FABRICATION OF GRAPHENE OXIDE AND GRAPHENE-OXIDE/PEPTIDE BIOFILMS: CHARACTERIZATION, CELL VIABILITY AND DIFFERENTIATION POTENTIAL OF WHARTON'S JELLY MESENCHYMAL STEM CELLS

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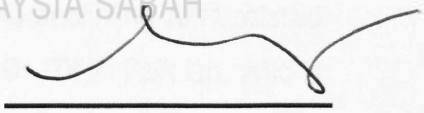

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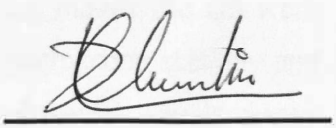

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ABSTRACT

Human Wharton's jelly mesenchymal stem cells (WJ-MSCs) treatments are being tested clinically for a range of disorders. Surface modification techniques have been instrumental in the development of biomaterials that promote cell-surface interactions. In this study, the surface of graphene oxide (GO) was modified to promote the proliferation and differentiation of Wharton's jelly mesenchymal stem cells (WJ-MSCs). Synthesized GO was prepared through modified Hummers method, fabrication of GO film using drop-casting method and attachment of peptide to GO film through non-covalent approach, π-π and electrostatic interactions. Synthesized GO were confirmed by UV-vis, XRD and FTIR. SEM and AFM images showed that synthesized GO has curled transparent thin film with thickness of 1.10 nm. Four peptide sequences, namely Pep1 ("N"-YIGSRWYQNMIRIKVAV-"C"), Pep2 ("N"-QHREDGSYIGSRIKVAV-"C"), Pep3 ("N"-WQPPRARIYIGSRIKVAV-"C") and Pep4 ("N"-DGEARGDSPKRSR-"C") were designed based short peptide sequences derived from extracellular matrix (ECM) adhesion peptides. AFM results revealed the thickness of GO biofilm (0.25 mg/mL) was 82.6 nm ± 10.4 nm, corresponding to 65 – 85 layers of single layer GO. The GO biofilm (0.25 mg/mL) treated with Pep1 shows decrease in thickness as compared to non-treated GO film and the present of peptide bond in GO/Pep biofilm was confirmed by modified Lowry method. Furthermore, GO biofilms with concentration lower than 0.25 mg/mL were able to maintain the cell viability at day 5 as compared to glass coverslip. The WJ-MSCs were able to attach and growth on GO film. Increased of cell viability at day 6 was observed for all the GO/Pep biofilms as compared to GO biofilm. GO and GO/Pep biofilm allowed WJ-MSCs attachment, proliferation and increased in osteogenic differentiation capacity. Besides, the cell cultured on GO and GO/Pep biofilm able to maintain its undifferentiated stem cell characteristic. The data obtained here collectively demonstrates that the GO/Peptide biofilm assemble via non-covalent approach is a potential substrate for the adhesion, proliferation and enhance osteogenic differentiation of human WJ-MSCs. In conclusion, GO/Pep biofilms can be utilized for designing and manipulating biomaterials for stem cell, biological and tissue engineering applications.

ABSTRAK

FABRIKASI BIOFILEM GRAPHENE OXIDE DAN GRAPHENE OXIDE/ PEPTIDA: PENCIRIAN, KEBOLEHAN HIDUPAN SEL DAN POTENSI PEMBEZAAN BAGI SEL STEM MESENKIMA WHARTON JELI

