

Characterisation of a newly-developed scaffold for tissue regeneration

Rina Haryani Binti Osman Basah

Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
School of Dentistry

June 2021

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

The right of Rina Haryani Binti Osman Basah to be identified as Author of this work has been asserted by her in accordance with the Copyright, Designs and Patents Act 1988.

© 2021 The University of Leeds and Rina Haryani Binti Osman Basah

Acknowledgements

First and foremost, I am very grateful to GOD The Almighty who gave me blessing, courage, strength and patience to complete this work.

I would like to convey all my acknowledgement, appreciation and gratitude to my supervisors. To my principal supervisor, Dr Simon Wood for his patience, kindness and guidance during my PhD, whom always open the door for me when I need it the most and helped me build my confidence along this roller coaster journey. To Dr Xuebin Yang that gave me training and incredible help during every step of my laboratory work especially in tissue culture. To Dr Mike Raxworthy, whom never missed my supervision meeting and facilitated me in fabricating the scaffolds. To Professor Deirdre Devine who always supportive and generous with her precious advice and comments throughout my PhD for microbiology work. I would never have been able to finish this research without my supervisors' exceptional guidance, support, supervision, comments and perseverance.

Extended thanks to all Oral Biology staff especially Mrs Jaqueline Hudson, Mrs Claire Godfrey, Mrs Ruth Kayman, Mrs Julie McDermott, Miss Sarah Mayers, Mr Gregory Baugh, Mr Matthew Percival, Mrs Shabnum Rashid for all their assistance in purchasing, ethical approval for teeth collection, conference booking, microbiology training and so much more.

I would like to offer my special appreciations to all my colleagues from Oral biology for the beautiful memories we have together. Especially to Mr Kenny Man (we might do the vlog about PhD student's real life in the laboratory someday), Mrs Manal Matoug-Elwerfelli, Mrs Rasha Albannaa, Mr Fahad Al-Dabbagh, Mrs Aseel Al-Jaboori, Miss Lucia Pontiroli, Miss Monika Naginyte, Mr Ali Marie, Mr Shane McGettrick and many more.

I would like to acknowledge the Ministry of Higher Education in Malaysia for funding my PhD and for their invaluable administrative and financial support. Massive thank you for giving me the opportunity to challenge myself and push me out from my comfort zone.

Finally, I would like to send my everlasting love with warm thanks to my dear husband Rashdan Ismail, who always being patient and never leave me alone in this lonesome journey. To my lovely and joyous sons, Muhammad Rafique, Muhammad Razique and Muhammad Rifqi, all of you are the greatest gifts that send by God to me as I learn to be resilient and strong from all of you. Thank you to my parents Osman Basah and Normah, and my family for always supporting and have faith in me, without your support I would not have made it on my own.

Abstract

Introduction: The introduction of antimicrobial agents into tissue engineering scaffolds to overcome infection in regenerative medicine has been of great interest. Erythrosine B (ERY) and Methylene Blue (MB) are well-known photosensitisers (PS) that have been used in studies of antibacterial photodynamic therapy (APDT) and are also present in agents revealing dental plaque. The combination of scaffolds, PS and light irradiation may provide a new way to overcome oral infections and maintain oral/dental health during regenerative medicine procedures used to replace tissue which has been lost as a result of trauma or disease.

Aim: The primary aim of this work was to fabricate, characterise and evaluate the cytotoxicity and photodynamic therapy (PDT) efficacy of electrospun poly-(glycolic acid) (PGA) scaffolds containing ERY or MB. The secondary aim was to test the scaffolds' antibacterial PDT efficacy in preventing the growth of bacteria such as *Enterococcus faecalis* (*E. faecalis*) and *Escherichia coli* (*E. coli*).

Results: Morphological assessment of PS-containing scaffolds showed that the scaffold fibre diameter decreased as PS was introduced. However, while the diameter of the scaffold fibres decreased as the ERY concentration increased, no further changes were caused by increasing the MB concentration incorporated in the scaffolds. The degradation rates, PS released, and hydrophilicity of the scaffold increased concurrently with the PS concentration.

The cytotoxicity of engineered scaffolds with respect to human dental pulp stromal cells (hDPSCs) was tested. ERY scaffolds were able to sustain the

attachment and proliferation of hDPSCs, but after 72 h in culture, MB scaffolds showed cytotoxicity to hDPSCs as cell metabolic activity and DNA content was reduced, contrary to the results found with ERY scaffolds.

APDT was carried out on *E. coli* and *E. faecalis* bacteria. The high ERY or MB concentrations showed an antibacterial effect on *E. faecalis*, both in planktonic and biofilm culture. In *E. coli*, an antibacterial effect was only exhibited at a high concentration of MB (5%M) among the four scaffolds used.

Conclusion: The addition of PS to the PGA polymer solution prior to electrospinning resulted in the production of nanofibre scaffolds containing incorporated PS. These scaffolds possessed antibacterial properties that could be activated by light irradiation, resulting in the killing of both planktonic and biofilm-grown bacteria.

Table of Contents

Acknowledgements	iii
Abstract	v
Table of Contents	vii
List of Tables	xii
List of Figures	xiv
Abbreviations	xix
Chapter 1 Introduction	1
1.1 Regenerative medicine.....	1
1.2 Scaffolds in regenerative medicine	5
1.2.1 Poly-(glycolic acid) (PGA) as a scaffold	20
1.2.2 Electrospinning method in scaffold fabrication	23
1.2.3 Incorporation of antimicrobial agents into scaffolds	30
1.3 Antimicrobial resistance	33
1.4 Photodynamic therapy.....	39
1.4.1 Mechanism of action	41
1.4.2 Photosensitiser.....	45
1.4.2.1 Methylene Blue (MB).....	53
1.4.2.3 Erythrosine B (ERY).....	56
1.4.3 Light sources	58
1.5 Photodynamic therapy in regenerative medicine	61
1.6 Aim and objectives:	66
Chapter 2 Fabrication and characterisation of the Poly-(glycolic acid) scaffold incorporating photosensitiser (Erythrosine B or Methylene Blue)	67
2.1 Introduction.....	67
2.2 Aim and objective	68
2.2.1 Aim	68
2.2.2 Objectives.....	68
2.3 Materials and methods	69
2.3.1 Poly-(glycolic acid) (PGA) scaffold with or without photosensitiser	69
2.3.2 Characterisation of scaffolds architecture	76
2.3.3 Water binding capacity	76
2.3.4 <i>In Vitro</i> degradation studies.....	77
2.3.5 Photosensitiser release profiles	78

2.3.6 pH changes during degradation studies.....	81
2.3.7 Statistical analysis	81
2.4 Results	82
2.4.1 Photosensitiser loading efficiency	82
2.4.2 Effect of Erythrosine B and Methylene Blue additions on architecture, fibre diameter and porosity of the scaffolds	84
2.4.3 Effect of Erythrosine B and Methylene Blue incorporation on water binding capacity of the scaffolds.....	89
2.4.4 Effect of Erythrosine B and Methylene Blue incorporation on scaffold <i>in vitro</i> degradation	92
2.4.4.1 Scanning Electron Microscopy	92
2.4.5 <i>In vitro</i> photosensitiser release from scaffolds	98
2.4.6 pH measurement of culture media	101
2.5 Discussion	103
Chapter 3 Biological assessment and characterisation of the fabricated scaffolds using human dental pulp stromal cells (hDPSCs).....	115
3.1 Introduction.....	115
3.2 Aim and objectives	116
3.2.1 Aim	116
3.3 Materials and methods	117
3.3.1 Human dental pulp extraction and isolation from third molar teeth	117
3.3.7 Statistical analysis	128
3.4 Results	129
3.4.1 Scanning electron microscope evaluation.....	129
3.4.2 Evaluation of cell seeding efficiency 24 h post-seeding on the scaffolds	133
3.4.3 Evaluation of hDPSC proliferation on scaffolds by DNA content quantification	134
3.4.4 Evaluation of metabolic activity of hDPSCs growing on the scaffolds	136
3.4.5 Morphological observation of hDPSCs using actin staining.....	142
3.5 Discussion	145
Chapter 4 Photodynamic therapy effect on human dental pulp stromal cells (hDPSCs).....	152
4.1 Introduction.....	152

4.2 Aim and objectives	154
4.2.1 Aim	154
4.2.2 Objectives.....	154
4.3 Materials and Methods	155
4.3.1 Light intensity measurement for tungsten lamp	155
4.3.2 Photodynamic therapy effect on hDPSCs growing on tissue culture plates and PGA scaffolds.....	158
4.3.2.1 Preparation of the PS released from the produced scaffold.....	158
4.3.2.2 Preparation of the PGA scaffold.....	158
4.3.2.3 Photodynamic therapy of hDPSCs.....	159
4.3.2.4 CyQuant® Direct Cell Proliferation assay	161
4.3.2.5 Live/ Dead Cell Imaging Kit.....	164
4.3.3 Statistical analysis	164
4.4 Results	165
4.4.1 Photodynamic therapy of hDPSCs on tissue culture plates.....	165
4.4.1.1 Effect of photodynamic therapy on hDPSCs metabolic activity.....	165
4.4.1.2 Effect of photodynamic therapy on hDPSCs proliferation	168
4.4.1.3 Fluorescence microscopy evaluation of the effect of photodynamic therapy on hDPSCs viability in monolayer culture	171
4.4.2 Photodynamic therapy of hDPSCs cultures on PGA scaffolds	175
4.4.2.1 Effect of photodynamic therapy on human dental pulp stromal cells metabolic activity	175
4.4.2.2 Fluorescence microscopy evaluation of the effect of photodynamic therapy on the viability of hDPSC growing on PGA scaffolds.....	177
4.4.2.3 Effect of photodynamic therapy on the morphology of hDPSCs morphology growing on PGA scaffolds	180
4.5 Discussion	186
Chapter 5 Antimicrobial photodynamic therapy on selective bacteria.....	190
5.1 Introduction.....	190
5.2 Aim and objectives	191
5.2.1 Aim	191

5.2.2 Objectives.....	191
5.3 Materials and Methods	192
5.3.1 Brain Heart Infusion (BHI) broth	192
5.3.2 Luria Bertani (LB) broth	192
5.3.3 Preparation of Columbia blood agar (CBA) plates	192
5.3.4 Preparation of Mueller Hinton agar (MHA) plates	193
5.3.5 Bacterial selection	193
5.3.6 Gram stain protocol	193
5.3.7 Standard curve of optical density (OD) versus colony- forming unit (CFU) for each bacterial strain	195
5.3.8 Preparation of photosensitisers.....	196
5.3.9 Photodynamic therapy on planktonic bacterial cultures	196
5.3.10 Photodynamic therapy of biofilms on the fabricated scaffold	199
5.3.11 LIVE/DEAD® <i>BacLight</i> ™ Bacterial Viability kits	202
5.3.12 Scanning electron microscopy evaluation	202
5.3.13 Statistical analysis	203
5.4 Results	204
5.4.1 Anti-microbial photodynamic therapy on planktonic cultures.....	204
5.4.1.1 Effect of photodynamic therapy on bacterial viable counts	204
5.4.1.3 Effect of PDT on bacterial metabolic activity	207
5.4.2 Anti-microbial photodynamic therapy on biofilm using the scaffold incorporated with the photosensitiser	210
5.4.2.1 Effect of PDT on bacterial viable counts	210
5.4.2.2 Confocal microscopy evaluation of the effect of photodynamic therapy on bacterial cell viability:.....	212
5.4.2.3 Effect of PDT on bacterial metabolic activity:.....	215
5.4.2.4 Scanning electron microscopy evaluation after the photodynamic therapy on bacterial cell viability ...	217
5.5 Discussion	222
Chapter 6 General discussion, future work and conclusions.....	226
6.1 General discussion	226
6.2 Conclusions.....	238
6.3 Recommendations for future work and clinical translation	241

Appendices	244
Appendix A: Tissue bank approval form for teeth collection and the use of dental pulp stem cells.	244
Appendix B: Presentations and awards achieved.	245
Appendix C: Neotherix Ltd extrusion process	247
Appendix D: DiameterJ Installation and user instruction	255
List of References	264

List of Tables

Table 1.1 Advantages and disadvantages of natural and synthetic polymers used in scaffold fabrication for tissue engineering (Filippi <i>et al.</i> , 2020; Garg, 2011).....	9
Table 1.2 Methods of fabricating scaffolds with advantages, disadvantages and application (Prasad, Sankar and Katiyar, 2017; Eltom, Zhong and Muhammad, 2019).....	12
Table 1.3 The effect of different parameters on fibre morphology (Hasan, Azim and Reza, 2014).....	29
Table 1.4 Reactions occurring during photodynamic action (Josefsen and Boyle, 2008).	44
Table 1.5 PS families with their basic molecular structure (Soukos and Goodson, 2011; Allison <i>et al.</i> , 2004).....	48
Table 1.6 Properties of some PS dyes approved for PDT treatment and used in PDT-related clinical and preclinical trials (Ormond and Freeman, 2013).....	52
Table 1.7 Advantages and disadvantages of laser, LED and lamp sources (Kim and Darafsheh, 2020)	60
Table 2.1 Electrospinning protocols for each of the three different types of scaffolds.....	71
Table 2.2 Mean fibre diameter (n=9) (+/- standard deviation) and porosity percentage of fabricated scaffolds with or without incorporation of PS.	87
Table 2.3 Mean fibre diameter (n=9) with standard deviation of PGA, PGA-E and PGA-M scaffolds during in vitro degradation studies in three different media.	93
Table 2.4 Representative SEM micrographs of fabricated scaffolds after incubation in dH ₂ O for 24 h, 72 h and 168 h. Scale is 50 µm.	94
Table 2.5 Representative SEM micrographs of fabricated scaffolds after incubation in PBS for 24 h, 72 h and 168 h. Scale is 50 µm.	95
Table 2.6 Representative SEM micrographs of fabricated scaffolds after incubation in PBS with 10% FBS for 24 h, 72 h and 168 h. Scale is 50 µm.	96
Table 2.7 pH reading of media retrieved from the <i>in vitro</i> degradation studies for each scaffold. Media used were dH ₂ O, PBS and PBS with 10% FBS (n=9).	102
Table 3.1 Preparation of DNA Stock Solutions.....	122
Table 3.2 Preparation of bisBenzimide H33258 Solution 2 µg/mL from the 1 mg/mL stock solution.....	123

Table 3.3 Conditions for standard curve 20-2000 ng DNA range used for comparison with DNA concentration within the samples.....	125
--	------------

List of Figures

Figure 1.1 Schematic illustration of progression to peri-implantitis.	3
Figure 1.2 Classification of biodegradable polymers used in biomedical applications based on their original source (natural, synthetic, or semi-synthetic).	6
Figure 1.3 Methods of polymer processing techniques	11
Figure 1.4 Molecular structure of PGA ($C_2H_2O_2$) _n	20
Figure 1.5 Illustration of electrospinning set up.	23
Figure 1.6 Main mechanisms of antibiotic resistance by bacteria (Alav, Sutton and Rahman, 2018).	35
Figure 1.7 The biofilm life cycle: individual cells attach to a surface followed by growth and cell-cell interaction resulting in the formation of colonies and 3D structures which can also further detach in clumps (Jamal <i>et al.</i> , 2015).....	37
Figure 1.8 The photophysical and photochemical mechanisms of PDT.	43
Figure 1.9 Basic structure of haematoporphyrin derivatives (HpD) (Rousset, Bourré and Thibaud, 2003).....	49
Figure 1.10 Chemical structure of MB $C_{16}H_{18}ClN_3S$ (Soukos and Goodson, 2011).	53
Figure 1.11 Redox equilibrium of MB (Wainwright, 2011).	55
Figure 1.12 Chemical structure of the ERY (Silva <i>et al.</i> , 2015).....	56
Figure 1.13 The advantages of PDT for localised infections compared to antibiotic drugs (Dai, Huang and Hamblin, 2009).	62
Figure 1.14 Structure of Gram-positive bacteria cell membrane.....	64
Figure 1.15 Structure of Gram-negative bacterial cell membrane.....	64
Figure 2.1 A diagram of solution preparation and scaffold fabrication using the electrospinning rig.....	74
Figure 2.2 Representative standard curves used in the quantitative analysis of ERY release profiles.....	79
Figure 2.3 Representative standard curves used in the quantitative analysis of MB release profiles.....	80
Figure 2.4 Photographs of the PGA scaffolds with/without different percentages of ERY and MB.	83
Figure 2.5 Scanning electron micrographs of electrospun PGA scaffolds containing different percentages of PS.....	85
Figure 2.6 Higher magnification scanning electron micrographs of electrospun PGA scaffolds containing different percentages of PS.	86

Figure 2.7 Mean water binding capacity of PGA-E scaffolds with different percentages of ERY incorporated.....	90
Figure 2.8 Mean water binding capacity of PGA-M scaffolds with different percentages of MB incorporated.....	91
Figure 2.9 Cumulative release of ERY from PGA-E scaffolds after incubation for 24 h, 72 h, 168 h and 240 h in dH ₂ O, PBS and PBS + 10%FBS.	99
Figure 2.10 Cumulative release of MB from PGA-M scaffolds after incubation for 24 h, 72 h, 168 h and 240 h in dH ₂ O, PBS and PBS + 10%FBS.	100
Figure 2.11 Micro-ribbon fibre production in ERY highest percentage of PGA-E scaffold.....	104
Figure 2.12 Micro-ribbon fibre production in PGA-M scaffold incorporating 7.5% MB.	105
Figure 2.13 The morphology of the PGA only scaffold after 168 h (7 days) in media.	108
Figure 2.14 The morphology of the PGA-E scaffold after 72 h (3 days) in media.	109
Figure 2.15 The morphology of the PGA-M scaffold after 72 h (3 days) in media.	110
Figure 3.1 Hoechst 33258 or bisBenzimide dye (magenta) bound to the minor groove of DNA (green and blue) (from http://www.rcsb.org/structure/264D).	121
Figure 3.2 Representative standard curves used in DNA concentration determinations.....	125
Figure 3.3 Steps of scaffold dehydration.....	126
Figure 3.4 Representative images of SEM analysis of cellular interaction with electrospun fibrous scaffolds at 24 and 72 h (100X). On both days (24 h and 72 h) cells attached and spread on the PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J). Scale bars at 500 µm.	131
Figure 3.5 Representative images of SEM analysis of cellular interaction with electrospun fibrous scaffolds at 24 and 72 h (1000X). On both days (24 h and 72 h) cells attached and spread on the PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J). Scale bars at 50 µm.	132
Figure 3.6 Seeding efficiency of hDPSCs after 24 h seeding on the produced scaffold.	133
Figure 3.7 DNA content of hDPSCs on different scaffolds at 24 h and 72 h after seeding.	135
Figure 3.8: Metabolic activity of hDPSCs after 24 h culture on the scaffolds.....	137

Figure 3.9: Metabolic activity of hDPSCs after 72 h culture on the scaffolds.....	139
Figure 3.10 Comparison of the metabolic activity of hDPSCs after 24 and 72 h culture on the different scaffolds.....	141
Figure 3.11 Actin staining using Alexa Flour 488 phalloidin stain (10X). PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J).	143
Figure 3.12 Actin staining using Alexa Flour 488 phalloidin stain (20X). PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J)	144
Figure 4.1 Diagram of the <i>in vitro</i> PDT experiment in this study.....	157
Figure 4.2 Process for the photodynamic therapy of hDPSCs cultured on tissue culture plates and PGA scaffold.....	160
Figure 4.3 Representative standard curve for live cell number calculation within the sample.	163
Figure 4.4 Percentage of cell killing of hDPSCs using the cell metabolic assay after incubation with PS and light irradiation (0-60 min).	166
Figure 4.5 Percentage of live cells based on live-cell DNA detected by fluorescence intensity after incubation of hDPSCs with PS and followed by light irradiation (0-60 min).....	169
Figure 4.6 Representative fluorescence microscopy images of Live (C AM, green)/ Dead (PI, red) staining of hDPSCs after 30 min incubation with photosensitisers followed by light irradiation for 15, 30 and 60 min. 10x magnification. Scale bars are 75 μ m.	173
Figure 4.7 Representative fluorescence microscopy images of Live (C AM, green)/ Dead (PI, red) staining of hDPSCs after 24 h incubation with PS followed by light irradiation for 15, 30 and 60 min. 10x magnification. Scale bars are 75 μ m.....	174
Figure 4.8 Percentage of cell killing of hDPSCs based on metabolic activity after incubation with PS and light irradiation (0-60 min).....	176
Figure 4.9 Fluorescence microscopy images of hDPSCs cultured on PGA scaffold. Images were taken after a 24 h recovery period (following 30 min incubation with PS and irradiation with visible light for 15, 30 and 60 min). Cell viability was indicated with the Live/Dead stain. Scale bars are at 75 μ m.....	178
Figure 4.10 Fluorescence microscopy images of hDPSCs seeded on PGA scaffold. Images were taken after a 24 h recovery period (following 24 h incubation with PS and irradiation with visible light for 15, 30 and 60min). Cell viability was indicated with the Live/Dead® stain. Scale bars are at 75 μ m.	179

Figure 4.11 Confocal microscopy of hDPSCs seeded on PGA scaffold. Images were taken after a 24h recovery period (following 30 min incubation with PS and irradiation with visible light for 15, 30 and 60 min). F-actin and nuclei were stained with AlexaFluor 488 (green) and DAPI (blue) respectively. Scale bars are at 50 μ m.	181
Figure 4.12 Confocal microscopy of hDPSCs seeded on PGA scaffolds. Images were taken after a 24 h recovery period (following 24 h incubation with PS and irradiation with visible light for 15, 30 and 60 min). F-actin and nuclei were stained with AlexaFluor 488 (green) and DAPI (blue) respectively. Scale bars are at 50 μ m.	182
Figure 4.13 SEM images of hDPSCs on PGA scaffolds. Images were taken after 24 h recovery period (after 30 min incubation with PS and irradiation with visible light for 15, 30 and 60 min).	183
Figure 4.14 SEM images of hDPSCs on PGA scaffolds. Images were taken after 24 h recovery period (after 24 h incubation with PS and irradiation with visible light for 15, 30 and 60 min).	184
Figure 5.1 Protocol for the PDT of planktonic cultures of bacteria.	198
Figure 5.2 Protocol for the PDT of biofilms on fabricated scaffolds. ..	201
Figure 5.3 The survival of bacterial cells based on calculation of log CFU/ mL.	205
Figure 5.4 Fluorescence measurement for bacterial metabolic activity after treatment with PDT for 0 min and 60 min.	208
Figure 5.5 The survival of bacterial cells recovered from the scaffolds based on the calculation of log CFU per mL.	211
Figure 5.6 Representative images showing the viability of <i>E. faecalis</i> . The results of CLSM observation after Live/Dead staining of <i>E. faecalis</i> on the scaffolds and treated with PDT for 0 and 60 min. Figure 6.4 (a-j) show the typical microscopy images of CLSM observation after LIVE/DEAD staining of <i>E. faecalis</i> with (60 min) or without (0 min) light irradiation on PGA (control) (a and f), PGA-1%E (b and g), PGA-5%E (c and h), PGA-1%M (d and i) and PGA-5%M (e and j) groups. Scale bars are at 20 μ m.	213
Figure 5.7 Representative images showing the viability of <i>E. coli</i> . The results of CLSM observation after LIVE/DEAD staining of <i>E. coli</i> on the scaffolds and treated with PDT for 0 and 60 min. Figure 6.5 (a-j) show the typical microscopy images of CLSM observation after LIVE/DEAD staining of <i>E. coli</i> with (60 min) or without (0 min) light irradiation on PGA (control) (a and f), PGA-1%E (b and g), PGA-5%E (c and h), PGA-1%M (d and i) and PGA-5%M (e and j) groups. Scale bars are at 20 μ m.	214

Figure 5.8 Fluorescent signal measurement of bacterial metabolic activity on the scaffolds after treatment with PDT for 0 min and 60 min.....	216
Figure 5.9 Representative SEM images of <i>E. faecalis</i> on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated control; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M (e).Scale is 50 μ m.	218
Figure 5.10 Representative SEM images in higher magnification of <i>E. faecalis</i> on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated control; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M (e). Scale is 10 μ m.	219
Figure 5.11 Representative SEM images of <i>E. coli</i> on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated group; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M(e). Scale is 50 μ m.	220
Figure 5.12 Representative SEM images in higher magnification of <i>E. coli</i> on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated group; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M (e). Scale is 10 μ m.	221
Figure 6.1 The diagram of cell interaction with nanofibre of the scaffold. Adapted from https://smart.servier.com/smart_image/cell-9/	231
Figure 6.2 The conformations of Methylene Blue-DNA intercalation. Methylene Blue (MB) is shown in blue and the DNA with alternating GC base sequence in green (Rohs <i>et al.</i> , 2005).	232

List of Abbreviations

ALA	5-aminolaevulinic acid
ANOVA	Analysis of variance
APDT	Antimicrobial photodynamic therapy
ASC	Adipose derived stem cell
ARG	Antibiotic resistant gene
BHI	Brain-heart infusion
BMSC	Bone marrow mesenchymal stem cell
BSA	Bovine serum albumin
CFU	Colony-forming unit
CBA	Columbia blood agar
CH	Chondral phase
CLSM	Confocal scanning laser microscope
D	Substrate
D ⁺	Oxidized substrate (donor)
DMSO	Dimethyl sulphoxide
ERY	Erythrosine B
ECM	Extracellular matrix
EPS	Extracellular polymeric substances
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FE ³⁺	Ferric ion
FE ²⁺	Ferrous ion
FITC	Fluorescein isothiocyanate
GTR	Guided tissue regeneration
hDPSCs	Human dental pulp stromal cells
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
HGT	Horizontal gene transfer
LB	Luria bertani

LED	Light-emitting diode
LPS	Lipopolysaccharide trimer
MB	Methylene Blue
MHA	Mueller-hinton agar
NCTC	The National Collection of Type Culture
$O_2^{\bullet -}$	Superoxide anion
$O_2^{\bullet}$	Superoxide radical
1O_2	Singlet oxygen
$^1O_2^{\bullet}$	Reactive singlet oxygen
3O_2	Ground state molecular oxygen
OD	Optical density
H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl radical
PS	Photosensitiser
PS^-	Reduced PS
$PS^{\bullet -}$	PS anion radical
$PS^{\bullet +}$	PS cation radical
1PS	Ground state PS
$^1PS^*$	Excited singlet state PS
$^3PS^*$	Excited triplet state PS
PB	Presto Blue
PBS	Phosphate-buffered saline
PCL	Poly- ϵ -caprolactone
PDT	Photodynamic therapy
PLA	Poly-(lactic acid)
PGA	Poly-(glycolic acid)
PLGA	Poly-(lactic-co-glycolic acid)
PGA-E	PGA scaffold incorporated with Erythrosine B
PGA-M	PGA scaffold incorporated with Methylene Blue

PGA-PS	PGA scaffold incorporated with photosensitiser
PHA	Poly-(hydroxyalkanoates)
PET	Poly-(ethylene terephthalate)
PETA	Pentaerythritol triacrylate-co-trimethylolpropane
PMMA	Poly-(methyl methacrylate)
PHB	Poly-(hydroxybutyrates)
PBAT	Poly-(butylene adipate-co-terephthalate)
SCPL	Solvent casting and particulate leaching
SNP	Silver nanoparticles
TCP	Tricalcium phosphate
PpIX	Protoporphyrin IX
ROS	Reactive oxygen species
r.p.m	Revolutions per minute
RS	¼-Strength Ringers Solution
NaOH	Sodium hydroxide
SEM	Scanning electron microscopy
2D	Two dimensional
3D	Three dimensional
1%E	1 percent of Erythrosine B
5%E	5 percent of Erythrosine B
1%M	1 percent of Methylene Blue
5%M	5 percent of Methylene Blue

Chapter 1

Introduction

1.1 Regenerative medicine

Regenerative medicine is an interdisciplinary field that is concerned with the replacement or regeneration of human tissue or organs, to restore or establish normal function (Mason and Dunhill, 2008). Classically, it involved tissue engineering which derived from a combination of cells, scaffolds and growth factors to achieve regeneration of the human tissues or organs (Badylak, 2005). FDA (Food and Drug Administration) approval of artificial skin and cartilage are examples of tissue engineering applications which are accepted for clinical use (Capo *et al.*, 2014).

Previous researchers had initial success in redeveloping and reconstructing bladder, blood vessels, skin, trachea and vision through tissue engineering and regenerative medicine techniques (Colombo *et al.*, 2016). In bone regeneration, a combination of bone marrow stromal cells and demineralised bone matrix exhibited osteogenic and angiogenic properties in a defect fibula bone model, thus promoting new fibular bone regeneration (LoGuidice, Houlihan and Deans, 2016). Previous research was also able to construct and implant a tissue-engineered vascular graft for small diameter arteries in mice (Kurobe *et al.*, 2015). Engineered skin was first reported in 1979 where human epidermal cells were used for skin grafting (Green, Kehinde and Thomas, 1979). In bladder repair, a bladder reconstructed using collagen scaffolds seeded with the patient's cells and engineered bladder biopsies showed adequate structural architecture and phenotype of a bladder (Atala *et al.*,

2006). Mooney and Vacanti achieved the development of liver tissue, cartilage and bone tissue as well as epithelial tissue for intestine using a synthetic polymer that was seeded with hepatocytes, chondrocytes/ osteocytes and uroepithelial cells (Mooney *et al.*, 1997; Mooney *et al.*, 1996; Vacanti *et al.*, 1995; Vacanti *et al.*, 1993). Recently, artificial intestine was developed using primary organoid cultures (mouse or human) and nonwoven scaffolds of poly-(glycolic acid) (PGA)/ poly-(lactic-co-glycolic acid) (PLGA) that was implanted for 4 weeks in mice and which demonstrated digestive and absorptive function upon extraction (Grant *et al.*, 2015).

Oral disease such as periimplantitis and trauma will result in tissue damage. In the repair of the oral tissues, implants such as bone substitutes and grafts, metallic dental implants, and guided tissue regeneration (GTR) are in common use (Thomas and Puleo, 2011). However, implants are susceptible to infection which is caused by microbial colonisation and can result in peri-implantitis (Fig. 1.1). The infection eventually leads to disruption of the osseointegration process and promotes implant failure (Bijukumar, McGeehan and Mathew, 2018; Pye *et al.*, 2009; Tanner *et al.*, 1997).

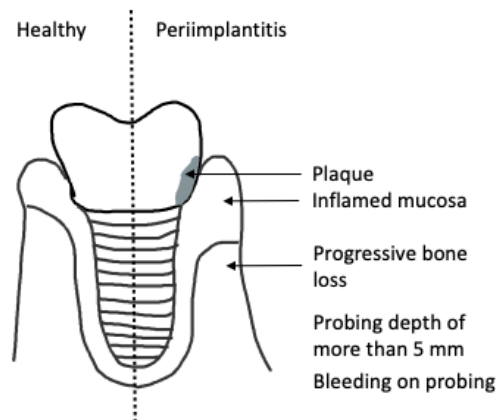


Figure 1.1 Schematic illustration of progression to peri-implantitis.

After implant introduction, the bacteria will colonise the soft tissue and dental implant which can lead to the development of peri-implant mucositis. Over time, the bacteria cause the gum to become inflamed with accompanying deterioration of the bone supporting the dental implant.

GTR is a periodontal regenerative technique that promotes cell repopulation and tissue regeneration in the periodontal defect area (Needleman *et al.*, 2006). Tissue regeneration is promoted by using a barrier material that will avoid any contact between the gingival epithelium and the root surface, thus, giving space and support needed for regeneration of the tissues (Thomas and Puleo, 2011). After any surgical placement, the barrier materials are rapidly colonised by periodontal pathogens (De Sanctis, Zucchelli and Clauser, 1996). Consequently, the prognosis for the treatment will be compromised if any deep pockets between the implant or tooth and bone or mucosa are present as these pockets serve as bacterial reservoirs that will expedite more microbial colonisation on the barrier materials (Nowzari and Slots, 1995). The common membrane barrier materials used in the GTR procedure are made from synthetic polymers (polytetrafluoroethylene and aliphatic polyesters), natural polymers (collagen, chitosan and alginate), metals (titanium alloy and cobalt-chromium alloy) and inorganic compounds (calcium sulfate and calcium phosphate) (Elgali *et al.*, 2017). The use of aliphatic polyesters such as PGA (poly-(glycolic acid)), PLA (poly-(lactic acid)) and PCL (poly- ϵ -caprolactone) as a bioresorbable membrane is also commonplace for the reconstruction of bone or tissue defects in the tissue regeneration field.

1.2 Scaffolds in regenerative medicine

Scaffolds play a significant role in regenerative medicine. They serve as three-dimensional (3D) tissue templates and are intended to provide synthetic extracellular matrix (ECM) microenvironments for cell attachment, proliferation, differentiation, and tissue neogenesis (Kim *et al.*, 2013; Kim and Mooney, 1998). The porous nature and 3D features of a scaffold also enable longer cell survival times by allowing the diffusion of nutrients, oxygen and bioactive factors (Hosseinkhani *et al.*, 2014). Scaffolds can be fabricated from polymers, bio ceramics and hybrid materials whether it is from natural or man-made sources. There are various kinds of polymers used to fabricate scaffolds in regenerative medicine including synthetic polymers, semi-synthetic polymers, naturally derived polymers and tissue-derived scaffolds (Fig. 1.2).

Scaffolds fabricated from synthetic polymers are reproducible and modifiable in order to refine their mechanical properties, porosity and their morphologies. PGA, PLA, PCL and polyester are examples of synthetic polymers (Mohiti-Asli and Lobo, 2016). Synthetic polymers are known for their good mechanical strength when compared to naturally-derived polymers. However, due to their hydrophobic surface and lack of cell-scaffold interaction, naturally-derived polymers may have more of an advantage (Armentano *et al.*, 2010). Cui and co-workers exhibited successful repair in articular cartilage regeneration using chondrogenic-induced adipose derived stem cells (ASCs) and PGA/PLA scaffold after implantation in a porcine model for 6 months (Cui *et al.*, 2009).

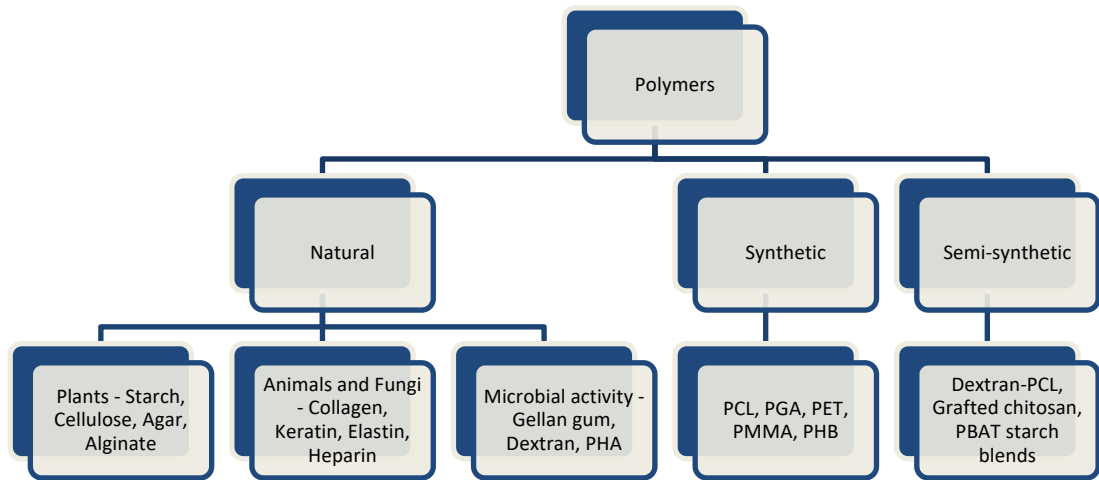


Figure 1.2 Classification of biodegradable polymers used in biomedical applications based on their original source (natural, synthetic, or semi-synthetic).

PHA (poly-(hydroxyalkanoates)); PET (poly-(ethylene terephthalate)); PMMA (poly-(methyl methacrylate)); PHB (poly-(hydroxybutyrates)); PBAT (poly-(butylene adipate-co-terephthalate)).

Another study showed that electrospun PCL membranes freeze-dried with a tricalcium phosphate (TCP) bone stratum were able to successfully regenerate osteochondral defects in a rabbit model when used as a periosteal scaffold. *In vitro* results also showed that the orientation and penetration of bone marrow mesenchymal stem cell (BMSCs) on the PCL-TCP membrane scaffold was promoted (Liu, Liu and Cui, 2014).

Naturally derived polymers are known to be more biocompatible than synthetic polymers (Shin, Jo and Mikos, 2003). However, their mechanical properties are weak and are not easily modified. Some of them are also insufficient in supply and not cost effective. Collagen, chitosan, fibrin and alginate fall under the naturally derived polymer category (Bao Ha *et al.*, 2013). Hydrogels that consist of collagen type I have been used as a dermo-epidermal skin substitutes by Hartmann-Fritsch and co-workers. They tested the dermo-epidermal skin substitutes *in vivo* using immune-incompetent rats and after a predetermined time, the substitutes were successfully grown into a well-stratified epidermis distributed homogenously over the skin grafts (Hartmann-Fritsch *et al.*, 2016). Electrospun gelatin-hydroxyphenylpropionic acid (Gel-HPA) also showed successful soft tissue regeneration which promotes cell adhesion, penetration and vascularisation after one month implanted subcutaneously into the dorsal region of rats (Nie *et al.*, 2020). Nevertheless, the particular source and extraction process of natural polymer can profoundly influence their qualities and their biological interactions. This means that it is difficult to directly compare those scaffolds that have been derived using different sources or extraction processes, a significant drawback in tissue engineering (Bonani *et al.*, 2018; Bhattarai *et al.*, 2018).

Tissue-derived scaffolds are obtained through the decellularization of a tissue or organ. Decellularization is achieved by a clearing of the cellular components from the tissues or organs so that the resulting template consists of extracellular matrix to be used as scaffolds (Tapias and Ott, 2014). Research by Kajbafzadeh's group demonstrated recellularization of the acellular rat colon-derived scaffold 9 months after grafting. Recellularization was shown by the presence of smooth muscle cells, endothelial progenitor cells, and neural elements in decellularized colon (Kajbafzadeh *et al.*, 2020). The decellularization method has the advantage of giving the similar mechanical and biological properties of the decellularized ECM that may promote cell growth and tissue regeneration. However, there is lack of homogeneous cell adhesion and spreading in decellularized tissue scaffolds. In addition, incomplete removal of cellular components during the production process may induce an immune response at the implantation site (Zheng *et al.*, 2005).

The advantages and disadvantages associated with the biomedical use of natural and synthetic polymers are listed in Table 1.1.

Table 1.1 Advantages and disadvantages of natural and synthetic polymers used in scaffold fabrication for tissue engineering (Filippi *et al.*, 2020; Garg, 2011)

Scaffold	Properties	Advantages	Disadvantages
Synthetic polymers	Mechanical and degradation properties can be adjusted by varying the polymer properties.	Excellent biocompatibility, good bioresorbable and degradation rate. Defined purity and reproducible chemical/mechanical properties. Low immune response. Low production cost. Off-the-shelf availability and production in large uniform quantities.	Produce local acidic condition from degradation products. Inflammatory response is possible. Poor cell adhesion, poor compression strength
Naturally derived polymers	Properties dependent on extraction and processing procedures.	Natural origin. Good biocompatibility, bioresorbable and degradation rate. Good cell recognition and adhesion sites. Not required harsh chemicals for processing.	Inadequate mechanical properties. Difficult to maintain structural integrity. Expensive production. Difficult processing. Susceptible to cross-contamination and have low stability.

Methods used in the fabrication of scaffolds for tissue engineering and regenerative medicine applications include emulsion freeze-drying (Sultana and Wang, 2012); foaming (Cho *et al.*, 2008); particle leaching (Xin *et al.*, 2016); electrospinning (MacKintosh *et al.*, 2015); sintering (Wiria *et al.*, 2007); and polymer casting and phase separation (Papenburg, 2009) as well as 3D printing (Fierz *et al.*, 2008). Figure 1.3 shows the various fabrication techniques of scaffolds that fall under conventional and rapid prototyping techniques (Eltom, Zhong and Muhammad, 2019).

Conventional fabrication techniques include solvent casting and particulate leaching, gas-foaming, emulsion freeze-drying, electrospinning, powder compaction and thermally induced phase separation. The classification of conventional and rapid prototyping scaffold fabrication techniques are summarised in Table 1.2.

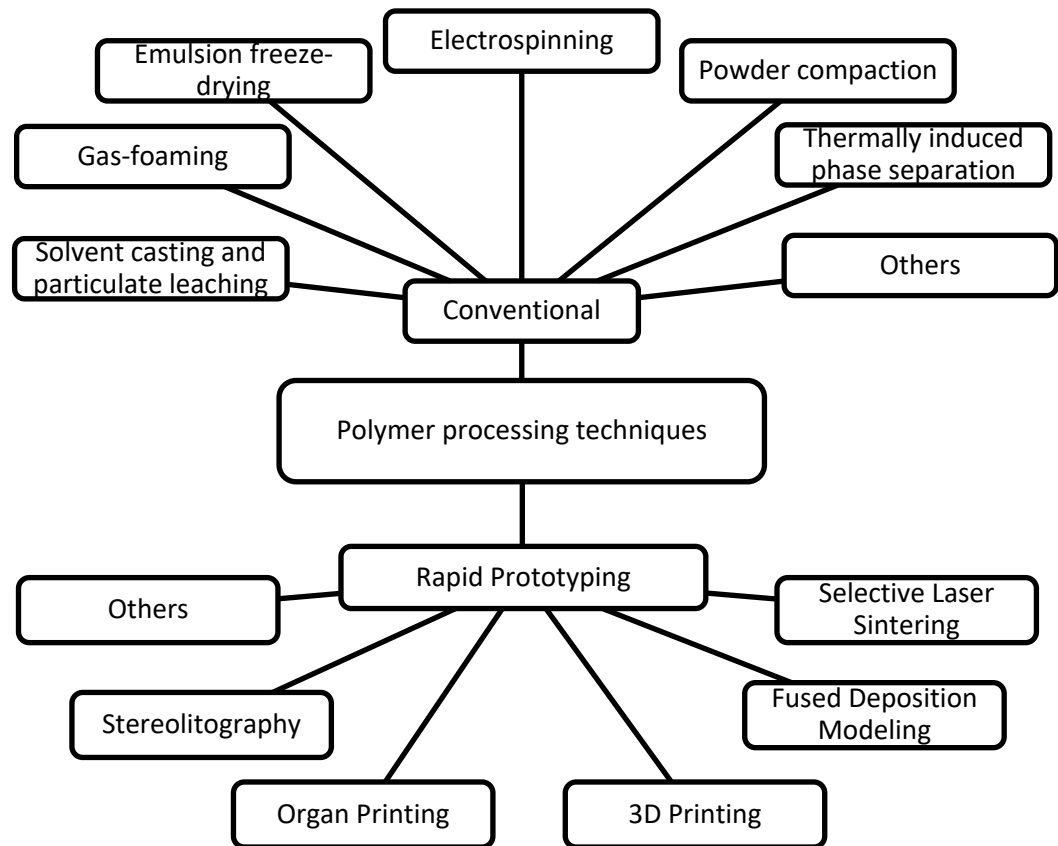


Figure 1.3 Methods of polymer processing techniques

Polymers were processed to produce scaffolds with different architectures to suit the application. This includes hydrogels, sponges, fibrous scaffolds, micro or nanoparticles, biofilms and membranes (Filippi *et al.*, 2020).

Table 1.2 Methods of fabricating scaffolds with advantages, disadvantages and application (Prasad, Sankar and Katiyar, 2017; Eltom, Zhong and Muhammad, 2019).

Fabrication technique	Advantages	Disadvantages	Applications
Solvent casting and particle leaching	Fits thin membrane of thin wall three-dimensional specimens High porosity (50%-90%) Low cost technique Biodegradable controlled porous scaffold	Time consuming since thin membranes are only used The widespread use of very toxic solvents, residual and porogen material Limited mechanical properties	Bone and cartilage tissue engineering (Sola <i>et al.</i>, 2019)
Gas foaming	Porosity up to 85% Controlled porosity and pore structure sponge	If the fabrication process is not combined with other processing techniques, the product obtained might have a closed pore structure or a solid polymeric skin Limited mechanical properties and inadequate pore interconnectivity Inconsistent production	Drug delivery and tissue engineering (Costantini and Barbetta, 2018)
Emulsion freeze-drying	Capability of obviating high temperature Pore size can be controlled by changing the freezing method 3D porous sponge structure, durable and flexible	High energy consumption Long-term timescale The use of cytotoxic solvents Small production Irregular pore sizes	Tissue engineering and regenerative medicine (Du <i>et al.</i>, 2020)
Electrospinning	Produce nanoscale fibrous scaffolds Homogenous mixture made of fibres with high tensile strength	Solvent used can be toxic Processing parameters depend on many variables	Drug delivery, wound healing, soft tissue synthetic skin, and scaffolds (Chong <i>et al.</i>, 2019)

	High surface area and good biomechanical properties	Pore size decreases with fibre thickness	
Thermally-induced phase separation	Low temperature can be utilised for the integration of bioactive molecules Highly porous scaffold with interconnected pore morphology	Limited used Difficult to precisely control scaffold morphology.	Thermoplastic (Han, 2019)
Stereolithography (SLA)	Reduces wastage in relation to the subtractive fabrication method High resolution Uniformity in pores and connectivity	Limitation in the process of photopolymerization Requiring massive amount of monomers and postpolymerization treatment to improve monomer conversion Production in small batches Cannot withstand high pressure forces	Cabin accessories (Najmon, Raeisi and Tovar, 2019)
Selective laser sintering	Uses ultrahigh-molecular-weight polyethylene Provides excellent control over the microstructures of the produced scaffold by adapting SLS process parameters Utilized to obtain the preferred properties of the created scaffold.	Steps required to remove injected powder High operating temperature Rapid cooling after heating effect causes deformation and shrinkage of structure	Bio-metal scaffold (Liu <i>et al.</i> , 2013)
Solvent-based extrusion freeforming (SFE)	Can be utilized to make ceramic, metal, and metal/ceramic composite parts It can directly or indirectly function in printing the actual part or a mould	Temperature extrusion The design includes a change to the parameters affecting extrusion pressure, including nozzle length-to-diameter ratio, a paste formulation and the extrusion velocity.	Ceramic 3D scaffolds (Lu <i>et al.</i> , 2010)

	Precise control of scaffold structure at the micron level Able to follow the structure of the natural tissue and the mechanical characteristics of the scaffold		
Bioprinting	Low costs Higher accuracy and greater shape complexity The high speed of printing with the capability of supporting parallel high cell viability (80-90%)	Depend on the existence of cells	Tissue regeneration (Rider <i>et al.</i> , 2018)
Fused deposition modelling (FDM)	Useful in scaffold design under the different aspects of scaffold fabrication Low temperature deposition	Limited application in biodegradable polymers	Additive manufacturing (Chacón <i>et al.</i> , 2017)

Solvent casting and particulate leaching (SCPL) uses solvents such as chloroform combined with salt particles and polymer to produce a scaffold. The salt particles are insoluble in solvent and thus, when the solvent evaporates and the scaffold is submerged in warm water, the salt particles dissolve, leaving the scaffold with a highly porous structure (Prasad, Sankar and Katiyar, 2017).

In the **gas foaming** process, a gas (typically CO₂) is applied at an elevated pressure in order to solidify the polymer discs until they reach saturation. The spongy structure of the scaffold created by the infiltration of the gas bubbles results from the high pressure gas becoming soluble in the polymeric solid phase (Gorth and J Webster, 2011). As the pressure is reduced to ambient

pressure, inducing instability, the gas begins to phase separate from the polymer and causes pore formation. However, the interconnectivity of the pore network formed by this technique is still limited due to the formation of a closed-pore structure due to the rapid depletion of the gas between pores (Carter and Bhattarai, 2013). In order to improve pore connectivity, this technique needs to be combined with other techniques such as particulate leaching (Hutmacher, Woodfield and Dalton, 2014).

A polymer solution is dissolved in an organic solvent and water for **emulsion freeze-drying**. It is promptly cooled down to maintain the liquid state structure after the solution is homogenised. To extract solvent and water and further generate a porous structure, the liquid state structure undergoes a freeze-drying phase (Walker and Santoro, 2017). Freeze drying is generally used for scaffold preparation, but some of the drawbacks associated with this technique include longer time needed, high energy consumption, the use of cytotoxic solvents and the creation of closed pores (Wahid *et al.*, 2018).

In the **thermally-induced phase separation** technique, high temperature is needed to dissolve the polymers in the solvent. After the polymers are dissolved, the solution is induced to a solid-liquid or liquid-liquid phase separation by decreasing the temperature. The porous structure of the scaffold is then produced by a sublimation process that eliminates the solidified solvent-rich phase (Martínez-Pérez *et al.*, 2011). The key drawbacks are that it is limited to certain polymers (such as PEG/PLLA and PLA-dextran blend) and its processing technique requires an organic solvent (dioxane) (Akbarzadeh and Yousefi, 2014).

More advanced processes such as stereolithography, solvent-based extrusion free forming, selective laser sintering, fused deposition modelling and bioprinting fall under rapid prototyping fabrication techniques.

The process of **stereolithography** is also known as manufacturing scaffolds with layers of polymer resin. A light-emitting device is used to cause photosensitive liquid polymerisation, accompanied by polymerized solid formation. In order to extract the residual resin, the post-polymerization process involves rinsing the produced scaffold in solvent (Walker and Santoro, 2017; Viera Rey and St-Pierre, 2019). This process, however, requires biodegradable and photocurable materials such as resin that are limited in supply and thus this method is typically used in small scale production (Kundu *et al.*, 2013; Salonitis, 2014).

Selective laser sintering (SLS) is a type of powder bed fusion process. A powder of polymer is sintered by controlled laser beam scanning that induces powder molecular interaction, followed by polymer solidification in layers (Muzaffar *et al.*, 2020). In **solvent-based extrusion freeforming**, the structure is fabricated from a paste of a polymer powder and a binder in a solvent. The paste undergoes extrusion and then is transformed into a solid state by solvent evaporation. After solvent removal, the fabricated polymer filaments undergo debinding and sintering processes (Lu *et al.*, 2010). The poor surface finishing of fabricated parts is one of the major limitations of SLS and the scaffold produced appears to have high shrinkage rates, which require post-processing procedures (Munir, Li and Wen, 2017).

Bioprinting is a technique that provide alternative ways to regenerate tissue. In bioprinting, the scaffolds are produced with bio-ink consisting of live cells,

biomaterials and additional biological substances such as growth factors (Semba, Mieloch and Rybka, 2020). Bio-ink modulation of, for example, cell types and biomaterials, are crucial in the production of the optimised scaffold. The bioprinting technique has been investigated using various types of cells in order to regenerate tissue (Mandla and Radisic, 2019; Williams, Yoo and Atala, 2019; Gillispie *et al.*, 2019). As well as being time consuming due to bio-ink preparation, production processes such as the layer by layer printing technique, also present difficulty in printing complex hollow 3D structures using this technique (Mukherjee, Rani and Saravanan, 2019).

The **fused deposition modelling (FDM)** technique uses a polymer material that is heated until it is transformed into a semi-liquid state. The heated polymer material is then extruded and solidifies rapidly to form layers of construct (Chacón *et al.*, 2017; Masood, 2014). Due to the high temperature, the inclusion of cells or biological materials in the process is not possible. The FDM technique also needs preformed fibres with consistent size and material properties due to the need to go through the rollers and nozzle (Altuntaş, Özkan and Yener, 2017).

In this study, the electrospinning method was chosen based on previous research with Neotherix Ltd. Basically, because of the manufacturing and planning process, all techniques have benefits and drawbacks. Scaffolds produced via the electrospinning method have a high surface to volume ratio, more tuneable porosity, and are easily adjustable to the desired fibre size, shape and composition to achieve the desired functionality.

Electrospinning produces a range of fibres from microfibres to nanofibers and smaller pores which are reflected in the higher surface area than fibres

produced from other methods (Cortez Tornello *et al.*, 2016). Electrospun fibres have been fabricated in various fields such as tissue engineering scaffolds, clothing fabrication and healthcare (Mo *et al.*, 2019). The characteristics and properties of the fibres can be changed by adjusting the solution and process parameters of electrospinning. Electrospinning is a simple and cost-effective method to produce scaffolds with a dense woven network of fibres compared to self-assembly and phase separation techniques (Shi *et al.*, 2015).

Some of the ideal characteristics of synthetic polymeric scaffolds were reviewed and listed (Hosseinkhani *et al.*, 2014; Kim and Mooney, 1998; O'Brien, 2011; Murphy, Haugh and O'Brien, 2010) as:

- 1) Promoting new tissue growth and providing proper surface properties that facilitate cell adhesion, proliferation and differentiation;
- 2) Biocompatibility – the cells can adhere, migrate, proliferate and interact on the scaffolds with no immunological rejection that can cause rejection by the host tissue;
- 3) Biodegradability – the scaffold must be able to degrade over time in order to allow cells to generate their own ECM and the by-product of degradation must be non-toxic and not interact with other organs during removal from the body;
- 4) Scaffold architecture - high porosity and high surface area to volume ratio is desired, with an integrated pore network for cell growth which also permits flow transport of nutrients and metabolic waste. The pore size needs to be optimised to allow the cells to penetrate the network of fibres and to permit efficient binding of cells on the fibres;
- 5) Adequate mechanical properties to withstand *in vivo* stresses – the fabricated scaffold must have adequate mechanical properties that are acceptable for the tissue properties at the implantation site;
- 6) Manufacturing technology – the manufactured scaffold must be capable of being produced cost-effectively in batch processing. Off-the-shelf availability is preferable for medical practitioners as it is less time consuming.

1.2.1 Poly-(glycolic acid) (PGA) as a scaffold

One example of a polymer used as a scaffold material is PGA (Fig. 1.4). PGA scaffolds made using electrospinning methods possess a dense mesh that has high porosity and surface-area-to-volume ratio, thus facilitating new tissue formation (MacKintosh *et al.*, 2015; Bonfield, 2006; Kim and Mooney, 1998). PGA is a rigid thermoplastic material with high crystallinity and is not soluble in most organic solvents (Generali, Dijkman and Hoerstrup, 2014). It is approved by the United States Food and Drug Administration (F.D.A) for clinical use and has previously been used to support the growth of human umbilical cord stromal cells, adipose stem cells and periodontal ligament cells (Mahmoudifar and Doran, 2010; Zhao and Detamore, 2010; Li *et al.*, 2006; Wang *et al.*, 2005). Recently, PGA scaffold was used to develop a functional 3D *in vitro* model of the endometrium with bovine stromal and epithelial cells (MacKintosh *et al.*, 2015). PGA is widely used in clinical settings such as in surgical sutures as it is biodegradable, hydrophilic and highly biocompatible with human tissue (Casagrande *et al.*, 2011; Pillai and Sharma, 2010; Frazza and Schmitt, 1971).

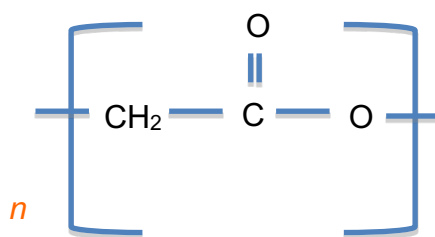


Figure 1.4 Molecular structure of PGA (C₂H₂O₂)_n.

Degradation of PGA is by a non-enzymatic hydrolysis process, with the eventual production and removal of carbon dioxide and water, which are non-toxic to the human (Giuliani *et al.*, 2014), and this provides advantages for PGA polymer when used in tissue engineering. The biodegradation of PGA is completed when PGA undergoes all four steps of the degradation mechanism: hydration, initial degradation, further degradation and solubilisation (Li *et al.*, 2017; Fredenberg *et al.*, 2011).

- (i) Hydration: The penetration of the aqueous medium into the polymer matrix will interfere with the secondary forces, which lead to the relaxation and reduction of the glass transition temperature (Chen *et al.*, 2020);
- (ii) Initial degradation: After hydrolysis, the cleavage of the covalent bonds in the polymer backbone starts in the hydrated region of the polymer, resulting in the polymer's molecular weight reduction. As the hydrolysis process proceeds, more and more carboxylic end-groups auto-catalyse the hydrolysis reaction within the polymer matrix, leading to a continuous decrease in the polymer's molecular weight. Along with the decrease in molecular weight, the polymer loses its mechanical strength, but the polymer's integrity remains (Siye *et al.*, 2014; Zhang *et al.*, 2014a).
- (iii) Further degradation: The polymer's molecular weight keeps decreasing to a threshold so that the polymer's integrity can no longer be maintained (Siye *et al.*, 2014) . A significant mass loss will be detected at this point.
- (iv) Solubilization or erosion: The polymer further loses its weight and

the fragments are further cleaved to monomer and oligomer which are soluble in the medium (Low *et al.*, 2020).

Inherent properties that may affect the PGA degradation rate include composition of the copolymer, molecular weight, shape, and indirect glass transition temperature and crystallinity factors that depend on the composition of the copolymer (Alexis, 2005).

1.2.2 Electrospinning method in scaffold fabrication

Electrospinning is one of the methods used to fabricate nanofibrous scaffolds. It is a process that is a combination of 2 techniques: electrospray and spinning (Agarwal, Wendorff and Greiner, 2009). This technique produces a scaffold with fibres that have a high surface to volume ratio and fibre diameter ranging from several microns to nanometres (Haider, Haider and Kang, 2015; Hasan, Azim and Reza, 2014). The basic electrospinning equipment consists of a syringe loaded with a polymer solution, metallic needle, power supply and metallic collector (Fig. 1.5).

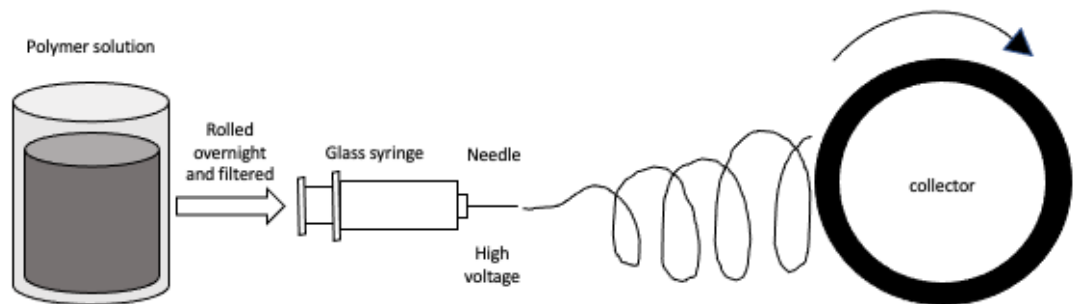


Figure 1.5 Illustration of electrospinning set up.

The polymer solution is loaded in the syringe and ejected from the needle when a high voltage is applied. The thin fibres are formed by the ejected solution and collected by the rotating mandrel.

Briefly, the electrospinning process starts when an electric charge is applied across the needle and pressure on the syringe causes the ejection of the polymer jet solution out of the needle. Repulsion of charges within the polymer solution produces a force that opposes and weakens the surface tension of the polymer. Increasing the voltage produces a cone-shape of the polymer solution (Taylor's cone) and stretches out the ultrafine fibres to be directed towards the metallic collector. During this process, the solvent evaporates from the polymer solution and deposition of dry polymer fibres can be seen on the rotating collector (Hasan, Azim and Reza, 2014; Bhardwaj and Kundu, 2010). Numerous studies have been performed to investigate the biomedical applications of the electrospun nanofibrous scaffold. These studies mostly focused on using the electrospun scaffold in tissue engineering, wound dressing, drug delivery and enzyme immobilisation (Jun *et al.*, 2018; Nwachukwu, Einstein and Tulp, 2017; Weng and Xie, 2015; Tran and Balkus Jr, 2012).

Many working parameters, such as the characteristics of the polymer solution, process parameters and ambient conditions, affect the electrospinning process. All of these parameters play an important role in the development of the required morphology and diameters of the scaffold fibres, (Hasan, Azim and Reza, 2014).

Molecular weight, concentration, viscosity, conductivity, and surface tension are polymer solution parameters. A high molecular weight polymer will result in the desired polymer solution concentration and viscosity. It is known that high molecular weight polymers possess a higher degree of polymer chain entanglement, thus creating larger and smoother fibre diameters during the

electrospinning process (Xue *et al.*, 2019).

Furthermore, the polymer concentration also directly affects the viscosity of the solution as the increase in the polymer concentration will lead to an increase in viscosity of the polymer solution. This will cause a further increase in entanglement of the polymer chains. As the chain entanglement is increased, it will overcome the surface tension of the polymer solution and produce spindle-like and uniform fibres (Haider, Haider and Kang, 2015). Meanwhile, low polymer concentration leads to low viscosity of the polymer solution which tends to cause the continuous jet of the polymer solution to break into droplets due to the instability of the solution (Rayleigh instability) (Hasan, Azim and Reza, 2014). An increase in polymer concentration results in increased viscosity which will eventually overcome the solution surface tension and lead to smooth fibre formation (Nezarati, Eifert and Cosgriff-Hernandez, 2013). However, too high a viscosity will result in polymer build up at the needle tip and prevent efficient polymer ejection with beaded fibre formation due to high cohesion of the polymer solution (Amariei *et al.*, 2017).

To form smooth fibres in the electrospinning process, an optimal solution concentration is vital. If the concentration of the polymer is too low, it will create an aggregate of beads and fibres (Motamedi *et al.*, 2017). However, if the concentration is too high, it may be possible to generate larger fibres. Spindle-like and uniform fibres will be produced by the optimum polymer concentration solution (Husain *et al.*, 2016). The close relationship between polymer molecular weight, concentration and viscosity can therefore be modified to create the diameter of choice, whether nanofibers or microfibres (Pillay *et al.*, 2013; Olkhov *et al.*, 2020).

The electrospinning process requires electrical charge transfer from the electrode to the polymer solution at the tip of the needle. Therefore, a minimum electrical conductivity is required to form the thread of fibres and initiate the electrospinning process. An increase in electrical conductivity will affect the elongation and thinning of the thread of fibres ejected from the needle thus controlling its tendency to form nanofibres (Pillay *et al.*, 2013). Conductivity-devoid solutions cannot be electrospun due to the lack of electrical charge in the solution. A substantial reduction in the diameter of the fibres results from an increase in the solution's electrical conductivity (Deshawar, Gupta and Chokshi, 2020). However, too high a conductivity will result in insufficient elongation of the polymer jets to produce uniform fibres and bead formation will also be observed (Haider, Haider and Kang, 2015).

The surface tension is the property of the solution that demonstrates strong cohesiveness in between molecules. It is strongly influenced by the nature of the solvent used to dissolve the polymer. The polymer solution can be ejected from the needle due to the result of the electrical conductivity action that overcome the solution's surface tension (Hasan, Azim and Reza, 2014). A surface tension that is either too high or too low will cause instability of the ejected polymer solution thus increasing the formation of droplets of polymer solution and the production of beaded fibres (Deshawar, Gupta and Chokshi, 2020). In order to ensure the uniformity of the fibre produced, the surface tension can be adjusted by altering the polymer to solvent ratio in the polymer solution.

Processing parameters for electrospinning include flow rate, applied voltage, types of collector mandrel, the distance between needle tip and mandrel,

needle tip design and placement and collector composition and geometry. The flow rate of the polymer to be ejected from the syringe determines the velocity of the solvent ejected and the transfer rate of the polymer. The flow rate must be kept to a minimum in order to provide adequate time for the solvent to evaporate. The fibres and pore diameter are found to increase as the flow rate increases, but too high a flow rate can result in beaded fibres because while residing on the mandrel, the polymer does not dry properly (Zong *et al.*, 2002).

An optimum applied voltage is needed to ensure that a jet of solution is ejected from the needle tip so the force of the electric field must be sufficient to overcome the surface tension of the solution and produce bead free fibres. However, as the voltage increases, the ejected solution will produce larger fibre diameters, and will eventually lead to bead formation and clogging of the needle tip (Motamedi *et al.*, 2017). Other than that, the type of collectors used to retrieve the ejected solution also play a crucial part in the electrospinning process. The collector acts as a conducting material, upon which the ejected solution settles. After the solvent evaporates, the newly formed fibres can be harvested from the collector (Xue *et al.*, 2019). Aluminium foil has been used as a collector material, but the disadvantage of aluminium foil is the difficulty in casting off the foil from the scaffold after the process. Other collectors have also been used such as conductive paper, conductive cloth, mesh wire and a rotating drum.

The needle tip to collector distance also determines the diameter and morphology of the fibres produced. A minimum distance is needed to provide an ample drying time for the fibres before they meet the collector. In the case that the distance is too close or too far, beading is rendered (Leach *et al.*,

2011).

Finally, environmental parameters like temperature and humidity also play a role in the electrospinning process. An elevated temperature can result in a decrease in the polymer's viscosity, thus decreasing the fibre diameter (Yang *et al.*, 2017a). Low humidity will result in the formation of many broken fibres during the electrospinning process due to increased stretching of the electrospun fibres after solvent evaporation (Nezarati, Eifert and Cosgriff-Hernandez, 2013). The electrospinning process has to be accomplished under vacuum in order to obtain higher electric fields and produce the favoured fibre diameter for the scaffold. Table 1.3 summarizes the effects of different parameters on fibre morphology in the electrospinning technique.

Table 1.3 The effect of different parameters on fibre morphology (Hasan, Azim and Reza, 2014).

Parameter	Effect on fibre morphology
Viscosity/ concentration	Low concentrations or viscosities lead to bead formation. Fibre diameter increases with increasing concentration or viscosity.
Conductivity	Increasing the conductivity aided in the production of uniform charge density, bead-free fibres. Increase in conductivity will result in decreased fibre diameter.
Surface tension	No conclusive relation is established between surface tension and fibre morphology.
Molecular weight of the polymer	The number of beads and droplets reduces with the increased molecular weight.
Flow rate	A decrease in fibre diameter with decreased flow rate and generation of beads with too high flow rate. High flow rates produces fibres that are not dry upon reaching the collector.
Applied voltage	Beading is observed at too high voltage. A decrease in fibre diameter results from a voltage increase.
Distance between needle tip and collector	A minimum distance was required to obtain dried fibres. Beading was observed at distances either too far or too close.
Collector composition and geometry	Smoother fibres are obtained from metal collectors; more porous fibre structure is obtained using porous collectors. Aligned fibres are obtained using a conductive frame, rotating drum, or a wheel-like bobbin collector.
Ambient parameters	Increased temperature causes a decrease in solution viscosity, thus resulting in a decrease in fibre diameter. High humidity may result in circular pore formation. Low humidity may result in fibre breakage.

All of these parameters will influence the morphology of the generated fibres so it is essential that they are all optimised as far as possible to enable the production of scaffolds with the desired morphology and physical characteristics for the chosen application. (Jun *et al.*, 2018; Haider, Haider and Kang, 2015).

1.2.3 Incorporation of antimicrobial agents into scaffolds

Recently, there has been much interest in the incorporation of antimicrobial agents into scaffolds to overcome infection in regenerative medicine (Chatzistavrou *et al.*, 2014; Furtos *et al.*, 2017; Saravanan *et al.*, 2011; Feng *et al.*, 2010). Along with their main function to supply cells with synthetic ECM microenvironments, these scaffolds also serve as a medium for sustained, localised drug release as well as having tuneable degradation properties to promote tissue integration and support cell delivery (Johnson and Garcia, 2015). Scaffolds provide an ideal medium to deliver long-term bactericidal doses of antibiotics to the injury site to treat infections (Dorati *et al.*, 2017). Some modification to the mode of drug release can also be achieved by encapsulating the drug within biodegradable polymer matrices used as a coating for orthopaedic implants that are prone to infections (Macha *et al.*, 2018).

Antimicrobial silver nanoparticles (SNPs) were used as a coating for hydroxyapatite and PETA (Pentaerythritol triacrylate-co-trimethylolpropane) composite scaffolds and significantly inhibited the proliferation of *Staphylococcus aureus* (*S. aureus*) and *E. coli* (Smoak *et al.*, 2014). Another example of antimicrobial activity engineered into scaffolds for tissue regeneration is Poly-(L-lactic acid) nano-composite scaffolds containing silver

nanoparticles, which have antibacterial properties when used as bone grafts (Marsich *et al.*, 2013). A previous research performed by Qian and co-workers which used a combination of Poly (lactic-co-glycolic acid) (PLGA) and PCL scaffold produced by electrospinning process with silver nanoparticle impregnation followed by polydopamine and collagen I coatings. The scaffold exhibited a wider antibacterial zone towards *S. aureus* and *Streptococcus mutans* (*S. mutans*) after 24 h inoculation (Qian *et al.*, 2019).

However, the previous studies showed that silver nanoparticles can be absorbed easily by the recipient and tend to accumulate in various organs such as liver (Yang *et al.*, 2017b) which is detrimental for the prognosis of the treatment. While the antibacterial effect of silver nanoparticles seems promising, previous experiments have shown that when the silver nanoparticles were used for wound treatment in mice, the histopathological effects on the liver were an increase in inflammatory cells, fatty degeneration, cell swelling and extension and congestion of the central vein after 14 days dermal exposure (Heydarnejad *et al.*, 2013). From *in vitro* studies, high amounts of silver nanoparticles (5 mg/ kg) are also known to interact with mammalian cells in a way which may disrupt cell membranes by altering membrane permeability and further, induce apoptosis (Wen *et al.*, 2017). Furthermore, silver nanoparticles can enter the cell by diffusion or endocytosis and accumulate in mitochondria which leads to reactive oxygen species (ROS) production and thus, initiation of oxidative stress that will lead to DNA damage, inhibition of cell proliferation and induced cell apoptosis (McShan, Ray and Yu, 2014).

In other studies by Albuquerque and co-workers, they constructed an

electrospun tubular-shaped scaffold made from polyester poly(p-dioxanone) that incorporated three antibiotics (metronidazole, ciprofloxacin, and minocycline). The scaffold was introduced to a multispecies biofilm consisting of *Actinomyces naeslundii*, *Streptococcus sanguinis* and *E. faecalis* grown on a dentin surface and inside dentinal tubules for 7 days. The elimination of viable bacteria was nearly accomplished after 7 days treatment when compared to antibiotic-free scaffold (Albuquerque, Nagata and Bottino, 2017).

Using a scaffold that has antimicrobial properties may help in the prevention of infection when the scaffold is introduced to the wound or surgical site, as the scaffold has an additional function to act as a medium for drug transfer to the local environment. However, the toxic effects on the mammalian cells of the antimicrobial agents within the scaffold should also be considered. This means that an antimicrobial scaffold is required to be toxic to bacteria but at the same time preserve the targeted cells and promote tissue regeneration.

1.3 Antimicrobial resistance

The World Health Organization (WHO) (2018) reported that bacterial resistance to common antibiotics has increased in many parts of the world (WHO, 2018). Furthermore, the WHO (2018) stated that between 8% and 65% of *E. coli* associated with urinary tract infections have developed resistance to standard antibiotics such as ciprofloxacin. This has increased in infections caused by multidrug-resistant bacteria worldwide (Blair *et al.*, 2015). Based on data reported in the Annual Report of the Chief Medical Officer (2011), approximately 25,000 people die annually due to multidrug-resistant infections in Europe. Therefore, alternative strategies to the use of antibiotics are needed for the treatment of bacterial infections.

Antibiotic resistance is either innate or acquired. Innate resistance is the ability of bacteria to resist the activity of an antimicrobial agent through their inherent structure or functional characteristics. Innate resistance may be due to a lack of affinity of the drug for the target in bacteria, the drug being unable to penetrate the bacteria due to the cell wall or outer membrane structure, an efflux pump or the bacteria producing enzymes that can inactivate the antibiotic. For example, some Gram-positive bacteria such as *E. faecalis* are known to have resistance to alpha beta-lactam antibiotics due to the lack of penicillin-binding protein, thus reducing the efficacy of this antibiotic (Arbeloa *et al.*, 2004). Most of the Gram-negative bacteria are resistant to vancomycin due to the inability of vancomycin to penetrate the outer layer of the cell membrane and thus fail to inactivate the bacteria (Munita and Arias, 2016). Efflux pumps are found in both types of bacteria. In Gram-negative bacteria, the efflux pump is located in the inner membrane and works in conjunction

with the periplasmic protein and outer membrane. Examples of efflux pumps are AcrB that can be found in *E. coli* and MexB in *Pseudomonas aeruginosa*. In Gram-positive bacteria, the efflux pump is solely mediated by pump proteins such as NorA in *S. aureus* and PmrA in *S. pneumoniae* (Cattoir, 2004; Li and Nikaido, 2004; Poole, 2004; Van Bambeke *et al.*, 2003).

For acquired resistance, the bacteria develop resistance after prolonged exposure to the drug. After being exposed to the antibiotic many times, the bacteria undergo mutation or modification in their DNA which alters the antibiotic target site resulting in resistance to the same antibiotic (Munita and Arias, 2016). This is more likely to happen due to overuse and misuse of the antibiotic in people and animals. Bacteria overcome the toxic effects of the antibiotic through physical removal of the antibiotic from the cell (efflux pump), chemical modification or prevention of access by binding or secretion of enzymes that inactivate or degrade the antibiotic (Blair *et al.*, 2015; Tenover, 2006). Figure 1.6 illustrates some examples of antibiotic resistance mechanisms for bacteria.

