EFFECT OF AGAROSE ON VIABILITY OF SEEDED CELLS

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Specially dedicated
to my beloved father and mother

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ABSTRAK

Agarose hidrogel sering digunakan dalam kajian kejuruteraan tisu dengan menyediakan persekitaran tiga dimensi untuk tumbesaran sel. Kajian ini bertujuan untuk mengkaji kesan kepekatan agar agarose yang berbeza untuk kebolehhidupan dan pertumbuhan sel insulin D11 dan juga menilai biopenguraian serta bioserasi agarose. Penguraian agarose yang mempunyai kepekatan yang berbeza iaitu 1, 2 and 3% dalam media RPMI diperhatikan dan kadar berat yang hilang serta kadar penguraian diukur selama 14 hari. Agar agarose yang mempunyai tahap kepekatan yang berbeza (1, 2, dan 3%) dikulturkan dengan 4 ‘seeding densities’ yang berbeza dan kebolehhidupan sel diperhatikan selama 7 hari (hari ke-2, 4, dan 7) melalui ujian MTT. Ujian ini dijalankan untuk melihat kesan kepekatan agarose dan ‘seeding densities’ yang berbeza atas kebolehhidupan sel. Keputusan bagi kajian penguraian menunjukkan kadar penguraian agarose agak rendah untuk kesemua kepekatan. Berat yang hilang dalam 1% kepekatan agarose merupakan yang paling banyak, menunjukkan penguraiannya diikuti 2% dan seterusnya untuk 3% kepekatan agarose. Keputusan ujian MTT menunjukkan perbezaan kebolehhidupan sel berdasarkan kepada kepekatan agarose dan juga ‘seeding densities’ yang berbeza. Kebolehhidupan sel dilihat rendah pada kepekatan agarose yang tinggi, manakala kepekatan agarose yang rendah iaitu 1% menunjukkan kebolehhidupan sel yang baik. ‘Seeding density’ yang lebih tinggi iaitu 1×10^{6} sel per ml dilihat lebih sesuai untuk penkulturan sel dan menunjukkan peratus kebolehhidupan sel paling tinggi (≈50%) pada kepekatan agarose yang rendah. Kesimpulannya, agar agarose ialah material yang sesuai dan serasi untuk pengkulturan sel D11 secara 3 dimensi dan melalui pengubahsuaian kepekatan agarose dan ‘seeding densitiy’, pertumbuhan sel dalam agaros dapat dikawal.

ABSTRACT

Agarose hydrogel is commonly used in tissue engineering studies to provide three dimensional environment for the cells to grow. This research was undertaken to study the effect of different concentrations of agarose gels on the viability of D11 insulin cells and thus evaluating agarose biodegradability and biocompatibility. The degradation of different concentrations of agarose 1, 2 and 3% in RPMI media were observed and the weight loss and degradation rate were measured for 14 days. To test agarose biocompatibility on D11 cells and optimize the seeding density, different concentrations of agarose (1, 2 and 3%) gel were seeded with 4 different seeding densities and the viability of the seeded cells were observed for 7 days (day 2, 4 and 7) by using MTT assay. This was done to see the effect of different agarose concentrations and different seeding densities on cells viability. Results for degradation study showed the degradation rate of agarose was relatively slow for all concentrations, and the weight loss in 1% agarose was the highest indicating that its degrading faster followed by 2% and 3% respectively. Results for MTT assay showed differences in cells viability with different agarose concentrations and different seeding densities. Cells viability found to be decreased at higher agarose concentrations, while lower agarose concentrations as 1% enhanced cell viability the best. Higher seeding densities as 1×10^{6}cell/ml found to be more suitable for seeding on agarose and showed the highest percentage viability (≈50%) at low agarose concentrations. This can be concluded that agarose hydrogel is suitable and a compatible material for 3 dimension culture of D11 cells and that by altering agarose concentration and seeding density cell proliferation in agarose can be controlled.