Rawatan sels stem mesenkima Wharton jeli manusia (WJ-MSCs) diuji secara klinikal untuk pelbagai penyakit. Teknik pengubahsuaian permukaan bagi menggalakkan interaksi sel dengan permukaan telah memainkan peranan penting dalam pembangunan biobahan. Dalam kajian ini, permukaan oksida grafit (GO) diubahsuai bagi menggalakkan kebolehan hidupan sel dan pembezaan WJ-MSCs. Oksida grafit yang disintesis melalui kaedah Hummers yang diubahsuai, fabrikasi filem GO dengan kaedah 'drop-casting' dan lekatan peptida ke filem GO melalui pendekatan bukan kovalen, π-π dan interaksi elektrostatik. GO yang disintesis disahkan oleh UV-vis, XRD, dan FTIR. Imej SEM dan AFM menunjukkan bahawa GO yang disintesis mempunyai ketebalan 1.10 nm. Empat urutan peptida, Pep1 ("N"-YIGSRWYQNMIRIKVAV-"C"), Pep2 ("N"-QHREDGSYIGSRIKVAV-"C"), Pep3 ("N"-WQPPRARIYIGSRIKVAV-"C") dan Pep4 ("DGEARGDSPKRSR-"C") direka berdasarkan urutan pendek peptida dari matriks ekstraselular (ECM). Hasil AFM mendedahkan ketebalan GO biofilem (0.25 mg/mL) adalah 82.6 nm ± 10.4 nm, bersamaan dengan 65 - 85 lapisan tunggal GO. Biofilem GO (0.25 mg/mL) yang dirawat dengan Pep1 telah menunjukkan penurunan dalam ketebalan berbanding filem GO yang tidak dirawat, dan kemunculan ikatan peptida dalam GO/Pep biofilem telah disahkan oleh kaedah Lowry yang diubah suai. Tambahan pula, biofilem GO dengan kepekatan lebih rendah daripada 0.25 mg/mL tidak menunjukkan ketara dalam daya tahan sel pada hari ke-5, berbanding dengan kawalan (kaca). WJ-MSCs dapat melampirkan dan berkembang pada filem GO. Peningkatan daya maju sel pada hari 6 diperhatikan bagi semua biofilem GO/Pep, berbanding dengan biofilem GO. GO dan GO / Pep biofilem membenarkan lekatan WJ-MSC, kebolehan hiduoan sel dan peningkatan keupayaan pembezaan osteogenik. Selain itu, sel yang berbudaya pada biofilem GO dan GO / Pep dapat mengekalkan ciri sel stem yang tidak dibezakan. Data yang diperoleh di sini secara kolektif telah menunjukkan bahawa biofilem GO/Pep dihasilkan melalui pendekatan bukan kovalen adalah substrat yang berpotensi untuk melekatkan, mempercepatkan dan meningkatkan pembezaan osteogenik WJ-MSC manusia. Kesimpulanya, biofilem GO/Pep boleh digunakan dalam mereka bentuk dan memanipulasi biobahan untuk aplikasi stem sel, biologi dan tisu.

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LIST OF SYMBOLS AND ABBREVIATIONS

&	And
ADSCs	Adipose-derived stem cells
AFM	Atomic force microscopy
BM-MSCs	Bone marrow derived mesenchymal stem cell
CD	Cluster of differentiation
ESC	Embryonic stem cell
FTIR	Fourier transformed infrared spectroscopy
GO	Graphene oxide
GO/Pep	Graphene oxide/Peptide
hESCs	Human embryonic stem cells
hMSCs	Human mesenchymal stem cells
hUC-MSCs	Human umbilical cord mesenchymal stem cells
iPSCs	Induced pluripotent stem cells
mg	Milligram
MHC	Major histocompatibility complexes
mL	Milliliter
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	number
nm	Nanometer
NSCs	Neural stem cells
PCL	Polycaprolactone
Pep1	Peptide-1
Pep2	Peptide-2
Pep3	Peptide-3
Pep4	Peptide-4
PXRD	Powder X-ray diffractometer
SD	Standard deviation
SEM	Scanning electron microscopy
UV	ultraviolet
WJ-MSCs	Wharton's jelly mesenchymal stem cells
µm	micrometer

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CHAPTER 1

INTRODUCTION

1.1 Research background

Recently, stem cell researches are expand rapidly with the potential to develop tissues/cells for transplantation, therapeutic agents to treat diseases as well as study disease development from early stages. Stem cells are an attractive prospect for tissue engineering and regenerative medicine due to their self-renewal capacity and multilineage differentiation (Higuchi *et al.*, 2012). Stem cell can be isolated from embryonic and adult stem cells. Embryonic stem cells (ESC) isolated from inner cell mass of a blastocyst are pluripotent (Bongso *et al.*, 1994), which able to differentiate into all types of cells from three germ layers namely, the ectoderm (epidermal tissue & nerves), mesoderm (muscle, bone & blood) and endoderm (liver, pancreas, gastrointestinal tract & lungs) (Thomas *et al.*, 2009). However, their clinical applications are limited by cellular immune rejection, tendency to form tumors and ethical issue (Edwards, 2007; Maitra *et al.*, 2005; Šarić *et al.*, 2008). Therefore, adult stem cells began to become a potential substitute source of stem cell. Mesenchymal stem cells derived from bone marrow show similar functions as ESC and at the same time without involving embryos destruction (Takahashi & Yamanaka, 2006). Unfortunately, when it comes to clinical applications, the bone morrow derived mesenchymal stem cell (BM-MSCs) also face challenges, such as genetic alterations occurring due to ageing, limitation of cell numbers, decreased growth and differentiation capacity changes and painful isolation process (Baksh *et al.*, 2007; Mimeault *et al.*, 2007; Mueller & Glowacki, 2001). Thus, extensive research have been done in proving mesenchymal stem cells isolated from human umbilical cord-derived stem cells as a new potential source of stem cells(Jin *et al.*, 2013; Nagamura-Inoue & He, 2014; Wang *et al.*, 2009; Yousefifard *et al.*, 2016).

