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THE DEVELOPMENT OF GELATIN COATED POLYSTYRENE MICROCARRIERS USING ULTRAVIOLET/OZONE SYSTEM FOR CELL CULTURE APPLICATIONS

BY

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy (Engineering)

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JUNE 2017

ABSTRACT

Microcarrier cell culture offers many advantages over conventional cell culture systems and has been widely used for the production of many important biological products. Nevertheless, the price of commercial microcarriers is quite expensive and can significantly increase the cost of culturing when used on an industrial scale. The present study attempts to address this problem by developing low cost, yet, efficient gelatin coated polystyrene (PS) microcarriers using an ultraviolet/ozone (UVO_3) system as the main surface modification method. In this study, polystyrene (PS) microspheres were prepared by using oil-in-water (O/W) emulsion solvent-evaporation method. In order to promote good cell attachment and proliferation on PS microspheres, their surfaces were first treated with UVO_3 system to introduce carboxyl (COOH) functional groups and subsequently coated with bovine gelatin using zero length cross-linker reagents. One-factor-at-a-time (OFAT) method and face centred central composite design (FCCCD) were employed to optimize UVO_3 treatment process conditions for maximal COOH concentration on the surface of PS microspheres. Factors affecting cross-linking conditions were also optimized using similar OFAT and FCCCD, to maximize the amount of immobilized gelatin on the surface of UVO_3 treated PS microspheres. The cytocompatibility of gelatin coated PS microspheres and the intermediate product, UVO_3 treated PS microspheres were evaluated by using them as attachment substrates in suspension cultures of Vero, CHO-K1 and AFSC in spinner vessel. Based on experiments, the highest amount of microspheres with appropriate mean size for microcarrier production could be generated at PS to $CHCl_3$ ratio of 1:5 (w/v), agitation speed of 300 rpm, surfactant PVA concentration of 0.25%, oil to water phase ratio of 1:5 (v/v) and agitation temperature of 80°C. Statistical analyses showed that the optimum UVO_3 process conditions; ozone concentration of ~64,603 ppm, exposure time of ~60 minutes and sample amount of 5.05 g resulted in the maximum COOH concentration of ~1,505 nmol/g. While at EDAC to COOH ratio of 2.5:1, NHS concentration of 0.5 mM and gelatin concentration of 40 mg/mL, the maximum gelatin immobilization at 2,524.74 µg/g was obtained. FTIR analysis revealed that UVO_3 treatment has successfully introduced COOH functional groups on the surface of PS microspheres whereas gelatin immobilization was proven by the presence of several amide peaks. The surface wettability and the dispersion stability of PS microspheres in liquid medium were also found to be highly improved after UVO_3 treatment and gelatin immobilization. Furthermore, UVO_3 treated and gelatin coated PS microspheres were also revealed as able to withstand different sterilization procedures without losing much of their surface functionalities. By using UVO_3 treated and gelatin coated PS microspheres, high cell density cultures of Vero and CHO-K1 cells were successfully achieved with results that were highly comparable to those obtained when using expensive commercial microcarriers. Additionally, EBs formation assay has confirmed that, AFSC cultured using both types of microspheres were able to maintain their 'stemness'. In conclusion, by using the UVO_3 system, this study has successfully developed two types of low cost and efficient microcarriers that have huge potentials for commercialization. Further studies involving the use of both types of microcarriers in the production of important biological products such as vaccines, recombinant proteins, gene therapy vector and etc. is highly encouraged.

