DETERMINATION OF MESENCHYMAL STEM CELL (MSC) ADHESION ON SURFACE MODIFIED WITH IONIC POLYMERS

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by

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CO_2	Carbon dioxide
Cy3	Cyanine 3
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
KCI	Potassium chloride
MSC	Mesenchymal stem cell
PAH	Poly (allylamine hydrochloride)
PBS	Phosphate buffer saline
PBT	Phosphate buffer with Tween 20
PSSCMA	Poly (4-styresulfonic acid-co-maleic-acid)

LIST OF SYMBOLS

%	percentage
nm	nanometer
μm^2	micrometer square
µl	microlitre
rpm	revolution per minute
ml	mililitre
g	gram
^{o}	temperature
mm^2	milimetre square
g	gram
mg/l	Miligram per litre

ABSTRAK

Daya kelekatan sel stem mesenkim dipengaruhi oleh ciri-ciri kimia and fizikal membran ekstraselular. Selain daripada itu, cas positif and cas negatif garam kalium juga mempengaruhi daya kelekatan sel. Namun begitu, kelekatan sel stem mesenkim pada polimer bercas positif and polimer bercas negatif masih kurang jelas. Jadi untuk mengetahui dengan lebih lanjut mengenai sel kelekatan pada permukaan yang disalutkan dengan kombinasi polimer bercas positif and juga negatif , kajian telah dilaksanakan. Kation seperti poli (allilamin hydroklorida) (PAH) and juga anion seperti poli (4-styresulfonik-acid-co-malleic-acid) (PSSCMA) telah digunakan. Kajian telah dilaksanakan dengan menggunakan 5 jenis permukaan yang disaluti dengan PAH:PSSCMA yang berlainan nisbah. Permukaan telah disaluti dengan menggunakan *spin coater*. Selain itu, permukaan yang tiada cas positif dan cas negatif telah digunakan sebagai kawalan kajian. Selain itu, permukaan yang disalutkan dengan polimer telah dianalisa dengan menggunakan 'goniometer'. Didapati nilai sudut sentuhan air pada permukaan 50:50 adalah tinggi. Ini menunjukkan bahawa kelekatan sel pada permukaan 50:50 adalah minimum. Kesan PAH:PSSCMA dengan berlainan nisbah pada kelekatan sel stem mesenkim telah dikaji. Didapati, ketumpatan cell pada permukaan 75:25 adalah 13 sel/mm^2 dan purata luas permukaan sel stem adalah yang paling tinggi. Ini menunjukkan sel stem mesenkim dapat mengadaptasi pada permukaan ini. Purata keamatan vinkulin juga tinggi pada permukaan 75:25. Manakala pada permukaan 0:100, terdapat 3 sel/mm^2 dan purata luas sel stem mesenkim adalah paling rendah.. Oleh itu, PAH dan PSSCMA boleh dijadikan bahan untuk mengkaji interaksi cell dengan polimer.

ABSTRACT

Mesenchymal stem cell (MSC) adhesion is influenced by the chemical and physical properties of extracellular membrane (ECM). For example, the electrostatic property of cationic and anionic sodium salt within ECM influence cell adhesion as well. However, the adhesion properties of MSC on surfaces constituting a combination of cationic and anionic polymer remains unclear. To uncover the complexity of cell-polymer interaction in *in vitro* settings, the ionic surfaces interaction with MSC was studied. Cationic such as poly (allylamine hydrochloride) (PAH) and anionic such as poly (4-styresulfonic-acid-co-malleic-acid) (PSSCMA) are used in designing the surfaces with different charges. In this study, five glass coverslips with different gradient of surface charges prepared by coating coverslip with varying ratio of PAH to PSSCMA using spin coater. Unmodified glass coverslip surfaces were used as control. The wettability testing of the surface were done using a goniometer. Surface with 50:50 gradient shows the highest contact angle , thus resisting the cell adhesion on the surface. Then the effects of effects of different PAH:PSSCMA gradient on MSC adhesion were investigated. Herein, cell numbers, cell density and the mean cell area determine the cell adhesion and spreadiness on different surfaces. Surfaces of 75:25 (PAH:PSSCMA) gradient have 13 cells/mm^2 and higher cell area while surfaces of 100:0 (PAH:PSSCMA) have 3 cells/mm^2 and with lowest cell area. In addition, the distribution of the vinculin in cell were determined as well. Vinculin was highly expressed on the 75:25 (PAH:PSSCMA) surface gradient followed by 25:75 (PAH:PSSCMA) while surfaces of 50:50 (PAH:PSSCMA) and 0:100 (PAH:PSSCMA) have lowest vinculin expression. Overall, MSC adhesion on ionic polymer surfaces has showed well expression of vinculin and better cell spreadiness.