Extensive investigations of Wharton's jelly mesenchymal stem cells (WJ-MSCs) as a potential source of stem cell have been done. WJ-MSCs shared several remarkable features that make these cells suitable as an alternative source of mesenchymal stem cells. WJ-MSCs have shown the differentiation potential of WJ-MSCs into adipogenic, osteogenic, chondrogenic, angiogenic, neurogenic and myogenic (Aristea *et al.*, 2013; Iwona *et al.*, 2013; Pires *et al.*, 2014; Xu *et al.*, 2017), high capacity of proliferation and no risk is associated with teratoma formation, do not elicit an immune reaction which may reduce the risks of rejection upon transplantation (Davies *et al.*, 2017; Karahuseyinoglu *et al.*, 2007; Kim *et al.*, 2013a; Medicetty *et al.*, 2004; Rachakatla *et al.*, 2007; Troyer & Weiss, 2008; Zhou *et al.*, 2011), abundance and easy accessibility during pregnancy from healthy mothers as they are discarded after delivery (Li *et al.*, 2017; Nagamura-Inoue & He, 2014), noninvasive isolation process and ethically free from controversial issues concerning human embryos as the stem cells sources (Edwards, 2007; Nagamura-Inoue & He, 2014). Due to these beneficial properties, WJ-MSCs shows ideal for clinical practice and good potential for cell therapy. However, WJ-MSCs have shown poor osteogenic and chondrogenic differentiation potential as compared to BM-MSCs (Hsieh *et al.*, 2010; Wang *et al.*, 2009). Hence, WJ-MSCs have been shown to increase its differentiation capacity by culturing in nano-scaffold (Gauthaman *et al.*, 2010; Hosseini *et al.*, 2015; Inthanon *et al.*, 2016). Despite the differences, future scientific and clinical application of WJ-MSCs will require a detailed understanding of the mechanisms and signals that maintain their undifferentiated or enhance their differentiation capacity.

The stem cell 'niche' refers to the specific microenvironment features that regulates the fate of stem cells (Fuchs *et al.*, 2004). The stem cell fate is manipulated by varying the niche, for example, by altering the signaling molecules, stromal support tissue with cell-cell interactions, integrins-mediated cell-matrix interaction with the surrounding ECM, biophysical stimuli (Brafman, 2013) . Recently, graphene-based nanomaterials are being applied to tissue engineering and induction of cell differentiations for *in vitro* stem cell culture.

Graphene-based biomaterials are able to modulate stem cell behaviors by providing a unique physical framework which proven to be comparable to natural ECM (Garcia-Alegria *et al.*, 2016; Kim *et al.*, 2013b; Marcela *et al.*, 2015). Due to the surface property of GO (hydrophilic) with the presence of oxygenated group enable them to bind with serum protein through electrostatic interactions and act as a pre-concentration platform to induce osteogenic and adipogenic differentiation (Lee *et al.*, 2011). Development of functionalized graphene oxide biomaterials is also necessary to improve their solubility and biocompatibility, and reduced cytotoxicity and genotoxicity in cellular application (Guo & Mei, 2014). The characteristic and biocompatibility of graphene-based nanomaterials may be controlled through surface functionalization either through covalent conjugation or noncovalent physisorption with other protein and peptides (Lu *et al.*, 2010; Sasidharan *et al.*, 2011).

To mimic the microenvironment of stem cell culture condition, extracellular matrix (ECM) components in important to enhance the growth and control stem cell fate (Ahmed & ffrench-Constant, 2016) Thus, synthetic biomaterials, such as peptides and polymers, are easily to fabricate and represent a reliable alternative for in vitro stem cell culture. Synthetic approaches for peptide materials, for example, short chain of amino acids, are quite different from protein materials, for example, long chains of amino acids. However, both strategies allow researchers the unique ability to program the exact monomer sequence within the biopolymer. Utilization of synthetic peptides as a component within engineered biomaterials is now a standard technique. For instances, peptides have been grafted to a variety of synthetic polymers to endow the material with cell-adhesive, enzymatically degradable and growth factor-binding properties.