خلاصة البحث

تقدم زراعة الخلايا بواسطة الحامل الدقيق (microcarrier) مزايا عديدة أكثر من النظم التقليدية لزراعة الخلايا ولذلك يتم استخدامها على نطاق واسع في العديد من المنتجات الحيوية الهامة. مع ذلك فإن أسعار الحاملات الدقيقة التجارية مرتفعة للغاية مما يسبب زيادة كبيرة في تكلفة زراعتها عند استخدامها على نطاق صناعي. لذلك تحاول هذه الدراسة التغلب على هذه المشكلة من خلال إنتاج حامل دقيق ذو كفاءة، ومنخفض التكلفة من الجيلاتين المغلف بمادة البوليستيرين (PS) وذلك باستخدام نظام الأشعة فوق البنفسجية / الأوزون (UVO3) كطريقة لتعديل السطح الرئيسي. في هذه الدراسة تم تحضير كريات دقيقة من البوليسترين (PS) باستخدام طريقة تبخير المذيب واستحلاب النفط في الماء (oil-in-water emulsion solvent evaporation method). ومن أجل تحسين تثبيت وتكاثر الخلايا على سطح الحاملات الدقيقة من PS، فقد تم أولاً معالجة سطحها بنظام UVO3، وذلك لإدخال المجموعة الوظيفية (كربوكسيل) ومن ثم تغليفها بالجيلاتين البقري باستخدام مواد تقاطع صفر طول. تم استخدام طريقة تغيير عامل واحد في الوقت الواحد (OFAT) وطريقة تصميم المركب المركزي (FCCD) لتحسين ظروف عملية معالجة UVO_3 من أجل تحقيق أقصى قدر من تركيز COOH على سطح البوليستيرين. وكذلك تم تحسين العوامل المؤثرة على روابط العبور باستخدام OFAT و FCCCD للحصول على أقصى كمية من الجيلاتين المثبت على سطح البوليستيرين المعالج بطريقة UVO_3. ومن أجل تقييم ملاءمة الوسط لقد تم استخدام كرات PS الدقيقة التي تمت معالجتها باستخدام UVO_3 والمغطاة بالجيلاتين لتنمية فيرو وCHO-K1 وAFSC في وسط زراعة الخلايا. تم انتاج اكبر قدر من كرات البوليسترين الدقيقة والمناسبة لانتاج الحاملات الدقيقة بمتوسط ملائم وذلك باستخدام نسبة PS الى $CHCL_3$ تعادل 1: 5 وسرعة تحريك تعادل 300 دورة في الدقيقة ونسبة PVA تساوي 0.25% ونسبة ماء لزيت تساوي 1:5 ودرجة حرارة 80 درجة مئوية. أظهرت التحاليل الإحصائية أن الظروف المثلى كانت كالتالي: تركيز الأوزون ~ 64603 جزء في المليون والتعريض لمدة 60 دقيقة واستخدام كمية عينة 5.05 جرام في أقصى تركيز COOH يعادل ~ 1505 نانومول / جرام. بينما باستخدام نسبة EDAC إلى COOH تساوي 2.5: 1، تم الحصول على NHS بتركيز 5 ميلمولار وتركيز جيلاتين يعادل 40 ملجم / مل،وقد ادى ذلك الى الحصول على أكبر قدر ممكن من الجيلاتين المثبت وقدره 2،524.74 ميكروجرام / غرام. وأظهر تحليل FTIR أن استخدام طريقة UVO_3 أدخلت بنجاح المجموعات الوظيفية COOH على سطح كرات PS في حين تجلى نجاح تثبيت الجيلاتين من خلال وجود عدة قمم للأميد. وقد تم ملاحظة تحسن كل من قابلية السطح للبلل وثبات انتشار كرات PS الدقيقة في الوسط السائل أيضا بعد معالجة UVO_3 وتثبيت الجيلاتين. وعلاوة على ذلك، تم الكشف عن أن كلا نوعي كرات PS الدقيقة قادرة على تحمل إجراءات التعقيم المعتادة دون أن تفقد الكثير من وظائف سطحها.وقد حقق نمو خلايا فيرو وCHO-K1 كثافة عالية مع استخدام كلا النوعين من الكرات الدقيقة مقارنة بالحاملات الدقيقة التجارية. بالإضافة إلى ذلك، أكد فحص EBs أن AFSC التي تمت زراعتها على كلا النوعين من الكرات الدقيقة كانوا قادرين على الحفاظ على جذعيتهم. وأخيرا يمكن استنتاج أن هذه الدراسة قامت بالتطوير الناجح لنوعين من الحاملات الدقيقة ذات الاسعار المنخفضة والامكانية العالية للتسويق مما يشجع على استخدامها في عمليات انتاج المواد الحيوية الهامة مثل التطعيمات, البروتينات المؤتلفة، المعالجة الجنية، إلخ.

APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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ACKNOWLEDGEMENTS

In the name of Allah, The Most Merciful and The Most Beneficent. All praises is to Allah S.W.T., the Almighty God, for His blessings and guidance have enabled me to accomplish this study.

Firstly, I would like to express my sincerest gratitude and appreciation to my main supervisor, Assoc. Prof. Dr. Maizirwan Mel for his patience, encouragement and continuous support of my PhD study. His valuable guidance and kind attention has allowed me to complete this research work and thesis writing smoothly and efficiently. He has been and will always be an important source of inspiration in my life. Besides him, my deepest appreciation also goes to my co-supervisors; Assoc. Prof. Dr. Yumi Zuhanis Has-yun Hashim, Prof. Hamzah Salleh and Prof. Iis Sopyan for their insightful ideas, teachings and often discussions despite their commitments in the administration works and lectures.