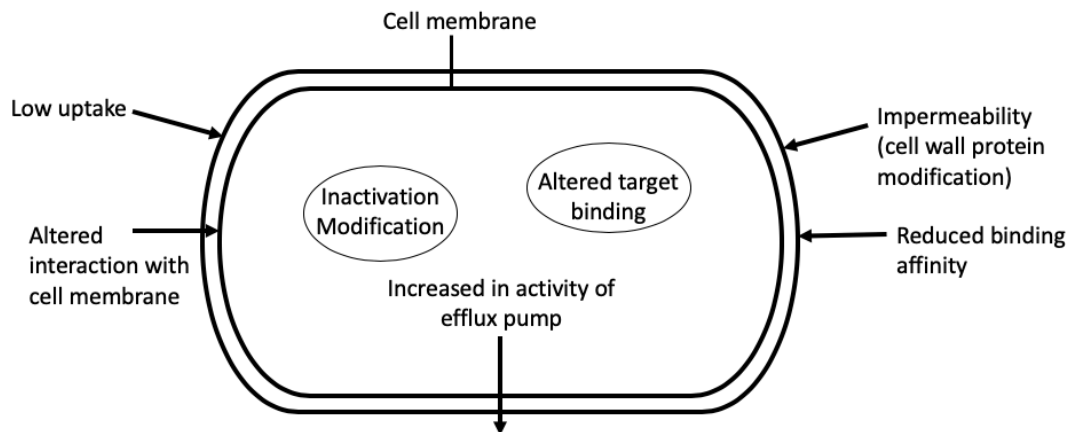


Figure 1.6 Main mechanisms of antibiotic resistance by bacteria (Alav, Sutton and Rahman, 2018).

Antibiotic resistance is either innate or acquired. An example of innate antibiotic resistance is reduced binding affinity by protein on the bacterial cell wall. Examples of acquired antibiotic resistance are the modification and increase of efflux pump activity.

Antimicrobial resistance is also affected by bacterial conditions such as environment, growth phase and whether the bacteria are growing planktonically or as a biofilm. Recent studies found a planktonic culture of *P. aeruginosa* was more susceptible to antibiotics than the biofilm retrieved from the patient (Aaron *et al.*, 2002; Ahmed *et al.*, 2020). In planktonic form, bacteria mostly do not form aggregates with each other and are present as individual cells. However, bacteria will form aggregates with each other after they settle on a suitable surface for attachment and thus form a biofilm (Fig 1.7). Biofilms are a mode of growth that is associated with increased levels of tolerance to antimicrobials such as antibiotics, disinfectants and host defences (Jamal *et al.*, 2015).

The biofilm is protected from the external environment by the production of extracellular polymeric substance (EPS) which surrounds the bacteria in the biofilm. EPS is composed of proteins, DNA, polysaccharides, RNA and water. The biofilm is protected from antimicrobial agent infiltration due to; (i) EPS matrix that inhibits the diffusion of antimicrobial substances; (ii) slower growth of bacteria in the biofilm, thus minimising the uptake of the antimicrobial agent into the bacteria; and (iii) the environment surrounding the bacteria promotes protection to the bacteria (Donlan, 2001).

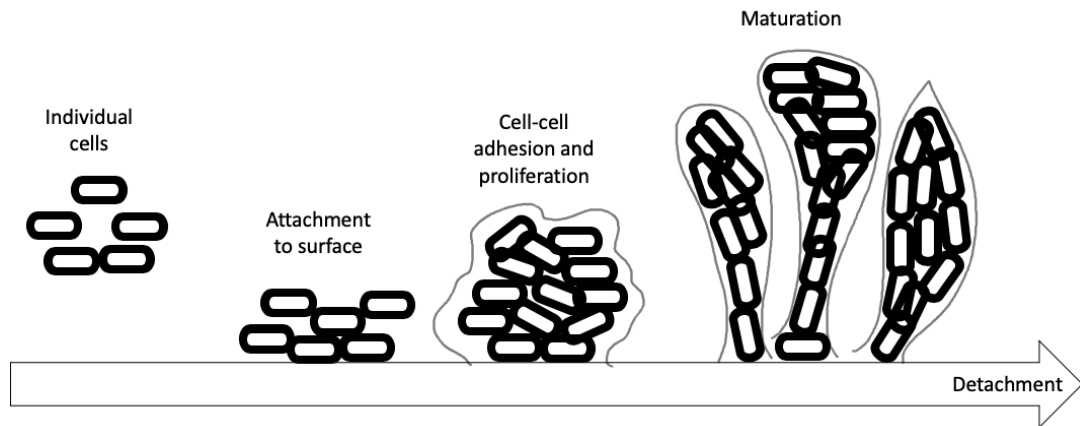


Figure 1.7 The biofilm life cycle: individual cells attach to a surface followed by growth and cell-cell interaction resulting in the formation of colonies and 3D structures which can also further detach in clumps (Jamal *et al.*, 2015).

Bacterial resistance to antibiotics can also be brought about by horizontal gene transfer (HGT). HGT is the mechanism whereby the bacteria acquire and transmit antibiotic-resistant genes (ARG) from outside their lineage. The three main mechanisms of HGT in bacteria are conjugation, transduction and natural transformation (Lerminiaux and Cameron, 2019). HGT makes use of plasmids, bacteriophages and extracellular DNA to transport the ARG within the human host and environment.

Plasmids spread in bacterial populations by a conjugation process which requires physical contact between cells in a mutually beneficial environment, followed by bridge formation that provides a route of transfer of a plasmid from a donor to the beneficiary cell in response to the presence of antibiotics (Sørensen *et al.*, 2005). Meanwhile, transduction occurs when infected bacterial DNA is transferred to the genome of the recipient cell by a bacteriophage. Both chromosomal- and plasmid-borne ARGs can be transferred by transduction (Lerminiaux and Cameron, 2019). Natural transformation occurs when naturally competent bacteria are able to uptake foreign extracellular DNA and integrate it into their genome by recombination (Johnston *et al.*, 2014).

1.4 Photodynamic therapy

Photodynamic therapy (PDT) is a treatment that comprises the combination of visible light and a photoactive molecule called a photosensitiser (PS). PDT has been employed for several years as a treatment for various types of cancer (Casas, 2020; Joubert *et al.*, 2020; Li *et al.*, 2020; Mpourazanis *et al.*, 2020; Xie *et al.*, 2020). More recently there has been increased interest and research on the use of PDT as an antibacterial therapy in different fields (St Denis *et al.*, 2011; Hamblin and Hasan, 2004; Boltes Cecatto *et al.*, 2020; Souza *et al.*, 2020; Shen *et al.*, 2020; Pantò *et al.*, 2020).

The principle of PDT is that a photoactive chemical known as a PS is introduced to the body. After a certain time or incubation time, the PS is absorbed by the diseased tissue or pathogen. After being irradiated with light at a wavelength corresponding to an absorption maximum of the PS, the PS will be excited or activated, and thus produce free radicals or reactive oxygen species (ROS) that eventually induce cell death in the abnormal or diseased tissue targeted. Either PS or light alone is non-toxic, which immediately provides the opportunity for targeting of the therapy to diseased areas (Yano *et al.*, 2011; Kalka, Merk and Mukhtar, 2000; Castano, Demidova and Hamblin, 2004).

The advantages of using PDT are that the PS used are selectively taken up by target cells or tissues. The precise application of the light causes the killing of target cells with minimal phototoxicity effects on the surrounding tissue (Gursoy *et al.*, 2013). The procedure can be performed in out-patient settings and repeated doses and treatment is acceptable. However, one drawback of PDT in cancer treatment is the possibility of persistent skin photosensitivity

that lasts more than a week for certain PS (Yano *et al.*, 2011). Nevertheless, antibacterial PDT treatment is more likely to be local, so skin photosensitivity should be minimal, depending on the mode of application of the PS. Furthermore, antimicrobial resistance to treatment does not develop with repeated PDT treatment, and no genotoxic or mutagenic effects have been observed after PDT (Konopka and Goslinski, 2007). In cancer treatment, PDT is usually used as local treatment and is not commonly used as a treatment for metastasised cancer (Capella *et al.*, 2003).

However, because of the depth of light penetration and poor tissue penetration of the PS, the efficacy of the PDT treatment is limited. The effectiveness of PS is decreased if the PS tends to self-aggregate and is thus impermeable to the lipid bilayer of the cell (Zhang *et al.*, 2018). The drawbacks of PDT are also due to the lack of complete selectivity in the diseased tissue and normal tissue and the localization of PS.

1.4.1 Mechanism of action

Figure 1.8 graphically illustrates the activation processes which occur during illumination and energy transfer in PDT. During illumination, the ground PS (^1PS) absorbs light energy and undergoes an electronic transition (an electron is excited to the lowest unoccupied molecular orbital) to the singlet excited state ($^1\text{PS}^*$) that has a short lifetime (Castano, Demidova and Hamblin, 2004). The singlet excited state, which is unstable and short-lived, may go through a radiative process, producing either heat or light (fluorescence) or can undergo intersystem crossing from the singlet to the excited triplet state ($^3\text{PS}^*$). The excited triplet state has a longer lifetime compared to the singlet excited state and allows a longer interaction time between the activated $^3\text{PS}^*$ with surrounding substrates (Nevřelová *et al.*, 2005; Kochevar and Redmond, 2000). The excited triplet state $^3\text{PS}^*$ can then interact with neighbouring molecules by either Type I or Type II mechanisms (Foote, 1991).

The Type I mechanism includes electron or hydrogen-atom transfer between the excited triplet state $^3\text{PS}^*$ and substrate (D) or molecular oxygen. Very highly reactive products known as free radicals or radical ions such as hydroxyl radicals ($\text{HO}^\bullet$) are formed by the direct interaction between the excited triplet state $^3\text{PS}^*$ and the substrate, which can then react with further substrates and thus intensify the oxidative damage within the cell or target. Superoxide anion $\text{O}_2^{\cdot-}$ can be formed from: (1) PS anion radical ($\text{PS}^{\cdot-}$) electron exchange with oxygen and (2) triplet excited state PS ($^3\text{PS}^*$) electron transfer with oxygen. Thus, the generation of singlet oxygen in the Type II mechanism will vie with the superoxide anion formation from the PS triplet excited state. Two superoxide anion molecules can combine with protons to produce hydrogen peroxide (H_2O_2). Then, the reduction of Fe^{3+} by superoxide anion

and subsequent Fe^{2+} reaction with hydrogen peroxide leads to the formation of a highly reactive hydroxyl radical. All these species can interfere with the biological functions of nucleic acids, fatty acids and certain amino acids. Table 1.4 lists the series of reactions occur in Type I and Type II during PDT.

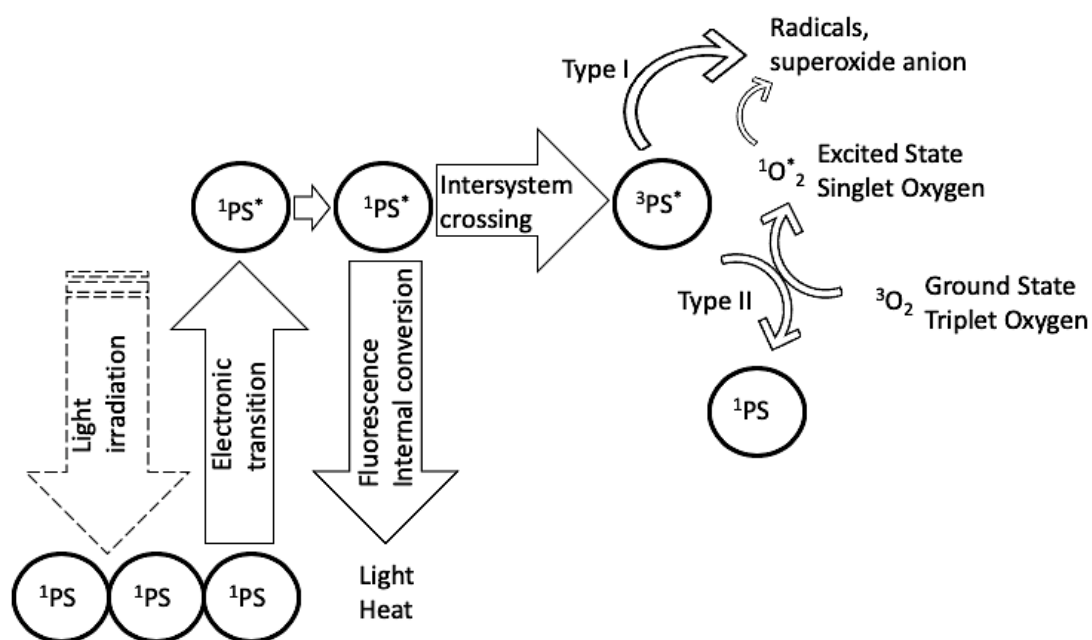


Figure 1.8 The photophysical and photochemical mechanisms of PDT.

The energy from light irradiation will excite the ground state PS (1PS) to the singlet excited state ($^1PS^*$). After intersystem crossing, the resulting triplet excited state ($^3PS^*$) can react with intracellular substrates, forming free radicals and ROS (Type I) or undergo energy transfer to ground state molecular oxygen (3O_2) thus producing reactive singlet oxygen (1O_2) (Type II) and further producing more ROS.

Table 1.4 Reactions occurring during photodynamic action (Josefsen and Boyle, 2008).

Photoprocess	Reaction	Product
Type I	$^3\text{PS}^* + ^1\text{PS} \rightarrow$	$\text{PS}^{\cdot-} + \text{PS}^{+\cdot}$
	$^3\text{PS}^* + \text{D} \rightarrow$	$\text{PS}^{\cdot-} + \text{D}^+$
	$\text{PS}^{\cdot-} + \text{O}_2 \rightarrow$	$^1\text{PS} + \text{O}_2^{\cdot-}$
	$^3\text{PS}^* + \text{O}_2 \rightarrow$	$\text{PS}^{+\cdot} + \text{O}_2^{\cdot-}$
	$2\text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow$	$\text{O}_2 + \text{H}_2\text{O}_2$
	$\text{FE}^{3+} + \text{O}_2^{\cdot-} \rightarrow$	$\text{FE}^{2+} + \text{O}_2$
	$\text{FE}^{2+} + \text{H}_2\text{O}_2 \rightarrow$	$\text{O}_2 + \text{OH}^- + \text{OH}^{\cdot}$
Type II	$^3\text{PS}^* + ^3\text{O}_2 \rightarrow$	$^1\text{PS} + ^1\text{O}_2^*$

^1PS = PS in ground state, $^3\text{PS}^*$ = PS in triplet excited state, D = electron donor molecule (NADH, cysteine), $\text{PS}^{\cdot-}$ = PS anion radical, $\text{PS}^{+\cdot}$ = PS cation radical, D^+ = oxidized donor, $\text{O}_2^{\cdot-}$ = superoxide anion, H_2O_2 = hydrogen peroxide, $\text{OH}^- / \text{OH}^{\cdot}$ = hydroxyl radical.

The Type II mechanism involves energy transfer between the excited triplet state $^3\text{PS}^*$ and ground-state molecular oxygen ($^3\text{O}_2$), resulting in the production of very highly reactive singlet oxygen ($^1\text{O}_2^*$). Reactive singlet oxygen $^1\text{O}_2^*$ is the primary cytotoxic agent in Type II mechanisms since it is extremely reactive and can interact with several biological molecules in the target tissue such as lipids, proteins and nucleic acids to cause oxidative damage (Mroz *et al.*, 2011). It has a very short lifetime of about 40 ns, so damage is typically limited to the region in which it is produced (Meisel and Kocher, 2005) – a diameter of approximately 10 nm (Macdonald and Dougherty, 2001).

Clinically, predominantly “Type II” mechanism PSs are currently used in the clinic. The Type II process involves only a limited number of molecules as reacting species must have a triplet state multiplicity. Both types of reaction can occur at the same time however, Type II is the dominant process in PDT and it is catalytic (Ormond and Freeman, 2013).

1.4.2 Photosensitiser

In PDT, the PS plays the crucial role of absorbing and translating light energy into Type I and II chemical reactions (Allison *et al.*, 2004), thus initiating the production of lethal cytotoxic agents (Sharman, Allen and van Lier, 1999). Type I and II chemical reactions both cause oxidation of biomolecules in the cell. Hundreds of natural and synthetic compounds have been found to have photosensitising potential (Rousset, Bourré and Thibaud, 2003; Castano, Demidova and Hamblin, 2004). Allison *et al.* (2004) have listed the characteristics that need to be considered in selecting the PS such as its dark toxicity, mutagenicity/ carcinogenicity, elimination time/ duration, selectivity, activation, sunlight precautions, route of administration, indications, reliability,

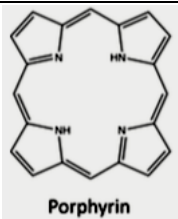
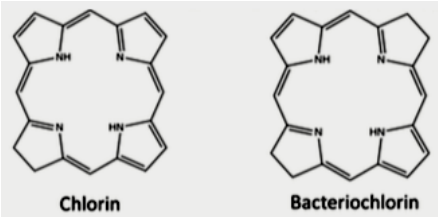
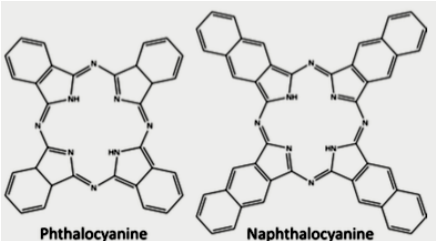
pain-free therapy, outpatient therapy, availability, cost, safety, biochemistry, the wavelength of activation, integrative ability, and transparency (Allison *et al.*, 2004).

For successful PDT, the PS must be able to generate sufficient singlet oxygen or reactive species to cause cell death. In particular, an ideal PS should also have the following characteristics (Josefsen and Boyle, 2008; DeRosa and Crutchley, 2002):

- a) have strong absorption with high extinction coefficient in the red or near infrared region of the electromagnetic spectrum, allowing deeper tissue penetration
- b) effectively generates the singlet oxygen and ROS
- c) has suitable photophysical characteristics:
 - a. high quantum yield of triplet formation ($\Phi_T \geq 0.5$);
 - b. high quantum yield of singlet oxygen ($\Phi_\Delta \geq 0.5$)
 - c. a longer triplet state lifetime (τ_T = microsecond range)
 - d. a high triplet state energy
- d) have no or minimum dark toxicity
- e) exhibit greater retention in target tissue over healthy tissue
- f) be water soluble for easy administration and clearance from the human body

Table 1.5 summarises some of the most common photosensitiser families with their basic molecular structure. Porphyrin families are based on haematoporphyrin (Hp) and its derivatives. Commercial products such as Photofrin® and Photosan are made by the modification and purification of molecules of hematoporphyrin derivatives (HpD) (Allison *et al.*, 2004). Fig. 1.9 represents the basic structure of HpDs made up of four methyl radicals (CH_3), two propanoyl radicals ($\text{CH}_2\text{CH}_2\text{COOH}$) and two hydroxyethyl radicals (CHOHCH_3) (Rousset, Bourré and Thibaud, 2003). Chlorophyll-like substances known as chlorins, also have photosensitising properties. Meso-tetra-hydroxyphenyl-chlorin (m-THPC, temoporfin, Foscan®), MACE and NpE6 (Bonnett, 2002) have all been used clinically. Foscan® was developed by Biolitec Pharma Ltd., Dublin, Ireland. Currently, Foscan® is used as a palliative treatment for patients with advanced head and neck cancer (Soukos and Goodson, 2011).

Table 1.5 PS families with their basic molecular structure (Soukos and Goodson, 2011; Allison *et al.*, 2004).

PS families	Basic molecular structure	PS families
Porphyrin platform	 Porphyrin	HpD (hematoporphyrin derivative) HpD-based BPD (benzoporphyrin derivative) ALA (5-aminolevulinic acid) Texaphyrins
Chlorophyll platform	 Chlorin Bacteriochlorin	Chlorins Purpurins Bacteriochlorins
Dyes	 Phthalocyanine Naphthalocyanine	Phthalocyanines Naphthocyanines

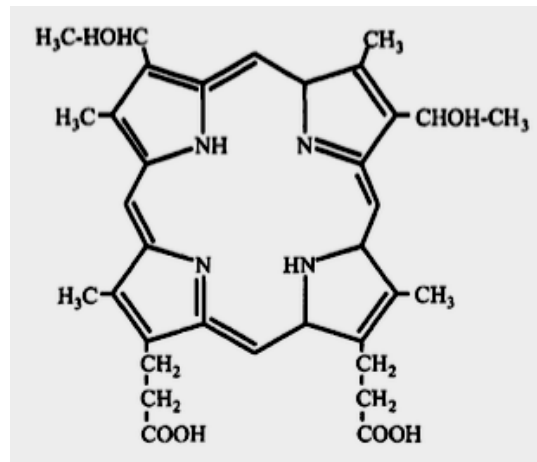


Figure 1.9 Basic structure of haematoporphyrin derivatives (HpD) (Rousset, Bourré and Thibaud, 2003).

Chemical dyes that have been used in treatment include phthalocyanines and naphthocyanines (Rosenthal, 1991). Phthalocyanines (PC) and naphthocyanines with maximum absorption wavelengths 680 nm and 780 nm, respectively, have strong absorption bands but are sparingly soluble in aqueous solvent (Bonnett, 2002). However, the addition of polar side groups, for example by sulphonation, is known to help to solubilise the phthalocyanines and has resulted in the production of effective compounds such as Photosens® (General Physics Institute) (Yano *et al.*, 2011; Bonnett, 2002). PDT-Photosens® has been investigated in clinical trials for primary tumours including lung, head and neck, oesophagus, stomach, vulva, bladder, breast cancer, skin (basalioma, melanoma, Kaposi's sarcoma) and breast cancer (Yano *et al.*, 2011).

Dyes such as PCs are relatively efficient and have a longer wavelength of radiation absorption due to more extensive conjugation of their ring structure compared to porphyrins (Josefsen and Boyle, 2008; Rosenthal, 1991). PCs are capable of forming metal complexes (metallophthalocyanines) with diamagnetic metal ions such as zinc (Zn^{2+}) and aluminium (Al^{3+}) that affect the photophysical properties of the PS by increasing the quantum yield and lifetime of the excited triplet state, thus enhancing the intersystem crossing process (Tedesco, Primo and Beltrame, 2016; DeRosa and Crutchley, 2002). Examples of PC-based PSs are CGP55847 developed by Ciba-Geigy that has undergone Phase I/II clinical trials and Photocyanine developed in China that has also undergone Phase II clinical trials (Lo *et al.*, 2020).

Table 1.6 provides the properties of some PS dyes approved for PDT treatment and used in PDT-related clinical and preclinical trials (Ormond and

Freeman, 2013). In this thesis, the PS that will be discussed further are Methylene Blue (MB) and Erythrosine B (ERY) as they were both used in this project.

Table 1.6 Properties of some PS dyes approved for PDT treatment and used in PDT-related clinical and preclinical trials (Ormond and Freeman, 2013)

Photosensitiser	Wavelength (nm)	ϵ_{max} ($\text{M}^{-1} \text{cm}^{-1}$)	Application
Porfimer sodium	632	3000	Bladder cancer, oesophageal cancer, lung cancer, Barrett's oesophagus, cervical cancer, endobronchial cancer
Methylene Blue	668	82000	Basal cell carcinoma, Kaposi's sarcoma, bladder carcinoma
5-Aminolaevulinic acid	632	5000	Actinic keratosis
Methyl aminolevulinate	-	-	Actinic keratosis
Tin etiopurpurin	664	30000	Breast adenocarcinoma, basal cell carcinoma, Kaposi's sarcoma, age-related macular degeneration
Toluidine blue	630	51000	Chronic periodontitis
Rose Bengal	549	100000	Breast carcinoma, metastatic melanoma
Merocyanine	556	110000	Leukemia, neuroblastoma
Curcuminoids	420	55000	Oral surgery via PDT
Hypericin	590	44000	Squamous cell carcinoma, basal cell carcinoma, Kaposi's sarcoma

1.4.2.1 Methylene Blue (MB)

One of the PS currently used in the clinic other than the HpDs is MB (Sharman, Allen and van Lier, 1999). MB falls under the category of phenothiazinium dyes that have a cationic character. Figure 1.10 shows the tricyclic chemical structure of MB ($C_{16}H_{18}ClN_3S$). The absorption maximum wavelength of MB (668 nm), makes it ideal for use in PDT as the long wavelength absorption provides better tissue penetration of light during irradiation (Allison *et al.*, 2004).

Some of the other relevant characteristics of Methylene Blue (MB) are:

- a. Molecular weight 319.85 g/mol
- b. Absorption maximum wavelength is 668 nm
- c. ϵ (extinction coefficient): 82000 at 668 nm
- d. $\Phi\Delta \sim 0.5$ (Lutkus, Rickenbach and McCormick, 2019)
- e. $\Phi T \sim 0.52$ (Junqueira *et al.*, 2002)

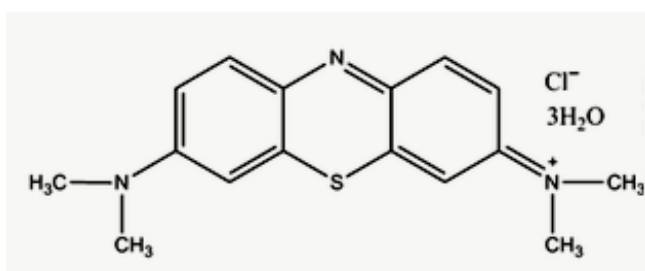


Figure 1.10 Chemical structure of MB $C_{16}H_{18}ClN_3S$ (Soukos and Goodson, 2011).

MB is usually used for staining purposes in molecular studies (Jori *et al.*, 2011) and is also known to have a low toxicity level when tested with cell lines (mouse mammary carcinoma, mouse melanoma, human melanoma and mouse fibrosarcoma) and also when given orally (100 mg and 500 mg) for anti-malarial treatment (Wainwright, 2011; Burhenne *et al.*, 2008). MB is also classified in WHO's List of Essential Medicines as an antidote used in poisonings (*WHO Model List of Essential Medicines (19th List)*, 2015).

Previously, it was shown that PDT with MB, using a laser (665 nm) as a light source, was able to inhibit the growth of the bacteria that cause prosthetic joint infections such as methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA) and *Staphylococcus epidermidis* (Briggs *et al.*, 2018). MB has also been tested at the preclinical and clinical level to treat nasal MRSA colonization; it eradicated MRSA from the nose with total treatment times less than 10 min (Street *et al.*, 2009). The photo-antimicrobial effect of MB was discovered when it was used to decontaminate freshly frozen plasma units by deactivating extracellularly enveloped viruses. MB was also reportedly used in the treatment of methaemoglobin anaemia as it can reduce the methaemoglobin to haemoglobin (Wainwright and Crossley, 2004). Clinically, in dentistry, a combination of MB and the commercially-available product, Periowave (<http://www.periowave.com>), was used to disinfect bacteria after scaling and root planning. Periowave utilises a non-thermal diode laser with light-activated antimicrobials agents (LAAAs) and has been approved for various dental disease treatment such as chronic periodontitis, gingivitis, endodontics and peri-implantitis.

The cationic PS such as MB are beneficial in deactivating Gram-negative bacteria as they can attach to the outer cell membrane of the bacteria due to the electrostatic attraction between positively-charged MB and the negatively-charged outer cell membrane, thus producing reactive singlet oxygen at effective target sites during illumination. Cationic PS may also improve the effectiveness of antibiotics and other hydrophobic molecules by disrupting the normal barrier properties of the outer cell membrane, thus facilitating the uptake of the antibiotics (Jori *et al.*, 2011). MB is hydrophilic (strong affinity for water) and reacts with guanine bases of DNA and hence, causes damage followed by cell death upon irradiation (Tardivo *et al.*, 2005). However, without any irradiation, MB showed no toxicity in humans even at high concentrations of 10 mg/ mL (Eichner, Gonzales and Maisch, 2013).

Cellular enzymes may reduce MB to a colourless (leuco) form *in vivo* which makes it photodynamically inactive (Sharman, Allen and van Lier, 1999). However, MB's properties can be altered by the inclusion of one or two methyl (- CH₃) groups to increase the lipophilicity of the molecule and hence the singlet oxygen yield to overcome the reduction process. This methylation process was found to inhibit the reduction of MB to its inactive leuco form (Fig. 1.11) (Wainwright, 2011). However, it is also known that the leuco form will be auto-oxidised to MB by the presence of any oxygen molecules in the environment (Liu *et al.*, 2017).

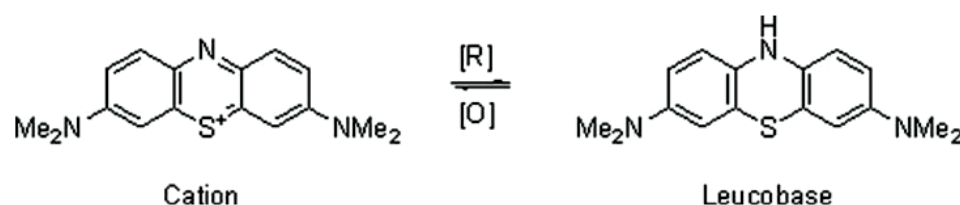


Figure 1.11 Redox equilibrium of MB (Wainwright, 2011).

1.4.2.3 Erythrosine B (ERY)

ERY is a xanthene dye used as a food colourant that can act as PS and has low toxicity to the host (Ergaieg and Seux, 2009; Allaker and Douglas, 2009). Its absorption maximum wavelength is 533 nm. ERY is an anionic PS and possesses an amphiphilic nature which has hydrophilic and lipophilic properties (affinity to both water and lipid) (Pellosi *et al.*, 2013). The presence of heavier halogens in ERY increases the yield of intersystem crossing to the triplet state of the dye, thus making it a more efficient PS than other halogenated derivatives such as eosin (DeRosa and Crutchley, 2002).

Erythrosine B (ERY) ($C_{20}H_6I_4Na_2O_5$):

- Molecular weights of 879.86 g/mol
- Absorption maximum wavelength (λ_{max}) is 525 nm
- ϵ (extinction coefficient): ≥ 82500 at 527-529 nm
- $\Phi\Delta \sim 0.59$ (Fracalossi *et al.*, 2016)
- $\Phi T \sim 0.55$ (Tomasini, Braslavsky and San Román, 2012)

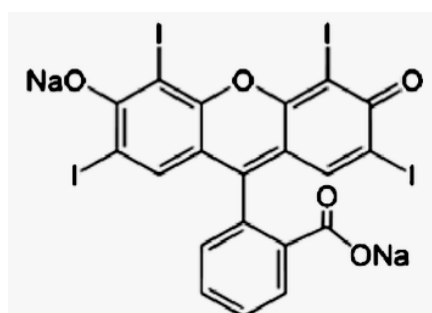


Figure 1.12 Chemical structure of ERY (Silva *et al.*, 2015).

Figure 1.12 shows the chemical structure of ERY, a 2,4,5,7-tetraiodofluorescein disodium salt ($C_{20}H_6I_4Na_2O_5$) (Silva *et al.*, 2015).

In the dental setting, ERY is used to disclose dental biofilms or plaque and it possesses antibacterial properties against oral bacteria (Garapati *et al.*, 2015). Previous studies found that PDT-ERY induced damage to the outer membrane of *Citrobacter freundii* bacteria after being irradiated with laser light at 532 nm (Silva *et al.*, 2015). PDT-ERY also showed antimicrobial effects in treating *Candida albicans* and *Candida dubliniensis* in buccal candidiasis infections without damaging the adjacent tissues using a light-emitting diode (LED) (Costa *et al.*, 2012; Costa *et al.*, 2011). Other than that, PDT-ERY showed excellent potential for oral plaque biofilm treatment by using visible light as a light source (Wood *et al.*, 2006). ERY also has the following benefits: it is nontoxic to the host cell, and has been approved by F.D.A as a food colourant and as a stain to visualize dental plaque in the form of disclosing solution or tablets.

According to Rolim *et al.* (2012), when the peak of light absorption is at 580 nm and the PS concentration is 163.5 μM , ERY effectively inhibited *Streptococcus mutans*, a cariogenic oral bacterium, when compared to other PS. However, they also reported that ERY exhibited some toxicity even without light irradiation (Rolim *et al.*, 2012). In other areas, ERY also has the potential for treating MRSA that is responsible for acute and chronic sinusitis inflammation (Garapati *et al.*, 2015). In oral cancer cells lines such as DOK and H357, ERY was also found to be effective in the killing of malignant and pre-malignant oral cancer cells (Garg *et al.*, 2012).

In previous work, ERY was used to induce photoinactivation of *S. aureus* and it was found that ERY enhanced the leakage of K^+ from bacteria, dissipating the membrane potential and further increasing membrane permeability (Kato

et al., 2012). It was suggested that *E. faecalis* and *Aggregatibacter actinomycetemcomitans* take up anionic PS through a protein transporter, not by simple diffusion, and increased uptake was observed under conditions of higher $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration thus suggesting that anionic PS may be suitable to inhibit bacteria in biofilm (George, Hamblin and Kishen, 2009).

1.4.3 Light sources

The light sources that are used to activate the PS during PDT, include lasers, lamps, and light-emitting diodes (LEDs) (Calin and Parasca, 2009; Nagata *et al.*, 2012). Argon and metal vapour lasers were initially used as a light source in PDT (Brancaleon and Moseley, 2002). Lasers were used due to their monochromaticity and efficiency of coupling into a single optical fibre when delivering the light to the targeted area of the body. Monochromaticity of laser light provides illumination at the specific wavelength of the absorption maximum of PS (Nagata *et al.*, 2012; Calin and Parasca, 2009; Brancaleon and Moseley, 2002). However, lasers have a low transparency range, high scattering effect and may induce damage and long-term degradation to the optical materials (Wood, Taylor and Rouse, 1975; Kiedrowski *et al.*, 2020). In addition, since lasers of an unspecified intent can burn and damage healthy tissue, the person who manipulates the laser must be fully qualified and professional. Lasers may also induce dysregulation by altering cell metabolism and transcription factors responsible for gene expression in the molecular mechanism of cell function. Scarring and hypopigmentation may occur several months after laser treatment (Khalkhal *et al.*, 2020).

Usually, one photon absorption is enough to activate a molecule of PS and the volume of tissue affected depends upon the area that has been irradiated, and the absorption and scattering properties of the tissue during illumination

at a specific wavelength (Wilson, 2003b). However, the illumination process in PDT may also be improved by utilising two-photon absorptions when using an ultrafast laser as a source of light (Wilson, 2003a). There are two main effects when two-photons are absorbed simultaneously. Firstly, each photon has a longer wavelength that increases penetration of light into the tissue in the area on which the beam is focused. Secondly, the intensity of light will determine the cross-sectional interaction between two photons and the interaction of photons only occurs in the immediate area of illumination so targeting is improved (Wilson, 2003a). However, due to the high cost of laser and ultrafast laser systems, alternative sources of light such as lamps and LEDs have been developed and used for PDT treatment. As far as possible, these light sources possess the ideal characteristics needed for excitation of the PS being used and to maximise the efficacy of the treatment. For example, for the effective penetration of light into human tissues, the wavelength of the light should be at least in the 400-700 nm range (Maisch, 2007).

A tungsten lamp has been used in previous studies as it has a continuous spectrum (350 – 850 nm) of visible light (Calin and Parasca, 2009) which corresponds well with the optimal therapeutic light window (600 – 800 nm) for effective PDT (Kim and Darafsheh, 2020). The maximum absorption wavelengths of ERY and MB are 533 nm and 668 nm, respectively, and these wavelengths fall under the broad spectrum of visible light from the tungsten lamp. The tungsten lamp was introduced for use in PDT by Kennedy and Pottier (Kennedy, Marcus and Pottier, 1996) when they used a slide projector lamp in animal experiments. The tungsten lamp has also been used clinically as a light source for topical ALA-PDT. Due to the broad emission wavelength of these lamps, several PSs with different absorption wavelengths can be

excited concurrently during illumination. Furthermore, the lamps are much more cost-effective than lasers, are more portable and are user-friendly (Brancaleon and Moseley, 2002).

As well as providing light of a wavelength that corresponds to an absorption maximum of the PS being used, one of the primary requirements for light source and delivery devices in PDT is to achieve adequate light illumination throughout the target tissue volume. This means having optical output at the appropriate wavelength for activation of the PS and optimum penetration into the target tissue (Star, 1990). Table 1.7 shows the advantages and disadvantages of laser, LED and lamp sources.

Table 1.7 Advantages and disadvantages of laser, LED and lamp sources (Kim and Darafsheh, 2020)

Light sources	Advantages	Disadvantages
Laser	Capable of high powers Highly monochromatic Adaptable to various source fibres	Expensive Can be large Requires high maintenance
LED	Small, adaptable Low cost Can be used directly for endoscopic/ interstitial applications	Thermal effects Low power Broad spectral width Large beam divergence
Lamp	Simple design Low cost Broad field irradiation	Can suffer from coupling losses with light guides Very broad spectrum Large beam divergence Needs optical filtering

1.5 Photodynamic therapy in regenerative medicine

Current therapeutic strategies to overcome infection include oral or systemic antibiotic therapy. However, an increase in bacterial resistance to antibiotics, as well as an increase in the number of immunocompromised patients and the fact that many infections are not caused by a single microorganism, mean that new ways to eradicate infection such as PDT (Fig 1.13) are needed (Meisel and Kocher, 2005; St Denis *et al.*, 2011). PDT was shown to be an efficient method for decontaminating dental implant surfaces by using MB as a PS (Marotti *et al.*, 2013). PDT also reduced the number of microorganisms in ligature-induced peri-implantitis in dogs and there was no significant difference when compared to conventional therapy (treated using mucoperiosteal flaps for scaling the implant surface and chlorhexidine irrigation) (Hayek *et al.*, 2005). Other researchers reported that PDT significantly reduced the bleeding on probing-positive sites compared to baseline. Thus, PDT represents an alternative approach to local drug delivery in the non-surgical treatment of initial peri-implantitis (Bassetti *et al.*, 2014). Furthermore, PDT is also suggested as a treatment for oral plaque biofilms and PDT using ERY which showed the highest cell killing efficacy when compared to Photofrin and MB in killing the cariogenic bacterium *S. mutans* after 15 min visible light irradiation (Wood *et al.*, 2006).

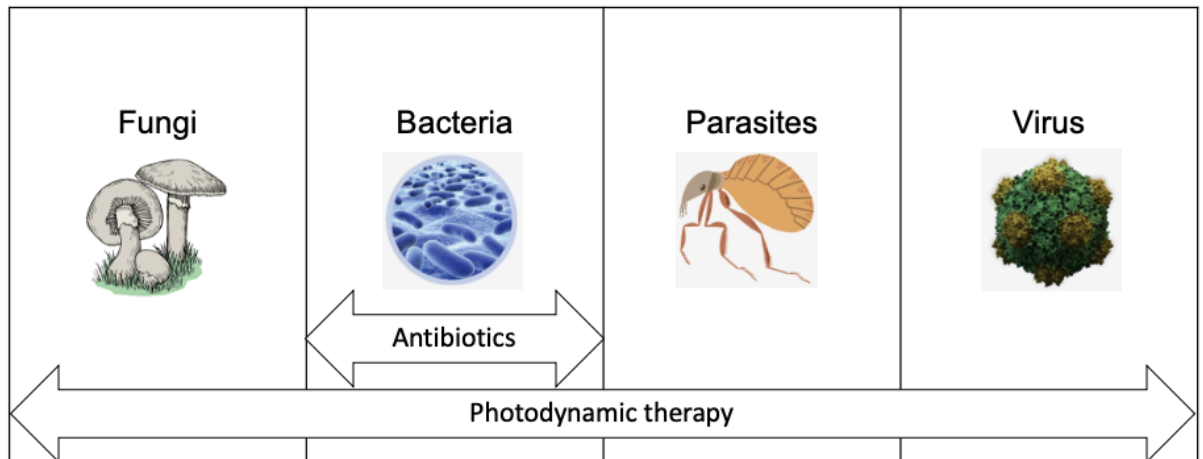


Figure 1.13 The advantages of PDT for localised infections compared to antibiotic drugs (Dai, Huang and Hamblin, 2009).

Adapted and modified (<https://www.seekpng.com/ipng>).

The advantages of PDT for the treatment of infections include: it is known to be safe for human tissue, instant results can be observed after treatment, has a broad therapeutic window, no development of resistance and destroys secreted virulence factors (Dai, Huang and Hamblin, 2009).

As described in section 1.4.2.1, PS such as MB can bind to the outer bacterial membrane, penetrate the membrane and interact with the molecules inside to produce reactive singlet oxygen and free radicals upon illumination. Resistance to PDT is unlikely to develop due to the action of free radicals and singlet oxygen that react with many molecular species in the target cell or tissue. The free radicals and singlet oxygen cause oxidative damage to bacterial cell membrane components such as lipids, amino-lipid, peptide and nucleic acid, thus drive the cell lysis (Wainwright, 1996).

There are some distinctive characteristics of Gram-positive (+) and Gram-negative (-) bacteria that govern the effectiveness of PDT. The cell wall of Gram-positive bacteria is 15-80 nm thick and is made up of a cytoplasmic membrane, which is surrounded by a layer of highly porous peptidoglycan that

allows the penetration of PS (Maisch, 2007; Jori *et al.*, 2011). Fig. 1.14 represents the structure of the Gram-positive bacterial cell wall (Hamblin and Hasan, 2004). Some of the neutral or anionic compounds such as ERY are known to penetrate the cell wall well and inactivate the Gram-positive bacteria due to the action of free radicals and reactive oxygen species after illumination (Kato *et al.*, 2012). Soukos and Goodson (Soukos and Goodson, 2011) showed that MB encapsulated within PLGA was able to induce 99% bacterial killing (*E. faecalis*) in planktonic form after red light irradiation.

Gram-negative bacteria have a thinner cell wall compared to Gram-positive bacteria but have an outer cell membrane, which surrounds the peptidoglycan layer (Fig 1.15). This outer cell membrane is relatively impermeable to anionic PS, thus restricting the uptake of anionic PSs into the cells (Nagata *et al.*, 2012; Jori *et al.*, 2011). However, the situation is different when using cationic PSs, such as Photofrin® which can penetrate the outer cell membrane and inactivate Gram-negative bacteria. Cationic PSs have a positive charge that promotes the binding of PS to the outer bacterial membrane, thus inducing localised damage, which helps in the penetration of PS and release of free radicals into the cell in a process known as self-promoted uptake (Eichner, Gonzales and Maisch, 2013; Sperandio, HUang and Hamblin, 2013; Melo *et al.*, 2011).

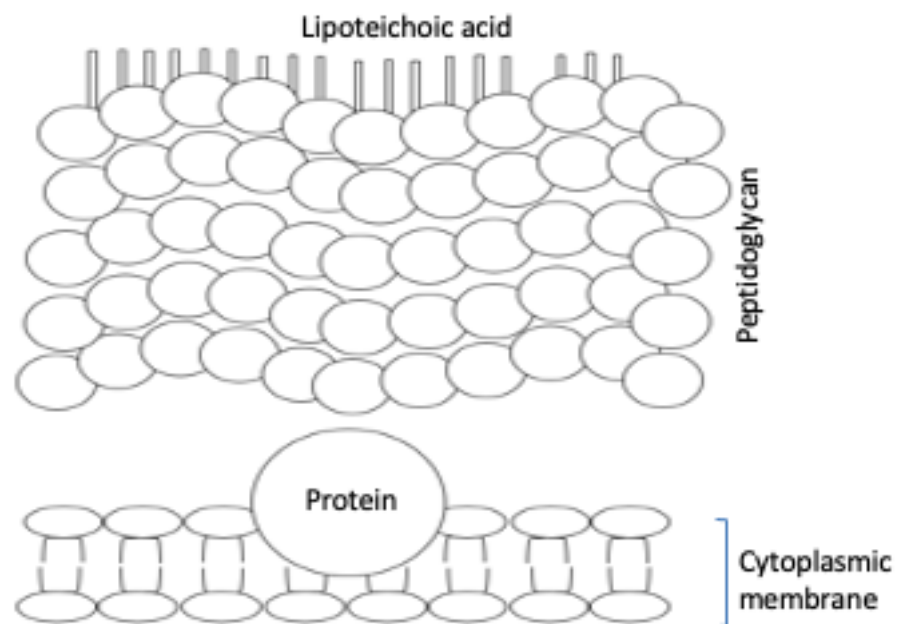


Figure 1.14 Structure of Gram-positive bacteria cell membrane.

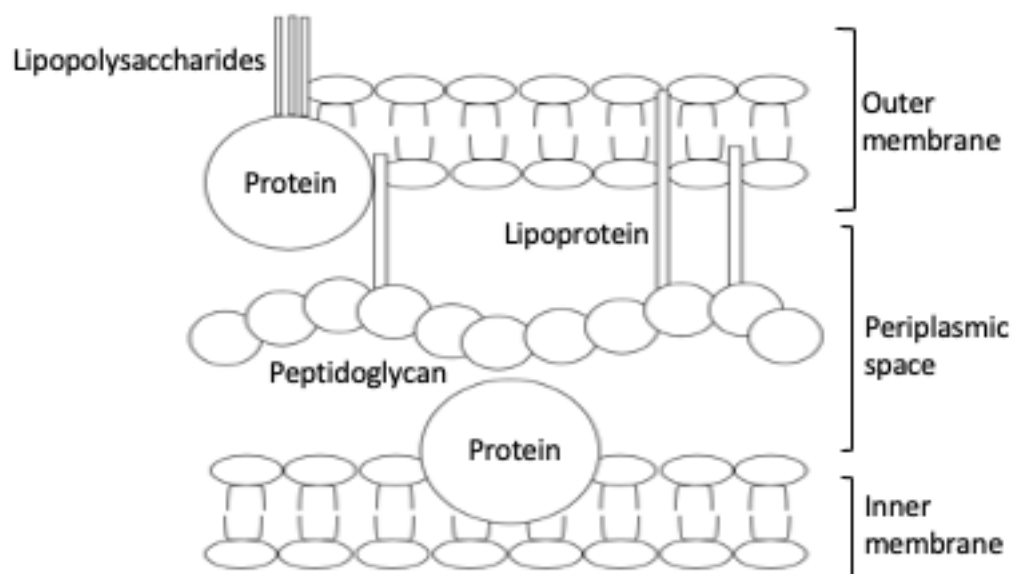


Figure 1.15 Structure of Gram-negative bacterial cell membrane.

There is much ongoing research involving antimicrobial PDT to overcome bacterial infections caused by oral biofilms (Soukos and Goodson, 2011). PDT has been used to treat microorganisms isolated from a deep carious lesion in 20 patients using Fotosan (Agent high) as PS and irradiated with a diode laser for 60 seconds ($\lambda = 632 \text{ nm}$). The bacterial number was reduced, thus eliminating the microbial infection after the treatment (Dogandzhiyska *et al.*, 2013). Then, the combination of PDT and scaling and root planning (SRP) significantly reduced the *A. actinomycetemcomitans* bacteria in the treatment of chronic periodontitis (Marta *et al.*, 2017).

In conclusion, PDT is an emerging field that has been used in several cancer treatments. However, the use of PDT in antimicrobial treatment is still being established, but more research needs to be performed to prove that PDT may be able to help to overcome the antibiotic resistance problem. Earlier work has shown that the level of toxicity of PSs against microbial species is much higher than against mammalian cells, thus making it less damaging to the host cells during PDT treatments at PS and light doses that are sufficient to kill bacteria (Fontana *et al.*, 2013; Costa *et al.*, 2012). It may be possible to utilise this “therapeutic window” to eradicate infections using PDT while sparing the surrounding host tissues, a process which will be of particular benefit in the field of regenerative medicine.

1.6 Aim and objectives:

We hypothesised that a newly fabricated scaffold that incorporates PS (MB and ERY) would support cell attachment and regeneration of dental pulp tissue and have capabilities as an antibacterial when activated by visible light.

The overall aim of this research was therefore to fabricate and characterise tissue regenerative scaffolds that incorporate PS (MB or ERY) and that can be activated by PDT to fight bacterial infection.

The objectives of this study are:

- 1) To fabricate and characterise the newly developed electrospun PGA scaffolds that contained a variety of PS.
- 2) To evaluate the biocompatibility of the selected scaffolds and to determine their effects on hDPSCs attachment and proliferation *in vitro*.
- 3) To evaluate the PDT effects on human dental pulp stromal cells seeded on tissue culture well-plates and the PGA scaffold in *in vitro* culture.
- 4) To investigate the potential anti-microbial effects of the scaffold and PDT on *Enterococcus faecalis* (a Gram-positive bacterium) and *Escherichia coli* (a Gram-negative bacterium).

Chapter 2

Fabrication and characterisation of the Poly-(glycolic acid) scaffold incorporating photosensitiser (Erythrosine B or Methylene Blue)

2.1 Introduction

A scaffold is one of the essential elements of regenerative medicine which is needed to provide a 3-dimensional site for the initial attachment of cells and to enhance the production of the extracellular matrix during cell proliferation and differentiation after cell adherence to the scaffold. As such, the structure and properties of the scaffold are crucial to the success of regenerative medicine (Chang and Wang, 2011). The electrospinning process was used to produce the scaffolds in this study (MacKintosh *et al.*, 2015). One of the advantages of using electrospinning is that the scaffold can be reproduced and maintain its character between different batches. The poly-(glycolic acid) (PGA) polymer has been widely accepted and used in the medical field, and the characteristics of the polymer have been extensively characterised (Ulery, Nair and Laurencin, 2011). However, the introduction of a photosensitiser (PS) into the PGA polymer scaffolds raises the question of whether or not the properties of the PGA polymer may be affected by the PS. Therefore, this chapter seeks to elaborate on the fabrication of the PGA scaffold that has PS incorporated during the production of the scaffold. The scaffolds produced were then characterised in terms of their morphology, water binding capacity and *in vitro* degradation studies (Li *et al.*, 2006; Park *et al.*, 2006).

2.2 Aim and objective

2.2.1 Aim

The aim of the work described in this chapter was to fabricate and characterise the newly developed electrospun PGA scaffolds that contain PS.

2.2.2 Objectives

The above aim will be achieved through the following objectives:

- To synthesise the PGA scaffolds using the electrospinning process both with and without the incorporation of different concentrations of PS (MB and ERY).
- To characterise the properties of these scaffolds and investigate the effects of incorporation of PS on the PGA scaffolds.
- To investigate the PS release profile from the scaffolds *in vitro* and the effect of incorporation of PS on the PGA scaffolds' degradation rate.

2.3 Materials and methods

All materials were purchased from Sigma-Aldrich, UK unless otherwise stated.

2.3.1 Poly-(glycolic acid) (PGA) scaffold with or without photosensitiser

Poly-(glycolic acid) (PGA) (PURAC Biomaterials, Netherland) with an approximate weight-average molecular weight 130,000 was used. PGA must be extruded (quenching process) before being used experimentally in order to improve the polymer chains' orientation and to increase their strength. This process was carried out by Neotherix Ltd (UK): a regenerative medicine company specialising in the development of novel bioresorbable scaffolds for tissue regeneration and repair.

The quenching and solution preparation by Neotherix Ltd. is explained in detail in Appendix C. Briefly, PGA granules were well dispersed across the bottom of an aluminium foil dish before the dish was placed in a fan assisted oven at 270°C for 150 seconds to be melted. After that, the dish with melted PGA granules was immediately removed from the oven and plunged into water at 2 to 5°C to rapidly cool the PGA granules. After 10 seconds the dish was removed from the water and placed upside down on a clean absorbent tissue paper to drain. The quenched PGA granules were then dried in a vacuum oven for 24 h at room temperature. The granules were then packed in an aluminium foil pouch and sealed until use in scaffold fabrication.

When used for scaffold fabrication, the extruded PGA was weighed out using an analytical balance (Avery-Weigh Tronix, USA) to prepare 11.5% (PGA only scaffold) and 12% (scaffolds incorporated with PS or PGA-PS scaffold) weight per volume (w/v) solutions of PGA in HFIP. Weighed PGA was dissolved in

1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (99.9%) (FluoroChem, UK) in a glass vial with a polymeric push on cap (VWR International Ltd, UK). For scaffolds containing PS, 1%, 3%, 5% and 7.5% weight per weight (w/w) of PS in PGA was then added. PS was added by percentage according to the protocol used in previous work by (Wood *et al.*, 2010) and the molar value was calculated as shown in Table 2.1. Two dyes were used as PS in this study:

1) Erythrosine B (ERY) ($C_{20}H_6I_4Na_2O_5$):

- a. Molecular weights of 879.86 g/mol;
- b. Absorption maximum wavelength (λ_{max}) is 525 nm;
- c. ϵ (extinction coefficient): ≥ 82500 at 527-529 nm

2) Methylene Blue (MB) ($C_{16}H_{18}ClN_3S$):

- a. Molecular weight 319.85 g/mol
- b. Absorption maximum wavelength is 668 nm;
- c. ϵ (extinction coefficient): 82000 at 668 nm

Table 2.1 Electrospinning protocols for each of the three different types of scaffolds.

Type of scaffold	PGA	PGA-PS							
		PGA-M				PGA-E			
Polymer %	11.5%	12.0%				12.0%			
PS %	-	1%	3%	5%	7.5%	1%	3%	5%	7.5%
PS loading concentration in Molar (mM)	-	6.8	20.4	34.2	51.6	2.5	7.4	12.4	18.6
Needle tip to mandrel distance (mm)	150	90				60			
Pump rate per needle (mL/min)	0.04	0.03				0.03			
Voltage (kV)	11	14	15	16	17.5	12	13.5	14.5	16
Total time for electrospinning (min)	50	63				63			
Lab temperature (°C)	21.6	22.2				21.5			
Relative humidity (%)	39.8	40.4				43.5			
Target scaffold thickness	100 μm								

$$Polymer \% = \left(\frac{PGA\ weight}{HFIP\ volume} \right) 100$$

$$PS \% = \left(\frac{PS\ weight}{PGA\ weight} \right) 100$$

The details of the settings for each scaffold are summarised in Table 2.1 which describes the polymer percentage concentrations used, PS concentration in percentage and molar, needle tip to mandrel distance, pump rate per needle, the voltage used, laboratory temperature, laboratory relative humidity, scaffold thickness and total time for electrospinning for each different scaffold. These settings had been optimised to ensure that the thickness of the scaffolds fabricated was 100 μm , on average.

The PGA-HFIP and PGA-PS-HFIP solutions in a vial (VWR International Ltd, UK) were covered with aluminium foil and placed on a roller (Thermo Scientific, UK) overnight to make sure all the PGA and PS were completely dissolved in HFIP. All solutions were then filtered through 10 μm polypropylene filters (WestGroup, UK) and were loaded into a syringe pump. The syringe pump was connected to the two 38 mm long flat-ended 21-gauge steel needles (Hamilton, UK) using HFIP resistant Nalgene 890 Teflon FEP tubing (Thermo Fisher Scientific, UK). These tubes and needles were supported by the needle arm which traverses along the length of the mandrel. The syringe pump flow rates were set accordingly so that the polymer solution was ejected from needles.

The needles were arranged in pairs and the needle tip to mandrel separation distance was set accordingly. The distance used between the needle tip to mandrel was dependent on the solution and PS used (type and concentration). This distance was optimised for each PS used so that the fibres formed were dispersed and settled on the white sterilization paper. The needles were perpendicularly aligned to the mandrel rotational axis. A 50 mm diameter mandrel with a rotation speed of 50 rpm was used as a target. The mandrel was covered by plain white sterilization paper (Westfield Medical,

UK) cut to 175×220 mm, which was fastened in place by using strips of double-sided tape between the overlapping paper surfaces to make sure there was no gap between the paper and the mandrel surface. The paper covered the mandrel surface approximately 10 mm past the front and rear edges of the metal surface of the mandrel to avoid the ejected fibres settling on the mandrel.

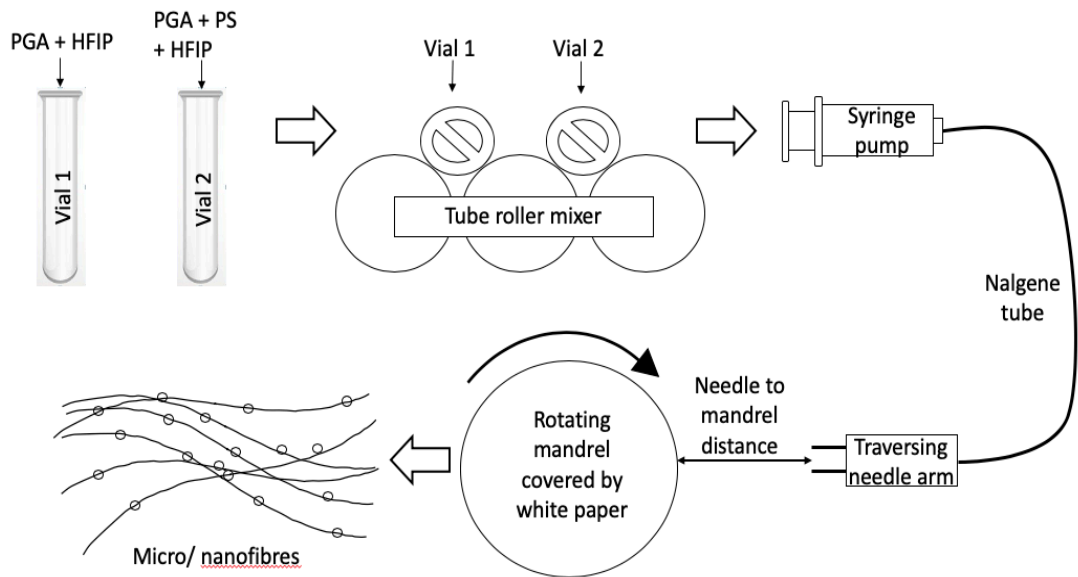


Figure 2.1 A diagram of solution preparation and scaffold fabrication using the electrospinning rig.

After solutions were prepared, the solutions with PGA, PS and HFIP were put on a roller to completely dissolve. The solution was filtered and loaded into a syringe pump that was connected to the needle arm by a tube. The solution was pumped from the syringe to the needles and ejected onto the paper covering the rotating mandrel when a high voltage was applied to the needles. The electrospun scaffold was then collected from the mandrel.

When a voltage was delivered to the needles, the solution was ejected and electrospun towards the paper to form the scaffold sheet (Fig. 2.1). The actual temperature and humidity values during the electrospinning process in the laboratory were recorded using a calibrated digital hygrometer (Pullman Digitron) that was positioned on top of the electrospinning rig. The voltage generator was switched off after the electrospinning process ended following the designated duration.

The scaffold was carefully removed from the mandrel by cutting the scaffold and paper together and releasing it from the mandrel. The scaffold was placed in a vacuum oven at room temperature for at least 72 h to remove any residual HFIP. Subsequently, the scaffold was stored in an aluminium foil pouch and sealed using a sealer machine (Amazon, UK). Desiccant such as silica gel was added to absorb any moisture present in the air. PGA scaffold was labelled as PGA meanwhile PGA-PS scaffolds were labelled differently according to the PS and the percentage used. PGA-PS scaffolds fabricated from ERY additions were labelled as PGA-E scaffolds (PGA-1%E, PGA-3%E, PGA-5%E and PGA-7.5%E) whereas PGA-PS scaffolds fabricated from MB additions were labelled as PGA-M scaffolds (PGA-1%M, PGA-3%M, PGA-5%M and PGA-7.5%M).

2.3.2 Characterisation of scaffolds architecture

The characterisation of scaffold architecture was performed using scanning electron microscopy (SEM). For SEM characterisation, the scaffold (1 cm) was attached to a 12 mm aluminium stub using double-sided carbon adhesive tape and sputter-coated with gold/palladium alloy for 40 s (Agar Scientific, UK). Coated samples were inspected using a S3400-N Hitachi SEM in high vacuum mode using a voltage of 20 kV. A series of images were captured at a magnification of x100 and x1000. The SEM images were then analysed using the DiameterJ software package (ImageJ software 1.49v, USA) to determine the average fibre diameter and porosity of all fabricated scaffolds. A detailed explanation of how to use the DiameterJ software is elaborated in Appendix D. The SEM images of the scaffold were also analysed qualitatively to observe any morphological changes due to incorporation of PS in the PGA polymer.

2.3.3 Water binding capacity

Water binding capacity influences the hydrolytic degradation of polymers and has been widely studied (Park *et al.*, 2006; Wang *et al.*, 2009; Aghdam *et al.*, 2012). The diffusion of water into the polymer can be determined by measuring the increase in mass during incubation in water (Hassan, Chong and Sultana, 2016). PGA, PGA-E (1%, 3%, 5% and 7.5%) and PGA-M (1%, 3%, 5% and 7.5%) scaffolds were cored out into small discs of 1 cm diameter using a cork borer. The mass of PGA, PGA-E (1%, 3%, 5% and 7.5%) and PGA-M (1%, 3%, 5% and 7.5%) discs were determined directly with an analytical balance (**W_{dry}**) (Thermo Fisher Scientific, UK). The scaffold discs were then immersed in distilled water (dH_2O) at 37°C in an incubator for 24 h. After 24 h, the hydrated scaffold discs were taken out of the water and immediately weighed (**W_{wet}**) after removing the excess surface water with a

filter paper. The water binding capacity was expressed as the increase in a mass percentage calculated using the equation below:

$$\text{Percentage of water binding capacity} = \left(\frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \right) 100$$

2.3.4 *In Vitro* degradation studies

PGA, PGA-E (1%, 3%, 5%, and 7.5%) and PGA-MB (1%, 3%, 5%, and 7.5%) scaffolds were cored into 1 cm diameter discs using a cork borer (R & L Enterprise, UK). Scaffolds were then placed in a 12-well tissue culture plate containing 2 mL of either phosphate buffered saline (PBS, pH 7.4) (Lonza BioWhittaker, UK), PBS with 10% foetal bovine serum (FBS) (Lonza BioWhittaker, UK) and dH₂O as a control. PBS was used to replicate the normal body fluid whereas FBS added to observe the degradation of the scaffolds with the serum present. The tissue culture plate containing scaffolds was then covered with aluminium foil to avoid light exposure and incubated at 37°C with 5% of CO₂ in the air. The PBS, PBS with 10% FBS and dH₂O was then collected, stored at 4°C and replaced with fresh solution at predetermined periods: 24 h, 72 h, 168 h and 240 h. After the predetermined periods the scaffolds were taken out, washed thoroughly with distilled water and vacuum dried at room temperature for 48 h (Li *et al.*, 2006). During the experiment, all tissue culture plates containing scaffolds and solution retrieved were covered with aluminium foil to avoid light exposure. Images of scaffolds were then captured using SEM and analysed to determine the changes in morphology and fibre diameter during the degradation process using the same methodology (DiameterJ) as in section 2.3.2.

2.3.5 Photosensitiser release profiles

The solutions (PBS, PBS with 10% FBS and dH₂O) retrieved from the degradation studies (section 2.3.4), were used to determine the concentration of ERY and MB released into the solutions during degradation. The fluorescence readings of each of the samples were then determined using a fluorescent microplate reader (Varioskan, Finland). Briefly, each solution retrieved was diluted in a series of dilutions to ensure the accuracy of the readings. The concentration of PS released from the scaffolds in the solution retrieved in degradation studies (section 2.3.4) was measured by fluorescence intensity emitted using excitation and emission wavelengths appropriate for each PS. For fluorescence detection, the excitation and emission wavelengths used for ERY detection were 500 nm and 540 nm respectively and the excitation and emission wavelengths for MB detection were 609 nm and 668 nm respectively. The concentration of PS released in each sample was determined by comparison of the sample fluorescence readings to a standard curve of known concentrations of either ERY or MB in dH₂O, PBS and PBS + 10% FBS.

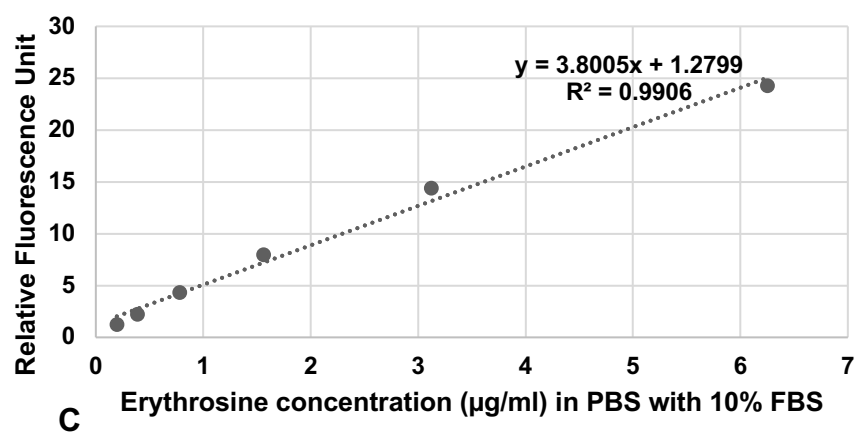
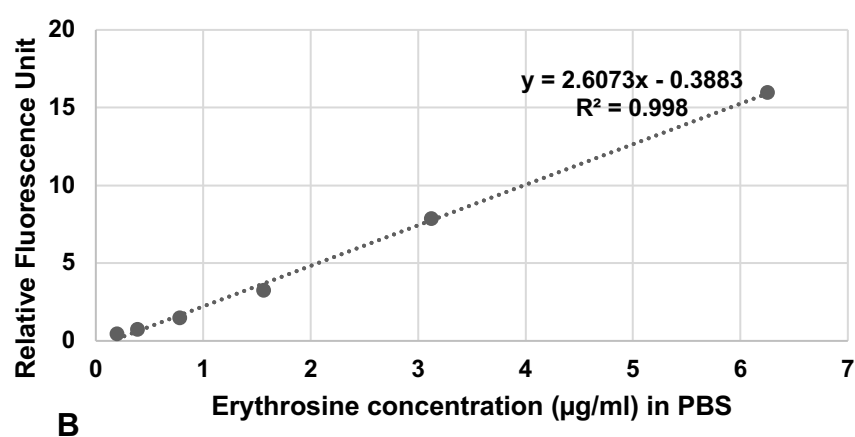
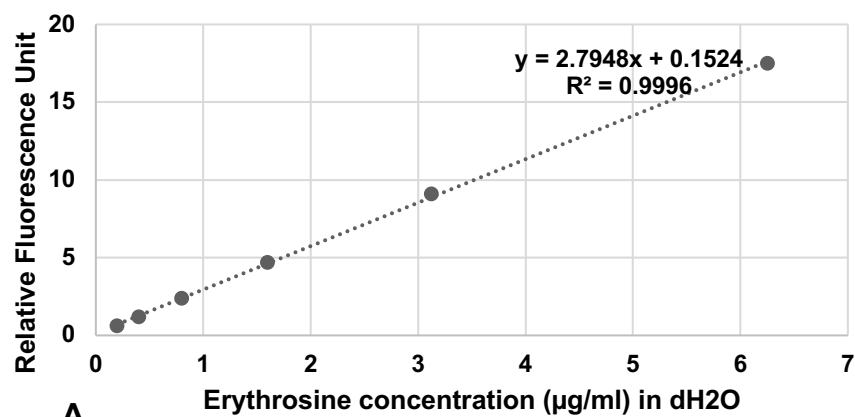
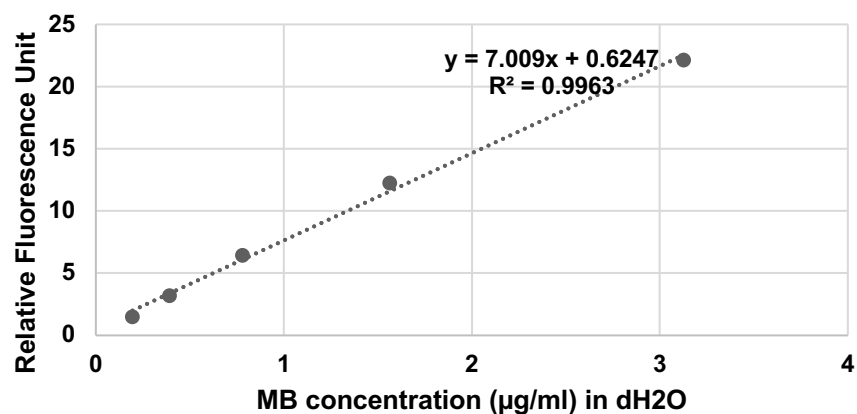


Figure 2.2 Representative standard curves used in the quantitative analysis of ERY release profiles.

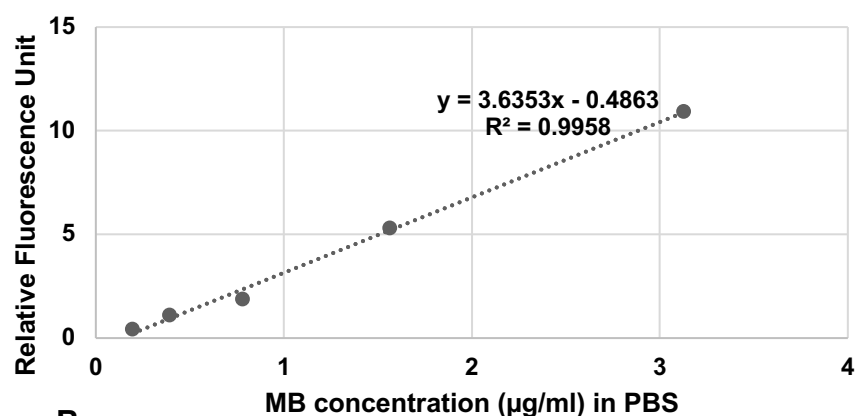
(A) Standard curve of ERY in dH₂O.

(B) Standard curve of ERY in PBS.

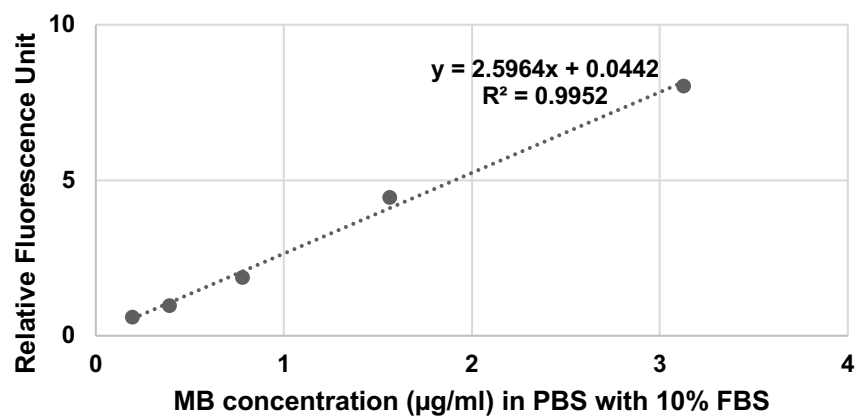
(C) Standard curve of ERY in PBS with 10% FBS.



A



B



C

Figure 2.3 Representative standard curves used in the quantitative analysis of MB release profiles.

(A) Standard curve of MB in dH₂O.

(B) Standard curve of MB in PBS.

(C) Standard curve of MB in PBS with 10% FBS.

2.3.6 pH changes during degradation studies

The pH of the solutions retrieved during degradation studies was checked using pH paper (Fisherbrand, UK) to broadly demonstrate any pH changes.

2.3.7 Statistical analysis

All experiments were carried out in triplicate with each experiment with each experiment being performed 3 times (n=9). The statistical analyses were carried out using IBM SPSS Statistics Version 23 software. Results were analysed using Students' t-test and one way analysis of variance (ANOVA) tests. P-values were determined, and $P < 0.05$ was considered significant. All results were demonstrated as mean $\pm$ standard error of mean (S.E.M) unless stated otherwise.

2.4 Results

2.4.1 Photosensitiser loading efficiency

Macroscopically, the scaffolds produced could be distinguished by their colour (Fig. 2.4). For PGA only scaffolds, the colour was yellowish white. Different percentages of PS were added to the PGA-PS-HFIP solution with a mass ratio range of 1%, 3%, 5% and 7.5%.

The electrospinning process for ERY scaffolds was smooth as there was no blockage inside the needle by the polymer. The highest percentage of ERY resulted in many polymers building up on the tips of the needles, but there was no observable blockage of the polymer solution during the electrospinning process. There were also a few polymer accumulations observed on the surface of the scaffold. While there was polymer build up on the needle, the spinnability of the solutions was not disturbed. The increased percentage of ERY in the polymer solution increased the pink colour intensity of the fabricated scaffold (Fig. 2.4 (b-e)).

Increased amounts of MB resulted in poor spinnability when the electrospinning process started. The needles had to be cleaned regularly to ensure the solutions did not obstruct the needles. The surface of the scaffold for 7.5% MB (PGA-7.5%M) was not as smooth as the other lower percentage MB incorporated scaffolds, and there were also many mesh fibres and fibres accumulated on the scaffold's surface. The increased percentage of MB in polymer solution led, as predicted, to an increase in the blue colour intensity of the produced scaffold (Fig. 2.4 (f-i)).

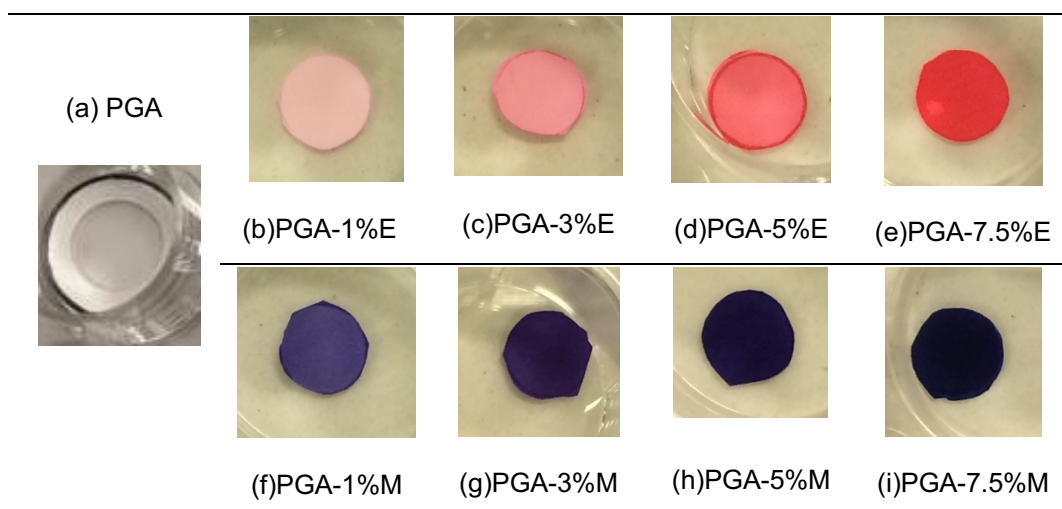


Figure 2.4 Photographs of the PGA scaffolds with/without different percentages of ERY and MB.