CHAPTER 1

INTRODUCTION

1.1 Overview of the study

Cells are constantly interacting with other cells and the extracellular matrix (ECM) for their physiological function, social communication and staying viable. One of the cell-ECM interactions is called cell adhesion. Integrins are transmembrane heterodimeric receptors that are involved in cell adhesion (Hynes, R.O., 2002). Downstream signalling from cell adhesion is essential for other cellular activities such as cell migration, cell proliferation and cell death. Mesenchymal stem cells (MSCs) can be used as a cell model for studying the role of cell adhesion on cell fate. MSCs are multipotent cells whereby they are able to differentiate into osteoblasts, adipocytes, and chondrocytes, among other mature cell types (Caplan A. et al., 2007). Hence, these cells are useful for development of tissue and organ regeneration (Ghaemi, S. R., 2012) by naturally or artificially manipulating the ECM constituents or properties.

The chemical and physical properties of the ECM influence the behavior of stem cells. For example, MSCs change their morphology and gene expressions in response to the angularity within confined regions (Kilian, K. et al., 2010) and the density (Firth, J. E. et al., 2012) of adhesive glycoproteins such as fibronection. Similar responses are also

reported on the cells cultured on the surface of bioactive polymers (Lih, E. et. al. 2015). Therefore, MSC conformation depends on the microenvironment of ECM.

As for the physiochemistry at interfaces within ECM or artificial systems, surface charges have been reported to have effects on cell adhesion (Curran, J. M. et. al. 2006). Polyelectrolytes such as cationic and anionic have been used in the modification of biomaterial and scaffold surfaces. Cationic and anionic salt have been introduced on surfaces to promote cell adhesion through electrostatic attraction with MSCs (Vleggert-Lankamp, C. L. et. al. 2004). However, the behavior of MSCs on a mixture of charged polymers remains unclear. Likewise, the effects of the surface charges and their gradient towards MSCs adhesion remain to be clarified.

To uncover the complexity of cell-surface charges in an *in vitro* setting, integration of techniques from inter-science disciplines are required. For example, cationic polymers like poly (allylamine hydrochloride) (PAH) and anionic polymers like poly (4 – styrenesulfonic acid – co – maleic- acid) sodium salt (PSSCMA) can be used in the design of cell interaction with surfaces with different charges and possibly different adhesiveness. Such approach enables us to understand cells as dynamic entities and to engineer intelligent system by using surface charges to control cell adhesion.

Therefore, studying cell adhesion using MSCs as amodel of a cell type alongside chemically-modified surfaces hopefully will open up plenty avenues in advancing applications in tissue regeneration and transplantation. For this study, surfaces are coated

with different gradient of PAH-PSSCMA in order to observe the usability of graduated charged- surfaces in controlling cell adhesion. Furthermore, the physiochemical interactions between the MSCs and the polymers are observed as well.

1.2 Cell adhesion

Cells adhere to ECM through specific classes of transmembrane receptor called integrin. Integrin binding to at least three units of the ECM leads to clustering of more ECM bound integrins in cell membranes (Boettiger, D. 2012). This in turn advances the recruitment of focal adhesion proteins that take part in intracellular signaling pathways (Geiger, B et.al. 2001). Cell adhesion has important roles in the normal function and survival of the cells. Cell adhesion also contributes in further cellular organisation and structure (Richard, O. H. 2013).

Adhesion receptors like integrins and several intracellular proteins mediates cell adhesion (Figure 1.1). Examples of adhesion proteins are taxin, paxilin, FAK, vinculin and α-actinin, (Dumbauld, D. W. et. al. 2010). These proteins are specialised for supporting cell adhesion via integrin between adjacent cells and between cells and ECM. Hence, these adhesion proteins support physical linkages between the ECM and the internal structures of cells, thus controls the cell shape.

Focal adhesion is highly regulated and plays critical roles in cell spreading (Wehrle-Haller, B. 2012). It begins from small, dot-like structures, called nascent adhesions and focal complexes, which are located at the periphery of a spreading cell. These structures are then mature into stabilized, force generating structure called focal adhesion. For stem cells, matrix stiffness influences the focal adhesion formation and as well as the cytoskeleton restructuring. Focal adhesion is one of the integrated networks for cell signaling and force transduction; it mediates cell attachment, spreading and motility in response to the ECM composition (Figure 1.1)

Focal adhesions are sensitive to the mechanics of the cell structure and the interaction with the surrounding ECM. Focal adhesion also exhibits continuous remodeling in focal complexes whereby they form or enlarge when the traction forces upon cell-ECM interaction increases and likewise, disassemble when the forces decreases (Grashoff, C. 2010). Focal adhesions serve as an indicator for cell-matrix interaction . Therefore, a cell responses to the matrix composition can be studied following the recruitment of mechanosensitive proteins like vinculin to the basal surface of adherent cells.