I would like to give special thanks to Dr. Norshariza Nordin and Sis. Norsaadah Hamzah, from the Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for willing to share their precious knowledge in handling stem cell culture and also for allowing me to use their laboratory, research facilities and chemicals for my experiments. I am also thankful to Prof. Wong Chiow San, Bro. Tay Wee Horng and Sis. Farah Nadia Dayana from the Department of Physics, Faculty of Science, University of Malaya for their scientific cooperation and useful assistance that have helped me in the construction of ultraviolet/ozone (UVO_3) system. I am greatly indebted to the Ministry of Higher Education (MOHE), Malaysia for financing the research project under Prototype Research Grant Scheme (PRGS) and also to Universiti Malaysia Pahang (UMP) for awarding me with a generous scholarship.

I wish to express my deepest gratitude to all my lab mates; Sis. Nurhusna, Bro. Phirdaous, Sis. Irmanisha, Sis. Norashikin, Sis. Faqihah and Sis. Rahilah, for their friendship, knowledge, support and also for all the problems and fun that have been shared together in the recent years. Also I thank my friend, Bro. Tamrin in The University of Nottingham Malaysia for his brilliant ideas and sincere help during sample observation using scanning electron microscopy (SEM). My sincerest gratefulness also extends to everyone, although not individually named here, who had contributed directly or indirectly to my research and thesis writing.

Last but not the least, I would like to thank my family: my very beloved parents, Arifin Bin Ahmad and Fatimah Binti Zainal, my sisters and brother in-laws as well as my wonderful nephews and nieces for their endless love, motivation, support and prayers that have provided me strength and determination throughout my PhD study.

TABLE OF CONTENTS

Abstract ii
Abstract in Arabic iii
Approval Page iv
Declaration v
Copyright Page vi
Acknowledgements vii
List of Tables xii
List of Figures xiv
List of Abbreviations xviii
List of Symbols xx

CHAPTER ONE: INTRODUCTION 1
1.1 Background of the Study 1
1.2 Problem Statement and Its Significance 5
1.3 Research Hypothesis 6
1.4 Research Objectives 8
1.5 Research Methodology 9
1.6 Scope of Research 10
1.7 Thesis Organization 10

CHAPTER TWO: LITERATURE REVIEW 12
2.1 Introduction 12
2.2 Polymers 12
2.3 Synthetic Polymers 13
2.3.1 Non-Biodegradable Polymers 14
2.3.1.1 Polycarbonate (PC) 15
2.3.1.2 Poly(ethylene terephthalate) (PET) 16
2.3.1.3 Polystyrene (PS) 17
2.3.2 Biodegradable Polymers 19
2.3.2.1 Poly(lactic acid) (PLA) 19
2.3.2.2 Poly(hydroxybutyrate) (PHB) 21
2.3.2.3 Polycaprolactone (PCL) 22
2.4 Natural Polymers 24
2.4.1 Dextran 25
2.4.2 Collagen 26
2.4.3 Gelatin 27
2.5 Animal Cell Culture 29
2.5.1 Types of Cell Cultures 30
2.5.1.1 Primary Cell Culture 31
2.5.1.2 Finite Cell Culture 32
2.5.1.2.1 Amniotic Fluid Stem Cells 33
2.5.1.3 Continuous Cell Lines 34
2.5.1.3.1 Vero Cells 36
2.5.1.3.2 Chinese Hamster Ovary (CHO) Cells 37

2.5.2 Adherence Dependence 38
2.5.2.1 Cell Adhesion to Surfaces 38
2.6 Microcarrier Cell Culture 40
2.6.1 Requirements for an Optimum Microcarrier 43
2.6.2 Applications of Microcarrier 44
2.6.2.1 Viral Vaccines Production 45
2.6.2.2 Recombinant Protein Production 47
2.6.2.3 Expansion of Stem Cells 49
2.6.3 Shapes of Microcarrier 53
2.7 Microsphere Preparation Techniques 55
2.7.1 Emulsion-Solvent Polymerization 55
2.7.2 Suspension Polymerization 57
2.8 Surface Modification of Polymers 59
2.8.1 Physical Modification Methods 60
2.8.1.1 Corona Discharge Treatment 61
2.8.1.2 Ion Beam Treatment 62
2.8.1.3 Plasma Treatment 64
2.8.1.4 Ultraviolet/Ozone (UVO_3) Treatment 66
2.8.2 Chemical Modification Methods 71
2.8.2.1 Wet Chemical Treatment 72
2.9 Biomolecule Immobilization 74
2.9.1 Techniques of Biomolecule Immobilization 76
2.9.1.1 Physical Adsorption 77
2.9.1.2 Covalent Bonding 79
2.9.1.2.1 Zero-length crosslinking 81
2.10 Summary 85

