. The light from the lamp is modified by internal optical filters to ensure the required spectrum (almost identical to Noon Summer Sunlight at the equator), and desired intensity of irradiance of the light at the sample is specified by the user, and controlled by sensors inside the Q-sun. Integrating the spectral distribution from 280nm-400nm yielded a total UV intensity of 63.63 Wm^{-2}, 62.30 Wm^{-2} in the UVA (315-400 nm) and 1.33 Wm^{-2} in the UVB (280-315 nm) region [26]. The Q-sun simulator is calibrated every 1000 hrs. The exposures are given in terms of exposure
time. However, they can be converted to UV dose, noting that 1 $Wm^{-2}$ equals 1 $Jm^{-2}s^{-1}$. For instance, a 30 min (1800 s) exposure with an irradiator providing UV irradiance of 63.60 $Wm^{-2}$ ($63.60 Jm^{-2}s^{-1}$) provides a dose of 114,480 ($63.6 X 1800$) $Jm^{-2}$ or 11.45 $Jcm^{-2}$.

### 6.3.4 Solar exposure

Prior to exposure to simulated solar radiation (SSR), the culture medium in the samples was removed and exchanged for phosphate buffered saline (PBS), thus avoiding cell death due to the formation of reactive oxygen species (ROS) via riboflavin photosensitisation and degradation present in the cell culture medium, as observed in previous studies [11,27]. The irradiation compartment of the Q-sun was sterilised with 100% methanol in order to perform the irradiation experiment without plastic lids, thus ensuring the samples are exposed to the full simulated solar spectrum. Previous studies have demonstrated that little or no differences in cell viability were exhibited by controls maintained in the incubator, or removed and "sham irradiated" in the solar simulator [27], and therefore control samples were treated in the same manner as the irradiated ones, with the exception of the irradiation exposure time while they were kept in the incubator. After ignition, the Q-sun was allowed to stabilise for 15 min. The temperature of the chamber is stabilised to 37°C. Exposed samples were irradiated for 30, 60, 90, 120 and 180 min. Post exposure, samples were removed from the compartment of irradiation and divided into two groups. Group one was used for cell viability assessment, Raman spectroscopy measurements or microscopy image acquisitions, immediately (taking into account sample preparation, approx. 10 min) after irradiation. From group two, the PBS was removed and replaced by pre-warmed medium, and samples were returned to the incubator at 5% $CO_2$ at 37°C before their further analysis, 24 hr post-exposure.
6.3.5 **Light microscopy imaging**

Samples were processed for morphological examination immediately after irradiation (within 10 min for sample preparation) and 24 h post exposure by using hematoxylin and eosin staining (H&E) [28]. All samples were cover slipped and observed under a light microscope (Olympus BX51) at 100× magnification (Olympus MPLN, NA 0.9) and photographed.

6.3.6 **Cell viability measurement with Alamar Blue**

Due to its non-toxic properties and sensitivity, the AB assay has become one of the most referenced methods to assess metabolic function and cellular health in research as well as in irradiation studies. [29–31] In the AB assay, the blue, non-fluorescent and cell membrane permeating reagent (Resazurin) is reduced to its pink highly fluorescent state (Resorufin), as an indicator of the metabolic activity of cells. [30] Thus, in this study, the AB reduction can serve as an indication of the presence of live cells in the biological system after exposure to UV/solar radiation. Cell viability was determined by AB for both experiments, immediately after irradiation (within 10 min for sample preparation) and 24 h incubation post-exposure. The experiment was performed in triplicates, dividing each plate into three parts and considering one 6-well plate per time point. Cells were seeded to a density of 1 X 10^5 cells per well. Untreated plates exposed to medium with no irradiation were also included in the experimental design as control. Post irradiation exposure, PBS was removed and samples were incubated in 3 ml of AB solution (5% [v/v] solution of AB dye) prepared in pre-warmed, un-supplemented (no FBS) medium and incubated for 3 h at 37°C, 5% CO2. AB conversion, as a measure of the metabolic activity of cells, was determined by
using a plate reading spectrometer (SpectraMax—M3) to monitor fluorescence with 540 nm excitation and 590 nm emission.

6.3.7 Raman Spectroscopy

A Horiba Jobin-Yvon LabRAM HR800 spectrometer with an external 300 mW diode laser operating at 785 nm as source was employed in this work. The power on the sample was ~70 mW. A 100× (Olympus LMPLFLNx100, NA 0.8) immersion objective was used for the measurements, providing a spatial resolution of ~1 µm at the sample. The water immersion environment acts as a heat sink, reducing the risk of photothermal damage of the cells. [32]

As part of the confocal operating settings, the confocal hole was set at 100 µm. The system was spectrally calibrated to the 520 cm⁻¹ line of silicon, and the intensity response function was corrected using the Standard Reference Material (SRM) No. 2243 of the national Institute of Standards, Boulder, Colorado, USA (NIST SRM 2243, 2242, 2241).[33] The LabRAM system is a confocal spectrometer that contains two interchangeable gratings (300 and 900 lines per mm respectively). In this experiment, the 300 lines/mm grating was used, providing a spectral dispersion of approximately 1.5 cm⁻¹ per pixel, resulting in a full width half maximum of the source 785 nm laser line of 6.16 cm⁻¹. The lower dispersion grating enables the spectral range of the fingerprint region, from 400 cm⁻¹ to 1800 cm⁻¹, to be captured in a single spectral window. The detector used was a 16-bit dynamic range Peltier cooled CCD detector. For Raman spectroscopic analysis, the HaCaT cells were seeded onto sterilised calcium fluoride (CaF₂) circular windows immersed in Petri dishes (30 mm diameter) at a density of 1×10⁵ 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 on the CaF₂ disc, after which 3 ml 10% FBS DMEM/F12 were added. After 24 h incubation with 5% CO₂ at 37°C, cells
were irradiated, as outlined in Section 1.4. The experiment was performed in triplicate, whereby each irradiation time point (30, 60, 90, 120 and 180 min) is represented by three individual Petri dish plates, as well as three control plates. Raman spectroscopic analysis was undertaken both immediately (within 2 min for sample preparation per sample) after irradiation and 24 h incubation post-exposure. After exposure in the Q-sun, the PBS in the samples was changed for pre-warmed DMEM/F12 (phenol red free) medium for the Raman spectroscopic analysis. Ten keratinocytes cells from each Petri dish were selected to acquire single Raman spectra, focusing on their nucleus to specifically explore DNA damage after simulated solar radiation exposure. The backscattered Raman signal was integrated for 30 s and accumulated two times to improve signal-to-noise ratio, and 30 spectra for control and irradiated samples were collected to perform pre-processing methods (baseline correction, smoothing and normalisation) to further improve the quality of the acquired spectra for their analysis. A number of other studies have used similar methodologies [34–36] demonstrating that the variability across the cell population is less than between populations which have undergone treatment with exogenous agents, enabling identification of significant changes as a result of treatment.

6.3.8 Data analysis

For AB, three independent experiments were conducted for each time point. Test results for each time point were expressed as percentage of the control +/- standard deviation (SD). Control values were set as 100%. Raman spectra were preprocessed and analysed using Matlab 2017 (Mathworks, USA) to remove the background signal and facilitate an accurate comparison among cell spectra. Extended Multivariate Signal Correction (EMSC) is a model-based multivariate data preprocessing method, based on linear statistical regression
modelling [37]. The EMSC method has been previously reported for baseline correction and background signal removal [22,38]. In this work, this approach is used to remove the background signal originating from the water in the un-supplemented DMEM/F12 (phenol red free) medium used in the Raman measurements. This water contribution, whose OH bending vibration appears at 1640 cm\(^{-1}\), overlaps in the Amide I region interfering with the spectral interpretation. The EMSC algorithm adapted from Kerr et al. [38] and used in this work, can be presented as follows: The raw spectrum, \(S\), consists of Raman spectrum of interest, \(R\), a baseline signal, \(B\), and the water signal, \(W\).

\[
S = R + B + W
\]  

(1)

The Raman spectrum, \(R\), can be described by a reference spectrum, \(r\), which can be the average spectrum of the sample data multiplied by a certain scalar weight, \(C_r\).

\[
R \sim C_r \times r
\]  

(2)

Similarly, the water signal, \(W\), is described by an average spectrum, \(w\), recorded directly from CaF\(_2\) immersed in DMEM/F12 medium and multiplied by a certain scalar weight, \(C_w\).

\[
B = [C_w \times w]
\]  

(3)

The slowly varying baseline, \(B\), can be represented using an appropriate N order polynomial expressed in eq. 4

\[
B_N = C_0 + C_1X + C_2X^2 + \cdots + C_NX^N
\]  

(4)

where \(N\) is the order of the polynomial, and \(C_m\) for \(m=0\rightarrow N\) represents the various coefficients in the polynomial. The EMSC algorithm will generate estimates values for the scalar weights \(C_r\), \(C_w\) and \(C_m\). These estimates are based on an optimal fit of the various vectors.
\[ S \sim [C_r \times r] + [C_w \times w] + [\sum_{m=0}^{N} C_m X^m] \quad (5) \]

The background corrected cell spectrum, \( T \), is given by

\[ T = \frac{S - [C_w \times w] - \sum_{m=0}^{N} C_m X^m}{C_r} \quad (6) \]

In this paper, the reference spectrum is the mean spectrum of 30 HaCaT cell nuclei spectra, acquired on \( CaF2 \) disc. The selected polynomial order was \( N=5 \), correcting the baseline and removing the water contribution from the spectra. Subsequently, the corrected spectra were smoothed using Savitzky–Golay method (polynomial order of 5 and window 13) and vector normalised to improve spectral quality and minimise the possible influences of measurement variability. Principal components analysis (PCA) and partial least squares regression (PLSR), combined with 10-fold cross-validation, were used as multivariate approaches to analyse the Raman spectra. PCA reduces the number of variables in a multidimensional data set (i.e. spectra) [11]. The extensively applied PLSR technique is a linear model that associates variations in the spectral data to a series of targets [22,39]. The targets used in this work are time of irradiation (e.g. 30 min, 60 min, 90 min, 120 min and 180 min) and AB assay response (% cell viability). 10-fold cross validation approach was used to validate the predictive model [40]. The goodness of fit \( R^2 \) (correlation between cell damage and spectral data) value and the mean squared error of prediction (MSEP) obtained from the 10 fold in cross validation were used to evaluate the performance of the regression model and select the optimal number of latent variables. The MSEP plots are shown in Supplementary Material. In this work, the PCA scores plot is used to visualise whether the spectra coming from cells irradiated at different time points are differentiated, while the PC
loadings provide information regarding the biochemical origin of the variability inside the data. Although the PLSR technique can be used to build prediction models based on the spectroscopic responses [22,39], in this work, the regression co-efficients were used to identify the direct effects of radiation on the cell nuclei as a function of (i) radiation exposure time and (ii) the toxicological response as measured by the AB assay. One-way ANOVA was performed on PC scores to verify the significance of group differences. A P value less than .05 was considered statistically significant.

6.4 Results

6.4.1 Light microscopy imaging

Visualisation of HaCaT cells before and after SSR exposure by light microscopy aims to elucidate the impact at a cellular level. In this study, hematoxylin and eosin (H&E) stains, which are frequently used for histologic examination [41], were employed to reveal the morphology of cells. The basic dye hematoxylin has an affinity for negatively charged molecules such as DNA and RNA, and thus it is used to reveal the location of the nucleus. The acidic dye eosin binds to positive charged molecules within the cytoplasm [28]. Figure 6.1 shows a series of images illustrating the progression from control (nonirradiated cells) to highly damaged irradiated HaCaT cells.