Cell adhesion is a complex process which affected by numerous factors such as cell types and the ionic charges eg . polyelectrolytes known to influence the phenomenon (Zhang, N. et. al. 2012). The focal adhesion of MSCs is mediated by its adhesion receptors mainly integrin (Vagaska, B. et. al. 2010). Interaction of cells with their niche

are through their cell receptors and proteins that the cell secretes including ECM of which containing soluble and fibrous proteins. These proteins are adsorbed in the ECM via the physicochemistry of the proteins. Therefore, this bio-functionalised and interwined niche is suitable for cell growth (Vladkova, T. G. 2010).

Given the importance of cell adhesion in the functionality and the viability of the cells, it would be beneficial to create a simple, artificial system that can regulate cell adhesion. Gradient of ionic charges, among other physicochemical factors in ECM, may play some roles in cell adhesion.

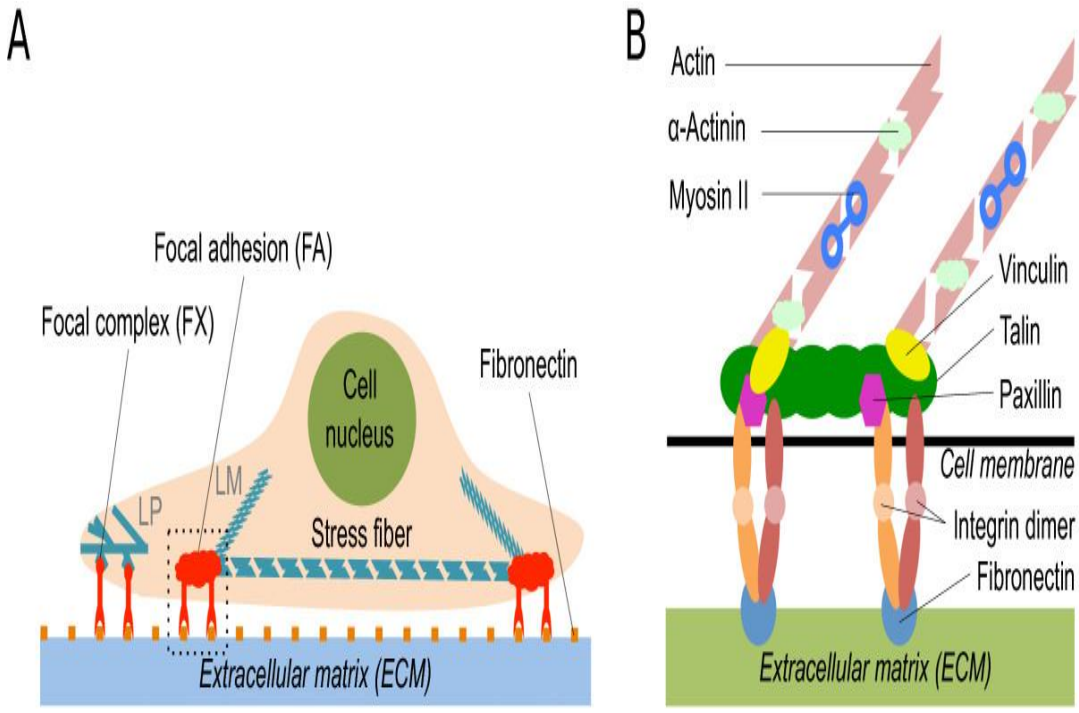

Figure 1.1 Schematic presentation of focal adhesions. (A) Cartoon of an adherent cell. During spreading and migration the cell adheres to ligands of the extracellular matrix (ECM), for example fibronectin, at the leading edge through nascent adhesions. They develop into focal complexes in the lamellipodium (LP), which can then mature into focal adhesions in the lamella (LM). (B) Enlarged view of a focal adhesion with the main molecular components. The transmembrane protein integrin binds to the fibronectin on the ECM.. This basic mechanical link is enhanced by proteins like vinculin, paxillin, or α-actinin. Figure adopted from (Hoffman, M & Schwarz, U. S. 2013)

1.3 Zwitterion

Zwitterions are electrically neutral ions. They contain nonadjacent regions of positively and negative charged groups (Chien, H. W. et. al. 2013). The prominence of zwitterionic materials in studying cell adhesion has increased in recent years (Kitano, H. et al. 2011). Previous studies have demonstrated that zwitterionic polymers have antifouling properties (Zhang, Z. et. al. 2006), which means the polymers inhibits non-specific protein adsorption (Tsai, W. B et. al. 2004). The antifouling phenomenon occurs as a result of high surface hydration on the opposing charges therefore more energy is required to remove the hydration layer to be replaced with the binding of non-specific proteins (Carr, R. L. et. al. 2011).