CHAPTER THREE: MATERIALS AND METHODS 87
3.1 Introduction 87
3.2 Experiment Materials 89
3.2.1 Cell Lines 89
3.2.2 Chemicals and Reagents 89
3.2.3 Equipment and Apparatus 90
3.2.4 Analytical Instruments 91
3.2.5 Setup of Ultraviolet/Ozone (UVO_3) Treatment System 91
3.3 Experimental Analyses 94
3.3.1 Particle Size Analysis 94
3.3.2 Quantification of Carboxyl (COOH) Functional Groups 95
3.3.3 Measurement of Immobilized Gelatin 96
3.3.4 Fourier Transform Infrared Spectroscopy (FTIR) Analysis 98
3.3.5 Contact Angle Analysis 99
3.3.6 Zeta Potential Analysis 99
3.3.7 Scanning Electron Microscopy (SEM) 100
3.3.8 Energy Dispersive X-Ray Spectroscopy (EDX) 100
3.3.9 Thermogravimetric Analysis (TGA) 101
3.3.10 Differential Scanning Calorimetry (DSC) 101
3.3.11 Gel Permeation Chromatography (GPC) 101
3.4 Experimental Methods 102
3.4.1 Production of Polystyrene (PS) Microspheres 102

3.4.2 Investigation of The Effect of Several Process Conditions of O/W Emulsification-Solvent Evaporation Technique on The Size of Microspheres and Production Scale-Up 103
3.4.3 Surface Oxidation of PS Microspheres Using UVO_3 Treatment System 105
3.4.4 Evaluation of Different Oxidation Methods for COOH Grafting on PS Microspheres 105
3.4.5 Optimization of UVO_3 Treatment Process Conditions for COOH Grafting on PS Microspheres 106
3.4.6 Gelatin Immobilization on PS Microspheres 108
3.4.7 Optimization of Cross-Linking Conditions for Gelatin Immobilization on PS Microspheres 109
3.5 Biological Testing 111
3.5.1 Medium Preparation 111
3.5.2 Thawing of Frozen Cells 112
3.5.3 Subculture 112
3.5.4 Cell Cryopreservation 113
3.5.5 Microcarrier Preparation 113
3.5.6 Suspension Cell Culture in Spinner Vessel 114
3.5.7 Cell Counting 115
3.5.8 Formation of Embryoid Bodies (EBs) 116
3.6 Summary 117

CHAPTER FOUR: RESULTS AND DISCUSSION 119
4.1 Introduction 119
4.2 Preparation of PS Microspheres 119
4.2.1 Effect of Process Conditions on Microsphere Size 120
4.2.1.1 Effect of Agitation Speeds 120
4.2.1.2 Effect of PVA Concentrations 121
4.2.1.3 Effect of PS to Chloroform ($CHCl_3$) Ratios 123
4.2.1.4 Effect of Oil to Water Phase Ratios 124
4.2.1.5 Effect of Agitation Temperatures 125
4.2.2 Scaled-up Production of Microspheres 128
4.3 Preparation of Gelatin Coated PS Microspheres 129
4.3.1 Evaluation of Different Oxidation Methods for COOH Grafting on PS Microspheres 130
4.3.2 Effect of Different COOH Concentration on Gelatin Immobilization on PS Microspheres 135
4.3.3 Optimization of UVO_3 treatment Process Conditions for COOH Grafting on PS Microspheres 137
4.3.3.1 Determination of Optimum Levels of UVO_3 Treatment Process Conditions by One-factor-at-a-time (OFAT) method 137
4.3.3.2 Optimization of UVO_3 Treatment Process Conditions by Face Centered Composite Design (FCCD) under Response Surface Methodology (RSM) 142
4.3.4 Optimization of Cross-Linking Conditions for Gelatin Immobilization on PS Microspheres 149

4.3.4.1 Determination of Optimum Levels of Cross-Linking Conditions by One-factor-at-a-time (OFAT) Method 149
4.3.4.2 Optimization of Cross-Linking Conditions by Face Centered Composite Design (FCCD) under the Response Surface Methodology (RSM) 153
4.4 Characterization of PS Microspheres 159
4.4.1 Fourier Transform Infrared (FTIR) Analysis 159
4.4.2 Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX) 164
4.4.3 Contact Angle Analysis... 167
4.4.4 Zeta Potential Analysis 169
4.4.5 Thermal Profiling 169
4.4.6 Gel Permeation Chromatography (GPC) 174
4.4.7 Effect of Different Sterilization Methods on PS Microcarriers ... 174
4.5 Cytocompatibility Evaluation of Microcarriers 176
4.5.1 Vero Cell Culture on Microcarriers 176
4.5.2 CHO-K1 Cell Culture on Microcarriers 181
4.5.3 Rat Amniotic Fluid Stem Cell (AFSC) Culture on Microcarriers 186
4.5.3.1 Embryoid Body Formation 189
4.6 Summary 191