Both column A and B display successive images, representing different times of exposure to simulated solar radiation. However, column A presents images of cells stained immediately after irradiation, while column B shows images of cells stained 24 hrs post exposure. In general, control cell images show large nuclei with dark-blue nucleoli and some cells in the mitotic phase. As the irradiation time increases, morphological and structural changes can be observed in both columns of images. In column A, even though
the changes seen from 30 to 120 min of exposure are not very dramatic, there are some common features, such as chromatic clumping or nuclear fragmentation.

**Figure 6.1** Microscopic examination of the H&E stained control and exposed samples (30, 60, 90, 120 and 180 min) to simulated solar radiation for both times of analysis A) immediately after irradiation and B) 24 h post exposure.
Mitotic cells, as in the control, are no longer evident and the appearance of some giant cells (red arrows) can be observed at all radiation times. Cells imaged after 180 min of exposure are seen to be the most damaged of all exposure times, as evidenced in column A by loss of cytoplasmic membrane and chromatin condensation. In column B, the radiation damage is more evident, from the first irradiation point time. The image corresponding to 30 min after exposure shows enlargement of cells and the presence of apoptotic bodies. Cells exposed for in excess of 60 min show common features, such as cytoplasmic degeneration and chromatin condensation, revealing the damage induced by the SSR.

6.4.2 Cell viability measurement with Alamar Blue

A more quantitative assessment of the effects on the cell culture of SSR exposure can be provided by evaluation of the cytotoxic response. The AB assay is a measure of mitochondrial metabolic activity of cells, [29] which can be impacted by exposure to SSR. In this study, the viability of HaCaT cells was examined after SSR exposure for variable times. The results for both analysis time points (immediately and 24hrs post exposure), as determined by the AB assay, are shown in Figure 6.2. In both analyses, HaCaT cells exhibit a monotonic decrease in AB fluorescence intensity, compared to control, as a result of the radiation. However, cells examined after 24 hrs present lower viability levels than cells analysed immediately after the radiation, especially those irradiated for in excess of 60 min, which exhibit a drastic drop in the viability levels below 5%, when compared to control. However, biochemical information concerning the effects of radiation within cells is still missing.
Figure 6. 2 Alamar blue response of HaCaT cells ($1 \times 10^5$ per well) to simulated solar irradiation for varying exposure times, measured immediately and 24 h post exposure.

3.3 Biochemical characterisation of HaCaT cells on CaF$_2$ with Raman spectroscopy

Raman spectroscopy is a powerful technique for analysing the chemical structure of matter [42]. In the case of biological samples, each of the constituent biomolecules has a unique molecular structure, which provides a distinctive, signature Raman spectrum [42]. Raman spectra of pure biomolecules (i.e. proteins, nucleic acids, lipids, carbohydrates) have been reported previously in several studies [10, 42–44]. These biomolecular signatures can be employed to assist in the interpretation of cellular and subcellular spectra [45]. (Table 1)
Figure 6.3 Average Raman spectrum of the nuclei of HaCaT cells cultured on a CaF2 disc. The highlighted bands correspond to molecular vibrations originating from nucleic acids within the nucleus of the cells. The red shaded area defines the standard deviation on the mean.

Table 1 Assignments of Raman Bands[3], [19], [21], [23], [42]. (A-Adenine, C-Cytosine, G-Guanine, T-Thymine, U-Uracil)

<table>
<thead>
<tr>
<th>Raman band (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>627</td>
<td>G, A</td>
</tr>
<tr>
<td>682</td>
<td>G</td>
</tr>
<tr>
<td>716-19</td>
<td>A</td>
</tr>
<tr>
<td>728</td>
<td>A (ring breathing mode of DNA/RNA bases)</td>
</tr>
<tr>
<td>748</td>
<td>T</td>
</tr>
<tr>
<td>777</td>
<td>U based ring breathing mode</td>
</tr>
<tr>
<td>783</td>
<td>T, C, O-P-O phosphodiester bands in DNA</td>
</tr>
<tr>
<td>792-4</td>
<td>C, U, T Nucleobase in DNA and RNA</td>
</tr>
<tr>
<td>813</td>
<td>O-P-O, Backbone, DNA</td>
</tr>
<tr>
<td>834-9</td>
<td>Amide III, Tyrosine</td>
</tr>
<tr>
<td>853</td>
<td>B-DNA</td>
</tr>
</tbody>
</table>
In this work, Raman spectroscopy was employed to provide a spectroscopic characterisation of the HaCaT cell line cultured on CaF$_2$ to further investigate the biochemical changes occurring when SSR interacts with cells, at different time points. In Figure 6.3 is presented the fingerprint region (400 $cm^{-1}$ to 1800 $cm^{-1}$) of the mean Raman spectrum for HaCaT cells grown on a CaF$_2$ disc. For illustrative purposes, the highlighted bands correspond to signatures of molecular vibrations originating from nucleic acids within the nucleus of the cells, extensively identified in literature. The spectrum of the nuclear region is also rich in bands associated with proteins and lipids which contribute to the nuclear function. [46, 47] Table1 summarises the spectral peak assignments employed in this work. [3,19,21,23,44]
The laser was focused on the nuclear region of the cells, thus, the spectrum contains, among others, characteristic bands of DNA and/or RNA, originating from various moieties like sugar, phosphate, bases, etc. For instance, the bands located at 719 cm$^{-1}$ and 783 cm$^{-1}$ are due to the ring breathing of adenine (A) and O-P-O Backbone, Thymine (T) and Cytosine (C) respectively, while the bands at 1093 cm$^{-1}$, 1255 cm$^{-1}$, 1340 cm$^{-1}$ and 1577 cm$^{-1}$ are due to backbone phosphate backbone vibrations of DNA, and ring modes of T, A, and guanine (G), respectively. Bands associated with proteins are also present in the spectrum, including those at 853 cm$^{-1}$, 1003 cm$^{-1}$, 1130 cm$^{-1}$, and 1650 cm$^{-1}$ [21,23,44]. PCA was performed to reduce the high-dimensional spectral data to scores on the PCs, thus highlighting the main molecular species responsible for the variation between spectra. Although some degree of clustering is evident in a PCA of all data, no clear trend on which to base a loadings analysis is obvious (Supplemental Figure 5S). Therefore, a binary (pairwise) scores analysis was conducted, which compares control with each time of irradiation, according to the methodology of Bonnier et al. [34] In all cases, PC3 accounted for 10% of the variance and did not contribute to differentiation of the datasets. Initially, the spectra of exposed cells were compared to those of control immediately after irradiation, to elucidate the direct biochemical effects of the radiation.

Figure 6.4 presents scatter plots (a) corresponding to the comparison between controls (green) and irradiated (blue) cells, at different exposure times and loading of the second PCs (b), associated to control and 180 min. The spectral acquisition was done immediately after irradiation.
Figure 6.4 PCA scatter plot (a) and loading of PC2 (b), corresponding to comparison of control versus 180 minutes of exposure. The Raman spectral acquisition was done immediately after irradiation. The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2. No significant differences were found for control vs 30 (p=0.051); 60 (p=0.156); 90 (p=0.986) and 120 (p=0.516), except for control vs 180 (p= 6.00 × 10^{-4}).
**Figure 6.5** PCA scatter plot (a) and loading of PC2 (b), corresponding to comparison of cells exposed for 180 min analysed immediately and 24 hrs post exposure. Red dotted line indicates the zero point in the PC2 loading. The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2. Statistical differences were observed for each score plot of irradiated cells. Significant statistical differences were found between groups: 30 and 30 (24 hrs) p = 3.00 × 10^-4; 60 vs 60 (24 hrs) p = 4.22 × 10^-8; 90 vs 90 (24 hrs) p = 4.62 × 10^-6; 120 vs 120 (24 hrs) p = 7.26 × 10^-6 and 180 vs 180 (24 hrs) p = 3.64 × 10^-11.
Although PC1 (explained variance 46%) accounts for the most variance in the data sets, it
does not show significant differentiation of the groups; however, it shows the diversity
across the groups, due to point to point intra-sample variability.

Among data for different irradiation exposure times, only cells exposed for 180 min show a
clear differentiation from control cells. However the separation between these two groups is
observed in PC2 (explained variance 14%), whereby control cells predominantly scored
negatively and approximately half of the irradiated cells scored positively. No significant
statistical differences were found between control and irradiated cells analysed immediately
after irradiation (p>0.05) except for cells irradiated for 180 min (p= 6.00X10^{-4}). In figure
4(b), is presented the PC2 loadings for 180 min versus control. Positive features of the
loading are manifest more strongly in spectra which score positively in the scatter plot, and
vice versa for negative features [34]. The PC2 loading is dominated mainly by positive
contributions of nucleic acids (792 cm^{-1}, 1093 cm^{-1}, 1132 cm^{-1}, 1216 cm^{-1}, 1298 cm^{-1}, 1375
cm^{-1}, and 1493 cm^{-1}) and proteins (834 cm^{-1}, 1064 cm^{-1}, 1270 cm^{-1} and 1657 cm^{-1}). The
prominent bands contributing to the negative loadings are due to proteins (1003 cm^{-1}) and
nucleic acids (716 cm^{-1} and 1339 cm^{-1}).

PCA was also carried out to compare spectra recorded from cells immediately after
irradiation and 24hrs post exposure. This comparison should elucidate the impact of the
irradiation on the cell metabolism, post exposure. Figure 6.5 shows the corresponding
scatter plots (a) and second loading of the PCA (b). PC1 (explained variance 55%), which
represent the most significant variance among the data, does not differentiate between the
datasets, but highlights the cell-to-cell variability. The spectra for the nucleus of cells
analysed immediately after irradiation are mostly clustered together, with the exception of
the 180 min time-point, while cells analysed 24 hrs post exposure tend to be more scattered, located on both the negative and positive side of PC1. However, compared to the results of Figure 6.4, the spectra related to cells analysed 24 hrs post exposure are relatively well differentiated from cells analysed immediately after irradiation according to PC2, with the exception of those cells exposed for only 30 min. The variance between the groups is highlighted by PC2 (explained variance of 20%). The predominant features, which differentiate the spectra of cells analysed immediately and 24 hrs post exposure in the PC loading comparing 180 min (immediate) and 180 min (24 hrs post exposure) are associated with nucleic acids (682 cm\(^{-1}\), 728 cm\(^{-1}\), 748 cm\(^{-1}\), 783 cm\(^{-1}\), 794 cm\(^{-1}\), 925 cm\(^{-1}\), 1091 cm\(^{-1}\), 1121 cm\(^{-1}\), 1298 cm\(^{-1}\) and 1376 cm\(^{-1}\)) and proteins (836 cm\(^{-1}\), 1650 cm\(^{-1}\)). In the negative aspect, associated with cells 24 hrs after irradiation, the dominant features are due to nucleic acids (627 cm\(^{-1}\), 813 cm\(^{-1}\), 1230 cm\(^{-1}\), 1323 cm\(^{-1}\) and 1480 cm\(^{-1}\)), and proteins (1683 cm\(^{-1}\)) (Table 1) [19,21,23,44]. Notably, the spectral profile of the differentiating PC loading 24 hrs after irradiation is significantly different to that immediately after exposure, indicating the spectroscopic profiles contain information related to the longer term responses of the cell, post irradiation, as well as to the direct impact of irradiation. There was statistical significant differences observed in the comparison between groups of cells analysed immediately and 24 hrs post exposure (p<0.005) as determined by ANOVA test.