Representative images showing PGA scaffolds with or without different percentages of ERY and MB. PGA only (a), PGA-E (1% (b), 3% (c), 5% (d), 7.5% (e)), PGA-M (1% (f), 3% (g), 5% (h), 7.5% (i)). Scale for the scaffolds is 1 cm in diameter.

2.4.2 Effect of Erythrosine B and Methylene Blue additions on architecture, fibre diameter and porosity of the scaffolds

The electrospun PGA scaffolds were fabricated as described in section 2.3.1.

To mimic the fibrous assembled structure of ECM, we chose PGA and produced scaffolds that incorporated either of two PS (ERY or MB). The scaffolds were produced as 100 μm thick sheets and consisted of dense mesh-like fibres.

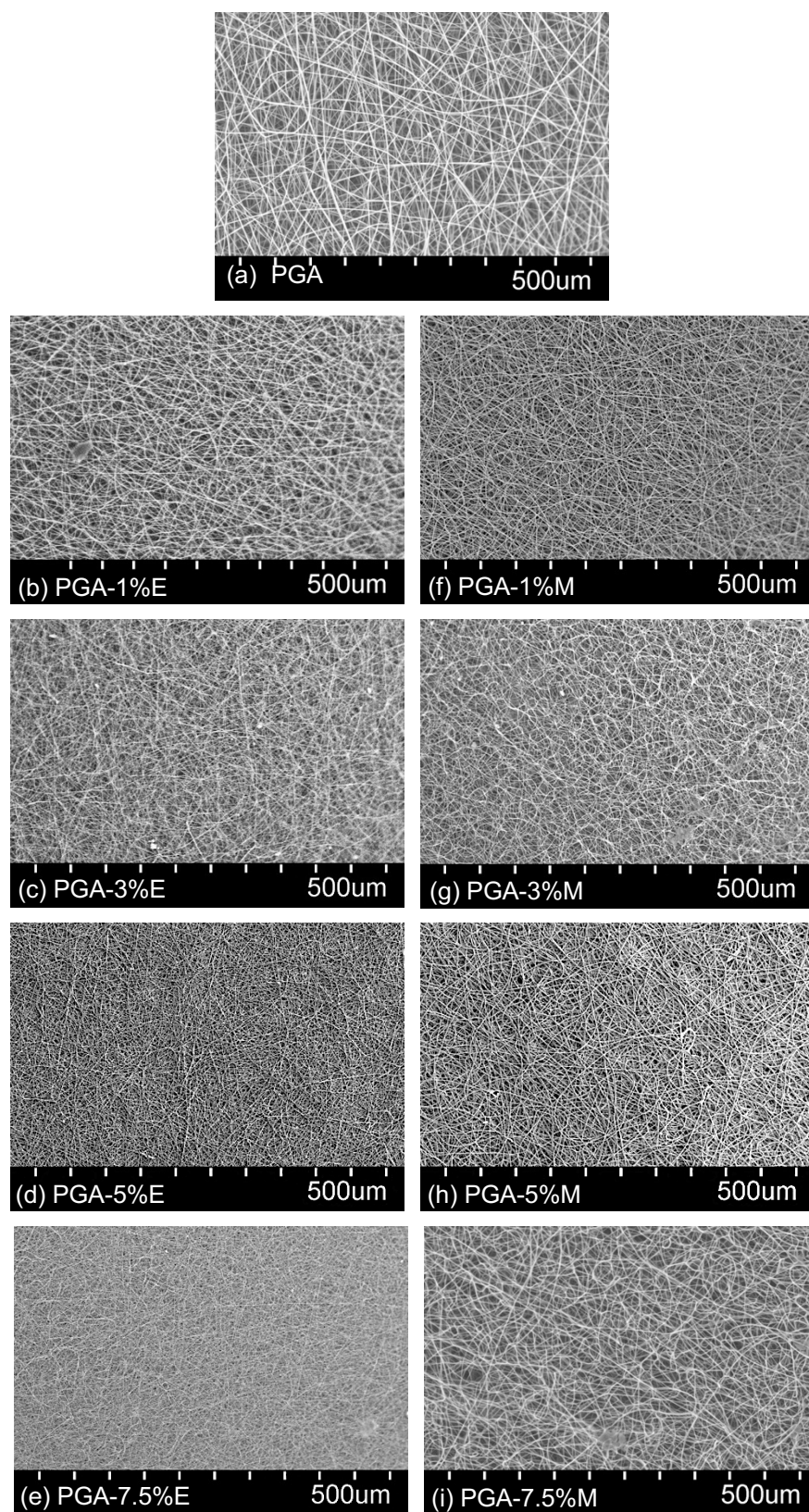


Figure 2.5 Scanning electron micrographs of electrospun PGA scaffolds containing different percentages of PS.

Representative images showing PGA scaffolds with or without different percentages of ERY and MB. PGA only (a), PGA-E (1%(b), 3%(c), 5%(d) and 7.5%(e)), PGA-M (1%(f), 3%(g), 5%(h) and 7.5%(i)). Scale is 500 μ m.

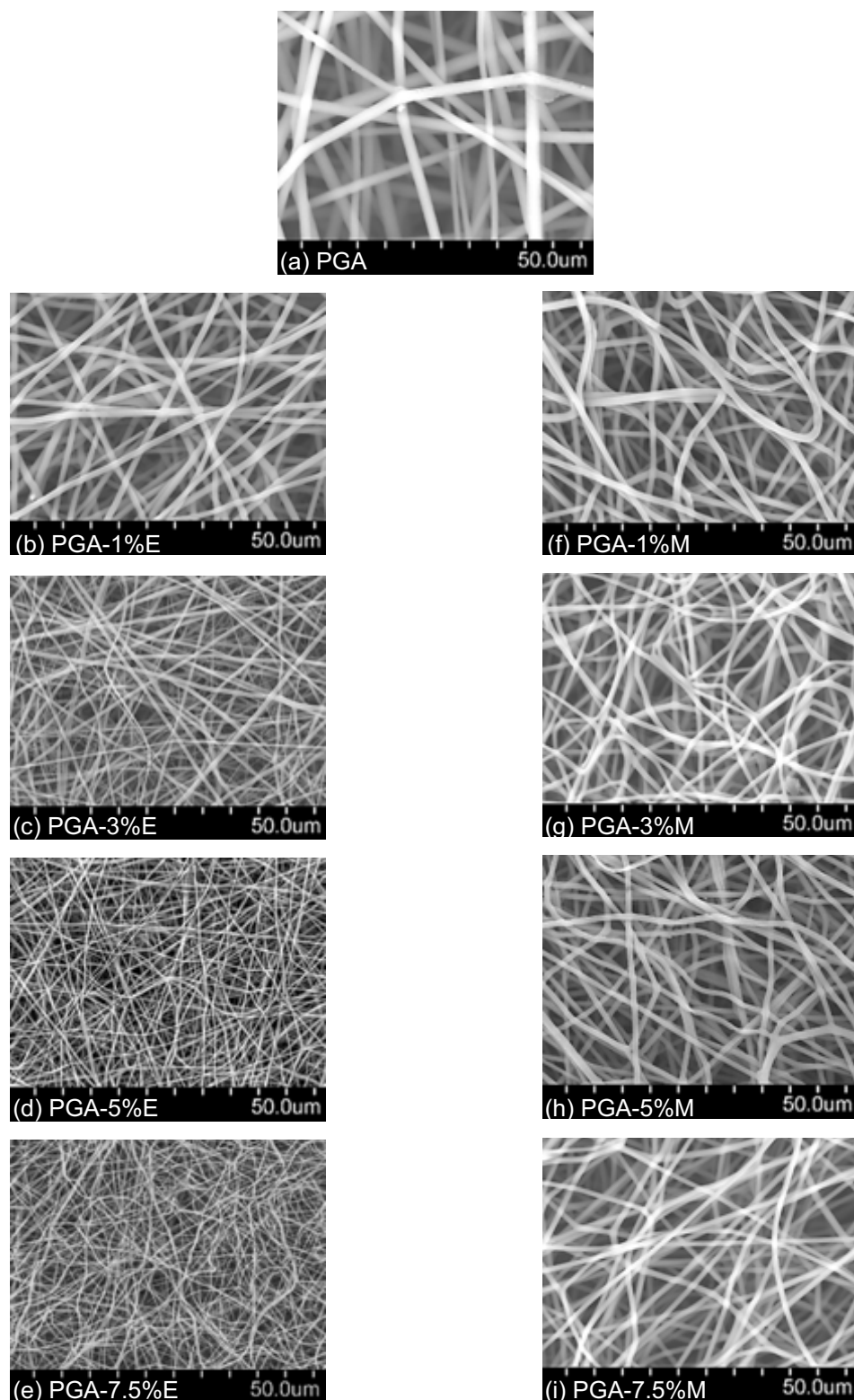


Figure 2.6 Higher magnification scanning electron micrographs of electrospun PGA scaffolds containing different percentages of PS.

Representative images showing PGA scaffolds with or without different percentages of ERY and MB. PGA only (a), PGA-E (1%(b), 3%(c), 5%(d) and 7.5%(e)) and PGA-M (1%(f), 3%(g), 5%(h) and 7.5%(i)). Scale is 50 μm .

Table 2.2 Mean fibre diameter μm (n=9) (+/- standard deviation) and porosity percentage of fabricated scaffolds with or without incorporation of PS.

PGA			PGA-PS			
			1%	3%	5%	7.5%
Fibre diameter	1.989 $\pm$ 0.505	E	0.966 $\pm$ 0.166	0.498 $\pm$ 0.173	0.332 $\pm$ 0.146	0.228 $\pm$ 0.167
		M	0.916 $\pm$ 0.186	0.834 $\pm$ 0.194	0.910 $\pm$ 0.142	0.931 $\pm$ 0.237
Porosity %	53	E	50	49	48	46
		M	57	53	52	50

The results (Fig. 2.5 and 2.6) showed that the appearance of the electrospun fibres in this study were PS type- and percentage-dependent. The SEM micrograph analysis revealed that all nine electrospun matrices were composed of mostly uniform, randomly orientated fibres. The diameters of the fibres ranged from 0.2 μm to 1.9 μm (Table 2.2), with PGA only being the largest, 1.989 μm (Fig. 2.6 (a)). PGA-E fibres diameter varied inversely with ERY percentage: 7.5% were the finest (0.228 μm) followed by 5% (0.332 μm), 3% (0.498 μm) and 1% (0.966 μm) (Fig. 2.6 (b)-(e)). PGA-M fibre diameters showed less variation relative to the percentage of MB incorporated: 1% fibres diameter was 0.916 μm , 3% was 0.834 μm , 5% was 0.910 μm and 7.5% was 0.931 μm (Fig. 2.6 (f)-(i)). The porosity percentage of the scaffolds produced ranged from 46% to 57%. The porosity percentage decreased as the PS percentage addition increased. However, the decrease was not significant.

2.4.3 Effect of Erythrosine B and Methylene Blue incorporation on water binding capacity of the scaffolds

The scaffold's ability to retain water within the matrix was investigated by measuring the water content of each scaffold after 24 h incubation in water as described in section 2.3.3. Figures 2.7 and 2.8 show the water binding capacity of PGA, PGA-E and PGA-M scaffolds with the different incorporated PS percentages (1%, 3%, 5% and 7.5%). The percentage of water binding capacity for PGA only scaffold was 298%. For PGA-M scaffolds, the percentage of water binding capacity increased with increasing MB percentage: PGA-1%M (282%) < PGA-3%M (383%) < PGA-5%M (426%) < PGA-7.5%M (451%). PGA-E scaffolds also showed an increase in the percentage of water binding capacity as the ERY percentage was increased: PGA-1%E (246%) < PGA-3%E (378%) < PGA-5%E (439%) except for the highest percentage PGA-7.5%E (367%).

There was a slight reduction in water binding capacity for PGA-1%E and PGA-1%M when compared to PGA only scaffold. However, this was not statistically significant. Conversely, there was a significant difference in the water binding capacity between PGA-E or PGA-M scaffolds with a higher percentage of ERY or MB (3%, 5% and 7.5%) when compared to PGA only scaffold. Therefore, the addition of PS (either ERY or MB) at a percentage greater than 1% to the PGA polymer increased the water binding capacity of the resultant scaffolds. These results demonstrated that the presence of PS led to an observed increase of the water binding capacity of the scaffolds.

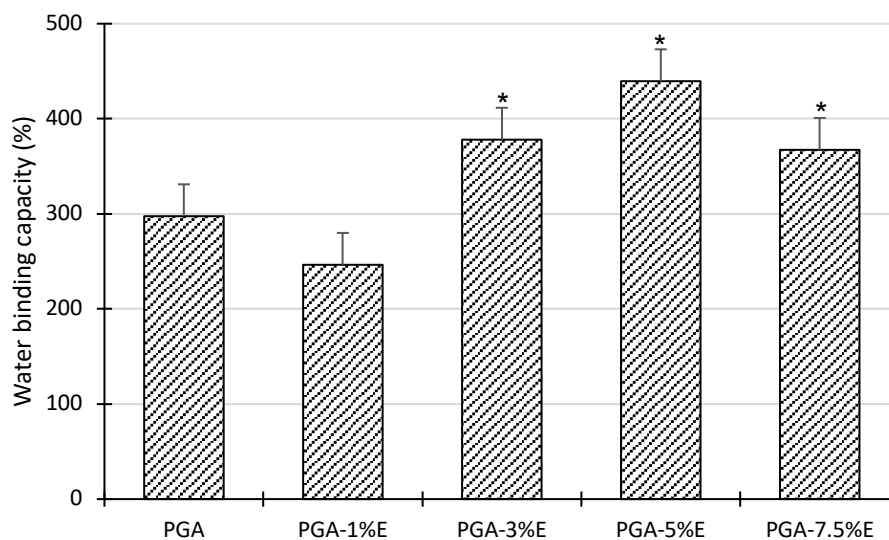


Figure 2.7 Mean water binding capacity of PGA-E scaffolds with different percentages of ERY incorporated.

The graph represents the mean percentage of water binding capacity of PGA-E scaffolds. Data are represented as the mean of percentage of the scaffold replicates ($n=9 \pm \text{S.E}$) in each group.

*Indicates a significant difference between the marked group and PGA group ($p < 0.05$).

$$\text{Percentage of water binding capacity} = \left(\frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \right) 100$$

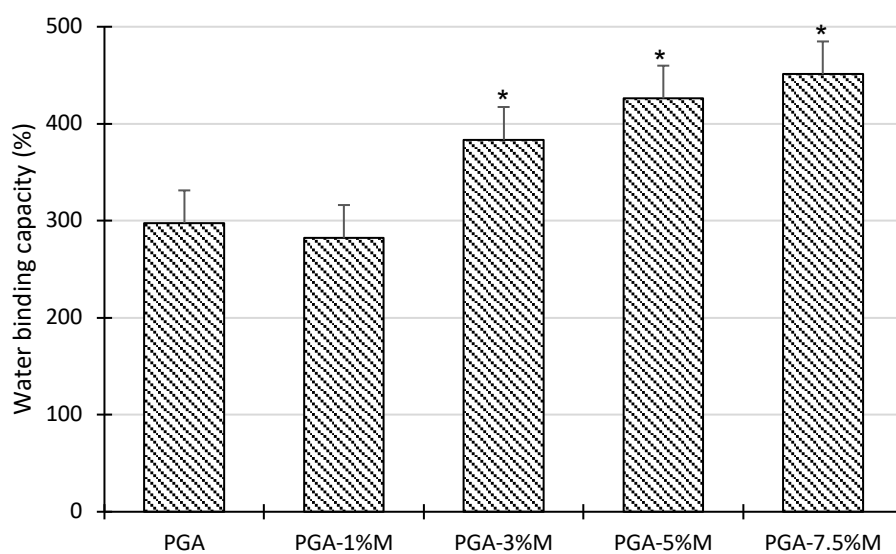


Figure 2.8 Mean water binding capacity of PGA-M scaffolds with different percentages of MB incorporated.

The graph represents the mean percentage of water binding capacity of PGA-M scaffolds. Data are represented as the mean of percentage of the scaffold replicates ($n=9$) $\pm$ S.E in each group.

*Indicates a significant difference between the marked group and PGA group ($p<0.05$).

$$\text{Percentage of water binding capacity} = \left(\frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \right) 100$$

2.4.4 Effect of Erythrosine B and Methylene Blue incorporation on scaffold *in vitro* degradation

2.4.4.1 Scanning Electron Microscopy

Changes in the fibre diameters of each scaffold obtained from *in vitro* degradation studies was calculated using SEM imaging in conjunction with DiameterJ software, as described in section 2.3.4. Each scaffold was incubated in selected media (dH₂O, PBS and PBS with 10% FBS) and collected at predetermined timepoints (24 h, 72 h and 168 h). Table 2.3 tabulates the mean fibre diameter of the PGA, PGA-E and PGA-M scaffolds. In all media tested, the fibre diameters of PGA scaffolds showed no significant changes during the degradation studies.

Table 2.3 Mean fibre diameter μm (n=9) with standard deviation of PGA, PGA-E and PGA-M scaffolds during *in vitro* degradation studies in three different media.

Media	Duration	PGA	PGA-E				PGA-M			
			1%	3%	5%	7.5%	1%	3%	5%	7.5%
dH ₂ O	24 h	1.81±0.42	1.03±0.27	0.53±0.23	0.43±0.14	0.33±0.13	1.02±0.27	0.84±0.15	0.94±0.17	1.45±0.44
	72 h	1.79±0.53	0.98±0.27	0.55±0.24	0.43±0.15	0.33±0.14	1.02±0.25	0.93±0.19	0.97±0.19	1.55±0.31
	168 h	1.89±0.50	1.14±0.31	0.55±0.26	0.43±0.17	0.35±0.13	0.98±0.39	0.91±0.23	1.04±0.23	1.46±0.45
PBS	24 h	1.73±0.70	0.96±0.27	0.63±0.29	0.45±0.16	0.30±0.14	1.10±0.33	0.87±0.22	0.91±0.14	1.41±0.45
	72 h	1.56±0.51	0.98±0.21	0.58±0.21	0.40±0.09	0.34±0.15	1.10±0.32	0.90±0.23	1.01±0.19	1.12±0.39
	168 h	1.80±0.37	0.99±0.17	0.57±0.17	0.40±0.15	0.31±0.13	0.96±0.26	0.82±0.15	1.08±0.47	1.18±0.33
PBS + 10%FBS	24 h	1.96±0.80	0.98±0.28	0.61±0.29	0.44±0.16	0.45±0.16	1.15±0.30	0.88±0.22	1.11±0.45	1.40±0.33
	72 h	1.80±0.70	1.01±0.21	0.59±0.23	0.41±0.14	0.31±0.15	1.11±0.38	0.88±0.14	1.09±0.46	1.45±0.33
	168 h	1.66±0.64	0.95±0.34	0.46±0.25	0.42±0.14	0.30±0.17	0.99±0.38	0.87±0.30	0.89±0.27	0.99±0.41

Table 2.4 Representative SEM micrographs of fabricated scaffolds after incubation in dH₂O for 24 h, 72 h and 168 h. Scale is 50 μ m.

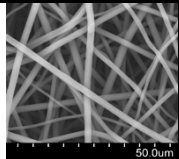
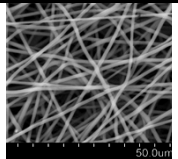
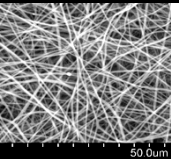
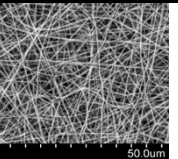
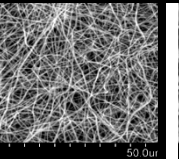
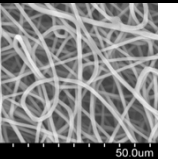
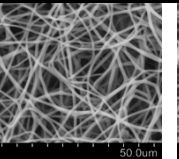
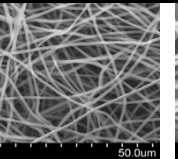
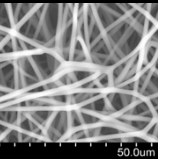
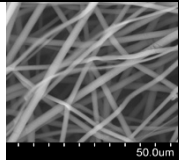
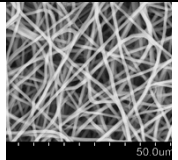
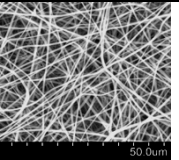
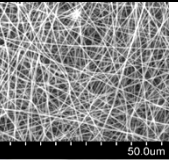
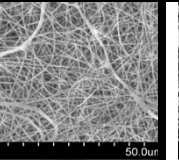
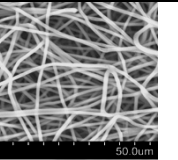
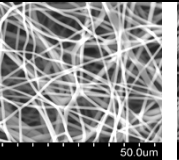
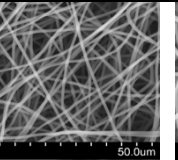
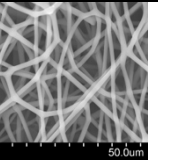
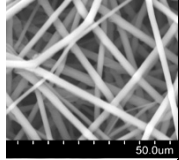
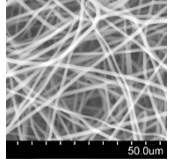
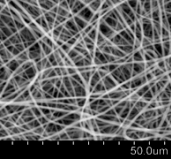
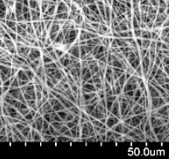
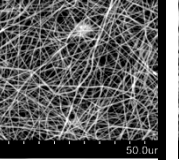
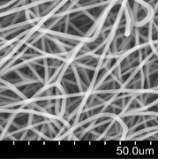
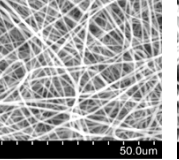
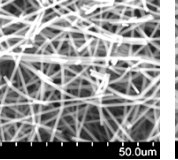
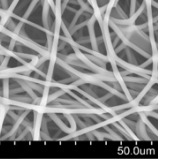
Media	Duration	PGA	PGA-E				PGA-M			
			1%	3%	5%	7.5%	1%	3%	5%	7.5%
dH ₂ O	24 h									
	72 h									
	168 h									

Table 2.5 Representative SEM micrographs of fabricated scaffolds after incubation in PBS for 24 h, 72 h and 168 h. Scale is 50 μ m.

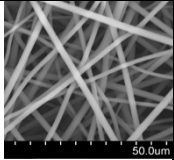
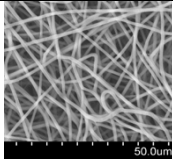
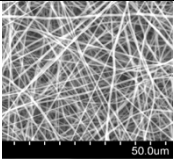
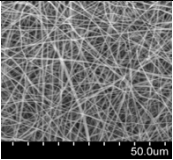
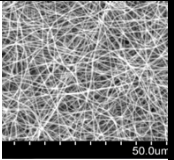
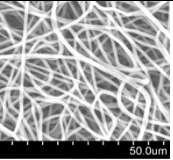
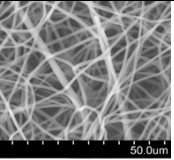
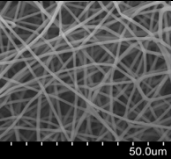
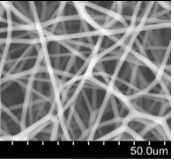
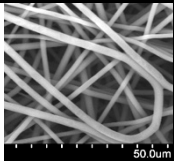
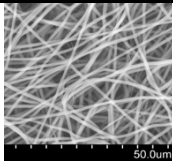
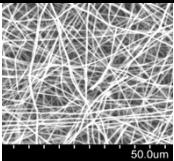
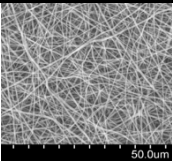
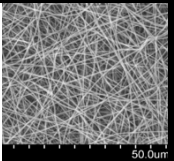
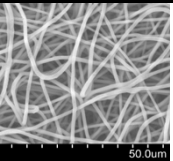
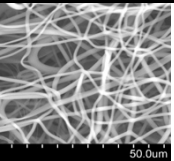
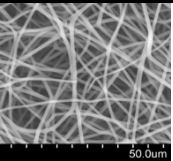
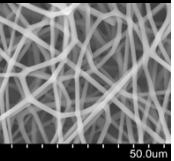
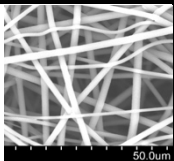
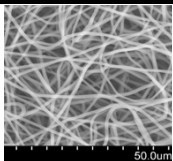
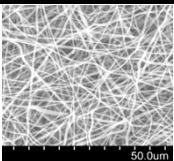
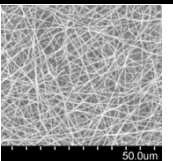
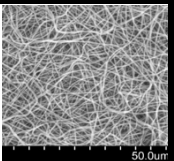
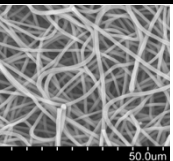
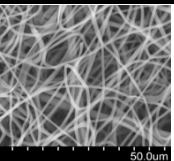
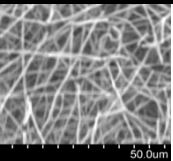
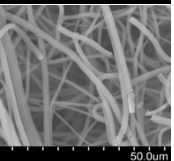
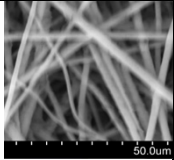
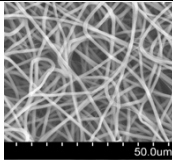
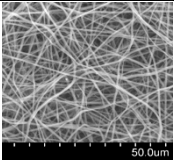
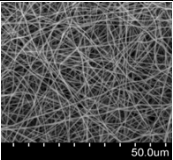
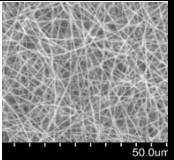
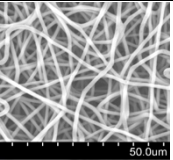
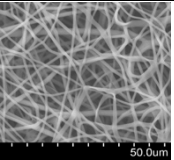
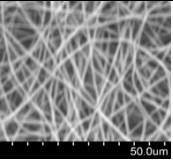
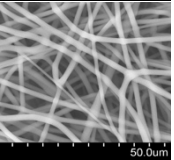
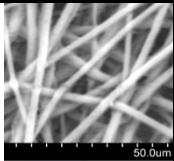
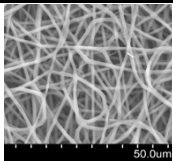
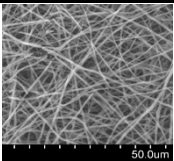
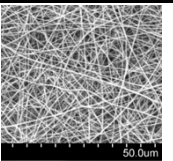
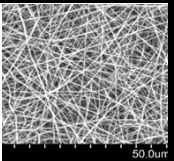
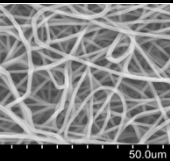
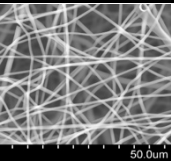
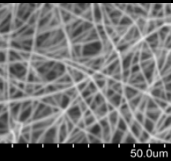
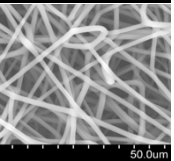
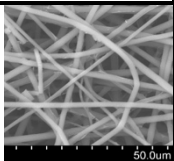
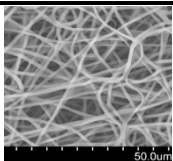
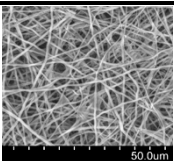
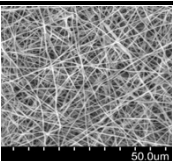
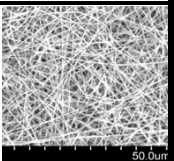
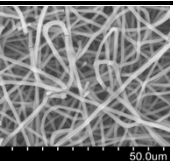
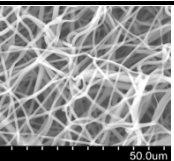
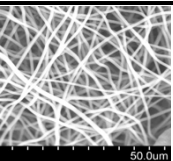
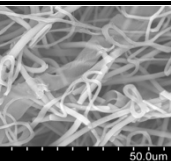
Media	Duration	PGA	PGA-E				PGA-M			
			1%	3%	5%	7.5%	1%	3%	5%	7.5%
PBS	24 h									
	72 h									
	168 h									

Table 2.6 Representative SEM micrographs of fabricated scaffolds after incubation in PBS with 10% FBS for 24 h, 72 h and 168 h. Scale is 50 μ m.

Media	Duration	PGA	PGA-E				PGA-M			
			1%	3%	5%	7.5%	1%	3%	5%	7.5%
PBS with 10% FBS	24 h									
	72 h									
	168 h									

SEM micrographs of the PGA, PGA-E (1%, 3%, 5% and 7.5%) and PGA-M (1%, 3%, 5% and 7.5%) scaffolds after various incubation times (24 h, 72 h and 168 h) are shown in Table 2.4, Table 2.5 and Table 2.6 respectively, in three different media (dH₂O, PBS and PBS with 10% FBS). A few disintegrated fibres and crack lines can be seen after 168 h in all solutions in the order of PBS with 10% FBS > PBS > dH₂O from the SEM micrographs of PGA only scaffold.

Meanwhile, in PGA-E scaffolds, crack lines and disintegrated fibres can be seen as early as 24 h for PGA-5%E and 72 h for PGA-1%E, PGA-3%E and PGA-7.5%E in all degradation solutions. The SEM micrographs showed that PGA-E scaffolds had a higher degradation rate than PGA only. The addition of ERY thus influences (accelerates) the rate of degradation of PGA polymer in the scaffolds.

Disintegrated fibres and crack lines can be seen in PGA-M scaffolds as early as 72 h for all PGA-M scaffolds in all degradation solutions. As the MB percentage increased, the number of disintegrated fibres and crack lines observed in the SEM micrograph were greater: 1% < 3% < 5% < 7.5%.

At 168 h degradation duration, there were several disintegrated fibres and crack lines observed in PGA-M scaffolds when compared to PGA and PGA-E scaffolds. The disintegrated fibres and crack lines observed at 168 h between all scaffolds were in the order: PGA-M > PGA-E > PGA only. Analysis of SEM micrographs showed that fibres with a smaller diameter appeared to have more crack lines compared to fibres with larger diameters within the scaffold. Some deposition was also seen on the fibres of PGA and PGA-E scaffolds

incubated in a solution of PBS+10%FBS. It is probable that the deposition was a serum protein (or proteins) from 10% FBS in the PBS degradation solution.

2.4.5 *In vitro* photosensitiser release from scaffolds

To investigate the release of PS from scaffolds into the media or environment, the scaffolds were incubated in three different solutions: distilled water (dH₂O), phosphate buffered saline (PBS) and PBS with the addition of 10% foetal bovine serum (FBS) for increasing periods from 0 h up to 240 h. The amount of PS released into the solutions were measured after 24 h, 72 h, 168 h and 240 h.

The cumulative release ($\mu\text{g/mL}$) of ERY from PGA-E scaffolds (containing 1%, 3%, 5% and 7.5% ERY) during 240 h of degradation are shown in figures 2.9. The highest release of ERY from scaffolds into the media was seen when using PBS with 10% FBS. The ERY released in PBS with 10% FBS after 240 h increased in the order of PGA-1%E (4.6 $\mu\text{g/mL}$) < PGA-3%E (6 $\mu\text{g/mL}$) < PGA-5%E (27 $\mu\text{g/mL}$) < PGA-7.5%E (36 $\mu\text{g/mL}$). After 24 h of incubation, all the PGA-E scaffolds released smaller amounts of ERY in all three solutions. After 72 h, the amount of ERY released from the PGA-E scaffolds increased significantly in PBS and PBS+10%FBS compared to dH₂O.

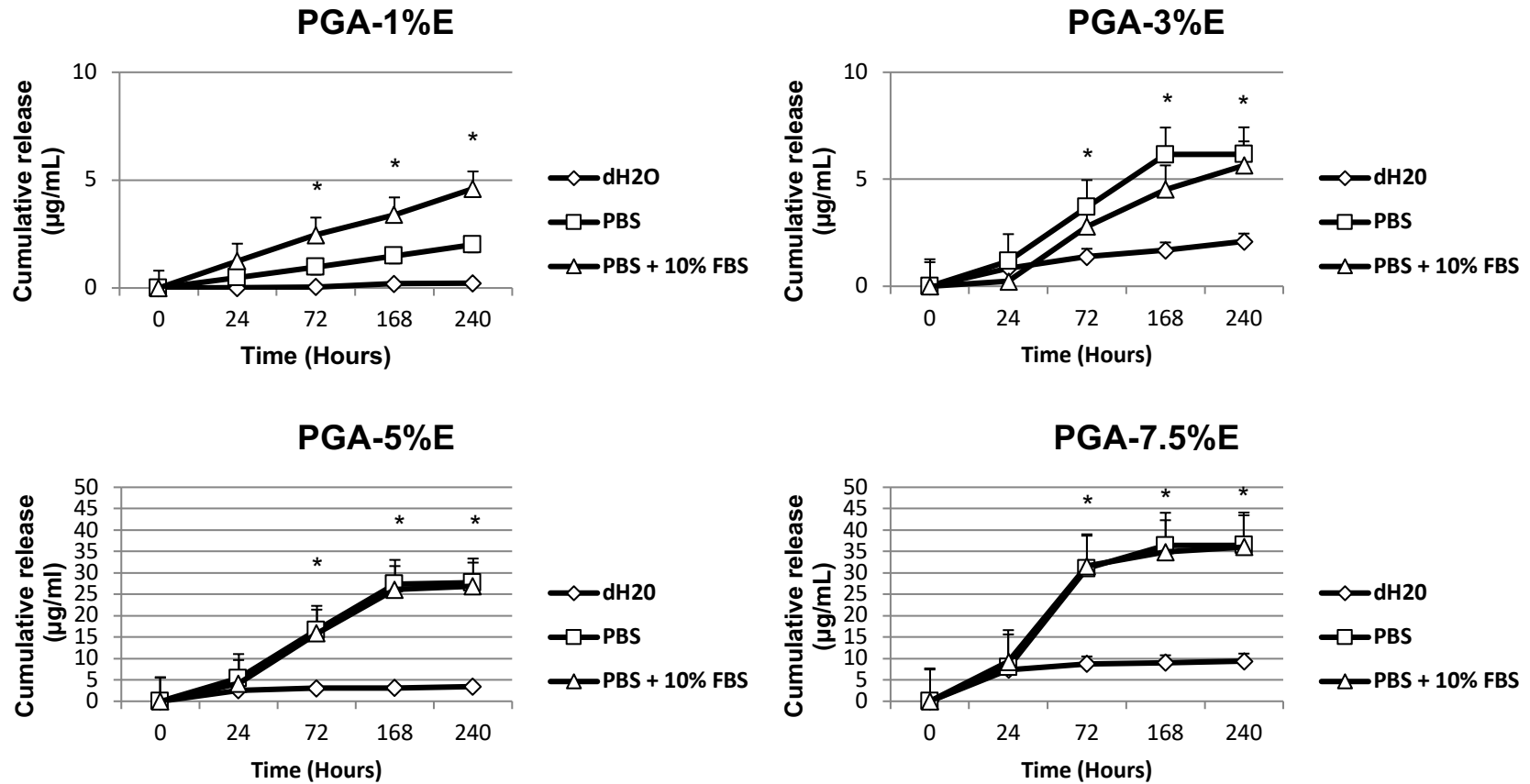


Figure 2.9 Cumulative release of ERY from PGA-E scaffolds after incubation for 24 h, 72 h, 168 h and 240 h in dH2O, PBS and PBS + 10%FBS.

The graphs represent the mean cumulative release of ERY from scaffolds. Data are presented as the mean cumulative release of ERY from scaffold replicates ($n=9$) $\pm$ S.E in each group. *Indicates a significant difference between cumulative release of ERY in PBS or PBS + 10%FBS group and dH2O group ($p<0.05$).

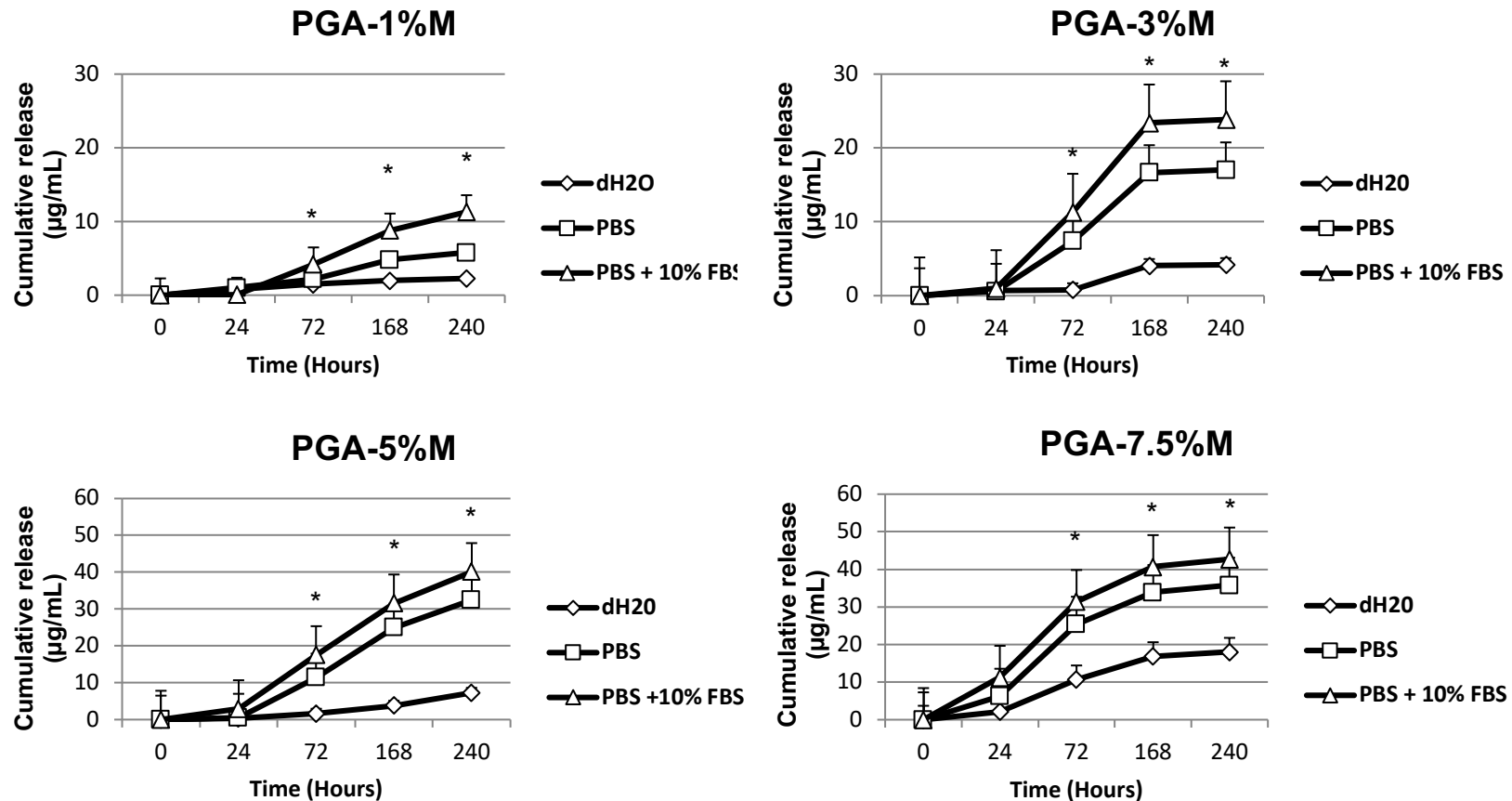


Figure 2.10 Cumulative release of MB from PGA-M scaffolds after incubation for 24 h, 72 h, 168 h and 240 h in dH2O, PBS and PBS + 10%FBS.

The graphs represent the mean cumulative release of MB from scaffolds. Data are presented as the mean cumulative release of MB from scaffold replicates ($n=9$) $\pm$ S.E in each group. *Indicates a significant difference between cumulative release of MB in PBS or PBS + 10%FBS group and dH2O group ($p<0.05$).

Figures 2.10 show the cumulative release ($\mu\text{g/mL}$) of MB from PGA-M scaffolds (containing 1%, 3%, 5% and 7.5% MB respectively) during 240 h of degradation. MB released during degradation was increased as the percentage of MB in PGA solution increased in order of 1% ($11.3 \mu\text{g/mL}$) < 3% ($23.8 \mu\text{g/mL}$) < 5% ($40 \mu\text{g/mL}$) < 7.5% ($42.7 \mu\text{g/mL}$). In the initial 24 h of incubation, the PGA-M scaffolds released a smaller amount of MB in all three solutions. After 72 h, the amount of MB released from the PGA-M scaffolds increased significantly in PBS and PBS+10%FBS compared to dH_2O .

The results showed that all PGA-E and PGA-M scaffolds released a smaller amount of PS in the initial 24 h of incubation in all three solutions and there was no significant difference between them. The degree of PS release from the scaffold can be seen in the order of high to low PS concentration from 7.5% > 5% > 3% > 1% PGA-E or PGA-M scaffolds. After 72 h, there was a significant difference of the amount PS released in PBS and PBS+10%FBS compared to dH_2O . It should be noted that serum proteins will adsorb to the biomaterial surface after implantation *in vivo* and play a role in regulating the host response to the scaffold biomaterial. Therefore, the solution that best reflects this situation when investigating the release of PS *in vitro* is PBS + 10 % FBS (Jose and Alagar, 2015).

2.4.6 pH measurement of culture media

The pH of the assay supernatant was checked at every time point (24 h, 72 h, 168 h and 240 h) to observe any changes in pH for each type of scaffold. From the observation at all time points shown in Table 2.7, the pH of PBS and PBS with 10% FBS solutions were approximately pH 7.0 for all type of scaffolds. However, in dH_2O , the pH level reduced from approximately pH 7.0 at 24 h to approximately pH 3.0 for all type of scaffolds at 72 h, 168 h and 240 h.

Media	Duration	PGA	PGA-E				PGA-M			
			1%	3%	5%	7.5%	1%	3%	5%	7.5%
dH ₂ O	24 h	4	4	4	4	4	4	4	3	3
	72 h	4	4	4	4	4	3	3	3	3
	168 h	4	4	3	3	3	3	3	3	3
	240 h	4	4	3	3	3	3	3	3	3
PBS	24 h	7	7	7	7	7	7	7	7	7
	72 h	7	7	7	7	7	7	7	7	7
	168 h	7	7	7	7	7	7	7	7	7
	240 h	7	7	7	7	7	7	7	7	7
PBS + 10%FBS	24 h	7	7	7	7	7	7	7	7	7
	72 h	7	7	7	7	7	7	7	7	7
	168 h	7	7	7	7	7	7	7	7	7
	240 h	7	7	7	7	7	7	7	7	7

Table 2.7 pH reading of media retrieved from the *in vitro* degradation studies for each scaffold. Media used were dH₂O, PBS and PBS with 10% FBS (n=9).

2.5 Discussion

In electrospinning technology, there are many parameters which affect the diameter of the fibres in the finished product. The important electrospinning parameters can be classified into solution parameters, process parameters and ambient parameters (Li and Wang, 2013). In this study, the process and ambient parameters for each of the PGA, PGA-E and PGA-M solutions were almost constant. Therefore, the difference in the fibres diameter of the produced scaffolds may be associated with the solution parameters that affect the nature of electrospinning scaffolds (Li and Wang, 2013). The characteristics and molecular weight of ERY (879.86 g/mol) and MB (373.90 g/mol) may possibly play a role in dissolving the PGA polymer, changing the viscosity, conductivity or surface tension of the polymer solution and hence resulting in the difference in morphology and the smaller fibre diameters observed with the PGA-E and PGA-M compared to PGA only scaffolds (Hasan, Azim and Reza, 2014). The increase in the content of ERY and MB may reduce the viscosity of polymer solution thus increasing the polymer conductivity of the polymer solution, resulting in smaller fibres by inducing the fibres to stretch by decreasing the surface tension (Li and Wang, 2013; Meng *et al.*, 2011; Park *et al.*, 2006). The higher molecular weight of the polymer may also increase the solution's molecular entanglement, leading to an increase in solution viscosity and final nanofibre size (Yousefzadeh, 2017).

As for the PGA-E scaffolds, their much-reduced fibre diameters suggest that the ERY may have a greater interaction with the PGA polymer and therefore cause significant changes to the electrospinning solutions, resulting in the

observed diameter decrease. Fig. 2.6 (b-e) showed that as the ERY percentage increased, fibres from smooth to micro-ribbon were produced and the fibre diameters were decreased.

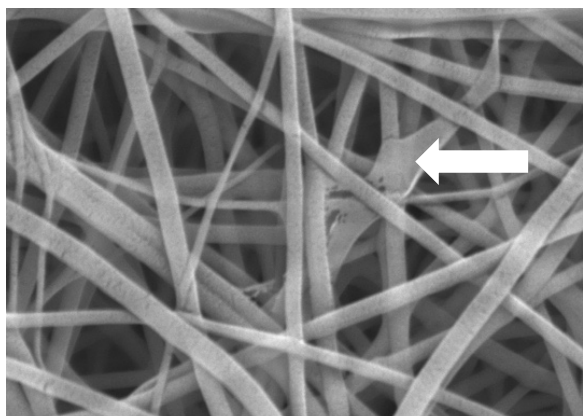


Figure 2.11 Micro-ribbon fibre production in ERY highest percentage of PGA-E scaffold.

White arrow indicating the position of the micro-ribbon fibre.

Figure 2.11 shows an example of the micro-ribbon present in the PGA-E scaffold, indicated by the white arrow.

The PGA-M scaffolds had smaller fibre diameters when compared to the PGA only scaffold. However, the fibre size was still larger when compared to PGA-E scaffolds except for PGA-1%E scaffold. When comparing the PGA-M scaffolds (1%, 3%, 5% and 7.5%), there was no significant difference in the fibre diameter measurements. Figure 2.6 (f-i) showed that the micrographs and fibre diameters of PGA-M scaffolds did not change significantly even when the percentage of MB added was increased more than 1%. The possible explanation for the insignificant changes in PGA-M fibres diameter is due to MB reaching its limit of maximum effect on the PGA polymer solution at a level

lower than 1%. Therefore, no changes in fibres diameter would be observed even though the MB percentage was increased.

Therefore, it can be concluded that the addition of PS to PGA polymer led to the formation of fibres with smaller diameters than PGA alone. For scaffolds with incorporated ERY, as the percentage of ERY was increased, the fibre diameters of the scaffolds decreased.

In contrast, while the fibre diameters of the MB-containing scaffolds were significantly less than the PGA alone, increasing the percentage of the MB did not appear to further reduce the diameter of the fibres. However, from the observation of scaffold morphology, the PGA-7.5%M scaffold showed that many micro-ribbons were produced along with smooth nanofibres (as indicated by the white arrow in Fig. 2.12).

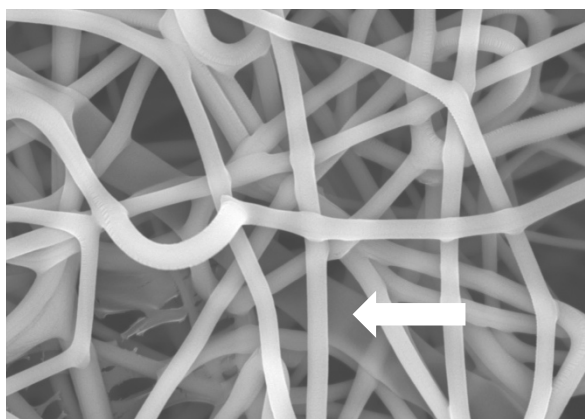


Figure 2.12 Micro-ribbon fibre production in PGA-M scaffold incorporating 7.5% MB.

The white arrow indicates the position of the micro-ribbon fibre.

Generally, in electrospun scaffolds, it is known that a decrease in the viscosity of the polymer solution will produce smaller fibre diameter (Nayak *et al.*, 2012). The addition of different solvents and materials will also change the polymer conductivity and surface tension. Higher conductivity or lower surface tension favours the formation of smaller fibres (Hasan, Azim and Reza, 2014; Bhardwaj and Kundu, 2010). It is likely that the PS have increased the polarity of the polymer as well as decreasing the viscosity and surface tension of the polymer solution, thus causing the fibres to stretch and become thinner as a result (You *et al.*, 2005; Li and Wang, 2013). The effect of these parameters on the physical characteristics of the scaffolds requires further investigation as it will potentially impact upon the ability of stem cells to grow on, and infiltrate the scaffolds.

The scaffold's ability to retain water within the matrix was determined by its water binding capacity. This has an important role for the absorption of body fluid and for the transfer of cell nutrients and metabolites through the scaffold (Vacanti and Langer, 1999). There was a slight difference in the water binding capacity, despite the fact that the fibre diameter of the different PGA-M scaffolds was consistent. This may be due to the hydrophilic nature of MB, which will further facilitate water absorption to the scaffolds (Park *et al.*, 2006). Therefore, with a higher amount of hydrophilic MB within the scaffolds, more water absorption will be expected. Other than that, the electrospun scaffolds' water uptake also depends primarily on their porosity and specific area to absorb water (Wu *et al.*, 2010). An increase in surface area to volume ratio means increased water absorption resulting in higher degradation rates. This can be seen in PGA-E scaffolds as ERY is not as hydrophilic as MB, but the

fibre diameters decreased as ERY concentration increased. The smaller fibre diameters will have a higher surface area to volume ratio and provide large areas for water contact and thus facilitate further absorption. However, for the PGA-7.5%E scaffold, the water binding capacity was lower than PGA-5%E scaffold. The decrease in water penetration and absorption may be due to the morphology of the PGA-7.5%E scaffolds which is dense due to the close proximity of the nanofibers leading to the scaffolds' small pore area. These dense nanofibers and small pore areas are likely to hinder water diffusion into the deeper areas of the scaffold.

The water binding capacity of the scaffolds provides information for the absorption of body fluids and for the transfer of metabolites and nutrients between cells and through the scaffolds. Therefore, a higher degree of water binding capacity will allow the scaffolds to have maximum probability of promoting and sustaining cell growth in 3D scaffolds (Vacanti and Langer, 1999).

Hydrophilicity of the scaffold is one of the important factors that will affect biological interactions such as protein adsorption, platelet adhesion and also attachment to the implanted scaffold (Xu and Siedlecki, 2007; Spriano *et al.*, 2017). It was found that a hydrophilic polymer has a good interaction with biological fluids and enables protein adsorption to the surface and promotes further interaction with cell receptors (Gittens *et al.*, 2014). As PGA polymer was known to be hydrophilic materials (Zhang *et al.*, 2014a), the scaffolds produced with the incorporation of the PS were shown to have higher hydrophilicity properties as their water binding capacity was significantly higher than PGA only scaffold.

The degradation rate of PGA is highly dependent on the accessibility of water to ester bonds in between glycolic acid groups to enable hydrolytic cleavage of the ester bond in the polymer backbone (Li *et al.*, 2006; Ju, Huang and Chen, 2014). The rate of degradation also relies on the crystallinity, molecular weight, copolymer composition, morphological structure of the scaffolds (Makadia and Siegel, 2011). PGA is also known to be hydrophilic and have larger fibre diameters compared to PGA-E or PGA-M. PGA only scaffold exhibited disintegrated fibre and initiation of disintegration after 168 h (7 days) as shown in figure 2.13.

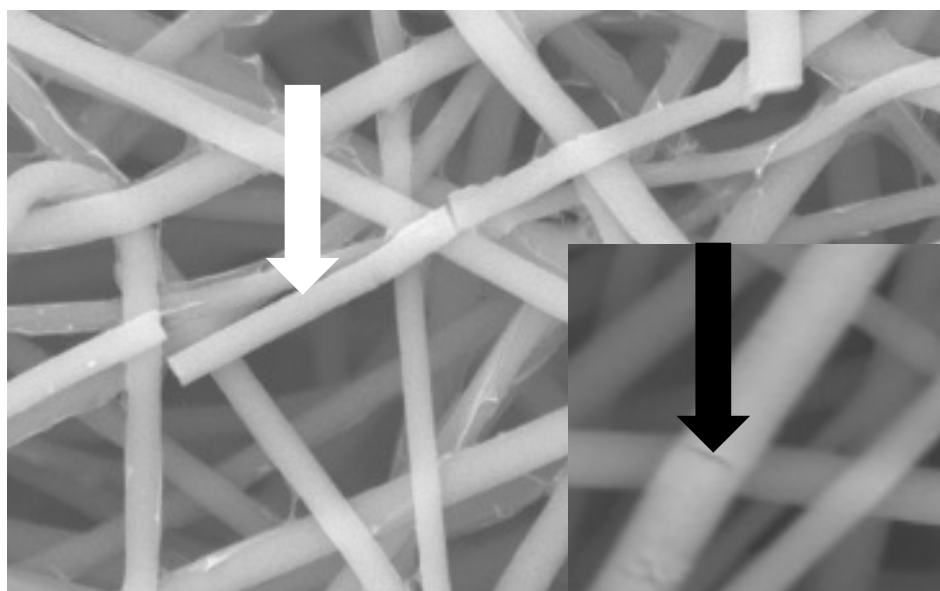


Figure 2.13 The morphology of the PGA only scaffold after 168 h (7 days) in media.

White arrow indicating disintegrated fibre and black arrow indicating the crack line (initiation of disintegration).

The degradation rates increased in PGA-E scaffolds as the percentage of ERY increased, as many disintegrated fibres were observed as early as 72 h which was in contrast to PGA only scaffold (168 h) (Fig. 2.14). This may be due to the smaller fibre diameters that provide a higher surface area to volume ratio, thus increasing the probability of fibres interacting with water and further increasing the degradation rates (You *et al.*, 2005).

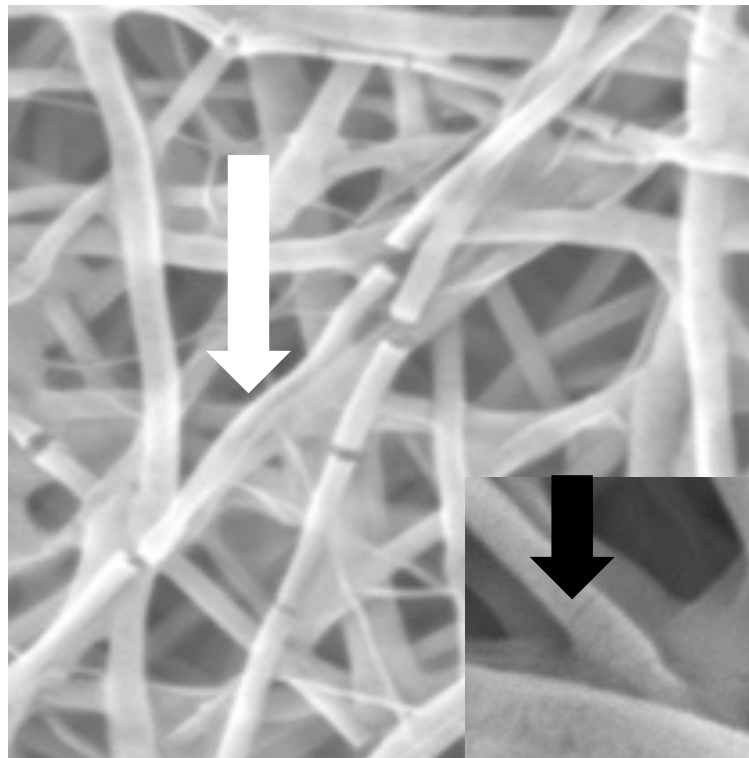


Figure 2.14 The morphology of the PGA-E scaffold after 72 h (3 days) in media.

White arrow indicating disintegrated fibre and black arrow indicating the crack line (initiation of disintegration).

In PGA-M scaffolds, when compared to PGA only scaffolds (168 h), the degradation rates increased as many disintegrated fibres and signs of initiation of disintegration (crack lines) could be seen as early as 72 h (Fig. 2.15). This may have been due to the hydrophilic nature of MB, that further increases the scaffold's water binding capacity and hydrophilicity, leading to the increased degradation rates of PGA-M compared to PGA only and PGA-E (Park *et al.*, 2006; Gittens *et al.*, 2014). The scaffold's morphology showed that by incorporating PS, the hydrolysis of the PGA polymer could be accelerated as the degradation rates are highest in PGA-M followed by PGA-E and PGA only scaffold.

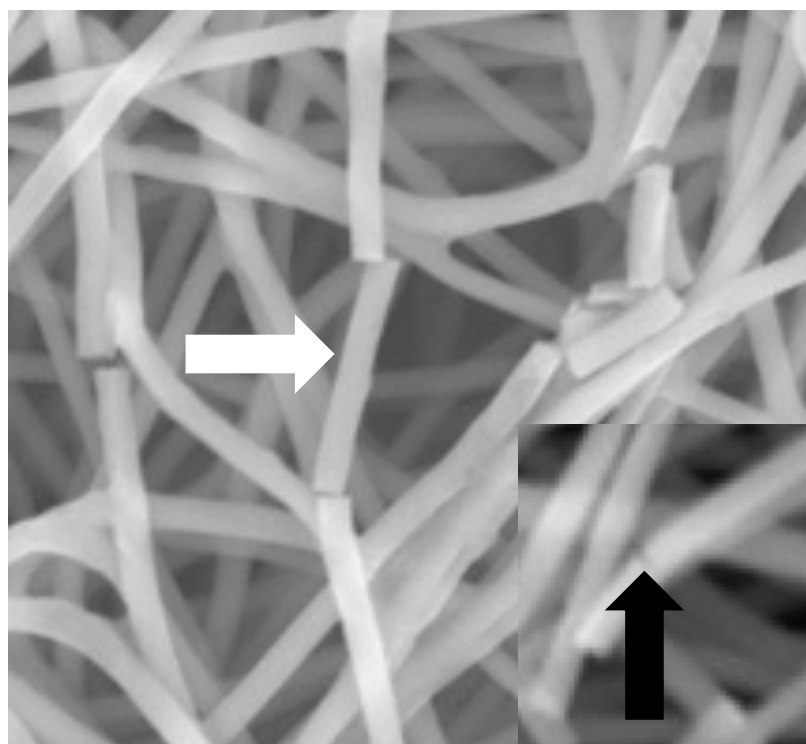


Figure 2.15 The morphology of the PGA-M scaffold after 72 h (3 days) in media.

White arrow indicating disintegrated fibre and black arrow indicating the crack line (initiation of disintegration).

The release of an antimicrobial agent from the scaffold or implant after surgery could prevent bacteria from invading the surgical site (Johnson and Garcia,

2015). Therefore, to provide antibacterial properties to the PGA scaffold, PS was used. Therefore, when an infection occurs, PS can be activated by visible light when antibacterial action is needed. The light should, however, be able to reach the infection site in order to be able to activate the PS. As the scaffold degrades, the released PS will be taken up by bacteria and will act as an antibacterial agent when light is applied. During degradation of the scaffolds in aqueous solutions, fibres were disintegrated and subjected to hydrolytic scission of the ester bonds of PGA (Ju, Huang and Chen, 2014), which permitted more PS to be released as polymer breakdown progressed (Obermeier *et al.*, 2015). The morphology of the scaffold, such as three-dimensional structure, fibre diameter, the orientation of the fibres, the location of the PS within the fibres, loading concentration and the presence of micro-ribbons and beads, will also play an important role in inducing more PS to be released from the scaffold (Obermeier *et al.*, 2015; Chou, Carson and Woodrow, 2015). PS release rate may possibly be modulated by changing PS percentages incorporated in the PGA polymer, as the result showed that a greater amount of PS was released as the incorporated PS percentage increased. This demonstrated the possibility of producing scaffolds with an appropriate amount of antibacterial agent by manipulating the PS percentage added to the polymer.

In addition, the rate and amount of PS released may also depend on the mechanism of PS release, such as simple diffusion, polymer erosion or polymer swelling followed by diffusion (Dorati *et al.*, 2017). From the results, as the percentage of PS increased, the PS released also increased.

Based on the findings, the addition of the PS into the PGA polymer modified the characteristics of the PGA polymer itself. The addition of ERY to the PGA polymer reduced the fibre diameters, increased the hydrophilicity and increased the degradation rates of the produced scaffold. Furthermore, PGA-E scaffolds release or deliver the PS in a manner dependent on the percentage incorporated, meaning more PS was released as more PS was added, which is favourable as the PS release amount can be easily tuned by manipulating the PS loading amount.

MB addition to the PGA polymer reduced the fibre diameters of the produced scaffold by approximately half compared to the PGA only scaffold. The addition of more than 1% of MB did not further affect the fibre diameters. However, as the percentage of added MB was increased, the hydrophilicity of the PGA-M scaffolds increased. The increase in hydrophilicity accelerates the rate of degradation and causes greater PS release.

The pH changes were only seen in dH₂O because the PBS and PBS with 10% FBS possess buffering capability. As the scaffolds degrade, the PGA polymer was released into the media and the accumulation of glycolic acid monomers cause the media to become acidic (Prieto and Guelcher, 2014). The release of acidic degradation products such as glycolic acid has been suggested to contribute to the late inflammatory response (Zhang *et al.*, 2014b).

However, glycolic acid accumulation is unlikely to occur as it is understood that glycolic acid is excreted in urine from the human body or as carbon dioxide and water after being processed in the tricarboxylic acid cycle (TCA) (Prieto and Guelcher, 2014).

In the buffered saline solution, degradation decreased PGA's tensile strength more than in the unbuffered solution. Thus, the higher rate of degradation observed in PBS may be due to the presence of disodium hydrogen phosphate (Na_2HPO_4), which eliminated the glycolic acids and shifted the reaction to increased hydrolysis in the PGA polymer (Chu, 1981). Furthermore, due to the buffering effect that maintains the media's pH, this effect cannot be seen in PBS dependent media. From the results, this buffering effect was not observed in unbuffered solution (dH_2O). In addition, the 10% FBS in PBS will further increase the rate of degradation above that seen in PBS alone because the FBS will increase the initial velocity of hydrolytic processes due to the higher activity of esterase enzymes present in the serum (Catiker, Gümüşderelioğlu and Güner, 2000).

An ideal scaffold for tissue growth should degrade rapidly while providing a suitable environment that enables cells to deposit ECM. Even if a polymer that degrades too rapidly dissolves before the tissue can form, it is suggested that the tissue organisation and mechanical properties of a scaffold that degrades too slowly also have adverse effects. Novel scaffold polymers that rapidly degrade while also stimulating collagen synthesis and new ECM deposition on the implant site will greatly promote the development of engineered connective tissue (Catiker, Gümüşderelioğlu and Güner, 2000).

Collectively, the results of this chapter demonstrate the fabrication of the PS incorporated scaffolds using the electrospinning method and characterisation of produced scaffolds by means of morphological, degradation rates, release profiles of PS and changes in pH.

In conclusion, the incorporation of PS into the PGA polymer led to the formation of fibres with smaller diameters and increased water binding capacity (with the exception for 1% PS). The change in the scaffold's characteristics depends on the type and percentage of the PS. The release of the PS from the scaffold can be tuned by varying the percentage of PS incorporated. The amount of ERY, however, must not be too high as it will produce too small fibre diameters (nanofibre) that may not be suitable for cell infiltration. Moreover, a high amount of MB will increase the rate of degradation, which would not be good for cell adhesion and proliferation. The quantity of PS released from the scaffolds during degradation studies correlated with the increase in the concentration of PS incorporated.

Chapter 3

Biological assessment and characterisation of the fabricated scaffolds using human dental pulp stromal cells (hDPSCs)

3.1 Introduction

A scaffold that is able to provide a good surface for cell attachment will promote more cells to adhere and migrate between the fibres of the scaffold. PGA scaffolds are synthetic polymers with good biocompatibility with human cells (Montes de OCa and Ward, 2006; Boland *et al.*, 2001; Kim *et al.*, 2011), but there is the possibility that the addition of PS to the PGA polymers may have altered the characteristics of the scaffold produced by the electrospinning process. These scaffolds are intended to be used for soft tissue repair in the oral cavity, so it is important to investigate whether these newly developed scaffolds are compatible and possess any cytotoxicity effects towards the cells in the oral cavity.

Several *in vitro* and *in vivo* studies have shown that human dental pulp stromal cells (hDPSCs) have the capability to act as multipotent stem cells in the formation of new tissue (Goldberg and Hirata, 2017; Abdullah *et al.*, 2014; Kawashima, 2012; Sakai *et al.*, 2011). In stem-cell based regenerative therapies, scaffolds applied to the site of wound or injury must demonstrate good biocompatibility with the host cell or tissue and provide an adequate environment and structural support for the regeneration of the targeted tissue.

Therefore, to verify the cytotoxicity of the scaffolds, hDPSC viability (based on metabolic activity and DNA quantitation) following seeding on PGA scaffolds (both with and without incorporated PS) and microscopic observation were performed to investigate whether the scaffolds were suitable to support the attachment and proliferation of the hDPSCs.

3.2 Aim and objectives

3.2.1 Aim

The aim of this chapter is to evaluate the cytotoxicity of the selected scaffolds and to determine their effects on hDPSCs attachment and proliferation *in vitro*.

3.2.2 Objectives

The above aim will be achieved through the following objectives:

- To evaluate the ability of hDPSCs to attach and spread on the produced scaffolds following seeding.
- To determine the ability of hDPSCs to proliferate on the produced scaffolds following seeding.
- To investigate the metabolic activity of hDPSCs after seeding on the produced scaffolds.
- To observe the morphology of hDPSCs on the produced scaffolds following seeding.

3.3 Materials and methods

All materials were purchased from Sigma-Aldrich, UK unless otherwise stated.

3.3.1 Human dental pulp extraction and isolation from third molar teeth

HDPSCs were isolated from extracted impacted third molars obtained with patient consent through the Leeds Dental Institute Research Tissue Bank (DREC No: 120615/RB/172). Teeth were immersed in 70% (v/v) ethanol (EtOH) (SLS, UK) for 20 s and shaken to detach any attached debris. The 70% ethanol was then discarded. Approximately, 8 mL of basal medium (Alpha Modified Eagle Medium (α -MEM) (Lonza BioWhittaker, UK) supplemented with 15 % FBS, 2 mM L-glutamine and 100 units/mL penicillin/100 μ g/mL streptomycin) were then added to the teeth. The teeth were then put on 70% ethanol-soaked tissue paper and any attached soft tissue was removed with a sterile scalpel blade. Then, teeth were carefully put in sterile gloves and fractured using a screw vice clamp to allow exposure of the dental pulp. Any debris from the fractured teeth was cleared up and dental pulp was pulled very lightly from the tooth fragments with sterile forceps. The extracted pulps were put in basal medium to avoid dehydration.

The extracted pulps were then incubated in an enzyme solution of 3 mg/mL collagenase type I and 4 mg/mL dispase type II (Roche, Germany) for 30 min at 37°C in an incubator. Extracted pulps were then cut into smaller fragments with a scalpel blade and returned to enzyme solution with gentle agitation on a tube rotator (Miltenyi Biotec, Germany) for 60 min at 37 °C in an incubator (Thermo Scientific, UK). The resulting suspension was centrifuged (Netheler-Hinz, Germany) at 200 x g for 5 min before resuspension of the cell pellet in

basal medium. Resuspended cells were incubated in T25 flasks (Corning, UK) (1 extracted pulp per flask) at 37°C in 5 % CO₂ in air for 10–14 days to adhere and proliferate (Tomlinson *et al.*, 2015). The initial passage of human dental pulp stromal cell culture was referred to as "Passage 0" (p0).

3.3.2 Human dental pulp stromal cells culture

The sub confluent flasks of p0 cells were passaged by digestion with 0.25 % trypsin/ 0.02 % EDTA and the resulting suspension was transferred to sterile T175 flasks (Corning, UK); this flask was designated as p1. Passaged cells were subsequently cultured in basal medium of α -MEM containing 10 % FBS, 2 mM L-glutamine and 100 units/mL penicillin/100 μ g/mL streptomycin at 37°C with 5 % CO₂ in air until 80 % confluency was reached. The medium was changed every two days and only cells of passages 4 to 6 were used in these studies.

3.3.3 Attachment and proliferation assays of human dental pulp stromal cells seeded on produced scaffolds

Each scaffold was sterilised under UV light for 20 min for each surface in the sterile laminar flow biosafety cabinet (Nuair, UK). For each group, n=3, and the experiment was performed in triplicate. The groups were assigned according to the percentage of incorporated PS, with PGA as the control group. PGA, PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M scaffolds were cored into discs of 1 cm in diameter using a cork borer and were placed in 48-well tissue culture plates (Corning, UK). Then, the scaffolds were static seeded with 1×10^5 cells per scaffold in basal media with 5 % CO₂ in air and maintained for 24 h and 72 h respectively. During the experiments all plates were covered with aluminium foil to avoid exposure to light. After each time point (24 h and 72 h), the cell-scaffold constructs were collected using sterile

small tweezers and washed twice with PBS to remove any unattached cells. The cell-scaffold construct was then put into fresh basal medium and further processed for the cell metabolic activity assay (section 3.3.4), DNA quantitation assay (section 3.3.5) and microscopy evaluation (section 3.3.6).

3.3.4 Cell metabolic activity assay

Metabolic activity of the attached cells was measured using the Presto Blue™ Cell Viability Reagent (ThermoFisher Scientific, UK). Presto Blue™ (PB) contains a compound that is known to be cell-permeant (resazurin) that is naturally blue in colour and non-fluorescent. When the reagent is added to the cells, the non-fluorescent resazurin compound will be modified by the reducing environment of the viable cells within their cytosol to the highly fluorescent, red in colour compound (resorufin). Reducing enzymes in the viable live cell will be able to reduce resazurin compound and the changes in colour is an indicator of the cell's energy metabolism. The rapid changes in colour can be detected by fluorescence measurement. Any dead cell will lose their ability to reduce the resazurin compound and thus no modification happens.

PB reagent was added to each cell-scaffold construct in basal medium at a ratio of 1:10 volume per well and properly mixed. The cell-scaffold constructs were then covered with foil and incubated for 1 h at 37°C with 5% CO₂ in air. Then, 100 µL of each cell-scaffold construct supernatant was transferred to a black microtiter plate with solid bottom for fluorescence measurement according to the PB reagent protocol provided by Thermo Fisher Scientific. Black microtiter plates (Thermo Scientific Nunc, UK) were used for fluorescence measurements as the black plates absorb light which reduces

scatter and background noise and minimises crosstalk compared to clear or white plates. Plates were read using a Varioskan Flash microplate reader (ThermoFisher Scientific, Finland). The fluorescence detection parameters for resorufin compound were: excitation wavelength = 560 nm and emission wavelength = 590 nm. A blank measurement of basal medium only was included to exclude the background autofluorescence of the basal medium.

3.3.5 DNA Quantitation assay

The DNA quantitation fluorescence assay (DNAQF) kit used in this study is based on quantitation of DNA using the fluorescent dye, bisBenzimide H33258 (Hoechst 33258). The kit consists of a DNA Standard, bisBenzimide H33258 solution, and 10X Fluorescent Assay Buffer. The bisBenzimide H33258 dye will bind to AT sequences in the minor grooves of double-stranded DNA and emit fluorescence when excited at 360 nm. The fluorescence emitted by the bisBenzimide H33258 dye will increase proportionately in the presence of DNA. Fig. 3.1 shows the interaction between the DNA and the bisbenzimidazole H33258 dye which has high-affinity binding due to its specific interaction with the DNA minor grooves (Guo *et al.*, 2016). The fluorescence intensity will increase ~30-fold when binding to DNA as a result of suppression of rotational relaxation and hydration reduction during binding.

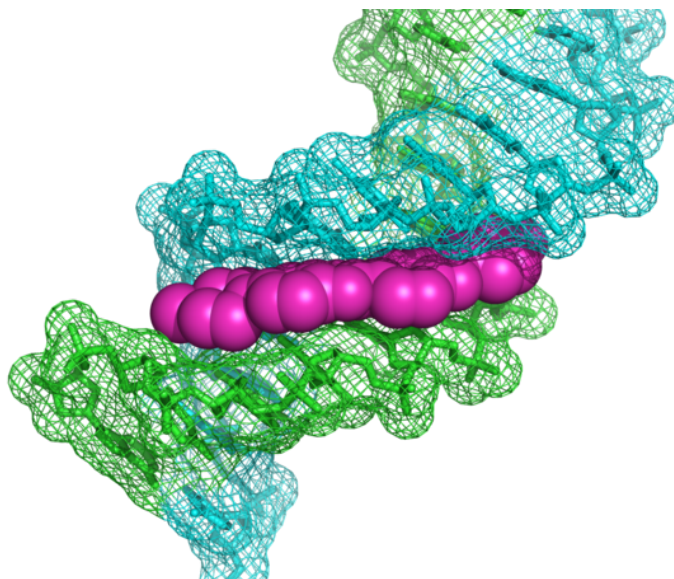


Figure 3.1 Hoechst 33258 or bisBenzimide dye (magenta) bound to the minor groove of DNA (green and blue) (from <http://www.rcsb.org/structure/264D>).

The reagents were prepared according to the kit instructions supplied. The DNA Standard was diluted to DNA concentrations of 100 µg/mL and 10 µg/mL (Table 3.1).

Table 3.1 Preparation of DNA Stock Solutions.

	DNA Stock Solution (100 µg/mL)	DNA Stock Solution (10 µg/mL)
DNA Standard, calf thymus DNA (1 mg/mL)	100 µL	10 µL
10X Fluorescent Assay Buffer	100 µL	100 µL
Molecular Biology Grade Water	800 µL	890 µL
Total Volume	1 mL	1 mL

The bisBenzimide dye solution was diluted by 10-fold dilution with molecular biology grade water to a concentration of 1 mg/mL. In order to prepare the standard curve, 1 mg/mL bisBenzimide H33258 was further diluted with molecular biology grade water and 10X Fluorescent assay buffer to make 2 µg/mL (Table 3.2). The bisBenzimide dye solution was stored in the dark at 4°C until ready to be used.