A polyelectrolyte (Figure 1.2) polymer has charged groups, subsist of either cationic groups, anionic groups or both. Thus, they can have a net positive, net negative or neutral charge depending on the ionic strength (Mah, E. & Ghosh, R. 2013). Polyzwitterions refer specifically to polymers that contain both positively and negatively charged groups.

The electrostatic repulsions between charged groups on polyelectrolyte chains force an extended chain formation. These repulsions can be disrupted by the addition of small electrolytes. This is the polyelectrolyte effect.

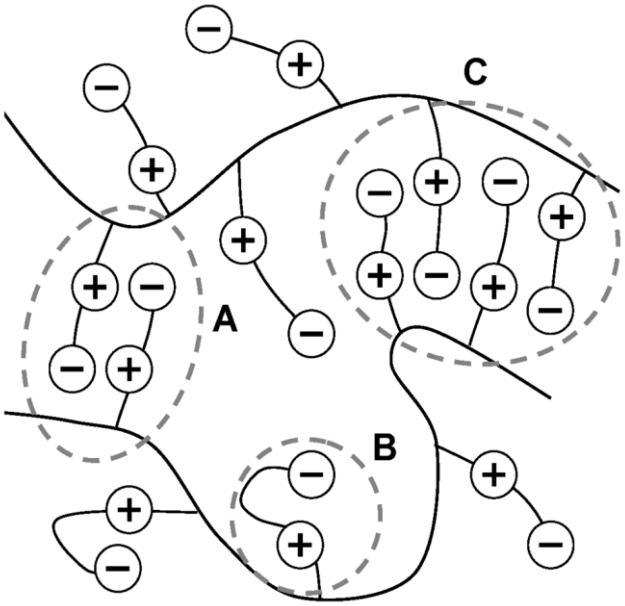

Figure 1.2 Short zwitterionic cluster (A), intragroup ion pair (B), long zwitterionic cluster (C), nucleated from a short cluster with entropy driven propagation outwards. Figure adopted from (Georgiev, G. S. et. al. 2001)

1.3.1 Charged polymers

Poly (allylamine hydrochloride) is a cationic polyelectrolyte (Figure 1.3). The preparation of this polymer is through the polymerization of allylamine. Previous study have used PAH in controlling the cell adhesion by changing the ratio of zwitterions (Estephan, Z. G. & Schlenoff, J. B. 2013).

It can be used together with an anionic polyelectrolyte which is poly (4– styrenesulfonic acid–co–maleic-acid) sodium salt (PSSCMA) (Figure 1.4). Previously, combination of PAH And PSSCMA forms layer by layer adsorbed film of positively and negatively charged polymers (Antipov, A. A. et al. 2003). However, no studies to date have shown a micture of cationic and anionic polymers on a single plane and their roles in cell adhesion.

Poly (allylamine hydrochloride), cations have been used for surface modification because of their electrostatic interaction with negatively charged cells (Gouping Chen et. al. 2008). Besides they did promote cell adhesion (Chen et. al 2009). On the other hand, poly (4 – styrenesulfonic acid – co – maleic- acid) sodium salt, anion interacts with the positively charged ECM protein, hence determines the cell-matrix adhesion (Lopez-Perez, P. M. et. al. 2010).

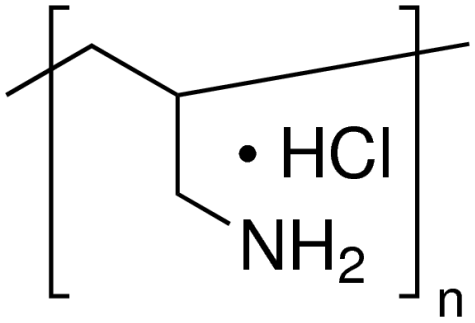

Figure 1.3 Chemical structure of PAH (cation)