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LIST OF ABBREVIATIONS

3 D	-	Three dimension
2 D	-	Two dimension
ECM	-	Extra cellular matrix
M monomer	-	Mannuronic acid monomer
G monomer	-	Guluronic acid monomer
DNA	-	Deoxyribonucleic acid
PGA	-	Polyglycolides acid
PLA	-	Polylactides acid
PLGA	-	Polylactic acid- co-glycolic acid
PCL	-	Polycaprolactone
PLLA	-	Poly-L-lactic acid
PDLA	-	Poly-D-lactic acid
PLDLLA	-	Poly-L-lactide- co –D,L-lactide
NEDH rat	-	New England Deaconess hospital rat
MTT	-	3 - [4,5 - dimethylthiazol-2-yl] -2,5-tetrazolium bromide diphenyl
CRFK	-	Crandall-Reese feline kidney cells
NOD mice	-	Non obese diabetic mice
ESC	-	Embryonic stem cells
cESC	-	Cynomolgus monkey embryonic stem cells
MSC	-	Marrow stem cells
hMSC	-	human mesenchymal stem cells
hBMSC	-	human bone marrow mesenchymal stem cells
ASCs	-	Adipose mesenchymal stem cells
HEK	-	Human embryonic kidney cells
MCF-7	-	Michigan Cancer Foundation-7
HEMA-MMA	-	Hydroxylethyl Methacrylate-co-methyl methacrylate

RPMI 1640	-	Roswell Park Memorial Institute Medium
FBS	-	Fetal Bovine Serum
PBS	-	Phosphate Buffer Saline
WST-1	-	Water soluble Tetrazolium salts assay
ml	-	millilitre
°C	-	degree Celsius (centigrade)
g	-	gram
mg	-	milligram
M	-	Molar
HCl	-	Hydrochloric acid
CO2	-	Carbon dioxide
cm	-	centimetre
mm	-	millimetre
µl	-	microliter
w/v	-	weight/volume
S.D	-	Seeding density

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Tissue engineering has emerged in late 1980's (Fergal, 2011). National science foundation workshop has defined it as the application of principles of engineering and life sciences to understand normal and pathological structure of mammalian tissues, and to develop biological substitutes that can restore, maintain and improve tissue function (Fergal, 2011). It is a multidisciplinary field which requires knowledge from material engineering, molecular and cell biology, physical science, medicine and life science to create artificial construct (Malafaya *et al*., 2007; Rezwan *et al*., 2006; Kneser *et al*., 2006).

The basic approach to tissue engineering is 3D culture system which involves the use of cells and scaffold to engineer the tissue *ex-vivo* (Rezwan *et al*., 2006). By isolating the specific cells through a small biopsy process and grow them in the flask. Then the specific seeding density that has been chosen will be seeded in a scaffold. Cells will grow and proliferate in the scaffold to form a construct which later can be implanted into the patient's body (Rezwan *et al*., 2006; Tejal, 2008).

An alternative approach in tissue engineering is by directly injecting the cells in *in-vivo* into the damaged area in the patient body (Koh and Atala, 2004). An advantage of this approach is to reduce the number of operation needed which will

lead to shorter recovery time but it's less controllable than *ex-vivo* approach and no additives such as (growth factor, proteins and DNA) can be added to enhance cell proliferation and relies on the body natural ability to regenerate (Narang *et al*., 2006; Godbey and Atala, 2002) while in *ex-vivo* approaches cell manipulation can be done *in-vitro* prior implantation and cell behavior and incubation conditions can be controlled (Godbey and Atala, 2002; Rezwan *et al*., 2006). Tissue engineering concepts have been successfully applied to generate different types of tissues such as bone, cartilage, skin, muscle, liver and others (Kenser *et al*., 2006).