Table 3.2 Preparation of bisBenzimide H33258 Solution 2 µg/mL from the 1 mg/mL stock solution.

	bisBenzimide H33258 Solution 2 µg/mL
bisBenzimide H33258 Solution 1 mg/mL	60 µL
10X Fluorescent Assay Buffer	3 mL
Molecular Biology Grade Water	27 mL
Total Volume	30 mL

Following the 24 h recovery period (section 3.3.3), the cell-scaffold constructs were washed twice with PBS and lysed in 200 μ L 0.2% (v/v) Triton X-100 for 30 min. They were then centrifuged at 12000 rpm at 4°C for 10 min. The supernatant (10 μ L) was added to 200 μ L of the prepared dye (bisbenzimidazole H33258 solution 2 μ g/mL) and incubated at 4°C for 1 h before the fluorescence of the dye bound to the DNA was measured using a microplate reader with 360 nm as excitation wavelength and 460 nm as emission wavelength. The DNA concentration of the samples was determined by comparison to the standard curve (Fig. 3.2). A standard curve was constructed based on Table 3.3.

Table 3.3 Conditions for standard curve 20-2000 ng DNA range used for comparison with DNA concentration within the samples.

Well	Total DNA	DNA Stock Solution	DNA Volume	2 µg/mL bisBenzimide Dye
1	2000 ng	1 mg/mL	2 µL	200 µL
2	1000 ng	100 µg/mL	10 µL	200 µL
3	500 ng	100 µg/mL	5 µL	200 µL
4	100 ng	10 µg/mL	10 µL	200 µL
5	50 ng	10 µg/mL	5 µL	200 µL
6	20 ng	10 µg/mL	2 µL	200 µL
7	10 ng	10 µg/mL	1 µL	200 µL
8	Blank	-	-	200 µL

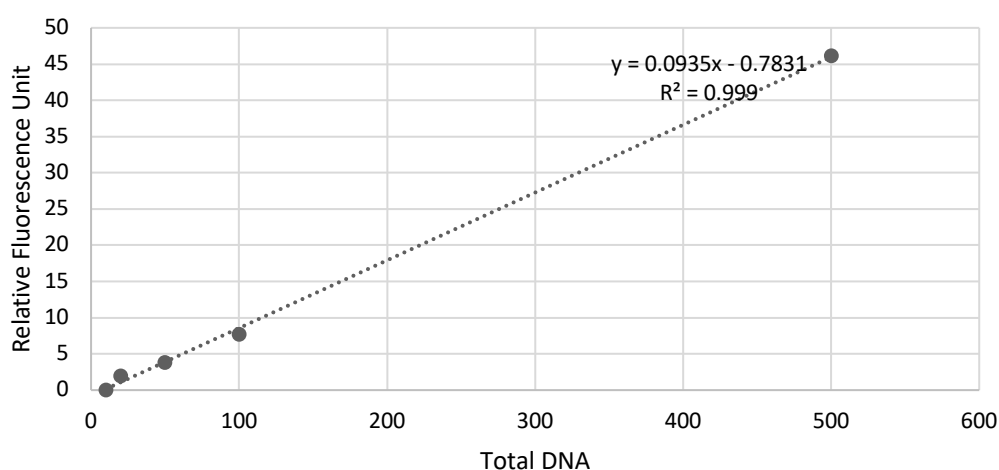


Figure 3.2 Representative standard curves used in DNA concentration determinations.

The DNA content was also used to determine the cell seeding efficiency on the scaffolds 24 h after seeding. Seeding efficiency is defined as the percentage ratio of the attached cells' DNA content on the scaffold to the of seeded cells' DNA content.

$$\text{Seeding efficiency \%} = \frac{\text{DNA of attached cells}}{\text{DNA of seeded cells}} \times 100$$

3.3.6 Evaluation of the cell-scaffold constructs using microscopy

3.3.6.1 Scanning electron microscopy evaluation

The cell-scaffold constructs were fixed in 10% neutral buffered formalin (Cellpath, UK) for 24 h at 4°C. The cell-scaffold constructs were then dehydrated through a series of ethanol concentrations as below (Fig. 3.3).

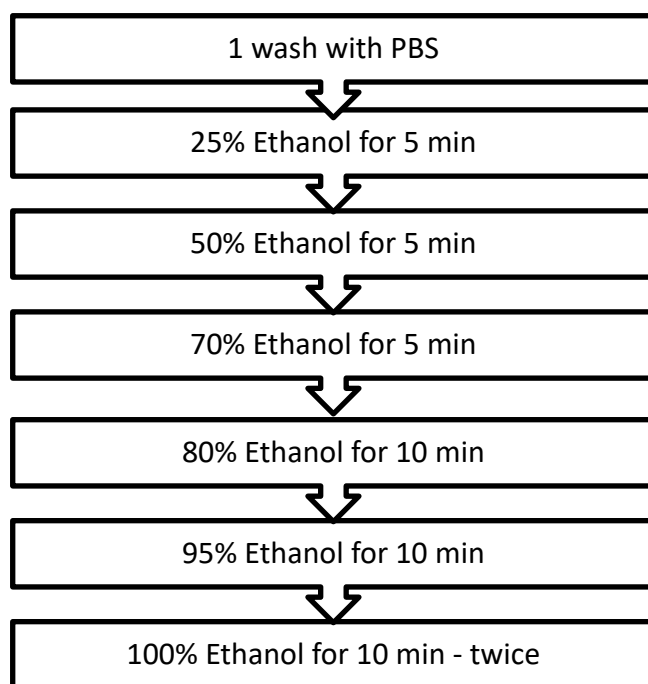


Figure 3.3 Steps of scaffold dehydration.

Constructs were then air-dried overnight. Fixed, dried cell-scaffold constructs were then attached to 12 mm aluminium stubs using double-sided carbon adhesive tape and sputter coated with gold/palladium alloy for 40 seconds. Coated samples were inspected using a S3400-N Hitachi SEM in high vacuum mode using a voltage of 20 kV. A series of images were captured at a magnification of 100x and 1000x.

3.3.6.2 Morphological staining using AlexaFluor™ 488 Phalloidin and DAPI

Two samples from each experimental group (sections 3.3.3) were collected and washed three times with PBS and fixed using 10% neutral buffered formalin (NBF) and kept at 4°C for 24 h. These samples were used for staining with AlexaFluor™ 488 phalloidin (Invitrogen, UK) to show hDPSCs morphology on the scaffold. After 24 h, the 10% NBF solution was aspirated and the cell-scaffold constructs were washed twice with PBS to remove any residual NBF. 500 µL of 0.1 % Triton X-100 in PBS was then added to each sample and incubated for 5 min at room temperature. Cell-scaffold constructs were washed two times with PBS after the 0.1% Triton X-100 was discarded. Processed cell-scaffold constructs were then incubated with 500 µL 0.5% Bovine Serum Albumin (BSA) (GE Healthcare Hyclone) in PBS for 30- to 60-min to block non-specific phalloidin binding. Any residual BSA was removed by washing twice with PBS.

AlexaFluor™ 488 phalloidin stain was added to cell-scaffold constructs (1:500 v/v in PBS) and incubated for 30 to 60 min. The constructs were then washed once with dH₂O to prevent any crystal salt formation and transferred to a microscope slide. The cell nuclei were then counterstained with DAPI and the slides were mounted with ProLong Gold antifade reagent (with DAPI) (ThermoFisher Scientific, UK) before viewing on the confocal laser scanning microscope (CLSM) (Leica GmbH, Germany). AlexaFluor™ 488 phalloidin stain was viewed at excitation wavelength 490 nm and emission wavelength 525 nm and visualised as green fluorescence. DAPI stain was viewed at excitation wavelength 345 nm and emission wavelength 455 nm and visualised as blue fluorescence.

3.3.7 Statistical analysis

All experiments were carried out in triplicate and each experiment was repeated 3 times (n=9), unless stated otherwise. The statistical analyses were carried out using IBM SPSS Statistics Version 23 software. Results were analysed using Students' t-test and one way analysis of variance (ANOVA) tests. P-values were determined, and $P < 0.05$ was considered significant. All results were demonstrated as mean $\pm$ standard error of mean (S.E.M) unless stated otherwise.

3.4 Results

Assessment of hDPSCs attachment and proliferation on fabricated scaffolds was performed by measuring the level of cell metabolic activity, DNA quantitation and through microscopy evaluation. All the experiments were conducted after fabricated scaffolds were cultured with hDPSCs for either 24 h or 72 h.

3.4.1 Scanning electron microscope evaluation

The surface topography of cell-scaffold constructs was observed using a high vacuum SEM. Figure 3.4 and Figure 3.5, respectively, show SEM generated images of hDPSCs cultured on scaffolds for 24 h and 72 h. Structural assessment of seeded scaffolds for both 24 h (Figure 3.4) and 72 h (Figure 3.5) revealed cell processes that bridge the fibres of the scaffold. After 24 h, a dense extracellular matrix (ECM) appeared in the form of a network covering the surface of the scaffolds.

Prominent intensification of ECM was observed after 72 h in culture on PGA only scaffolds (Fig. 3.4 (B) and Fig. 3.5 (B)). For PGA-1%E (Fig. 3.4 (D) and Fig. 3.5 (D)) and PGA-5%E (Fig. 3.4 (F) and Fig. 3.5(F)), a similar finding can be seen where ECM was extended on the surface of the scaffolds as the duration of the culture increased.

On the PGA only and PGA-E (1% and 5%) scaffolds, the cells proliferated rapidly and the ECM was secreted and covered some pores of the scaffolds. However, relative to PGA only and PGA-1%E, there was more hDPSC attachment and ECM secretion on the surface of PGA-5%E. The ECM production was abundant and dense. However, hDPSCs were not able to infiltrate the PGA-5%E scaffold fibres, and thus proliferated on the surface or

top few layers of the scaffold fibres. In contrast, when compared to 24 h in culture, PGA-1%M and PGA-5%M showed less ECM formation after 72 h in culture. The cell morphology showed that some cells had shrunk and the cell morphology was not satisfactory. There was the presence of cell aggregates on the scaffolds that showed that the cells were still adhering to the scaffold fibres. However, it was not known whether the cells were alive, dying or dead.

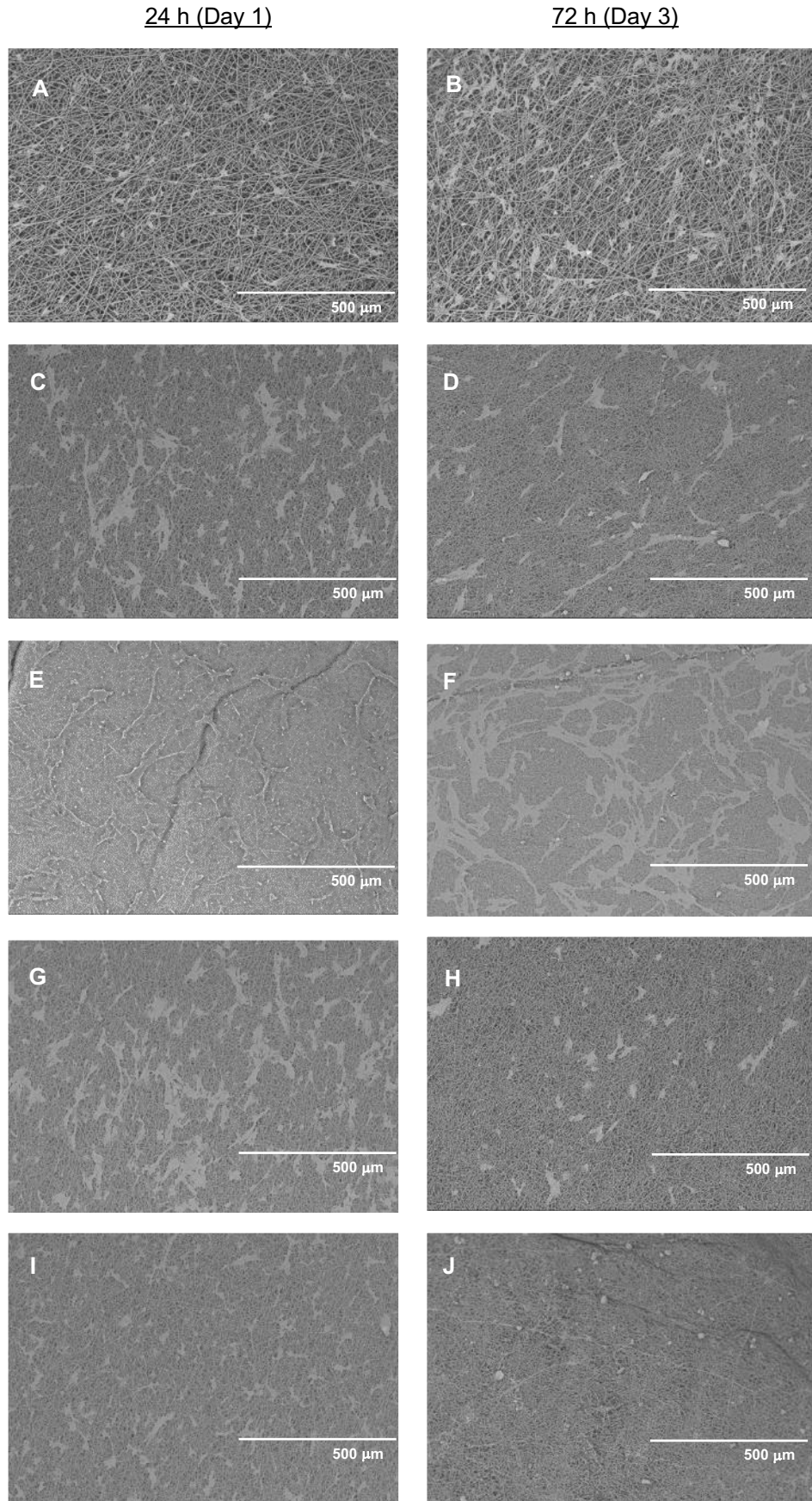


Figure 3.4 Representative images of SEM analysis of cellular interaction with electrospun fibrous scaffolds at 24 and 72 h (100X). On both days (24 h and 72 h) cells attached and spread on the PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J). Scale bars at 500 μm .

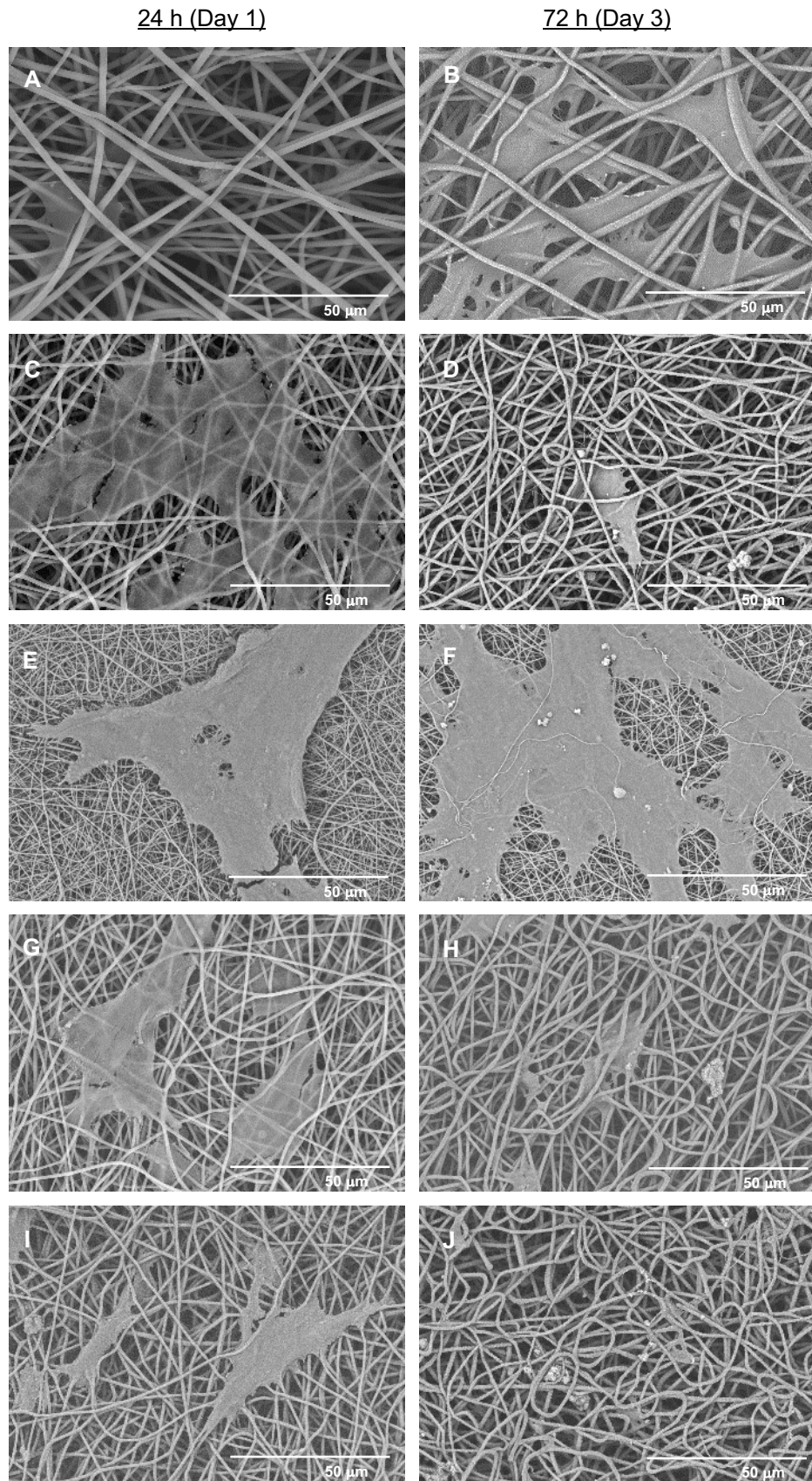


Figure 3.5 Representative images of SEM analysis of cellular interaction with electrospun fibrous scaffolds at 24 and 72 h (1000X). On both days (24 h and 72 h) cells attached and spread on the PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J). Scale bars at 50 μm.

3.4.2 Evaluation of cell seeding efficiency 24 h post-seeding on the scaffolds

After culturing for 24 h, the DNA content of hDPSCs attached on the scaffolds was quantitated using the DNAQF kit as defined in section 3.3.5. In order to evaluate the impact of the scaffold structure and PS percentage on hDPSCs attachment, the scaffolds incorporated with PS (PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M) were compared to the PGA only scaffold.

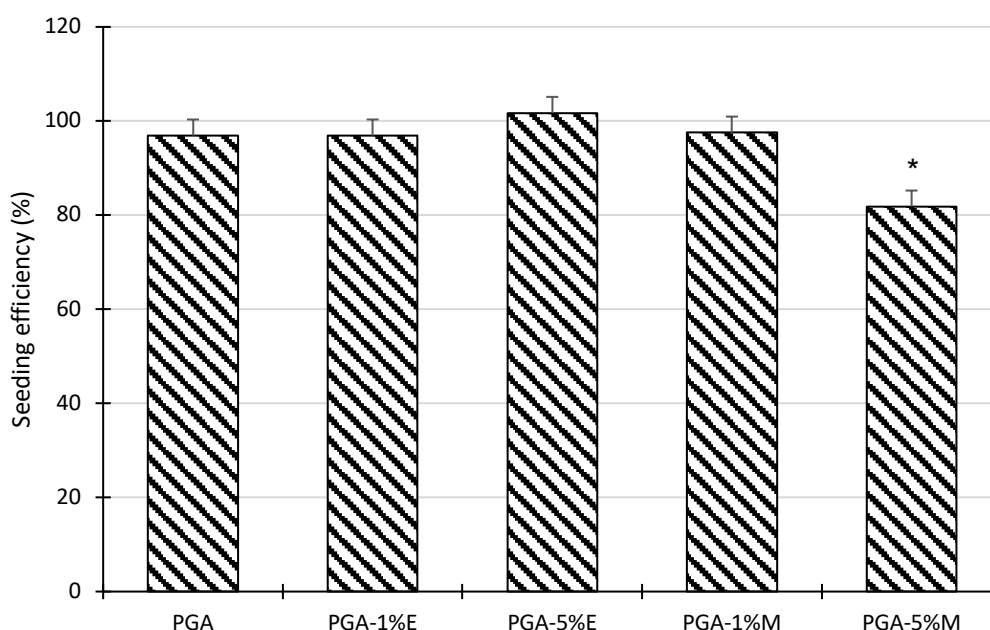


Figure 3.6 Seeding efficiency of hDPSCs after 24 h seeding on the produced scaffold.

The graph represents the mean hDPSC seeding efficiency percentage on scaffolds. Data are represented as the mean hDPSC seeding efficiency percentage of the scaffold replicates (n=9) $\pm$ S.E in each group.

*Indicates a significant difference between different scaffolds tested including PGA only scaffold (p<0.05).

$$\text{Seeding efficiency \%} = \frac{\text{DNA of attached cells}}{\text{DNA of seeded cells}} \times 100$$

At 24 h, a seeding efficiency of more than 90% was achieved with hDPSCs on PGA, PGA-1%E, PGA-5%E and PGA-1%M, whereas a seeding efficiency of 82% was observed with hDPSCs on PGA-5%M (Fig. 3.6).

3.4.3 Evaluation of hDPSC proliferation on scaffolds by DNA content quantification

Cell proliferation was investigated based on the amount of DNA retrieved from the scaffold at 24 h and 72 h in culture shown in figure 3.7. The DNA content of hDPSCs on PGA scaffold was significantly increased after culturing for more than 24 h. However, a significant reduction in DNA concentration on PGA-5%E, PGA-1%M and PGA-5%M compared to PGA scaffolds was observed after 72 h in culture. Meanwhile, the hDPSCs on PGA-1%E and PGA-5%E maintained their DNA content over 72 h. However, the hDPSCs seeded onto PGA-1%M and PGA-5%M showed a significant decrease in DNA concentration over 72 h in culture when compared to the DNA concentration at 24 h. No significant difference in DNA concentration was observed between PGA-E scaffolds when compared to the DNA concentration for 24 h. In contrast, there was a significant difference in DNA concentration between the PGA-M scaffolds.

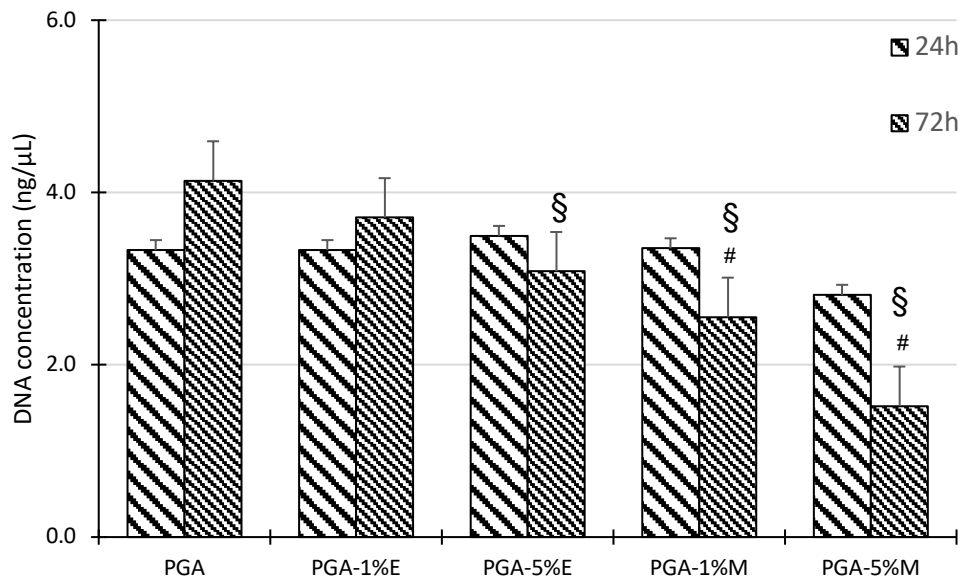


Figure 3.7 DNA content of hDPSCs on different scaffolds at 24 h and 72 h after seeding.

The graph represents the mean hDPSCs DNA content on the scaffolds. Data are represented as the mean of hDPSCs DNA concentration (ng/μL) ($n=9$) $\pm$ S.E in each group.

§Indicates a significant difference between the marked group and PGA group (72 h).

#Indicates a significant difference between 24 h and 72 h (same scaffold group) ($p<0.05$).

3.4.4 Evaluation of metabolic activity of hDPSCs growing on the scaffolds

The cell metabolic activity assay of hDPSCs following seeding on the scaffolds was evaluated using the Presto Blue cell viability reagent following 24 h and 72 h culture. Figure 3.8 shows that the metabolic activity of hDPSCs was significantly lower in all scaffolds after 24 h culture in basal medium relative to the PGA only scaffold. In comparison to the PGA only scaffold, PGA-E scaffolds maintained more than 70% of cell metabolic activity, while PGA-M scaffolds had less than 70% activity. When a comparison was made between the scaffold with PS group, a significant difference in cell metabolic activity was only found between PGA-5%E and PGA-5%M. In contrast with the other PGA-PS scaffolds, PGA-5%E has the highest level of cell metabolic activity relative to PGA-5%M. This showed that hDPSCs still favour attaching to the scaffolds with PS whether PGA-E and PGA-M even though the scaffolds produced have a nano-scale fibre compared to PGA only scaffold.

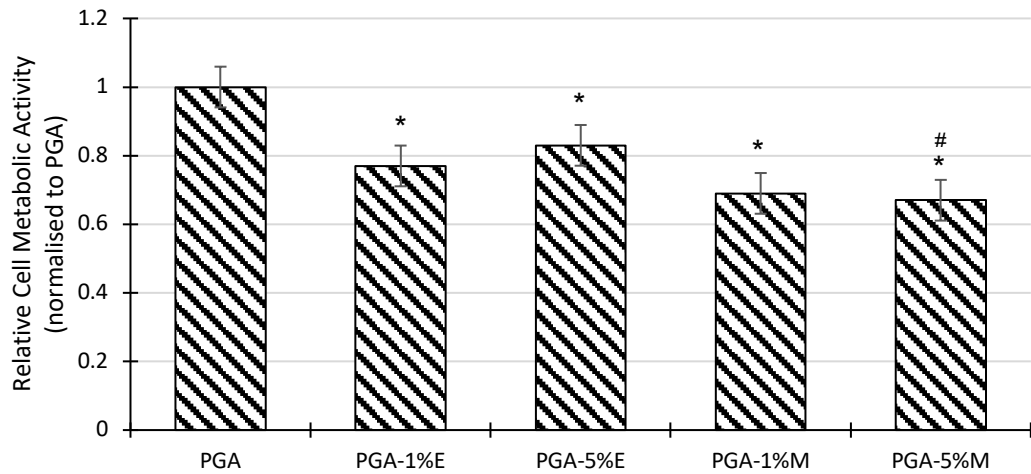


Figure 3.8: Metabolic activity of hDPSCs after 24 h culture on the scaffolds.

The graph represents the relative cellular metabolic activity of hDPSCs attached on the scaffolds after 24 h. Data are represented as the mean of relative hDPSC metabolic activity ($n=9 \pm$ S.E in each group).

Cell viability assay (Presto Blue) was performed at 24 h after initial seeding.

*Indicates a significant difference between the marked group and PGA group ($p<0.05$).

Indicates a significant difference between the marked group and PGA-5%E ($p<0.05$).

The results from 72 h culture of hDPSCs on the scaffolds showed some varied results (Figure 3.9). Scaffolds incorporated with PS (PGA-E and PGA-M) showed that hDPSCs had significantly less metabolic activity relative to PGA only scaffold. Compared to PGA only scaffold, the most prominent reduction of metabolic activity was seen for hDPSCs on PGA-5%M scaffolds. Furthermore, PGA-5%M cell metabolic activity was significantly reduced when compared to the PGA-E scaffold groups (PGA-1%E and PGA-5%E). However, the reduction was not significant between the PGA-M groups (PGA-1%M and PGA-5%M).

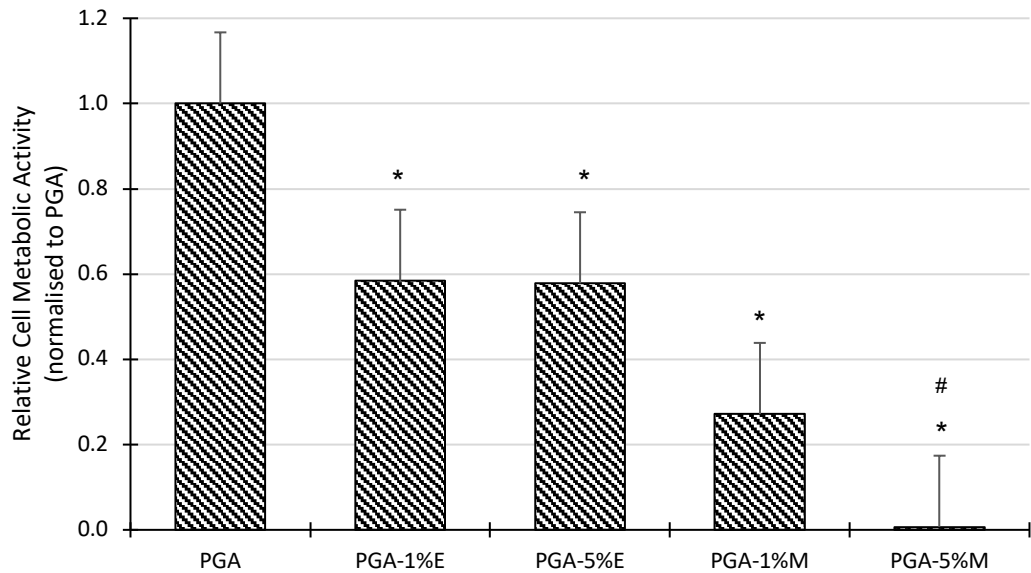


Figure 3.9: Metabolic activity of hDPSCs after 72 h culture on the scaffolds.

The graph represents the relative cellular metabolic activity of hDPSCs attached on the scaffolds after 72 h. Data are represented as the mean of relative hDPSC metabolic activity ($n=9 \pm$ S.E in each group).

Cell viability assay (Presto Blue) was performed at 72 h after initial seeding.

*Indicates a significant difference between the marked group and PGA group ($p<0.05$).

#Indicates a significant difference between the marked group and PGA-E scaffolds ($p<0.05$).

Figure 3.10 shows that the cell metabolic activity on scaffolds incorporated with PS significantly increased after 72 h culture time in comparison to PGA only scaffold (24 h) that acts as a control. The only exception was PGA-5%M which showed a significant reduction in cell activity relative to the control. Cell metabolic activity was significantly increased in the PGA-E group after 72 h culture, compared to 24 h in the same scaffold group. Within the same scaffold group, even though there is some increment of cell metabolic activity on PGA-1%M, it was not significant. PGA-5%M did not show significantly different metabolic activity between 24 h and 72 h culture.

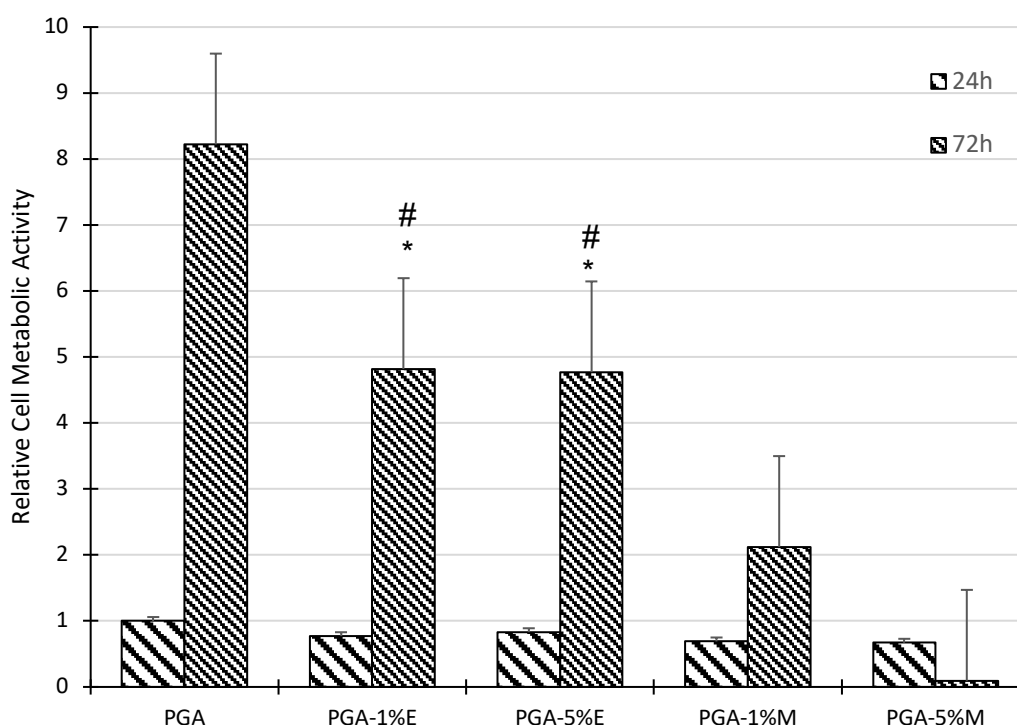


Figure 3.10 Comparison of the metabolic activity of hDPSCs after 24 and 72 h culture on the different scaffolds.

The graph represents the relative cellular metabolic activity for hDPSCs attached on the scaffolds after 24 h and 72 h. Data are represented as the mean of relative hDPSC metabolic activity ($n=9 \pm S.E$) in each group. Cell viability assay (Presto Blue) was performed at 24 h and 72 h after initial seeding. PGA culture exhibited the highest hDPSC metabolic activity followed by PGA-1%E, PGA-5%E and PGA-1%M. However, PGA-5%M exhibited reduced cellular metabolic activity.

*Indicates a significant difference between the marked group and PGA group (24 h) ($p<0.05$).

#Indicates a significant difference between 24 h and 72 h (same scaffold group) ($p<0.05$).

3.4.5 Morphological observation of hDPSCs using actin staining

The morphology of the hDPSCs attached to the scaffold was visualised using commercially-available actin staining, AlexaFlour 488 (green). Fluorescence images of hDPSCs attached to the scaffolds in 10x and 20x are presented in Figure 3.11 and 3.12, respectively. After 24 h in culture, the cell-scaffold constructs showed that most areas of the scaffolds were populated by cells with elongated actin cytoskeletons. There were no differences in cell morphology observed between cells cultured on scaffolds with or without incorporated PS after 24 h in culture. However, after 72 h in culture, the hDPSCs on the PGA-1%M scaffold exhibit a less elongated, shrunken cell size with blurred cytoplasm and a lack of spreading activity between the cells. Meanwhile, a lack of F-actin cytoskeleton staining and condensed cytoplasm, which is typical of apoptotic cells, were shown prominently in hDPSCs growing on the PGA-5%M scaffold. These results indicate that the effect of the incorporation of MB into the scaffolds on the cells was dependent on concentration, a higher percentage of PS inducing a severe effect on the cells. However, compared to PGA only scaffold, the hDPSCs growing on PGA-E exhibited similarly organised and elongated F-actin structures, which is a sign of optimal status of the hDPSCs.

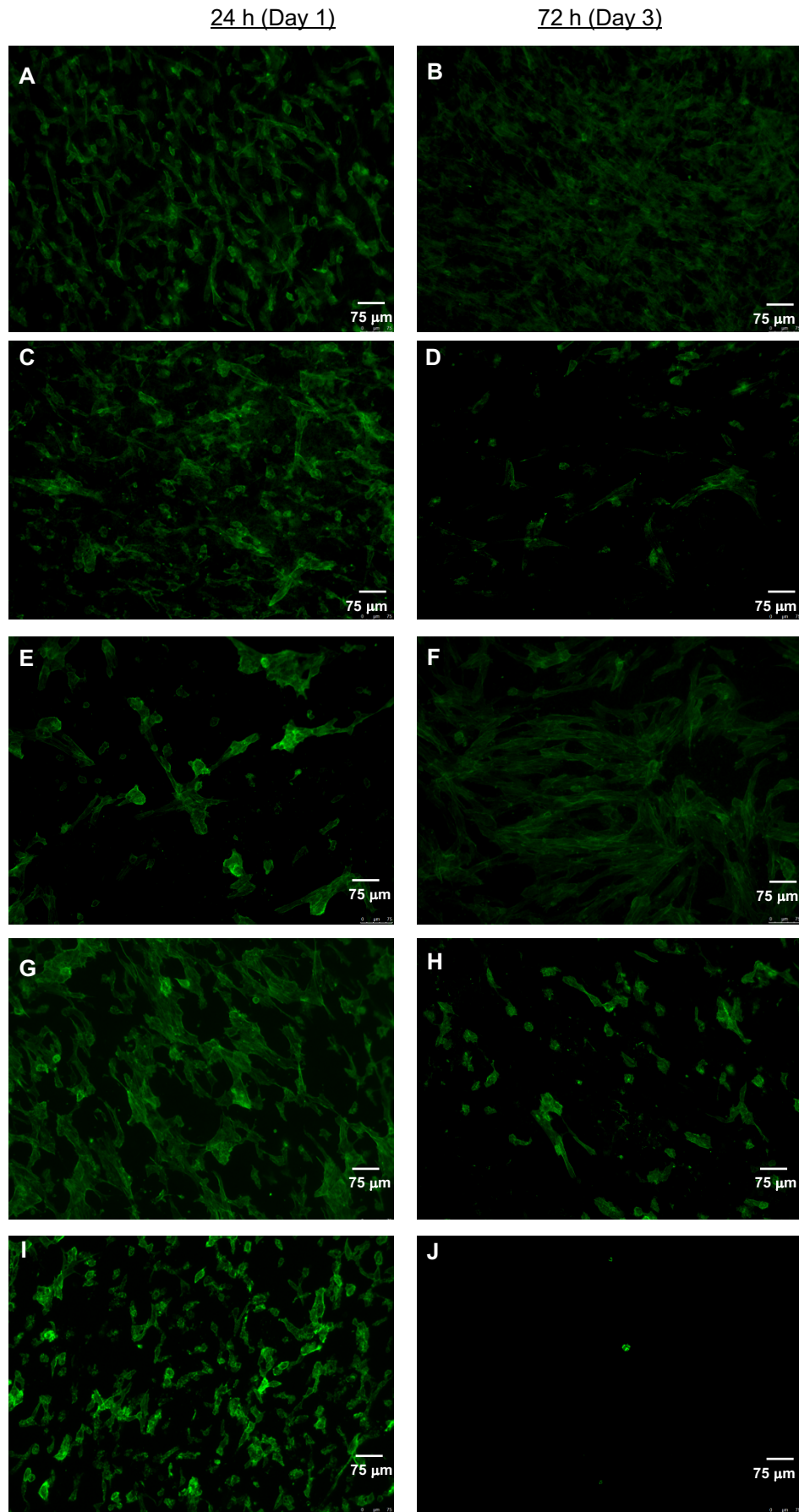


Figure 3.11 Actin staining using Alexa Fluor 488 phalloidin stain (10X). PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J).

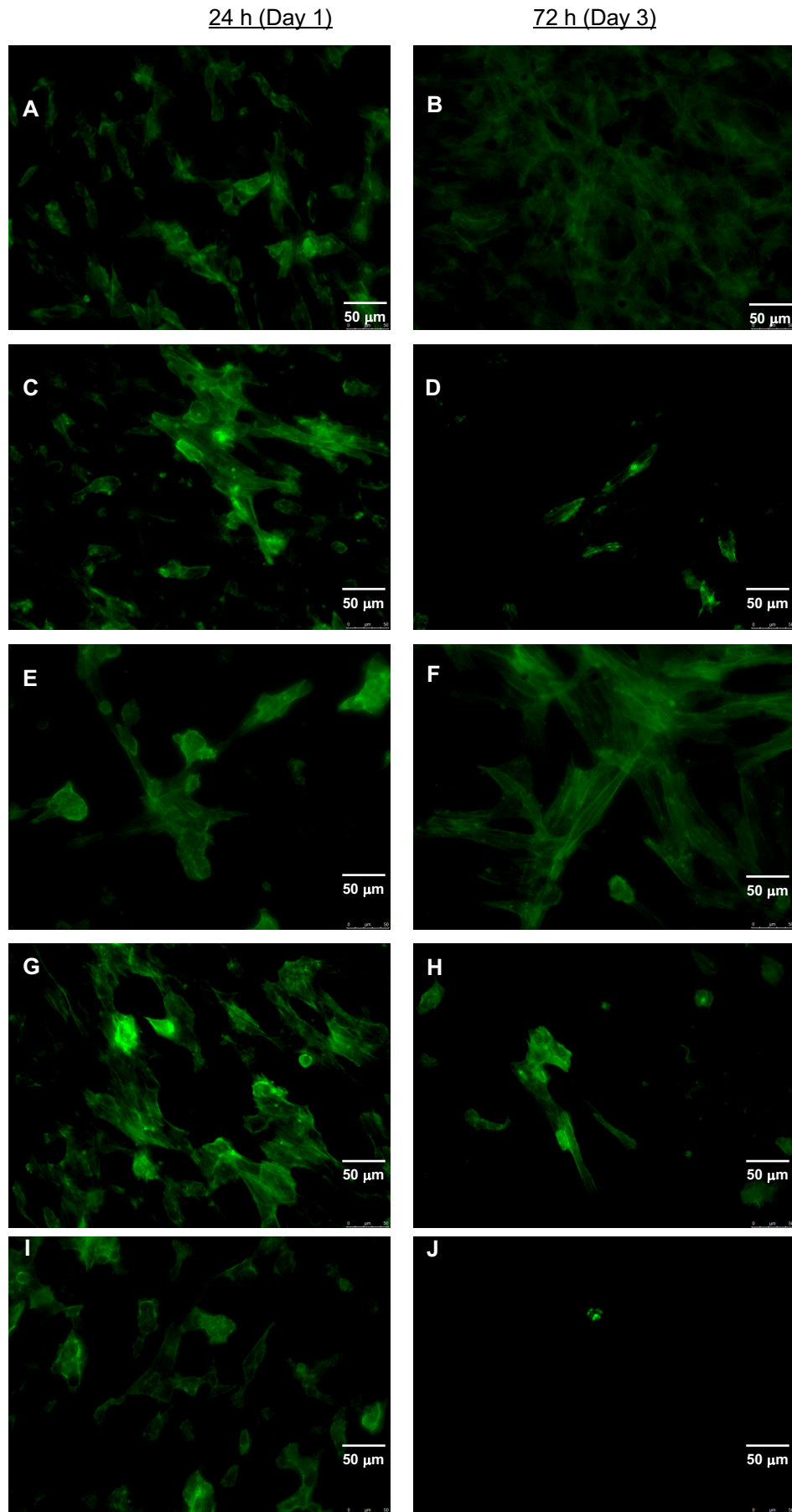


Figure 3.12 Actin staining using Alexa Fluor 488 phalloidin stain (20X). PGA (A, B), PGA-1%E (C, D), PGA-5%E (E, F), PGA-1%M (G, H) and PGA-5%M (I, J).

3.5 Discussion

In tissue regeneration, initial cell adherence and attachment are important factors in assessing whether the scaffolds will be favourable for cell growth, differentiation and eventual tissue formation. There are many factors that can contribute to the different cell proliferation rates of tested scaffolds. The structural variations between the scaffolds are one of the factors that will influence the fate of the seeded cells (Chang and Wang, 2011). The differences between the micro and nanoscale of fibre diameter affects the cells' behaviour and whether they attach on the surface and proliferate or are able to infiltrate through the pores in between the fibres (Bruzauskaite *et al.*, 2016). Previous work demonstrated that endothelial cells seeded on nanofibre PLGA scaffolds with a 200 nm fibre diameter did show rapid cell attachment and spread in comparison to a microfibre PLGA scaffold. In addition, they also tested the cell infiltration, and found that the endothelial cells did not penetrate into the 200 nm PLGA scaffolds, whereas for 600 nm and 1.5 μ m PLGA scaffolds, endothelial cells were able to infiltrate and increase in proliferation after 14 days in culture (Ko *et al.*, 2016).

The data presented above shows a similar result: on PGA only scaffolds, hDPSCs were found to be located within the pores and attached to the junction where the fibres crossed. After being in culture for 24 h, hDPSCs produced thick and dense ECM and migrated in multiple fibre directions. However, for the scaffolds with incorporated PS, the cell-scaffold interaction was greater on PGA-E scaffolds compared to PGA-M scaffolds. This may be due to the effect of the smaller diameter of fibre and the dense mesh of PGA-E scaffold fibres. As the size of the fibre diameter decreased, many hDPSCs

were attached on the surface of the scaffold. However, hDPSCs attached to the PGA-1%E scaffold were still able to infiltrate the scaffold as some cells and ECM were found a few layers of fibres below the surface. In contrast, with the PGA-5%E scaffold, the scaffold produced a very dense mesh of fibres (due to it having the smallest mean fibre diameter) and most of the hDPSCs attached and proliferated on the surface of the scaffold and hence produced thicker ECM in comparison to the other fabricated scaffolds (Ko *et al.*, 2016). Previous researchers reported similar observations as there was an increase in neural stem cell proliferation and spreading as the fibre diameter of polyethersulfone (PES) fibre meshes was decreased (Christopherson, Song and Mao, 2009).

DNA quantitation shows that after seeding on the scaffold, hDPSCs managed to attach and spread for the first 24 h. This was confirmed by the SEM images that showed hDPSCs attachment to the fibres, and spreading on the surface and stretching of the cell cytoskeleton across multiple fibres and directions in all tested scaffolds. Other investigations on periodontal ligament cell attachment and proliferation on random fibres found that the DNA content was significantly higher than on film scaffolds. They also observed that the cells on random scaffolds grew in a polygonal shape and were attached to multiple fibres (Shang *et al.*, 2010).

However, for the PGA-5%M, the DNA content of hDPSCs attached to the scaffold was significantly reduced at 72 h compared to the control group. The key point here is the percentage of MB used in the manufacture of scaffold. During the initial 24 h of culture of the cells on the PGA-5%M scaffolds, the positively-charged groups of MB will improve the cell-scaffold interaction (the

hDPSC cells carry a negative charge, and will thus be attracted and adhere to the scaffolds). This is supported by previous studies which demonstrated that incubation of high-density polyethylene (HDPE) scaffolds with a positively-charged Arginine substance increased the attachment and proliferation of HeLa cells compared to untreated scaffold (without Arginine incubation) (Sengupta, Surwase and Prasad, 2018). However, as the percentage of MB released from the scaffold increased with increased time in culture, hDPSCs were vulnerable as they were exposed to a higher, cytotoxic dose of MB in the media, causing cell damage, reduction in viability and a resultant decrease in DNA content. Previous research has also found a reduced viability of breast cancer cells (4T1) when incubated with 8 μ M MB for 24 h (Wu *et al.*, 2018). This supports our findings for PGA-5%M scaffolds, particularly as the concentration of MB used in this study was approximately 67.93 μ M.

Proliferation of hDPSCs on the scaffolds was measured after 72 h in culture. PGA scaffold showed the highest proliferation rates after 72 h, based on DNA concentration, relative to those of scaffolds incorporated with PS. There was no significant difference in DNA concentration between the PGA-1%E and PGA-5%E groups, despite an increase in cell metabolic activity after 72 h in culture. Based on the metabolic activity of the cells, hDPSCs attached to the scaffolds incorporated with PS might interact differently compared to the PGA only scaffold (Ko *et al.*, 2016; Christopherson, Song and Mao, 2009; Shang *et al.*, 2010). Therefore, hDPSCs on PGA-E scaffolds may not actively proliferate, but because of the increase in metabolic activity, the cells may

actively participate in ECM production. This is supported by the SEM images of PGA-5%E that showed robust ECM production on the scaffold's surface.

PGA-M scaffolds, on the other hand, are more cytotoxic, most likely due to the MB released from the scaffolds during 24 h in culture. The results from this study showed that the concentration of MB released from scaffolds were 6.91 μM (PGA-1%M) and 67.93 μM (PGA-5%M) respectively. Furthermore, when the culture duration was increased to 72 h, more severe cytotoxicity was observed for both PGA-M scaffolds. Quantitation of DNA concentration confirmed that MB really had affected the cells, as the DNA found on both PGA-M scaffolds was significantly decreased compared to the control. In PGA-1%M scaffold, the concentration of MB released was cumulatively 45 μM after 72 h in culture, which was significantly increased compared to that of the same scaffold at 24 h of culture.

The results of the cell metabolic activity assays were supported by the results of DNA quantitation, as both decreased significantly when the concentration of MB released into the media was increased (for PGA-5%M, cumulative value $\sim 400 \mu\text{M}$) after 72 h. Previous research has found that MB has a cytotoxic effect on dental pulp cells at concentrations as low as 0.025 mg/ml ($\sim 28 \mu\text{M}$) and 0.050 mg/ml ($\sim 57 \mu\text{M}$) after 5 mins exposure (Diniz *et al.*, 2014). Thus, as in this research, using a higher concentration and longer incubation period causes a stronger cytotoxicity impact on the hDPSCs. The severe impact is reflected in reduced DNA concentration and metabolic activity, thus indicating a decrease in cell viability.

MB is known to self-aggregate in aqueous solution and this property will increase proportionately to the concentration of MB at concentrations higher

than $\sim 20 \mu\text{M}$. As a positively charged PS, MB is known to accumulate in mitochondria and lysosomes and to easily aggregate in the presence of negatively-charged surfaces such as cell membranes leading to the promotion of membrane partition and facilitation of the induction of apoptosis and necrosis (Bacellar *et al.*, 2015; Bacellar *et al.*, 2014; Oliveira *et al.*, 2011). As can be seen from the SEM images of hDPSCs after 72 h in culture, shrunken cells and reduction in ECM production are evident when using PGA-M scaffolds. These cellular morphologies are particularly associated with cells undergoing apoptosis.

Conversely, other research using human gingival fibroblasts and osteoblasts showed no severe cytotoxic effects after 10 min incubation with MB at a concentration of 0.050 mg/ml (approximately $57 \mu\text{M}$) (Xu *et al.*, 2009). In addition, a recent study using human non-malignant immortalised keratinocytes (HaCaT) showed no toxicity at MB concentrations ranging from 10-20 nM and 1-3 μM after exposure for one hour (Martins *et al.*, 2018). These results indicate that the cytotoxicity of MB is cell type and MB concentration dependent. However, even though the MB concentration used in the previous study was higher than in this study, the duration used was shorter compared to this study (24 h). Thus, the reaction and cytotoxicity level may be different due to the exposure duration, cell type and MB concentration.

Phalloidin staining was used in this study to observe the actin filaments and shape of the cytoskeleton within the cytoplasm of the cells. Cellular actin filaments are the important structure that affects the interaction between cells and the extracellular matrix through processes such as adhesion, migration and differentiation of the cells (Jones *et al.*, 2015). Based on the microscopy

images, hDPSCs morphologies were homogeneous with well-spread attached morphologies on both PGA only and PGA-E scaffolds. However, after 72 h of incubation, the hDPSCs on PGA-M scaffolds again showed signs of a severe cytotoxicity effect. In the PGA-M scaffold images, detrimental effects such as blurred cytoplasm, shrunken cells and loss spreading filaments could be seen. Recent work by Silva *et al.* (2018) found that 1%MB (in saline solution) did not have an adverse effect on the mice's normal skin tissue morphology and activity up to 24 h and 15 days after MB was introduced (Silva *et al.*, 2018b). The research was performed *in vivo* and showed that the cytotoxic effect of MB can be reduced at low MB concentration, different cell types and in the *in vivo* environment.

The results for the PGA-E groups, on the other hand, showed that the hDPSCs were not affected when the percentage of ERY was increased, and so may provide a better option than MB in terms of cytotoxicity.

The difference in the results seen between ERY and MB demonstrate the importance of ensuring that optimal conditions for hDPSCs are determined for each PS and scaffold as this will impact on cell migration, proliferation and, ultimately, the success of tissue repair.

In conclusion, this chapter described the effect of PS incorporation on cell-scaffold interactions when using scaffolds incorporating different concentrations of PS and of different fibre diameters. Cell attachment and morphology were studied, as were proliferation and metabolic activity. The scaffolds with incorporated ERY (PGA-1%E and PGA-5%E) were able to support hDPSCs viability, attachment, morphology and growth in *in vitro* culture. However, those scaffolds incorporating MB (PGA-1%M and PGA-

5%M) exhibited cytotoxicity to hDPSCs, particularly after being in culture for more than 24 h with resultant negative effects on morphology, growth and viability.

Chapter 4

Photodynamic therapy effect on human dental pulp stromal cells (hDPSCs)

4.1 Introduction

Photodynamic therapy (PDT) is known as a treatment for cancers (Yano *et al.*, 2011; Yoon, Li and Shim, 2013), precancerous lesions (Nava *et al.*, 2011) and dermatological problems (Wan and Lin, 2014). Recently, there was more researches conducted to investigate the potential of antibacterial photodynamic therapy (aPDT) for its ability to effectively destroy multidrug-resistant pathogenic bacteria and for its low propensity to cause drug resistance that can be rapidly formed by bacteria against traditional antibiotic therapy (Liu *et al.*, 2015).

In order to reduce potential side effects and the damaging of non-diseased tissue, it is important to validate that PDT has minimal or low toxicity effects on the host cells before PDT is applied to the target tissue or cell *in vivo*. The severity of the effect of PDT is also dependent on the type of cell, and several studies have investigated the effect of light and PS on mammalian cells (Silva *et al.*, 2018b; Kushibiki *et al.*, 2015; Kamocki, Nor and Bottino, 2015; Fontana *et al.*, 2013). For example, previous research found that the combination of MB (50 µg/ mL) and light did not cause any significant cytotoxic effect in terms of cell viability and mitochondrial activity on human gingival fibroblast but exhibited a significant reduction in osteoblast viability and mitochondrial activity (Xu *et al.*, 2009). Furthermore, when a red laser (60 J/ cm² for 1 min) was applied in the presence of the PS, at a concentration 0.025 and 0.050 mg/mL of MB, PDT was seen to kill dental pulp cells as cellular viability was reduced (Diniz *et al.*, 2014). In this study, the highest concentration used was

0.021 mg/mL (5%M), thus similar effects on cytotoxicity were anticipated when using this PS.

The main purpose of this study was to evaluate the effects of antibacterial PDT (aPDT) when directly applied on human dental pulp stromal cell cultures. Experiments were performed to compare PDT effects on hDPSCs in two different growth conditions: monolayer culture (tissue culture plates) and culture on PGA scaffolds. This chapter will further elaborate on how hDPSCs react to treatment with light only, light with PS and PS only. PGA scaffold without incorporated PS was used as a standard against which to compare the PS-incorporated scaffolds.

4.2 Aim and objectives

4.2.1 Aim

The aim of this chapter is to evaluate the PDT effects on hDPSCs seeded on both tissue culture plates and the PGA scaffold in *in vitro* culture.

4.2.2 Objectives

The above aim will be achieved through the following objectives:

- To determine the effects of PDT on the metabolic activity, proliferation rate, viability and morphology of hDPSCs in culture on tissue culture plates.
- To determine the effects of PDT on the metabolic activity, viability and morphology of hDPSCs seeded on the PGA scaffolds.

4.3 Materials and Methods

All materials were purchased from Sigma-Aldrich, UK unless otherwise stated.

4.3.1 Light intensity measurement for tungsten lamp

Human dental pulp stromal cells (hDPSCs) grown on tissue culture plates and PGA scaffolds were irradiated using the set-up shown in Figure 4.1.

A 400W tungsten filament lamp (Aurora, UK) was used for illumination of the samples. The power output of the light source used in this study had been previously measured in the Dept of Medical Physics, University of Leeds using a light meter (Wood *et al.*, 2006; Metcalf *et al.*, 2006). With the water bath in place, and at the set distance of irradiation from the sample (30 cm), the intensity of the light did not vary significantly across the area of the field of irradiation (unpublished data). Intensity readings were taken both above and immediately beneath the sample plate, and did not vary significantly.

The measured average intensity of the lamp was 22.7 mW/cm² in the wavelength range 500-550 nm (the ERY maximum absorption region) and 22.5 mW/cm² in the wavelength range 600-680 nm (the MB maximum absorption region). Light is known to interact with materials by being reflected, refracted, absorbed and transmitted (Kanyathare *et al.*, 2020). Reflection occurs as light bounces off of a material; absorption occurs when the energy of light is captivated by a material; and transmission occurs as light travels through a material without losing any energy. The clear water bath was used in this study to absorb and remove infrared light (which would otherwise cause heating of the sample), but allow transmission of the wavelengths of light absorbed by the PS. With the water bath in place, previous measurements (unpublished data) showed that there was no increase in temperature in the

plane of the sample when the light source was set at 30 cm above the samples.

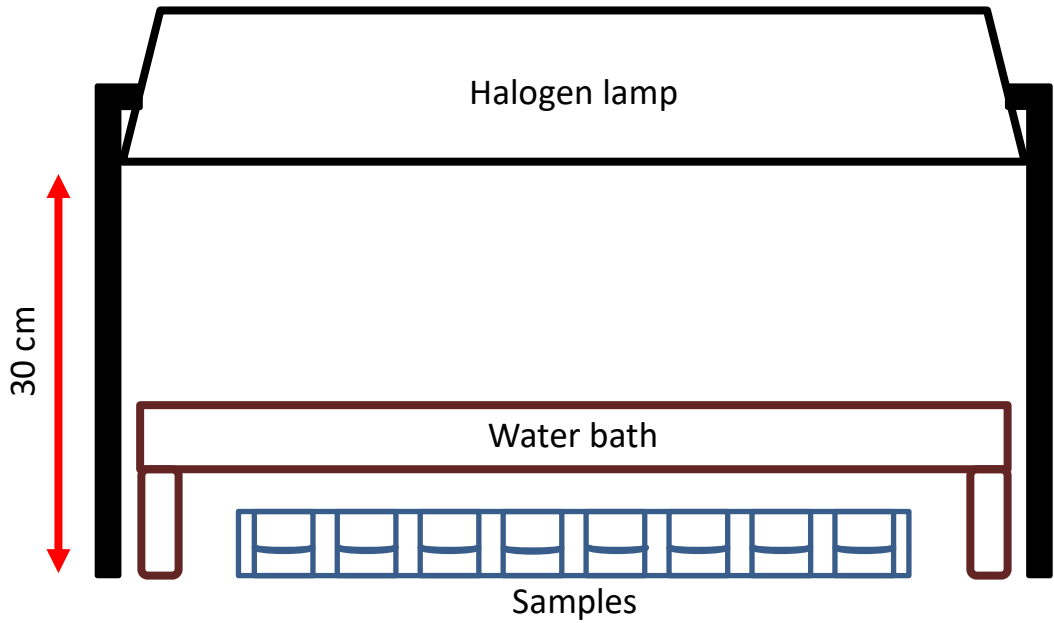


Figure 4.1 Diagram of the *in vitro* PDT experiment in this study.

The samples were placed in multi-well tissue culture plates and irradiated 30 cm from a halogen lamp for various irradiation times. The water bath was placed in between the samples and the halogen lamp to avoid any heating effects.

Light fluence rate or irradiance is defined as power per unit area (W/m^2 or mW/cm^2). The amount of the light dose absorbed by the PS is known to be proportionate to the energy fluence rate or light dose (Ke *et al.*, 2012).

$$\text{Energy fluence rate} = \text{Irradiance} \times \text{irradiation time}$$

$$\text{Energy fluence rate } \left(\frac{\text{J}}{\text{cm}^2} \right) = \frac{(\text{mW}/\text{cm}^2) \times s}{1000}$$

4.3.2 Photodynamic therapy effect on hDPSCs growing on tissue culture plates and PGA scaffolds

4.3.2.1 Preparation of the PS released from the produced scaffold

Five types of scaffold were used in this assay: PGA, PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M. Although scaffolds incorporating 7.5% PS had also originally been synthesised, they were not selected for ongoing studies due to their high degradation rates and potentially high cytotoxic effects. For each scaffold, a cork borer was used to cut a 1 cm diameter disc which was then placed into a 48-well plate. All the scaffolds were sterilised under UV light for 20 min for each surface in the fume hood. For each group, n=3, and the experiment was performed three times. Each scaffold was then covered with basal medium and incubated for 24 h at 37°C in 5 % CO₂ in the air. After 24 h, the basal medium containing released PS was collected and stored at -80°C for further use. The average concentrations of the released photosensitiser were calculated and used in this experiment. The concentrations were: 1%E = 1.09 µM, 5%E = 56.37 µM, 1%M = 6.91 µM, 5%M = 67.93 µM.

4.3.2.2 Preparation of the PGA scaffold

PGA scaffold was used in this experiment as a model for cell growth on the scaffold to be compared with the monolayer cells (tissue culture plate) model. PGA scaffolds were cored into 1 cm diameter discs using a cork borer and sterilised under UV light in the fume hood for 20 min. PGA scaffolds were placed in 48 well-plates before being seeded with human dental pulp cells as described (Section 4.3.2.3).

4.3.2.3 Photodynamic therapy of hDPSCs

PDT was performed using the medium containing the PS released from the scaffolds, as prepared in section 4.3.2.1 and the PS incubation time with cells was performed for either 30 min or 24 h (Fig. 4.2). Briefly, 1×10^5 cells were seeded per well in basal medium (α -MEM containing 10 % FBS, 2 mM L-glutamine and 100 units/mL penicillin/100 μ g/mL streptomycin) with or without PGA scaffolds. Cell culture media are known to have comparable water transmission properties and good optical properties that can be used as optical medium for various biophotonic system (Hoang *et al.*, 2019). Cells were cultured for 24 h at 37°C with 5 % CO₂ in the air. The basal medium was then discarded and replaced with basal medium containing PS collected as described in section 4.3.2.1. All the plates were then covered with foil to avoid any light exposure and returned to the incubator. One group was incubated with basal medium containing PS for 30 min and the other group was incubated for 24 h. After that, the basal medium containing PS was discarded and replaced with fresh basal medium without PS. Cells were then irradiated using the tungsten lamp for 15 min, 30 min and 60 min. One group did not receive any light irradiation to investigate the effect without any light exposure. The plates were then returned to the incubator for a 24 h recovery period. For each group, n=3, and the experiment was performed in triplicate (total n=9). All the samples (cells on tissue culture plates and cell-scaffold constructs) were then processed, as described earlier, for the cell metabolic activity assay (Chapter 3, section 3.3.4), DNA quantitation assay (CyQuant) for monolayer cells (4.3.2.4) and microscopy evaluation (Chapter 3, section 3.3.6).

For the cell metabolic assay, the percentage of cell killing following PDT was determined using the following equation:

$$\text{Percentage (\%) of cell killing} = 100 - \left(100 \times \frac{\text{Fluorescence sample}}{\text{Fluorescence control}} \right)$$

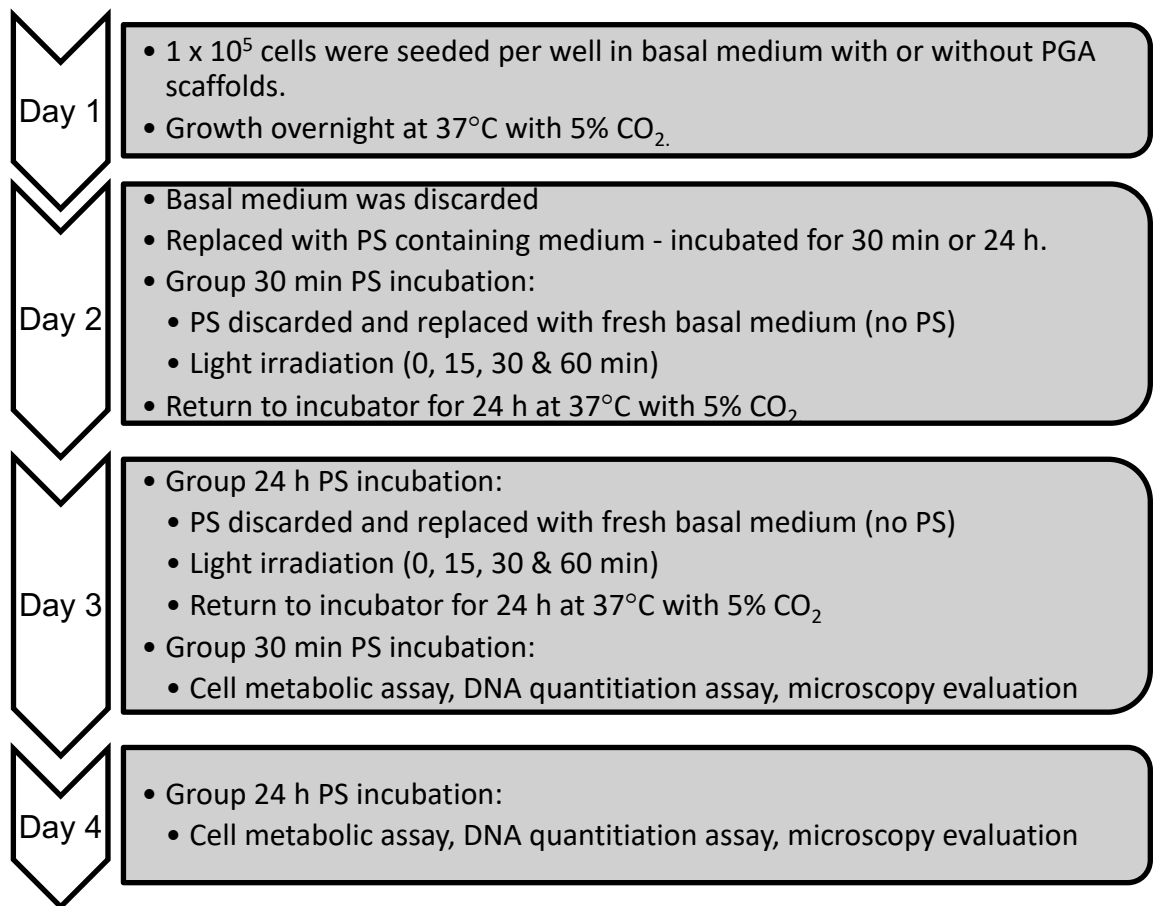


Figure 4.2 Process for the photodynamic therapy of hDPSCs cultured on tissue culture plates and PGA scaffold.

4.3.2.4 CyQuant® Direct Cell Proliferation assay

The reagents supplied with the CyQuant® Direct Cell Proliferation assay kit (ThermoFisher Scientific, UK) were CyQuant® Direct nucleic acid stain (Component A) and CyQuant® Direct background suppressor I (Component B). The assay is based on the amount of fluorescence detected using a microplate reader.

The CyQuant Direct Cell Proliferation assay is known to be useful in estimating live cell numbers based on DNA content and assessing the membrane integrity of the cells. The CyQuant Direct nucleic acid stain reagent only emits green fluorescence once it binds to the DNA in cells that are healthy and possess intact cell membranes. The suppressor reagent in this kit is permeable to dead and damaged cells that have lost their membrane integrity. It will therefore block the CyQuant Direct nucleic acid stain reagent from binding to DNA in dead cells which would lead to false positives. Therefore, only live cells will be stained with the CyQuant Direct nucleic acid stain reagent, and a reduction in staining is indicative of disruption in the integrity of the cell membranes.

Prior to the assay, 2X detection reagent was prepared by adding 48 µL of Cyquant® Direct nucleic acid stain and 240 µL CyQuant® Direct background suppressor I to 11.7 mL basal medium (α -MEM) supplemented with 10 % FBS, 2 mM L-glutamine and 100 units/mL penicillin/100 µg/mL streptomycin). 2X detection reagent was properly mixed by vortexing. The procedure described in section 4.3.2.3 was then performed with the following modifications: 2X detection reagent was added to the cells in a ratio of 1:1

(v/v) with basal medium and incubated for 1 h at 37°C with 5% CO₂ in the air and covered with aluminium foil to avoid light exposure. The fluorescence was then measured using a bottom-read plate reader with an excitation wavelength of 480 nm and an emission wavelength of 535 nm. A blank measurement of basal medium alone was included to provide a background value which was subtracted from the test sample measurements. The number of live cells within the sample was determined by comparison with the standard curve (Figure 4.3).

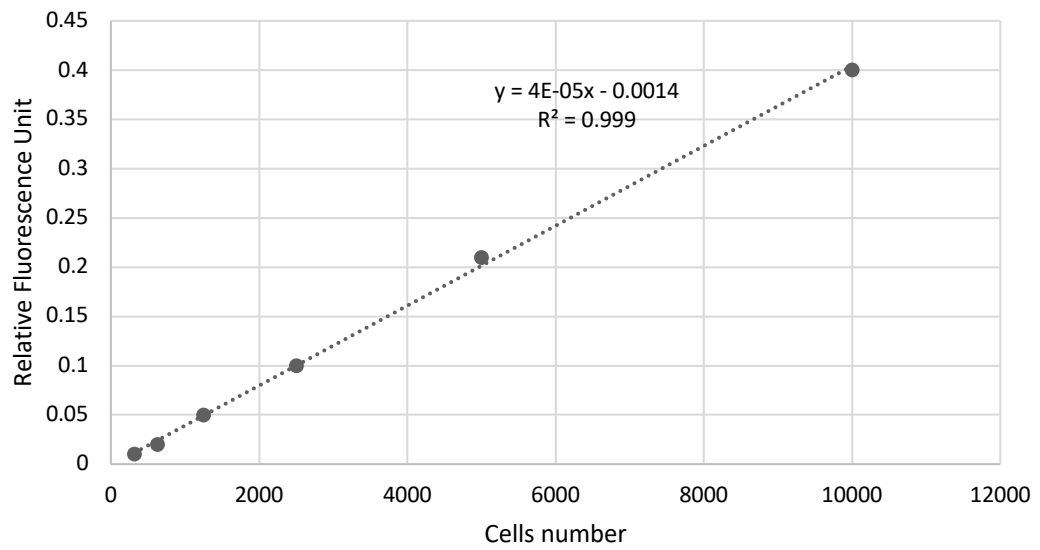


Figure 4.3 Representative standard curve for live cell number calculation within the sample.

The standard curve was used to calculate the number of live cells based on the fluorescence detected from the live-cell DNA.

4.3.2.5 Live/ Dead Cell Imaging Kit

The cell-scaffold constructs were stained using the Live/Dead™ Cell Imaging Kit (ThermoFisher Scientific, UK) which stains live cells with green fluorescence and dead cells with red fluorescence. The kit consists of two components: component A (Live Green/ Calcein AM) and component B (Dead Red/ Propidium Iodide). The vials of these components were thawed to room temperature before the start of the staining procedure. Live Green was transferred into Dead Red and the mixture was mixed to create a 2X working solution according to the kit instructions supplied. An equal volume of 2X working solution was then added to the cells with basal media. The cell-scaffold constructs and working solution were incubated for 15 min at room temperature before images were captured using an inverted fluorescence microscope (DM16000 B, Leica GmbH, Germany). FITC filters (excitation wavelength = 488 nm and emission wavelength = 515 nm) and Texas Red filters (excitation wavelength = 570 nm and emission wavelength = 602 nm) were used to visualise the cells. The microscope objectives used to visual the cells were 10x and 20x magnification (air immersion medium).

4.3.3 Statistical analysis

All experiments were carried out in triplicate and each experiment was repeated 3 times (total n=9) unless stated otherwise. The statistical analyses were carried out using IBM SPSS Statistics Version 23 software. Results were analysed using Students' t-test and one way analysis of variance (ANOVA) tests. P-values were determined, and $P < 0.05$ was considered significant. All results were presented as mean $\pm$ standard error of mean (S.E.M).

4.4 Results

Assessment of the PDT effects on hDPSCs that were cultured on either tissue culture plates or PGA scaffolds was performed by measuring the cell metabolic activity level, using a cell proliferation assay and through microscopy evaluation. All of the experiments were performed after hDPSCs were cultured with basal media for 24 h (whether on tissue culture well-plate or PGA scaffold).

4.4.1 Photodynamic therapy of hDPSCs on tissue culture plates

4.4.1.1 Effect of photodynamic therapy on hDPSCs metabolic activity

Figure 4.4 (a) shows the percentage of cell killing for hDPSCs after being incubated for 30 min with the PS followed by light irradiation for 0, 15, 30 and 60 min. Cell culture exposure to PS for 30 min without any light irradiation showed that cell killing with 5%M (70%) was the highest followed by 1%M (27%) and 5%E (9%). However, 1%E did not show any cell killing and the cellular metabolic activity was increased slightly. However, this was not significant when compared to the control (no PS and no light). Cells incubated with 1%E showed no significant changes even when the visible light irradiation times were increased accordingly. In the 5%E group, the cells that were irradiated with visible light significantly increased in percentage kill when compared to the non-irradiated 5%E group. The percentage cell killing for 5%E was significantly increased for the highest irradiation time (60 min ~ 27%) when compared to the PDT groups which had received 15 min (19%) and 30 min (18%) irradiation.