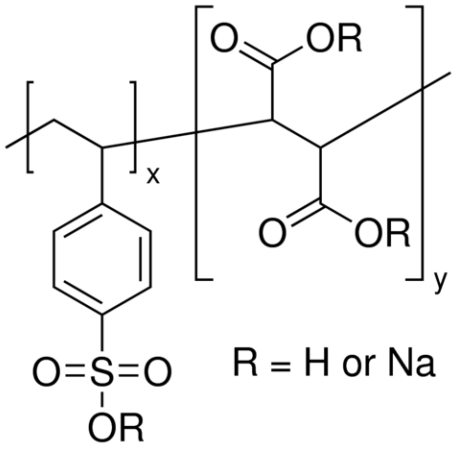

Figure 1.4 Chemical structure of PSSCMA (anion)

1.4 Mesenchymal Stem Cell

Stem cells are unspecialised precursor cells. They are capable of self renewal and differentiation into various connective tissue lineages via the production of various cytokines, chemokines and adhesion molecules (Zhao, W. et al. 2014).

Mesenchymal stem cells (MSCs) are adult stem cells found primarily in bone marrow. However, MSCs can also be isolated from other tissues including cord blood, peripheral blood, fetal liver and lung. MSCs differentiate to form adipocytes, cartilage, bone, tendons, muscle and skin (Mendez-Ferrer, S. et al. 2010). Morphologically MSCs have long thin cell bodies with one large nucleus. MSCs have a high capacity for self renewal while maintaining multipotency.

To our knowledge, there lack of study on the efficiency of MSC adhesion on the PAH-PSSCMA gradient surface. Thus, this study is aimed to examine on PAH-PSSCMA gradient formation the MSC adhesion and on the surface.

1.5 Vinculin

Vinculin is an ubiquitously expressed cytoskeletal protein that involves in cell adhesion. Vinculin is phosphorylated at several residue sites within the tail domain. The vinculin protein consists of a globular head domain connected to an elongated tail region by proline-rich domain. The head region contains binding sites for two cytoskeletal proteins, alpha-actinin and talin. Besides, the head region act as binding sites for tail region of vinculin. The tail region contains binding sites for actin and paxilin. Vinculin head controls integrin dynamics whereas the tail control the functional link between the focal adhesions and the actin cytoskeleton (Humphries, J. D. et. al. 2007).

Cell spreading occurs through the binding of cell surface integrin receptors to the extracellular matrix adhesion molecules. Vinculin is associated with focal adhesion and adherent junctions. These complexes nucleate actin filaments and form crosslinkers between the external medium (eg. Surface coated polymers), plasma membrane, and actin cytosleleton.

Cell adhesion requires the coordinated formation and release of focal adhesions. During this process, the exchangeable vinculin turnover rises during movement and declines upon maturation. The lack of vinculin decreases cell adhesion by inhibiting focal adhesion assembly. Vinculin is able to interact with integrin and cytoskeleton at the focal adhesion. This interaction controls the cell spreading and lamellipodia formation

(Figure 1.5). Thus, vinculin controls cell shape by modulating focal adhesion structure and function.

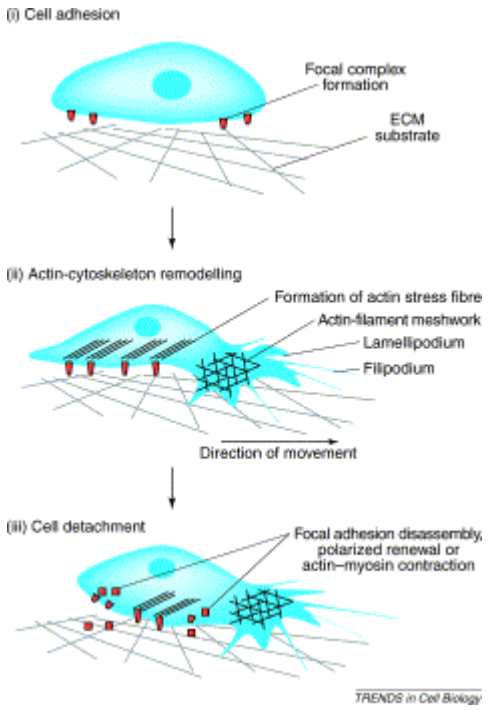

Figure 1.5 Schematic diagram lamellopodium formation (i) Cell adhesion, (ii) actin-cytoskeleton remodelling and (iii) cell detachment. Abbreviation: ECM, extracellular matrix. Figure adapted from (Carragher, N.O. & Frame, M. C. 2004)

1.6 Surface modification

Surface modification is the act of modifying the surface by bringing physical, chemical or biological characteristics different from the ones originally found on the surface. Modification can be done by different methods with a view to altering a wide range of characteristics of the surface such as roughness (Lapshin, R. V. et. al. 2010), surface charge, surface energy, biocompatibility and reactivity.