In order to explore this further, the spectroscopic data was subjected to PLSR using the target of (i) exposure time immediately after irradiation, to identify signatures of direct radiation damage, and (ii) the AB cell viability 24 hrs after irradiation, to identify signatures of cellular response. For (i), the MSEP plot, presented in Figure S2.b of the Supplementary Information, shows that 3 or 4 components can be sufficient to account for 90% of the variance. However, 5 components were selected to fit the model, since it gives a
better linear prediction plot than using fewer components. A correlation accuracy $R^2$ of 0.91 was obtained for the model, which is illustrated in Figure S4 confirming the linear trend of the regression and the intrasample variability. The PLSR co-efficient plot is displayed in Figure 6, compared with the PC2 loading of Figure 4.

![Figure 6](image)

**Figure 6.6 PLSR against exposure time for Raman spectra of cells analysed immediately after irradiation.** (PLSR C) plot of PLSR coefficient for regression against exposure time and (PCA L) PCA loadings of control vs 180min. The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2 and PLSR co-efficient.

Similar to PC loadings [34], PLSR co-efficient can exhibit positive and negative features, which either increase, or decrease as a function of the external target variable [48]. Negative spectral features, associated with decreased contributions as a function of irradiation time, are mainly due to nucleic acids (716 cm$^{-1}$, 1121 cm$^{-1}$, 1339 cm$^{-1}$ and 1420 cm$^{-1}$).
which are also present as negative loadings in the PC2 loading, and characterise control cells. The positive spectral feature in Figure 6 due to O-P-O phosphodiester (792 cm$^{-1}$, 1093 cm$^{-1}$) is also present in the PC2 loading as a feature of the spectra of irradiated cells [19,21,23,44]. The spectroscopic data, acquired immediately after exposure, was also regressed against cell viability, although the correlation coefficient proved to be almost identical to that of the regression against exposure time (albeit inverted) (Figure S3a), which is understandable as the viability and time are quite closely (inversely) correlated (Figure 1). The PLSR technique was also applied to the Raman spectra of cells analysed 24 hrs post exposure.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6_7.png}
\caption{PLSR modeling versus cell viability for Raman spectra of cells analysed 24hrs post exposure, (PLSR C) plot of PLSR coefficient for regression against cell viability and (PCA L) PCA loadings of 180 min vs 180 min (24hrs). The red vertical dotted lines in the spectra highlights the regions where conformational and biochemical changes are occurring due to the action of SSR in cells while horizontal red dotted line represents the zero point in PC2 and PLSR co-efficient.}
\end{figure}
The Raman data was regressed against cell viability to elucidate spectral features associated to metabolic changes within cells. Seven components were used to fit the model, (see MSEP plot in Figure S2.b) yielding an $R^2$ value of 0.91. The PLSR co-efficient plot is shown in Figure 6.7, along with the PC2 loading of Figure 6.5. It should be noted that positive PLSR features are those which are associated with decreased cell viability whereas positive PCA loadings are those which are stronger in the spectra 24hrs post 180 hrs post irradiation, than immediately. The PLSR co-efficient exhibits positive spectral features associated with nucleic acids (783 cm$^{-1}$, 813 cm$^{-1}$ and 1480 cm$^{-1}$) and proteins (981 cm$^{-1}$), which also feature as negative bands of the PC2 loading (also shown in Figure 6), and correspond to spectra of cells analysed immediately after irradiation. The negative spectral features in the PLSR co-efficient are related to nucleic acids (794 cm$^{-1}$, 1091 cm$^{-1}$, 1375 cm$^{-1}$) and proteins (836 cm$^{-1}$ and 1670cm$^{-1}$). These bands can also be identified on the positive side of PC2 loading which corresponds to spectra of cells analysed 24 hrs post exposure. (table1) [19,21,23,44]. The spectroscopic data, acquired 24 hr after exposure, was also regressed against exposure time, although the correlation co-efficient proved to be almost identical to that of the regression against viability (although inverted) (Figure S3b), which is understandable as the viability and time are quite closely (inversely) correlated.

### 6.5 Discussion

Solar radiation is considered one of the principal factors associated with life on earth. A myriad of vital functions carried out by humans, plants and animals are connected with the energy given off by the sun [49]. However, solar radiation is also the most abundant human carcinogen [50]. This mutagenic capacity is mostly attributed to the UV region of the solar spectrum which can be absorbed by DNA and chromophores in the cell [10,51]. This
interaction can result in DNA damage, intracellular lipid and protein peroxidation and a dysfunction of the moderating inflammatory and apoptotic cell responses [5]. These events could lead to a final scenario: skin carcinogenesis [52]. The effects of UVR on cells can be seen immediately, and in a delayed manner [53]. The induction of inflammation is one of the most obvious effects on skin [54]. This process occurs in response to an excessive dose of UVR, whereby keratinocyte cells with irreparable UVR damage activate apoptotic pathways and die [54]. Apoptosis is considered to be a regulated and controlled process of cell death [55]. It can be characterised by specific morphological cell patterns, such as nuclear and cytoplasmic condensation and cellular fragmentation into membrane-bound fragments or apoptotic bodies [56]. In this work, the morphological cell analysis after H&E staining reveals, as general features, cell cycle arrest, chromatin condensation and some enlarged cells without complete loss of membrane integrity (Figure 1, column A). These morphological alterations can be associated with the apoptotic pathway as a result of SSR insult [56,57], and can be more clearly observed in cells 24 hrs post irradiation (Figure 1, column B), including the presence of apoptotic bodies and the loss of contact with their neighbouring cells. The process of progressive cell death can be visualised and quantified using the cytotoxicity assay, AB, as shown in Figure 2. The cell death process induced by SSR can be seen as a monotonic drop in cell viability (Figure 2), indicating that, the longer the exposure the higher the cell damage. Notably, the loss of cell viability is significantly more pronounced 24 hrs after SSR exposure. Ali et al., and others [11,58], have also reported a similar decrease in cell viability due to solar radiation in a time dependent manner. To gain further insight into the deleterious effects of SSR in HaCaT cells, Raman spectroscopy was used to characterize and identify biochemical signatures within the nucleus of the cells, before, immediately after and 24 hrs after
irradiation [11,20,21]. Figure 3 presents the average Raman spectrum of a HaCaT cell, highlighting the characteristic bands related to nucleic acids, which confirm the Raman spectra were acquired in the nucleus and not in another subsection of the cell [59,60]. The comparison of the average spectrum of control and irradiated cells (immediately and 24hrs post exposure) respectively do not clearly elucidate DNA damage in cells after irradiation (Figure S1). Therefore, the multivariate techniques of PCA and PLSR were used to more clearly elucidate changes to the spectral signatures of the biochemical composition of the cells. The spectral variations varied linearly with exposure time and with the measured cell viability response, as measured by AB assay. Figure 6.4a suggests that only cells irradiated for 180 min analysed immediately after irradiation were clearly differentiated from control cells. The prominence in both the PC2 loading and PLSR co-efficient of bands originating from the DNA backbone moieties at 1093 cm\(^{-1}\) and deoxyribose phosphate vibrations at 1121 cm\(^{-1}\) and 1460 cm\(^{-1}\) suggest possible alterations in the main chain spatial structure of the DNA, [61] while bands located at 792 cm\(^{-1}\), 1375 cm\(^{-1}\) and 1657 cm\(^{-1}\) indicate that the cells have already embarked upon an apoptotic pathway [62]. The co-efficient of PLSR against exposure time, presents positive features attributed DNA backbone moieties, indicating an increase and/or a conformational change of the biological constituent, while the negative features associated with nucleic acids and deoxyribose indicates direct damage upon exposure time. Each of these features may signify single strand breaks, leading to formation of pyrimidine photoproducts which are associated to replication arrest, as seen in the H&E images, and the formation of reactive oxygen species, which in turn attack the genome [3,5,7,63]. In order to better visualise the biological mechanism response after SSR exposure, spectra recorded from cells immediately after irradiation and 24hrs post exposure were analysed. In both PCA and PLSR analysis, the band centered at 794 cm\(^{-1}\), can be
correlated with the progress of nucleosomal DNA cleavage for cells in the late apoptotic phase [61]. Structural changes in the biochemical constituents of lipids and proteins are associated with features of amide I (1670 cm\(^{-1}\)), amide III, tyrosine (836 cm\(^{-1}\)) and 1438 cm\(^{-1}\) (CH2 in lipids/proteins), possibly related to a high concentration of free radicals produced as a primary mechanism of SSR molecular response [62,64]. The presence of the band at 1375 cm\(^{-1}\) for cells analysed 24 hrs post exposure indicates progression of nuclear condensation. [61] The amide I signal, previously seen at 1657 cm\(^{-1}\) in cells analysed immediately after irradiation, is shifted to 1670 cm\(^{-1}\) in cells 24 hrs post exposure, indicating modifications in proteins, and an increased prominence of β-sheet as a secondary structure. The results suggest SSR exposure gives rise directly to modifications to the conformational structure of DNA, whereas changes in protein features are mostly seen as a longer term metabolic response to radiation. These results are similar to those reported by Ali et. al. [11], who identified DNA damage occurring at early stages of cytotoxicity and protein modifications related to metabolic changes in cells due to radiation, in reconstructed skin culture models. It should be noted that the contributions of cell repair mechanisms should also be considered, and could potentially be explored by Raman microspectroscopy. For the case of the effects of the nuclear targeting chemotherapeutic agent doxorubicin, Farhane et al. have attempted to associate differences of in vitro cell-line drug sensitivities with differing levels of activation of anti-apoptotic proteins and DNA repair processes, and identify trends in Raman spectral changes [65]. In a study of the effects of gamma-ray irradiation on HaCaT cells in vitro, Meade et al. explored the manifestations of hyper-radiosensitivity and increased radioresistance at low doses in the dose and time dependent infrared and Raman signatures, and extended the study to analyses of targeted and non-targeted effects of the radiation [66]. Verification of the associated
spectral signatures requires correlation with other cytological assay for repair mechanisms (e.g. gamma-H2AX) and anti-apoptotic processes (e.g. BCL-2) and ideally the dose dependence of repair and pro- and anti-apoptotic processes should be systematically mapped out, potentially employing more sophisticated data-mining methods, such as kinetically constrained Multiple Curve Resolution-Alternating Least Squares Regression [67-68]. The potential of Raman microspectroscopy to monitor such effects at a cellular or subcellular level opens up the path to more relevant real life exposures scenarios of repeated doses, of varying duration, and varying periods of intermission.

### 6.6 Conclusions

This work has illustrated the short term and longer term effects of SSR on the human keratinocyte HaCaT cell line, in vitro, differentiating the cell culture characteristics immediately and 24 hrs post exposure, at a biochemical level. While histological staining can help visualise important changes to cell structure after irradiation, it gives no insight into the impact of SSR at a molecular level, where complex biological processes are involved such as photochemical damage and the subsequent cellular response. In this context, Raman spectroscopy information concerning the biochemical content in HaCaT cells nuclei, and changes to it, due to SSR exposure. The additional incorporation of multivariate techniques such as PCA and PLSR in parallel with conventional cytotoxicity assays helped to investigate whether differences among spectra of control and irradiated cells were inherently due to cell-to-cell variability or a direct photochemical effect or a metabolic response of cell to SSR.

**ACKNOWLEDGMENTS**
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**Author contributions**

UL-G performed all experiments, analysis and drafting of the manuscript. Alan Casey advised on the experimental protocol. Hugh J. Byrne contributed to data analysis and drafting of the manuscript.

**Conflict of interest**

The authors declare no potential conflict of interests.

**SUPPORTING INFORMATION**

The following supporting information is available as part of the online article:

**Figure S1.** Mean Raman spectra of ctrl (i) and 30 min (ii), 60 min (iii), 90 (iv), 120 min (v) and 180 min (vi). Analysed immediately after irradiation (A) and 24 hrs post exposure (B).