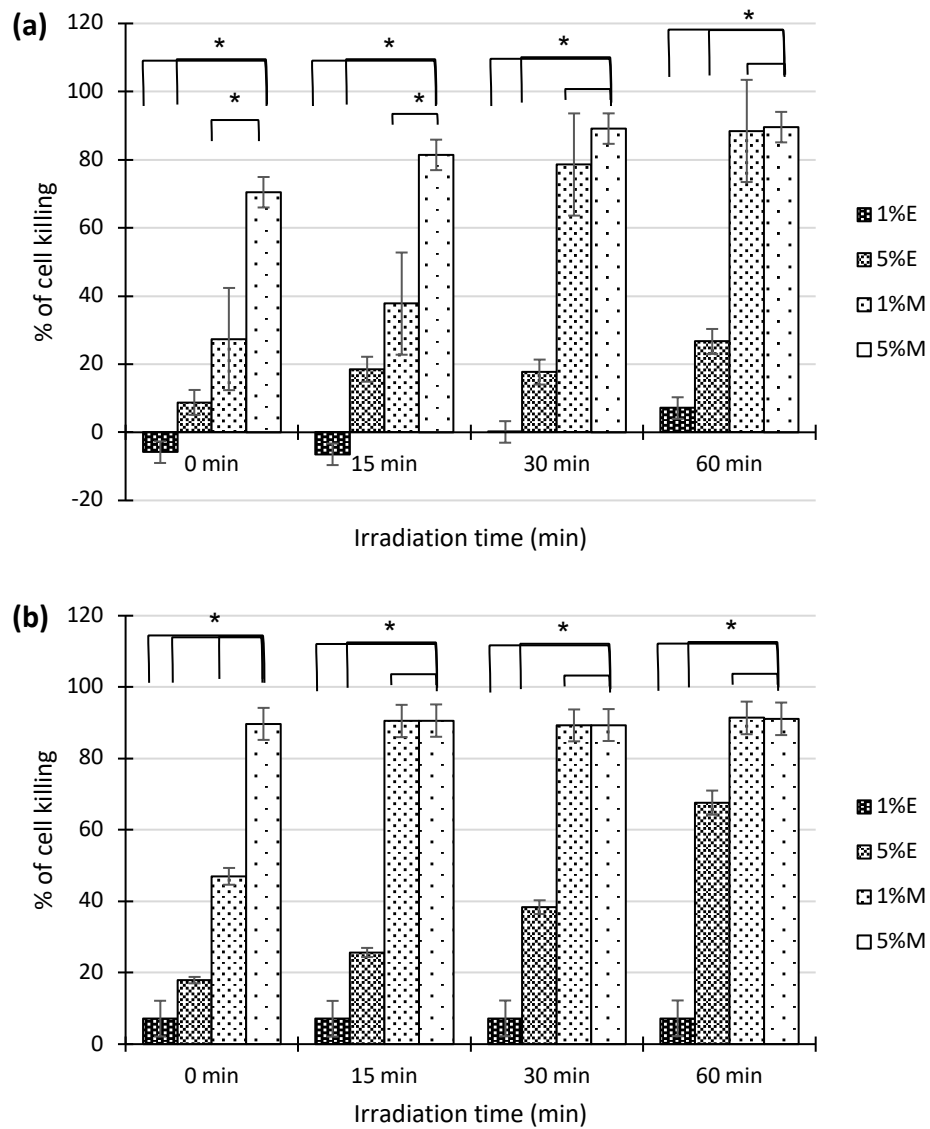


Figure 4.4 Percentage of cell killing of hDPSCs using the cell metabolic assay after incubation with PS and light irradiation (0-60 min).

The graphs represent the percentage of hDPSC killing after incubation with PS for either 30 min or 24 h. Data are represented as the mean percentage of hDPSC killing ($n=9$) $\pm$ S.E in each group.

(a) HDPSCs were incubated for 30 min with a series of PS.

(b) HDPSCs were incubated for 24 h with a series of PS.

* Indicates a significant difference $p < 0.05$.

$$\text{Percentage (\%)} \text{ of cell killing} = 100 - \left(100 \times \frac{\text{Fluorescence sample}}{\text{Fluorescence control}} \right)$$

Cells incubated with 1%M for 30 min and which received no light irradiation showed 27% of cell killing. Furthermore, at lower irradiation time (15 min), the cell-killing percentage did not increase when compared to non-irradiated cells. However, the cell-killing percentage increased significantly when the light irradiation time was increased as the percentage of killing rose to 79% (30 min) and 88% (60 min). The cell-killing percentage was more prominent in 5%M even in non-irradiated cells (70% cell-killing) and increased significantly in a light dose-dependent manner; 15 min (81%) < 30 min (89%) < 60 min (90%).

Figure 4.4 (b) shows the percentage of cell killing for hDPSCs after being incubated for 24 h with the PS followed by light irradiation times varying from 0 to 60 min. Cells incubated with 1%E showed no changes in percentage cell killing between irradiated and non-irradiated cells. However, a more gradual increasing trend of percentage cell killing can be observed in 5%E when the light irradiation times were increased; 0 min (17.9%) < 15 min (25.7%) < 30 min (38.4%) < 60 min (67.6%). Cell incubation in 1%M resulted in 47% of cell killing in the non-irradiated group and increased to 90.5% (15 min), 89.3% (30 min) and 91.4% (60 min) when the light irradiation times were increased. In 5%M, the percentage of cell killing was extremely high as it displayed 89.7% cell killing even in the dark and the trend was maintained when the light was introduced to the cells; 15 min (90.6%), 30 min (89.14%) and 60 min (91.1%).

4.4.1.2 Effect of photodynamic therapy on hDPSCs proliferation

Figure 4.5 illustrates the percentage of live hDPSCs after incubation with PS for either 30 min or 24 h, and followed by light irradiation, as measured by the CyQuant assay.

Non-irradiated cells: In the non-irradiated group, cells exposed to PS maintained their live cell numbers compared to control (no PS and no light) for both PS incubation times, 30 min (Fig. 4.5 (a)) (1%E = 103%, 5%E = 97%, 1%M = 109%) and 24 h (Fig. 4.5 (b)) (1%E = 103%, 5%E = 101%, 1%M = 92%). The only exception was seen with the higher MB concentration (5%M), where the percentage of live cells was significantly reduced to 62% after 30 min incubation time and further reduced to 6% when incubated for 24 h.

Irradiated Cells: For 30 min incubation time with PS (Figure 4.5 (a)), the cells incubated with 1%E (89%) and 5%E (85%) showed a significant decrease in the percentage of live cells after being exposed to the highest light irradiation time (60 min) compared to cells with no irradiation.

For 30 min and 60 min irradiation times, cells incubated with 1% M showed a significant decrease in the percentage of live cells after irradiation (86% and 63% respectively). Cells incubated in 5%M showed an even greater decrease in the percentage of live cells for all light irradiation times compared to unirradiated controls (15 min = 7%, 30 min = 8%, 60 min = 5%).

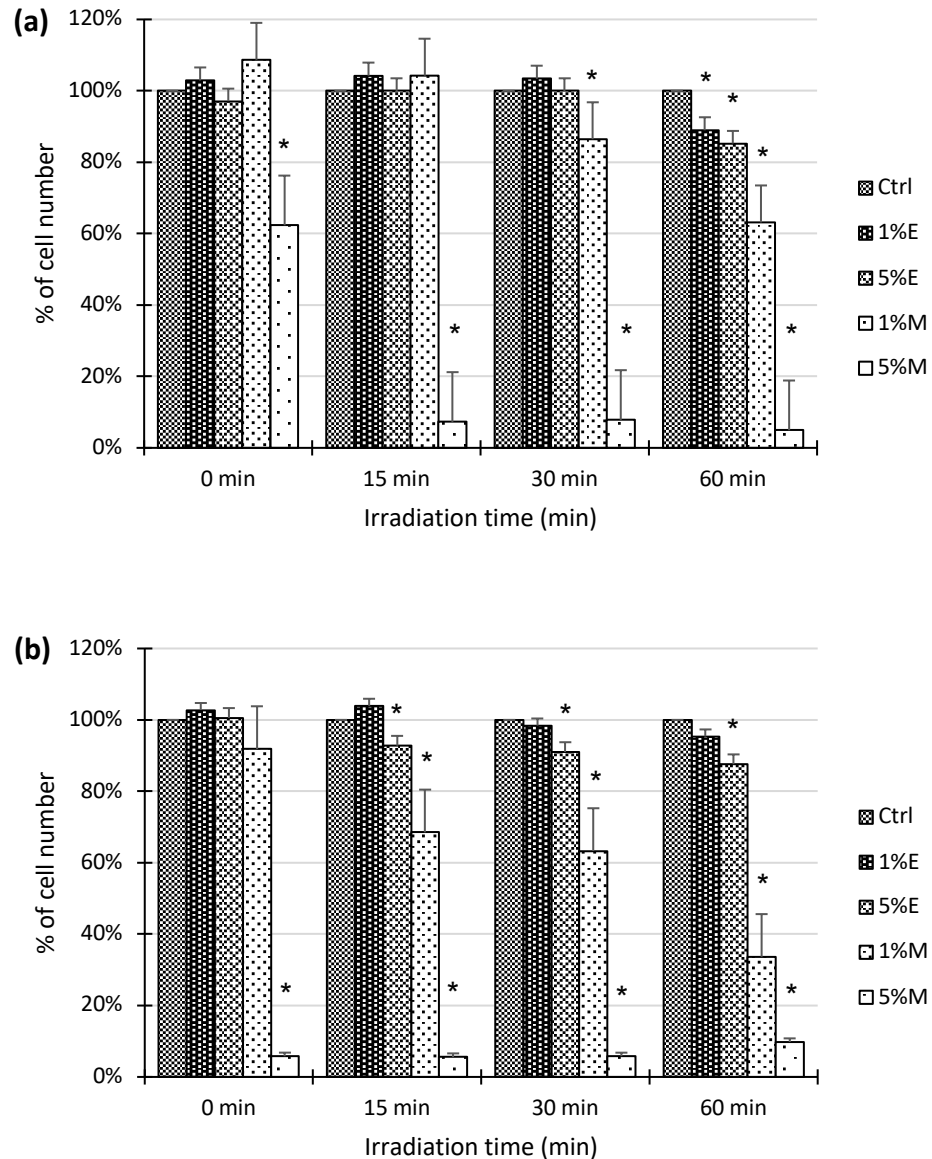


Figure 4.5 Percentage of live cells based on live-cell DNA detected by fluorescence intensity after incubation of hDPSCs with PS and followed by light irradiation (0-60 min).

The graphs represent the percentage of live hDPSCs after incubation with PS for either 30 min or 24 h. Data are represented as the mean percentage of hDPSCs ($n=9$) $\pm$ S.E in each group.

(a) Percentage of live cells after 30 min incubation time with PS.

(b) Percentage of live cells after 24 h incubation time with PS.

* Indicates a significant difference compared to Ctrl ($p<0.05$).

$$\text{Percentage (\%)} \text{ of cell number} = 100 \left(\frac{\text{Fluorescence sample}}{\text{Fluorescence control}} \right)$$

In the 24 h incubation experiment (Figure 4.5 (b)), at the highest irradiation time (60 min), the cells incubated with 1%E (95%) displayed a slight decrease in live cell number when compared to the control (no PS, no light). The cells incubated with a higher concentration of erythrosine (5%E), showed a significant reduction in the percentage of live cells in all the irradiated groups compared to the non-irradiated group (0 min) (15 min = 93% > 30 min = 91% > 60 min = 88%). The live cell number percentage in 1%M showed a significant light dose-dependent decrease with increasing light exposure times when compared to the control (no PS and no light) (15 min = 69% > 30 min = 63% > 60 min = 34%). As expected, the 5%M live cell number percentage was significantly decreased when compared to the control (no PS and no light) in all of the light treated samples, irrespective of light dose (0 min = 6%, 15 min = 6%, 30 min = 6% and 60 min = 10%).

4.4.1.3 Fluorescence microscopy evaluation of the effect of photodynamic therapy on hDPSCs viability in monolayer culture

The cell viability of hDPSCs following PDT was evaluated after a 24 h recovery period using a commercial Live/Dead[®] stain. The cells that had not been incubated with any PS but irradiated with various light doses revealed normal viability and morphology compared control which had received no PS and were non-irradiated.

Cell viability: Figure 4.6 shows the images of the hDPSCs on the tissue culture plate after 30 min incubation with a range of PS and followed by irradiation (0 min, 15 min, 30 min and 60 min). The cells incubated with PS, but which received no light irradiation exhibited similar staining and viability compared to the control except for the 5%M group. Cells incubated with 1%E exhibited similar viability compared to controls, irrespective of light dose. A similar observation can be seen for the 5%E group except for the highest light dose (60 min) where there was some reduction in the number of cells present. A gradual decrease of cell viability was seen in the 1%M group with increasing light irradiation time. The cells incubated with a high concentration of MB (5%M) and irradiated were affected severely, where most of the staining was red and indicative of dead cells.

The effect of longer exposure (24 h) to PS can be seen in Figure 4.7. Cells in the 1%E group showed no effect from the light irradiation when compared to the control. However, the 5%E group showed a gradual decrease in cell viability as irradiation time increased and more dead cells were observed in the highest light dose (60 min). A similar trend can be seen in the 1%M group where less cell viability was observed in both the non-irradiated and irradiated

groups. Following prolonged exposure to 5%M for 24 h, very few viable cells could be seen, even in the samples that had not received any light irradiation.

Cell Morphology: HDPSCs without PS incubation (control) showed normal fibroblastic morphology, high expression of F-actin and confluency irrespective of the light irradiation time. The cells exhibited elongated morphology, with stretched and dense actin assemblies which indicated that the hDPSCs were in an optimal state. For 5%E and 1%M, the cell cytoplasm was more shrunken with loss of cell-to-cell linkage after light irradiation. This effect was light dose-dependent. With 5%M, the PDT effect on cell morphology was prominent as there was no defined arrangement of actin to be seen. The nuclei seemed intact, however, as only the debris of the cytoplasm was stained. No cells could be detected on the plates after 60 min light irradiation for 5%M. The severity of the effect was again light dose-dependent.

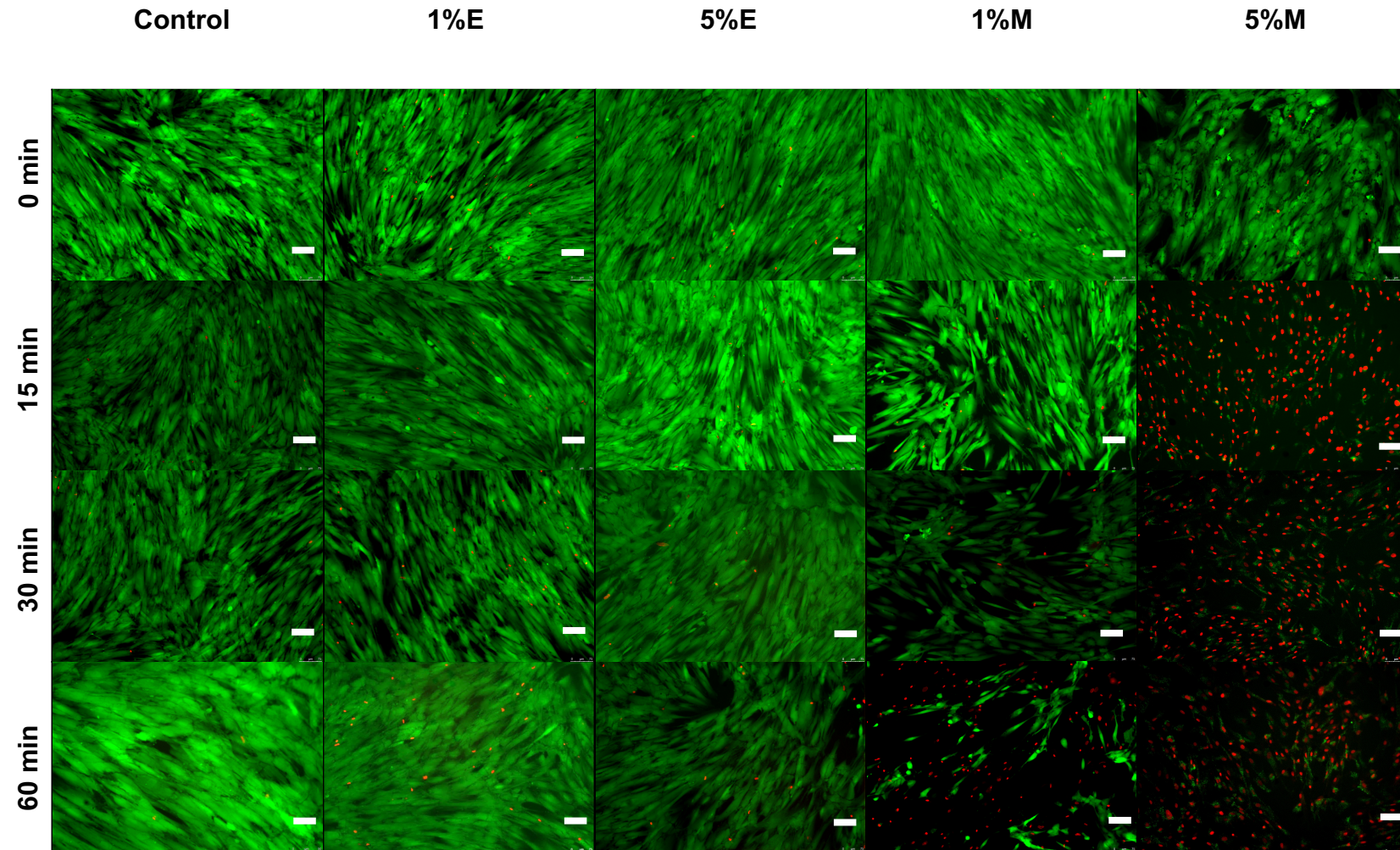


Figure 4.6 Representative fluorescence microscopy images of Live (C AM, green)/ Dead (PI, red) staining of hDPSCs after 30 min incubation with PS followed by light irradiation for 15, 30 and 60 min. 10x magnification. Scale bars are 75 μ m.

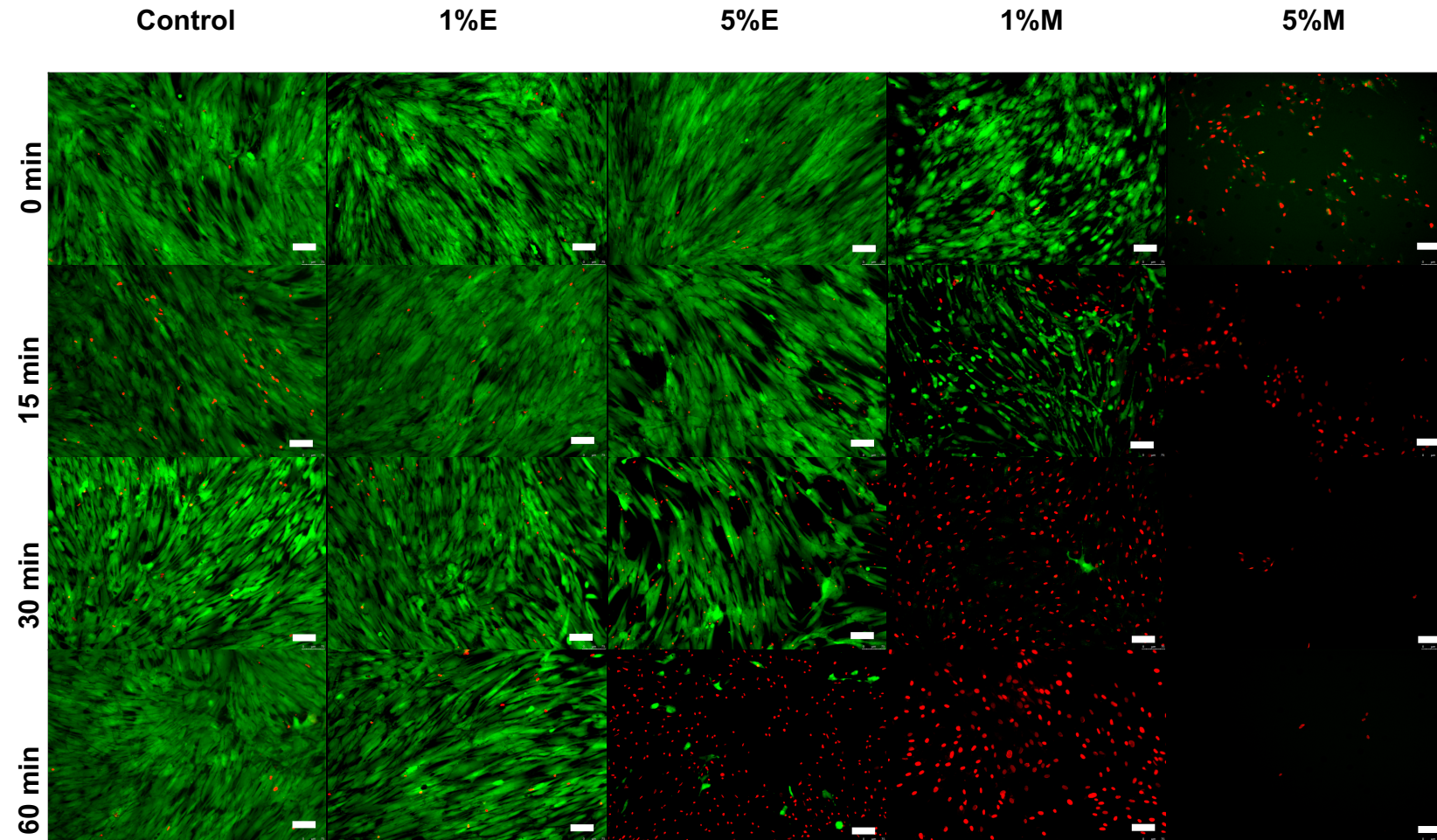


Figure 4.7 Representative fluorescence microscopy images of Live (C AM, green)/ Dead (PI, red) staining of hDPSCs after 24 h incubation with PS followed by light irradiation for 15, 30 and 60 min. 10x magnification. Scale bars are 75 μ m

4.4.2 Photodynamic therapy of hDPSCs cultures on PGA scaffolds

4.4.2.1 Effect of photodynamic therapy on hDPSCs metabolic activity

Figure 4.8 (a) shows the percentage of cell killing for hDPSCs after being incubated for 30 min with the PS followed by light irradiation for 0, 15, 30 and 60 min.

The cell killing percentage was less than 30% for the cells incubated for 30 min with 1%E and 5%E for all irradiation groups (1%E :15 min = 14%, 30 min = 6% and 60 min = 5%; 5%E: 15 min = 22%, 30 min = 15% and 60 min = 13%). The effect of longer exposure of PS (24 h) can be seen in figure 4.8 (b). When the incubation time for the 5%E group was increased to 24 h a greater amount of cell killing was observed for the 30 min and 60 min irradiation times (30 min = 27%, 60 min = 57%). However, the 24 h incubation time for 1%E did not affect the percentage of cell killing regardless of irradiation time (15 min = 9%, 30 min = 6% and 60 min = 9%).

Cells incubated with MB (1%M and 5%M) showed a noticeably higher cell killing percentage for both concentrations and incubation times in all irradiation groups. Furthermore, MB also exhibited 39% and 78% cell killing even in the absence of light irradiation for 1%M and 5%M respectively after incubated for 24 h.

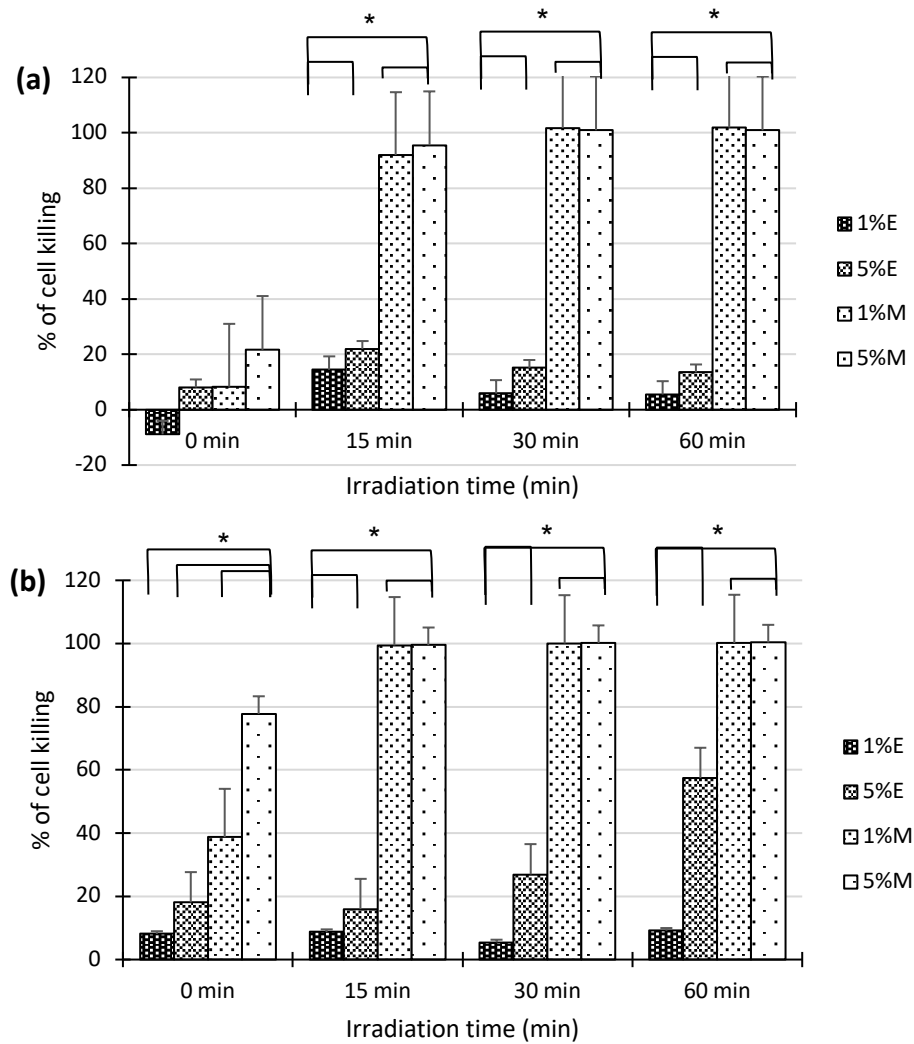


Figure 4.8 Percentage of cell killing of hDPSCs based on metabolic activity after incubation with PS and light irradiation (0-60 min).

The graphs represent the percentage of hDPSC killing on PGA scaffold after incubation with PS for either 30 min or 24 h. Data are represented as the mean of percentage of hDPSC killing ($n=9$) $\pm$ S.E in each group.

(a) Percentage of cell killing after 30 min incubation with PS and irradiated with visible light.

(b) Percentage of cell killing after 24 h incubation with PS and irradiated with visible light.

* Indicates a significant difference $p < 0.05$.

$$\text{Percentage (\%)} \text{ of cell killing} = 100 - \left(100 \times \frac{\text{Fluorescence sample}}{\text{Fluorescence control}} \right)$$

4.4.2.2 Fluorescence microscopy evaluation of the effect of photodynamic therapy on the viability of hDPSC growing on PGA scaffolds

The viability of hDPSCs seeded on PGA scaffolds was evaluated following a 24 h post-irradiation recovery period using a commercial Live/Dead® stain. Fluorescence microscopy images of hDPSCs after incubation with PS for 30 min or 24 h and treatment with a range of light doses are presented in figures 4.9 and 4.10 respectively. Green fluorescent staining in these images showed live cells and red fluorescent staining exhibited dead cells. The cells that received PS, but no light irradiation, displayed similar cell viability compared to the control group (no PS and no light) for both incubation times.

From the images, it was evident that the hDPSCs did not survive after light irradiation in both concentrations of MB (1%M and 5%M) and incubation times (30 min and 24 h) as there was more red fluorescence observed from the captured images. For the 1%M concentration, there was increased red fluorescence in all the irradiated groups for both incubation times and the increase in red fluorescence was dependent on MB percentage. As expected, the 5%M concentration induced more cell death (red fluorescence) even without application of light especially at the longer incubation time of 24 h. However, the cells incubated with ERY (1%E and 5%E) exhibited no obvious difference when compared to control (no PS) for the 30 min incubation time in all light doses. A similar result was seen for the 24 h incubation time for 1%E and 5%E except for the highest light dose (60 min) in 5%E where there was a reduction in green (live) cell staining.

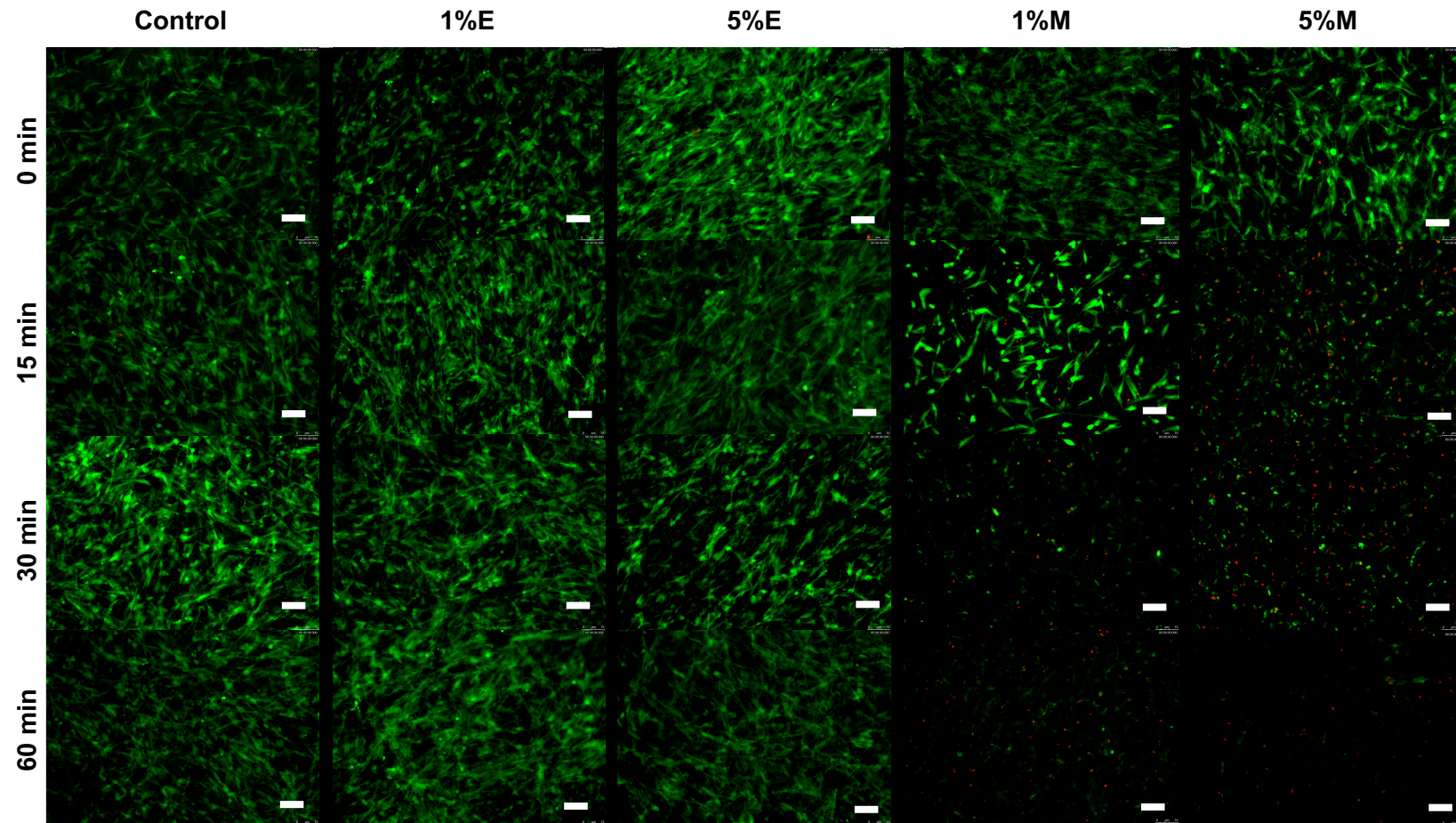


Figure 4.9 Fluorescence microscopy images of hDPSCs cultured on PGA scaffold. Images were taken after a 24 h recovery period (following 30 min incubation with PS and irradiation with visible light for 15, 30 and 60 min). Cell viability was indicated with the Live/Dead stain. Scale bars are at 75 μ m.

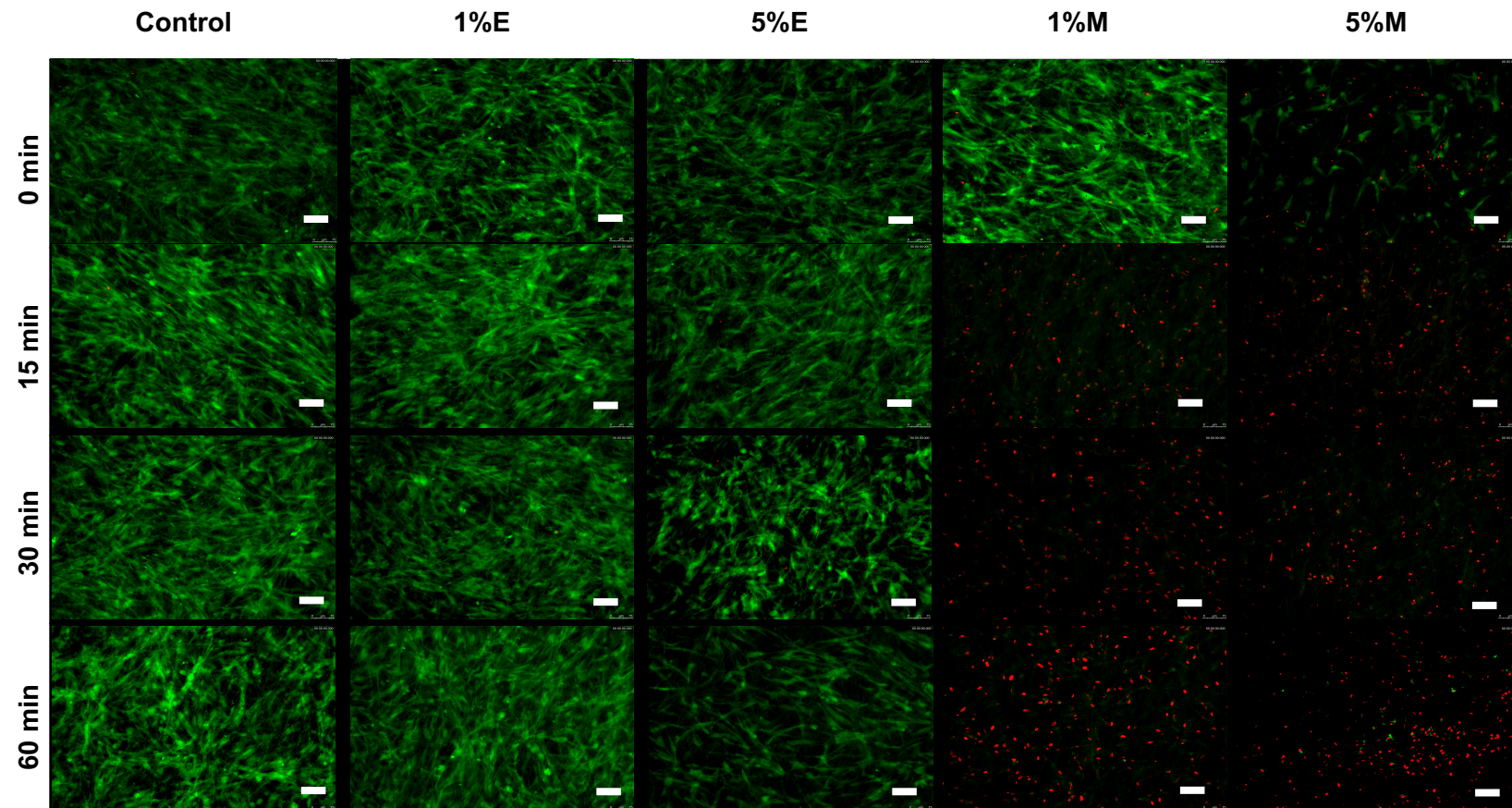


Figure 4.10 Fluorescence microscopy images of hDPSCs seeded on PGA scaffold. Images were taken after a 24 h recovery period (following 24 h incubation with PS and irradiation with visible light for 15, 30 and 60min). Cell viability was indicated with the Live/Dead[®] stain. Scale bars are at 75 μ m.

4.4.2.3 Effect of photodynamic therapy on the morphology of hDPSCs morphology growing on PGA scaffolds

The cell morphology of hDPSCs seeded on PGA scaffolds, both with and without PDT, was evaluated after a 24 h recovery period. The effect of PDT on hDPSCs cellular morphology and the cytoskeletal arrangement was investigated using the commercial stains Alexa Fluor® 488 phalloidin and DAPI as described in Chapter 3 (section 3.3.6.2). Control (no PS) hDPSCs showed normal fibroblastic morphology, high expression of F-actin and confluency irrespective of the light irradiation time (Fig. 4.11 and Fig. 4.12). The cells were elongated, stretched and exhibited dense actin assemblies, indicating the hDPSCs optimal state. For 5%M, the PDT effect on cell morphology was prominent as no defined arrangement of actin could be seen. The nuclei seemed intact, but only debris of the cytoplasm was stained. For 1%M, there was significant cell shrinkage and a loss of cell-to-cell linkages. There was also a reduction in actin staining when compared to the control group.

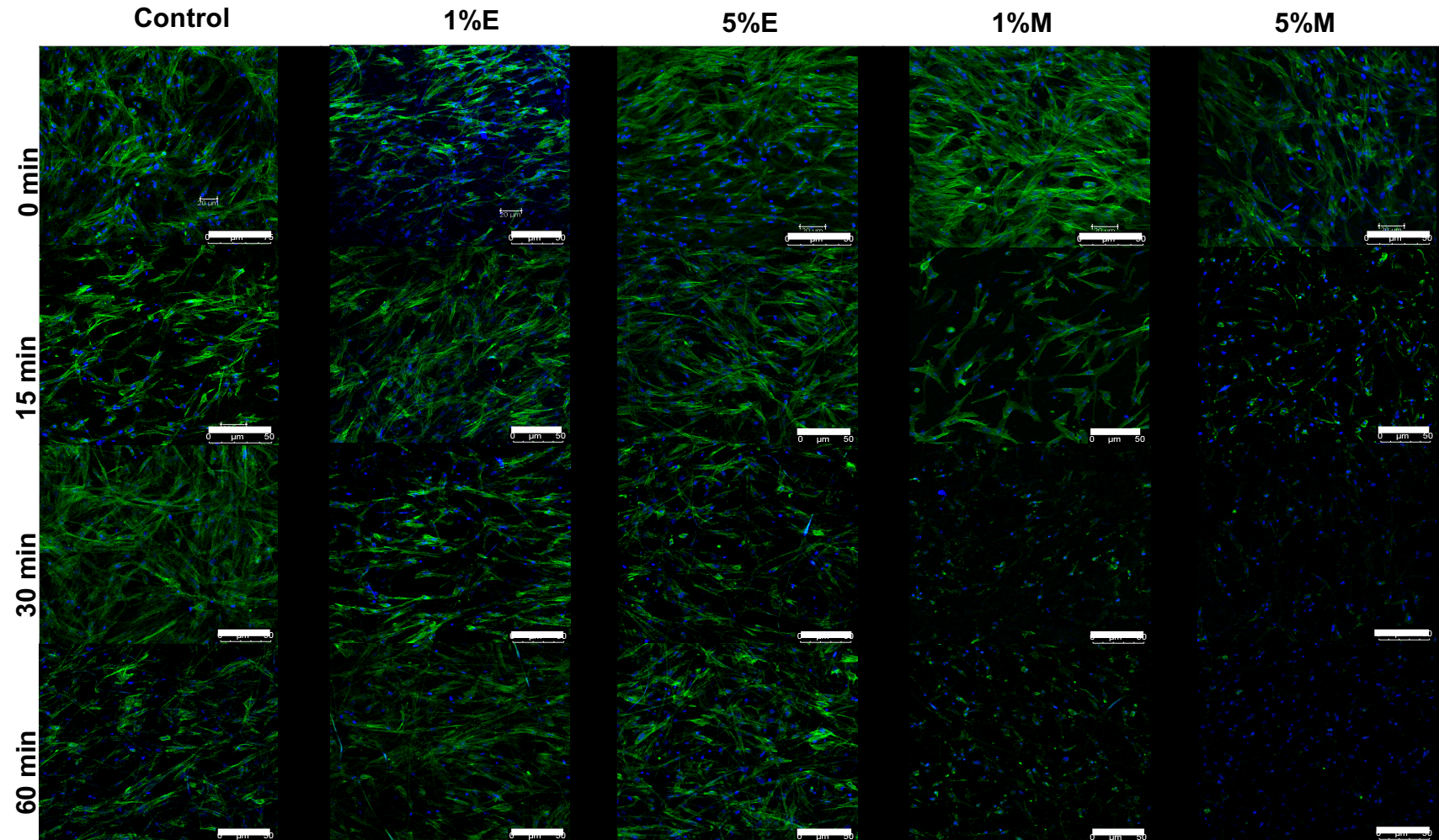


Figure 4.11 Confocal microscopy of hDPSCs seeded on PGA scaffold. Images were taken after a 24h recovery period (following 30 min incubation with PS and irradiation with visible light for 15, 30 and 60 min). F-actin and nuclei were stained with AlexaFluor 488 (green) and DAPI (blue) respectively. Scale bars are at 50 μm.

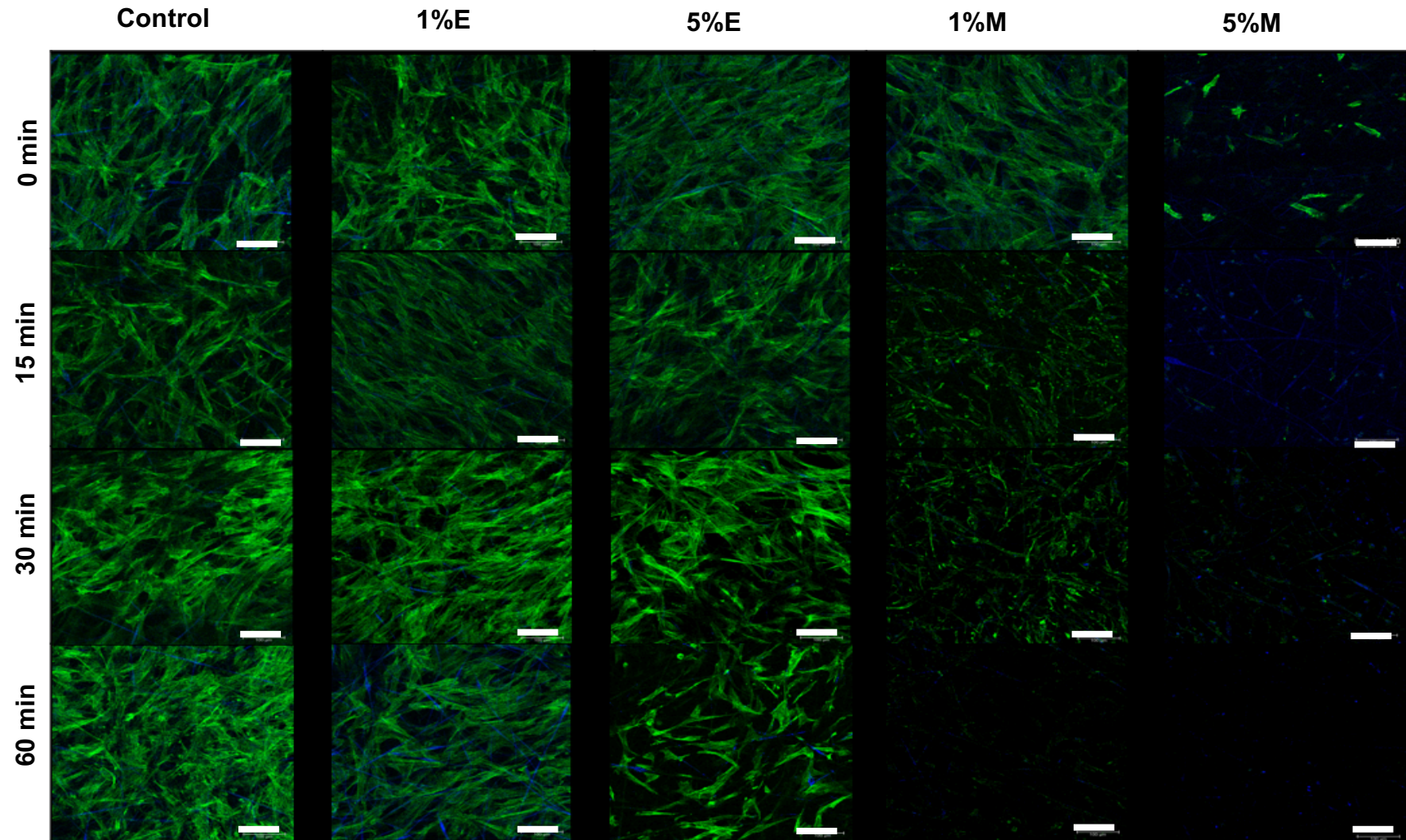


Figure 4.12 Confocal microscopy of hDPSCs seeded on PGA scaffolds. Images were taken after a 24 h recovery period (following 24 h incubation with PS and irradiation with visible light for 15, 30 and 60 min). F-actin and nuclei were stained with AlexaFluor 488 (green) and DAPI (blue) respectively. Scale bars are at 50 μm .

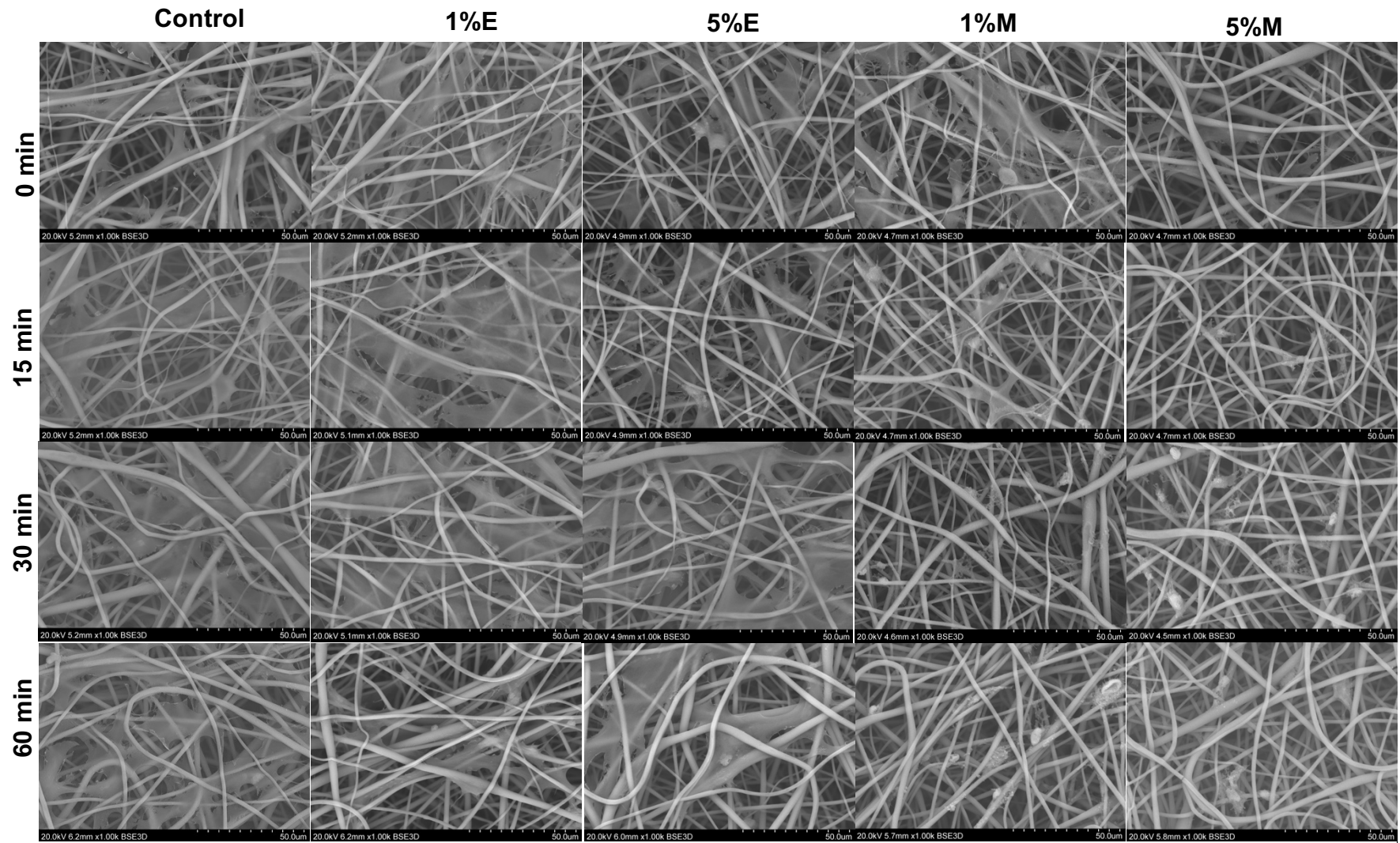


Figure 4.13 SEM images of hDPSCs on PGA scaffolds. Images were taken after 24 h recovery period (after 30 min incubation with PS and irradiation with visible light for 15, 30 and 60 min).

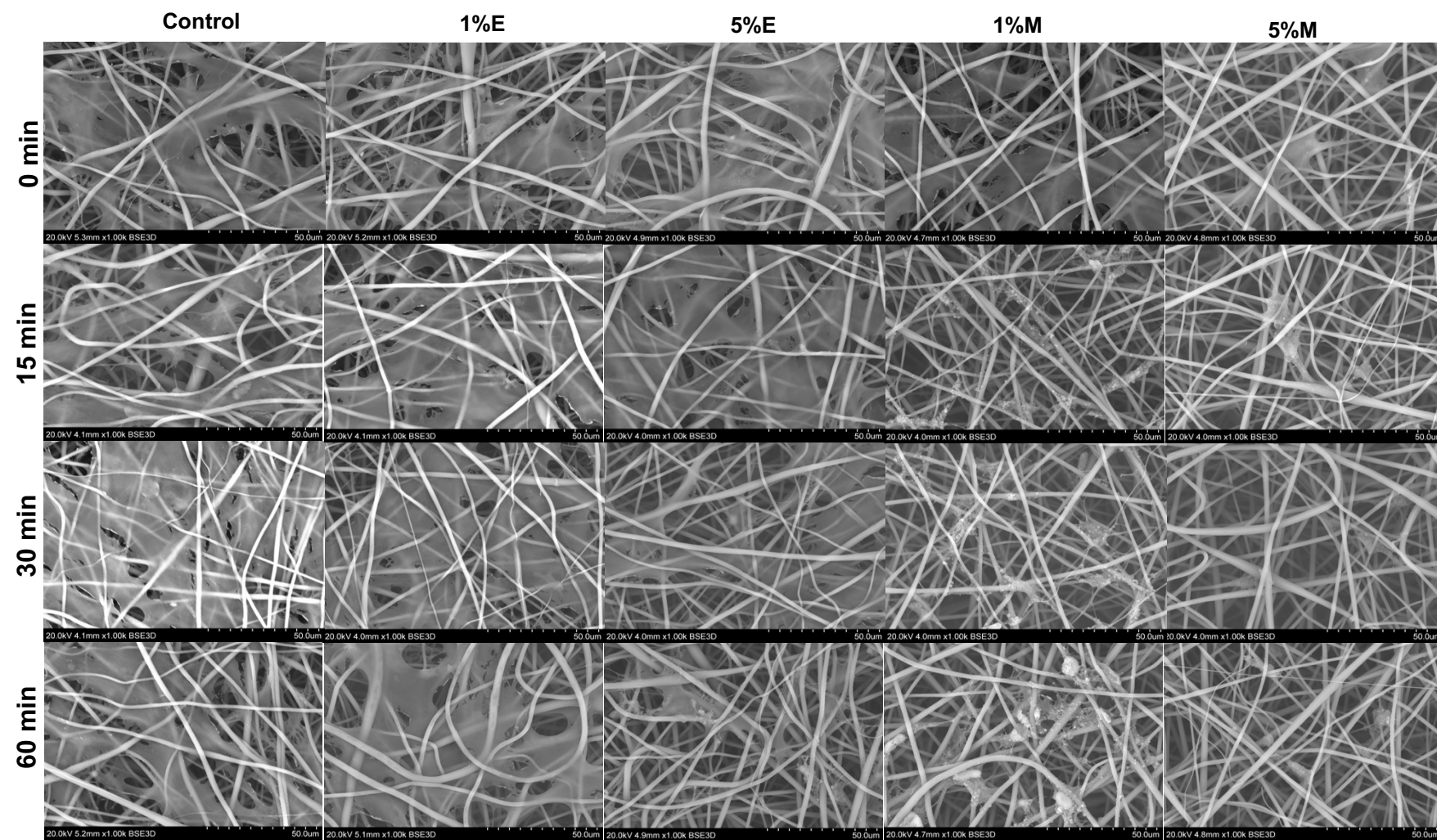


Figure 4.14 SEM images of hDPSCs on PGA scaffolds. Images were taken after 24 h recovery period (after 24 h incubation with PS and irradiation with visible light for 15, 30 and 60 min).

Further observation with SEM imaging (Chapter 3 section 3.3.6.1) confirmed what had been observed using fluorescence and confocal microscopy (Fig. 4.13 and 4.14). After the 24 h recovery period, normal cells and ECM production could be seen on the PGA scaffold without PS incubation. A similar cell morphology and ECM production covering the surface of PGA scaffolds could be seen in cells that had been incubated with 1%E and 5%E compared to control (no PS) despite light irradiation. However, a reduction in ECM and cell number were found on PGA scaffolds following 24 h incubation with 5%E and which had received 60 min light irradiation. Cells were still attached to the fibres of the scaffold, but the morphology of the cells was not satisfactory. Cells on PGA scaffolds and incubated with 1%MB showed no sign of toxicity in the non-irradiated group. However, after light irradiation was introduced, the PDT effect reduced the number of cells with healthy morphology with many cells being rounded but with cytoplasmic extensions still attached to the fibres of the scaffold. Cells on PGA scaffolds and incubated with 5%M revealed a greater toxicity effect, as fewer cells were seen on the scaffolds as the incubation time and light doses were increased.

4.5 Discussion

The effect of PDT on hDPSCs in terms of culture method, incubation time, type of PS, the concentration of PS and light dose were investigated in this chapter. The purpose of these studies was to investigate the potential side effects of using PDT toward the hDPSCs growing on multi-well plates or on PGA scaffold culture with two different incubation times (30 min and 24 h) with PS. It is important to investigate this to identify possible effects on the hDPSCs before any clinical trial is conducted. The protocol used in this study is a modification of that used in previous research (Garg *et al.*, 2012; Wood *et al.*, 2010). The average concentration of PS (either MB or ERY) released from the scaffolds were as follows; 1%E = 1.09 μ M, 5%E = 56.37 μ M, 1%M = 6.91 μ M, 5%M = 67.93 μ M. Therefore, these were the concentrations of PS added to the samples.

As a result, the ERY concentrations used in our study were lower than the concentration used in the previous research (Garg *et al.*, 2012) which was 22 μ M. However, our findings for the shorter incubation time seem to align with their results which showed no obvious cell killing (less than 30%) in hDPSCs when incubated for 30 min despite light irradiation and culture condition. Conversely, when the incubation time was extended to 24 h, ERY did induce cell killing gradually in a light dose-dependent manner. There was no observed difference between the monolayer and PGA scaffold cultures suggesting that the percentage of cell killing induced by ERY-PDT was not influenced by the structure of the scaffold. Garg *et al.* (2012) stated that at 71.03 μ M of ERY concentration, an incubation time of 30 min followed by 90 min irradiation also did not induce significant cell killing for pre-malignant

(DOK) oral epithelial cells. However, despite the lack of cell killing, they found that ERY did decrease cell mitochondrial trans-membrane potential that may lead to permeabilization of mitochondrial membranes and induce apoptosis.

The CyQuant assay was performed on monolayer cultures of hDPSCs to establish whether PDT affects the cell number and membrane integrity of the cells. The apparent significant reduction of live cell number for hDPSCs incubated for 30 min in 1%E after irradiation with the highest light irradiation time (60 min) showed that cellular membrane integrity had begun to be impaired even though the cellular metabolic activity was not affected. However, when the incubation time with PS was extended to 24 h, the 1%E group did not show any significant reduction in live cell number and metabolic activity. This indicates that the membrane integrity is not compromised and may reflect the relocalisation of ERY from the cell membrane to other sites within the cell during 24 h incubation. As an anionic PS, ERY might be taken up by the endocytic pathway and end up in lysosomes (Castano, Demidova and Hamblin, 2004). The relative PDT efficacy for cell killing induced by PS localised in lysosomes is significantly lower compared to other sites of localisation such as mitochondria (MacDonald *et al.*, 1999). However, cells in 5%E did show an increase in cell killing and a significant decrease in membrane integrity, and the results demonstrated that the severity of the PDT effect is incubation time and light dose-dependent.

For MB, both percentages (1%M = 6.91 μ M and 5%M = 67.93 μ M) had a severe toxicity effect (more than 80% cell-killing) towards the hDPSCs at both incubation times and in both culture conditions (monolayer and PGA scaffold) when the light was applied. The cell-killing increased correspondingly to the

MB concentration, incubation time and irradiation time. The killing was greater in cell culture on the scaffold when compared to cell culture on tissue culture multi-well plates.

Diniz *et al.* (2014) found that MB also exerts a dark toxicity effect and that the effect was enhanced when the light was applied where most of the cells died by necrosis at a MB concentration of 0.05 mg/mL ($\approx 135 \mu\text{M}$) (Diniz *et al.*, 2014). The MB concentration used here was much lower than this previous study but more cell killing was observed in our studies, probably due to the longer incubation and irradiation times compared to the previous research. Martins *et al.* (2018) found that photoactivation of MB in low concentration ($0.5 \mu\text{M}$), was able to cause mild damage to the lysosomes and induce accumulation of acidic vacuoles while preserving mitochondria and cell viability. However, photoactivation of $2 \mu\text{M}$ MB did promote the generation of oxidative species and was usually localised in mitochondria and lysosomes of mammalian cells (Martins *et al.*, 2018).

PS such as MB and ERY tend to accumulate in mitochondria of mammalian cells and photoactivation will produce reactive oxygen species and further propagate oxidative damage, thus initiating mechanisms of cell death such as necrosis and apoptosis (Costa *et al.*, 2016; Bacellar *et al.*, 2015; Bacellar *et al.*, 2014). Light dose, photosensitiser concentration and incubation time are the important factors that govern the effect of PDT on cells (Austin and Jagdeo, 2018; Nesi-Reis *et al.*, 2018). A longer incubation time allows more interaction between cells and photosensitiser where cells might take up more PS due to its activity (Yu *et al.*, 2011). Longer irradiation times will produce

more reactive oxygen species that will induce the cell to undergo apoptotic and necrotic processes (Babilas *et al.*, 2003).

In tissue culture plates, the cells were seeded and grown on a flat surface and cell to cell contact was only at the edge of the cells and did not represent the conditions of the cells during the tissue regeneration process. In contrast, cells grown on 3D materials will induce more cell-matrix interaction, ECM production and have greater stability compared to monolayer culture (Langhans, 2018).

In conclusion, hDPSCs were found to be sensitive to the photodynamic action of MB at both percentages (1%M = 6.91 μ M and 5%M = 67.93 μ M). Photodynamic action of ERY only induced cell killing in high concentration (5%E = 56.37 μ M) and with the longer incubation time (24 h). The use of monolayer or scaffold culture did affect the percentage of cell killing for hDPSCs. Cells on PGA scaffolds with ERY incubation did show less susceptibility to the PDT effect compared to the monolayer cell culture. However, cells exposed to MB were severely damaged irrespective of monolayer or PGA scaffold culture conditions.

Chapter 5

Antimicrobial photodynamic therapy on selective bacteria

5.1 Introduction

The effectiveness of PDT in bacterial inhibition is affected by differences in cell wall structure, growth rates, modes of uptake, type of bacteria, type of PS and PS accumulation site (Jori *et al.*, 2011; Jori *et al.*, 2006). Most previous research has concentrated on the use of PS as an aqueous solution (Tokubo *et al.*, 2018), nano-particles (Junqueira Garcia *et al.*, 2018; Wang *et al.*, 2018) and nano-encapsulation (Rout, Liu and Wu, 2018) to be applied in PDT. In this study, we tested a scaffold into which had been incorporated a PS as a means to deliver and release the anti-microbial agent that must then be photoinitiated to be effective in bacterial inhibition. To date, most anti-microbial scaffold research has been based on antibiotics or drug delivery (Ong *et al.*, 2018; Khorshidi and Karkhaneh, 2018; Dorati *et al.*, 2017), which might result in some antibiotic resistance and toxicity problems (Marsich *et al.*, 2013).

It is crucial to develop an anti-microbial scaffold that not only inhibits the bacterial infection but also provides support for host tissue growth. The advantage of incorporation of the PS in the scaffold is that with this combination, anti-microbial PDT can be initiated when there is infection during the tissue regeneration process. If the infection occurs after scaffold implantation, the PS within and released from the anti-microbial scaffold can be photoinitiated, thus, inducing bacteria killing. This is important to inhibit bacterial infection on the implanted scaffold as infection results in tissue break down and failure in tissue regeneration. If no infection occurs, the PS released will be discharged naturally from the body and no accumulation will take place.

5.2 Aim and objectives

5.2.1 Aim

This chapter aims to investigate the potential anti-microbial effects of the scaffold and PDT on *Enterococcus faecalis* (*E. faecalis*) (a Gram-positive bacterium) and *Escherichia coli* (*E. coli*) (a Gram-negative bacterium).

5.2.2 Objectives

The above aim will be achieved through the following objectives:

- To evaluate the antibacterial PDT effect towards *E. faecalis* and *E. coli* planktonic suspensions by measuring viable bacterial count, metabolic activity and Live/ Dead staining.
- To determine the antibacterial PDT effects of this newly developed scaffold and PDT against bacterial biofilms formed on the scaffolds.

5.3 Materials and Methods

All materials were purchased from Sigma-Aldrich, UK unless otherwise stated.

5.3.1 Brain Heart Infusion (BHI) broth

Brain heart infusion broth was prepared by dissolving 37 g of BHI powder (ThermoFisher Scientific, UK) in one litre of dH₂O. The broth was mixed well to make sure that all the powder was dissolved properly. The BHI broth was sterilised by autoclaving (Prestige Medical, UK) at 121°C for 15 min. BHI broth was used to culture *E. faecalis* bacteria in this study.

5.3.2 Luria Bertani (LB) broth

Luria Bertani broth was prepared by dissolving 25 g of LB powder (ThermoFisher Scientific, UK) in one litre of dH₂O. The media was mixed and sterilised by autoclaving (Prestige Medical, UK) at 121°C for 15 min. LB broth was used to culture *E. coli* bacteria in this study.

5.3.3 Preparation of Columbia blood agar (CBA) plates

Columbia blood agar plates for bacterial growth experiments were prepared by adding 39 g of CBA base (ThermoFisher Scientific, UK) to one litre of dH₂O. The media were dissolved completely by heating until boiling. The media were then sterilised by autoclaving (Prestige Medical, UK) at 121°C for 15 min. The media were cooled to 50°C in a water bath (SLS-Grant Instrument, UK) and 5% v/v (50 mL) sterile defibrinated horse blood (ThermoFisher Scientific, UK) was then added to the medium and gently mixed to avoid bubble formation. The media were then poured into petri dishes and left to set for 24 h. The agar was kept at 4°C until use.

5.3.4 Preparation of Mueller Hinton agar (MHA) plates

Mueller Hinton agar plates for antimicrobial and PDT experiments were prepared by dissolving 38 g of MHA powder (ThermoFisher Scientific, UK) in one litre of dH₂O. The media were dissolved completely by heating to boiling. The media were then sterilised by autoclaving (Prestige Medical, UK) at 121°C for 15 min. The media were then poured into petri dishes and left to set for 24 h. The agar was kept at 4°C until use.

5.3.5 Bacterial selection

The aerobic bacteria selected for this study were: *Enterococcus faecalis* NCTC 755 (*E. faecalis*); Gram-positive and *Escherichia coli* NCTC 11954 (*E. coli*); Gram-negative. *E. faecalis* was cultured in BHI broth and *E. coli* was cultured in LB broth. These bacteria were reported to develop resistance to a wide range of antibiotics (Cieplik *et al.*, 2018; Laxminarayan *et al.*, 2016; Renwick, Simpkin and Mossialos, 2016) thus it is important to investigate the effect of alternative approach such as PDT to eliminate infection from these bacteria. Cultures were maintained by weekly subculture on Columbia agar, supplemented with 5% defibrinated horse blood. Cultures were grown aerobically at 37°C for 24 to 48 h until used in the experiments. The bacteria used in antibacterial PDT were in both planktonic and biofilm cultures. We used ERY and MB solutions that had been released from scaffolds in order to determine the efficacy of the antibacterial PDT when PS had been integrated and released from the scaffold (Misba, Zaidi and Khan, 2017; Silva *et al.*, 2018a).

5.3.6 Gram stain protocol

Gram staining was performed to check the purity of the bacterial samples before any experiments were started (Smith and Hussey, 2005). Microscope

slides were prepared and labelled with bacterial identification. A bacterial smear was then prepared from the bacterial broth. Heat fixing was applied by passing the microscopic slides through a Bunsen burner flame 2 or 3 times. Crystal violet was then added to the slides for 1 minute before rinsing with tap water. The Gram's iodine was applied for 1 minute before rinsed again with tap water. The slides were decolourised using 95 % ethyl alcohol before rinsed with tap water. Safranin O was added to counterstain for 1 minute before rinsing with tap water. The microscope slides were then blotted dry with clean tissue paper and viewed under the light microscope.

5.3.7 Standard curve of optical density (OD) versus colony-forming unit (CFU) for each bacterial strain

From the freezer stock (-80°C), the bacterial suspensions of either *E. faecalis* or *E. coli* were thawed to room temperature. One loop of bacterial suspension was streaked on the CBA and left to grow for 24 h for *E. coli* and 48 h for *E. faecalis*. Then, one loop of bacterial colonies was inoculated into 10 mL fresh BHI or LB broth. The new bacterial suspension was incubated overnight at 37°C with 10% CO_2 . 1 mL of bacterial suspension was then added to 9 mL fresh BHI or LB broth and the initial optical density ($\text{OD}_{600\text{nm}}$) was measured using a spectrophotometer. Serial dilutions were made from that suspension: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512. 1 mL of each dilution was added to cuvette and the optical density was measured using a spectrophotometer (Jenway 6305) at a wavelength of 600 nm. The optical density reading was then corrected with the $\text{OD}_{600\text{nm}}$ of BHI or LB broth without inoculated bacteria (blank).

1 mL of the same overnight bacterial culture was added to 9 mL of fresh BHI or LB broth. Further serial dilutions were made from each bacterial suspension for colony-forming unit (CFU) counting: 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} . From each dilution, 100 μL was pipetted and spread on MHA plates (as prepared in section 5.3.4). The agar plates were incubated for 24 h for *E. coli* and 48 h for *E. faecalis* at 37°C with 10% CO_2 in the air before colony counting (Stuart SC6). The optical density reading was then plotted against CFU counting to get the standard curve for each bacterial strain.

5.3.8 Preparation of photosensitisers

ERY and MB were prepared as 1 mg/mL stock solutions in BHI or LB broth and filtered using 0.22 μm filter (Merck Milipore, UK). The stock solution was covered with foil and kept at 4°C. The stock solutions were diluted with BHI or LB to the desired concentration when needed; 1%E = 1.09 μM ERY solution; 5%E = 56.37 μM ERY; 1%M = 6.91 μM MB; and 5%M = 67.93 μM MB. These concentrations are the same concentrations used in PDT with hDPSCs (Chapter 4, section 4.3.2.1).

5.3.9 Photodynamic therapy on planktonic bacterial cultures

The bacterial suspensions were inoculated with one inoculation loop from daily culture onto CBA plates and incubated for 24 h at 37°C with 10% CO₂ in the air. After 24 h, the bacteria were inoculated into 10 mL of fresh BHI or LB broth using one sterile inoculation loop, vortexed and incubated at 37°C with 10% CO₂ in the air. The bacterial suspensions were grown for a further 24 h before being diluted 1:10 dilution in fresh BHI or LB broth. The new bacterial suspensions were allowed to grow until they reached a mid-exponential phase in a shaking incubator (New Brunswick 4450) (200 rpm) at 37°C. The optical density at 600 nm (OD_{600nm}) was recorded at various time points until the early-log phase was reached (as determined on species-specific temporal proliferation curves). After the bacterial suspension reached mid-exponential phase, it was centrifuged (Netheler-Hinz, Germany) (4000 r.p.m/ 4 min) and the BHI or LB broth was discarded. Bacterial pellets were resuspended with PS supplemented media prepared in section 5.3.8 as the following: 1%E group samples were incubated with 1.09 μM ERY solution; 5%E group were incubated with 56.37 μM ERY; 1%M group were incubated with 6.91 μM MB;

and 5%M group were incubated with 67.93 μ M MB. The PS concentrations used were equivalent to the average PS released from the fabricated scaffolds (PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M) in basal medium (α -MEM containing 10 % FCS, 2 mM L-glutamine and 100 units/mL penicillin/100 μ g/mL streptomycin) as used in tissue culture conditions. All samples were covered with foil and incubated with their respective broth and PS for 30 min in a shaking incubator (200 rpm) at 37°C. The samples were then centrifuged (4000 r.p.m/ 4 min) and bacterial pellets resuspended with ¼ strength Ringer solution (RS) to remove any unbound PS. All samples were centrifuged (4000 r.p.m/ 4 min) again to remove any residual PS and resuspended again in ¼ strength RS. 1 mL from each sample was then added to each well of a 24-well plate and irradiated with light of the appropriate wavelength for 60 min. Control group samples for each species consisted of cultures exposed to PS alone, light alone or neither PS nor light. After irradiation, all samples were covered with foil at room temperature for a 30 min recovery period to avoid any light.

Bacterial samples were diluted by 10-fold serial dilutions in ¼ strength RS and 100 μ L of each dilution was spread on MHA plates for colony counting. After 24 h (*E. coli*) and 48 h (*E. faecalis*) incubation at 37°C with 10% CO₂, agar plates with 30 to 300 colonies were selected for counting. Bacterial samples were also stained using the Live/ Dead BacLight Bacterial Viability kit for qualitative evaluation as explained in section 5.3.11. The summary of the protocol is shown in Fig. 5.1.

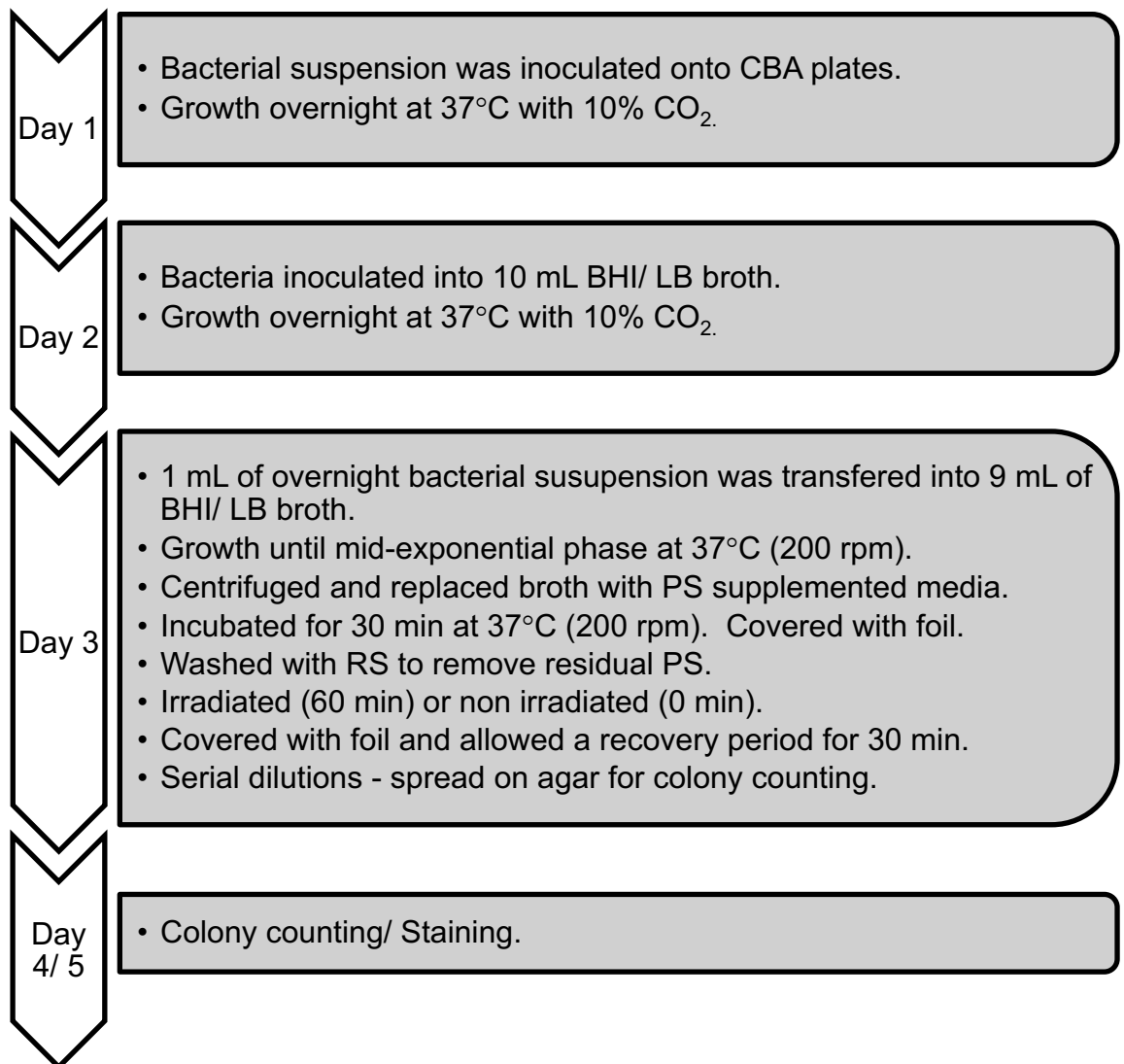


Figure 5.1 Protocol for the PDT of planktonic cultures of bacteria.