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS 193
5.1 Conclusions 193
5.2 Recommendations 199

REFERENCES 200

LIST OF PUBLICATIONS 239

APPENDIX A: ACHIEVEMENTS 241
APPENDIX B: THE COST OF MICROCARRIERS 242
APPENDIX C: THE DEVELOPMENT OF MICROCARRIERS 247
APPENDIX D: LIST OF GLASSWARES AND CONSUMABLES 252
APPENDIX E: PREPARATION OF REAGENTS 253

LIST OF TABLES

Table No.		Page No.
2.1	Advantages of microcarrier cell culture	41
2.2	Requirements of an optimum microcarrier	44
2.3	Virus/vaccines produced using microcarriers	47
2.4	Commercial recombinant proteins produced using microcarrier	49
2.5	Stem cell types, type of diseases and injuries and the number of stem cells required for each transplantation per patient	51
2.6	Propagation of different types of stem cells using microcarriers	53
2.7	Commercial microcarriers and their properties	54
2.8	Objectives of polymer surface modification	60
2.9	List of selected previous works of UVO_3 treatment on various polymers	68
2.10	Major features of several immobilization techniques	77
2.11	Selected recent researches that utilized EDAC/NHS (or sulfo-NHS) reagents in bioconjugation procedures	83
3.1	Intensities of UV radiation measured at sample location	94
3.2	Experimental structure for evaluating the effects of individual process conditions on the mean size of microspheres	104
3.3	Experimental design for approximate optimization of process conditions by OFAT method	107
3.4	Experimental conditions according to the Face Centered Central Composite Design (FCCD) generated using statistical software Design Expert 7.0	108
3.5	Experimental design for approximate optimization of cross-linking conditions by OFAT method	110
3.6	Face Centered Central Composite Design (FCCD) for the optimization of cross-linking conditions	110
4.1	Summary of the effects of process conditions on the mean size of microspheres	127

4.2 Experimental and predicted results for surface COOH concentration obtained by Face Centered Central Composite Design (FCCD) 143

4.3 Analysis of Variance (ANOVA) for the second order polynomial model 144

4.4 Validation experiments designed by Design Expert 7.0 148

4.5 Experimental and predicted results for the amount of immobilized gelatin obtained by Face Centered Central Composite Design (FCCD) 154

4.6 Analysis of Variance (ANOVA) for the second order polynomial model 155

4.7 Validation experiments designed by Design Expert 7.0 158

4.8 EDX data showing carbon and oxygen content on PS microspheres 167

4.9 Contact angle and surface energy measurements on untreated PS, UVO_3 treated PS and gelatin coated PS films 168

4.10 Effect of different sterilization methods on PS microspheres 176

LIST OF FIGURES

Figure No.		Page No.
1.1	Production of Gelatin Coated PS microcarriers	9
2.1	Chemical Structure of Polycarbonate (PC)	16
2.2	Chemical Structure of Poly (ethylene terephthalate) (PET)	17
2.3	Chemical Structure of Polystyrene (PS)	19
2.4	Chemical Structure of Poly(lactic acid) (PLA)	21
2.5	Chemical Structure of Poly(hydroxybutyrate) (PHB)	22
2.6	Synthesis of Polycaprolactone (PCL)	24
2.7	Chemical Structure of Dextran	26
2.8	Coupling mechanism of two different molecules by using EDAC/NHS reagents	85
3.1	An overview of experimental studies	89
3.2	Schematic diagram of ultraviolet/ozone (UVO_3) system	92
3.3	Assembly of ultraviolet/ozone (UVO_3) system	92
3.4	Internal view of ultraviolet/ozone (UVO_3) system	93
3.5	Calibration curve for determination of carboxyl surface concentration on PS microspheres.	96
3.6	Calibration curve for determination of amount of immobilized gelatin on PS microspheres	97
3.7	Schematic diagram of preparation of PS microspheres by emulsification solvent-evaporation technique	103
3.8	Counting grids of Neubauer Improved cell counting chamber	116
4.1	Effect of agitation speeds on the size of microspheres	121
4.2	Effect of PVA concentrations on the size of microspheres	123
4.3	Effect of PS to $CHCl_3$ ratios on the size of microspheres	124
4.4	Effect of oil to water phase ratios on the size of microspheres	125