**Figure S2.** Cross-validated MSEP curve for PLSR of Raman spectra of cells analysed immediately after irradiation regressed against exposure time (a) and cells analysed 24 hrs post exposure regressed against cell viability (b). The MSEP have units of minutes (a) and units of cell viability (b).

**Figure S3.** PLSR modelling of spectroscopic data, acquired immediately after and 24 hrs post exposure regressed against cell viability (a) and time of exposure (b) respectively.
**Figure S4.** 4S Linear predicted response in PLSR of Raman spectra of cell analysed (a) immediately after irradiation versus time of exposure and (b) 24 hrs post exposure versus cell viability. The solid line depicts the idealised 1:1 correlation.

**Figure S5.** PCA of Raman spectra of cells analysed (a) immediately after irradiation versus control and b) 24 hrs post exposure vs immediate.

**Figure S6.** Raw Raman spectra acquired from the nucleus of HaCaT cells irradiated for 30 min, 60 min, 90 min, 120 min and 180 min and analysed immediately and 24 hrs post exposure.

**Table S1.** Exact contribution for PC1 and PC2, between control and spectra of irradiated cells analysed immediately after irradiation.

**Table S2.** Exact contribution for PC1 and PC2, between spectra of cells analysed immediately and 24 hrs after irradiation.
6.7 References


6.6 Supplementary information

Figure. S1 Mean Raman spectra of ctrl (i) and 30 min (ii), 60 min (iii), 90 (iv), 120 min (v) and 180 min (vi). Analysed immediately after irradiation (A) and 24 hrs post exposure (B). The coloured shaded area in each spectrum defines the standard deviation on the mean.
Figure. S2 Cross-validated MSEP curve for PLSR of Raman spectra of cells analysed immediately after irradiation regressed against exposure time (a) and cells analysed 24 hrs post exposure regressed against cell viability (b). The MSEP have units of minutes (a) and units of cell viability (b).

Figure. S3 PLSR modelling of spectroscopic data, acquired immediately after and 24 hrs post exposure regressed against cell viability (a) and time of exposure (b) respectively.
Figure. S4 Linear predicted response in PLSR of Raman spectra of cell analysed (a) immediately after irradiation versus time of exposure and (b) 24 hrs post exposure versus cell viability. The solid line depicts the idealised 1:1 correlation.

![Figure S4](image)

Figure. S5 Principal Component Analysis (PCA) of Raman spectra of cell analysed (a) immediately after irradiation versus control and (b) 24 hrs post exposure versus control. The explained variance in a) PC1=41%, PC2=23% and PC3=8% and b) PC1=48%, PC2=14 and PC3=13%.

![Figure S5](image)
Raw Raman Spectra

30 min

30 min (rec)

60 min

60 min (rec)
Figure. S6 Raw Raman spectra acquired from HaCaT cells irradiated for 30 min, 60 min, 90 min, 120 min and 180 min and analysed immediately and 24 hrs post exposure. (imm= immediately after irradiation; rec= 24 hrs post exposure)
**Table S1.** Exact contribution for PC1 and PC2, between spectra of control and spectra of irradiated cells analysed immediately after irradiation.

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**Table S2.** Exact contribution for PC1 and PC2, between spectra of cells analysed immediately and 24 hrs post exposure.

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<td>PC2 (%)</td>
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Chapter VII

Biochemical changes occurring to human keratinocytes exposed to solar radiation by Raman spectroscopy; effects of cell culture environment

The following chapter has been adapted from the published journal article:


Author list: Ulises Lopez Gonzalez, Alan Casey and Hugh J. Byrne

Ulises Lopez Gonzalez performed all experiments, analysis and drafting of the manuscript. Alan Casey advised on the experimental protocol. Hugh J. Byrne contributed drafting of the manuscript.
7.1 Abstract

Understanding and amelioration of the effects of solar radiation exposure are critical in preventing the occurrence of skin cancer. Towards this end, many studies have been conducted in 2D cell culture models under simplified and unrealistic conditions. 3D culture models better capture the complexity of in vivo physiology, although the effects of the 3D extracellular matrix have not been well studied. Monitoring the instantaneous and resultant cellular responses to exposure, and the influence of the 3D environment, could provide an enhanced understanding of the fundamental processes of photocarcinogenesis. This work presents an analysis of the biochemical impacts of simulated solar radiation (SSR) occurring in immortalised human epithelial keratinocytes (HaCaT), in a 3D skin model, compared to 2D culture. Cell viability was monitored using the Alamar Blue colorimetric assay (AB), and the impact of the radiation exposure, at the level of the biomolecular constituents (nucleic acids and proteins), were evaluated through the combination of Raman microspectroscopy and multivariate statistical analysis. The results suggest that SSR exposure induces alterations of the conformational structure of DNA as an immediate impact, whereas changes in the protein signature are primarily seen as a subsequent response.

Keywords: Principal Components Analysis, Partial Least Squares Regression, Raman spectroscopy, 3D Cell culture models, solar radiation,

Abbreviations: AB1, alamar blue 1; SSR2, simulated solar radiation 2; ECM3, extracellular matrix 3; IR4, Infrared spectroscopy 4; PBS5, phosphate buffered saline 5;
HDF6, human dermal fibroblast 6; PCA7, principal components analysis 7; PLSR8, partial least squares regression 8; EMSC9, extended multivariate signal correction 9.

7.2 Introduction

Cell culture systems, both two-dimensional (2D) and three-dimensional (3D) models, are invaluable tools commonly employed to provide a better understanding of the mechanisms that underlie in vivo cell behaviour.[1] Traditionally, 2D cell cultures have been accepted and used to study cellular responses to stimulations from biochemical and biophysical signals of the microenvironment [2]. However, this practice of culturing cells on flat, synthetic and rigid substrates does not reproduce the in vivo cellular microenvironment, leading to results which are questionably representative of true cellular behaviour [1], [3], [4]. As an alternative, 3D models provide cells with an extracellular matrix (ECM) which allows cellular proliferation, differentiation, mechano-responses and communication [1], [2], [5]. A wide variety of biomaterials for supporting and guiding 3D culture and tissue formation exists on the market. Scaffold type substrates can be derived from animal (Matrigel®, Collagen) or plant (QGel® Matrix, 3-D Life Biomimetic, Puramatrix) sources; whereas, scaffold-free options range from adhesion plates, hanging drop models, magnetic levitation techniques etc [6]–[8]. Reconstructed artificial models of skin have been developed to mimic the 3D organisation of human skin [9], [10]. However, such models present limitations in their barrier function, primarily presented by the outermost, stratum corneum layer [11], limiting observations in the development of the responses to external stimuli, which is of interest in for example, studies of skin damage and toxicity.

In previous studies, it was shown that simulated solar radiation (SSR) exposure can produce short and long-term detrimental effects on keratinocytes (HaCaT) cultured in 2D
models [12], [13]. The radiation and cell interaction induces a series of immediate and later biochemical responses through the interaction with endogenous photosensitisers, which can be translated in the formation of reactive oxygen and nitrogen species (ROS and RNS), single strand break, DNA-protein cross links and the formation of cyclobutane pyrimidine dimers [12], [14], [15]. Such reactive species can be generated by radiation across the solar spectrum, highlighting the importance of not only the UV wavelengths in the study of the effects of solar radiation [16], [17]. Moreover, it is important to examine whether the environment of cell culture impacts on the observations of the effects of SSR on the cell characteristics, both in the short and long-term post exposure, and to understand any protective effects which may be inferred by the ECM environment.

In a previous study of SSR on HaCaT cells, in addition to conventional cytotoxicity assay screening of cellular responses, Raman microspectroscopy was demonstrated to be an ideal technique to identify variations in cellular metabolism as a result of the external insult [12], [18], [19]. This technique allows rapid, non-destructive and high spatial resolution measurements (~0.5-1.5 µm) in tissues or single cells. The Raman spectra exhibit information about cellular components (e.g. proteins, lipids, nucleic acids) or specific molecules in these groups (e.g. phenylalanine, amide I, adenine, cytosine, tyrosine) which can be altered upon exposure to external stimuli such as solar radiation [12], [19]–[21]. Raman spectroscopy is relatively insensitive to water, compared to, for example, the complementary technique of infrared absorption spectroscopy, and little or no sample preparation is required [22].

In this study are evaluated the effects of culturing HaCaT cells in a 3D microenvironment upon SSR exposure at different points in time. Raman spectroscopy, coupled with
multivariate statistical analysis techniques, is employed as a powerful tool to investigate the immediate and longer term cell responses to solar radiation. Comparison of the spectral signatures of HaCaT cells exposed to SSR in 2D and 3D models is explored to provide information regarding the differences and similarities between the two cell culture systems under the same exposure conditions.

7.3 Experimental section

7.3.1 Materials

Cell culture media, foetal bovine serum and trypsin were sourced from Sigma Aldrich Ltd. (Arklow, Co. Wicklow, Ireland). Collagen I Rat-Tail (Gibco)- LOT Number 1851583, Geltrex® hESC-qualified Ready-to-Use Reduced Growth Factor Basement Membrane Matrix, Catalogue Number A1569601, as well as Alamar Blue (AB) were sourced from Biosciences (Dublin, Ireland). 35 mm glass bottom Petri dishes were obtained from MatTek Life for Science (Boston, USA). Phenol-red free cell culture media were purchased from Thermo Fisher Scientific (Dublin, Ireland).

7.3.2 HDF and HaCaT cell lines

Adult human dermal fibroblast (HDF) cells (106-05A) were obtained from Sigma Aldrich Ltd. (Arklow, Co. Wicklow, Ireland), and immortalised human dermal keratinocytes (HaCaT) from the Leibnitz Institute, DSMZ-German Collection of Microorganisms and Cell Cultures. Both were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% foetal bovine serum under standard conditions.
of 5% CO2 at a temperature of 37 °C and humidity of 95% [6]. The cell cultures were maintained until they reached a confluency of approximately 80-90%. They were then detached by trypsin and seeded in co-culture, as described in section 2.5. All the experiments were performed in triplicate.

7.3.3 Co-culture model preparation

7.3.3.1 Collagen substrate preparation (dermal substrate)

Collagen I Rat Tail (Gibco) was utilised to replicate the ECM found in the dermis of the skin. In the substrate preparation, 3 mg/ml solution was mixed with 1 M sodium hydroxide (1 M NaOH), 10X phosphate buffered saline (PBS) and distilled water (dH20). All constituents were previously sterilised. The relative quantities of these components are determined by the final concentration of 2.5 mg/ml and the volume required. [6] After mixing, 500 µl of the solution were placed into a 35 mm glass bottom Petri dish, before incubation at a temperature of 37 °C degrees in a 95% humidity incubator in 5% CO2 conditions, until a solid gel was seen to form (45 – 60 min). All preparation steps were performed on ice to avoid premature gelation.

7.3.3.2 Geltrex substrate preparation

Geltrex was used to replicate the basement membrane found in the epidermis of the skin and it served as a base to seed keratinocytes cells on top of the co-culture system. Geltrex is a ready to use, reduced growth factor basement membrane matrix, which means no thawing or dilution is required. Similar to Matrigel, it is derived from the Engelbreth-Holm-Swarm tumour [6]. To avoid gelation, the Geltrex stock was placed on ice and 200 µl of the
solution were placed on top of each previously prepared collagen substrate. The samples were then incubated for ~1 h until the basement membranes were seen to form.