In this study, GO biofilm were fabricated in various concentration and treated with bioactive peptide sequences via non-covalent approach as a biomaterials for culture WJ-MSCs. Then, direct contact of WJ-MSCs with various concentration of GO biofilm for certain cultured period via cell viability assay and morphological changes of cells were determined. The effect of bioactive peptide grafted on GO biofilm at different time point via cell morphology, cell viability assay and also the differentiation potential towards osteogenic and adipogenic lineages were studied.

1.2 Objectives

The objectives of this research are:

(i) To synthesize and characterize GO flakes, GO biofilms and GO/Peptides biofilms.

(ii) To evaluate the cell viability of various concentrations of GO biofilms and proliferation potential of GO/Peptide biofilm on Wharton Jelly's mesenchymal stem cells (WJ-MSCs).

(iii) To investigate the osteogenic and adipogenic differentiation potential of GO and GO/Peptides biofilms on WJ-MSCs.

1.3 Scope of Work

This research focus on the fabrication of biocompatible substrate for growing Wharton's Jelly mesenchymal stem cells (WJ-MSCs). Graphene oxide (GO) flake is first synthesize through the modified Hummers method from graphite flake. The fabrication of GO biofilms with varying ratio is prepare by drop-casted the synthesize GO solution on APTES-treated glass coverslip. For the fabrication of GO/Pep biofilm, peptide sequences is first design based on the bio-active short peptides. Then, GO/Peptide biofilms are prepare by assembly the different peptide sequences onto the surface of GO biofilm using non-covalent method. The surface of GO and GO/Pep biofilms are characterize using scanning-electron microscope (SEM) and atomic force microscope (AFM). The present of peptide bond on GO/Pep biofilm is measure using modified Lowry's method and Fourier transform infrared spectroscopy (FTIR). The cell viability of varying concentrations of GO biofilms are studied by culture with WJ-MSCs for 5 days using MTT assay. The osteogenic and adiposgenic differentiation potential of both GO and GO/Pep biofilms are also studied with culture WJ-MSCs. The main objective is to evaluate the cell viability and differentiation potential of WJ-MSCs growth on the GO film before and after treated with peptide using non-covalent approach. The expected outcome and hypothesis is that the GO biofilm treated with peptide will allow better stem cell proliferation and increase the differentiation capacity.

CHAPTER 2

LITERATURE REVIEW

2.1 Stem Cell

Stem cells are defined as cells that have two main characteristic: capability to proliferate indefinitely and multilineage differentiation (Leeb *et al.*, 2010; Zhang & Fu, 2008). These properties of human stem cells have been extensively studied for their regeneration processes, cell-replacement therapies, use for disease modeling and drug screening (Srivastava & Ivey, 2006; Wu & Izpisua Belmonte, 2016). Basically, stem cell can be classified based the differentiation capacity. Stem cell produced from the fusion of an egg and sperm cell are totipotent cells which is the most important stem cells that capable to differentiate into all types of cell, including cell for development of placenta, umbilical cord, and any other cell found in the adult body (Baker & Pera, 2018; Forraz & McGuckin, 2011). Embryonic stem cells isolated from inner cell mass of a blastocyst are pluripotent cells and potential to give rise to any differentiated cell in the body, except the placenta and umbilical cord (Hima & Srilatha, 2011; Thomson *et al.*, 1998). Induced pluripotent stem cells (iPSC) discovered by Yamanaka and coworkers by inducing the somatic terminally differentiated cell to express a number of genes that are normally present in embryonic stem cell to produce stable lines of embryonic-like pluripotent stem cells (Takahashi *et al.*, 2007; Takahashi & Yamanaka, 2006). Adult or somatic stem cells can also be isolated from various adult human tissues, such as brain, bone marrow, skin, liver, gut, fat and more. These adult stem cells can be isolated from several tissue sources, such as bone marrow, the central nervous system and skeletal. They are multipotent stem cells which can proliferate and differentiate into those closely related family of cells (Toma *et al.*, 2001). In the context of clinical applications of stem cells, totipotent stem cells, pluripotent stem cells (embryonic stem cell) and iPSC are currently constrained by cellular immune rejection (Šarić *et al.*, 2008),

tendency to form tumors (Maitra *et al.*, 2005; Shih *et al.*, 2011), ethical issue concerning human embryos as the sources (Edwards, 2007; Nagamura-Inoue & He, 2014) and low efficiency induced gene expression (for iPSC) (Forraz & McGuckin, 2011; Yamanaka, 2012). In contrast, the use of adult stem cells such as mesenchymal stem cells does not involve destruction of human embryos and at the same time show similar functions as ESC (Okolicsanyi *et al.*, 2015; Takahashi & Yamanaka, 2006). Adult stem cells have been used for many years for leukemia and blood/bone cancers treatment though bone marrow transplants (Moorthy, 2011; Radhika & Laxmi, 2011).