Previous studies indicated that parameters such as the chemistry of the surface affects cellular behavior such as the cell adhesion (Yang, J. et. al. 2010). Such surface gradient are one of the format that can be employed to examine the interaction of the cells with surface (Zonca Jr, M. R. et. al. 2013). In this study, I will introduce PAH-PSSCMA gradient to study the effect of such chemistry on cell adhesion.

1.7 Goniometer

Goniometer is used to measure the static contact angle of a surface. The method of measurement is based on Young-Laplace equation and the program designed to handle few measurements and one such is the sessile drop or better known as contact angle measurement. Young-Laplace equation is a nonlinear partial differential equation that describes the capillary pressure difference sustained across the interface between two static fluids such as water.

Sessile drop (Figure 1.6) are static drops that rest on a substrate below or they are bubbles that rise up against a substrate below. Sessile drop is utilized by an optical subsystem that snaps the profile of a static fluid on the surface (coated coverslip). Sessile drop is established by measurement of tangent of a liquid drop with a solid surface at the base (Della Volpe, C. et al. 2006).

The purpose of measuring contact angle in this study is to determine whether the surface coated with PAH and PSSS are hydrophilic or hydrophobic. In addition, the relationship of the polymers and the cell adhesion can be obtained as well.

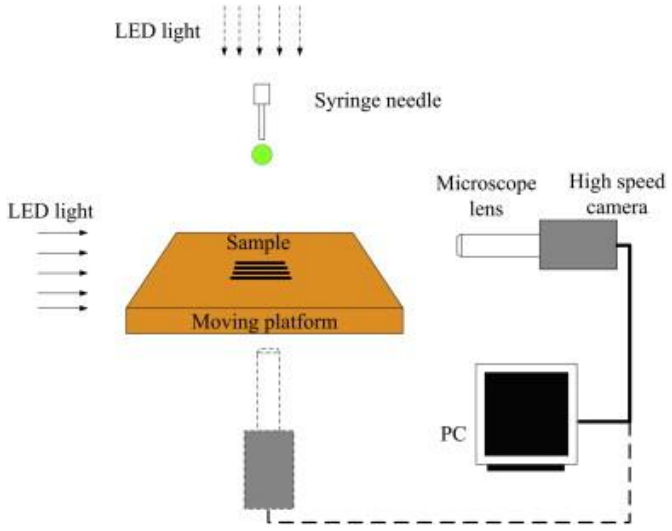

Figure 1.6 Schematic diagram of goniometer setup

(Song, D. et. al. 2015)

1.8 Confocal Microscopy

Fluorescence confocal microscopy most commonly used for in vitro and in vivo studies. The fluorescence microscopy uses dyes that fluoresce when stimulated by fluorophores. Fluorophores improve sensitivity and specificity by increasing the signal-to-noise and allows sharper detection of the image or target (Nwanieshiudu, A. et. al. 2012)

The principle behind the confocal laser microscopy is that it uses point illumination via spatial pinhole to eliminate out focus signals. The excitation light is usually provided by a laser to generate high intensities of fluorescence from the focal spot.

Upon hitting the target tissues, the excitation laser generates high intensities of fluorescence at well defined focal point. Laser light and the resultant emission fluorescence pass through the dichroic mirror. Dichroic mirror reflects the incoming, higher-energy laser light and at the same time allows the lower energy fluorescenct light to pass through the light detector (Figure 1.7).

Besides that, a pinhole is used to eliminate scattered light. As the end result, the light will be collected from a highly focused point. Images scanned can be reconstructed point by point as well.