7.3.3.3 Co-culture preparation

Co-cultures were established by embedding $1 \times 10^6$ HDF cells in a solid collagen and Geltrex covered substrate and then incubating for 24 hrs to form a dermal substrate. After that time, to replicate the epidermis of the skin, $1 \times 10^5$ HaCaT cells were incorporated into the co-culture system. HaCaT cells were seeded on top of the dermal substrate and grown submerged in DMEM F-12 medium (2 ml) until they formed a complete layer (13 days). The co-culture model was monitored and the medium was changed every 2-3 days. Once the co-culture models were ready to use, they were exposed to simulated solar radiation and subjected to cell viability assessment, morphological examination by hematoxylin and eosin (H&E) staining and Raman spectroscopic analysis. All experiments were performed in triplicates, 3 Petri dishes for control and 3 for each exposure time point.

7.3.4 Dosimetry

To produce the damage caused by full-spectrum sunlight to cells, irradiation of the samples was performed using a full spectrum Q-sun solar simulator (Q-panel, Cleveland, USA) [13], [23]. The instrument simulates exposure to the full solar spectrum, including UVA and UVB regions [13]. Internal optical filters modify the lamp output to deliver a spectrum which is equivalent to summer sunlight at noon at the equator. The irradiance intensity at the sample is specified by the user, and controlled by internal sensors. The instrument is routinely calibrated every ~1000 hrs. The integrated spectral distribution over the range 280 nm-400 nm constitutes a total UV intensity of 63.63 Wm$^{-2}$, proportioned as 62.30 Wm$^{-2}$
within 315-400 nm (UVA) and 1.33 Wm\(^{-2}\) in the range 280-315 nm (UVB). [12] The Q-sun simultaneously delivers ~ 400 Wm\(^{-2}\) over the range 400-700 nm. [23] In the NIR region, although a similar dose is delivered, it will be attenuated by the water immersion environment. In the presentation of the results, the exposures are given in terms of exposure time. These values can be easily converted to UV, or full spectral dose, noting that 1 W m\(^{-2}\) equals 1 J m\(^{-2}\) s\(^{-1}\). [12].

7.3.5 Solar Exposure

In previous studies, Maguire et al.[24] reported death of keratinocytes after similar full spectral SSR exposure due to the formation of ROS, via riboflavin photosensitisation and degradation within the in vitro cell culture medium. Therefore, in the current study, the culture medium was removed and exchanged for PBS, prior to exposure to SSR. In order to perform the irradiation exposure without plastic lids, ensuring exposure of the cells to the full simulated solar spectrum, the irradiation compartment of the Q-sun was sterilised with 100% methanol. The instrument was allowed to stabilise for 15 min after ignition. The temperature inside the chamber was set to 37 oC. Samples were irradiated for varied periods of 30, 60, 90, 120 and 180 min. Little or no difference was reported by Maguire et al. in the cellular viability of controls which were maintained in the incubator, or removed and “sham irradiated” in the solar simulator [24]. Thus, control samples received the same treatment as the irradiated ones, except that they were kept in the incubator while the exposed samples underwent irradiation. Post exposure, the samples were removed from the Q-sun irradiation compartment and were split into two groups. The first group was used for immediate (taking into account sample preparation, approx. 10 min) assessment of cell viability, and Raman spectroscopic evaluation. Samples of the second group were returned
to the incubator at 5% CO2 and 37 °C before their further analysis, 24 hr post- exposure, after the PBS was removed and replaced by pre-warmed medium.

7.3.6 Light microscopy imaging

The co-culture model was fixed in 4% formaldehyde for 3 hrs. Then, the model was cut vertically, perpendicular to the surface of the sample, in 4 pieces, embedded in paraffin wax, and subsequently dewaxed. Cross-sectional samples of 10 µm thickness were microtomed, mounted on glass slides and then dried. The samples were dewaxed by immersion in a series of baths; two baths of xylene (Lennox, Dublin) for 5 and 4 min, respectively, two of absolute ethanol (Lennox, Dublin) for 3 and 2 min, and finally a bath of 95% Industrial Methylated spirits (Lennox, Dublin) for 1 min. The samples were then stained routinely using H&E, enabling visualisation of the general morphology of the co-culture model. All samples were cover slipped for microscopic observation (BX51 Olympus) at a magnification of 100× (Olympus MPLN, NA 0.9) and then photographed.

7.3.7 Cell viability measurement with Alamar Blue

The Alamar Blue (AB) assay is commonly employed as a method to quantitatively assess cellular proliferation. [18] Due to its sensitivity and non-toxic properties, this bioassay is one of the preferred methods in analysis of metabolic function, cytotoxicity and in irradiation studies [7], [25]–[27]. The AB assay acts as an indicator of the metabolic activity of cells by the reduction of the blue, non-fluorescent and cell membrane permeating reagent (Resazurin) to its pink, highly fluorescent state (Resorufin) [26]. In this study, for consistency with previous studies, the colorometric AB reduction assay was conducted to elucidate the presence of live cells in the co-culture model, post exposure to SSR. The
assay was performed for the first group, immediately after irradiation (within 10 min for sample preparation) and for the second, incubated for 24 h post-exposure. Unexposed coculture models were included as controls in the experimental design. Post irradiation exposure, the PBS was removed from the samples, and they were incubated in AB solution (3 ml of 5% [v/v] solution of AB dye) prepared in un-supplemented (no FBS) medium which was pre-warmed, and subsequently incubated at 37 °C, 5% CO2 for 3 h. As a measure of the metabolic activity of cells, AB conversion was determined using a spectroscopic plate reader (SpectraMax—M3) to monitor the fluorescence, excited by 540 nm and emitted at 590 nm.

7.3.8 Raman Spectroscopy

This work employed a Horiba Jobin-Yvon LabRAM HR800 spectrometer, with a 16-bit dynamic range Peltier cooled CCD detector. It has an external 300 mW 785 nm diode laser as source, producing ~ 70 mW at the sample. For the measurements, an Olympus LMPLFLN100 immersion objective (NA 0.8) was employed, resulting in a spatial resolution at the sample of approximately 1µm. Following the protocols established by previous studies of live and fixed cells [6], [12], [18], [28], the water immersion environment reduces the risk of photothermal damage of the cells by acting as a heat sink [29]. The confocal hole was set at 100 µm. The instrument was spectrally calibrated to the 520 cm⁻¹ line of silicon. Correction of the intensity response function was performed using the Standard Reference Material (SRM) No. 2243 of the US National Institute of Standards, Boulder, Colorado, USA (NIST SRM 2243, 2242, 2241) [3]. A 300 lines/mm grating was used, providing a spectral dispersion of approximately 1.5 cm⁻¹ per pixel (6.16 cm⁻¹ full width half maximum of the source 785 nm laser line). The spectral range of the fingerprint
region, from 400 cm$^{-1}$ to 1800 cm$^{-1}$ was captured in a single spectral window.

For the Raman spectroscopy measurements, the co-culture models were prepared and irradiated as described in sections 2.3 and 2.5. All experiments were performed in triplicates, such that each irradiation time point (30, 60, 90, 120 and 180 min) is represented by three control plates and three individual Petri dish samples. Raman microspectroscopic analysis was performed for both the first group, immediately after irradiation and the second, incubated for 24 h post-exposure. After SSR exposure, the PBS was exchanged for pre-warmed DMEM/F12 (phenol red free) medium for the Raman spectroscopic analysis of the samples. The samples were measured en-face, and ten keratinocytes, visible on the surface, were selected to acquire single Raman spectra for each co-culture skin model, focusing on their nuclei to specifically elucidate DNA damage as a result of SSR exposure. The backscattered Raman signal was integrated for 30 s and accumulated twice to improve the signal-to-noise ratio. 30 spectra were collected from both irradiated and control samples, which were then subjected to pre-processing (baseline correction and smoothing) to improve the quality of the acquired spectra for further analysis.

7.3.9 Data analysis

For the AB assay for each time point, three independent experiments were conducted. Test results for control samples were set at 100%, and those for each time point were expressed as percentage of the control +/- standard deviation (SD).

Raman spectral data were pre-processed before analysis to remove the spectral background using Matlab 2017 (Mathworks, USA). The Extended Multivariate Signal Correction
(EMSC) protocol, previously reported for baseline correction and background signal removal [19], [30], [31] was employed throughout. The EMSC algorithm adapted from Kerr et al. [31], also described in detail in [12], is used in this work to remove the background signal originating from the collagen I rat-tail and Geltrex extracellular matrices employed to produce the co-culture model. As reference spectrum the average spectrum of the sample data was employed.

The mean spectrum, recorded directly from the ECM immersed in DMEM/F12 medium (phenol red free) represents the spectral contribution of ECM. The slowly varying baseline is represented by an appropriate $N^{th}$ order polynomial. $N=3$ was chosen as the most appropriate polynomial order, correcting the baseline and removing the ECM contribution from the spectra. The corrected spectra were subsequently smoothed using the Savitzky–Golay method (polynomial order of 5 and window 13) to improve spectral quality. No significant contributions from the underlying glass to the recorded spectra was observed, and thus, no correction was deemed necessary.

Raman spectra were subjected to principal components analysis (PCA) and partial least squared regression (PLSR), combined with 10-fold cross-validation, to analyse the spectral variation in the co-culture model. PCA aims to reduce the number of variables in a multidimensional data set (i.e. spectra) [32], keeping most of the variance within the data set. PCA is a multivariate technique which analyses the data set by reducing multiple variables to a small number of a significant linear combination (Principal components). In PCA, two new set of axes, called principal components (PC), are generated by forming linear combinations of the original axes. The first PC is the linear combination containing
the maximal variance contained within the data; PC2 is the subsequent linear combination which has maximal variance perpendicular to the first PC, and so on. As part of the PCA, two new matrices are generated, called scores and loadings, from which the variability within a dataset, as well as the spectral origins can be visualised. PLSR is a technique which constructs a linear model which associates variations in the spectral data to a target dataset [26], [33]. In this work, the targets are the times of irradiation (e.g. 30 min, 60 min, 90 min, 120 min and 180 min) and the values of the AB assay response (% cell viability). The predictive models were developed using a 10-fold cross validation approach. [34] The optimal number of latent variables for the calibration model was determined using the goodness of fit $R^2$ value and the mean squared error of prediction (MSEP), 10 fold in cross validation.

PCA score plots show whether spectra collected from irradiated cells at different time points can be differentiated, whereas the PC loadings identify spectral features which are changing due to the action of simulated solar radiation on cells. Although the PLSR methodology is commonly employed to build models to predict the cellular response based on their spectroscopic profiles [26], [33], in this work, the regression co-efficients are analysed to identify the direct effects of radiation on the nuclei of cells as a function of (i) duration of radiation exposure and (ii) the cytotoxicological response as registered by the AB assay. One-way ANOVA of the PC scores was employed to verify the significance of differences between groups. A P value was considered to be statistically significant if it was less than 0.05.
7.4 Results and discussion

7.4.1 Light microscopy imaging

The co-cultured model was constructed to assess the SSR damage to keratinocytes cells in a 3D environment and the biochemical differences between 2D and 3D cultures were compared. The organisation of the model consists of a bottom layer composed of HDF embedded in collagen I coated with an upper layer of Geltrex where keratinocytes are seeded to be on top of the co-culture. The co-culture forms a gelatinous mass in the center of the Petri dish of 20 mm (glass diameter) as presented in Figure 7.1a. The surface of the model is completely covered by keratinocytes on the 13\textsuperscript{th} day and it can then be used to undertake the radiation studies. Histological assessment of cross-sectional samples of 10 µm thickness was achieved using standard H&E staining. Hematoxylin, a positively charged basic dye, stains cell nuclei in blue, whereas eosin, a negatively charged acidic dye, stains the ECM and most cellular organelles in pink. [35] Figure 7.1 shows the spatial arrangement of HaCaT cells in co-culture with HDF in a 3D model. Figure 7.1(b) shows a cross-sectional (10µm) picture of the reconstructed HaCaT epithelium on top of the dermis layer. The double-layer of HaCaT cells grown over the ECM is clearly visible, with large nuclei stained in dark-blue and the cytoplasm in pink colour. A consistency of 2 to 3 layers of keratinocytes growing on top of each other was observed across different samples. Similar to HaCaT cells, the nuclear compartments of the less dense HDF (red arrow) cells are stained dark blue and their elongated cytoplasm is stained in pink, as shown in Figure 7.1 c,d. Figure 7.1(c) highlights 2 or 3 layers of keratinocytes growing on top of eachother. Moreover, in the bottom layer, a human dermal fibroblast is observed within the dermis, coloured in light pink. Figure 7.1(d) presents the same organisation of HaCaT cells growing
on top of the ECM as in figure b and c. The elongated shape of a human dermal fibroblast is also observed within the ECM.