4.5 Effect of agitation temperatures on the size of microspheres 126

4.6 Size distribution of PS microspheres at PS to $CHCl_3$ ratio of 1:5 129

4.7 Effect of different oxidation treatments on COOH grafting on PS microspheres 131

4.8 Formation of POO• from the interaction of polystyrene with ozone 132

4.9 General interaction mechanism between PS and UV radiation; R represents carbon containing substituents 133

4.10 Effect of different COOH concentrations on PS microspheres on the amount of immobilized gelatin 136

4.11 Effect of different ozone concentrations on COOH concentration on PS microspheres 138

4.12 Effect of exposure time on the COOH concentration on PS microspheres 139

4.13 Effect of sample amount on the COOH concentration on PS microspheres 140

4.14 Effect of UV radiation on the COOH concentration on PS microspheres 141

4.15 Response surface and contour plots showing the effect of ozone concentration and exposure time on COOH concentration on the surface of PS microspheres 145

4.16 Response surface and contour plots showing the effect of ozone concentration and the amount of sample on COOH concentration on the surface of PS microspheres 146

4.17 Response surface and contour plots showing the effect of exposure time and the amount of sample on COOH concentration on the surface of PS microspheres 146

4.18 Effect of EDAC to COOH ratio on the amount of immobilized gelatin on PS microspheres 150

4.19 Effect of NHS concentration (mM) on the amount of immobilized gelatin on PS microspheres 151

4.20 Effect of gelatin concentration (mg/mL) on the amount of immobilized gelatin on PS microspheres 152

4.21 Response surface and contour plots showing the effect of EDAC to COOH ratio and NHS concentration on the amount of immobilized gelatin on PS microspheres 157

4.22	Response surface and contour plots showing the effect of EDAC to COOH ratio and gelatin concentration on the amount of immobilized gelatin on PS microspheres	157
4.23	Response surface and contour plots showing the effect of NHS concentration and gelatin concentration on the amount of immobilized gelatin on PS microspheres	158
4.24	ATR/IR spectra of; a) untreated PS microspheres, b) UVO_3 treated PS microspheres and c) gelatin coated PS microspheres	161
4.25	Subtraction result of ATR/IR plot; UVO_3 treated PS minus untreated PS microspheres	162
4.26	Subtraction result of ATR/IR plot; gelatin coated PS minus UVO_3 treated PS microspheres	163
4.27	SEM photographs of untreated PS (a and b), UVO_3 treated PS (c and d) and gelatin coated PS microspheres (e and f) at different magnifications (100x and 500x)	166
4.28	Water droplet images contact on a) untreated PS; b) UVO_3 treated PS and c) gelatin coated PS films	168
4.29	TG curves of untreated PS, UVO_3 treated PS and gelatin coated PS microspheres	171
4.30	DSC curve of untreated PS microspheres	172
4.31	DSC curve of UVO_3 treated PS microspheres	173
4.32	DSC curve of gelatin coated PS microspheres	173
4.33	Growth profile of Vero cells grown on untreated PS microspheres, UVO_3 treated PS microspheres, gelatin coated PS microspheres, SoloHill and Cytodex™ 3 microcarriers	178
4.34	Attachment and proliferation of Vero cells on five different microcarriers at peak concentration	179
4.35	SEM photographs of Vero cell attachment on UVO_3 treated PS microcarriers	180
4.36	Growth profile of CHO-K1 cells grown on untreated PS microspheres, UVO_3 treated PS microspheres, gelatin coated PS microspheres, SoloHill and Cytodex™ 3 microcarriers	182
4.37	Attachment and proliferation of CHO-K1 cells on five different microcarriers at peak concentration	183