The key components in tissue engineering *ex-vivo* are cells and scaffold. Normally body cells are residing in solid matrix called extra cellular matrix (ECM). ECM consists of mixture of components such as glycoproteins, proteoglycans and glycosaminoglycan that are organized in a network to which cells adhere (Rosso *et al*., 2004). ECM provides cells with rigidity and elasticity that also play an important role in cell differentiation and proliferation (Adams and Watt, 1993). So to successfully engineer the tissue, cells need to relay on scaffold material which is similar to ECM of tissue in native state (Chan and Leong, 2008).

Scaffold serve as a temporary ECM that entrap cells in 3 dimensional environment (3D) and provide frame work and support for the cells to attach, proliferate and differentiate at the same time forms their own ECM when the temporary ECM degrade by time in culture (Shoichet *et al*., 1996). Despite the advance in tissue engineering there still a number of obstacles, one of them is the reduce number of renewable cells (donors) that are immunologically compatible with patient, and lack of biomaterials (scaffolds) that can mimic mechanical, biological and chemical properties of ECM (Khademhosseini *et al*., 2007).

Scaffold need to be semi- permeable, which allows movement of nutrients, oxygen and growth factor but should not be permeable for immune components (Orive *et al*., 2003; Lahooti and Sefton, 2000). Biocompatible; must not elicit unresolved inflammatory, immunity or cytotoxicity response. Also need to be mechanically and structural support to the cells (Hutmacher, 2001), biodegradable

and easily fabricated into variety of shapes and sizes (Rezwan *et al*., 2006; Chung *et al*., 2008).

Scaffold material can be either from natural or synthetic polymers. Natural polymers include polysaccharides and proteins (Rezwan *et al*., 2006). Polysaccharide polymers such as (cellulose, agarose, alginate, and chitosan) compose of sugar ring building blocks and are commonly used in tissue engineering. Cellulose is the most abundant polysaccharide in nature and composed of glucose based repeated units linked by β glycosidic bonds (Ko *et al*., 2010). Chitosan is derived from chitin which found in exoskeleton of crustaceans and it is soluble at low pH (less than 5.5) and the gelling occurs by rising the pH, while alginate is derived from brown algae and it is structure composed of M monomers (mannuronic acid) and G monomers (guluronic acid) and gels via ionic cross linking in presence of divalent ions (Ko *et al*., 2010).

Protein polymers such as collagen, gelatin and fibrin which their building blocks are amino acids and they can mimic ECM and can induce direct cell growth during the tissue regeneration (Rezwan *et al*., 2006). Collagen is a major protein component in ECM of connective tissues, there are about 27 types of collagen but type I is the most used in medical applications, collagen mainly isolated from animals tissues and must be purified to eliminate immunogenicity problems (Malafaya *et al*., 2007). Gelatin is derived from collagen and commonly used in medical applications due to its low antigenicity in contrast to collagen. Fibrin is produced from fibrinogen which can be harvested from the patient providing immune-compatible carrier to the cells (Malafaya *et al*., 2007).

Synthetic polymers can be organic such as: PGA (polyglycolides), PLA (polylactides), PLGA (polylactic acid- co-glycolic acid), PCL (polycaprolactone), or inorganic: (bioactive ceramic, glass and hydroxyapatite) (Elif, 2010; Rezwan *et al*., 2006). Organic synthetic polymers belong to polyesters family and they are attractive in tissue engineering due to their biocompatibility and biodegradability through hydrolysis of ester bonds and their degradation products are metabolites that can be removed naturally through body pathways (Rezwan *et al*., 2006). PGA is a rigid thermoplastic material with high crystallinity that can be fabricated into various

forms and their degradation product is glycolic acid which is natural metabolite (Gunatillake and Adhikari, 2003). PLA is semi crystalline solid and has three isomers (PLLA, PDLA and PLDLLA), PLLA is the mostly used form because its metabolized best by the body and its degradation product is lactic acid (Gunatillake and Adhikari, 2003). PCL is also semi crystalline polymer has low melting point (59°C) and used mainly in drug delivery. PLGA is a copolymer of PLA/PGA which can be easily processed and their degradation rate and mechanical properties are adjustable (Rezwan *et al*., 2006). Bioactive ceramics and glasses are mainly used for bone tissue engineering; they can react with physiological fluids and form a bond with the bone but their biodegradability and biocompatibility are insufficient which limit their uses (Brahatheeswaran *et al*., 2011). Hydroxyapatite (HA) also shown to have good ability to bind to bones and their degradation products found to regulate gene expression that control osteogenesis (Rezwan *et al*., 2006).