Figure 7.1. Microscopic examination of the H&E stained co-culture model. The morphology of fibroblast and keratinocytes is similar to that in normal human skin.

7.4.2 Cell viability measurement with Alamar Blue

The viability levels of HaCaT and HDF cells in a 3D matrix were evaluated with the commonly used AB cytotoxicity assay. Resazurin, the active ingredient in the AB assay, is reduced to resorufin, due to the cellular respiration metabolic reactions [7], [26].

Figure 7.2 Alamar Blue response of the co-culture model to solar radiation for varying exposure times analysed immediately and 24 h post exposure.
This change from oxidised to reduced state allows a quantification of the effects of SSR on the 3D cell culture model via fluorometric detection [12]. Figure 7.2 displays the AB fluorescence measured immediately and 24 hrs post exposure for the co-culture model. When measured immediately after irradiation, no systematic reduction in the viability of the cell population, compared to control, is observed. Indeed, a slight increase in cell viability after 90-120 minutes irradiation is suggested, although the values fall with the standard deviation of the shorter exposure times. When analysed 24hrs after irradiation, however, the AB fluorescence intensity, compared to control, is observed to decrease monotonically. After 60 min of cell exposure, the cell viability value has reduced by more than 50%.

7.4.3 Raman analysis

Raman microspectroscopic analysis was used to acquire molecular information regarding the mechanisms of action of the SSR on HaCaT cells in co-culture with HDF cells. Raman spectroscopy elucidates a detailed spectroscopic profile of the cells and monitors the biochemical response in a time dependent manner. Thirty-point spectra per time of exposure (e.g. 30, 60, 90, 120 and 180 min) including control were acquired (Supplementary Figure S6), specifically focusing on the nuclei of HaCaT cells seeded on the top of the co-culture models. The spectra were averaged for each time of exposure, and are shown in Supplementary Figure S1. Literature derived, typical band assignments of cellular spectral features employed in further analysis are detailed in Table 1. [12], [21], [22], [33], [36] Notably, any differences between the spectra of the SSR exposed cells are not striking, and therefore PCA was employed in an attempt to elucidate more subtle changes.
<table>
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<tr>
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<td>A (ring breathing modes of the DNA/RNA bases)</td>
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<td>G (B, Z marker)</td>
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<td>T,A,G (ring breathing mode DNA/RNA)</td>
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Immediately after irradiation, PCA of all the data display some degree of clustering, although, there is no clear trend on which to base a loadings analysis (supplementary Figure S2). A pairwise analysis was therefore performed, comparing control with each time of exposure [37]. Figure 7.3 presents the scores plots (a) comparing control (green) versus exposed cells (blue) analysed immediately after irradiation. PCA examines and seeks to reduce the variance within the dataset (i) within cell groups, and (ii) between cell groups. If the variance within cell groups is dominant, the cell groups are not differentiated according to the first PC, but may be partially differentiated by PC2, and vice versa. The profile of the scatter plots therefore depends on the relative intra-group and inter-group variances. In the case of the PCA of control and cells analysed immediately after irradiation, a clear differentiation according to PC1 was observed for the case of 180 min (Figure 3(a), the loading of which is shown in Figure 3(b), and therefore the evolution according to PC1 (explained variance 42%) was monitored, and quantified by ANOVA. Using ANOVA of the PC scores, significant differences are indicated for control vs 30 min (P = 0.0018); 120 min (P = 0.0486) and 180 min (P = $1.324 \times 10^{-13}$), although not for control vs 60 min (P = 0.0772) and 90 min (P =0.410). The loading of PC1 for control vs 180 min (Figure 7.3b), which shows the spectral features relevant for the discrimination, highlights positive peaks
related to exposed cells, whereas negative to control. At shorter irradiation times, the control vs irradiated cells show a tendency to separate according to PC2, and, for comparison, the PC2 loadings are displayed in Figure S9.

![PCA scatter plots (a) and first loadings (b) derived from comparison of control and irradiated cells (180 min). ANOVA indicates significant differences for control vs 30 min (P = 0.0018); 120 min (P = 0.0486) and 180 min (P = 1.324 × 10^{-13}), although not for control vs 60 min (P = 0.0772) and 90 min (P = 0.410). The PC1 loading is mainly dominated by positive contributions of nucleic acids (750 cm\(^{-1}\), 675 cm\(^{-1}\), 716 cm\(^{-1}\), 750 cm\(^{-1}\), 791 cm\(^{-1}\), 850 cm\(^{-1}\), 874 cm\(^{-1}\), 918 cm\(^{-1}\), 951 cm\(^{-1}\), 974 cm\(^{-1}\), 1006 cm\(^{-1}\), 1047 cm\(^{-1}\), 1080 cm\(^{-1}\), 1097 cm\(^{-1}\), 1210 cm\(^{-1}\), 1240 cm\(^{-1}\), 1251 cm\(^{-1}\), 1323 cm\(^{-1}\), 1343 cm\(^{-1}\), 1375 cm\(^{-1}\), 1438 cm\(^{-1}\), 1507 cm\(^{-1}\), 1520 cm\(^{-1}\), 1583 cm\(^{-1}\), 1608 cm\(^{-1}\), 1630 cm\(^{-1}\), 1640 cm\(^{-1}\), 1672 cm\(^{-1}\), 1625 cm\(^{-1}\)].
791 cm\(^{-1}\), 1097 cm\(^{-1}\), 1240 cm\(^{-1}\), 1251 cm\(^{-1}\), 1323 cm\(^{-1}\), 1343 cm\(^{-1}\), 1375 cm\(^{-1}\) and 1583 cm\(^{-1}\)\), proteins (1006 cm\(^{-1}\), 1210 cm\(^{-1}\), 1608 cm\(^{-1}\), 1630 cm\(^{-1}\), 1640 cm\(^{-1}\) and 1672 cm\(^{-1}\)\) and peptides (625 cm\(^{-1}\) and 675 cm\(^{-1}\)).

**Figure 7.4** PCA scatter plots (a) and second loadings (b) derived from comparison of cells analysed immediately (180 min) and 24 hrs post exposure (180 min). ANOVA indicates significant differences for control vs 60 min (P = 1.921 \(\times\) 10\(^{-11}\)); vs 90 min (P = 5.125 \(\times\) 10\(^{-5}\)), 120 min (P = 6.672 \(\times\) 10\(^{-9}\)) and 180 min (P = 3.622 \(\times\) 10\(^{-13}\)), but not for control vs 30 (P = 0.059).
The prominent bands identifiable in the negative loadings are due to nucleic acids 716 cm\(^{-1}\), \(850\) cm\(^{-1}\), \(874\) cm\(^{-1}\), \(918\) cm\(^{-1}\), \(974\) cm\(^{-1}\), \(1080\) cm\(^{-1}\), \(1507\) cm\(^{-1}\) and \(1520\) cm\(^{-1}\)) and proteins (\(951\) cm\(^{-1}\) and \(1438\) cm\(^{-1}\)). For comparison, the loading of the first principle component for PCA of control vs 30 min is illustrated in Figure S7. Although prominent peaks are evident at \(786\) cm\(^{-1}\), attributed to DNA/RNA ring breathing, and at \(1436\) cm\(^{-1}\), related to vibrations of lipids and proteins, the cell groups are not statistically differentiated by these features, which should be therefore considered to derive from variance across the cell cultures.

**Figure 7.5** Partial least squared regression (PLSR) of Raman spectra of cells analysed immediately after irradiation against exposure time. Exposure time regression co-efficient (a) and principal component loading (b) of control versus 180 min. The horizontal red dashed lines represent the zero point of PC1 and PLSR co-efficient. The black vertical dashed lines highlight regions of conformational and biochemical changes due to the action of simulated solar radiation in cells.
Raman spectra of cells which were analysed immediately and 24 hrs post exposure, for each exposure time, were subjected to PCA to elucidate biochemical relevant information concerning the influence of the irradiation on the metabolism of the cell. Figure 7.4 presents the score plots (a) comparing these two groups and the second PC loadings (b). In contrast to the PCA analysis of the results immediately post irradiation (Figure 7.3), the cluster separation is observed to be primarily according to PC2 (explained variance 16%), whereas PC1, accounts for the most variance in the data set (45%), and describes the diversity of the groups due to intra-sample variability of the sampled points. Significant differences were indicated for control vs 60 min ($P = 1.921 \times 10^{-11}$); vs 90 min ($P = 5.125 \times 10^{-5}$), 120 min ($P = 6.672 \times 10^{-9}$) and 180 min ($P = 3.622 \times 10^{-13}$), but not for control vs 30 ($P = 0.059$).

![Raman Spectra](image)

**Figure 7.6** Partial least squared regression (PLSR) against cell viability for Raman spectra of cells analysed 24 hrs after irradiation. Regression co-efficient against exposure time (a) and PCA loading (b) of 180 min immediate versus 180 min 24 hrs post exposure. The
horizontal red dashed lines represent the zero point of PC1 and PLSR co-efficient. The black vertical dashed lines in the spectra highlight the regions of conformational and biochemical changes due to the action of simulated solar radiation in cells.