5.3.10 Photodynamic therapy of biofilms on the fabricated scaffold

Five scaffold groups were tested in the antimicrobial assay: PGA, PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M. 1 cm sample of each scaffold were prepared using a cork borer and sterilised under UV light in the fume hood for 20 min before being placed in a 48-well plate.

The bacterial suspension was prepared by inoculating one inoculation loop onto CBA plates and incubating for 24 h at 37°C with 10% CO₂. After 24 h, the bacteria were inoculated into fresh 10 mL BHI or LB broth using one or two sterile inoculated loops, vortexed and incubated at 37°C with 10% CO₂. The bacterial suspension was further grown for 24 h before further dilution (1:10) in new fresh BHI or LB broth.

The inoculum of the bacterial suspension was prepared by adjusting the bacterial culture to 1×10^6 colony forming units (CFUs)/ mL. A 1 mL aliquot containing 1×10^6 CFUs/ mL was added to each scaffold and incubated overnight at 37°C with 10% CO₂ in the air. All plates were covered with aluminium foil. After 24 h, the scaffolds were washed twice with $\frac{1}{4}$ strength RS to remove any unbound bacteria. All scaffolds were transferred to a new plate that contained $\frac{1}{4}$ strength RS and irradiated for 60 min at room temperature. Scaffolds with no irradiation were used as the control group. After irradiation, all scaffolds were kept in the dark at room temperature for 30 min as a recovery period.

The adherent bacteria on the scaffolds were removed by ultrasonication (Advantage-Lab) at 37 kHz with a power output of 30 W for 10 min at 37°C. The solutions were collected and diluted by 10-fold serial dilutions in $\frac{1}{4}$

strength RS. 100 μ L from each dilution was then spread on MHA for colony counting. After 24 h (*E. coli*) and 48 h (*E. faecalis*) incubation at 37°C with 10% CO₂, agar plates with 30 to 300 colonies were selected for counting.

Scaffolds were also processed for qualitative evaluation of biofilm via SEM and also stained using the Live/Dead® BacLight Bacterial Viability kit (see section 5.3.11 and 5.3.12). The summary of the protocol is shown in Fig. 5.2.

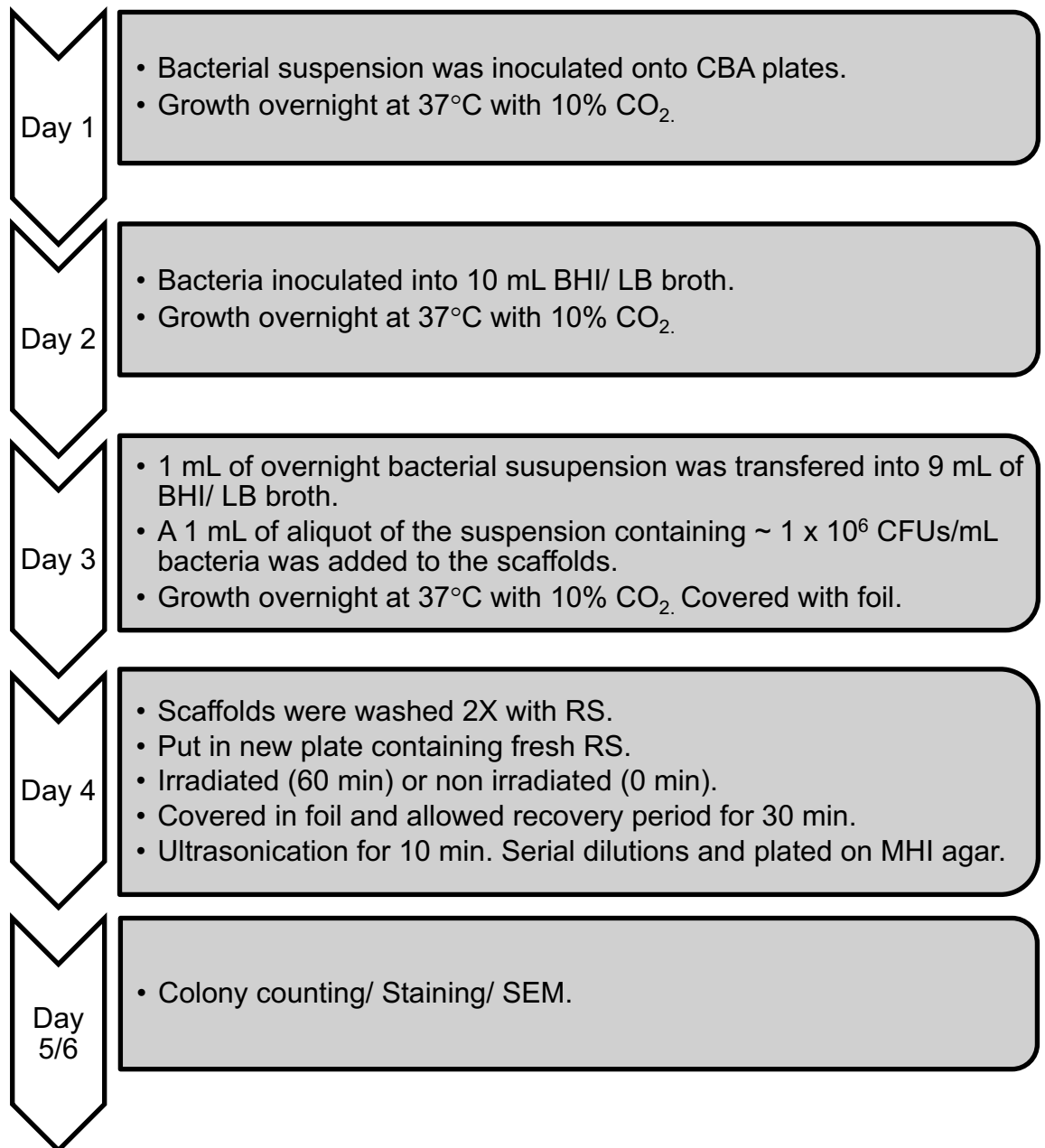


Figure 5.2 Protocol for the PDT of biofilms on fabricated scaffolds.

5.3.11 LIVE/DEAD® *BacLight*™ Bacterial Viability kits

The kit consisted of SYTO® 9 dye (Component A), Propidium iodide (Component B) and *BacLight* mounting oil (Component C). The kit provides a two-colour fluorescence assay for bacterial viability. SYTO® 9 is a green fluorescent dye and propidium iodide is red fluorescent dye; both dyes will stain nucleic acid in a bacterial cell. SYTO® 9 dye is permeable to healthy and live cells. However, Propidium iodide is only permeable to dead bacteria with compromised membranes. A 2X stock solution of the LIVE/DEAD *BacLight* staining reagent mixture was prepared in 5 mL of filter sterilised dH₂O. An equal volume of the 2X stock solution was then added to the bacterial sample or bacterial-scaffold sample. The final concentration of each dye was 6 µM SYTO 9 stain and 30 µM propidium iodide as stated by the kit instruction. After thorough mixing, all samples were covered with foil and incubated for 15 min at room temperature. For the bacterial sample, 5 µL of the stained bacterial sample was trapped between the slide and a coverslip and observed under the fluorescence microscope. For bacterial-scaffold samples, the scaffold was transferred onto the slide and covered with a coverslip and observed using the CLSM using FITC filter for live bacteria (Excitation/ Emission = 480 nm/ 500 nm) and Texas Red® filter (Excitation/ Emission = 490 nm/ 635 nm).

5.3.12 Scanning electron microscopy evaluation

The bacterial-scaffold samples were dehydrated through a series of ethanol concentrations and prepared as described in Chapter 3 section 3.3.6.1. A series of images were captured at a magnification of 100x and 1000x.

5.3.13 Statistical analysis

All experiments were carried out in triplicate and each experiment was repeated 3 times unless stated otherwise (n=9). Statistical analyses were carried out using IBM SPSS Statistics Version 23 software. Results were analysed using Students' t-test and one way analysis of variance (ANOVA) tests. P-values were determined, and $P < 0.05$ was considered significant. All results were presented as mean $\pm$ standard error of mean (S.E.M) unless stated otherwise.

5.4 Results

Assessment of the PDT effects on two bacterial species that were cultured on either planktonic suspensions or scaffolds (PGA, PGA-E (1% and 5%), PGA-M (1% and 5%)) was performed by measuring the cell metabolic activity level, viable bacterial count and through microscopy evaluation. All of the experiments were performed after bacteria were cultured with broth (LB or BHI) for 24 h.

5.4.1 Anti-microbial photodynamic therapy on planktonic cultures

5.4.1.1 Effect of photodynamic therapy on bacterial viable counts

The photodynamic efficiency of MB and ERY as antimicrobials were studied by determining reductions in viable counts, expressed as CFU per mL, of bacteria in suspensions exposed to two percentages of each PS. Figure 5.3 presents the PDT effect of ERY and MB on suspensions of *E. faecalis* (Figure 5.3 (a)) and *E. coli* (Figure 5.3 (b)).

It can be seen that visible light irradiation of controls without PS did not affect the viability of either bacterial species. The dark control PS groups for *E. coli* that received no light irradiation also showed no dark toxicity (Figure 5.3 (b)). A similar trend was seen for dark control PS groups of *E. faecalis* with the exception of the 5%M group which showed dark toxicity shown by a 1-log (90% reduction) difference compared to no PS dark control (Fig. 5.3 (a)).

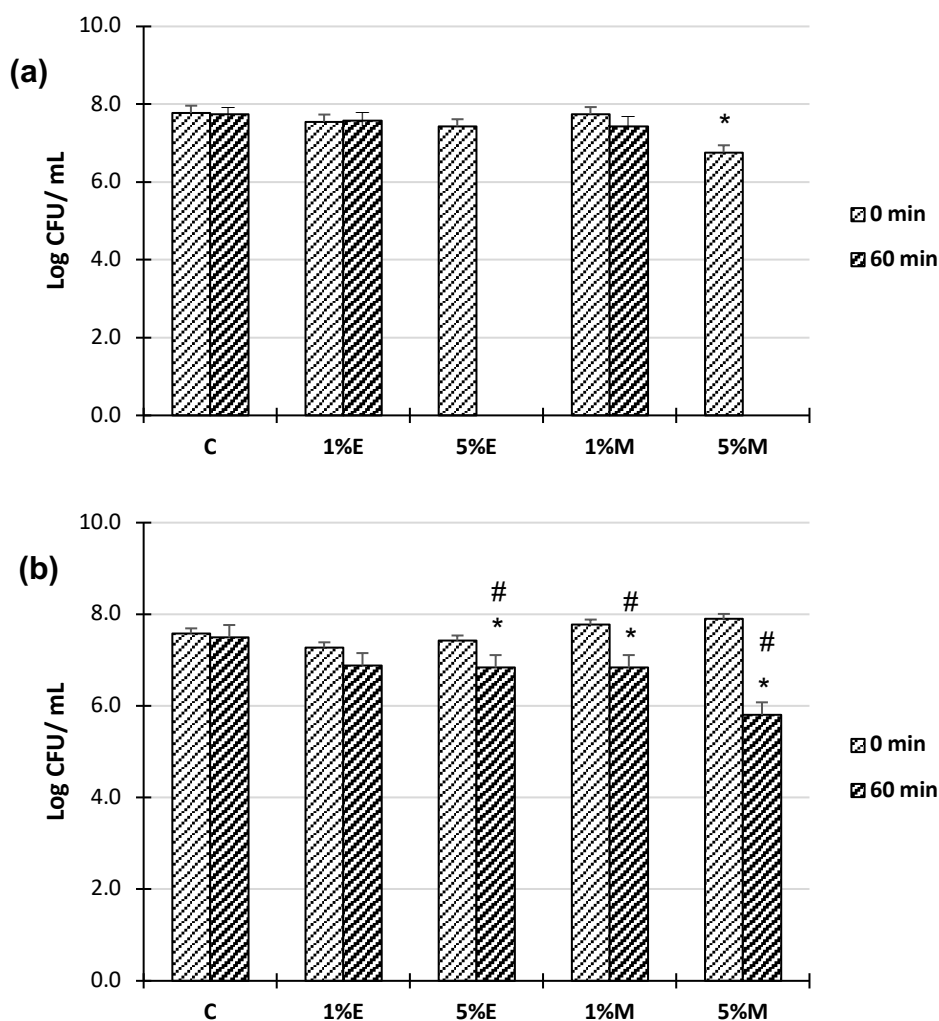


Figure 5.3 The survival of bacterial cells based on calculation of log CFU/ mL.

Cells suspensions and controls with no PS (C) were irradiated for 0 min (dark controls) or 60 min after incubation with 1%E = 1.09 μ M; 5%E = 56.37 μ M; 1%M = 6.91 μ M; 5%M = 67.93 μ M PS (M = MB or E = ERY). The graphs represent the log CFU/ mL of bacteria after incubation with PS for 30 min and irradiated with visible light for 0- or 60-min. Data are represented as the mean of log CFU/ mL of bacteria replicates (n=9) $\pm$ S.E in each group.

(a) *E. faecalis* (NCTC 755) bacteria

(b) *E. coli* (NCTC 11954) bacteria

* Indicates a significant difference compared to Ctrl (p<0.05).

Indicates a significant difference compared to non-irradiated PS group (p<0.05).

The complete photoinactivation (no viable counts) (more than 99.9999% reduction) of *E. faecalis* was observed after exposure to high percentages (5%) of both ERY and MB and irradiation, whereas there was no PDT effect relative to the non-irradiated samples when low percentages (1%) of PS were used. However, no statistical value could be derived as CFU/mL of the *E. faecalis* bacteria did not form colonies on the agar plates.

When the assays were carried out with *E. coli*, the combination of MB and light irradiation reduced the viable bacterial count compared to the non-irradiated group; with 1%M the reduction was 1-log (90% reduction) and with 5%M it was 2-log (99% reduction). However, 1%E did not show any significant inhibitory effect toward *E. coli*. Bacterial cells in 5%E did show a significant reduction when compared to the control (C) and non-irradiated groups. There was no significant difference between control (C) and the bacterial cells incubated with PS and no light irradiation (dark control).

5.4.1.3 Effect of PDT on bacterial metabolic activity

Cell metabolic activity of the bacteria was tested using the resazurin based reagent, Presto Blue as described in Chapter 3 section 3.3.4. Viable cells reduce resazurin to resorufin, a red fluorescent substance that can be detected spectrophotometrically. The fluorescence measurement was expressed as relative fluorescence unit (r.f.u).

Figure 5.4 (a) shows that the irradiation of *E. faecalis* suspensions after incubation with higher concentrations of PS (5%E and 5%M) resulted in low detection of bacterial viability or activity (5%E = 10 r.f.u., 5%M = 9 r.f.u). There was a significant reduction in the metabolic activity of *E. faecalis* after incubation with the high concentration of MB (5%) even when the suspension received no irradiation (dark control group). When incubated with low concentrations of PS, the metabolic activity of *E. faecalis* were 74 r.f.u and 70 r.f.u for 1%E and 1%M, respectively after irradiation.

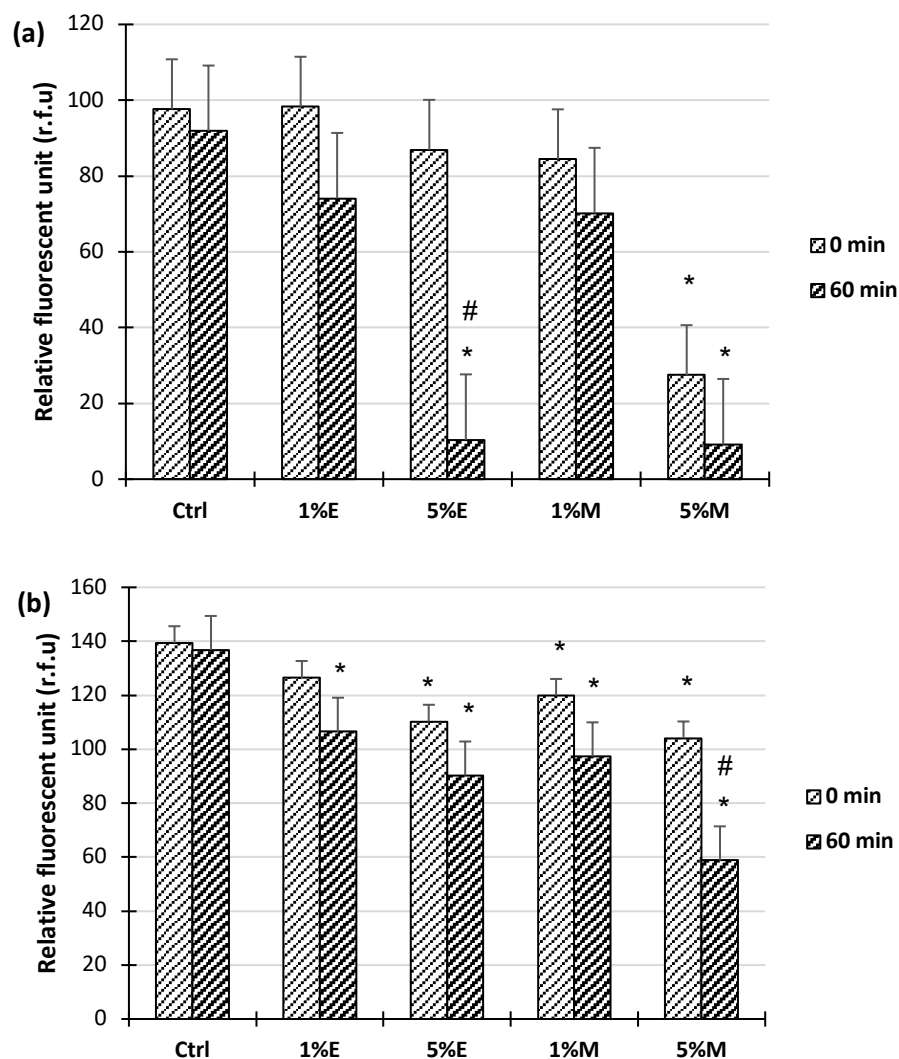


Figure 5.4 Fluorescence measurement for bacterial metabolic activity after treatment with PDT for 0 min and 60 min.

Cells suspensions with PS were irradiated for 0 min (dark controls) or 60 min after incubation with 1%E = 1.09 μ M; 5%E = 56.37 μ M; 1%M = 6.91 μ M; 5%M = 67.93 μ M PS (M = MB or E = ERY). The control group (Ctrl) which contain no PS was also included. The graphs represented the bacterial metabolic activity after incubation with PS for 30 min and irradiated with visible light for 0- or 60-min. Data are represented as the mean of r.f.u of bacterial replicates ($n=9$) $\pm$ S.E in each group.

(a) *E. faecalis* (NCTC 755) bacteria

(b) *E. coli* (NCTC 11954) bacteria

* Indicates a significant difference compared to Ctrl ($p<0.05$).

Indicates a significant difference compared to 0 min (between the same PS concentration group) ($p<0.05$).

Figure 5.4 (b) shows that both PS used in this assay did show some dark toxicity as evidenced by a reduction in cell metabolic activity even when present at low concentration (the fluorescent value for *E. coli* in the dark control PS group was 126 for 1%E; 110 for 5%E; 120 for 1%M; and 104 for 5%M). As irradiation was applied, all groups showed a more significant reduction in bacterial metabolic activity compared to the control: 106 for 1%E; 90 for 5%E; 97 for 1%M; and 59 for 5%M. However, only cells that were incubated with 5%MB showed a significant reduction after the light was applied when compared to the non-irradiated group.

5.4.2 Anti-microbial photodynamic therapy on biofilm using the scaffold incorporated with the photosensitiser

5.4.2.1 Effect of PDT on bacterial viable counts

The photodynamic efficiency of MB and ERY (incorporated within the scaffold) as antimicrobials were studied. Fabricated scaffolds (PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M) were inoculated with *E. faecalis* or *E. coli* and biofilms were allowed to form for 24 h. The viable counts (CFU/ mL) of bacteria recovered from scaffolds that had been irradiated and non-irradiated controls were calculated and are presented in Figure 5.5. Bacterial viability was not affected by the scaffold without PS (PGA) after irradiation. The dark control groups for scaffolds incorporated within PS also exhibited no dark toxicity against either bacterial species. In the experiment with *E. faecalis* (Figure 5.5 (a)), more groups demonstrated statistically significant reductions compared to the non-irradiated groups; PGA-1%E, PGA-5%E and PGA-5%M exhibited reductions of 1.8 log (more than 90% reduction), 2.3 log (more than 99% reduction) and 2.5 log (more than 99% reduction), respectively. Meanwhile, the PGA and PGA-1%M groups showed no significant reduction.

In the experiment with *E. coli* (Figure 5.5 (b)), only the PGA-5%M group showed a statistically significant reduction (2.5 log) (more than 99% reduction) relative to the non-irradiated group. The other groups, PGA-1%E, PGA-5%E and PGA-1%M, showed no significant reduction even though some reduction was observed especially for PGA-5%E scaffold.

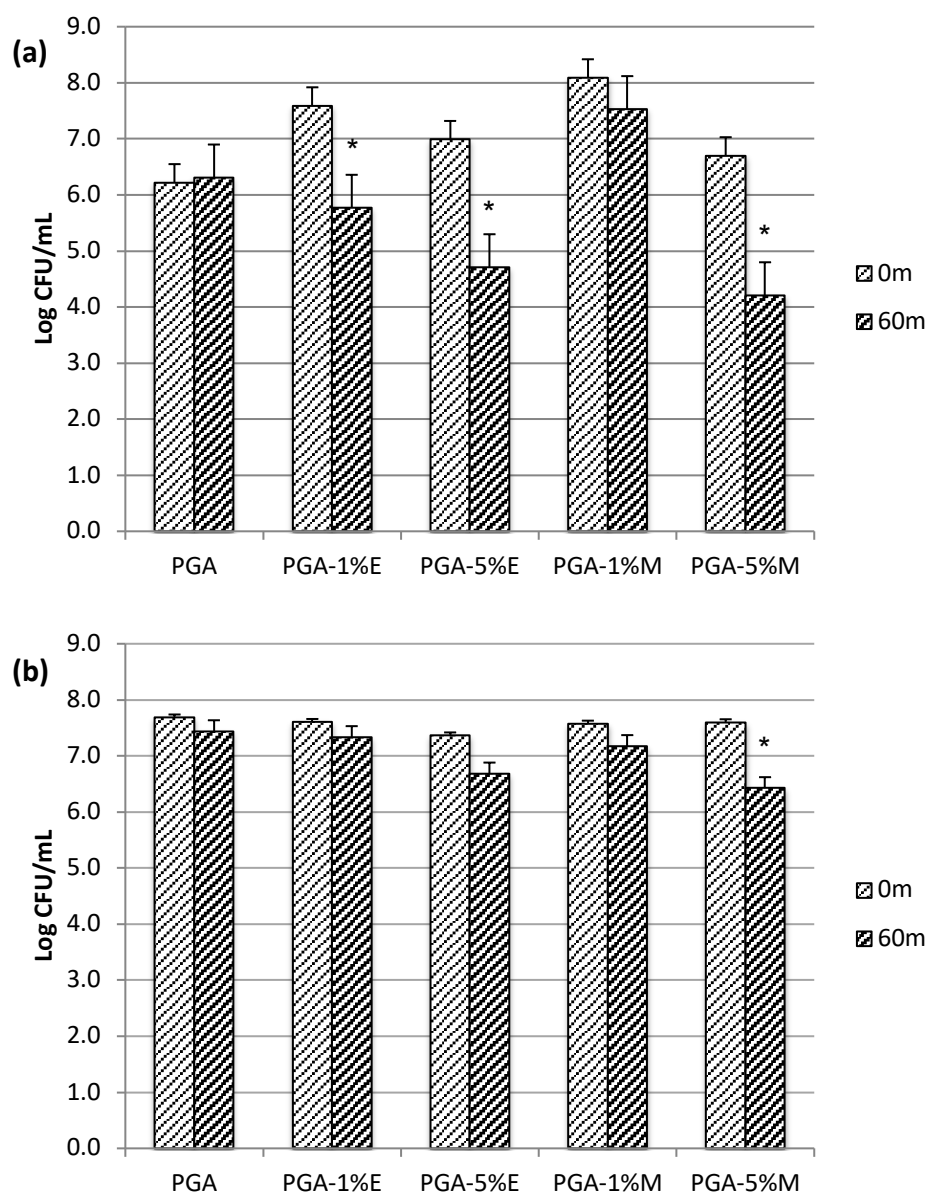


Figure 5.5 The survival of bacterial cells recovered from the scaffolds based on the calculation of log CFU per mL.

Cells on these scaffolds (PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M) and controls with no PS (PGA) were irradiated for 0 min (dark controls) or 60 min after 24 h in culture. The graphs represent the log CFU/ mL of bacteria after inoculation on scaffolds for 24 h and irradiation with visible light for 0- or 60-min. Data are represented as the mean of log CFU/ mL ($n=9$) $\pm$ S.E in each group.

(a) *E. faecalis* (NCTC 755) bacteria

(b) *E. coli* (NCTC 11954) bacteria

* Indicates a significant difference compared to 0 min (between same PS concentration group) ($p < 0.05$).

5.4.2.2 Confocal microscopy evaluation of the effect of photodynamic therapy on bacterial cell viability:

The confocal laser scanning microscope (CLSM) was used to examine bacteria stained with LIVE/DEAD *BacLight* staining kit (SYTO9/ PI) (section 5.3.11). The inactivation of the bacteria on the scaffold with incorporated PS, when irradiated with visible light, can be seen in Figures 5.6 and 5.7 for *E. faecalis* and *E. coli*, respectively.

It can be seen that the biofilm on the PGA only scaffold was not affected by irradiation for both selected bacteria (*E. faecalis* or *E. coli*). The difference between the irradiated groups and non-irradiated groups are evident for PGA-1%E, PGA-5%E and PGA-5%M for *E. faecalis* as fewer cells were stained green indicating more cell death after irradiation.

Furthermore, based on microscopy images of *E. coli*, irradiated groups showed less green staining relative to non-irradiated groups for all scaffolds incorporated with PS.

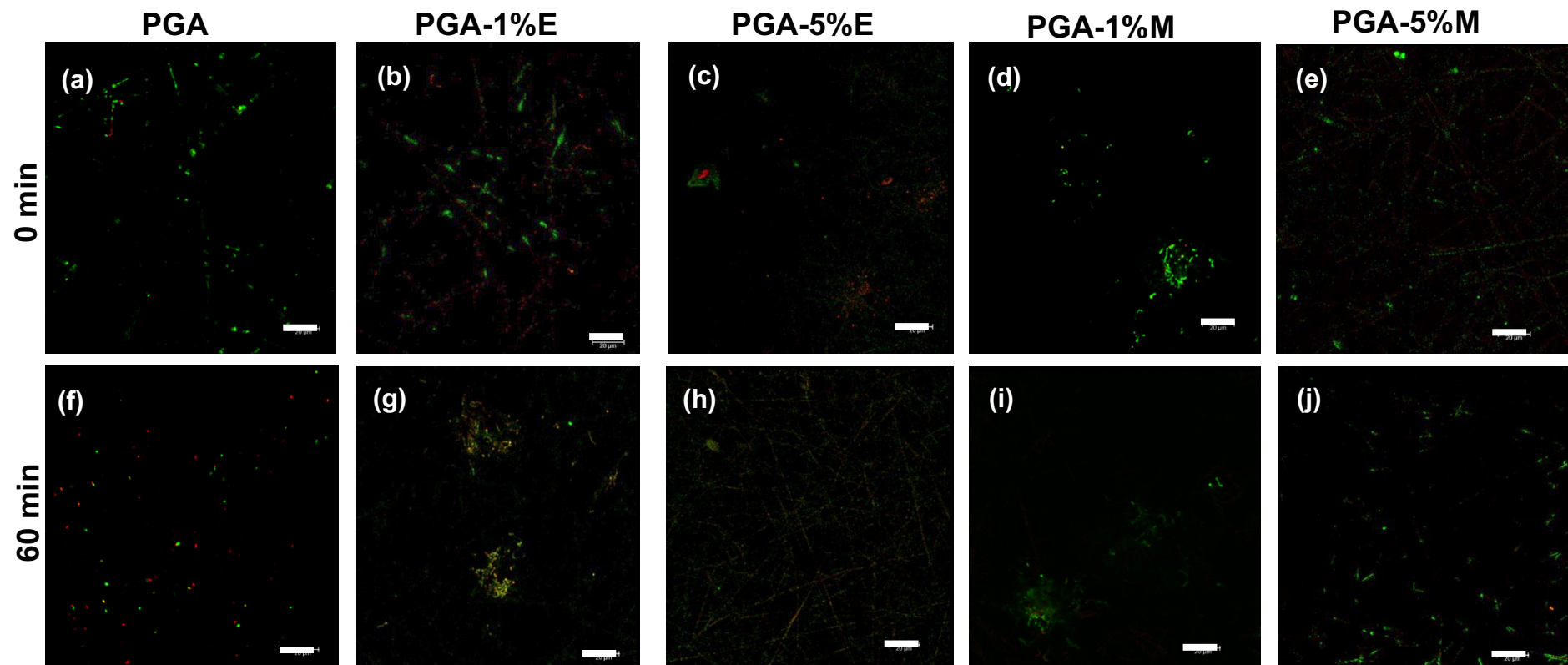


Figure 5.6 Representative images showing the viability of *E. faecalis*. The results of CLSM observation after Live/Dead staining of *E. faecalis* on the scaffolds and treated with PDT for 0 and 60 min. PGA (control) (a and f), PGA-1%E (b and g), PGA-5%E (c and h), PGA-1%M (d and i) and PGA-5%M (e and j) groups. Live cell (green), dead cell (red). Scale bars are at 20 μm.

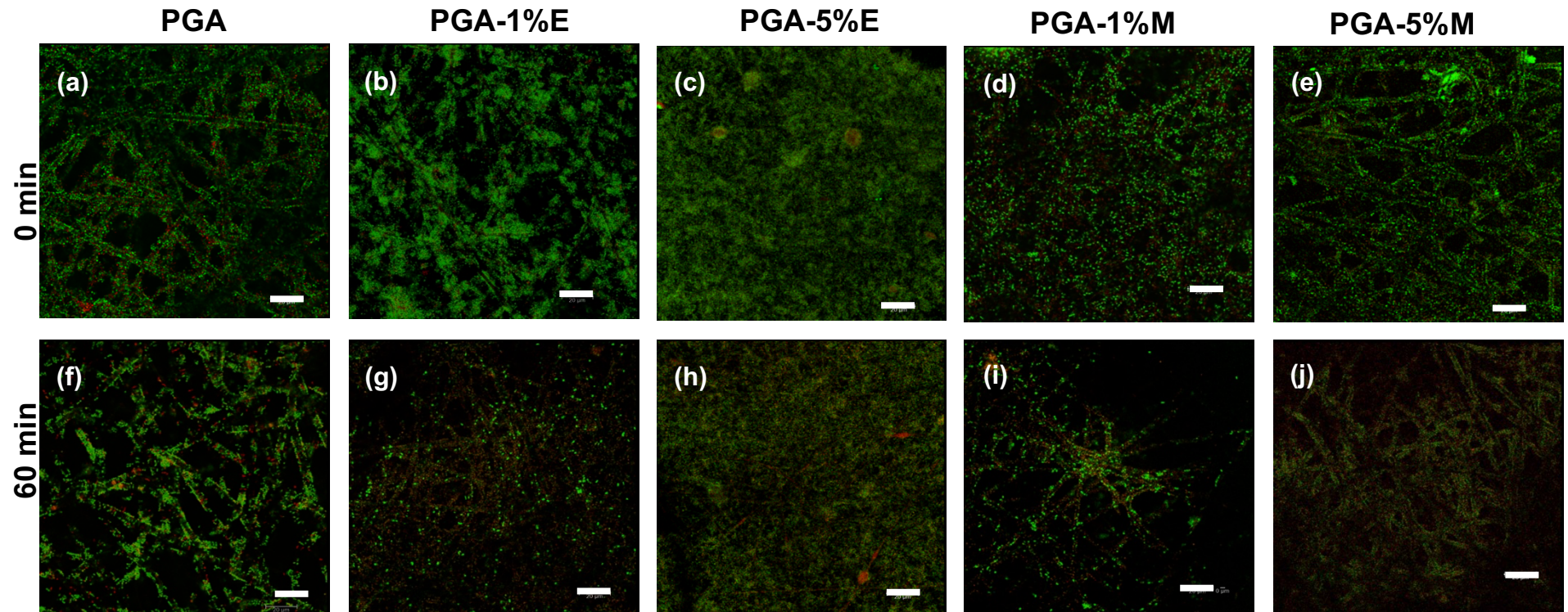


Figure 5.7 Representative images showing the viability of *E. coli*. The results of CLSM observation after LIVE/DEAD staining of *E. coli* on the scaffolds and treated with PDT for 0 and 60 min. PGA (control) (a and f), PGA-1%E (b and g), PGA-5%E (c and h), PGA-1%M (d and i) and PGA-5%M (e and j) groups. Live cell (green), dead cell (red). Scale bars are at 20 μm .

5.4.2.3 Effect of PDT on bacterial metabolic activity:

Figure 5.8 (a) and (b) display the photoinactivation of *E. faecalis* and *E. coli* biofilms on the scaffold, respectively. The results showed that the relative fluorescent units detected for all groups tested was variable. As expected, visible light irradiation without PS did not affect the cell metabolic activity, thus indicating that the viability of both bacterial species on PGA scaffold (control group) was unaffected by light irradiation. Significant reductions in cell metabolic activity were seen in PGA-1%E, PGA-5%E and PGA-5%M irradiated groups for both species of bacteria relative to the non-irradiated group. Conversely, the PGA-1%M irradiated group showed no reduction in metabolic activity relative to the non-irradiated group for both types of bacteria.

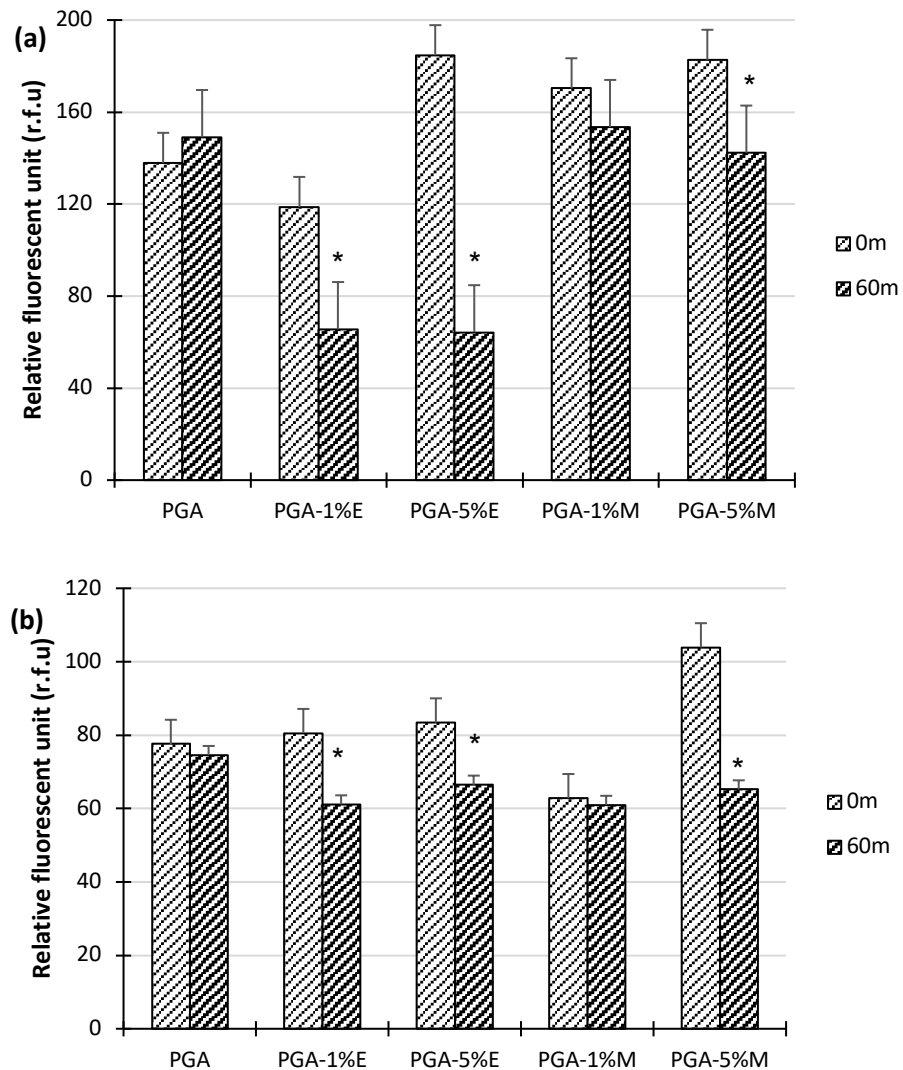


Figure 5.8 Fluorescent signal measurement of bacterial metabolic activity on the scaffolds after treatment with PDT for 0 min and 60 min.

Cells on these scaffolds (PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M) and controls with no PS (PGA) were irradiated for 0 min (dark controls) or 60 min after 24 h in culture. The graphs represented bacteria metabolic activity on the scaffold after incubated with PS for 24 h and irradiated with visible light for 0- or 60-min. Data are represented as the mean r.f.u of bacteria replicates (n=9) $\pm$ S.E in each group.

(a) *E. faecalis* (NCTC 755) bacteria

(b) *E. coli* (NCTC 11954) bacteria

* Indicates a significant difference compared to 0 min (between same PS concentration group) (p<0.05).

5.4.2.4 Scanning electron microscopy evaluation after the photodynamic therapy on bacterial cell viability

SEM imaging revealed a significant amount of bacterial biofilm formation 24 h after inoculation of scaffolds with *E. faecalis* (Figure 5.9 and 5.10) or *E. coli* (Figure 5.11 and 5.12). All scaffolds demonstrated the presence of bacteria on the surface and adhered to the scaffold fibres. Therefore, the SEM images indicated that both of the bacterial species were able to attach to the scaffold after inoculation. After 24 h incubation, the irradiated group was exposed to visible light for 60 min. SEM images show that light irradiation for 60 min in the presence of PS from the scaffold reduced qualitatively the numbers of *E. faecalis* bacteria attached to scaffolds incorporated with a high concentration of PS. The most prominent reduction of *E. faecalis* bacteria was seen on PGA-5%E and PGA-5%M scaffolds that received 60 min irradiation compared to the same scaffold that received no light irradiation (Fig. 5.9 and 5.10 (h and j)). Most of the bacteria were found as single cells or in short chain arrangements.

E. coli showed some resistance to PDT, although fewer cells were attached on PGA-1%M and PGA-5%M scaffolds when irradiated with visible light for 60 min. Most of the bacteria were present as individual cells and less aggregation was observed on PGA-1%M and PGA-5%M when comparing irradiated with non-irradiated scaffolds (Fig. 5.11 and 5.12 (i-j)). *E. coli* bacteria on other scaffolds (PGA-1%E and PGA-5%E) showed fewer bacterial aggregation when irradiated for 60 min (Fig. 5.12 g-h).

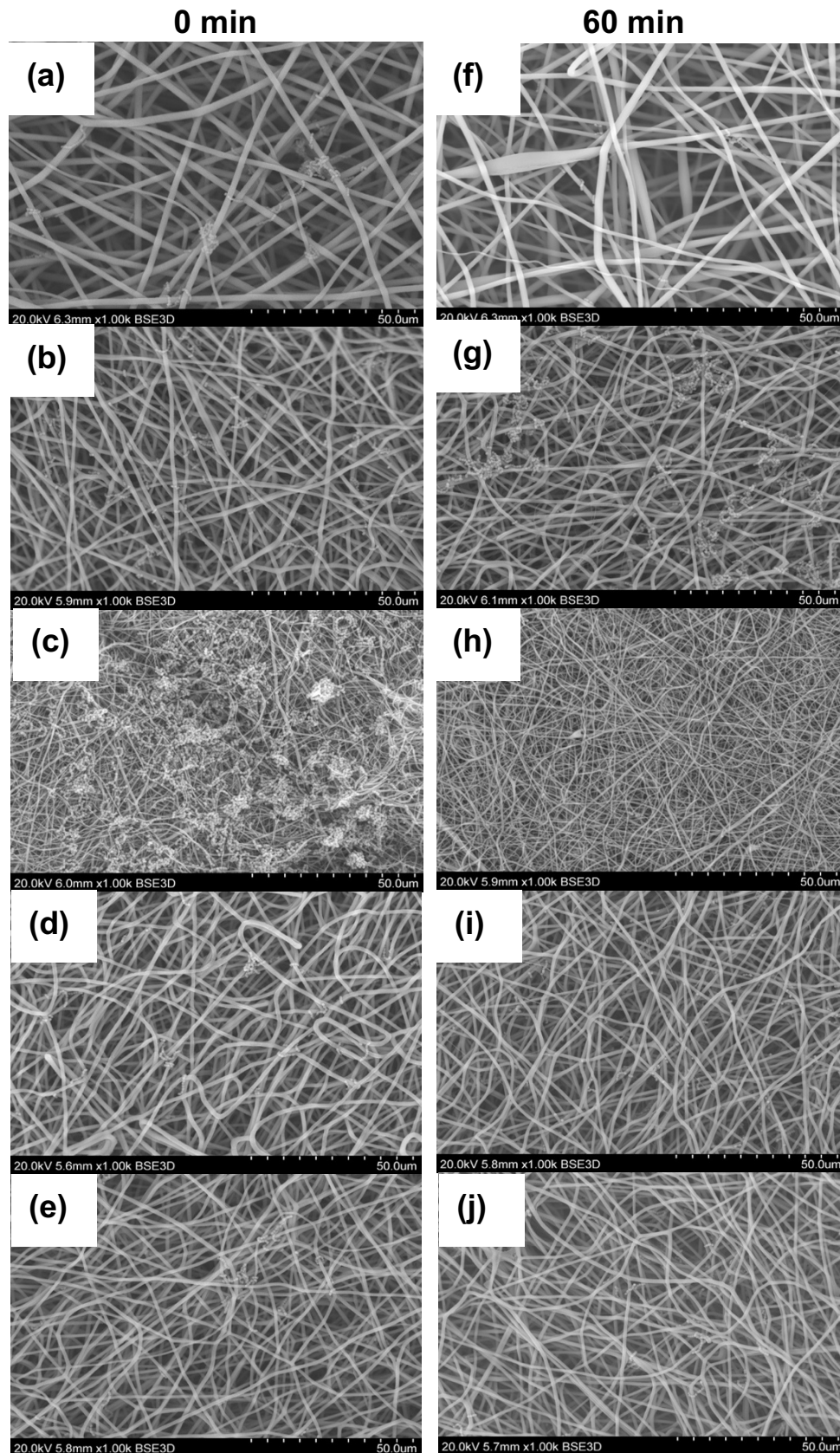


Figure 5.9 Representative SEM images of *E. faecalis* on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated control; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M (e). Scale is 50 μ m.

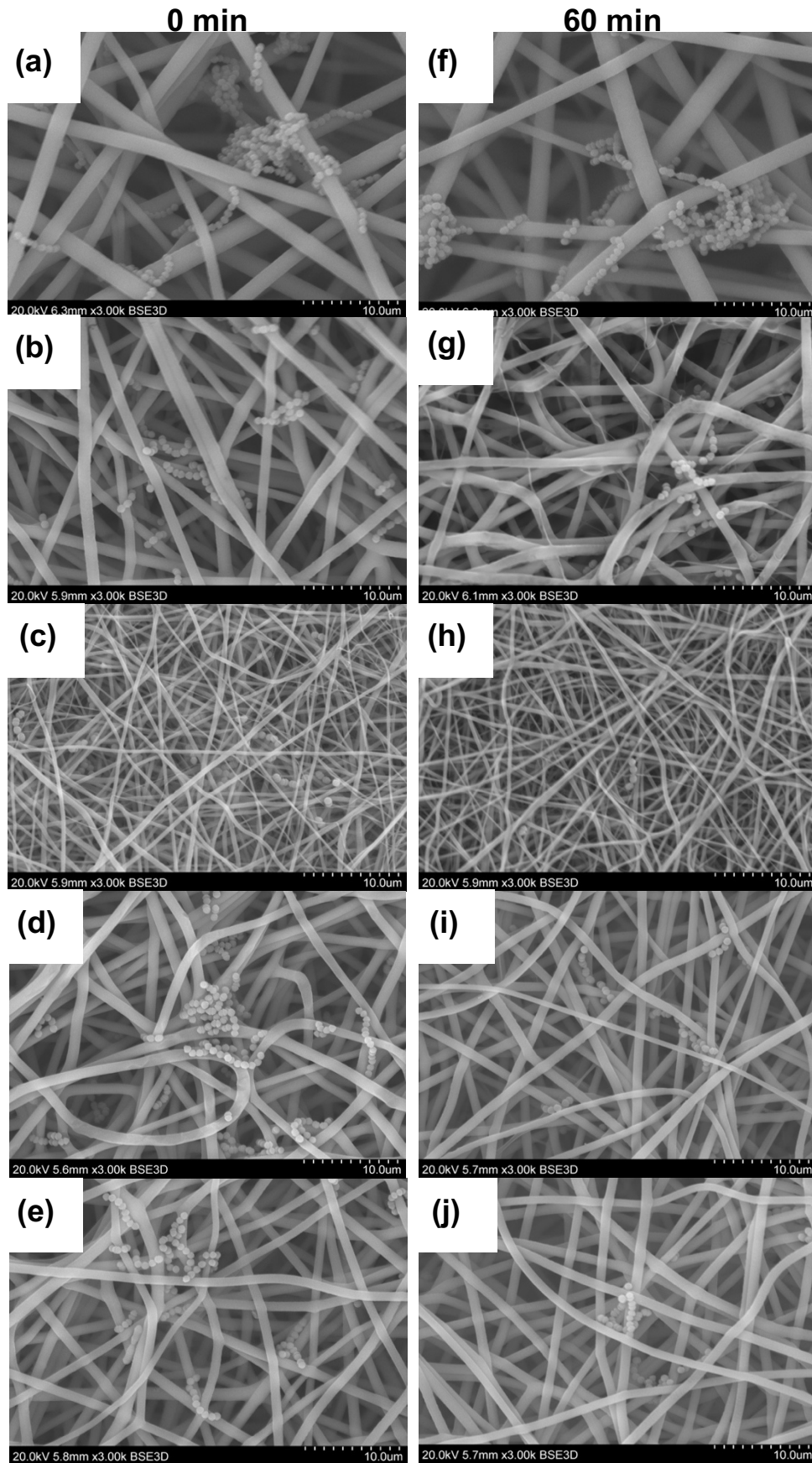


Figure 5.10 Representative SEM images in higher magnification of *E. faecalis* on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated control; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M (e). Scale is 10 μ m.

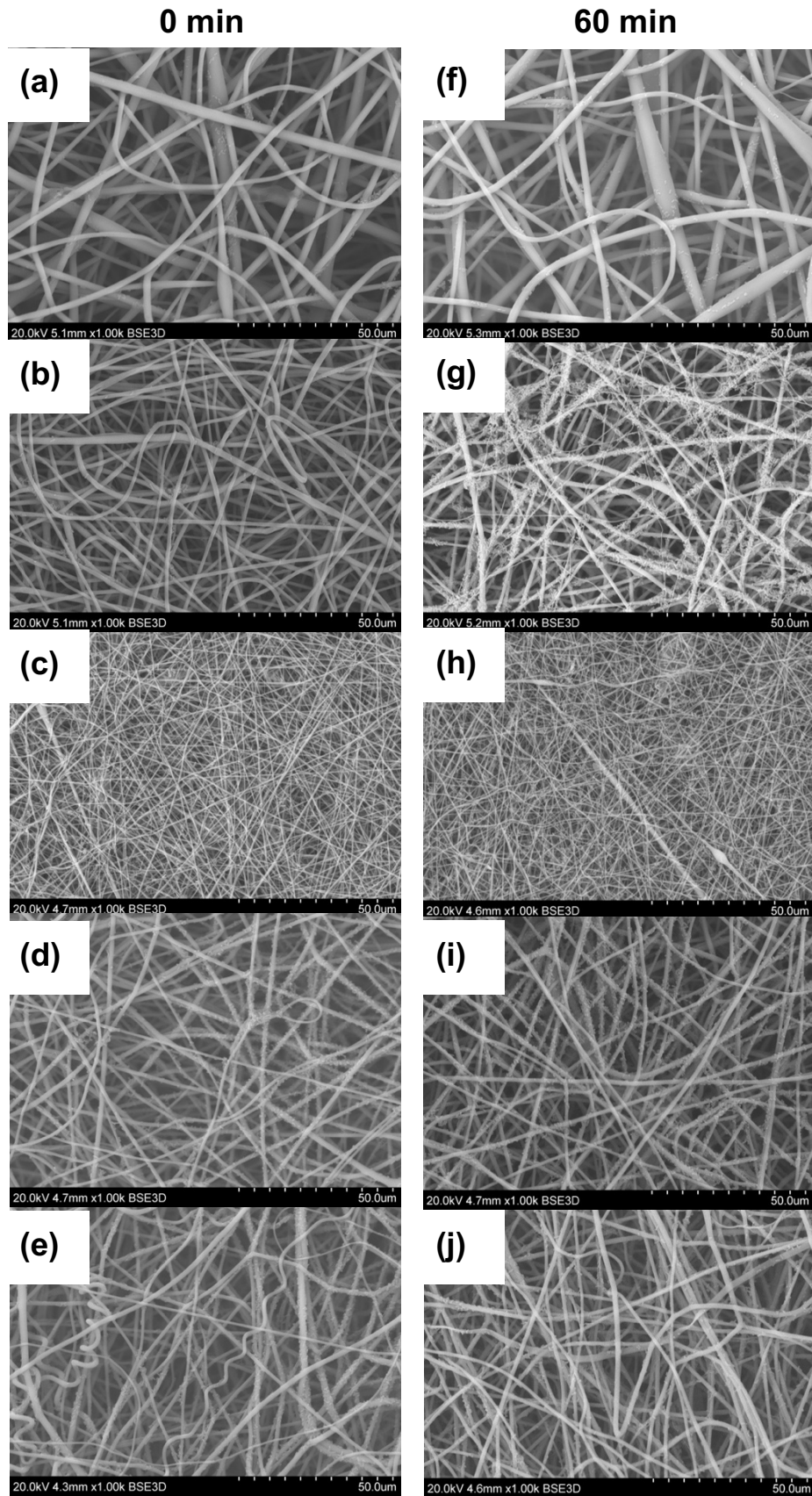


Figure 5.11 Representative SEM images of *E. coli* on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated group; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M (e). Scale is 50 μm .

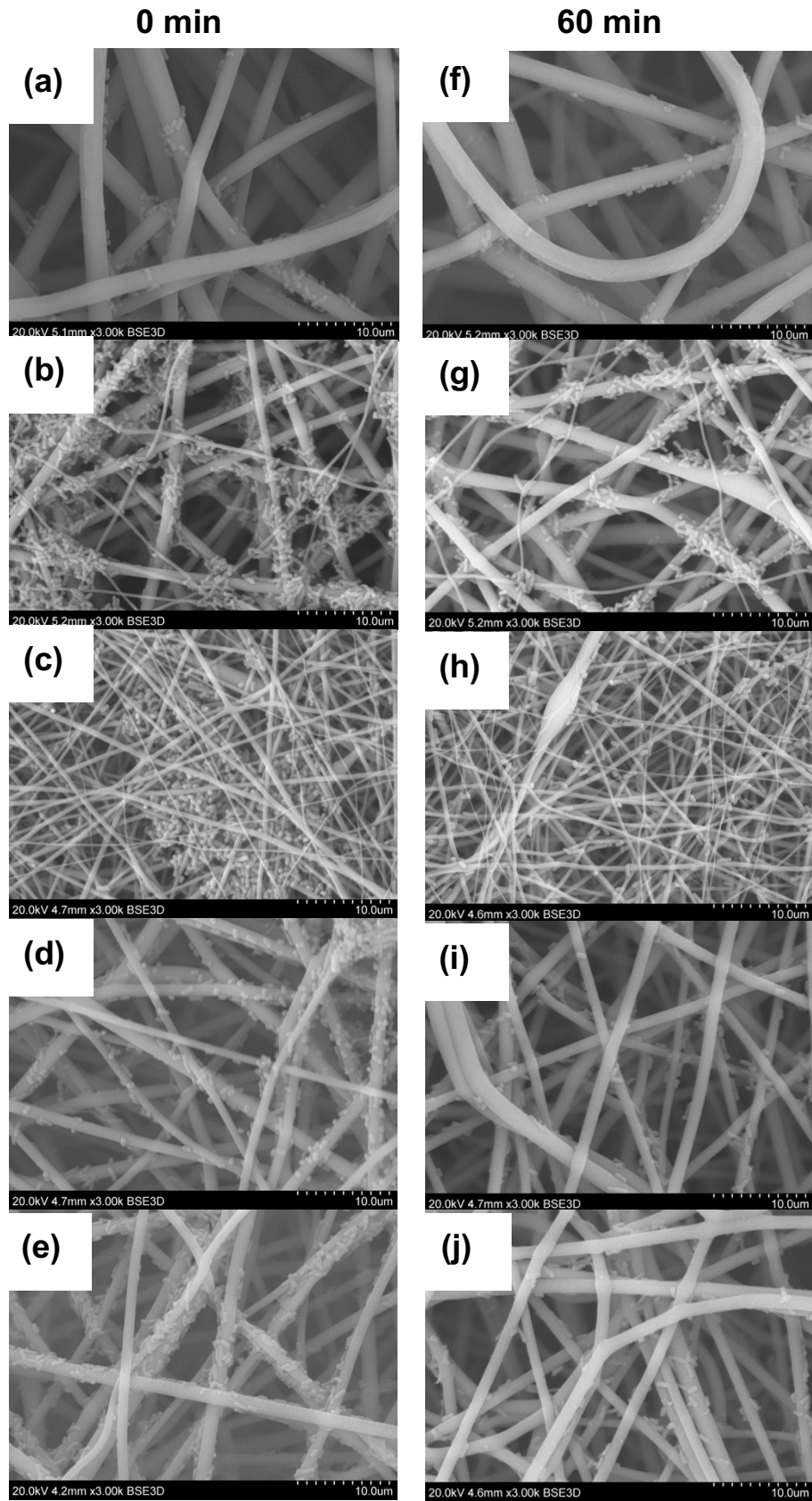


Figure 5.12 Representative SEM images in higher magnification of *E. coli* on PGA (f), PGA-1%E (g), PGA-5%E (h), PGA-1%M (i) and PGA-5%M (j) scaffolds after treated with PDT for 60 min. Non-irradiated group; PGA (a), PGA-1%E (b), PGA-5%E (c), PGA-1%M (d) and PGA-5%M (e). Scale is 10 μ m.

5.5 Discussion

The efficacy of anti-microbial PDT relies on the reaction between the PS and the bacteria, which is influenced by the ability of the PS to bind to the cell, to penetrate the cell membrane and to form singlet oxygen upon activation with light (Tokubo *et al.*, 2018). From the experiments presented in this chapter, the reduction in bacterial colonies showed that the photoinactivation of planktonic bacteria was higher than biofilms.

In the experiments using the planktonic form of *E. faecalis* and high concentrations of PS (5%E and 5%M), the bacterial counts were reduced to undetectable levels using colony counting. On the other hand, based on the quantification of the biofilm-growing *E. faecalis* from the scaffold surfaces, PDT with PGA-5%E and PGA-5%M reduced viable bacterial numbers by 2-log (99%). It also is interesting to note that the susceptibility of the Gram-negative bacterium, *E. coli*, was much lower than for *E. faecalis*, and was not affected to the same extent by the mode of growth (whether in planktonic or biofilm culture).

While the concentration of the PS used was the same for both the planktonic and biofilm cultures, it is probable that the reduction in killing seen for the biofilm-grown *E. faecalis* was due to the characteristics of the biofilm structure which would have a protective effect on the bacteria (Soukos and Goodson, 2011). The gradual release of the PS from the scaffolds could also affect the uptake of the PS into the biofilm when compared to the readily available PS in the planktonic medium.

Additionally, the differences in cell wall structure between Gram-positive and Gram-negative bacteria also play a role in the efficiency of the PDT. The thick

layer of the Gram-positive bacterial cell wall consists of peptidoglycan layers which are traversed by negatively-charged lipoteichoic and teichuronic acid that will attract positively-charged substances such as MB (Kato *et al.*, 2012). The abundance of MB in a solution will promote self-aggregation that occurs in the presence of negatively-charged surfaces. Photoactivation will then induce respiratory inhibition and disrupt the electron transfer system leading to cell membrane potential dissipation thereby triggering cell death. This mechanism helps to explain the high levels of bacterial killing seen in experiments with *E. faecalis*.

However, lower amounts of cell killing were observed with the Gram-negative bacterium, *E. coli* (a significant reduction of bacterial numbers was only observed when using the PGA-5%M scaffold, where a 1-log reduction was calculated). This smaller/lower reduction of *E. coli* numbers may be due to the presence of membrane-localised multidrug resistance pumps (MDRs) that pump the PS out of the cells (Blanco *et al.*, 2016). Tegos and Hamblin (2006) found 2 to 4 logs more killing in MDR-deficient *E. coli* compared to wild-type strains when MB and red light were used for PDT. Furthermore, they added that uptake of MB by the cells was concomitant with MDR expression levels and that MDR therefore affected the uptake of MB (Tegos and Hamblin, 2006). Since MB is internalised by *E. coli* and is considered to be a substrate in *E. coli* efflux pumps, it could be fairly expected that aPDT may face greater resistance when being used to treat Gram-negative bacteria. In addition, resistance may also be expected due to factors such as decreased penetrability to antimicrobial agents, increased activity of antimicrobial efflux

from the bacteria and enzymatic changes, that may prevent the effective accumulation of PS at target sites in the bacteria (Rineh *et al.*, 2018).

However, Wainwright *et al.*, (2002) found that photoactivation of MB (1 mM) was efficient at breaking down the extracellular polymeric substance (EPS) that encapsulated biofilms of Gram-negative bacteria after irradiation for 30 min. They suggested that MB induced damage to EPS by oxidation processes, thus affecting the integrity of the biofilm (Wainwright *et al.*, 2002).

Conversely, they also tested rose Bengal (RB), which is in the same class of xanthene dyes as ERY, and found that there was no difference in the extent of cell killing comparing the tested group and controls of *E. coli* bacteria and confirmed that the uptake of RB was not affected and not recognised by MDRs.

Previously, Kato *et al.* (2012) found that ERY engaged near the polar group of the lipid bilayer of the bacteria cell membrane, and exposure to light caused the membrane potential to dissipate and membrane permeability was increased due to production of ROS (Kato *et al.*, 2012). This raises the point that xanthene dyes might not be taken up into the cell but remain attached on the cell membrane within the lipid bilayer constituent. Thus, ROS production following light irradiation on ERY will disrupt membrane integrity, triggering cell death.

Furthermore, the membrane disruption due to PDT might escalate the PS penetration into the cell, allowing it to induce more damage by binding to the cellular proteins and DNA (Awad *et al.*, 2016). The singlet oxygen produced by photoactivation will further interact with close targets, further disrupting cell metabolism and inducing cell death (Castano, Demidova and Hamblin, 2004).

This can be seen from our data as the metabolic activity of cells on the scaffolds was significantly decreased compared to the non-irradiated group.

In conclusion, this study showed that scaffold incorporated with ERY in combination with visible light irradiation exerted an anti-microbial effect against *E. faecalis* regardless of the percentages of PS; however, for the *E. coli* only high concentrations of scaffold incorporated with ERY (PGA-5%E) demonstrated anti-microbial effects after photoactivation by visible light. Meanwhile, scaffold incorporated with a high concentration of MB (PGA-5%M) combined with light irradiation demonstrated greater anti-microbial effects compared to the scaffold with a low concentration of incorporated MB (PGA-1%M) regardless of bacterial species.

Chapter 6

General discussion, future work and conclusions

6.1 General discussion

The incorporation of antibacterial into electrospun scaffolds to prevent/treat infection is a relatively new area of research. For example, Zhang *et al.* (2015) incorporated antibiotics into electrospun scaffold made from a mix of PLGA (Poly(_{D,L}-lactic-co-glycolide) polymer and co-polymer (PELA (poly(ethylene glycol)-*b*-poly(_{D,L}-lactic-co-glycolide)/ PGA/ PDO (poly(dioxanone))) to investigate the antibacterial activity against *Staphylococcus aureus* (Gram-positive) and *E. coli* (Gram-negative) (Zhang *et al.*, 2015). However, a scaffold that incorporates antibacterial properties other than antibiotics is needed due to the increase in antimicrobial resistance seen in recent years (WHO, 2018). Photodynamic therapy is well studied in the treatment of cancer. However, there has also been an increase of interest in applying PDT to bacterial inactivation or killing (Stájer *et al.*, 2020). PDT is comprised of three components: a light source, PS and the target cells. Previous research has mainly focused on the developing or testing of PS in suspension or liquid form (Tokubo *et al.*, 2018; Soares *et al.*, 2018; Rout, Liu and Wu, 2018). However, in order to improve the targeting and delivery of PS to potentially infected tissue during tissue regeneration, we developed scaffolds incorporating PS that can provide antibacterial properties and at the same time support normal cell growth. In this study, we were able to fabricate PGA scaffolds that incorporate PS (ERY and MB) in different percentages. The effects of PS addition on the architecture, biocompatibility, PDT effects and antibacterial properties of PGA scaffolds were investigated and discussed in the previous chapters of this thesis.

In order to understand whether PS addition into the PGA polymer will affect the architecture and character of the scaffold produced, four concentrations for each PS were initially used. The method used in this work was adapted from previous research that produced a scaffold that contains PS (Wood *et al.*, 2010). The two different dyes that were used as PS in this study were ERY and MB, and they were incorporated into the PGA scaffolds at concentrations of 1%, 3%, 5% and 7.5% for each PS. The characteristics of the PS such as molecular weight, hydrophilicity and concentration will define the morphology of the produced scaffold (Li and Wang, 2013). Park *et al.* (2006) showed that the addition of a more hydrophilic substance to the hydrophobic PGA polymer did change the scaffold fibre diameters from microfibres to nanofibers, thus increasing the degradation rates. The blending of hydrophilic substances such as MB into the PGA polymer enhance the water absorption ability and it is expected to increase the hydrolysis rate of scaffold, thus inducing faster degradation rates (Meng *et al.*, 2011). This chain reaction will further promote more release of the added substance, as the degradation process will release the substance into their surroundings (Cui *et al.*, 2006).

However, ERY is an anionic (negatively-charged) dye with amphiphilic character, but which also has a high molecular weight compared to MB (Kato *et al.*, 2012). Thus, the increase in degradation rates in ERY scaffolds might not be due to the hydrophilic properties of the incorporated ERY, but are more likely due to the increased ERY concentration in the loaded PGA polymer which will result in smaller fibre diameters, and a higher surface to volume ratio. This will enhance contact between fibres and the surrounding water molecules, thus promoting increased scaffold degradation rates (Cunha-Reis

et al., 2007) which is then further increased, in a concentration-dependent manner, by the ERY released into the surrounding environment.

The PS release profiles were biphasic in character which consisted of an initial burst effect, followed by more gradual release of PS. The burst effect was mainly affected by the type and concentration of the PS added. Immediate contact of a scaffold with a solution will cause release of PS due to the solubility effect. A more gradual release then occurs when the solution hydrolyses the polymer into monomer and creates more available area for PS to be released by diffusion and erosion until the polymer is completely solubilised (Kamaly *et al.*, 2016; Makadia and Siegel, 2011).

Based on the initial studies of scaffold morphology, PS release profiles and degradation rates, four scaffolds incorporated with PS (PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M) were selected for further study and testing with hDPSCs and bacteria. The PGA-7.5%M scaffold was not selected due to its high degradation rates and unsuitability for sustaining cell growth for any length of time. PS release from the 3% PS scaffolds was not very different to the 1% scaffolds, so the 1% scaffolds were taken forward for further study in order to keep the PS concentration as low as possible.

ERY has been used as plaque disclosing agents in dentistry and approved by the F.D.A to be used as a food colouring agent. MB is used clinically as a surgical marker in surgery such as the endoscopic marking of intestinal lumen (Ginimuge and Joythi, 2010). The scaffolds produced in this study that contained ERY or MB were further tested with hDPSCs for biocompatibility which will govern whether the cells are able to adhere, attach, proliferate and thus form tissue or organs (O'Brien, 2011). In the present work, the effect of

incorporation of PS into the scaffold varied, depending on the type and concentration of PS used during scaffold fabrication. Due to differences in the structure/morphology and chemistry of the different scaffolds produced, the attachment of the cells to the scaffold also varied as the cells will act differently in adapting to the different scaffold structure and materials (Chang and Wang, 2011; Jun *et al.*, 2018). Scaffold properties such as hydrophobicity, charge, roughness, hardness and chemical composition will all also affect the cell attachment on the scaffold (Chang and Wang, 2011).

In this study, the addition of PS within the scaffold did change the hydrophilicity, charge, fibre diameters and chemical composition of the fabricated scaffold. Based on water binding capacity, PS loaded into the PGA polymer increased the hydrophilicity of the fabricated scaffolds. A previous study showed that scaffold modification by chemical treatment with NaOH to increase the hydrophilicity of the scaffold, did enhance cell attachment and proliferation rates of human adipose-derived stem cells (hADSCs) (Wang *et al.*, 2016). The charge of the polymer scaffold whether it is cationic (positively-charged), anionic (negatively-charged) or neutral also affects the cellular responses to the scaffold. Courtenay *et al.* (2017) demonstrated that cationic modification of cellulose films did increase human osteosarcoma cancer cell line (MG-63) attachment on the surface of the film. They suggested that there is an attraction between the positively-charged scaffold and the negatively-charged mammalian cell membrane and proved that cell attachment on cationic cellulose scaffolds is surface charge driven (Courtenay *et al.*, 2017). In other words, the negatively-charged phospholipids comprising the plasma membrane of the cells is more attracted to adhere and attach to the cationic scaffold (Li *et al.*, 2014). In our work, the addition of the cationic PS, MB,

further promoted the attachment of hDPSCs to the produced scaffold after 24 h incubation.

Moreira and co-workers (2004) performed anionic modification on collagen scaffolds by alkaline treatment, and found that the negatively-charged surface interacts with osteoblasts by a series of morphological modifications which resulted in a change in the metabolism of the cells. The osteoblasts cultured on the negatively-charged surface exhibited an active metabolism resulting in promotion of cell proliferation and ECM production (Moreira *et al.*, 2004). In our study, there was an increase in hDPSCs metabolic activity after 72 h culture on the PGA-ERY scaffolds that also indicated the cellular response of promotion of proliferation. Other research using HeLa cells, fibroblasts and osteoblasts cultured with anionically-modified scaffolds also showed an increase in cell adhesion and proliferation (Cui *et al.*, 2018; Chaterji and Gemeinhart, 2007).

Furthermore, nanofibers have also been found to be a co-founding factor that encourages cells to attach to the scaffold. The addition of PS during scaffold fabrication did change the diameter of the fibres from micrometres to nanometres fibres. Scaffold incorporated with ERY showed a reduction in their fibres diameter as the loaded ERY concentration was increased. Tian *et al.* (2007) reported that nanofibrous PGA/ collagen scaffolds with fibre diameters of 500 nm have the highest affinity for fibroblast cell adhesion, and cell spreading was more elongated on the fibres. The cells initially attach by wrapping around the cylindrical surface of the scaffold fibres and then spread along the fibres' orientation (Fig. 6.1) (Tian *et al.*, 2008). The small fibre diameters and interwoven mesh of nanofiber scaffolds will better mimic the

nature of the ECM, and thus further enhance cell proliferation (Donnelly *et al.*, 2018).

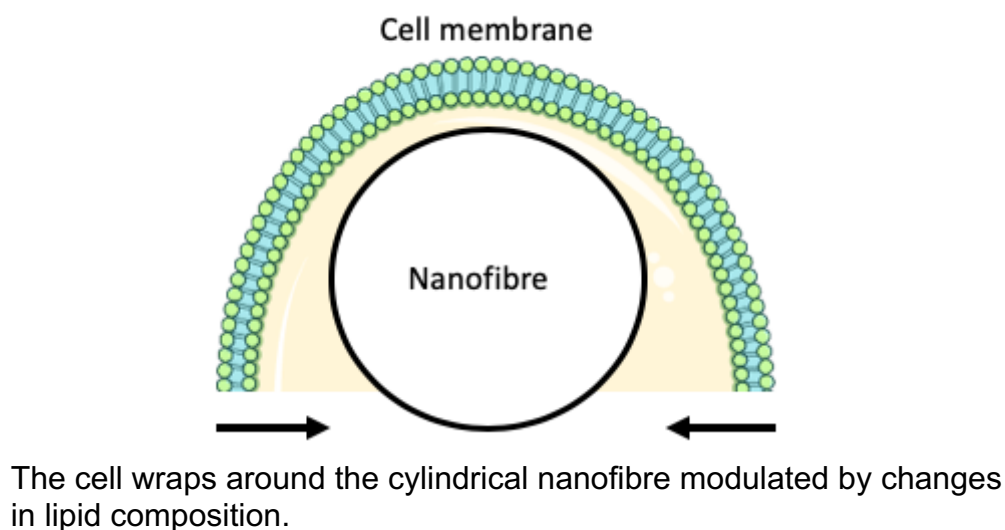


Figure 6.1 The diagram of cell interaction with nanofibre of the scaffold.
Adapted from https://smart.servier.com/smart_image/cell-9/

Cytotoxicity data showed that the cytotoxic effect of MB on the cells is greatest after 72 h of culture time. This cytotoxic effect was demonstrated in the lower ECM production, DNA amount, reduced metabolic activity and was supported by SEM and actin staining. MB is known to be attracted to negatively-charged cell membranes due to its cationic charge and the uptake into the cells is induced by endocytic pathways. Once inside the cell, MB will then accumulate in lysosomes, mitochondria and is capable of interacting with nucleic acids by stacking between two consecutive base pairs (Fig. 6.2) and triggering DNA damage by oxidation process and cell death by apoptosis (Costa *et al.*, 2016).

Ultimately, the cell attachment, spreading and proliferating onto the PGA-ERY scaffolds by hDPSCs exhibited a promising result at both concentrations of ERY (1% and 5%). This is due to the better support for cell attachment, proliferation and the reduction in cytotoxicity compared to PGA-MB scaffolds.

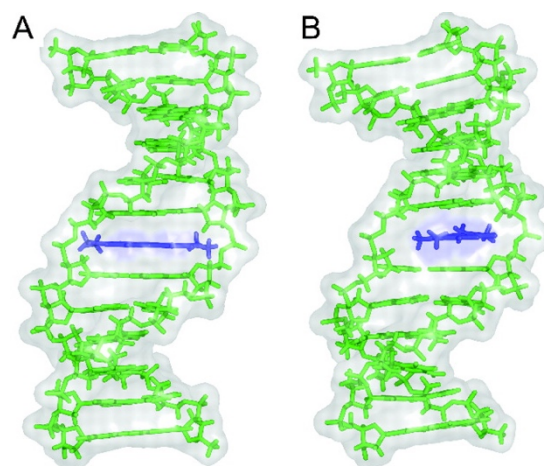


Figure 6.2 The conformations of Methylene Blue-DNA intercalation. Methylene Blue (MB) is shown in blue and the DNA with alternating GC base sequence in green (Rohs *et al.*, 2005).

As discussed above, the effect of PS towards hDPSCs was greater in the presence of light irradiation. After the 24 h recovery period from PDT, most of the cells were triggered to undergo either apoptosis or necrosis. Depending on the PDT dose and PS subcellular localisation, the treatment may trigger the cells to die directly by the induction of apoptosis (lower dose PDT) or by non-apoptotic pathways such as necrosis and autophagy (higher dose PDT) (Chen *et al.*, 2008; Diniz *et al.*, 2014). PDT can cause damage to cellular targets such as plasma membrane, mitochondria, lysosomes, Golgi apparatus and endoplasmic reticulum (ER) (Buytaert, Dewaele and Agostinis, 2007). PDT has been reported to induce apoptosis when the PS localizes to the mitochondria and cytoplasm. However, a PS localized within the plasma membrane will induce cell membrane disintegration and necrosis (Mroz *et al.*, 2011). MB-PDT is known to induce apoptosis by activation of caspases or alternative pathways independently of caspase activation (dos Santos *et al.*, 2017; Chen *et al.*, 2008). Previous research investigated the effect of MB-PDT (1% MB/ 100 J/cm²) on a mouse model and normal skin and found that MB increased apoptotic activity by caspase activation, more reactive fibroblasts and reduced epithelial cell proliferation (Silva *et al.*, 2018b). It has also been shown that lysosomal photodamage by MB-PDT (2 µM/ 11 J/cm²), did initiate the intrinsic apoptotic caspase-dependent pathway and mitophagy as there was a decrease in mitochondrial mass suggesting that photodamaged mitochondria were being removed (Martins *et al.*, 2018).

The key to minimising the PDT effect towards hDPSCs is to limit the incubation time, improve selectivity of uptake by bacteria and to use shorter irradiation times. By limiting the exposure of PS on the cell, the uptake of PS

into the cell will be decreased. A study performed PDT by using aluminium-chloride-phthalocyanine (AlClPc) in liposomes and irradiated by red laser found that bacterial cells selectively uptake the dye compared to dental pulp cells (Longo *et al.*, 2012). Therefore, we can make use of the faster binding and increased uptake of PS by bacteria to further target PDT, as by limiting the exposure time to PS we will be able to preserve the hDPSCs while maintaining the cell killing effect on bacteria that are present.