Natural polymers can be applied mostly in growth of soft tissues such as (skin, tendons, muscles and nerves) (Sachlos and Czernuszka, 2003; Brun *et al*., 1999) because some of them are the main components of ECM of these tissues (*e.g*. collagen and gelatin), in addition they cannot support hard tissues like bone due to their poor mechanical properties (Hayashi and Toshio, 1994). Investigation into synthetic polymer and inorganic ceramic material mostly aimed for bone tissue engineering (Burg *et al*., 2000) because they resemble natural components of bone and have osteo- conductive properties.

Hydrogels are a promising scaffold option due to their structural similarity of ECM of many tissues. They are hydrophilic polymers that can swell in presence of water (Kong *et al*., 2003; Huang *et al*., 2006), and their high water content allow cell attachment and diffusion of nutrients which enhance cell viability (Nisbet *et al*., 2008). Hydrogels can provide a minimum invasive vehicle for tissue transplantation due to their elasticity, stability and biodegradability (Bryant and Anseth, 2001).

Agarose is a linear polysaccharide extracted from marine red algae; it’s a thermosetting hydrogel that undergoes gelation in response to reduction in temperature (Buckley *et al*., 2009). Agarose consist of alternating 1,3-linked β.D-

Galactopyranose and 1,4-linked 3,6 anhydro-α-L.galactopyranose units and contain a few ionized sulfate groups, the propensity to form gels increases with increase desulfation (Labropoulos *et al*., 2002; Malafaya *et al*., 2007). Gelling of agarose occur when agarose chains joined together forming a double helix closed tightly and trapping the water inside (Malafaya *et al*., 2007). These double strained helices are the result of specific intermolecular hydrogen bonding that cause the rigidity of polymer chain. Gelation occurs at temperature below 40°C where the melting temperature is 90°C (Malafaya *et al*., 2007).

Agarose hydrogel was used in this research based on its mechanical stiffness and its ability to distribute cells more uniformly (Lahooti and Sefton, 2000) which ensures nutrient availability for the growing cells allowing them to continue differentiation (Michael *et al*., 2010; Balgude *et al*., 2001), in addition to its biodegradability which will give space for the cells to grow and proliferate (Hunt and Grover, 2010). All of these properties have made agarose a good candidate for this research.

1.2 Problem Statement

Biodegradability of scaffold is a critical requirement for tissue engineering since it's difficult to remove scaffold surgically after implantation, also degradation of the scaffold will give space for the cells to grow and proliferate forming a new tissue (Zhang *et al*., 2012).

Degradation of different agarose gel concentrations in medium will be observed to see if the degradation rate of agarose can be controlled. Also a range of seeding densities will be used to optimize the suitable seeding density for insulin secreting cells (BRIN-D11) on agarose gel and also to find out which agarose concentration is more suitable and can enhance cell viability, thus determining the possibility of using agarose in the studies of insulin secretion, tissue engineering and transplantation in insulin dependent diabetes mellitus patients in future.

1.3 Objectives

- To study the degradation process on different concentrations of agarose gel in culture.
- To optimize the cell seeding densities for seeding on agarose gel.
- To observe the viability of seeded cells by using MTT assay.

1.4 Scope of Study

The scope of the study is to observe the degradation rate of agarose gel over time. Also to see the effect of different seeding densities on agarose gel and at the same time to observe the viability of the cells on different concentrations of agarose gel in culture.

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