The positive features in the PC2 loading are related to spectra of cells exposed for 180 min (immediate) and are associated with nucleic acids (718 cm\(^{-1}\), 766 cm\(^{-1}\), 813 cm\(^{-1}\), 1238 cm\(^{-1}\), 1280 cm\(^{-1}\) and 1323 cm\(^{-1}\)), and proteins (1004 cm\(^{-1}\), 1036 cm\(^{-1}\), 1605 cm\(^{-1}\), 1626 cm\(^{-1}\), 1640 cm\(^{-1}\), 1655 cm\(^{-1}\) and 1677 cm\(^{-1}\)). Negative features related to 180 min (24 hrs post exposure) are derived from nucleic acids (680 cm\(^{-1}\), 794 cm\(^{-1}\), 893 cm\(^{-1}\), 1093 cm\(^{-1}\), 1375 cm\(^{-1}\), 1492 cm\(^{-1}\) and 1515 cm\(^{-1}\)) and proteins (839 cm\(^{-1}\) and 1438 cm\(^{-1}\)). (Table 1). The target used in the PLSR applied in the spectroscopic data to identify signatures of direct radiation damage was (a) exposure time, immediately after irradiation, whereas regression against (b) AB cell viability, 24 hrs post exposure identified signatures of later cellular responses. The number of components selected to fit the model in (a) were obtained from the MSEP plot, which is presented in Figure S3a of supplementary material. 5 components were found to account for 89% of the variance. The model provides a linear trend of regression with a correlation accuracy (R\(^2\)) of 0.89 (Figure S4a). The regression coefficient plot presented in Figure 7.5 is compared with the PC1 loading of Figure 7.3(b). The spectral features show increases (positive bands) or decreases (negative bands) in the intensity of a specific vibrational response, due to changes in the biomolecular content, conformation or morphology [38]. Negative spectral features related mainly to nucleic acids (716 cm\(^{-1}\), 850 cm\(^{-1}\), 918 cm\(^{-1}\), 1179 cm\(^{-1}\), 1338 cm\(^{-1}\) and 1417 cm\(^{-1}\)) are also present as negative features in the PC1 loading, which characterise control cells. Positive spectral features, derived from nucleic acids (600 cm\(^{-1}\), 791 cm\(^{-1}\), 994 cm\(^{-1}\), 1097 cm\(^{-1}\) and 1240 cm\(^{-1}\)
and proteins (1210 cm$^{-1}$ and 1640 cm$^{-1}$) are present in the PC1 loading as spectral features of irradiated cells. Raman spectra of cells analysed 24 hrs post exposure were also subjected to PLSR using the target of cell viability to obtain information regarding metabolic changes within cells. Although the MSEP plot (Figure S3b) suggests that 75% of the variance is accounted for by 3 - 4 components, 5 were selected to fit the model. The model yielded a correlation accuracy ($R^2$) of 0.81 thus providing a better linear prediction (Figure S4b). Figure 7.6 shows the regression co-efficient plot, which also displays the PC2 loading of Figure 7.4. The positive spectral features in the PLSR are related to decreased cell viability and are also associated to those bands in PCA loading coming from spectra of cells analysed 180 min immediately after irradiation. The positive bands are associated to nucleic acids (680 cm$^{-1}$, 718 cm$^{-1}$, 766 cm$^{-1}$, 813 cm$^{-1}$, 874 cm$^{-1}$, 1323 cm$^{-1}$ and 1480 cm$^{-1}$) and proteins (981 cm$^{-1}$). Features of the negative side of the PLSR are derived from nucleic acids (680 cm$^{-1}$, 794 cm$^{-1}$ and 1093 cm$^{-1}$) and proteins (1640 cm$^{-1}$). (Table1). The Raman data concerning spectra of cells analysed 24 hrs post exposure was also regressed against time of exposure. Figure S5b (supplementary material) presents the regression co-efficient, which, although inverted, is almost identical to that of the regression against viability.

7.4.4 Discussion

In this study, the results of co-culturing HaCaT, keratinocytes with HDF, in a 3D extracellular matrix to produce a simplistic 3D in vitro model of skin is reported. Moreover, the impact of SSR on the co-culture model, specifically on the nuclear compartment of the HaCaT cells, monitored with conventional AB assay and Raman microspectroscopy are reported. The two commercial products, collagen I and Geltrex, provided the cells with a 3D culture microenvironment to grow and proliferate [6], as depicted in Figure 7.1. The
HaCaT cells attached rapidly to the surface of the co-culture, forming confluent layers (2 to 3) within 13 days, and have the capacity to differentiate, as reported in previous studies [39]. It is noted that several types of similar and more sophisticated artificial skin models which mimic human skin tissue have been successfully reconstructed in vitro [9], [32], [40]. These approaches can represent a multi-layered epithelium, from dermis, mainly composed of collagen fibres, to the stratified epidermal layer. Such models are less than ideal, however, and have been demonstrated to be limited in their barrier function, for example, determined by lipid packing in the stratum corneum [9]. Moreover, commercially available models are delivered full differentiated, and it is therefore not possible to investigate the effects of external insults such as SSR on the evolution processes. Rather than develop a stratified epidermis, the aim of this work was to elucidate the effect of the 3D environment of a simplistic co-culture model on the biochemical changes in HaCaT cells induced by SSR, in comparison to those previously observed in 2D cultures of these cells under the same conditions [12]. A striking effect of the translation from 2D culture to 3D culture can be observed in the cell viability results assessed by the colorimetric cytotoxicity assay, AB. The results suggest that cells in a 3D environment, analysed immediately after irradiation, were not affected by the SSR with increasing time. This is in contrast to the observations for cells cultured in a 2D environment, which were seen to exhibit a clear monotonic reduction of viability levels due to exposure under the same conditions [12]. When analysed 24 hrs post exposure, a clear exposure time dependent reduction of culture viability was observed, and this more pronounced reduction of viability post exposure is similar to that observed in studies of 2D cultures [12], as well as in artificial skin models [30] exposed to time dependent solar radiation. It should be noted, however, that the differences in the observed responses may be related to the performance
of the AB assay in different cell culture environments [6], [18]. The effective surface area of each cell is different in the different culture environments, and the absorptive nature of the ECM can reduce the bioavailability of the assay dye, reducing the uptake rate [6], [7]. The results of the conventional cytotoxicity assay in the two environments are therefore not directly comparable. Notably, the difference in the half maximal effective concentrations (EC50) for 2D (0.66 Jcm⁻²) [12] and 3D (0.45 Jcm⁻²) models 24 hrs post irradiation is consistent with a dilution factor of 25%, previously observed in collagen matrices [7]. Accounting for such factors, therefore, the results suggest that there is little or no difference in cell viability response to SSR in both 2D and 3D cell cultures (24 hrs post exposure). Significant differences have been reported, however, between the cycle of cells in 2D and 3D culture environments.[21], [41] Gargotti et al. showed that cells cultured in 2D (CaF2 substrates) manifest higher cell number in the G0/G1 phase and fewer in the G2/M and S and phases, compared to those cells cultured in 3D (collagen matrices) [6]. Notably, cell cycle can also be affected by SSR exposure, and, in turn, the sensitivity of cells to radiation exposure has been demonstrated [12]. Sandra et al. demonstrated that low levels of exposure to UV radiation are not likely to produce DNA strand breaks, but cell cycle arrest in the G2 phase, due to the induction of high levels of the p16 protein, whereas levels of the p53 protein are enhanced after high doses of UV. An apoptotic rather than cell cycle response is implicated [39], [41]. The observations suggests that the translation from 2D to 3D environments not only affects cell cycle but also cell interactions with their surroundings. Moreover, other studies [42] suggest that cell morphology and geometry is also modified in this transition. As conventional cytotoxicity assays do not enable a direct comparison of 2D and 3D cultures, the ability of Raman microspectroscopy to investigate
the molecular alterations in the nucleus of cells by an external insult by SSR insult was explored. Raman microspectroscopic analysis enables a direct analysis of the biochemical alterations in HaCaT cells due to SSR impact in the 3D model system, which can be directly compared to those observed in a 2D culture [12], [32]. Raman spectroscopic analysis provided clear signatures of the characteristic biochemical content of the nuclei of the cells. Notably, no strong background, attributable to auto-fluorescence emission was observed, although it has been demonstrated that such emission, at lower excitation wavelengths of 640nm, can be used to analyse oxidative effects of UV radiation [43]. The spectroscopic signatures related to SSR impact on cell nuclei are not clearly discernible in a plot of the averaged Raman spectra acquired from the nucleus of cells analysed immediately, or 24 hrs post exposure (Figure S1), and therefore, Raman spectra were subjected to the multivariate statistical techniques of PCA, to better visualise differences between exposed and non-exposed groups, and PLSR, to identify progressive spectral variations which are correlated with exposure time and cell viability. According to the PCA of figure 7.3, immediately after exposure, spectra of cells irradiated for 180 min were clearly differentiated from those of control cells. PLSR also indicates that these differentiating features are progressive over the period of SSR, consistent with the observations of the AB assay. The spectral features of both the PC loading and regression co-efficient are associated with DNA backbone moieties (1097 cm\(^{-1}\)) and C-O ribose (994 cm\(^{-1}\)), which suggests possible alterations to the main chain conformation of the DNA. [12] The co-efficient of regression against exposure time exhibits negative features related to nucleic acids (716 cm\(^{-1}\), 850 cm\(^{-1}\) and 1338 cm\(^{-1}\)), ribose and deoxyribose structures (918 cm\(^{-1}\) and 1417 cm\(^{-1}\)) which suffered direct damage upon exposure, while positive
features associated with DNA ($791\text{ cm}^{-1}$ and $1097\text{ cm}^{-1}$) and phenylalanine structure ($1006\text{ cm}^{-1}$ and $1210\text{ cm}^{-1}$) indicate modifications in these biological constituents. The bands related to ring breathing vibrations of phenylalanine ($1006\text{ cm}^{-1}$ and $1210\text{ cm}^{-1}$) and bending vibrations of guanine or adenine residues of DNA ($1583\text{ cm}^{-1}$) have been reported to be markers for UVR induced apoptosis in cells [44]. The bands assigned to glutathione ($625\text{ cm}^{-1}$ and $675\text{ cm}^{-1}$), corresponding to cells analysed immediately after irradiation, are considered a protective cell response to oxidative stress generated by UVR [45]. All these observations can suggest induction of single strand breaks, formation of bipyrimidine photoproducts and oxidative damage of bases, as a direct effect of SSR on cells [12], [44], [45]. To further investigate the biological mechanisms response to SSR exposure, the spectral profiles of cells analysed immediately and 24 hrs post exposure were compared using PCA and PLSR. Figure 6 shows Raman signals attributed to O-P-O stretching vibrations in DNA ($794\text{ cm}^{-1}$) and DNA backbone ($1093\text{ cm}^{-1}$). These bands can be correlated with internucleosomal DNA fragmentation in apoptotic cells [12], [33], [34]. In addition, the appearance of two bands at $791\text{ cm}^{-1}$ and $813\text{ cm}^{-1}$ may be related to non-coding RNA formation due to the ROS formation [21]. Associated with the disintegration of the DNA strands, a decrease in the protein content as presented in the negative bands associated with amide III ($839\text{ cm}^{-1}$) and amide I ($1640\text{ cm}^{-1}$) in the regression co-efficient can suggest activation of the caspase cascade in apoptotic cells [33].

These observations are consistent with those previously reported for 2D models and artificial skin models, in which DNA damage is mainly seen, immediately after irradiation, as an early stage of cytotoxicity and protein damage is mostly seen, 24 hrs after irradiation,
as a late response to radiation [12], [32]. Apart from the similarities between the two cell culture systems, there are signatures which were only identified in spectra of HaCaT cells cultured in 3D models. The bands located at 625 cm$^{-1}$ and 675 cm$^{-1}$, associated with an immediate cellular response to UVR insult [38], are absent in spectra of HaCaT cells cultured in 2D models. It has been reported that nuclear glutathione possess antioxidant properties which protects the DNA and DNA-binding proteins from external insults as ionising radiation [46]. However, it is also implicated in the reduction of the nuclear environment as cells pass from G1 to G2/M phases to prevent DNA damage upon breakdown of the nuclear membrane which is affected during solar radiation exposure [46], [47]. Note, that such mechanisms may account for an increased cell viability/proliferative capacity, as suggested by the AB responses in Figure 7.2. The absence of these two bands in 2D models can be attributed to an altered cell response to drugs, compounds or external stimuli (UVR) due to their unnatural microenvironment [4], [48], [49]. In contrast, cells cultured in a 3D environment acquire a spatial arrangement which better reproduces in vivo-like conditions which favours cellular responses to external stimuli and cellular functions such as proliferation, differentiation, gene and protein expression [4].