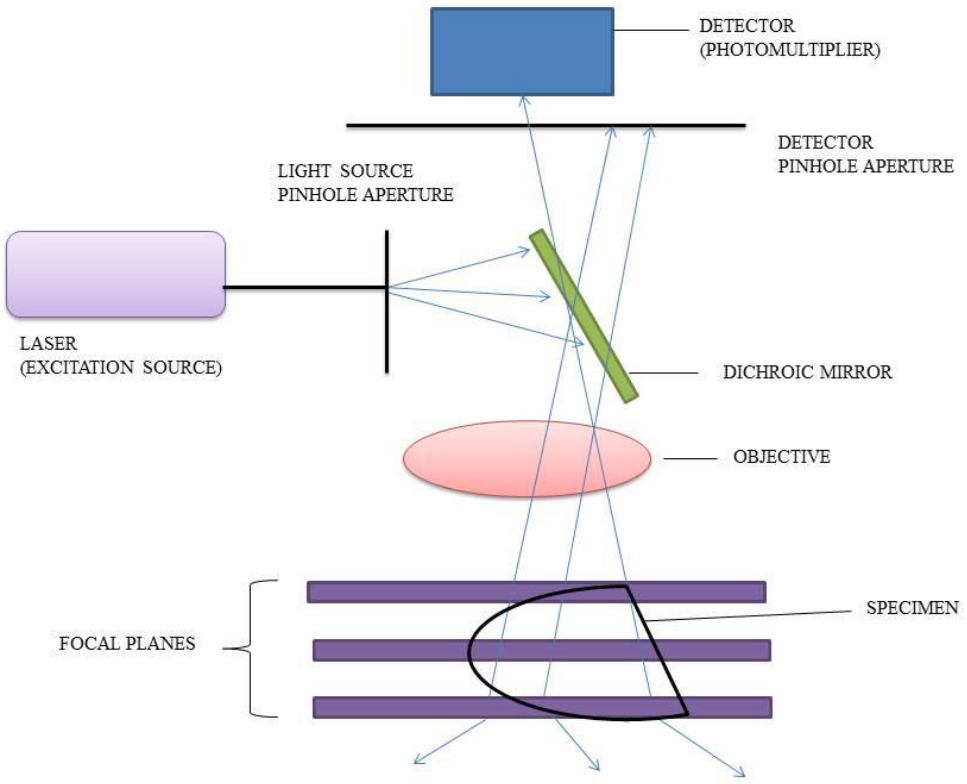

Figure 1.7 Schematic diagram of laser scanning confocal microscope

1.9 Research Questions

To date, many studies use cationic for cell adhesion study; however, very little knowledge of zwitterionic polymers reaction for similar purposes. There is also uncertainty on the effects of cell adhesion and its morphology on the cationic and anionic coated surface. Therefore, our proposed research questions:

i) gradient of PAH (cationic) and PSSCMA (anionic) and surface properties

ii) whether PAH (cationic) coated surface can promote cell adhesion

iii) whether PSSCMA (anionic) coated surface can promote cell adhesion

iv) the cell density on ionic surface

1.10 Hypothesis

i. MSCs on cation to anion surface or otherwise are well spread

ii. Less MSCs adhere on the 50:50 gradient surface as it serves as antifouling

1.11 Objective

1.11.1 General objective

To investigate whether gradient of cationic:anionic polymers can influence mesenchymal stem cell adhesion

1.11.2 Specific Objectives

a) To quantify the configuration of the polymers coated surface

b) To investigate MSC morphology and adhesion on PAH-PSSCMA gradient surface

c) To quantify fluorescent intensity of MSC on the PAH:PSSCMA gradient

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals, reagents and consumables

All chemicals were reagent grade or higher and purchased from Sigma-Aldrich, Life Technology and general consumables were obtained from Central Research Laboratory (CRL), IPPT, USM.

For surface coating, sulphuric acid (QRec) and hydrogen peroxide (QRec) were used to clean the coverslip. MiliQ water was used to wash the surface thoroughly after the cleaning.

For tissue culture, cell lines were taken from the Scientific Officer of Regenerative Medicine Cluster, Puan Siti Maisurah. Human mesenchymal stem cell (hMSC) was used as the cell models throughout this work. Dulbecco's Modified Eagle Medium (DMEM, Gibco, 11885-084) were used for cell culture. Foetal bovine serum (FBS, Gibco, 10270) and penstrep were used in the addition to the cell culture media. 0.25% Trypsin was used to lift off the adherent cells. Dulbecco's Phosphate Buffer Saline (DPBS, Gibco, 1627696) was used to rinsing cells in culture.

The following items were used in cell fixation and staining. Triton X- 100 (Sigma , X 100-500ml) was used for permeabilisation of the cell. TWEEN 20 (Sigma, P2287-100 ml) and Bovine Serum Albumin (BSA, Sigma, A9647) were used for washing and as blocking agent for the cells. Paraformaldehyde 4% was used to fix the cells on the coverslips. Vinculin Rb recombinant monoclonal Ab (Life Technologies, 700062) and Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Life Technologies, 1616933) were used to stain the vinculin. Alexa Fluor 555 phalloidin (Life Technologies, A34055) were used to stain the actin and Dapi (Invitrogen) was used to stain the cell nucleus of fixed cells.

2.2 Cell culture

Aseptic techniques were practiced in biological safety cabinets for all cell culturing. The cell were always cultured in a humidified , 37ºC incubator with 5% carbon dioxide (CO_2) and 95% air. The media and buffer were warmed in a water bath at 37º C prior to use.