In this *in vitro* study, antibacterial PDT was tested on planktonic and biofilm cultures of *E. faecalis* and *E. coli*. Planktonic bacteria were incubated for 30 min with PS followed by light irradiation for 60 min. The concentration of the PS used for planktonic culture were 1%E = 1.09 μ M, 5%E = 56.37 μ M, 1%M = 6.91 μ M, 5%M = 67.93 μ M. The concentrations used were the average release of PS from the scaffold for 24 h. For biofilms, bacteria were inoculated on the scaffold incorporated with PS for 24 h followed by light irradiation for 60 min. Appropriate controls (no PS and PS with no light (dark control)) were included. There are several factors that affect the photoinactivation of bacteria such as the bacterium itself (bacterial structure, cellular target), PS (type, dose, incubation time, localisation of PS) and light energy (Fontana *et al.*, 2013; Jori *et al.*, 2011).

Bacterial cells have different cell membrane structures that will define their susceptibility to PDT. To increase PDT efficiency, PS must be able to bind to or traverse through the bacteria cell membrane. Gram-positive bacteria are known to have a thick layer of inter-connected peptidoglycan on their cell walls. However, this porous layer does not serve as a strict permeability barrier. On the other hand, Gram-negative bacteria have an additional cell

wall known as the outer cell membrane that consists of lipid, lipopolysaccharides (LPS), teichoic and lipoteichoic acids that makes this outer cell membrane more organised and able to inhibit the permeability of a substance or compound (Jori *et al.*, 2011; Jori *et al.*, 2006). Due to the porosity of the Gram-positive cell membrane, PS is able to cross the cell wall. However, anionic PS (negatively-charged) such as ERY have difficulty in crossing the lipid layer, resulting in selective accumulation within the cell membrane of Gram-positive bacteria. During light irradiation, the selective accumulation of PS leads to damage of the lipid and protein component within the membrane. This oxidative damage is confined to the cell membrane and alters the lipid and protein function, leading to a dissipation of membrane integrity. Loss of membrane integrity will further enhance the diffusion of PS into the bacteria (Kato *et al.*, 2012) leading to a cascade in cell killing.

Cationic PS like MB have a greater affinity for binding to the Gram-positive bacterial cell wall due to its overall negatively-charged surface which is attributed to the teichoic acid within the cell wall (Eichner, Gonzales and Maisch, 2013). Due to the electrostatic interaction between cationic PS and the cell wall, the cationic PS will be further induced to accumulate at the surface of the Gram-positive bacteria. The accumulation will also be enhanced as MB will form self-aggregates in solution. Light irradiation of the cationic PS bound to the cell wall will produce ROS which attack bacterial respiration and electron transfer systems, thus inducing cell death.

In Gram-negative bacteria, permeability to anionic PS is low due to the complexity of the cell wall and presence of the outer membrane. However, in our study, a high concentration of ERY (5%) in the planktonic or scaffold-

grown cultures was able to cause a reduction of *E. coli* viable counts after 60 min irradiation. A study conducted by Silva *et al.* (2018) showed that *E. coli* was more tolerant to PDT than Gram-positive bacteria whether in planktonic or biofilm culture. However, similar to our study, the authors were able to use a high concentration of ERY and high light dose (50 $\mu\text{Mol/L}$ / 486.40 J/cm^2) to completely inactivate *E. coli* biofilms. Furthermore, they found that a protein transporter on the outer cell wall transported ERY into the Gram-negative bacteria. ERY is known to have a lipophilic tendency and has an affinity for getting between lipid layers of the cell membrane. Therefore, during irradiation, ROS produced will further damage any molecule nearby within the cell membrane inducing instability and breakdown of the permeability barrier. The disruption of the cell membrane will allow more anionic PS to diffuse into the bacteria and further enhance the PDT effect (Silva *et al.*, 2018a; Kato *et al.*, 2012; George, Hamblin and Kishen, 2009).

A high concentration of MB in planktonic (5%M) and biofilm (PGA-5%M scaffold) cultures also reduced the *E. coli* viable counts. Previous research found a similar result as viable counts in *E. coli* planktonic cultures reduced when incubated with a 10 μM MB concentration for 10 min, followed by irradiation (10 J/cm^2) (Melo *et al.*, 2011). MB (cationic PS) is able to displace divalent cation by binding to the LPS. Divalent cation (Ca^{2+} and Mg^{2+}) serves as the stabiliser of the outer cell wall via electrostatic bonds. Displacement by MB therefore triggers instability of the LPS which decreases the permeability barrier of the cell wall of the Gram-negative bacteria. This mechanism is called “self-promoted uptake” pathway (George, Hamblin and Kishen, 2009; Jori *et al.*, 2006). Once inside the bacteria, MB is also known to interact with guanine

bases of DNA, hence inducing damage upon irradiation and causing cell death (Awad *et al.*, 2016).

Based on the results, PDT using high concentrations of both ERY (5%E) and MB (5%M) caused more cell killing in planktonic culture than in biofilm culture for *E. faecalis* and *E. coli* bacteria. This showed that biofilm bacteria are more resistant toward PDT which might be a consequence of promotion of microbial efflux pumps that expel foreign substances outside the bacterial cell membrane (Shrestha and Kishen, 2012). Biofilms also have an extra protective layer made up of extracellular polymeric substances (EPS) that are able to act as a filter for substances before they are able to enter bacteria. Bacteria in biofilms therefore have enhanced potential to resist and neutralise antimicrobial agents such as antibiotics (Jamal *et al.*, 2015). However, further research needs to be done in order to fully understand and maximise the effect of PDT on the bacteria.

6.2 Conclusions

The data and evidence presented in this thesis described further characterisation of a novel antibacterial scaffold fabrication using PGA polymer with the incorporation of photosensitiser (ERY/ MB) using the electrospinning technique. The four main objectives for this research were outlined in the introduction (i) to fabricate and characterise electrospun PGA scaffolds that contained a variety of concentrations of PS (1%, 3%, 5% and 7.5%); (ii) to evaluate the biocompatibility of the selected scaffolds (PGA-1%E, PGA-5%E, PGA1%M and PGA-5%M) and their effects on hDPSC attachment and proliferation *in vitro*; (iii) to evaluate the PDT effects on hDPSCs seeded on tissue culture plates and on the PGA scaffold in *in vitro* culture; and (iv) to investigate the potential anti-microbial effects of the scaffold and PDT on *E. faecalis* (a Gram-positive bacterium) and *E. coli* (a Gram-negative bacterium).

The addition of PS into PGA polymer did change the physical characteristics and degradation rates of the produced scaffolds depending on the type and percentages of PS added. The release of the PS from the scaffold could be tuned by varying the percentage of PS incorporated. However, there is a limit to the amount of PS that can be incorporated into the polymer: the amount of PS incorporated must not be too high as the modification may be cytotoxic to the cells and inhibit their ability to attach and infiltrate (MB) or the scaffold strength will be reduced (MB) meaning that it would not maintain mechanical integrity for long enough to support tissue regeneration.

Cytotoxicity tests were performed with hDPSCs to examine cell attachment, proliferation and metabolic activity of four of these scaffolds incorporated with

PS (PGA-1%E, PGA-5%E, PGA-1%M and PGA-5%M). PGA-E (PGA-1%E and PGA-5%E) was able to support hDPSCs attachment and growth following *in vitro* culture, as well as resulting in cells with similar morphologies to control scaffolds. However, for both percentages of (PGA-1%M and PGA-5%M) cytotoxic effects were observed after being in culture for longer than 24 h.

The PDT effect on hDPSCs was dependent on the type of PS, PS concentration and light dose used in the treatment. The use of monolayer or scaffold culture also affected the percentage of cell killing for hDPSCs. Cells on PGA scaffolds with ERY incubation had similar susceptibility to the PDT effect compared to the monolayer cell culture. However, cells exposed to MB were severely damaged irrespective of the culture conditions. In addition, hDPSCs were found to be more sensitive to the photodynamic action of MB compared to ERY. This data strongly suggests that PGA-ERY scaffolds will be suitable for use in antibacterial PDT during tissue regeneration, whereas the MB scaffolds would result in cytotoxicity to normal tissues and would therefore be a less ideal choice.

In vitro studies showed that PGA-5%E scaffold could exhibit antibacterial PDT effects, whilst at the same time, enhancing and supporting hDPSCs attachment and proliferation. PGA-1%E scaffolds did have good biocompatibility with hDPSCs. However, it did not possess enough antibacterial PDT effect on *E. coli* (Gram-negative) bacteria in this study. PGA-1%M scaffold was found to cause cytotoxicity on hDPSCs but at the same time did not cause any significant bacterial reduction when light was applied. Conversely, even though it was an effective antibacterial PS, the MB concentration in the PGA-5%M scaffold caused cytotoxicity to the hDPSCs.

Overall, the results from this thesis may lay a firm foundation for further developing antibacterial PDT scaffolds for use in tissue regeneration. The bacterial killing during infection must be greater and at the same time preserve normal cells from the PDT effect.

More research needs to be done in manipulating the concentration of PS and light irradiation time to get a perfect combination of low cytotoxicity and an efficient antibacterial effect on the same PGA-PS scaffold. One suggestion is to photoactivate the scaffold early which will limit the bacterial infections in the short term and after that, promotes cell proliferation on the scaffolds that are freed from bacteria. In this *in vitro* study, the burst released of the PS occurs during 72 h after the scaffold introduction, thus the effect of the high concentration or percentages of PS released from the scaffold when implanted in the oral cavity or soft tissue might be different, as a buffering system in the oral cavity or body fluid will remove the PS from the body within 24 h (Peter *et al.*, 2000).

Therefore, the development of scaffolds with antibacterial properties for use in regenerative medicine is extremely important to prevent infection at the surgical sites. ERY might be useful to be further investigated as it showed less toxicity on human dental pulp stromal cells and also exhibited antibacterial properties when irradiated with light. However, more research needs to be done to find the accurate PDT doses that balance killing of bacteria and preservation of the normal human cells or tissue.

6.3 Recommendations for future work and clinical translation

For the future use of anti-bacterial PDT and scaffold technology, the following aspects should be addressed in developing this scaffold further:

- I. Scaffold structure: The work presented in this thesis included studying the morphological properties, *in vitro* degradation rates and water binding capacity of the fabricated scaffolds. Additional physicochemical properties of the fabricated scaffolds also need to be investigated such as tensile strength, rheology (viscosity), crystallinity to further understand the interaction between PS and PGA polymer. Polymer viscosity (Li and Wang, 2013) and crystallinity (You *et al.*, 2005) affect the physical properties of the scaffold and thus directly impact cell attachment and proliferation. The addition of PS definitely induced changes in the physicochemical properties of the polymer and it should be investigated further to understand the interaction better. Modification of the incorporation of PS can then be made based on the type of scaffold needed.
- II. PS cellular localisation: In this study, only PS released into the media was retrieved. The cellular localisation of the PS will define the biological responses from target cells and the induced mechanism of cell death (Bacellar *et al.*, 2015). Therefore, determination of cellular localisation should be carried out by staining cellular targets such as lysosomes (LysoTracker Green) and mitochondria (Rhodamine 123) and comparing with the localisation of the fluorescent PS.
- III. *In vitro* Co-culture of bacteria and human DPSCs on the scaffold: Mono-cultures can only exhibit the interaction of human cells or

bacteria with produced scaffold. However, during infection the interaction is not only one to one but in between human cells, bacteria and scaffold. Thus, PDT on co-cultures should be explored further to support the antibacterial data presented in this thesis.

- IV. Scaffold material: The synthetic polymer used in this thesis was PGA. The addition of PS into PGA scaffolds increased the degradation rate of the scaffolds. In order to modulate the degradation rate of the scaffold, other types of synthetic polymer or combination with another polymer should be tested. For example, poly-(lactic acid) (PLA) and poly-(lactic-co-glycolic acid) (PLGA) are known to have slower degradation rates than PGA. Thus, it should be possible to produce a scaffold that degrades more slowly than PGA alone, and thus support cell growth for longer. Further work in our group on this aspect of the technology has recently been published (Contreras *et al.*, 2019; Contreras *et al.*, 2020).
- V. *In vivo* approach: The efficacy of the produced scaffold to interact with host cells in the *in vivo* setting should be further investigated. Synthesised scaffolds should be tested *in vivo* using gold standard functional animal models. The biocompatibility of the scaffold toward host cells and its environment will also need to be more fully investigated.
- VI. Anti-bacterial PDT: The anti-bacterial PDT work presented in this study involved two selected bacteria: *E. faecalis* (Gram positive) and *E. coli* (Gram negative). While the results were encouraging, further work is needed using complex multi-species biofilms such as those found in the oral cavity.

- VII. Seeding: In this study, cell seeding was performed statically. To fully understand the cell-material interaction, dynamic seeding should be tested as the cells will have more surface area to attach to the scaffold. However, as stated above, modification to the scaffold polymer to induce slower degradation rates would also improve the efficacy of dynamic seeding by providing a more stable scaffold that would not degrade as quickly during seeding.
- VIII. Cell types: Different cell types should be studied as the sensitivity of the cells will be different. For example, fibroblasts and skin tissue are known to tolerate a higher concentration of MB. Thus, further exploration is also needed on the use of the PS-incorporated scaffolds at varied sites of implantation where the host cells may better tolerate the PS and PDT treatment.

Appendices

Appendix A: Tissue bank approval form for teeth collection and the use of dental pulp stem cells.



Tissue Sample Form for School of Dentistry Research Tissue Bank

Applicant: Rina Osman Basah

DREC No: 120615/RB/172

Research Tissue:

Bank ID <u>No</u> :.....
Age of patient (time of collection):.....
Sex:
Ethnic <u>Origin</u> :.....
Signed (Recipient):
Signed (Supplier):.....
Date sample <u>taken</u> :.....

Please ensure Bank ID number is recorded on all samples created from tissue received from the LDI Tissue Bank.

This is a requirement of the Human Tissue Act. By receiving tissue from the bank you are agreeing to abide by the HTA regulations.

Useful links:

<http://www.hta.gov.uk/> Human Tissue Authority website

http://www.opsi.gov.uk/acts/acts2004/ukpga_20040030_en_1 Human Tissue Act 2004

Appendix B: Presentations and awards achieved.

Poster awards:

1st Place in Best Poster Awards – The IV International Conference on Humanities, Sciences, Social Sciences and Technologies (ICHSSST) 2017

Other presentation/ posters:

Image A2:

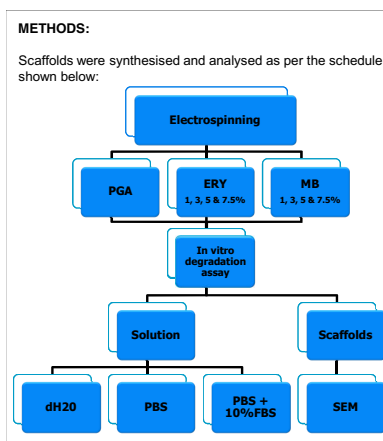
School of Dentistry
FACULTY OF MEDICINE AND HEALTH

UNIVERSITY OF LEEDS

**MORPHOLOGICAL CHARACTERISATION OF THE IN VITRO DEGRADATION OF
POLY-(GLYCOLIC ACID) SCAFFOLDS INCORPORATING EITHER
ERYTHROSINE OR METHYLENE BLUE.**

Rina Osman Basah¹, Simon R. Wood¹, Xuebin Yang¹ & Mike Raxworthy²
¹ School of Dentistry, University of Leeds, LS9 7TF, UK; ² Neotherix Ltd, York.

There has been much interest in the incorporation of antimicrobial agents into tissue engineering scaffolds to overcome infection in regenerative medicine. Erythrosine (ERY) and Methylene Blue (MB) are well-known photosensitisers (PS) which have been employed in antibacterial photodynamic therapy (APDT) studies and are also contained in dental plaque disclosing agents. The combination of scaffolds, PS and PDT may provide a new way to overcome infections such as peri-implantitis.



RESULTS:

Table 1: Mean fiber diameter (μm) $\pm$ S.D

Scaffolds		1%	3%	5%	7.5%
PGA	1.9895 $\pm$ 0.5045				
ERY		0.9656 $\pm$ 0.1658	0.498 $\pm$ 0.1725	0.3317 $\pm$ 0.1463	0.2281 $\pm$ 0.167
MB		0.9162 $\pm$ 0.1859	0.8342 $\pm$ 0.1937	0.9103 $\pm$ 0.1424	0.9308 $\pm$ 0.2367

Figure 1: SEM images of PGA only, Erythrosine and Methylene Blue (1, 3, 5 & 7.5%) scaffolds (50 μm).

Table 2: SEM images of scaffolds after day 7 in three different solution.

DAY 7 (50 μm)	dH ₂ O	PBS	PBS+10%FBS
PGA			
5% ERY			
5% MB			

Acknowledgements:

We would like to thank Dr Peter Iddon, Neotherix Ltd for his help and advice in making the scaffolds.
Malaysian Government for their sponsorship of this PhD studentship.

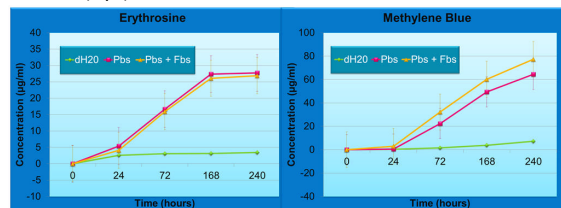


Figure 2: Erythrosine and Methylene Blue released profile over 10 days degradation time in three different solution.

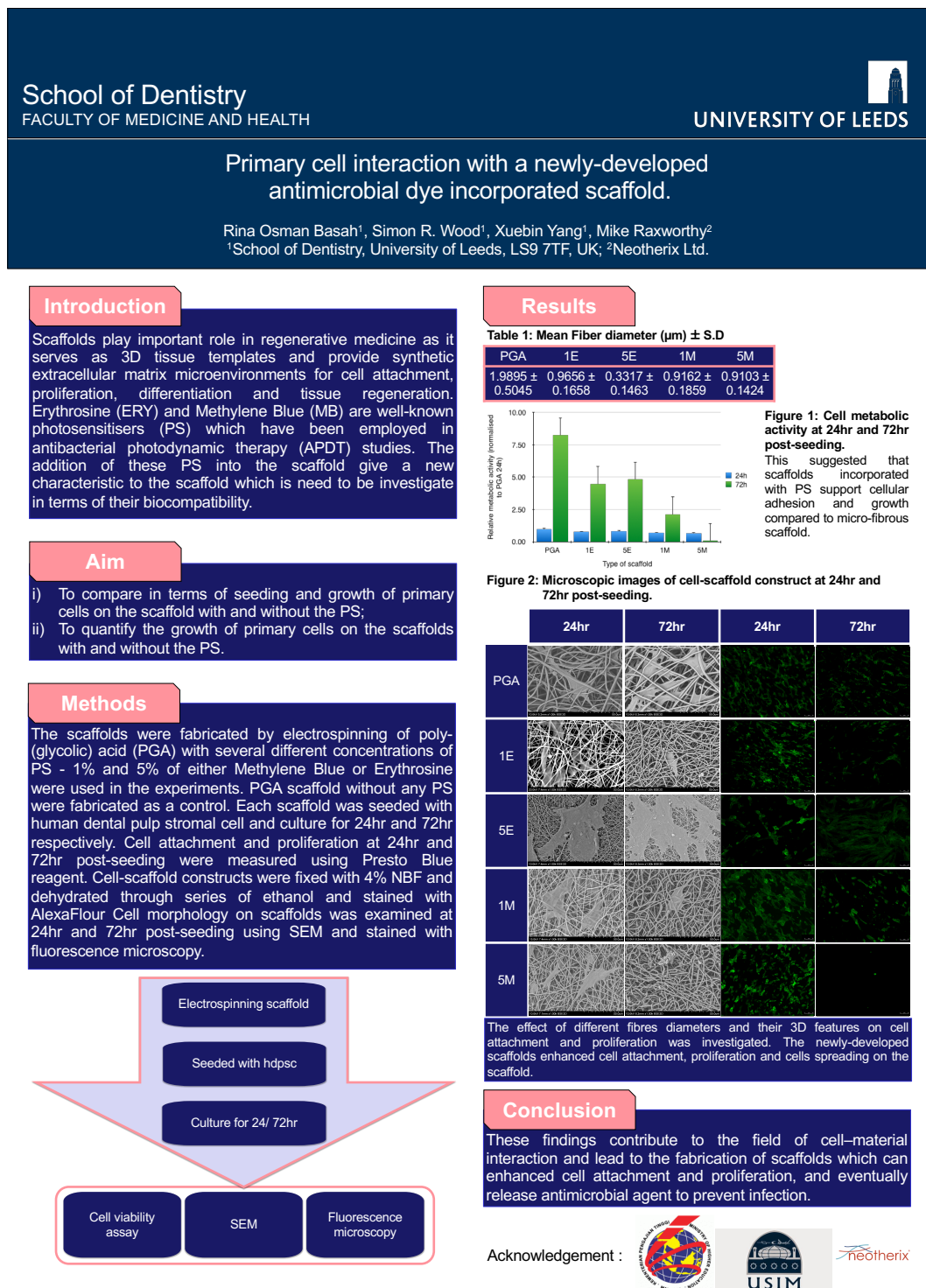
RESULTS:

The scaffolds fabricated by electrospinning showed different fiber diameters when visualized by SEM, PGA having the largest fiber diameter followed by Methylene Blue (MB) and Erythrosine (ERY)-incorporated scaffolds. The fiber diameter also decreased as the concentration of incorporated PS increased. As degradation time increased, more fibers were disintegrated and microcracks were found along the fibers.

CONCLUSIONS:

The addition of ERY or MB to the PGA, caused the scaffold morphologies to change, and as degradation time increased, SEM images showed the fibers become more scattered and disintegrated with increasing time in each solution. More studies are required to further characterise the effects of the incorporation of PS into PGA scaffolds for use in regenerative medicine strategies.

Image A3:



Appendix C: Neotherix Ltd extrusion process

CONFIDENTIAL

Neotherix Ltd

PGA Quenching & Solution Preparation

R&D Methods

Last Updated 2 November 2016

These methods cannot be used for Controlled Manufacturing. For preparation of quenched PGA and PGA solutions for Controlled Manufacturing refer to the relevant SOPs.

PGA Quenching

1. Equipment and Materials Required

PGA	Crystalline poly(glycolic acid) polymer used as purchased (without further purification).
Scissors	Metal scissors
Quenching fluid	Water used to cool down the melted PGA granules.
Spatula	Clean spatula for measuring out PGA granules.
Forceps	Clean thumb forceps for measuring out PGA granules.
Dishes	Disposable aluminium foil dishes for melting the PGA granules. <i>what size?</i>
Trays	Disposable plastic or metal trays with lids, large enough to hold at least one melting dish and labelled with details of the PGA granules contained within.
Tongs	Metal pincers used for grasping and lifting the melting dish.
Oven	Fan assisted oven for melting the PGA granules.
Bucket	Stainless steel bucket or pail for quenching the melted PGA granules
Bucket lid	Stainless steel bucket or pail lid used to cover the bucket or pail during the process when the bucket or pail is not used.
Cooling water bath	Metal tray containing water plus dry ice (or ice or cold packs) used to cool down the bucket containing the quenching fluid.
Temperature meter	Probe immersed in the cooling fluid used to continuously record the temperature of the cooling fluid.
Quenched PGA	Poly(glycolic acid) polymer as collected after quenching prior to any drying step.
Vacuum oven	One or more of the vacuum ovens described in SOP 002.
Vacuum pump	One of the vacuum pumps described in SOP 002.
Quenched dried PGA	Poly(glycolic acid) polymer after the drying step.
Foil pouches	Foil/polymer laminate heat-sealable pouches/bags.
Vacuum packaging machine	AudionVac VM151 vacuum packaging unit, asset number 3971
Inert gas	Nitrogen used during the packaging process.
Detergent spray	General cleaning purpose neutral detergent spray to be used in conjunction with dry wipes.
Dry wipes	General cleaning purpose dry wipe.

2. Preparation

- 2.1. Remove one or more pouches of PGA material from the freezer which have at least 100 g PGA granules to process, and allow the sealed pouch(es) to warm to room temperature. Ensure that all the other materials are also clean and ready.
- 2.2. Record the batch number of the PGA (as supplied), the date of weighing and the balance details, including instrument number and calibration expiry date on the Work Record Sheet. Separate Work Record Sheets must be used for PGA granules that are weighed and processed on different days.
- 2.3. Turn the oven on and set the temperature at 270 °C; ensure that the oven safety thermostat is set to 280-290 °C. Ensure there is one shelf positioned in the middle of the oven.
- 2.4. 100 g of the same PGA batch (usually one or more pouches) is to be split equally between 7 melting dishes, which will contain a minimum of 13.4 g of PGA granules per dish.
- 2.5. Label the required number of melting dishes and trays; the trays must be labelled with at least the Quenched PGA Reference number and name of operator. One tray per dish is required for the whole melting & quenching process.
- 2.6. Once the PGA has warmed to room temperature, for each dish in turn:
 - 2.6.1. Place the empty dish onto the balance, close the balance doors and zero the balance.
 - 2.6.2. Weigh out between (13.4 g and 13.6 g) of PGA into the dish using a spatula or forceps.
 - 2.6.3. Place the dish containing the weighed PGA granules into a tray.
 - 2.6.4. Record the measured weight of PGA added to the dish on the Work Record Sheet.
- 2.7. The remaining PGA can be retained for analysis if required. If so, place remaining PGA into a pouch and store in the vacuum oven with the quenched PGA dishes once they are completed (see Section 4). It will then be sealed when the contents of the dishes are sealed into pouches. The labelled sample must then be stored in the freezer until required (-15 °C or below).
- 2.8. When all the dishes have been completed, move them to the melting & quenching working area.

3. Melting & Quenching

- 3.1. Several parameters in the melting & quenching process are critical. Ensure that:
 - 3.1.1. The quenching water does not reside in the bucket for longer than 2 hours;
 - 3.1.2. The quenching bath is located close enough to the oven used to melt the PGA and in such a position that the operator can handle the dish containing the PGA comfortably enough to minimise the time taken to place the dish into the oven, remove the dish from the oven and quench the melted PGA;
 - 3.1.3. The tray lid is kept on the tray containing the dish containing the weighed out PGA granules until the dish is to be removed and placed into the oven;
 - 3.1.4. The cooling water and the quenching water never come into contact with each other during the entire melting & quenching process;
 - 3.1.5. The bucket lid is always placed on the bucket when the bucket is not in use;
 - 3.1.6. When the bucket lid is removed, it is always placed on a clean surface;
 - 3.1.7. When the dish containing the melted PGA is removed from the quenching bucket, it is always placed on clean piece of absorbent paper to drain;

- 3.1.8. A minimum volume of cooling water is maintained in the cooling water bath in order to prevent water splashes/aerosols produced by air being sucked into the cooling water bath agitator;
- 3.1.9. A maximum volume of cooling water is not exceeded in the cooling water bath in order to prevent water overtopping the edges of the quenching bucket or cooling bath;
- 3.1.10. The dry ice is added to the cooling water gradually and in moderate quantity;
- 3.1.11. A minimum volume of 8 L of quenching water at a temperature of about 2-5 °C is added into the bucket;
- 3.1.12. A gap of 2 °C between the temperature of the cooling water and that of the quenching water is maintained to ensure that the quenching water is below 5 °C;
- 3.2. When not in use, the tongs are always placed on a clean surface. Position the cooling water bath close to the oven.
- 3.3. Make or obtain dry ice and transfer the dry ice to the melting & quenching area.
- 3.4. Fill the cooling water bath with deionised water.
- 3.5. Add dry ice to the cooling water to decrease the temperature.
- 3.6. Once the cooling water has reached a temperature of below 3 °C, place the bucket into the cooling water bath (measure using temperature meter).
- 3.7. The bucket can be filled with quenching water before or after placing in the cooling water bath (if placed in prior to filling, one operator will need to hold the bucket down while another operator fills it with the quenching water). Record the time the bucket is filled on the Work Record Sheet.
- 3.8. Ensure that the cooling water bath is below 3 °C and the oven is at 270 °C, and that both temperatures are stable, before starting the melting & quenching phases.
- 3.9. For each dish in turn:
 - 3.9.1. Record the time and temperature of the cooling water bath.
 - 3.9.2. Take the dish using the tongs, ensuring a secure grip.
 - 3.9.3. Ensure the PGA granules are well dispersed across the bottom of the dish.
 - 3.9.4. Place the dish in the oven, ideally using a second operator to open and close the door in order to minimise the time that the door is open.
 - 3.9.5. The dish must be placed on the oven shelf at the front so that it is located on the right-hand half of the shelf but not touching the side of the oven (the oven shelf should already be located in the middle of the oven).
 - 3.9.6. The second operator must start recording the melting duration from when the dish is placed in the oven and the door closed.
 - 3.9.7. When the melting duration has reached **2 minutes 30 seconds (150 s)** exactly, immediately remove the dish from the oven and plunge into the quenching water without delay, ideally using a second operator to open and close the oven door in order to minimise the time the dish is cooling down in the air. It is important that submersion of the PGA occurs as rapidly as possible.
 - 3.9.8. After at least 10 seconds, remove the dish from the quenching water and place upside down on a clean absorbent surface to drain (see Step 3.1.7).
 - 3.9.9. Clean and dry the tongs.
- 3.10. The maximum duration that the quenching water can reside in the bucket before it must be disposed of is **2 hours**.

cooling < 3°C
oven 270°C

2 mins 30 sec

- 3.11. Following the completion of the melting & quenching of all the PGA granules, the dishes should be placed back into the lidded trays.
- 3.12. The trays and any samples for analysis are then to be transferred to the vacuum oven for drying according to Section 4.

4. Drying quenched PGA granules

- 4.1. Load the lidded plastic trays into the vacuum ovens (according to the appropriate SOP), ensuring that the content of the trays are kept covered by the lids (the trays are vented allowing vapour to escape even when lidded).
- 4.2. For each tray, record the Quenched PGA Reference number and the time loaded into the vacuum oven on the Work Record Sheet.
- 4.3. Label the oven so that other users are aware of the contents.
- 4.4. The vacuum pump should obtain a pressure of 25 mbar (2.5 kPa) or lower inside the vacuum oven within 30 minutes of loading the plastic trays (as read from the digital pressure display on the pump). Record the time at which pressure reaches 25 mbar (2.5 kPa) on the Work Record Sheet.
- 4.5. The vacuum should be maintained at a maximum pressure of 25 mbar (2.5 kPa) for the duration of drying, except for limited periods to load or unload other plastic trays containing more quenched PGA (interruption periods). Minimise the number of such interruption periods.
- 4.6. The quenched PGA granules (i.e. each output of one melting & quenching session) must be retained in the vacuum oven (under dynamic vacuum at room temperature) for at least 24 h but no more than 72 h.
- 4.7. Remove the plastic trays containing the dried quenched PGA granules (i.e. the output of one melting & quenching session) from the vacuum oven (according to the appropriate SOP), ensuring lids remain on trays. Record the time each tray is removed from the vacuum oven on the Work Record Sheet.
- 4.8. Ensure that the quenched PGA looks substantially homogenous in terms of appearance. If not, samples from the different areas of PGA will need to be sampled for inherent viscosity to ensure that it has melted evenly.
- 4.9. Take the necessary samples to allow for any required testing (e.g. inherent viscosity).
- 4.10. The dried quenched PGA material can be packed according to Section 5 or used to prepare PGA solutions in HFIP for electrospinning.

5. Packing Quenched PGA

- 5.1. Before starting this procedure, ensure that the vacuum packaging machine and surrounding working surface areas are available.
- 5.2. Two pouches are required per dish of quenched PGA - an inner and an outer pouch.
- 5.3. Label the outer foil pouches prior to packing. The label must contain the following information as a minimum:
 - 5.3.1. Quenched PGA Reference number (NXxxx-yyy-zz-Qnm where NXxxx is the Project Number, yyy is the Activity Number, zz is the Work Request Number, *n* is the number of the Melting & Quenching Session [1-9] and *m* is a letter designating the specific dish in the order in which they are quenched [A-Z, starting at A for each new session]).
 - 5.3.2. Name of operator.

5.3.3. Date of quenching.

- 5.4.** The content of one dish must be individually collected into one unlabelled foil pouch (the inner pouch).
- 5.5.** The dried quenched PGA granules will be stuck to the surfaces of the foil dish; therefore, (the use of forceps or spatula may be required in addition to the use of gloved fingers to detach them.)
- 5.6.** Place the whole contents of one dish into the foil pouch, using a spatula to push the granules towards the end of the foil pouch.
- 5.7.** The inner pouch will need to be folded over in order to fit sideways within the outer pouch. A maximum of one fold is allowed between the open end of the inner pouch and the quenched PGA stored within, therefore it may be necessary to shorten the inner pouch using scissors. This pouch must not be sealed (see Figure 1).
- 5.8.** Cut three slits down each side of the inner pouch to provide vents for venting and purging with nitrogen. The slits must completely cross the side seals and penetrate into the pouch a few millimetres in order to perforate it, although they must not be so long that they permit quenched PGA to easily fall out. The six slits are in case the fold in the pouch prevents proper venting/purging, so must be made adjacent to where the quenched PGA is located within the inner pouch (see Figure 1).
- 5.9.** Place the inner pouch inside the outer pouch (see Figure 1).

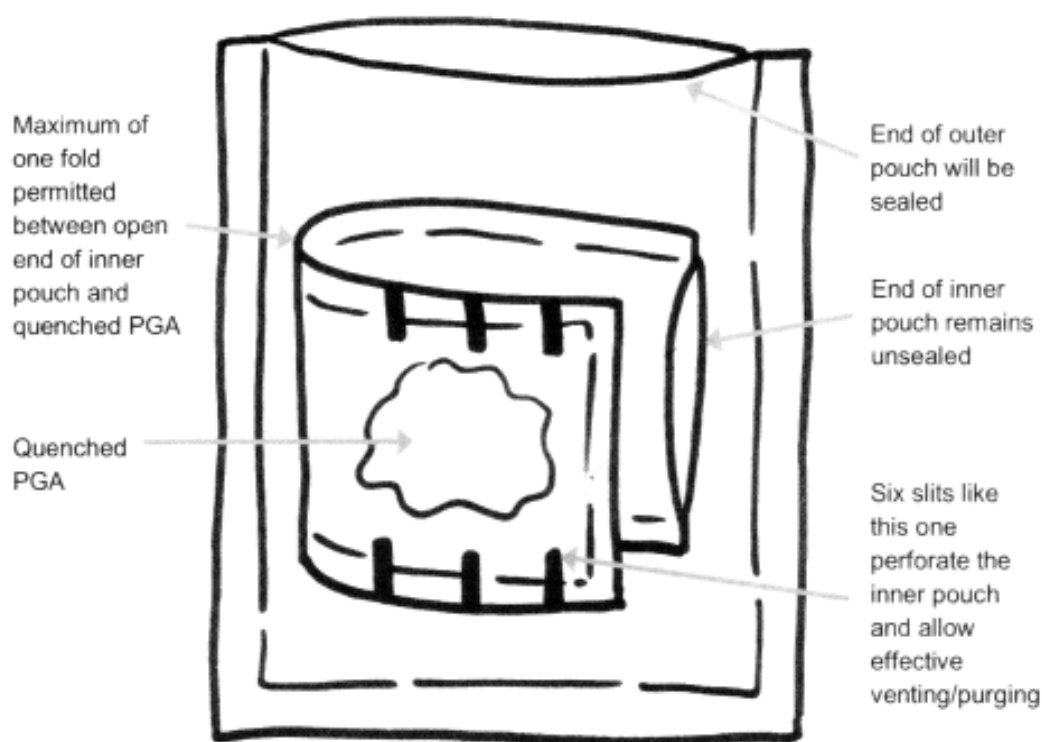


Figure 1. Arrangement of inner unsealed pouch inside the outer sealed pouch, showing the location of the six side slits that ensure ventilation when venting/purging.

- 5.10.** Place the assembled pouches back in the empty tray and replace the lid.

- 5.11.** Repeat Steps 5.5 and 5.6 for all the dishes containing the dried quenched PGA material.
- 5.12.** When all the dried quenched PGA material has been collected into the pouches, they are ready to be sealed. Turn on the vacuum packaging machine, ensure that the main valve on the nitrogen cylinder is also open, and check that the pouches will be appropriately supported during sealing.
- 5.13.** Select program 2 and run a test without anything to seal, in order to ensure that the nitrogen pressure is set to 0.9-1.1 bar (read from the regulator while the system is not filling the sealer), that the correct required program is running and the correct sealing phase is set. Program 2 consist of five consecutive phases:
- Vacuum phase: 25 s (phase during which pressure is reduced in the chamber)
 - Nitrogen phase: 10 s (phase during which nitrogen is introduced into the chamber)
 - Vacuum phase: 25 s (phase during which pressure is reduced in the chamber)
 - Nitrogen phase: 10 s (phase during which nitrogen is introduced into the chamber)
 - Sealing phase: 2.8 s (phase during which the pouches are heat-sealed)
- 5.14.** Place one outer pouch around each nozzle (a total of two pouches can be packed at the same time). Ensure that the pouches are placed in a way such that the rubber grips on the chamber lid will hold the pouch when the lid is closed. The inner pouches remain unsealed.
- 5.15.** Run program 2 by closing the lid.
- 5.16.** When the program terminates and the number 2 returns on the machine display, the lid can be completely opened.
- 5.17.** Collect the sealed foil pouches in the lid of the open tray.
- 5.18.** Repeat Steps 5.14 to 5.17 to seal all the foil pouches and the pouch containing the retained PGA from the previous day.
- 5.19.** Store the sealed foil pouches under the same conditions as for the PGA raw material.

Solution Preparation

6. Equipment and Materials Required

Equipment or Material	Details
PGA	Poly(glycolic acid) polymer (PGA) with sufficient solution viscosity for the preparation of electrospun fibres of the required diameter (SurModics PGA or extruded PURAC PGA).
HFIP	1,1,1,3,3,3-hexafluoropropan-2-ol solvent (spectrophotometric grade).
Beaker	Glass beaker into which HFIP is dispensed for solution preparation.
Vial and cap	Glass vial with polymeric push on cap (e.g. vials with a 28.5 mL capacity)
Pipette	Glass Pasteur pipette
Pipette bulb	Bulb to be fitted to mouth of glass pipette
Balance	Three or four figure enclosed balance located within a fume cupboard.
Spatula	Clean spatula for measuring out PGA granules (if used).
Scalpel	Clean, sharp scalpel for cutting PGA fibre (if used).

Roller	Laboratory bottle roller located within a fume cupboard.
Roller bottles	Glass or plastic labelled bottles large enough to hold a vial for rolling.
Lubricant-free syringes	Disposable Luer lock lubricant-free polypropylene syringes (e.g. 20.0 mL capacity).
Syringe caps	Disposable Luer tip polyethylene syringe cap.
Syringe filters	Disposable polypropylene syringe filter device suitable to remove particulates with size $\geq 10 \mu\text{m}$ (e.g. all polypropylene $10 \mu\text{m}$ filter unit, 50 mm diameter).

7. Safety

- 7.1.** HFIP is a harmful and corrosive volatile solvent. Appropriate personal protective equipment must be worn. See the relevant risk assessment for further information before use.

8. General Solution Preparation Procedure

- 8.1.** Remove PGA from the freezer and allow the sealed package to warm to room temperature.
- 8.2.** Record the batch number of the PGA, the date of weighing and the balance details on the Work Record Sheet. Separate Work Record Sheets must be used for solutions that are weighed on different days.
- 8.3.** Determine the total required weight of PGA in HFIP solution, and thereby the weight of PGA required in each vial, according to SOP 021. The use of a spreadsheet to take into account excess solution required for filtration and priming the rig is advised.
- 8.4.** Label the required number of 30 mL glass vials.
- 8.5.** Once the PGA has warmed to room temperature, for each vial in turn:
- 8.5.1.** Place the empty vial (without the lid) onto the balance, close the balance doors and then zero the balance.
- 8.5.2.** Weigh the required amount of PGA into the vial by either using a spatula (for PGA granules) or by cutting lengths from rolls using a sharp scalpel (for PGA fibre), to within the following limits of accuracy:
- 8.5.2.1.** The minimum weight must be the calculated weight of PGA.
- 8.5.2.2.** The maximum weight should not exceed 100 mg more than the calculated weight of PGA (to avoid overfilling of the vial and wastage of PGA). This limit can be exceeded as long as the solution volume in the vial does not exceed 25 mL.
- 8.5.3.** Record the measured weight of PGA added to the vial on the Work Record Sheet.
- 8.5.4.** Divide the measured weight of PGA by the solution concentration to calculate the solution weight.
- 8.5.5.** Then:
- 8.5.5.1.** Either add HFIP to the vial to the required total solution weight;
- 8.5.5.2.** Or subtract the PGA weight from the solution weight to obtain the HFIP weight, zero the balance, and then add HFIP to the vial to the required HFIP weight.
- 8.5.6.** If too much HFIP is added, allow HFIP to evaporate until the required weight is achieved.
- 8.5.7.** Since HFIP is volatile, cap the vial as soon as the required solution weight is achieved.

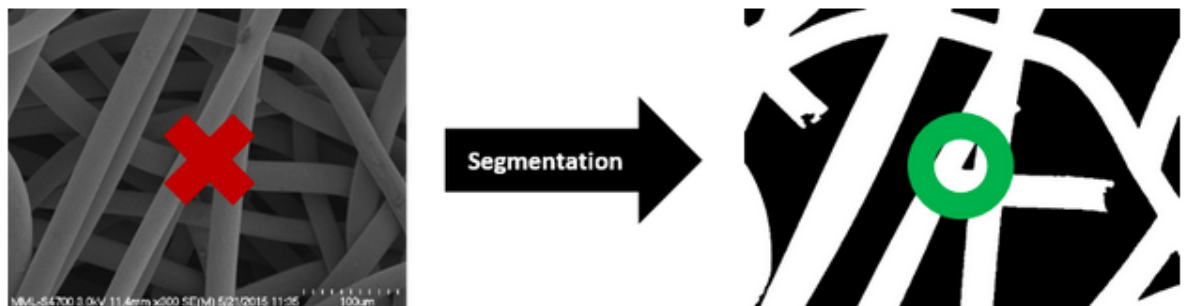
- 8.5.8.** Record the total solution weight and calculated solution concentration on the Work Record Sheet.
- 8.6.** When all the vials have been completed, individually place them inside lidded roller bottles, and then place these onto the roller.
- 8.7.** Start the roller. Record the starting time for each solution on the Work Record Sheet.
- 8.8.** Reseal the PGA back into a foil pouch, purging with nitrogen first if desired, and with fresh desiccant sachets included if needed.
- 8.9.** Return the PGA and HFIP to storage.
- 8.10.** When the solutions have rolled for the required period of time (usually overnight), record the finish time for each solution on the Work Record Sheet.
- 8.11.** At the end of the rolling time, visually inspect the solution to ensure that no large particles of undissolved PGA remain and that no leakage has occurred from the vial. The solution should be pale yellow to pale brown, and may contain very small undissolved particles of crystalline PGA from the ends of the cut fibres or granules.
- 8.12.** For each vial, quickly pour the solution into an empty 20 mL lubricant-free syringe fitted with a 10 μ m syringe filter, taking care to avoid significant solvent vapour loss. Immediately replace the syringe plunger.
- 8.13.** Filter the solution into a second empty 20 mL lubricant-free syringe fitted with a syringe cap, maintaining pressure on the first syringe to prevent detachment of the filter and loss of solution and to maintain close contact between the filter and second syringe to reduce solvent vapour loss. After filtration, immediately replace the syringe plunger.
- 8.14.** Stand the filled syringes on their plungers to allow any air bubbles to rise to the surface, before removing resulting single air bubble.
- 8.15.** The resulting solutions should be clear and free from visible particulates.

1) Plastic development tray
www.sigmaaldrich.com/catalog/product/sigma/p2915

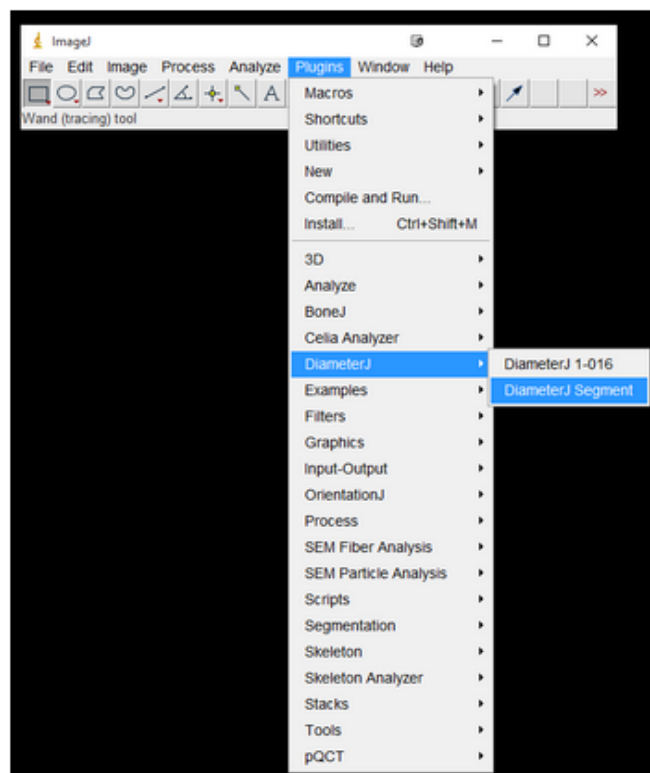
Appendix D: DiameterJ Installation and user instruction

1. Download and install ImageJ or Fiji.
2. Download the DiameterJ plugin http://fiji.sc/DiameterJ#Download_Link
3. Unzip the files and move or copy the three folders into the “plugins” folder of ImageJ/FIJI.
4. Restart ImageJ

Image Segmentation: Image segmentation is needed to convert image from SEM to a binary image before performing DiameterJ analysis. The white pixels represent the portions of the original image that going to be measured and the black pixels represent the background that not going to be measured.



1. Open ImageJ.
2. Go to “Plugins→DiameterJ→DiameterJ Segment” to segment images.



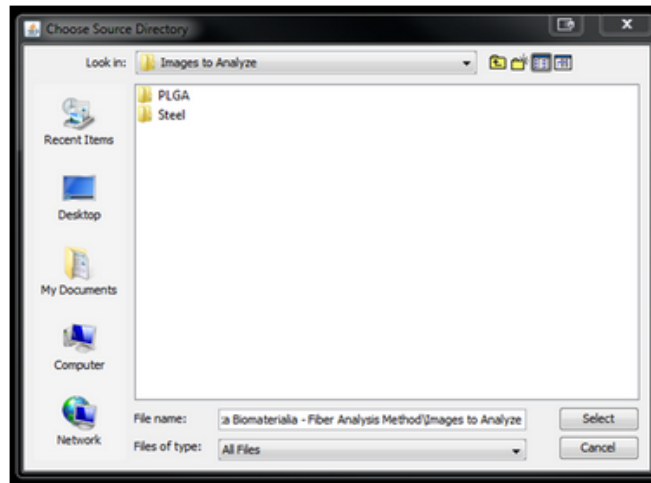
3. A pop-up window will appear asking for several pieces of information.

The screenshot shows a dialog box titled "Location of Cropping Field" with a close button (X) in the top right corner. The dialog is organized into four sections:

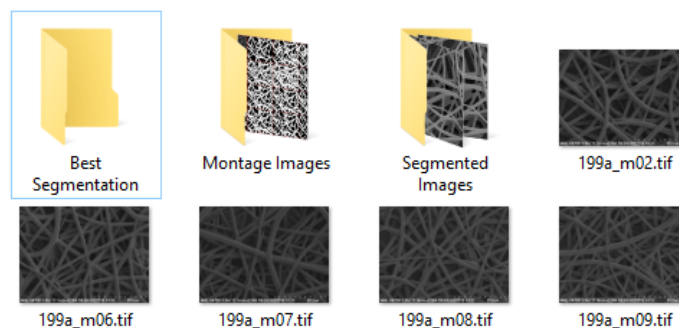
- Basic Image Information:** Contains two input fields: "Image Width (Uncropped)" with the value "1280" and "Image Height (Uncropped)" with the value "960". Both fields have a "Pixels" label to their right.
- Cropping Location:** Starts with a question "Do you want to crop your image?" followed by a dropdown menu set to "Yes". Below this are four input fields for coordinates: "Top Left - X coordinate" (0), "Top Left - Y coordinate" (0), "Bottom Right - X coordinate" (1280), and "Bottom Right - Y coordinate" (880).
- Segmentation Algorithms to Use:** Contains four checkboxes: "None", "Traditional", "Stat. Region Merged" (checked), and "Mixed" (checked).
- Batch Processing:** Contains the question "Do you want to analyze more than one image?" followed by two radio buttons: "Yes" (selected) and "No".

At the bottom right of the dialog are "OK" and "Cancel" buttons.

4. Enter in the dimensions of original image in pixels.
5. Select whether image needs to be cropped and what pixels coordinates of the box (the selected area after the cropped).
6. DiameterJ Segment can use up to 24 different segmentation algorithms to segment your image. Each of the check boxes under "Segmentation Algorithms to Use" represent 8 of these algorithms. Select as many or as few needed.
7. Batch processing for analysing more than one image is enabled by default but if the user only would like to analyse one image select the "No" radio button. If the user only wants to analyse one image that image must be opened in DiameterJ prior to starting the DiameterJ Segment or DiameterJ 1.XXX plugin.
8. Click the "OK" button after you have made all the selections you would like to make.
9. If you chose to batch analyse the images, a pop-up window will appear asking what folder you would like to analyse images from. If you chose not to batch analyse, the pop-up window will ask where you would like to save the output of the analysis of the image that is open in ImageJ. If batch was selected, output files will be saved in the same location that the images the user selected are in.



10. The code will produce three new folders within the folder where the image that you selected for analysis located. One folder is called “Best Segmentation”, another is called “Montage Images”, and the third is called “Segmented Images”.
- The Best Segmentation folder contains no images.
 - The Montage Images contains a montage comparison image of the original image to the 8, 16, or 24 different segmentation methodologies. Look at each of the montage images and find the name of the image with the most accurate representation of your fibres.
 - The Segmented Images folder has all of the segmented images from the montage images. Copy and paste the images you have determined to have the best segmentation into the “Best Segmentation” folder.



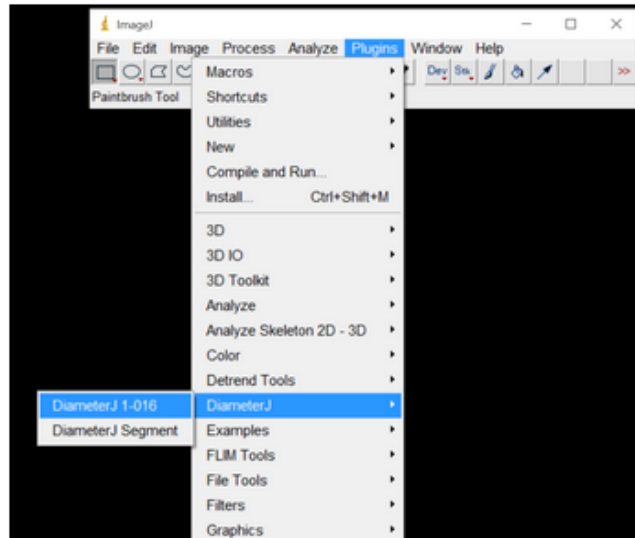
11. Visually inspect these images to make sure that the segmentation worked well and that the segmentation represents the image you want to analyse. If it does not, try the other segmentation algorithms.

12. If none of the 124 algorithms segment your image well, you can take the best segmentation results from running the algorithm and manually correct any remaining errors. Other methods include creating your own segmentation or using your own segmentation algorithm to create the black and white images. DiameterJ only needs a black and white (binary) image to analyse and thus it does not matter how that image is created.
13. After copying the best image into the “Best Segmentation” folder, the user can delete all other unused images/ montage images in the other folders. This step is not required it just saves a lot of hard drive space.

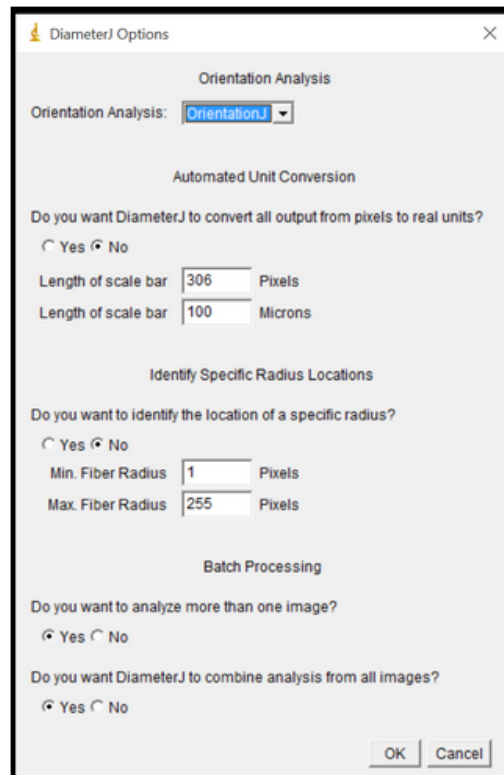
DiameterJ Analysis:

In ImageJ, go to:

“Plugins→DiameterJ→DiameterJ 1-XXX” to analyse the segmented image.



1. A pop-up menu will appear that gives the user several options for analysis.



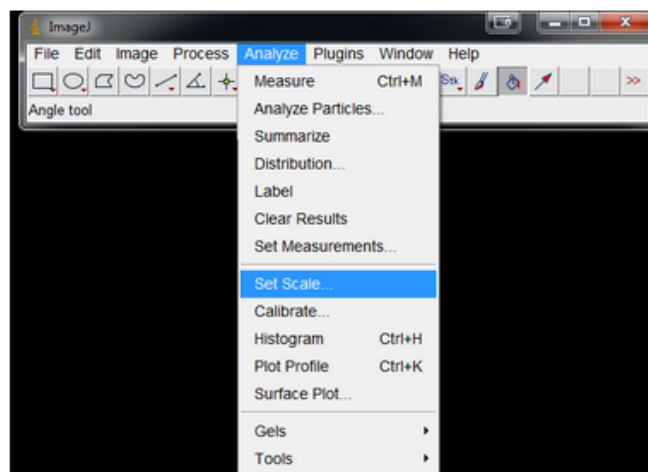
2. First choose what type of orientation analysis you would like to perform on your images.
 - a. OrientationJ is the default because it is fast and provides a good comprehensive graph of fibre orientation. However, it will not provide raw data for the user. If the user requires the raw data for the orientation graph, they will have to either run OrientationJ manually, OR use FIJI and select the “Directionality” plugin for analysis. The Directionality plugin only works for FIJI, is very slow and leaves windows open that cannot be closed in any ways but manually.
3. Next, the user must decide if they want DiameterJ to report all outputs in pixels (default if no unit conversion is selected) or if they want to convert pixels into real units such as micrometers or nanometers. If the user would like DiameterJ to convert units they will need to know the distance to pixel transformation.
4. If the user would like to know where a particular radius of fibre is occurring the user has the option for DiameterJ to create a separate graphic showing the location of radii in an image specified in the range specified by the user. The user must select the “Yes” button in “Identify Specific Radius Location” heading and enter in the lower and upper bounds of the radii they would like to see for this option to work.
5. Batch processing is enabled by default but if the user only would like to analyse one image select the “No” button under the “Batch Processing” heading. If the user only wants to analyse one image that image must be opened in DiameterJ prior to starting the DiameterJ Segment or DiameterJ 1.XXX plugin.
6. Additionally, DiameterJ can now combine the analysis of multiple files into single summary sheets if the “Yes” button is selected under the question “Do you want DiameterJ to combine analysis from all images?”. Note this combines all images analysed into summary files. Thus, if multiple samples are contained in the same folder and DiameterJ is analysing that folder It will combine the analysis from multiple samples into the same summary files.

7. Click the OK button after you have made all of the selections you would like to make.
8. A pop-up window will appear asking what folder you would like to analyse images from if you chose to batch analyse images, or asking where you would like to save the output of the analysis of the image that is open in ImageJ if no batch analysis was selected. If batch was selected output files will be saved in the same location that the images the user selected are in.
9. DiameterJ will then analyse all the images in the specified directory and will separate its analysis into three folders: “Diameter Analysis Images”, “Histograms”, and “ Summaries”. These folders will be located in the directory where the analysed image is located. The file labelled “DiameterJ Output Description” describes what the outputs are in each of these folders. All the raw data regarding measurement can be found in these folders.

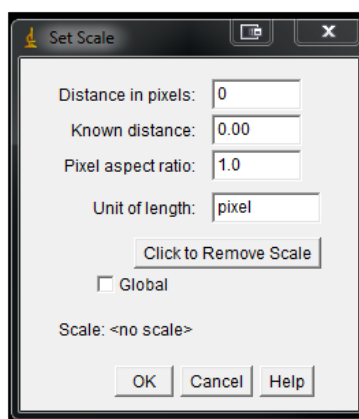
Pixel to Unit Distance Transformation:

All measurements given by DiameterJ are in pixels by default.

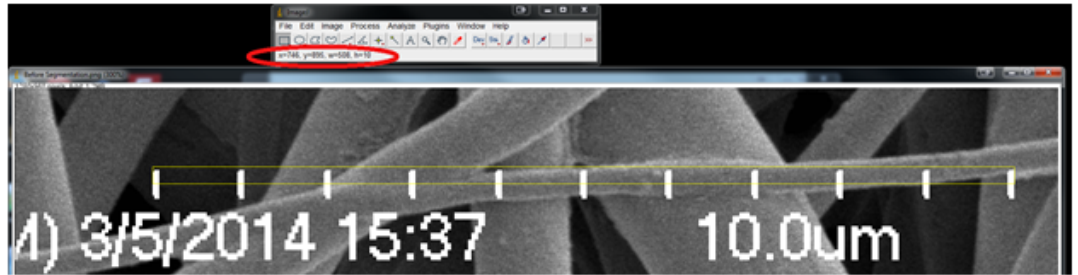
1. To determine the pixel to unit distance (nanometer/ micrometer/ millimeter) conversion, there are two options depending on how much metadata your imaging software stores with your image. It is possible that you already have the pixel to distance conversions in the metadata and thus getting the distance transformation number is easy.
 - a. Simply go to: a) Analyse→Set scale



2. Look at what the unit of length is. If it is inches, then your SEM does not store the necessary metadata information with the micrograph and you will have to do the conversion by hand.
3. In the case that you have to calculate by hand, open any micrograph (at the magnification that you have taken your SEM images) in ImageJ.
4. In ImageJ go to menu:
 - a. a) Analyse→Set scale
5. Select “Click to Remove Scale”.



6. Close the scale window.
7. Place your mouse over the scale bar and hot the “+” key to zoom-in.
8. Continue to zoom-in on the scale bar until it fills the screen. After zooming in, you can widen the default width of the window that ImageJ chooses just like any normal window.
9. Next select the “Rectangular” tool from the menu bar. If the rectangular tool is not available, go to the tile that has “>>” and click “Restore startup tools” from the drop down menu.
10. Next, draw a square or a line that spans the scale bar.
11. To get the length or width of this rectangle, simply look under the tool bar of ImageJ as you are drawing the rectangle. As soon as you stop drawing the line, the measurements will disappear, so do not let go of the cursor until you have seen and noted the rectangle width.



12. Record the number that “w” is equal to.
13. Then, it is simply a matter of entering in the length of the scale bar in real units (10 microns above) and the number of pixels you just recorded (“w”) into the DiameterJ 1.XXX menu and selecting “Yes” to unit conversion.
14. All units in all output files will then be in terms of microns, microns², etc.

List of References

- Aaron, S. D., Ferris, W., Ramotar, K., Vandemheen, K., Chan, F. and Saginur, R. (2002) 'Single and combination antibiotic susceptibilities of planktonic, adherent, and biofilm-grown *Pseudomonas aeruginosa* isolates cultured from sputa of adults with cystic fibrosis', *J Clin Microbiol*, 40(11), pp. 4172-9.
- Abdullah, M. F., Abdullah, S. F., Omar, N. S., Mahmood, Z., Fazliah Mohd Noor, S. N., Kannan, T. P. and Mokhtar, K. I. (2014) 'Proliferation rate of stem cells derived from human dental pulp and identification of differentially expressed genes', *Cell Biol Int*, 38(5), pp. 582-90.
- Agarwal, S., Wendorff, J. H. and Greiner, A. (2009) 'Progress in the field of electrospinning for tissue engineering applications', *Adv Mater*, 21(32-33), pp. 3343-51.
- Aghdam, R. M., Najarian, S., Shakhesi, S., Khanlari, S., Shaabani, K. and Sharifi, S. (2012) 'Investigating the effect of PGA on physical and mechanical properties of electrospun PCL/PGA blend nanofibers.', *J. Appl. Polym. Sci.*, 124, pp. 123-131
- Ahmed, M. N., Abdelsamad, A., Wassermann, T., Porse, A., Becker, J., Sommer, M. O. A., Høiby, N. and Ciofu, O. (2020) 'The evolutionary trajectories of *P. aeruginosa* in biofilm and planktonic growth modes exposed to ciprofloxacin: beyond selection of antibiotic resistance', *NPJ Biofilms Microbiomes*, 6(1), pp. 28.
- Akbarzadeh, R. and Yousefi, A. M. (2014) 'Effects of processing parameters in thermally induced phase separation technique on porous architecture of scaffolds for bone tissue engineering', *J Biomed Mater Res B Appl Biomater*, 102(6), pp. 1304-15.
- Alav, I., Sutton, J. M. and Rahman, K. (2018) 'Role of bacterial efflux pumps in biofilm formation', *The Journal of antimicrobial chemotherapy*, 73.
- Albuquerque, M. T. P., Nagata, J. and Bottino, M. C. (2017) 'Antimicrobial Efficacy of Triple Antibiotic-eluting Polymer Nanofibers against Multispecies Biofilm', *J Endod*, 43(9S), pp. S51-S56.

Alexis, F. (2005) 'Factors affecting the degradation and drug-release mechanism of poly(lactic acid) and poly[(lactic acid)-co-(glycolic acid)]', *Polymer International*, 54, pp. 36-46.

Allaker, R. P. and Douglas, C. W. (2009) 'Novel anti-microbial therapies for dental plaque-related diseases', *Int J Antimicrob Agents*, 33(1), pp. 8-13.

Allison, R. R., Downie, G. H., Cuenca, R., Hu, X.-H., Childs, C. J. H. and Sibata, C. H. (2004) 'Photosensitizers in clinical PDT', *Photodiagnosis and Photodynamic Therapy*, 1(1), pp. 27-42.

Altuntaş, E., Özkan, B. and Yener, G. (2017) '3 - Porous scaffolds', in Razavi, M. and Thakor, A. (eds.) *Nanobiomaterials Science, Development and Evaluation*: Woodhead Publishing, pp. 27-59.

Amariei, N., Manea, L. R., Berteau, A. P., Berteau, A. and Popa, A. 'The Influence of Polymer Solution on the Properties of Electrospun 3D Nanostructures.', *International Conference on Innovative Research (ICIR EUROINVENT 2017)*: **IOP PUBLISHING**.

Arbeloa, A., Segal, H., Hugonnet, J. E., Josseaume, N., Dubost, L., Brouard, J. P., Gutmann, L., Mengin-Lecreulx, D. and Arthur, M. (2004) 'Role of Class A Penicillin-Binding Proteins in PBP5-Mediated β -Lactam Resistance in *Enterococcus faecalis*', *Journal of Bacteriology*, 186(5), pp. 1221-1228.

Armentano, I., Dottori, M., Fortunati, E., Mattioli, S. and Kenny, J. M. (2010) 'Biodegradable polymer matrix nanocomposites for tissue engineering: A review', *Polymer Degradation and Stability*, 95(11), pp. 2126-2146.

Atala, A. J., Bauer, S. B., Soker, S., Yoo, J. J. and Retik, A. B. (2006) 'Tissue-engineered autologous bladders for patients needing cytoplasty. ', *The Lancet*, 367, pp. 1241-1246.

Austin, E. and Jagdeo, J. (2018) 'An *In Vitro* Approach to Photodynamic Therapy', *Journal of Visualized Experiments*, (138).

Awad, M. M., Tovmasyan, A., Craik, J. D., Batinic-Haberle, I. and Benov, L. T. (2016) 'Important cellular targets for antimicrobial photodynamic therapy', *Appl Microbiol Biotechnol*, 100(17), pp. 7679-88.

Babilas, P., Schacht, V., Liebsch, G., Wolfbeis, O. S., Landthaler, M., Szeimies, R. M. and Abels, C. (2003) 'Effects of light fractionation and

different fluence rates on photodynamic therapy with 5-aminolaevulinic acid in vivo', *British Journal of Cancer*, 88(9), pp. 1462-1469.

Bacellar, I., Tsubone, T., Pavani, C. and Baptista, M. (2015) 'Photodynamic Efficiency: From Molecular Photochemistry to Cell Death', *International Journal of Molecular Sciences*, 16(9), pp. 20523-20559.

Bacellar, I. O., Pavani, C., Sales, E. M., Itri, R., Wainwright, M. and Baptista, M. S. (2014) 'Membrane damage efficiency of phenothiazinium photosensitizers', *Photochem Photobiol*, 90(4), pp. 801-13.

Badylak, S. F. (2005) 'Regenerative medicine and developmental biology: the role of the extracellular matrix', *Anat Rec B New Anat*, 287(1), pp. 36-41.

Bao Ha, T. L., Minh, T., Nguyen, D. and Minh, D. (2013) 'Naturally Derived Biomaterials: Preparation and Application', *Regenerative Medicine and Tissue Engineering*.

Bassetti, M., Schar, D., Wicki, B., Eick, S., Ramseier, C. A., Arweiler, N. B., Sculean, A. and Salvi, G. E. (2014) 'Anti-infective therapy of peri-implantitis with adjunctive local drug delivery or photodynamic therapy: 12-month outcomes of a randomized controlled clinical trial', *Clin Oral Implants Res*, 25(3), pp. 279-87.

Bhardwaj, N. and Kundu, S. C. (2010) 'Electrospinning: a fascinating fiber fabrication technique', *Biotechnol Adv*, 28(3), pp. 325-47.

Bhattarai, D. P., Aguilar, L. E., Park, C. H. and Kim, C. S. (2018) 'A Review on Properties of Natural and Synthetic Based Electrospun Fibrous Materials for Bone Tissue Engineering', *Membranes (Basel)*, 8(3).

Bijukumar, D. R., McGeehan, C. and Mathew, M. T. (2018) 'Regenerative Medicine Strategies in Biomedical Implants', *Curr Osteoporos Rep*, 16(3), pp. 236-245.

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O. and Piddock, L. J. (2015) 'Molecular mechanisms of antibiotic resistance', *Nat Rev Microbiol*, 13(1), pp. 42-51.

Blanco, P., Hernando-Amado, S., Reales-Calderon, J., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Sanchez, M. and Martinez, J. (2016)

'Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants', *Microorganisms*, 4(1).

Boland, E. D., Wnek, G. E., Simpson, D. G., Pawlowski, K. J. and Bowlin, G. L. (2001) 'Tailoring Tissue Engineering Scaffolds Using Electrostatic Processing Techniques A Study Of Poly Glycolic Acid Electrospinning.', *Journal of Macromolecular Science*, 38(12), pp. 1231-1243.

Boltes Cecatto, R., Siqueira Magalhães, L., Fernanda Setúbal Destro Rodrigues, M., Pavani, C., Lino-Dos-Santos-Franco, A., Teixeira Gomes, M. and Fátima Teixeira Silva, D. (2020) 'Methylene blue mediated antimicrobial photodynamic therapy in clinical human studies: The state of the art', *Photodiagnosis Photodyn Ther*, pp. 101828.

Bonani, W., Singhatanadgige, W., Pornanong, A. and Motta, A. (2018) 'Natural Origin Materials for Osteochondral Tissue Engineering', *Adv Exp Med Biol*, 1058, pp. 3-30.

Bonfield, W. (2006) 'Designing porous scaffolds for tissue engineering', *Philos Trans A Math Phys Eng Sci*, 364(1838), pp. 227-32.

Bonnett, R. (2002) 'Progress with Heterocyclic Photosensitizers for the Photodynamic Therapy (PDT) of Tumours', *J. Heterocyclic Chem.*, 39, pp. 455-470.

Brancalion, L. and Moseley, H. (2002) 'Laser and Non-laser Light Sources for Photodynamic Therapy', *Lasers Med Sci* 17, pp. 173-186.

Briggs, T., Blunn, G., Hislop, S., Ramalhet, R., Bagley, C., McKenna, D. and Coathup, M. (2018) 'Antimicrobial photodynamic therapy-a promising treatment for prosthetic joint infections', *Lasers Med Sci*, 33(3), pp. 523-532.

Bruzauskaite, I., Bironaite, D., Bagdonas, E. and Bernotiene, E. (2016) 'Scaffolds and cells for tissue regeneration: different scaffold pore sizes-different cell effects', *Cytotechnology*, 68(3), pp. 355-69.

Burhenne, J., Riedel, K. D., Rengelshausen, J., Meissner, P., Muller, O., Mikus, G., Haefeli, W. E. and Walter-Sack, I. (2008) 'Quantification of cationic anti-malaria agent methylene blue in different human biological matrices using cation exchange chromatography coupled to tandem mass spectrometry', *J Chromatogr B Analyt Technol Biomed Life Sci*, 863(2), pp. 273-82.

Buytaert, E., Dewaele, M. and Agostinis, P. (2007) 'Molecular effectors of multiple cell death pathways initiated by photodynamic therapy', *Biochim Biophys Acta*, 1776(1), pp. 86-107.

Calin, M. A. and Parasca, S. V. (2009) 'Light sources for photodynamic inactivation of bacteria', *Lasers Med Sci*, 24(3), pp. 453-60.

Capella, M., aacute, rcia Alves, M. and Capella, L. S. (2003) 'A Light in Multidrug Resistance: Photodynamic Treatment of Multidrug-Resistant Tumors', *Journal of Biomedical Science*, 10(4), pp. 361-366.

Capo, J. T., Kokko, K. P., Rizzo, M., Adams, J. E., Shamian, B., Abernathie, B. and Melamed, E. (2014) 'The use of skin substitutes in the treatment of the hand and upper extremity', *Hand (N Y)*, 9(2), pp. 156-65.

Carter, P. and Bhattarai, N. (2013) 'Chapter 7 - Bioscaffolds: Fabrication and Performance', in Lakhtakia, A. and Martín-Palma, R.J. (eds.) *Engineered Biomimicry*. Boston: Elsevier, pp. 161-188.

Casagrande, L., Cordeiro, M. M., Nor, S. A. and Nor, J. E. (2011) 'Dental pulp stem cells in regenerative dentistry', *Odontology*, 99(1), pp. 1-7.

Casas, A. (2020) 'Clinical uses of 5-aminolaevulinic acid in photodynamic treatment and photodetection of cancer: A review', *Cancer Lett*, 490, pp. 165-173.

Castano, A. P., Demidova, T. N. and Hamblin, M. R. (2004) 'Mechanisms in photodynamic therapy: part one—photosensitizers, photochemistry and cellular localization', *Photodiagnosis and Photodynamic Therapy*, 1(4), pp. 279-293.

Catiker, E., Gümüşderelioğlu, M. and Güner, A. (2000) 'Degradation of PLA, PLGA homo- and copolymers in the presence of serum albumin: A spectroscopic investigation', *Polymer International*, 49, pp. 728-734.

Cattoir, V. (2004) '[Efflux-mediated antibiotics resistance in bacteria]', *Pathol Biol (Paris)*, 52(10), pp. 607-16.

Chacón, J. M., Caminero, M. A., García-Plaza, E. and Núñez, P. J. (2017) 'Additive manufacturing of PLA structures using fused deposition modelling: Effect of process parameters on mechanical properties and their optimal selection', *Materials & Design*, 124, pp. 143-157.

Chang, H. and Wang, Y. (2011) 'Cell Responses to Surface and Architecture of Tissue Engineering Scaffolds.', in Eberli, D. (ed.) *Regenerative Medicine and Tissue Engineering - Cells and Biomaterials*.

Chaterji, S. and Gemeinhart, R. A. (2007) 'Enhanced osteoblast-like cell adhesion and proliferation using sulfonate-bearing polymeric scaffolds', *J Biomed Mater Res A*, 83(4), pp. 990-8.

Chatzistavrou, X., Fenno, J. C., Faulk, D., Badylak, S., Kasuga, T., Boccaccini, A. R. and Papagerakis, P. (2014) 'Fabrication and characterization of bioactive and antibacterial composites for dental applications', *Acta Biomater*, 10(8), pp. 3723-32.

Chen, S., Zhang, X., He, M. and Li, J. (2020) 'Degradation of PGA, prepared by reactive extrusion polymerization, in water, humid, and dry air, and in a vacuum', *Journal of Materials Research*, 35(14), pp. 1846-1856.

Chen, Y., Zheng, W., Li, Y., Zhong, J., Ji, J. and Shen, P. (2008) 'Apoptosis induced by methylene-blue-mediated photodynamic therapy in melanomas and the involvement of mitochondrial dysfunction revealed by proteomics', *Cancer Sci*, 99(10), pp. 2019-27.

Cho, M. H., Kim, K. S., Ahn, H. H., Kim, M. S., Kim, S. H., Khang, G., Lee, B. and Lee, H. B. (2008) 'Chitosan gel as an in situ-forming scaffold for rat bone marrow mesenchymal stem cells in vivo', *Tissue Eng Part A*, 14(6), pp. 1099-108.

Chong, C., Wang, Y., Fathi, A., Parungao, R., Maitz, P. K. and Li, Z. (2019) 'Skin wound repair: Results of a pre-clinical study to evaluate electrospun collagen-elastin-PCL scaffolds as dermal substitutes', *Burns*, 45(7), pp. 1639-1648.

Chou, S., Carson, D. and Woodrow, K. A. (2015) 'Current strategies for sustaining drug release from electrospun nanofibers', *Journal of Controlled Release*, 220, pp. 584-591.