In terms of significance of the solar model, the full spectrum solar dose delivered by the Q-sun can be compared to equivalent doses in for example Naples, Italy (40°N, 12 noon, July 11th), Albuquerque, USA (38°N, noon, July 3rd) and Melbourne, Australia (38°S, solar noon, January 17th) [13], [50] It should of course be noted that, in vivo skin exposure is modulated by the melanin in the skin. Ultimately, monitoring similar effects using in vivo Raman microspectroscopy [51] may be of interest.
7.5 Conclusion

In this work, the effects of culturing HaCaT cells in a 3D microenvironment on the impact of SSR are evaluated. The combination of two commercial products for 3D culture showed the potential to reproduce a viable microenvironment for cell growth and proliferation. This 3D in vitro model served to study replicative cellular functions mimicking in vivo-like skin responses to SSR. Although the conventional cytotoxicity assay indicated a significant difference between the cellular responses in 3D compared to 2D culture environments, the assay responses cannot be directly compared, due to the differing bioavailability of the dye. Raman microspectroscopy provides more direct evidence of the similarities in cellular response, as well as the differences, which may derive from enhanced cellular protection mechanisms associated with the antioxidant glutathione. Thus, coupled with multivariate statistical analysis, Raman microspectroscopy has been demonstrated to be an ideal tool to investigate molecular changes in the nuclear compartment of HaCaT cells irradiated with SSR. Apart from cell cycle, the spectral analysis showed that the cellular response to SSR is modified when cells are transferring from 2D to 3D environments.

ACKNOWLEDGMENTS

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7.6 References


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Figure. S1 Mean Raman spectra of ctrl (i) and 30 min (ii), 60 min (iii), 90 (iv), 120 min (v) and 180 min (vi). Analysed immediately after irradiation (A) and 24 hrs post exposure (B). The coloured shaded area in each spectrum defines the standard deviation on the mean.
**Figure. S2** Principal Component Analysis (PCA) of Raman spectra of cell analysed (a) immediately after irradiation versus control and (b) 24 hrs post exposure versus control. The explained variance in a) PC1=41%, PC2=23% and PC3=8% and b) PC1=48%, PC2=14 and PC3=13%.
Figure. S3 Cross-validated MSEP curve for PLSR of Raman spectra of cells analysed immediately after irradiation regressed against exposure time (a) and cells analysed 24 hrs post exposure regressed against cell viability (b). The MSEP have units of minutes (a) and units of cell viability (b).

Figure. S4 Linear predicted response in PLSR of Raman spectra of cell analysed (a) immediately after irradiation versus viability and (b) 24 hrs post exposure versus cell viability. The solid line depicts the idealised 1:1 correlation.

Figure. S5 PLSR modelling of spectroscopic data, acquired 24 hrs post exposure regressed against time of exposure.
Raw Raman Spectra
Figure. S6 Raw Raman spectra acquired from HaCaT cells irradiated for 30 min, 60 min, 90 min, 120 min and 180 min and analysed immediately and 24 hrs post exposure. (imm= immediately after irradiation; rec= 24 hrs post exposure)
Table S1. Exact contribution for PC1 and PC2, between spectra of control and spectra of irradiated cells analysed immediately after irradiation.

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Table S2. Exact contribution for PC1 and PC2, between spectra of cells analysed immediately and 24 hrs post exposure.

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<td>PC2 (%)</td>
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Chapter VIII

Conclusions

8.1 Introduction

Cell culture technologies are invaluable tools employed in understanding cell biology. Traditionally, cell culturing has been carried out in two dimensions, without the structure and stroma of a real tissue. Cell morphology and functions such as viability, proliferation, differentiation, response to stimuli, gene and protein expression are thus limited or modified in the conventional 2D cell culture [1]. Alternative approaches of animal models became popular as they present anatomical and physiological similarities with humans [2]. However, a new challenge emerged when animal models showed limitations in mimicking complex process of human carcinogenesis, physiology and progression [3]. Moreover, new regulations against animal testing prompted the development of improved in vitro models [4]. The development of 3D cell culture offers a possibility to reproduce a microenvironment which mimics that of a real tissue [3], [5], [6]. To date, this technology has led to the development of skin models finding applications in pharmacology and toxicology [5]. However these models present limitations in their lipidic barrier function, [7] and commercially available options are delivered fully differentiated, limiting options for many fundamental studies. The field of development of suitable skin models to overcome the limitations of 2D models following the new EU regulations against human and animal testing is therefore still active [4]. In this thesis, the effects of culturing cells in
an elaborated 3D culture model, compared to a conventional 2D model, exposed to simulated solar radiation were explored. Rather than develop a fully stratified epidermis, the aim was to reveal the effects of using 3D matrices in a co-culture system on the biochemical changes in immortalised keratinocyte cells (HaCaT) exposed to SSR in comparison with the effects observed in HaCaT cells cultured in 2D models under the same conditions.

### 8.2 The 3D Model

Understanding the basic biology of skin and its function as the first line of body defence can prevent the development of diseases by the influence of external stimuli. The construction of a 3D in vitro skin model to study the photobiological effects of solar radiation at the molecular level lies within the field of multidisciplinary sciences. Different techniques, which have applications in the field of biology, chemistry and physics are combined in this study to accomplish the aims of this work. This human skin model, comprised of keratinocytes co-cultured with fibroblasts, served to gain further understanding of the cell-solar interaction at the level of the nucleus. Cell culture techniques served to build the skin layers (epidermis and dermis) by using two extracellular matrices and epidermal and dermal cells. The cell morphology and alterations to it due to the SSR insult were studied with histological staining techniques (H&E). The viability levels of cells exposed to SSR were monitored with the AB assay, whereas the proliferative capacity of cells to form colonies cultured in media supplemented with HS and FBS was examined with the clonogenic assay. Finally, the main technique for this study, Raman spectroscopy, coupled with multivariate analysis, provided the molecular characterisation
of human keratinocytes (HaCaT) and, in particular, changes to the biochemical content in
the nuclear compartment as a result of the irradiation.

8.3 The 3D model vs 2D cultures; effects of cell microenvironment.

Multiple studies have reported the use of extracellular matrices to culture cells in three
dimensions to have a closer approximation of in vivo-like conditions [5], [8], [9]. However,
it has been reported that cells can exhibit different behaviour when cultured in 3D in
comparison with 2D models. Although viability levels are not affected when translating
from 2D to 3D models, as verified via flow cytometry, the cell cycle can be altered when
cells are embedded in 3D membranes, as a lower proliferation rate was observed in
comparison with cells grown in monolayers [10]. Cell responses to external stimuli also
vary in different culture microenvironments. In radiation studies, 3D membranes were
reported to provide a protective effect due to the 3D nature of the cell growth environment
[11]. In the case of drug assessment, it was shown that 3D membranes affect the
concentration and dilution of the chemotherapeutic agent [12]. This could be related to the
absorptive nature of the matrix and a difference in the uptake rate of the cytotoxicity assay
by cells. The results support the use of 3D cultures in cytotoxicity assays to improve the
relevance of drug or toxin screening protocols, as there is no loss in cellular viability.

In contrast with previous studies, [12] this work reports that using human serum to
supplement the culture media can affect the proliferation capacity and health of HaCaT
cells in both 2D and 3D models. The Alamar blue assay and the clonogenic assay
monitored the viability and colony formation of HaCaT cells cultured in media with
different percentages of human serum respectively. As a future work, flow cytometry could
be an appropriate method to investigate the cell cycle alteration when HaCaT cells are supplemented with HS.

8.4 Effects of Simulated Solar Radiation

In the study of the effects of simulated solar radiation on epithelial human immortalised keratinocyte cells, histological staining (H&E) proved to be a suitable technique to visualise morphological changes in HaCaT cells as a result of SSR exposure. The AB assay was used to monitor the changes in proliferative capacity, in 2D compared with 3D, and in both cases immediately and 24 hours post irradiation. However, a direct comparison of the viability results is not appropriate, as cells were cultured in different microenvironments. Previously reported [10], [13], the performance of the cytotoxicity assay is affected when cells are embedded in a 3D matrix. A dilution factor attributed to the nature of the matrix must be considered when comparing the AB results in both culture systems.

8.5 Raman Microspectroscopic analysis

Raman spectroscopy as a bioanalytical tool was employed to compare the biochemical alterations occurring at the genomic level in both culture systems as a result of the exposure. Apart from improving the quality of the spectra, the multivariate analysis served to associate spectral variations to specific targets (cell viability and time of exposure). The spectral results suggests that solar radiation effects can be manifested immediately in modifications to the conformational structure of DNA, whereas changes in protein features are mostly seen as a later metabolic response. Moreover, 3D microenvironments better
reproduce in vivo-like conditions, favouring cellular responses to external stimuli, which are altered in 2D models due to their unnatural microenvironment.

8.6 Future Perspectives

The innovation of in vitro models has led to the development of three dimensional systems by embedding or culturing cells in 3D membranes. However, the use of these 3D matrices has been reported to modify cell cycle, signalling pathway, proliferation and responses to external stimuli in comparison to 2D models [10], [12], [14], [15]. It is important to critically assess the real impact of the 2D environment, compared to the more natural 3D environment of a cell. In chemotherapeutic studies, the apparent cell resistance to chemotherapeutic agents was seen to be due to different bioavailability, due to a reduced effective concentration of the chemotherapeutic agent within the matrix of the cell culture microenvironment in 3D models [12] [16]. Much of our understanding of drug efficacy and mechanisms of action is based on 2D in vitro models, and therefore further investigation and comprehensive assessment of the drugs performance within 3D cell culture models may be appropriate.

As reported in this work, 3D models have shown to better reproduce cellular responses to solar radiation insult compared to 2D models. However, a model which better reproduces the lipidic organisation within the stratum corneum is still needed. A human skin equivalent model which presents the full thickness of the stratum corneum, cell proliferation, differentiation markers and the right amount of proteins and lipids in the epidermis would serve to do toxicological and pharmacological studies in agreement with the ethical issues against animal tissue-based. Moreover, solar radiation related effects on the skin integrity
and its main function as a barrier against environmental stressors can be investigated, providing further insight in the development of skin cancer. In this context, Raman spectroscopy as a non-invasive technique has been employed to study human skin [17], animal derived skin and artificial skin models [18]. Various research works applied Raman spectroscopy for skin layers characterization, hydration levels measurements, chemotherapeutic agents permeation, cutaneous photodamage, etc [19]. Therefore, Raman spectroscopy is an ideal tool to perform a compositional analysis of skin in vitro and in vivo and modifications to it as results of external stimuli.

In this thesis, apart from the widely employed cytotoxicity assays, Raman microspectroscopy was proposed and used to reveal spectral similarities and differences between the two cell culture models. The combination of non-invasiveness, molecular specificity and high spatial resolution enabled the characterisation of cells and the identification of the immediate photo-induced response and metabolic later-term response caused by the radiation exposure. Thus, this study has demonstrated the versatile application of Raman spectroscopy in two different cell culture formats and the capacity to monitor biochemical modifications at a microscopic scale in the nuclear cell compartment upon the action of external stimuli as the solar radiation. The next step to improve the 3D in vitro model presented here would be to raise the culture to the air-liquid interface and keep it for at least one week to induce keratinocyte stratification and differentiation. [20] Then, the introduction of melanocytes in the co-culture and the production of melanin are of great importance in solar radiation effects studies. Melanin serves as a natural photoprotective pigment against solar radiation and it provides antioxidant and radical scavenging properties [21]. In this simplistic 3D in vitro model, melanocyte cells can be co-cultured to
study their protective role against solar radiation damage and repair signals that induce melanogenesis or to evaluate the cytotoxicity of drugs and chemicals.
8.7 Bibliography


Appendix 1: Publications


Appendix 2: Conferences and Modules

Conferences:

• CLIRCON, April 2017 – Poster presentation
• CLIRSPEC Summerschool, July 2017
• SPEC, April 2018 – Poster presentation
• Biophotonics Summerschool, June 2018
• ECSBM, August 2019 – Poster presentation
• Skin models in Cosmetic science: Bridging Established Methods and Novel Technologies, December 2019 – Oral presentation

Modules taken:

• Research Methods
• Multivariate analysis & Data preprocessing
• MATLAB course
• Applied Optics – Polarised Light
• Bioinformatics and Data analysis
• Work – based Learning and Employability Skills
• Advanced Chemistry