2.2.1 Culturing of MSC

The mesenchymal stem cell (MSC) were cultured and seeded into two 25cm^2 flasks. Three ml of DMEM were added into 25 cm^2 flask. Non adherent cells were eliminated by changing the entire medium at day 3, then were replaced with fresh DMEM. The cells reach 80% confluency on the 5th day. The medium will be removed and the adherent cells were washed once with DPBS.

The cells were incubated with 1ml Trypsin for 3 minutes at 37ºC until the cells detach from the flask. Trypsin were transferred into 15ml falcon. About 4ml of DMEM were added into the 15ml falcon tube. The neutralisation of trypsin followed by collection and centrifugation of the cells for 3 minutes with the speed of 1000 rpm at 4ºC. The supernatant were discarded and about 1ml of fresh DMEM well be added to mix the cell pellet. Next the cell counting were proceeded using the haemocytometer.

2.2.2 Cell counting

The cell counting were carried out using hemocytometer (Neubauer). Before starting, the hemocytometer and its coverslip were cleamed by removing the dust particle using lens tissue. The coverslip were placed over the counting surface before loading the cell suspension. About 10 microlitre (µl) of cell suspension were introduced onto the surface. The loaded hemocytometer were placed on the microscope stage and the counting grid were brought into focus (Grigoryev, Y. 2014).

The full grid on the hemocytometer has nine squares, each of which is $1mm^2$. The central counting are of the hemocytometer contains 25 large squares and each large square has 16 smaller squares. Those cells on the lines of two sides of the large square were counted. (Figure 2.1).

The formula used for calculating total number of cells were as below:

Total cells/ml= Total cells counted/ # of squares x dilution factor x 10, 000 cells/ ml

Total number of cells obtained and its calculation

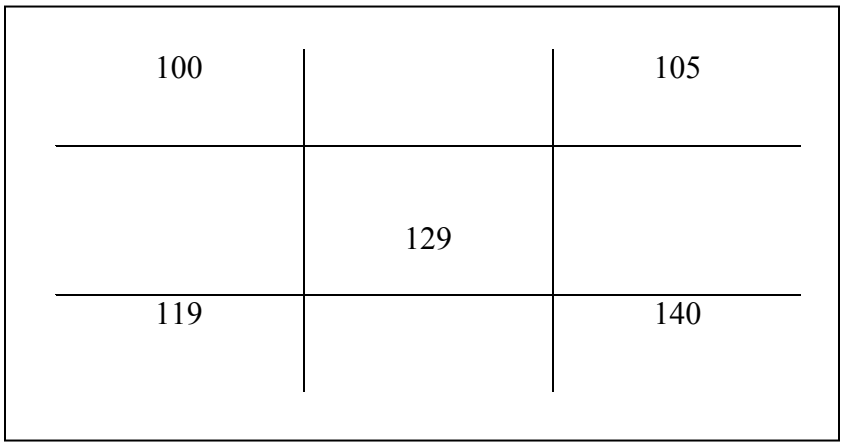

Figure 2.1 Example of cell counting

$(100 + 105 + 119 + 140 + 129) / 5 \times 10^4 \times 1 \text{ ml} = 1.186 \times 10^6$ cells

Per coverslip requires 150 000 cells, thus for 6 coverslips, total of 900, 000 cells required.

2.2.3 Cell seeding

After the cell counting, about 1874µl of DMEM were added into the each well plate containing the polymers coated coverslip. For each coverslip, desirable number of mesenchymal stem cells were $1.5x10^5$ cells which is about 126 µl. Then the cells were incubated in chamber with 5% carbon dioxide and temperature 37ºC. The incubation took about 4 hours. The cells reaches confluency after 4 hours of incubation. Now the cells are ready for fixation and immunostaining.

2.2.4 Immunostaining

The human mesenchymal stem cells (hMSC) were fixed with 4% paraformaldehyde (PFA) for 20 minutes, rinsed with PBS for three times and permeated with 0.2 % Triton X-100 in PBS (PBT) for 20 minutes (Wang PH et al. 2012). Then, the cells were blocked with 1% BSA-PBT solution for one hour.

After one hour of blocking with 1% BSA PBT solution, the cells were incubated overnight with primary antibody which is the vinculin in a humidified chamber. On the next day, the cells were washed three times, 5minutes each with BSA-PBT. Secondary antibody were added on the coverslip, incubated for 1 hour. The cells were washed again three times with BSA-PBT , 5 minutes each.