Mesenchymal stem cells (MSC) are adult stem cells that can proliferate as undifferentiated cells and able to potentially differentiate to lineages of mesenchymal tissues, such as cartilage, bone, tendon, fat, muscle and marrow stroma (Pittenger *et al.*, 1999) (Okolicsanyi *et al.*, 2015). Mesenchymal stem cell can be isolated mainly from adult tissues, including bone marrow (Wexler *et al.*, 2003) and adipose tissue (Zuk *et al.*, 2002). To a lesser extent, MSC can also isolated from dental pulp (Gronthos *et al.*, 2000), trabecular bone (Nöth *et al.*, 2002), tendon (Bi *et al.*, 2007), placenta (Fukuchi *et al.*, 2004), umbilical cord (Romanov *et al.*, 2003), amniotic fluid, synovia (De Bari *et al.*, 2001) and others. Due to the lack of standard isolation, culture and characterizing protocols, results in many difficulties when comparing the study outcomes. Thus, in year 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposes minimal criteria to define human mesenchymal stem cells (hMSCs). Firstly, hMSCs must be plastic-adherent in classical culture conditions. Secondly, under specific *in vitro* differentiation conditions, hMSCs must be able to consistently differentiate into trilineage differentiation, including adipocytes, osteoblasts and chrondroblasts. Additional requirements included hMSC population must express high levels (≥ 95% positive) of CD105, CD73 and CD90, and the low expression (≤ 2% positive) of the hematopoietic markers, CD 34, CD11b or CD14, CD 45, CD19 or CD79α and HLADR (Dominici *et al.*, 2006; Keating, 2012). For multipotentcy plastic adherent cells with lack of characterization data, the term 'multipotent mesenchymal stromal cell' with the same acronym MSC can be used to indicate these unique properties without ascribing homogeneity or stem cell activity (Davies *et al.*, 2017; Horwitz *et al.*, 2005).

For the past few years, clinical application of autologous bone marrow mesenchymal stem cells (BM-MSCs) was reported for conditions, including Crohn's disease (Duijvestein *et al.*, 2010), cardiac infarction (Minguell *et al.*, 2011), bone tissue engineering (Kagami *et al.*, 2011) and graft-versus-host disease (GVHD) (Muroi *et al.*, 2013; Muroi *et al.*, 2016; Weng *et al.*, 2010). Besides, BM-MSCs also face challenges like limitation of cell numbers, decreased growth and differentiation capacity due to age-related changes when it applied for clinical applications (Baksh *et al.*, 2007; Mueller & Glowacki, 2001). In recent years, extensive studies focus on human umbilical cord mesenchymal stem cells (hUC-MSCs) with similar gene expression profile of hESCs and faster self-renewal as compared to BM-MSCs as the alternative source of stem cell (Baksh *et al.*, 2007; Jin *et al.*, 2013; Malgieri *et al.*, 2010; Nagamura-Inoue & He, 2014; Wang *et al.*, 2009; Yousefifard *et al.*, 2016).

2.2 Wharton's Jelly Mesenchymal Stem Cells (WJ-MSCs)

2.2.1 WJ-MSCs as the Alternative Source of Mesenchymal Stem Cells

Human umbilical cord is considered as medical waste has been collected and used as the alternative source of stem cells. Isolation of mesenchymal stem cell from umbilical cord is noninvasive and does not encounter ethical problems (Li *et al.*, 2017; Nagamura-Inoue & He, 2014). Human umbilical cord with an inner tissue contain two arteries and one vein and is surrounded by a connective tissue called Wharton's jelly (Figure 2.1). Mesenchymal stem cells which directly obtained from Wharton's jelly, and are known as human Wharton's jelly mesenchymal stem cells (WJ-MSCs) (Forraz & McGuckin, 2011). Fibroblast-like cells were first isolated about 20 years ago from Wharton's jelly (McElreavey *et al.*, 1991). In 2004, WJ-MSCs were finally proved to be MSCs, as the cells expressed CD 105, CD 73, CD 51, CD 44 and CD 29, lacked expression of CD 45 and CD 34, and proved to be able to differentiate into osteogenic and adipogenic lineages (Wang *et al.*, 2004). Currently, WJ-MSCs can be isolated either using the explant method or the enzymatic digestion method and most likely consist of a heterogeneous population (Seshareddy *et al.*, 2008).