DEVELOPMENT OF POLYCAPROLACTONE MICROCARRIER WITH SURFACE TREATMENT USING ULTRAVIOLET/OZONE SYSTEM

BY

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ABSTRACT

Growing cells on microcarriers may have overcome the limitation of conventional cell culture system. However, the main challenge remains at ensuring the surface biocompatibility with cells. Polycaprolactone (PCL), a biodegradable polymer, has received considerable attention because of its good mechanical properties and degradation kinetics that suit various applications, but its non-polar hydrocarbon moiety renders it sub-optimal for cell attachment. In this present study, microcarrier was developed using emulsion-solvent evaporation method where the factors that affect the particle size distribution was studied. The factors studied were stirring speed, surfactant concentration and ratio of solute to solvent. Next is the modification of the microcarrier surface to improve the polarity and hydrophilicity of PCL microcarrier using ultraviolet/ozone (UV/O_3) treatment and followed by immobilisation of Halal gelatin on the microcarrier surface. Optimise condition of UV/O_3 that give maximum carboxylic functional group concentration (1509.19nmol/g) was determine by varying the parameters such as oxygen concentration, amount of samples and exposure time. Introduced oxygen functional group on the surface made the microcarrier susceptible for the biomolecular immobilisation as well as the attachment of certain cell (immortalised cell). The immobilisation of gelatin was statistically optimised by varying parameters such as EDAC ratio to carboxylic group, NHS concentration and gelatin concentration. The improvement of modified surface was determined by series of characterisation such as contact angle analysis, ATR-FTIR and SEM analysis. The last stage was the biocompatibility study of the developed PCL microcarrier (untreated, UV/O_3 treated, gelatin coated). The microcarriers were tested with few cell lines namely human keratinocytes cells (HaCaT), rat amniotic fluid stem cell (AFSC) and human skin fibroblast cell (HSFC) that represent immortalised cells, stem cells and primary cells respectively. All cells show favourable biocompatibility towards gelatin coated microcarrier surface except for HaCaT cell that achieved high cell density towards both gelatin coated PCL and UV/O_3 treated PCL. This study has successfully developed two functional microcarriers which are i) UV/O_3 microcarrier (charge microcarrier) that are meant for immortalized and continuous cell lines and ii) gelatin coated microcarrier for low plating efficiency cells that require supplementary growth factor to attach and proliferate in-vitro. To this end, this study provides a novel model system for the in vitro study of cell proliferation ability on PCL microcarrier.

ملخص البحث

إنّ نمو الخلايا على الناقلات الدقيقة قد تحدّ من النظام التقليدي لزراعة خلايا الثدييات. ومع ذلك، يبقى التحدي الرئيسي في ضمان توافق سطح الناقلات مع الخلايا. وقد حظي عديد الكابرولاكتون (PCL)، وهو بوليمر قابل للتحلل، باهتمام كبير بسبب خواصه الميكانيكية الجيدة وحركية الانحلال التي تناسب التطبيقات المختلفة، إلا أن طبيعته الهيدروكربونية غير القطبية تجعله دون المستوى الأمثل لمرافقة الخلية. والهدف من هذه الدراسة هو تطوير ناقل دقيق عديد الكابرولاكتون المغلف بالجيلاتين مع خصائص جيدة باستخدام نظام مكون من الأشعة فوق البنفسجية / الأوزون (O_3 / UV). تم تطوير الناقل الدقيق باستخدام طريقة استحلاب المذيبات والتبخر. تم دراسة العوامل (سرعة التحريك، تركيز مخفضات التوتر السطحي ونسبة المذاب إلى المذيب) التي تؤثر على توزيع حجم الجسيمات. وأعقب ذلك تعديل سطح الناقل الدقيق لتحسين قطبية الناقل الدقيق PCL و ميله للماء باستخدام المعالجة بنظام الأشعة فوق البنفسجية / الأوزون (O_3 / UV) ثُمّ لاحقاً تثبيت الجيلاتين الحلال على سطح الناقل الدقيق. تم تحديد الحالة المثلى للأشعة فوق البنفسجية / الأوزون والتي أعطت أقصى تركيز لمجموعة الكربوكسيل (1509.19 نانومول/ جرام) لتكون في تركيز الأوزون عند 75،328،71 جزءًا في المليون، وكمية من العينات 5.2 جرام ووقت التعرض عند 53 دقيقة. وفي الوقت نفسه، تم الحصول على التثبيت الأمثل للجيلاتين (1,830 ميكروجرام / جرام) في نسبة EDAC إلى مجموعة الكربوكسيل عند 1.5: 1، وتركيز NHS بقيمة 10 ميللي مولار وتركيز الجيلاتين عند 78 ملجرام / مللتر. تم التأكد من نجاح تثبيت الجيلاتين على سطح الناقل الدقيق PCL باستخدام التحليل الطيفي للأشعة تحت الحمراء (FTIR)، حيث سُجلت أعلى قمة عند 1654 سم$^{-1}$ و 1544 سم$^{-1}$ والذي يعزى إلى أميد I و أميد II. وعلاوة على ذلك، فإنّ انخفاض زاوية اتصال الماء (86.93 درجة لPCL غير المعالجة إلى 49.34 درجة بعد تثبيت الجيلاتين) أكّد تحسين ميل السطح للماء. تم اختبار التوافق الحيوي للناقل الدقيق PCL الذي تم تطويره (غير المعالج، والمعالج بالأشعة فوق البنفسجية / الأوزون ، و المغلف بالجيلاتين) مع الخلايا الكيراتينية البشرية (HaCaT)، الخلايا الجنينية الجذعية للفئران (AFSC) وخلايا الجلد البشرية الليفية (HSFC) والتي تمثل كلاً من الخلايا الخالدة (المستمرة) والخلايا الجذعية والخلايا الأولية على التوالي. أظهرت خلايا HaCaT توافقاً حيويّا مماثلاً نحو كلٍّ من PCL المغلف بالجيلاتين (. $17.6 \times 10^5 \pm 14.1$ خلية / مللتر) و المعالجة بالأشعة فوق البنفسجية / الأوزون ($18.5 \times 10^5 \pm 9.8$ خلية / مللتر) من الناقلات الدقيقة. ومع ذلك، كانت خلايا HSFC و AFSC أكثر ملاءمةً تجاه الناقلات الدقيقة المغلفة بالجيلاتين (20.6