4.38 SEM photographs of CHO-K1 cell attachment on UVO_3 treated PS microcarriers 184

4.39 Growth profile of amniotic fluid stem cells (AFSC) grown on UVO_3 treated and gelatin coated PS microspheres 187

4.40 Attachment and proliferation of AFSC on two different microcarriers at peak concentration 188

4.41 SEM photographs of AFSC attachment on UVO_3 treated PS microcarriers 189

4.42 Spontaneous differentiation of AFSC into embryoid bodies (EBs) by the hanging drop method 191

LIST OF ABBREVIATIONS

AFSC	Amniotic Fluid Stem Cells
ANOVA	Analysis of Variance
ATR/IR	Attenuated Total Reflection-Infrared
CH_3	Methyl
$CHCl_3$	Chloroform
CHO	Chinese Hamster Ovary
CO	Carbonyl
CO_2	Carbon dioxide
COOH	Carboxyl/carboxylic acid
DEAE	Diethylaminoethyl
df	Degree of freedom
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DOE	Design of Experiments
DSC	Differential Scanning Calorimetry
DTG	Differential Thermogravimetric Curve
EB	Embryoid body
ECM	Extracellular Matrix
EDAC	1-Ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride
EDX	Energy-Dispersive X-Ray Spectroscopy
FBS	Fetal Bovine Serum
FCCD	Face Centered Central Composite Design
FTIR	Fourier Transform Infrared Spectroscopy
GGFY	Girifalco-Good-Fowkes-Young
GMEM	Glasgow Minimum Essential Medium
GMP	Good Manufacturing Practice
GPC	Gel Permeation Chromatography
H_2	Hydrogen
HCl	Hydrochloric Acid
MES	2-(N-morpholino)-ethanesulfonic acid
Mw	Molecular Weight
N_2	Nitrogen
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NH_2	Primary amine
NHS	N-Hydroxysuccinimide
O/O	Oil-in-Oil
O/W	Oil-in-Water
O_2	Oxygen
OFAT	One-Factor-At-A-Time
OH	Hydroxyl
PBS	Phosphate Buffered Saline

PCL	Polycaprolactone
PCR	Polymerase Chain Reaction
PEG	Poly(ethylene glycol)
POO•	Polymer Peroxy Radicals
PS	Polystyrene
PTFE	Polytetrafluoroethylene
RGD	Arg-Gly-Asp
RPMI	Roswell Park Memorial Institute
RSM	Response Surface Methodology
S/V	Surface Area to Volume Ratio
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
TBO	Toluidine Blue O
T_c	Crystallization Temperature
TCPS	Tissue Culture Polystyrene
T_g	Glass Transition Temperatures
TGA	Thermogravimetric Analysis
THF	Tetrahydrofuran
T_{max}	Maximum Degradation Rate
tPA	Tissue Plasminogen Activator
UV	Ultraviolet
UVC	Ultraviolet C
UVO_3	Ultraviolet/ozone
W/O/W	Water-in-Oil-in-Water
WCA	Water Contact Angle

LIST OF SYMBOLS

%	percentage
$<$	less than
±	plus-minus
°	degrees
° C	degree Celsius
$^1\Delta_g$	singlet oxygen (lower singlet state)
$^1\Sigma_g^+$	singlet oxygen (higher singlet state)
$O(^1D)$	atomic oxygen
$O(^3P)$	ground state atomic oxygen atoms
p	probability
ppm	parts per million
v/w	volume per weight
w/w	weight per weight

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Animal cell culture has emerged as one of the most important tools used in life sciences today. Techniques of cell culture are essential for studying biochemical and physiological processes, and large-scale cultures of animal cells have become the preferred system for commercial production of various biological products (Kwon and Peng, 2002; Costa et al., 2013; Ayyildiz-Tamis et al., 2014). Although there are cells that can naturally grow in suspension, a large number of animal cell lines with industrial potentials require or prefer attachment to solid substratum for their survival and replication (Swiech et al., 2007; Rodrigues et al., 2013). Such cells include Vero (African green monkey kidney epithelial cells), CHO (Chinese hamster ovary cells), WI-38 (human lung fibroblast cells) and MDCK (Madin-Darby canine kidney epithelial cells) which constitute the few cell lines that have been approved by the World Health Organization (WHO) as safe cell substrates for biological production (Knezevic et al., 2008).

At the laboratory scale, adherent cells can be grown in two-dimensional tissue culture surfaces such as those provided by T-flasks, Petri dishes or roller bottles. However, these conventional culture systems provide restricted surface area for cell growth and become increasingly inefficient when the scale of culture is increased (Butler, 2004; Yadav and Tyagi, 2008). In order to overcome these limitations, an economical and efficient culture system with extensive surface area must be established. Several systems that have been examined to fulfill such requirements include spiral films, multiple plates, hollow fiber beds and small beads. Among these systems,

microcarrier suspension culture, which first conceived by Dr. Anton L. van Wezel in 1976, was regarded as the most successful approach for increasing cell yields (van Wezel, 1967; Sarkar, 2009; Chen et al., 2013; Rodrigues et al., 2013). This revolutionary breakthrough in cell culture technology offers an extremely high surface area to volume ratio that enables adherent cells to grow to high density in suspension cultures while maintaining their normal adherent growth mode (Card et al., 2005; Goh et al., 2013).