Christopherson, G. T., Song, H. and Mao, H. (2009) 'The influence of fibre diameter of electrospun substrates on neural stem cell differentiation and proliferation.', *Biomaterials*, 30(4), pp. 556-564.

Chu, C. C. (1981) 'An in-vitro study of the effect of buffer on the degradation of poly(glycolic acid) sutures', *J Biomed Mater Res*, 15(1), pp. 19-27.

Cieplik, F., Deng, D., Crielaard, W., Buchalla, W., Hellwig, E., Al-Ahmad, A. and Maisch, T. (2018) 'Antimicrobial photodynamic therapy – what we know and what we don't', *Critical Reviews in Microbiology*, 44(5), pp. 571-589.

Colombo, F., Sampogna, G., Coccozza, G., Guraya, S. Y. and Forgione, A. (2016) 'Regenerative medicine: Clinical applications and future perspectives.', *Journal of microscopy and ultrastructure*, 5(1), pp. 1-8.

Contreras, A., Raxworthy, M. J., Wood, S., Schiffman, J. D. and Tronci, G. (2019) 'Photodynamically Active Electrospun Fibers for Antibiotic-Free Infection Control', *ACS Applied Bio Materials*, 2(10), pp. 4258-4270.

Contreras, A., Raxworthy, M. J., Wood, S. and Tronci, G. (2020) 'Hydrolytic Degradability, Cell Tolerance and On-Demand Antibacterial Effect of Electrospun Photodynamically Active Fibres', *Pharmaceutics*, 12(8), pp. 711.

Cortez Tornello, P. R., Ballarin, F. M., Caracciolo, P. C. and Abraham, G. A. (2016) 'Chapter 8 - Micro/nanofiber-based scaffolds for soft tissue engineering applications: Potential and current challenges', in Grumezescu, A.M. (ed.) *Nanobiomaterials in Soft Tissue Engineering*: William Andrew Publishing, pp. 201-229.

Costa, A. C., Campos Rasteiro, V. M., da Silva Hashimoto, E. S., Araujo, C. F., Pereira, C. A., Junqueira, J. C. and Jorge, A. O. (2012) 'Effect of erythrosine- and LED-mediated photodynamic therapy on buccal candidiasis infection of immunosuppressed mice and *Candida albicans* adherence to buccal epithelial cells', *Oral Surg Oral Med Oral Pathol Oral Radiol*, 114(1), pp. 67-74.

Costa, A. C., de Campos Rasteiro, V. M., Pereira, C. A., da Silva Hashimoto, E. S., Beltrame, M., Jr., Junqueira, J. C. and Jorge, A. O. (2011) 'Susceptibility of *Candida albicans* and *Candida dubliniensis* to erythrosine- and LED-mediated photodynamic therapy', *Arch Oral Biol*, 56(11), pp. 1299-305.

Costa, S. R. d., Monteiro, M. d. C., da Silva Júnior, F. M. R. and Sandrini, J. Z. (2016) 'Methylene blue toxicity in zebrafish cell line is dependent on light exposure', *Cell Biology International*, 40(8), pp. 895-905.

Costantini, M. and Barbetta, A. (2018) '6 - Gas foaming technologies for 3D scaffold engineering', in Deng, Y. and Kuiper, J. (eds.) *Functional 3D Tissue Engineering Scaffolds*: Woodhead Publishing, pp. 127-149.

Courtenay, J. C., Deneke, C., Lanzoni, E. M., Costa, C. A., Bae, Y., Scott, J. L. and Sharma, R. I. (2017) 'Modulating cell response on cellulose surfaces; tunable attachment and scaffold mechanics', *Cellulose*, 25(2), pp. 925-940.

Cui, L., Wu, Y., Cen, L., Zhou, H., Yin, S., Liu, G., Liu, W. and Cao, Y. (2009) 'Repair of articular cartilage defect in non-weight bearing areas using adipose derived stem cells loaded polyglycolic acid mesh', *Biomaterials*, 30(14), pp. 2683-93.

Cui, W., Li, X., Zhu, X., Yu, G., Zhou, S. and Weng, J. (2006) 'Investigation of Drug Release and Matrix Degradation of Electrospun Poly(dl-lactide) Fibers with Paracetamol Inoculation.', *Biomacromolecules*, 7(5), pp. 1623-1629.

Cui, X., Hartanto, Y., Wu, C., Bi, J., Dai, S. and Zhang, H. (2018) 'Tuning microenvironment for multicellular spheroid formation in thermo-responsive anionic microgel scaffolds.', *Journal of Biomedical Materials Research Part A*, 106(11), pp. 2899-2909.

Cunha-Reis, C., TuzlaKoglu, K., Baas, E., Yang, Y., El Haj, A. and Reis, R. L. (2007) 'Influence of porosity and fibre diameter on the degradation of chitosan fibre-mesh scaffolds and cell adhesion', *J Mater Sci Mater Med*, 18(2), pp. 195-200.

Dai, T., Huang, Y. Y. and Hamblin, M. R. (2009) 'Photodynamic therapy for localized infections--state of the art', *Photodiagnosis Photodyn Ther*, 6(3-4), pp. 170-88.

De Sanctis, M., Zucchelli, G. and Clauser, C. (1996) 'Bacterial colonization of barrier material and periodontal regeneration.', *J Clin Periodontol*, 23, pp. 1039-1046.

DeRosa, M. C. and Crutchley, R. J. (2002) 'Photosensitized singlet oxygen and its applications', *Coordination Chemistry Reviews*, 233-234, pp. 351-371.

Deshawar, D., Gupta, K. and Chokshi, P. (2020) 'Electrospinning of polymer solutions: An analysis of instability in a thinning jet with solvent evaporation', *Polymer*, 202, pp. 122656.

Diniz, I. M., Teixeira, K. I., Araujo, P. V., Araujo, M. S., Marques, M. M., Poletto, L. T. and Cortes, M. E. (2014) 'Evaluation of antibacterial

photodynamic therapy effects on human dental pulp cell cultures', *Photodiagnosis Photodyn Ther*, 11(3), pp. 300-6.

Dogandzhiyska, V., Gergova, R., Dimitrov, S. and Doychinova, M. (2013) 'Antimicrobial activity of Photodynamic therapy against microorganisms isolated from deep carious lesions.', *J of IMAB*, 19 (4), pp. 430-434. .

Donlan, R. M. (2001) 'Biofilm Formation: A Clinically Relevant Microbiological Process.', *Clinical Infectious Diseases*, 33, pp. 1387-92.

Donnelly, H., Dalby, M. J., Salmeron-Sanchez, M. and Sweeten, P. E. (2018) 'Current approaches for modulation of the nanoscale interface in the regulation of cell behavior', *Nanomedicine*, 14(7), pp. 2455-2464.

Dorati, R., DeTrizio, A., Modena, T., Conti, B., Benazzo, F., Gastaldi, G. and Genta, I. (2017) 'Biodegradable Scaffolds for Bone Regeneration Combined with Drug-Delivery Systems in Osteomyelitis Therapy', *Pharmaceuticals (Basel)*, 10(4).

dos Santos, A. F., Terra, L. F., Wailemann, R. A. M., Oliveira, T. C., Gomes, V., Mineiro, M. F., Meotti, F. C., Bruni-Cardoso, A., Baptista, M. S. and Labriola, L. (2017) 'Methylene blue photodynamic therapy induces selective and massive cell death in human breast cancer cells', *BMC Cancer*, 17(1).

Du, B., Yin, H., Chen, Y., Lin, W., Wang, Y., Zhao, D., Wang, G., He, X., Li, J., Li, Z., Luo, F., Tan, H. and Fu, Q. (2020) 'A waterborne polyurethane 3D scaffold containing PLGA with a controllable degradation rate and an anti-inflammatory effect for potential applications in neural tissue repair', *J Mater Chem B*, 8(20), pp. 4434-4446.

Eichner, A., Gonzales, F. P. and Maisch, T. (2013) 'Antimicrobial PDT for Clinical Infectious Diseases', in Hamblin, M.R. and Huang, Y. (eds.) *Handbook of Photomedicine*: Taylor & Francis, pp. 385-393.

Elgali, I., Omar, O., Dahlin, C. and Thomsen, P. (2017) 'Guided bone regeneration: materials and biological mechanisms revisited', *Eur J Oral Sci*, 125(5), pp. 315-337.

Eltom, A., Zhong, G. and Muhammad, A. (2019) 'Scaffold Techniques and Designs in Tissue Engineering Functions and Purposes: A Review', *Advances in Materials Science and Engineering*, 2019, pp. 3429527.

- Ergaieg, K. and Seux, R. (2009) 'A comparative study of the photoinactivation of bacteria by meso-substituted cationic porphyrin, rose Bengal and methylene blue', *Desalination*, 246 pp. 353-362.
- Feng, K., Sun, H., Bradley, M. A., Dupler, E. J., Giannobile, W. V. and Ma, P. X. (2010) 'Novel antibacterial nanofibrous PLLA scaffolds', *J Control Release*, 146(3), pp. 363-9.
- Fierz, F. C., Beckmann, F., Huser, M., Irsen, S. H., Leukers, B., Witte, F., Degistirici, O., Andronache, A., Thie, M. and Muller, B. (2008) 'The morphology of anisotropic 3D-printed hydroxyapatite scaffolds', *Biomaterials*, 29(28), pp. 3799-806.
- Filippi, M., Born, G., Chaaban, M. and Scherberich, A. (2020) 'Natural Polymeric Scaffolds in Bone Regeneration', *Frontiers in Bioengineering and Biotechnology*, 8(474).
- Fontana, C. R., Lerman, M. A., Patel, N., Grecco, C., Costa, C. A., Amiji, M. M., Bagnato, V. S. and Soukos, N. S. (2013) 'Safety assessment of oral photodynamic therapy in rats', *Lasers Med Sci*, 28(2), pp. 479-86.
- Foote, C. S. (1991) 'DEFINITION OF TYPE I AND TYPE II PHOTSENSITIZED OXIDATION', *Photochemistry und Photobiology*, 54(5), pp. 659.
- Fracalossi, C., Nagata, J. Y., Pelloso, D. S., Terada, R. S., Hioka, N., Baesso, M. L., Sato, F., Rosalen, P. L., Caetano, W. and Fujimaki, M. (2016) 'Singlet oxygen production by combining erythrosine and halogen light for photodynamic inactivation of *Streptococcus mutans*', *Photodiagnosis Photodyn Ther*, 15, pp. 127-32.
- Frazza, E. J. and Schmitt, E. E. (1971) 'A new absorbable suture.', *J. Biomed. Mater. Res.*, 5(2), pp. 43-58.
- Fredenberg, S., Wahlgren, M., Reslow, M. and Axelsson, A. (2011) 'The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems—A review', *International journal of pharmaceuticals*, 415, pp. 34-52.
- Furtos, G., Rivero, G., Rapuntean, S. and Abraham, G. A. (2017) 'Amoxicillin-loaded electrospun nanocomposite membranes for dental

applications', *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 105(5), pp. 966-976.

Garapati, C., Clarke, B., Zadora, S., Burney, C., Cameron, B. D., Fournier, R., Baugh, R. F. and Boddu, S. H. (2015) 'Development and characterization of erythrosine nanoparticles with potential for treating sinusitis using photodynamic therapy', *Photodiagnosis Photodyn Ther*, 12(1), pp. 9-18.

Garg, A. D., Bose, M., Ahmed, M. I., Bonass, W. A. and Wood, S. R. (2012) 'In vitro studies on erythrosine-based photodynamic therapy of malignant and pre-malignant oral epithelial cells', *PLoS One*, 7(4), pp. e34475.

Garg, T. (2011) 'Scaffold: Tissue engineering and regenerative medicine', *International Research Journal of Pharmacy*, 2.

Generali, M., Dijkman, P. E. and Hoerstrup, S. P. (2014) 'Bioresorbable Scaffolds For Cardiovascular Tissue Engineering', *EMJ Int Cardiol.*, 1, pp.:91-99.

George, S., Hamblin, M. R. and Kishen, A. (2009) 'Uptake pathways of anionic and cationic photosensitizers into bacteria', *Photochemical & Photobiological Sciences*, 8(6).

Gillispie, G. J., Park, J., Copus, J. S., Pallickaveedu Rajan Asari, A. K., Yoo, J. J., Atala, A. and Lee, S. J. (2019) 'Chapter 47 - Three-Dimensional Tissue and Organ Printing in Regenerative Medicine', in Atala, A., Lanza, R., Mikos, A.G. and Nerem, R. (eds.) *Principles of Regenerative Medicine (Third Edition)*. Boston: Academic Press, pp. 831-852.

Ginimuge, P. R. and Joythi, S. D. (2010) 'Methylene Blue: revisited', *Journal of Anaesthesiology, Clinical Pharmacology*, 26(4), pp. 517-520.

Gittens, R. A., Scheideler, L., Rupp, F., Hyzy, S. L., Geis-Gerstorfer, J., Schwartz, Z. and Boyan, B. D. (2014) 'A review on the wettability of dental implant surfaces II: Biological and clinical aspects', *Acta Biomater*, 10(7), pp. 2907-18.

Giuliani, A., Moroncini, F., Mazzoni, S., Belicchi, M. L., Villa, C., Erratico, S., Colombo, E., Calcaterra, F., Brambilla, L., Torrente, Y., Albertini, G. and Della Bella, S. (2014) 'Polyglycolic acid-polylactic acid scaffold response to different progenitor cell in vitro cultures: a demonstrative and comparative X-

ray synchrotron radiation phase-contrast microtomography study', *Tissue Eng Part C Methods*, 20(4), pp. 308-16.

Goldberg, M. and Hirata, A. (2017) 'The Dental Pulp: Composition, Properties and Functions.', *JSM Dent*, 5(1), pp. 1079.

Gorth, D. and J Webster, T. (2011) '10 - Matrices for tissue engineering and regenerative medicine', in Lysaght, M. and Webster, T.J. (eds.) *Biomaterials for Artificial Organs*: Woodhead Publishing, pp. 270-286.

Grant, C. N., Mojica, S. G., Sala, F. G., Hill, J. R., Levin, D. E., Speer, A. L., Barthel, E. R., Shimada, H., Zachos, N. C. and Grikscheit, T. C. (2015) 'Human and mouse tissue-engineered small intestine both demonstrate digestive and absorptive function', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 308(8), pp. G664-G677.

Green, H., Kehinde, O. and Thomas, J. (1979) 'Growth of cultured human epidermal cells into multiple epithelia suitable for grafting', *Proc. Natl. Acad. Sci. USA*, 76(11), pp. 5665-5668.

Guo, Y., Xu, L., Hong, S., Sun, Q., Yao, W. and Pei, R. (2016) 'Label-free DNA-based biosensors using structure-selective light-up dyes', *The Analyst*, 141(24), pp. 6481-6489.

Gursoy, H., Ozcakir-Tomruk, C., Tanalp, J. and Yilmaz, S. (2013) 'Photodynamic therapy in dentistry: a literature review', *Clin Oral Investig*, 17(4), pp. 1113-25.

Haider, A., Haider, S. and Kang, I. (2015) 'A comprehensive review summarizing the effect of electrospinning parameters and potential applications of nanofibers in biomedical and biotechnology', *Arabian Journal of Chemistry*, 11(8), pp. 1165-1188.

Hamblin, M. R. and Hasan, T. (2004) 'Photodynamic therapy: a new antimicrobial approach to infectious disease?', *Photochem. Photobiol. Sci.*, 3, pp. 436-450.

Han, E. X. (2019) 'Arterial Grafts for Transplantation', in Reis, R.L. (ed.) *Encyclopedia of Tissue Engineering and Regenerative Medicine*. Oxford: Academic Press, pp. 169-177.

Hartmann-Fritsch, F., Biedermann, T., Braziulis, E., Luginbühl, J., Pontiggia, L., Böttcher-Haberzeth, S., van Kuppevelt, T. H., Faraj, K. A., Schiestl, C., Meuli, M. and Reichmann, E. (2016) 'Collagen hydrogels strengthened by biodegradable meshes are a basis for dermo-epidermal skin grafts intended to reconstitute human skin in a one-step surgical intervention', *J Tissue Eng Regen Med*, 10(1), pp. 81-91.

Hasan, M. M., Azim, A. Y. M. A. and Reza, M. S. (2014) 'Effect of Electrospinning Parameters on Fiber Morphology of Tissue Engineering Scaffolds: A Review', *Journal of Fashion Technology & Textile Engineering*, 02(03).

Hassan, M. I., Chong, L. H. and Sultana, N. (2016) 'Wettability and water uptake properties of PLA and PCL/ Gelatin-based electrospun scaffolds.', *ARPJ Journal of Engineering and Applied Sciences*, 11(23), pp. 13604-13607.

Hayek, R. R. A., Araújo, N. S., Gioso, M. A., Ferreira, J., Baptista-Sobrinho, C. A., Jr., A. M. Y. and Ribeiro, M. S. (2005) 'Comparative Study Between the Effects of Photodynamic Therapy and Conventional Therapy on Microbial Reduction in Ligature-Induced Peri-Implantitis in Dogs', *J Periodontol* 76, pp. 1275-1281.

Heydarnejad, M. S., Yarmohammadi-Samani, P., Dehkordi, M. M., Shadkhast, M. and Rahnema, S. (2013) 'Histopathological effects of nanosilver (Ag-NPs) in liver after dermal exposure during wound healing.', *Nanomedicine Journal*, 1(3), pp. 191-197.

Hoang, V. T., Stepniewski, G., Czarnecka-Chrebelska, K., Kasztelan, R., Van, C., Khoa, D., Shao, L.-Y., Smietana, M. and Buczynski, R. (2019) 'Optical Properties of Buffers and Cell Culture Media for Optofluidic and Sensing Applications', *Applied Sciences*, 9, pp. 1145.

Hosseinkhani, M., Mehrabani, D., Karimfar, M. H., Bakhtiyari, S., Manafi, A. and Shirazi, R. (2014) 'Tissue Engineered Scaffolds in Regenerative Medicine', *World J Plast Surg.*, 3(1), pp. 3-7.

Husain, O., Lau, W., Edirisinghe, M. and Parhizkar, M. (2016) 'Investigating the particle to fibre transition threshold during electrohydrodynamic atomization of a polymer solution', *Mater Sci Eng C Mater Biol Appl*, 65, pp. 240-50.

Hutmacher, D. W., Woodfield, T. B. F. and Dalton, P. D. (2014) 'Chapter 10 - Scaffold Design and Fabrication', in Blitterswijk, C.A.V. and De Boer, J. (eds.) *Tissue Engineering (Second Edition)*. Oxford: Academic Press, pp. 311-346.

Jamal, M., Tasneem, U., Hussain, T. and Andleeb, S. (2015) 'Bacterial biofilm: its composition, formation and role in human infections.', *Research & Review: Journal of Microbiology and Biotechnology*, 4(3), pp. 1-14.

Johnson, C. T. and Garcia, A. J. (2015) 'Scaffold-based anti-infection strategies in bone repair', *Ann Biomed Eng*, 43(3), pp. 515-28.

Johnston, C., Martin, B., Fichant, G., Polard, P. and Claverys, J. P. (2014) 'Bacterial transformation: distribution, shared mechanisms and divergent control', *Nat Rev Microbiol*, 12(3), pp. 181-96.

Jones, T. D., Naimipour, H., Sun, S., Cho, M. and Alapati, S. B. (2015) 'Mechanical changes in human dental pulp stem cells during early odontogenic differentiation', *J Endod*, 41(1), pp. 50-5.

Jori, G., Camerin, M., Soncin, M., Guidolin, L. and Coppellotti, O. (2011) 'Chapter 1. Antimicrobial Photodynamic Therapy: Basic Principles', 0, pp. 1-18.

Jori, G., Fabris, C., Soncin, M., Ferro, S., Coppellotti, O., Dei, D., Fantetti, L., Chiti, G. and Roncucci, G. (2006) 'Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications.', *Lasers Surg Med*, 38(5), pp. 468-81.

Jose, A. J. and Alagar, M. (2015) '3 - Preparation and characterization of polysulfone-based nanocomposites', in Mittal, V. (ed.) *Manufacturing of Nanocomposites with Engineering Plastics*: Woodhead Publishing, pp. 31-59.

Josefsen, L. B. and Boyle, R. W. (2008) 'Photodynamic Therapy and the Development of Metal-Based Photosensitisers', *Metal-Based Drugs*, 2008, pp. 276109.

Joubert, K. S., George, B. P., Razlog, R. and Abrahamse, H. (2020) 'A Review of Preclinical Research on the Effects of Photodynamic Therapy and Homeopathic Medicine on Cancer Cells', *Altern Ther Health Med*.

- Ju, S., Huang, W. and Chen, C. (2014) 'The hydrolysis mechanism of polyglycolic acid under tensile mechanical loading: a density functional theory study.', *RSC Adv.*, 4(68), pp. 35862-35867.
- Jun, I., Han, H. S., Edwards, J. R. and Jeon, H. (2018) 'Electrospun Fibrous Scaffolds for Tissue Engineering: Viewpoints on Architecture and Fabrication', *Int J Mol Sci*, 19(3).
- Junqueira Garcia, M. T., Pedralino Goncalves, T., Sao Felix Martins, E., Silva Martins, T., Carvalho de Abreu Fantini, M., Regazi Minarini, P. R., Costa Fernandez, S., Cassone Salata, G. and Biagini Lopes, L. (2018) 'Improvement of cutaneous delivery of methylene blue by liquid crystals', *Int J Pharm*, 548(1), pp. 454-465.
- Junqueira, H. C., Severino, D., Dias, L. G., Gugliotti, M. S. and Baptista, M. S. (2002) 'Modulation of methylene blue photochemical properties based on adsorption at aqueous micelle interfaces', *Physical Chemistry Chemical Physics*, 4(11), pp. 2320-2328.
- Kajbafzadeh, A. M., Fendereski, K., Khorramirouz, R., Daryabari, S. S., Masoomi, A., Moosavi, S., Ataei, M. and Arshadi, H. (2020) 'In vivo application of decellularized rat colon and evaluation of the engineered scaffolds following 9 months of follow-up', *Cell Biol Int*.
- Kalka, K., Merk, H. and Mukhtar, H. (2000) 'Photodynamic therapy in dermatology', *J Am Acad Dermatol* 42, pp. 389-413.
- Kamaly, N., Yameen, B., Wu, J. and Farokhzad, O. C. (2016) 'Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release', *Chem Rev*, 116(4), pp. 2602-63.
- Kamocki, K., Nor, J. E. and Bottino, M. C. (2015) 'Effects of ciprofloxacin-containing antimicrobial scaffolds on dental pulp stem cell viability-In vitro studies', *Arch Oral Biol*, 60(8), pp. 1131-7.
- Kanyathare, B., Asamoah, B. O., Ishaq, U., Amoani, J., Rätty, J. and Peiponen, K.-E. (2020) 'Optical transmission spectra study in visible and near-infrared spectral range for identification of rough transparent plastics in aquatic environments', *Chemosphere*, 248, pp. 126071.

- Kato, H., Komagoe, K., Nakanishi, Y., Inoue, T. and Katsu, T. (2012) 'Xanthene dyes induce membrane permeabilization of bacteria and erythrocytes by photoinactivation', *Photochem Photobiol*, 88(2), pp. 423-31.
- Kawashima, N. (2012) 'Characterisation of dental pulp stem cells: a new horizon for tissue regeneration?', *Arch Oral Biol*, 57(11), pp. 1439-58.
- Ke, E., Nazzal, S., Tseng, Y. H., Chen, C. P. and Tsai, T. (2012) 'Erythrosine-Mediated Photodynamic Inactivation of Bacteria and Yeast Using Green Light-Emitting Diode Light', *Journal of Food and Drug Analysis*, 20.
- Kennedy, J. C., Marcus, S. L. and Pottier, R. H. (1996) 'Photodynamic therapy (PDT) and photodiagnosis (PD) using endogenous photosensitization induced by 5-aminolevulinic acid (ALA): mechanisms and clinical results', *J Clin Laser Med Surg*, 14(5), pp. 289-304.
- Khalkhal, E., Razzaghi, M., Rostami-Nejad, M., Rezaei-Tavirani, M., Heidari Beigvand, H. and Rezaei Tavirani, M. (2020) 'Evaluation of Laser Effects on the Human Body After Laser Therapy', *Journal of lasers in medical sciences*, 11(1), pp. 91-97.
- Khorshidi, S. and Karkhaneh, A. (2018) 'Particle-coated electrospun scaffold: A semi-conductive drug eluted scaffold with layered fiber/particle arrangement', *Journal of Biomedical Materials Research Part A*, 106(12), pp. 3248-3254.
- Kiedrowski, K., Jakobs, F., Kielhorn, J., Johannes, H.-H., Kowalsky, W., Kracht, D., Balasa, I. and Ristau, D. (2020) *Laser-induced degradation and damage morphology in polymer optical fibers. SPIE Photonics Europe: SPIE*, p. EPE.
- Kim, B. and Mooney, D. J. (1998) 'Development of biocompatible synthetic extracellular matrices for tissue engineering', *TIBTECH* 16, pp. 224-230.
- Kim, J. A., Choi, J. H., Kim, M., Rhee, W. J., Son, B., Jung, H. K. and Park, T. H. (2013) 'High-throughput generation of spheroids using magnetic nanoparticles for three-dimensional cell culture', *Biomaterials*, 34(34), pp. 8555-63.

- Kim, M. M. and Darafsheh, A. (2020) 'Light Sources and Dosimetry Techniques for Photodynamic Therapy', *Photochem Photobiol*, 96(2), pp. 280-294.
- Kim, M. S., Kim, J. H., Min, B. H., Chun, H. J., Han, D. K. and Lee, H. B. (2011) 'Polymeric Scaffolds for Regenerative Medicine', *Polymer Reviews*, 51(1), pp. 23-52.
- Ko, Y., Park, J. H., Lee, J. B., Oh, H. H., Park, W. H., Cho, D. and Kwon, O. H. (2016) 'Growth behavior of endothelial cells according to electrospun poly(D,L-lactic-co-glycolic acid) fiber diameter as a tissue engineering scaffold.', *Tissue Engineering and Regenerative Medicine*, 13(4), pp. 343-351.
- Kochevar, I. E. and Redmond, R. W. (2000) 'Photosensitized Production of Singlet Oxygen', *METHODS IN ENZYMOLOGY* 319, pp. 20-28.
- Konopka, K. and Goslinski, T. (2007) 'Photodynamic Therapy in Dentistry', *J Dent Res*, 86(8), pp. 694-707.
- Kundu, J., Pati, F., Hun Jeong, Y. and Cho, D.-W. (2013) 'Chapter 2 - Biomaterials for Biofabrication of 3D Tissue Scaffolds', in Forgacs, G. and Sun, W. (eds.) *Biofabrication*. Boston: William Andrew Publishing, pp. 23-46.
- Kurobe, H., Maxfield, M. W., Tara, S., Rocco, K. A., Bagi, P. S., Yi, T., Udelsman, B., Zhuang, Z. W., Cleary, M., Iwakiri, Y., Breuer, C. K. and Shinoka, T. (2015) 'Development of small diameter nanofiber tissue engineered arterial grafts', *PLoS One*, 10(4), pp. e0120328.
- Kushibiki, T., Tu, Y., Abu-Yousif, A. O. and Hasan, T. (2015) 'Photodynamic activation as a molecular switch to promote osteoblast cell differentiation via AP-1 activation', *Sci Rep*, 5, pp. 13114.
- Langhans, S. A. (2018) 'Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning', *Frontiers in Pharmacology*, 9.
- Laxminarayan, R., Matsoso, P., Pant, S., Brower, C., Røttingen, J. A., Klugman, K. and Davies, S. (2016) 'Access to effective antimicrobials: a worldwide challenge', *Lancet*, 387(10014), pp. 168-75.

- Leach, M. K., Feng, Z. Q., Tuck, S. J. and Corey, J. M. (2011) 'Electrospinning fundamentals: optimizing solution and apparatus parameters', *J Vis Exp*, (47).
- Lerminiaux, N. A. and Cameron, A. D. S. (2019) 'Horizontal transfer of antibiotic resistance genes in clinical environments', *Can J Microbiol*, 65(1), pp. 34-44.
- Li, L., Shi, X., Guo, X., Li, H. and Xu, C. (2014) 'Ionic protein-lipid interaction at the plasma membrane: what can the charge do?', *Trends Biochem Sci*, 39, pp. 130-140.
- Li, W. J., Cooper, J. A., Jr., Mauck, R. L. and Tuan, R. S. (2006) 'Fabrication and characterization of six electrospun poly(alpha-hydroxy ester)-based fibrous scaffolds for tissue engineering applications', *Acta Biomater*, 2(4), pp. 377-85.
- Li, X., Lovell, J. F., Yoon, J. and Chen, X. (2020) 'Clinical development and potential of photothermal and photodynamic therapies for cancer', *Nat Rev Clin Oncol*.
- Li, X. Z. and Nikaido, H. (2004) 'Efflux-mediated drug resistance in bacteria', *Drugs*, 64(2), pp. 159-204.
- Li, Y., Chu, Z., Li, X., Ding, X., Guo, M., Zhao, H., Yao, J., Wang, L., Cai, Q. and Fan, Y. (2017) 'The effect of mechanical loads on the degradation of aliphatic biodegradable polyesters', *Regenerative biomaterials*, 4(3), pp. 179-190.
- Li, Z. and Wang, C. (2013) 'Chapter 2 Effects of Working Parameters on Electrospinning', *One-Dimensional Nanostructures Electrospinning Technique and Unique Nanofibres: SpringerBriefs in Materials*, pp. 15-28.
- Liu, F.-H., Lee, R.-T., Lin, W.-H. and Liao, Y.-S. (2013) 'Selective Laser Sintering of Bio-Metal Scaffold', *Procedia CIRP*, 5, pp. 83-87.
- Liu, X., Liu, S. and Cui, W. (2014) 'Evaluation of oriented electrospun fibers for periosteal flap regeneration in biomimetic triphasic osteochondral implant', *J Biomed Mater Res B Appl Biomater*, 102(7), pp. 1407-14.
- Liu, Y., Qin, R., Zaat, S. A. J., Breukink, E. and Heger, M. (2015) 'Antibacterial photodynamic therapy: overview of a promising approach to

fight antibiotic-resistant bacterial infections', *Journal of clinical and translational research*, 1(3), pp. 140-167.

Liu, Y., Zhou, X., Wang, X., Liang, K., Yang, Z., Shen, C., Imran, M., Sahar, S. and Xu, A. (2017) 'Hydrogenation/oxidation induced efficient reversible color switching between methylene blue and leuco-methylene blue', *RSC Advances*, 7(48), pp. 30080-30085.

Lo, P.-C., Rodríguez-Morgade, M. S., Pandey, R. K., Ng, D. K. P., Torres, T. and Dumoulin, F. (2020) 'The unique features and promises of phthalocyanines as advanced photosensitisers for photodynamic therapy of cancer', *Chemical Society Reviews*, 49(4), pp. 1041-1056.

LoGuidice, A., Houlihan, A. and Deans, R. (2016) 'Multipotent adult progenitor cells on an allograft scaffold facilitate the bone repair process', *J Tissue Eng*, 7, pp. 2041731416656148.

Longo, J. P., Leal, S. C., Simoni, A. R., de Fatima Menezes Almeida-Santos, M., Tedesco, A. C. and Azevedo, R. B. (2012) 'Photodynamic therapy disinfection of carious tissue mediated by aluminium-chloride-phthalocyanine entrapped in cationic liposomes: an in vitro and clinical study.', *Lasers Med Sci*, 27(3).

Low, Y. J., Andriyana, A., Ang, B. C. and Zainal Abidin, N. I. (2020) 'Bioresorbable and degradable behaviors of PGA: Current state and future prospects', *Polymer Engineering & Science*, n/a(n/a).

Lu, X., Lee, Y., Yang, S., Hao, Y., Evans, J. R. G. and Parini, C. G. (2010) 'Solvent-based paste extrusion solid freeforming', *Journal of the European Ceramic Society*, 30(1), pp. 1-10.

Lutkus, L., Rickenbach, S. and McCormick, T. (2019) 'Singlet Oxygen Quantum Yields Determined by Oxygen Consumption', *Journal of Photochemistry and Photobiology A: Chemistry*, 378.

Macdonald, I. J. and Dougherty, T. J. (2001) 'Basic principles of photodynamic therapy', *Journal of Porphyrins and Phthalocyanines*, 05(02), pp. 105-129.

MacDonald, I. J., Morgan, J., Bellnier, D. A., Paszkiewicz, G. M., Whitaker, J. E., Litchfield, D. J. and Dougherty, T. J. (1999) 'Subcellular Localization Patterns and Their Relationship to Photodynamic Activity of

Pyropheophorbide-a Derivatives', *Photochemistry and Photobiology*, 70, pp. 789-797.

Macha, I. J., Karacan, I., Ben-Nissan, B., Cazalbou, S. and Müller, W. H. (2018) 'Development of antimicrobial composite coatings for drug release in dental, orthopaedic and neural prostheses applications', *SN Applied Sciences*, 1(1).

Mackintosh, S. B., Serino, L. P., Iddon, P. D., Brown, R., Conlan, R. S., Wright, C. J., Maffeis, T. G., Raxworthy, M. J. and Sheldon, I. M. (2015) 'A three-dimensional model of primary bovine endometrium using an electrospun scaffold', *Biofabrication*, 7(2), pp. 025010.

Mahmoudifar, N. and Doran, P. M. (2010) 'Chondrogenic differentiation of human adipose-derived stem cells in polyglycolic acid mesh scaffolds under dynamic culture conditions', *Biomaterials*, 31(14), pp. 3858-67.

Maisch, T. (2007) 'Anti-microbial photodynamic therapy: useful in the future?', *Lasers Med Sci*, 22(2), pp. 83-91.

Makadia, H. K. and Siegel, S. J. (2011) 'Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier', *Polymers*, 3(3), pp. 1377-1397.

Mandla, S. and Radisic, M. (2019) 'Chapter 61 - Cardiac Tissue', in Atala, A., Lanza, R., Mikos, A.G. and Nerem, R. (eds.) *Principles of Regenerative Medicine (Third Edition)*. Boston: Academic Press, pp. 1073-1099.

Marotti, J., Tortamano, P., Cai, S., Ribeiro, M. S., Franco, J. E. and de Campos, T. T. (2013) 'Decontamination of dental implant surfaces by means of photodynamic therapy', *Lasers Med Sci*, 28(1), pp. 303-9.

Marsich, E., Bellomo, F., Turco, G., Travan, A., Donati, I. and Paoletti, S. (2013) 'Nano-composite scaffolds for bone tissue engineering containing silver nanoparticles: preparation, characterization and biological properties', *J Mater Sci Mater Med*, 24(7), pp. 1799-807.

Marta, S., Sol, G., Lilian, S. V., Andrés, L., María, D. M., Martín, A., Francisco, A. and Jose, M. V. (2017) 'Effects of photodynamic therapy in periodontal treatment: A randomized, controlled clinical trial.', *J Clin Periodontol.*, 44, pp. 915-925.

Martins, W. K., Santos, N. F., Rocha, C. S., Bacellar, I. O. L., Tsubone, T. M., Viotto, A. C., Matsukuma, A. Y., Abrantes, A. B. P., Siani, P., Dias, L. G. and Baptista, M. S. (2018) 'Parallel damage in mitochondria and lysosomes is an efficient way to photoinduce cell death', *Autophagy*, pp. 1-21.

Martínez-Pérez, C. A., Olivas-Armendariz, I., Castro-Carmona, J. S. and García-Casillas, P. E. (2011) 'Scaffolds for tissue engineering via thermally induced phase separation', *Advances in regenerative medicine*, pp. 275-294.

Mason, C. and Dunhill, P. (2008) 'A brief definition of regenerative medicine.', *Regen Med*, 3(1), pp. 1-5.

Masood, S. H. (2014) '10.04 - Advances in Fused Deposition Modeling', in Hashmi, S., Batalha, G.F., Van Tyne, C.J. and Yilbas, B. (eds.) *Comprehensive Materials Processing*. Oxford: Elsevier, pp. 69-91.

McShan, D., Ray, P. C. and Yu, H. (2014) 'Molecular toxicity mechanism of nanosilver', *J Food Drug Anal*, 22(1), pp. 116-127.

Meisel, P. and Kocher, T. (2005) 'Photodynamic therapy for periodontal diseases: state of the art', *J Photochem Photobiol B*, 79(2), pp. 159-70.

Melo, W. C. M. A., Castro, L. F., Dal'Mas, R. M. M. T. S. and Perussi, J. R. 'Effectiveness of photodynamic therapy on Gram-negative bacteria.'. *Science against Microbial Pathogens: Communicating Current research and Technological Advances.*, *FORMATEX 2011*: Formatex Research Centre.

Meng, Z. X., Zheng, W., Li, L. and Zheng, Y. F. (2011) 'Fabrication, characterization and in vitro drug release behavior of electrospun PLGA/chitosan nanofibrous scaffold', *Materials Chemistry and Physics*, 125(3), pp. 606-611.

Metcalf, D., Robinson, C., Devine, D. and Wood, S. (2006) 'Enhancement of erythrosine-mediated photodynamic therapy of *Streptococcus mutans* biofilms by light fractionation', *J Antimicrob Chemother*, 58(1), pp. 190-2.

Misba, L., Zaidi, S. and Khan, A. U. (2017) 'A comparison of antibacterial and antibiofilm efficacy of phenothiazinium dyes between Gram positive and Gram negative bacterial biofilm', *Photodiagnosis and Photodynamic Therapy*, 18, pp. 24-33.

- Mo, X., Sun, B., Wu, T. and Li, D. (2019) 'Chapter 24 - Electrospun Nanofibers for Tissue Engineering', in Ding, B., Wang, X. and Yu, J. (eds.) *Electrospinning: Nanofabrication and Applications*: William Andrew Publishing, pp. 719-734.
- Mohiti-Asli, M. and Lobo, E. G. (2016) '23 - Nanofibrous smart bandages for wound care.', in Agren, M.S. (ed.) *Wound Healing Biomaterials*: Woodhead Publishing, pp. 483-499.
- Montes de OCa, H. and Ward, I. M. (2006) 'Structure and mechanical properties of PGA crystals and fibres', *Polymer*, 47, pp. 7070-7077.
- Mooney, D. J., Mazzoni, C. L., Breuer, C., McNamara, K., Hern, D., Vacanti, J. P. and Langer, R. (1996) 'Stabilized polyglycolic acid fibre-based tubes for tissue engineering', *Biomaterials*, 17(2), pp. 115-24.
- Mooney, D. J., Sano, K., Kaufmann, P. M., Majahod, K., Schloo, B., Vacanti, J. P. and Langer, R. (1997) 'Long-term engraftment of hepatocytes transplanted on biodegradable polymer sponges', *J Biomed Mater Res*, 37(3), pp. 413-20.
- Moreira, P. L., An, Y. H., Santos, A. R., Jr. and Genari, S. C. (2004) 'In vitro analysis of anionic collagen scaffolds for bone repair', *J Biomed Mater Res B Appl Biomater*, 71(2), pp. 229-37.
- Motamedi, A. S., Mirzadeh, H., Hajiesmaeilbaigi, F., Bagheri-Khoulenjani, S. and Shokrgozar, M. (2017) 'Effect of electrospinning parameters on morphological properties of PVDF nanofibrous scaffolds', *Prog Biomater*, 6(3), pp. 113-123.
- Mpourazanis, G., Mpourazanis, P., Stogiannidis, G. and Ntritsos, G. (2020) 'The effectiveness of photodynamic therapy and cryotherapy on patients with basal cell carcinoma: A systematic review and meta-analysis', *Dermatol Ther*, pp. e13881.
- Mroz, P., Yaroslavsky, A., Kharkwal, G. B. and Hamblin, M. R. (2011) 'Cell death pathways in photodynamic therapy of cancer', *Cancers (Basel)*, 3(2), pp. 2516-39.
- Mukherjee, P., Rani, A. and Saravanan, P. (2019) 'Chapter 4 - Polymeric Materials for 3D Bioprinting', in Ahmad, N., Gopinath, P. and Dutta, R. (eds.) *3D Printing Technology in Nanomedicine*: Elsevier, pp. 63-81.

Munir, K. S., Li, Y. and Wen, C. (2017) '1 - Metallic scaffolds manufactured by selective laser melting for biomedical applications', in Wen, C. (ed.) *Metallic Foam Bone*: Woodhead Publishing, pp. 1-23.

Munita, J. M. and Arias, C. A. (2016) 'Mechanisms of Antibiotic Resistance', *Microbiol Spectr*, 4(2).

Murphy, C. M., Haugh, M. G. and O'Brien, F. J. (2010) 'The effect of mean pore size on cell attachment, proliferation and migration in collagen–glycosaminoglycan scaffolds for bone tissue engineering', *Biomaterials*, 31(3), pp. 461-466.

Muzaffar, A., Ahamed, M. B., Deshmukh, K., Kovářík, T., Křenek, T. and Pasha, S. K. K. (2020) 'Chapter 4 - 3D and 4D printing of pH-responsive and functional polymers and their composites', in Sadasivuni, K.K., Deshmukh, K. and Almaadeed, M.A. (eds.) *3D and 4D Printing of Polymer Nanocomposite Materials*: Elsevier, pp. 85-117.

Nagata, J. Y., Hioka, N., Kimura, E., Batistela, V. R., Terada, R. S., Graciano, A. X., Baesso, M. L. and Hayacibara, M. F. (2012) 'Antibacterial photodynamic therapy for dental caries: evaluation of the photosensitizers used and light source properties', *Photodiagnosis Photodyn Ther*, 9(2), pp. 122-31.

Najmon, J. C., Raeisi, S. and Tovar, A. (2019) '2 - Review of additive manufacturing technologies and applications in the aerospace industry', in Froes, F. and Boyer, R. (eds.) *Additive Manufacturing for the Aerospace Industry*: Elsevier, pp. 7-31.

Nava, H. R., Allamaneni, S. S., Dougherty, T. J., Cooper, M. T., Tan, W., Wilding, G. and Henderson, B. W. (2011) 'Photodynamic therapy (PDT) using HPPH for the treatment of precancerous lesions associated with Barrett's esophagus', *Lasers Surg Med*, 43(7), pp. 705-12.

Nayak, R., Padhye, R., Kyratzis, I. L., Truong, Y. B. and Arnold, L. (2012) 'Effect of viscosity and electrical conductivity on the morphology and fiber diameter in melt electrospinning of polypropylene', *Textile Research Journal*, 83(6), pp. 606-617.

Needleman, I. G., Worthington, H. V., Giedrys-Leeper, E. and Tucker, R. J. (2006) 'Guided tissue regeneration for periodontal infra-bony defects', *Cochrane Database Syst Rev*, (2), pp. CD001724.

- Nesi-Reis, V., Lera-Nonose, D., Oyama, J., Silva-Lalucci, M. P. P., Demarchi, I. G., Aristides, S. M. A., Teixeira, J. J. V., Silveira, T. G. V. and Lonardoni, M. V. C. (2018) 'Contribution of photodynamic therapy in wound healing: A systematic review', *Photodiagnosis Photodyn Ther*, 21, pp. 294-305.
- Nevřelová, P., Kolářová, H., Bajgar, R., Maceček, J., Tomečka, M., Tománková, K. and Strnad, M. (2005) 'Measurement of reactive oxygen species after photodynamic therapy in vitro', *SCRIPTA MEDICA (BRNO)*, 78(5), pp. 281-290.
- Nezarati, R. M., Eifert, M. B. and Cosgriff-Hernandez, E. (2013) 'Effects of humidity and solution viscosity on electrospun fiber morphology', *Tissue Eng Part C Methods*, 19(10), pp. 810-9.
- Nie, K., Han, S., Yang, J., Sun, Q., Wang, X., Li, X. and Li, Q. (2020) 'Enzyme-Crosslinked Electrospun Fibrous Gelatin Hydrogel for Potential Soft Tissue Engineering', *Polymers (Basel)*, 12(9).
- Nowzari, H. and Slots, J. (1995) 'Microbiologic and clinical study of polytetrafluoroethylene membranes for guided bone regeneration around implants.', *International Journal of Oral & Maxillofacial Implants*, 10(1), pp. 87-104.
- Nwachukwu, C. C., Einstein, G. P. and Tulp, O. L. (2017) 'Electrospun Nanofibers as Scaffolds for Wound Healing', *The FASEB Journal*, 31(1).
- O'Brien, F. J. (2011) 'Biomaterials & scaffolds for tissue engineering', *Materials Today*, 14(3), pp. 88-95.
- Obermeier, A., Schneider, J., Fohr, P., Wehner, S., Kuhn, K. D., Stemberger, A., Schieker, M. and Burgkart, R. (2015) 'In vitro evaluation of novel antimicrobial coatings for surgical sutures using octenidine', *BMC Microbiol*, 15, pp. 186.
- Oliveira, C. S., Turchiello, R., Kowaltowski, A. J., Indig, G. L. and Baptista, M. S. (2011) 'Major determinants of photoinduced cell death: Subcellular localization versus photosensitization efficiency', *Free Radic Biol Med*, 51(4), pp. 824-33.
- Olkhov, A. A., Staroverova, O. V., Kuherenko, E. L. and Iordanskii, A. L. (2020) 'Effect of electrospinning solution parameters on the

properties of nonwoven fibrous material based on

polyhydroxybutyrate', *Journal of Physics: Conference Series*, 1431(0120209).

Ong, Y. X. J., Lee, L. Y., Davoodi, P. and Wang, C.-H. (2018) 'Production of drug-releasing biodegradable microporous scaffold using a two-step micro-encapsulation/supercritical foaming process', *The Journal of Supercritical Fluids*, 133, pp. 263-269.

Ormond, A. B. and Freeman, H. S. (2013) 'Dye Sensitizers for Photodynamic Therapy', *Materials (Basel)*, 6(3), pp. 817-840.

Pantò, F., Adamo, L., Giordano, C. and Licciardello, C. (2020) 'Efficacy and safety of photodynamic therapy with RLP068 for diabetic foot ulcers: a review of the literature and clinical experience', *Drugs Context*, 9.

Papenburg, B. (2009) *Design Strategies for Tissue Engineering Scaffolds*. PhD, University of Twente, Gildeprint Drukkerijen, Enschede, The Netherlands.

Park, K. E., Kang, H. K., Lee, S. J., Min, B. and Park, W. H. (2006) 'Biomimetic Nanofibrous Scaffolds: Preparation and Characterization of PGA/Chitin Blend Nanofibers', *Biomacromolecules*, 7(2), pp. 635-643.

Pellosi, D. S., Estevão, B. M., Freitas, C. F., Tsubone, T. M., Caetano, W. and Hioka, N. (2013) 'Photophysical properties of erythrosin ester derivatives in ionic and non-ionic micelles', *Dyes and Pigments*, 99(3), pp. 705-712.

Peter, C., Hongwan, D., Kupfer, A. and Lauterburg, B. H. (2000) 'Pharmacokinetics and organ distribution of intravenous and oral methylene blue.', *Eur J Clin Pharmacol.*, 56(3), pp. 247-50.

Pillai, C. K. and Sharma, C. P. (2010) 'Review paper: absorbable polymeric surgical sutures: chemistry, production, properties, biodegradability, and performance', *J Biomater Appl*, 25(4), pp. 291-366.

Pillay, V., Dott, C., Choonara, Y. E., Tyagi, C., Tomar, L., Kumar, P., du Toit, L. C. and Ndesendo, V. M. K. (2013) 'A Review of the Effect of Processing Variables on the Fabrication of Electrospun Nanofibers for Drug Delivery Applications', *Journal of Nanomaterials*, 2013, pp. 789289.

Poole, K. (2004) 'Efflux-mediated multiresistance in Gram-negative bacteria', *Clin Microbiol Infect*, 10(1), pp. 12-26.

Prasad, A., Sankar, M. R. and Katiyar, V. (2017) 'State of Art on Solvent Casting Particulate Leaching Method for Orthopedic ScaffoldsFabrication', *Materials Today: Proceedings*, 4(2, Part A), pp. 898-907.

Prieto, E. M. and Guelcher, S. A. (2014) '5 - Tailoring properties of polymeric biomedical foams', in Netti, P.A. (ed.) *Biomedical Foams for Tissue Engineering Applications*: Woodhead Publishing, pp. 129-162.

Pye, A. D., Lockhart, D. E., Dawson, M. P., Murray, C. A. and Smith, A. J. (2009) 'A review of dental implants and infection', *J Hosp Infect*, 72(2), pp. 104-10.

Qian, Y., Zhou, X., Zhang, F., Diekwisch, T. G. H., Luan, X. and Yang, J. (2019) 'Triple PLGA/PCL Scaffold Modification Including Silver Impregnation, Collagen Coating, and Electrospinning Significantly Improve Biocompatibility, Antimicrobial, and Osteogenic Properties for Orofacial Tissue Regeneration', *ACS Appl Mater Interfaces*, 11(41), pp. 37381-37396.

Renwick, M. J., Simpkin, V. and Mossialos, E. (2016) 'Targeting innovation in antibiotic drug discovery and development: The need for a One Health – One Europe – One World Framework'.

Rider, P., Kačarević, Ž. P., Alkildani, S., Retnasingh, S. and Barbeck, M. (2018) 'Bioprinting of tissue engineering scaffolds', *Journal of tissue engineering*, 9, pp. 2041731418802090-2041731418802090.

Rineh, A., Bremner, J. B., Hamblin, M. R., Ball, A. R., Tegos, G. P. and Kelso, M. J. (2018) 'Attaching NorA efflux pump inhibitors to methylene blue enhances antimicrobial photodynamic inactivation of Escherichia coli and Acinetobacter baumannii in vitro and in vivo', *Bioorganic & medicinal chemistry letters*, 28(16), pp. 2736-2740.

Rohs, R., Bloch, I., Sklenar, H. and Shakked, Z. (2005) 'Molecular flexibility in ab initio drug docking to DNA: binding-site and binding-mode transitions in all-atom Monte Carlo simulations', *Nucleic Acids Research*, 33(22), pp. 7048-7057.

Rolim, J. P., de-Melo, M. A., Guedes, S. F., Albuquerque-Filho, F. B., de Souza, J. R., Nogueira, N. A., Zanin, I. C. and Rodrigues, L. K. (2012) 'The

antimicrobial activity of photodynamic therapy against *Streptococcus mutans* using different photosensitizers', *J Photochem Photobiol B*, 106, pp. 40-6.

Rosenthal, I. (1991) 'YEARLY REVIEW PHTHALOCYANINES AS PHOTODYNAMIC SENSITIZERS', *Photochemistry and Photobiology*, 53(6), pp. 859-870.

Rousset, N., Bourré, L. and Thibaud, S. (2003) 'Sensitizers in photodynamic therapy', pp. 59-80.

Rout, B., Liu, C.-H. and Wu, W.-C. (2018) 'Increased anti-biofilm efficacy of toluidine blue on *Staphylococcus* species after nano-encapsulation', *Photodiagnosis and Photodynamic Therapy*, 21, pp. 190-200.

Sakai, V. T., Cordeiro, M. M., Dong, Z., Zhang, Z., Zeitlin, B. D. and Nor, J. E. (2011) 'Tooth slice/scaffold model of dental pulp tissue engineering', *Adv Dent Res*, 23(3), pp. 325-32.

Salonitis, K. (2014) '10.03 - Stereolithography', in Hashmi, S., Batalha, G.F., Van Tyne, C.J. and Yilbas, B. (eds.) *Comprehensive Materials Processing*. Oxford: Elsevier, pp. 19-67.

Saravanan, S., Nethala, S., Pattnaik, S., Tripathi, A., Moorthi, A. and Selvamurugan, N. (2011) 'Preparation , characterization and antimicrobial activity of a bio-composite scaffold containing chitosan/ nano-hydroxyapatite/nano-silver for bone tissue engineering.', *Int J Biol Macromol*, 49(2), pp. 188-93.

Semba, J. A., Mieloch, A. A. and Rybka, J. D. (2020) 'Introduction to the state-of-the-art 3D bioprinting methods, design, and applications in orthopedics', *Bioprinting*, 18, pp. e00070.

Sengupta, P., Surwase, S. S. and Prasad, B. L. (2018) 'Modification of porous polyethylene scaffolds for cell attachment and proliferation', *Int J Nanomedicine*, 13, pp. 87-90.

Shang, S., Yang, F., Cheng, X., Walboomers, X. F. and Jansen, J. A. (2010) 'The effect of electrospun fibre alignment on the behaviour of rat periodontal ligament cells', *European Cells and Materials*, 19, pp. 180-192.

- Sharman, W. M., Allen, C. M. and van Lier, J. E. (1999) 'Photodynamic therapeutics: basic principles and clinical applications', *DDT* 4(11), pp. 507-517.
- Shen, J. J., Jemec, G. B. E., Arendrup, M. C. and Saunte, D. M. L. (2020) 'Photodynamic therapy treatment of superficial fungal infections: a systematic review', *Photodiagnosis Photodyn Ther*, pp. 101774.
- Shi, X., Zhou, W., Ma, D., Ma, Q., Bridges, D., Ma, Y. and Hu, A. (2015) 'Electrospinning of Nanofibers and Their Applications for Energy Devices', *Journal of Nanomaterials*, 2015, pp. 140716.
- Shin, H., Jo, S. and Mikos, A. G. (2003) 'Biomimetic materials for tissue engineering', *Biomaterials*, 24(24), pp. 4353-4364.
- Shrestha, A. and Kishen, A. (2012) 'Polycationic chitosan-conjugated photosensitizer for antibacterial photodynamic therapy', *Photochem Photobiol*, 88(3), pp. 577-83.
- Silva, A. F., Borges, A., Freitas, C. F., Hioka, N., Mikcha, J. M. G. and Simoes, M. (2018a) 'Antimicrobial Photodynamic Inactivation Mediated by Rose Bengal and Erythrosine Is Effective in the Control of Food-Related Bacteria in Planktonic and Biofilm States', *Molecules*, 23(9).
- Silva, A. P., Neves, C. L., Silva, E. D. A., Portela, T. C. L., Iunes, R. S., Cogliati, B., Severino, D., Baptista, M. D. S., Dagli, M. L. Z., Blazquez, F. J. H. and Silva, J. (2018b) 'Effects of methylene blue-mediated photodynamic therapy on a mouse model of squamous cell carcinoma and normal skin', *Photodiagnosis Photodyn Ther*, 23, pp. 154-164.
- Silva, J. R., Cardoso, G., Maciel, R. R. and de Souza, N. C. (2015) 'Morphological alterations on *Citrobacter freundii* bacteria induced by erythrosine dye and laser light', *Lasers Med Sci*, 30(1), pp. 469-73.
- Siye, T., Guilian, L., Rui, Z., Leilei, H. and Hui, T. (2014) 'In vitro degradation of polyglycolic acid synthesized by a one-step reaction', *Journal of Polymer Engineering*, 34(7), pp. 591-596.
- Smith, A. C. and Hussey, M. A. (2005) 'Gram stain protocols', *American Society for Microbiology*.

Smoak, M., Chen, C., Qureshi, A., Garber, L., Pojman, J. A., Janes, M. E. and Hayes, D. J. (2014) 'Antimicrobial cytocompatible pentaerythritol triacrylate-co-trimethylolpropane composite scaffolds for orthopaedic implants', *Journal of Applied Polymer Science*, 131(22), pp. n/a-n/a.

Soares, J. A., Soares, S., de Jesus Tavares, R. R., de Castro Rizzi, C., Vaz Rodrigues, S. C. G., Maia Filho, E. M., Brito-Junior, M., Pereira, R. D., Magalhaes, P. P. and de Macedo Farias, L. (2018) 'Exploring different photodynamic therapy parameters to optimize elimination of *Enterococcus faecalis* in planktonic form', *Photodiagnosis Photodyn Ther*, 22, pp. 127-131.

Sola, A., Bertacchini, J., D'Avella, D., Anselmi, L., Maraldi, T., Marmioli, S. and Messori, M. (2019) 'Development of solvent-casting particulate leaching (SCPL) polymer scaffolds as improved three-dimensional supports to mimic the bone marrow niche', *Mater Sci Eng C Mater Biol Appl*, 96, pp. 153-165.

Soukos, N. S. and Goodson, J. M. (2011) 'Photodynamic therapy in the control of oral biofilm', *Periodontology 2000*, 55, pp. 143-166.

Souza, E. Q. M., da Rocha, T. E., Toro, L. F., Guiati, I. Z., Ervolino, E., Garcia, V. G., Wainwright, M. and Theodoro, L. H. (2020) 'Antimicrobial photodynamic therapy compared to systemic antibiotic therapy in non-surgical treatment of periodontitis: Systematic review and meta-analysis', *Photodiagnosis Photodyn Ther*, pp. 101808.

Sperandio, F. F., HUang, Y. and Hamblin, M. R. (2013) 'Antimicrobial Photodynamic Therapy to Kill Gram-negative Bacteria.', *Recent Pat Antiinfect Drug Discov*, 8(2), pp. 108-120.

Spriano, S., Sarath Chandra, V., Cochis, A., Uberti, F., Rimondini, L., Bertone, E., Vitale, A., Scolaro, C., Ferrari, M., Cirisano, F., Gautier di Confiengo, G. and Ferraris, S. (2017) 'How do wettability, zeta potential and hydroxylation degree affect the biological response of biomaterials?', *Mater Sci Eng C Mater Biol Appl*, 74, pp. 542-555.

St Denis, T. G., Dai, T., Izikson, L., Astrakas, C., Anderson, R. R., Hamblin, M. R. and Tegos, G. P. (2011) 'All you need is light: antimicrobial photoinactivation as an evolving and emerging discovery strategy against infectious disease', *Virulence*, 2(6), pp. 509-20.

Star, W. M. (1990) 'Light delivery and light dosimetry for photodynamic therapy', *Lasers in Medical Science*, 5(2), pp. 107-113.

Street, C. N., Pedigo, L., Gibbs, A. and Loebel, G. N. 'Antimicrobial photodynamic therapy for the decolonization of methicillin-resistant *Staphylococcus aureus* from the anterior nares.'. *12th World Congress of the International Photodynamic Association*, Seattle, Washington, United States.

Stájer, A., Kajári, S., Gajdács, M., Musah-Eroje, A. and Baráth, Z. (2020) 'Utility of Photodynamic Therapy in Dentistry: Current Concepts', *Dent J (Basel)*, 8(2).

Sultana, N. and Wang, M. (2012) 'Fabrication of Tissue Engineering Scaffolds Using the Emulsion Freezing/ Freeze-drying Technique and Characteristics of the Scaffolds', in M. Ramalingam, Z.H., S. Ramakrishna, H. Kobayashi and Y. Haikel (ed.) *In Integrated Biomaterials in Tissue Engineering*: Scrivener Publishing LLC.

Sørensen, S. J., Bailey, M., Hansen, L. H., Kroer, N. and Wuertz, S. (2005) 'Studying plasmid horizontal transfer in situ: a critical review', *Nat Rev Microbiol*, 3(9), pp. 700-10.

Tanner, A., Maiden, M. F. J., Lee, K., Shulman, L. B. and Webe, H. P. (1997) 'Dental Implants Infection.', *Clinical Infectious Diseases*, 25(2), pp. S213-S217.

Tapias, L. F. and Ott, H. C. (2014) 'Decellularized scaffolds as a platform for bioengineered organs', *Current Opinion in Organ Transplantation*, 19(2), pp. 145-152.

Tardivo, J. P., Del Giglio, A., de Oliveira, C. S., Gabrielli, D. S., Junqueira, H. C., Tada, D. B., Severino, D., de Fátima Turchiello, R. and Baptista, M. S. (2005) 'Methylene blue in photodynamic therapy: From basic mechanisms to clinical applications', *Photodiagnosis and Photodynamic Therapy*, 2(3), pp. 175-191.

Tedesco, A. C., Primo, F. L. and Beltrame, M. (2016) 'Phthalocyanines: Synthesis, Characterization and Biological Applications of Photodynamic Therapy (PDT), Nanobiotechnology, Magnetohyperthermia and Photodiagnosis (Theranostics)', *Reference Module in Materials Science and Materials Engineering*: Elsevier.

Tegos, G. P. and Hamblin, M. R. (2006) 'Phenothiazinium antimicrobial photosensitizers are substrates of bacterial multidrug resistance pumps', *Antimicrob Agents Chemother*, 50(1), pp. 196-203.

- Tenover, F. C. (2006) 'Mechanisms of antimicrobial resistance in bacteria', *American Journal of Infection Control*, 34(5), pp. S3-S10.
- Thomas, M. V. and Puleo, D. A. (2011) 'Infection, inflammation, and bone regeneration: a paradoxical relationship', *J Dent Res*, 90(9), pp. 1052-61.
- Tian, F., Hosseinkhani, H., Hosseinkhani, M., Khademhosseini, A., Yokoyama, Y., Estrada, G. G. and Kobayashi, H. (2008) 'Quantitative analysis of cell adhesion on aligned micro- and nanofibers', *Journal of Biomedical Materials Research Part A*, 84A(2), pp. 291-299.
- Tokubo, L. M., Rosalen, P. L., de Cassia Orlandi Sardi, J., Freires, I. A., Fujimaki, M., Umeda, J. E., Barbosa, P. M., Tecchio, G. O., Hioka, N., de Freitas, C. F. and Suga Terada, R. S. (2018) 'Antimicrobial effect of photodynamic therapy using erythrosine/methylene blue combination on *Streptococcus mutans* biofilm', *Photodiagnosis Photodyn Ther*, 23, pp. 94-98.
- Tomasini, E. P., Braslavsky, S. E. and San Román, E. (2012) 'Triplet quantum yields in light-scattering powder samples measured by laser-induced optoacoustic spectroscopy (LIOAS)', *Photochemical & Photobiological Sciences*, 11(6), pp. 1010-1017.
- Tomlinson, M. J., Dennis, C., Yang, X. B. and Kirkham, J. (2015) 'Tissue non-specific alkaline phosphatase production by human dental pulp stromal cells is enhanced by high density cell culture', *Cell and Tissue Research*, 361(2), pp. 529-540.
- Tran, D. N. and Balkus Jr, K. J. (2012) 'Enzyme Immobilization via Electrospinning', *Topics in Catalysis*, 55(16), pp. 1057-1069.
- Ulery, B. D., Nair, L. S. and Laurencin, C. T. (2011) 'Biomedical Applications of Biodegradable Polymers', *J Polym Sci B Polym Phys*, 49(12), pp. 832-864.
- Vacanti, C. A., Kim, W., Upton, J., Mooney, D. and Vacanti, J. P. (1995) 'The efficacy of periosteal cells compared to chondrocytes in the tissue engineered repair of bone defects', *Tissue Eng*, 1(3), pp. 301-8.
- Vacanti, C. A., Kim, W., Upton, J., Vacanti, M. P., Mooney, D., Schloo, B. and Vacanti, J. P. (1993) 'Tissue-engineered growth of bone and cartilage', *Transplant Proc*, 25(1 Pt 2), pp. 1019-21.

Vacanti, J. P. and Langer, R. (1999) 'Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation', *The Lancet*, 354, pp. S32-S34.

Van Bambeke, F., Glupczynski, Y., Plésiat, P., Pechère, J. C. and Tulkens, P. M. (2003) 'Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy', *J Antimicrob Chemother*, 51(5), pp. 1055-65.

Viera Rey, D. F. and St-Pierre, J.-P. (2019) '6 - Fabrication techniques of tissue engineering scaffolds', in Mozafari, M., Sefat, F. and Atala, A. (eds.) *Handbook of Tissue Engineering Scaffolds: Volume One*: Woodhead Publishing, pp. 109-125.

Wahid, F., Khan, T., Hussain, Z. and Ullah, H. (2018) '30 - Nanocomposite scaffolds for tissue engineering; properties, preparation and applications', in Inamuddin, Asiri, A.M. and Mohammad, A. (eds.) *Applications of Nanocomposite Materials in Drug Delivery*: Woodhead Publishing, pp. 701-735.

Wainwright, M. (1996) 'Non-porphyrin Photosensitizers in Biomedicine', *Chemical Society Reviews*, pp. 351-359.

Wainwright, M. (2011) 'Chapter 2. Phenothiazinium Salts as Antimicrobial Photosensitizing Agents', 0, pp. 19-43.

Wainwright, M. and Crossley, K. B. (2004) 'Photosensitising agents—circumventing resistance and breaking down biofilms: a review', *International Biodeterioration & Biodegradation*, 53(2), pp. 119-126.

Wainwright, M., Phoenix, D. A., Nickson, P. B. and Morton, G. (2002) 'The Use of New Methylene Blue in *Pseudomonas aeruginosa* Biofilm Destruction', *Biofouling*, 18(4), pp. 247-249.

Walker, J. L. and Santoro, M. (2017) '9 - Processing and production of bioresorbable polymer scaffolds for tissue engineering', in Perale, G. and Hilborn, J. (eds.) *Bioresorbable Polymers for Biomedical Applications*: Woodhead Publishing, pp. 181-203.

Wan, M. T. and Lin, J. Y. (2014) 'Current evidence and applications of photodynamic therapy in dermatology', *Clin Cosmet Invest Dermatol*, 7, pp. 145-63.

Wang, K. K., Shin, E. P., Lee, H. J., Jung, S. J., Hwang, J. W., Heo, I., Kim, J. H., Oh, M. K. and Kim, Y. R. (2018) 'Target-oriented photofunctional nanoparticles (TOPFNs) for selective photodynamic inactivation of Methicillin-resistant Staphylococcus aureus (MRSA)', *J Photochem Photobiol B*, 183, pp. 184-190.

Wang, L., Tran, I., Seshareddy, K., Weiss, M. L. and Detamore, M. S. (2009) **'A Comparison of Human Bone Marrow–Derived Mesenchymal Stem Cells and Human Umbilical Cord–Derived Mesenchymal Stromal Cells for Cartilage Tissue Engineering'**, *TISSUE ENGINEERING: Part A*, 15, pp. 2259-2266.

Wang, W., Caetano, G., Ambler, W. S., Blaker, J. J., Frade, M. A., Mandal, P., Diver, C. and Bartolo, P. (2016) 'Enhancing the Hydrophilicity and Cell Attachment of 3D Printed PCL/Graphene Scaffolds for Bone Tissue Engineering', *Materials (Basel)*, 9(12).

Wang, Y., Xia, H., Zhao, Y. and Jiang, T. (2005) 'Three-Dimensional Culture of Human Periodontal Ligament Cells on Highly Porous Polyglycolic Acid Scaffolds in vitro', *Proceedings of the 2005 IEEE*, pp. 1-4.

Wen, H., Dan, M., Yang, Y., Lyu, J., Shao, A., Cheng, X., Chen, L. and Xu, L. (2017) 'Acute toxicity and genotoxicity of silver nanoparticle in rats', *PLoS One*, 12(9), pp. e0185554.

Weng, L. and Xie, J. (2015) 'Smart Electrospun Nanofibers for Controlled Drug Release: Recent Advances and New Perspectives', *Curr Pharm Des*, 21(15), pp. 1944-1959.

WHO (2018) *Antimicrobial resistance*.

WHO Model List of Essential Medicines (19th List), Organization, W.H. (2015).

Williams, J. K., Yoo, J. J. and Atala, A. (2019) 'Chapter 59 - Regenerative Medicine Approaches for Tissue Engineered Heart Valves', in Atala, A., Lanza, R., Mikos, A.G. and Nerem, R. (eds.) *Principles of Regenerative Medicine (Third Edition)*. Boston: Academic Press, pp. 1041-1058.

Wilson, B. C. (2003a) 'Potential Applications of Photodynamic Therapy in Regenerative Medicine', *THE JOURNAL OF CRANIOFACIAL SURGERY*, 14(3), pp. 278-283.

Wilson, B. C. (2003b) 'Technologies and biophysical techniques for PDT', pp. 127-158.

Wiria, F. E., Leong, K. F., Chua, C. K. and Liu, Y. (2007) 'Poly-epsilon-caprolactone/hydroxyapatite for tissue engineering scaffold fabrication via selective laser sintering', *Acta Biomater*, 3(1), pp. 1-12.

Wood, R. M., Taylor, R. T. and Rouse, R. L. (1975) 'Laser damage in optical materials at 1.06 μm ', *Optics & Laser Technology*, 7(3), pp. 105-111.

Wood, S., Metcalf, D., Devine, D. and Robinson, C. (2006) 'Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms', *J Antimicrob Chemother*, 57(4), pp. 680-4.

Wood, S., Yang, X., De Matas, M., Iddon, P. and Raxworthy, M. (2010) *Scaffold*. [Online].

Wu, L., Li, H., Li, S., Li, X., Yuan, X., Li, X. and Zhang, Y. (2010) 'Composite fibrous membranes of PLGA and chitosan prepared by coelectrospinning and coaxial electrospinning', *J Biomed Mater Res A*, 92(2), pp. 563-74.

Wu, P., Lin, C., Lin, C., Chang, N., Tsai, W. and Yu, J. (2018) 'Methylene-Blue-Encapsulated Liposomes as Photodynamic Therapy Nano Agents for Breast Cancer Cells', *Nanomaterials*, 9(1).

Xie, Z., Fan, T., An, J., Choi, W., Duo, Y., Ge, Y., Zhang, B., Nie, G., Xie, N., Zheng, T., Chen, Y., Zhang, H. and Kim, J. S. (2020) 'Emerging combination strategies with phototherapy in cancer nanomedicine', *Chem Soc Rev*.

Xin, X., Liu, Q., Chen, C., Guan, Y. and Yao, S. (2016) 'Fabrication of bimodal porous PLGA scaffolds by supercritical CO₂foaming/particle leaching technique', *Journal of Applied Polymer Science*, 133(27).

Xu, L. C. and Siedlecki, C. A. (2007) 'Effects of surface wettability and contact time on protein adhesion to biomaterial surfaces', *Biomaterials*, 28(22), pp. 3273-83.

Xu, Y., Young, M. J., Battaglino, R. A., Morse, L. R., Fontana, C. R., Pagonis, T. C., Kent, R. and Soukos, N. S. (2009) 'Endodontic antimicrobial

photodynamic therapy: safety assessment in mammalian cell cultures', *J Endod*, 35(11), pp. 1567-72.

Xue, J., Wu, T., Dai, Y. and Xia, Y. (2019) 'Electrospinning and Electrospun Nanofibers: Methods, Materials, and Applications', *Chemical Reviews*, 119(8), pp. 5298-5415.

Yang, G. Z., Li, H. P., Yang, J. H., Wan, J. and Yu, D. G. (2017a) 'Influence of Working Temperature on The Formation of Electrospun Polymer Nanofibers', *Nanoscale Res Lett*, 12(1), pp. 55.

Yang, L., Kuang, H., Zhang, W., Aguilar, Z. P., Wei, H. and Xu, H. (2017b) 'Comparisons of the biodistribution and toxicological examinations after repeated intravenous administration of silver and gold nanoparticles in mice', *Sci Rep*, 7(1), pp. 3303.

Yano, S., Hirohara, S., Obata, M., Hagiya, Y., Ogura, S.-i., Ikeda, A., Kataoka, H., Tanaka, M. and Joh, T. (2011) 'Current states and future views in photodynamic therapy', *Journal of Photochemistry and Photobiology C: Photochemistry Reviews*, 12(1), pp. 46-67.

Yoon, I., Li, J. Z. and Shim, Y. K. (2013) 'Advance in photosensitizers and light delivery for photodynamic therapy', *Clin Endosc*, 46(1), pp. 7-23.

You, Y., Min, B.-M., Lee, S. J., Lee, T. S. and Park, W. H. (2005) 'In vitro degradation behavior of electrospun polyglycolide, polylactide, and poly(lactide-co-glycolide)', *Journal of Applied Polymer Science*, 95(2), pp. 193-200.

Yousefzadeh, M. (2017) '12 - Modeling and simulation of the electrospinning process', in Afshari, M. (ed.) *Electrospun Nanofibers*: Woodhead Publishing, pp. 277-301.

Yu, Z., Huang, K.-h., Zhong, W., Yang, L.-q., Chen, Q.-k. and Zhu, Z.-h. (2011) 'In vitro lethal effect of photodynamic therapy on human pancreatic cancer cells and its major influencing factors', *Clinical Oncology and Cancer Research*, 8(3), pp. 155-162.

Zhang, J., Jiang, C., Figueiró Longo, J. P., Azevedo, R. B., Zhang, H. and Muehlmann, L. A. (2018) 'An updated overview on the development of new photosensitizers for anticancer photodynamic therapy', *Acta Pharm Sin B*, 8(2), pp. 137-146.

- Zhang, Z., Ortiz, O., Goyal, R. and Kohn, J. (2014a) 'Biodegradable Polymers', *Principles of Tissue Engineering*, pp. 441-473.
- Zhang, Z., Ortiz, O., Goyal, R. and Kohn, J. (2014b) 'Chapter 23 - Biodegradable Polymers', in Lanza, R., Langer, R. and Vacanti, J. (eds.) *Principles of Tissue Engineering (Fourth Edition)*. Boston: Academic Press, pp. 441-473.
- Zhang, Z., Tang, J., Wang, H., Xia, Q., Xu, S. and Han, C. C. (2015) 'Controlled Antibiotics Release System through Simple Blended Electrospun Fibers for Sustained Antibacterial Effects', *ACS Applied Materials & Interfaces*, 7(48), pp. 26400-26404.
- Zhao, L. and Detamore, M. S. (2010) 'Chondrogenic differentiation of stem cells in human umbilical cord stroma with PGA and PLLA scaffolds', *J Biomed Sci Eng*, 3(11), pp. 1041-1049.
- Zheng, M. H., Chen, J., Kirilak, Y., Willers, C., Xu, J. and Wood, D. (2005) 'Porcine small intestine submucosa (SIS) is not an acellular collagenous matrix and contains porcine DNA: Possible implications in human implantation', *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 73B(1), pp. 61-67.
- Zong, X., Kim, K., Fang, D., Ran, S., Hsiao, B. S. and Chu, B. (2002) 'Structure and process relationship of electrospun bioabsorbable nanofiber membranes', *Polymer*, 43(16), pp. 4403-4412.