4.7 ±10^5 × خلية/ مللتر في حالة HSFC ؛ بينما شوهد 24 ± 10^5 ×16.5 خلية/ مللتر في حالة AFSC) مقارنة بالناقلات المعالجة بالأشعة فوق البنفسجية /الأوزون (10^5 ×6.1 8.4 ± خلية /مللتر في حالة HSFC ؛ و 7.01 ± 10^5 ×4.3 خلية / مللتر في حالة AFSC). في الختام، فقد حددت هذه الدراسة بنجاح الظروف المثلى (معلمات العملية) وطورت اثنين من الناقلات الدقيقة الوظيفية) 1) الناقلات الدقيقة المعالجة بالأشعة فوق البنفسجية / الأوزون (نواقل مشحونة) والتي تناسب الخلايا الخالدة والمستمرة و 2) الناقلات الدقيقة المغلفة بالجيلاتين المغلفة للخلايا ذات الكفاءة المنخفضة مثل الخلايا الجذعية والخلايا الأولية.

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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LIST OF ABBREVIATION

µ	micro unit
2-D	Two dimension
3-D	Three dimension
AFSC	Amniotic Fluid Stem Cells
ANOVA	Analysis of Variance
ATR/IR	Attenuated Total Reflection-Infrared
CO	Carbonyl
CO_2	Carbon dioxide
COOH	Carboxyl/carboxylic acid
$D_{3,2}$	Sauter mean diameter
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DOE	Design of Experiments
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
FCS	Fetal calf serum
FTIR	Fourier Transform Infrared
GMEM	Glasgow Minimum Essential Medium
HaCaT	Human keratinocytes cell
HCl	Hydrochloric Acid
HSFC	Human Skin Fibroblast Cell
MC	Microcarrier
MES	2-(N-morpholino)-ethanesulfonic acid
MSCs	Mesenchymal Stem Cells
N_2	Nitrogen
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NH_2	Primary amine
NHS	N-Hydroxysuccinimide
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
PVA	Polyvinyl alcohol
RGD	Arg-Gly-Asp
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SKUT-1	Human uterine mixed leiomyodarcoma
TBO	Toluidine Blue O
THF	Tetrahydrofuran
UV	Ultraviolet
UV/O_3	Ultraviolet/ozone
WCA	Water Contact Angle
XPS	X-ray photoelectron

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Microspheres are spherical particles with a size range between 0.1 to 200 µm (Sahil et al., 2011). Due to its small size, microspheres can be catagorise as microparticle, microcarrier and microcapsule. It has a wide variety of applications for example in drug delivery system, cell culture and tissue engineering, protein immobilisation, and gene delivery. In each of the application, there are different terms used to indicate the used of microspheres specifically. The term microparticle is commonly used in drug delivery system where the consistent and predictable particle surface area is important (Nikam et al, 2012). The microsphere that may entrap cells in the inner compartment is called microcapsule (Tan et al, 2009). Meanwhile, microsphere that is use to support cell in mammalian cell culture which grow as monolayer on the surface of microcasphere are called microcarrier (Brun-Graeppi et al, 2011; Tan et al, 2009). In this thesis, the term microcarrier is use since this particle is applied to cultivate and propagate mammalian cells.