Microcarriers can generally be described as microscopic-sized particles (normally with a diameter of about 100 to 300µm), either solid (non-porous or microporous) or macroporous, that function as the attachment substrate for adherent cells in suspension cultures (Griffiths, 2013; Hervy et al., 2014; Eisenkraetzer, 2015). Most microcarriers have a spherical shape (due to the ease of manufacturing) with densities that are slightly higher than the culture medium (1.02 to 1.04 g/cm^3), allowing them to suspend easily when subjected to low-speed agitation (Lundgren and Blüml, 1998; Chen et al., 2013). The first microcarriers were based on cross-linked dextran, or more precisely DEAE-Sephadex A-50 beads which are normally used as column packing for ion-exchange chromatography (Hu, 1992; Kiremitçi, 1992; Blüml, 2007). Nowadays, a wide range of microcarriers is commercially available, prepared from a variety of materials including DEAE-dextran, gelatin, cellulose, polyacrylamide, polyethylene and polystyrene, among others (Halsall and Davis, 2011; Zhou et al., 2013; Pörtner. 2015). The surface of microcarriers is usually derivatized by the incorporation of extracellular matrix proteins, recombinant proteins, peptides or charged molecules (positive or negative) in order to facilitate cell attachment and proliferation on these materials (Chen et al., 2013). In microcarrier culture, cells attach, spread and gradually propagate until confluence, on the surface of solid microcarriers

or within the pores of macroporous microcarriers, that are maintained in suspension by gentle stirring (Blüml, 2007; Sinha and Kumar, 2008; Zhou et al., 2013). When the agitation is stopped, microcarriers sedimented easily due to their relatively large size, thus facilitate the harvesting procedure (Veliz et al., 2008). Cells may be detached from microcarriers by using proteolytic enzymes such as trypsin or collagenase though, in cases which only require cell secreted products, the removal of cells from microcarriers may be unnecessary (Butler, 2004).

Apart from their ability in producing high density culture of adherent cells in various types of bioreactors (such as spinner flask, stirred tank, airlift and Wave bioreactors), microcarriers also offer numerous other advantages including reduced requirement for labour and culture medium, lower risk of contamination, improved process monitoring and control, simpler harvesting and downstream processing procedures and easier culture scale up; when compared to conventional culture systems (Markvicheva and Grandfils, 2004; Trabelsi et al., 2011). At present day, microcarriers are highly employed in the production of numerous viral vaccines (Wu et al., 2004; Chen et al., 2011a; Kurokawa and Sato, 2011; Yu et al., 2012), recombinant proteins (Bleckwenn et al., 2005; Swiech et al., 2007; Tharmalingam et al., 2008), monoclonal antibodies (Voigt and Zintl, 1999; Makimoto et al., 2002; Rodrigues et al., 2011a; Costa et al., 2013) and gene therapy vectors (McTaggart and Al-Rubeai, 2000; Wu et al., 2002). In recent years, microcarriers are increasingly used for more biomedically oriented applications such as stem cell therapies (Rodrigues et al., 2011b; Chen et al., 2013; Goh et al., 2013) tissue regeneration (Malda and Frondoza, 2006) as well as *in vitro* toxicology studies (Sharfstein and Kaisermayer, 2013). These new applications have invigorated the use of microcarriers in cell culture and has led to the development

of more novel microcarriers, new culture modalities, new cell lines and also new reactor designs (Markvicheva and Grandfils, 2004; Chen et al., 2011a; Griffiths. 2013).

Despite their many advantages, usability and diversity, the price of microcarriers is highly expensive, reaching up to RM 8,455 per 100 grams for solid microspheres (Cytodex™ 3) and RM 11,295 per 100 grams for macroporous microspheres (Cultispher G). Until now the use of microcarriers is largely dominated by major pharmaceutical companies such as Merck, Baxter, GlaxoSmithKline, Sanofi, etc. for the production of vaccines and high-value pharmaceutical products. In Southeast Asia, there is only one company that has been identified as using microcarriers for commercial production of their pharmaceuticals. PT Bio Farma (Indonesia) which is the only human vaccine manufacturer in Indonesia and the biggest in Southeast Asia uses Cytodex™ microcarriers for producing vaccines for diseases such as measles, polio and hepatitis B (Maryama, 2010).

Meanwhile in Malaysia, local pharmaceutical companies still largely depend on herbal extracts, semi-synthetic products and suspension cell cultures for the production of generic drugs, biosimilar and other basic pharmaceutical products (MIDA, 2013). Moreover, the country's biggest vaccine manufacturer, Malaysia Vaccines and Pharmaceuticals (MVP) also still use the cumbersome embryonated eggs method to produce their vaccines. Most pharmaceutical products in the local market are imported products and more worryingly, many of these products contain non-halal ingredients, derived from porcine or parts of animals that have not been slaughtered according to the Shariah ("Malaysia: CCM Leading the way in Halal Pharmaceuticals", 2013). The current scenario is believed to have resulted from the lack of appropriate infrastructures, weak financial capacities and most importantly limited expertise in the pharmaceutical sector in Malaysia. But recently, the government has taken an initiative by collaborating