Microcarrier cell culture system served two significant purposes. First, it is to mass produce large amounts of certain bioproducts such as recombinant protein, hormone, and vaccine, where animal cells are routinely cultured in a bioreactor to meet industrial demand (van der Velden-de Groot, 1995) and clinical trial stage (Goh et al., 2013). The second purpose is to serve as delivery of cultured cells, and transplantation of biodegradable microcarrier loaded with the cultured cell into the body (Seland et al., 2011). Therefore, materials with appropriate degradation rate are

favourable in microcarrier cell culture system to reduce the effect of toxic degradation product.

Formerly, in cell culture work, anchorage dependent cells are commonly been activated on the wall of roller bottles or non-agitated vessels such as tissue culture flasks (White and Ades, 1990). This system is perfectly suit for research and lab scales. Moving to industrial production scale, those systems were no longger relevant due to the limitation in culture space, cell yield, control in culture condition and sterility. Particularly, industrial production is to produce large amount of mammalian and its bioproduct. Microcarrier is among the most establish technological platforms for industrial production to increase productivity (Chu and Robinson, 2001). Microcarrier acts as a substrate for cells to attach and is cultured in suspension in bioreactor. There are different types of bioreactors that are utilised microcarrier to grow mammalian cells such as stirred tank and fluidised bed bioreactor.

Cell culture using microcarrier beads as three-dimensional substrate was first introduced by Van Wezel (1967). This three-dimensional cell culture has undergone extensive modifications with the goal of optimising conditions for cell propagation and simulating in vitro and in vivo conditions (Overstreet et al., 2003; Blum, 2007). Under proper conditions, cells attach and spread onto the carrier beads and gradually grow out to a confluent monolayer (van der Velden-de Groot, 1995).

Physical and chemical interactions between the cell, microcarrier and the suspending medium have been shown to improve cell attachment and productivity. The bulk chemistry, surface charge, wettability and geometrical properties including surface roughness of the engineered matrix are the primary factors in determining cell behaviour for in vitro cultures (Ramsay et al., 1997; Zhu et al., 2004; Chen et al., 2004; Li et al., 2005; Langer, 1999; Hersel et al., 2003; Benhabbour et al., 2008;

Howe et al., 2002; Davis et al., 2002; Rebollar et al., 2008). Therefore, selection of materials as the carrier system is important since every aspect of material preparation can introduce variables that are known to influence cell interactions.

Biomaterials such as biodegradable polymers are particularly suited for the development of microcarrier. The surface can be further improved as desired by introducing functional groups such as hydroxyl and carbonyl onto the surface of polymer microparticle. For instance, a combination of ozone aeration and UV irradiation was reported to improve immobilisation of gelatin onto the microcarrier, therefore, enhancing the anchorage-dependent cell proliferation (Yusilawati et al., 2010).

This study was set to fabricate biodegradable microcarrier beads using solvent evaporation method and identify the controlling parameters that affect particle size. Ultraviolet ozone (UV/O_3) process conditions were then optimised to improve the surface of the microcarrier beads by introducing functional groups prior to optimisation of gelatin immobilisation on the surface of the microcarrier. Finally, the fabricated microcarrier beads were tested for their cytocompatibility by culturing mammalian cell lines on the microcarrier in spinner vessel culture system.

1.2 PROBLEM STATEMENT

Conventional culture systems for growing anchorage-dependent animal cells such as T-flasks and roller bottles have many limitations. This includes the requirement of a large area for growth with a high volume of culture medium. Such system also poses a high risk of contamination, yield low cell densities, difficult monitoring, control and scale-up; and often does not meet the requirements of industrial production of biological products. A microcarriers culture system provide a solution to the problem, the main challenge is to select the best material that is biocompatible and able to suspend in culture medium. Polycaprolactone (PCL), a biodegradable polymer, has received great attention because of its good mechanical properties and degradation kinetics, thus suitable to various applications. However, PCL is a non-polar material rendering it not readily suitable for cell attachment; the surface of PCL needs to be modified with the addition of functional groups such as carbonyl (CO), hydroxyl (OH) and carboxyl (COOH). Another major challenge is related to the coating of most commercial microcarriers with doubtful and non-halal materials such as porcine gelatin or collagen. Mammalian cells such as primary and epithelial cells have preferences towards surfaces that are coated with biological material like collagen or gelatin and have a weak attachment to polymeric culture surfaces that only provide positive charges. The products (enzymes, hormones, vaccines) which are produced using the microcarrier with these dubious materials may contain traces of non-halal material that are not suitable for Muslim consumers.