

**THREE DIMENSIONAL GLYCOSAMINOGLYCAN MIMETIC
PEPTIDE AMPHIPHILE HYDROGELS FOR REGENERATIVE
MEDICINE APPLICATIONS**

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN
MATERIALS SCIENCE AND NANOTECHNOLOGY

By

Yasin Tümtaş

May, 2015

THREE DIMENSIONAL GLYCOSAMINOGLYCAN MIMETIC PEPTIDE
AMPHIPHILE HYDROGELS FOR REGENERATIVE MEDICINE
APPLICATIONS

By Yasin Tümtaş

May, 2015

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Asst. Prof. Dr. Ayşe Begüm Tekinay
(Advisor)

Assoc. Prof. Dr. Mustafa Özgür Güler

Assoc. Prof. Dr. Çağdaş Devrim Son

Approved for the Graduate School of Engineering and Science:

Prof. Dr. Levent Onural

Director of the Graduate School of Engineering and Science

ABSTRACT

THREE DIMENSIONAL GLYCOSAMINOGLYCAN MIMETIC PEPTIDE AMPHIPHILE HYDROGELS FOR REGENERATIVE MEDICINE APPLICATIONS

Yasin Tümtaş

M.Sc. in Materials Science and Nanotechnology

Advisor: Asst. Prof. Dr. Ayşe Begüm Tekinay

May, 2015

Defects and impairments of tissues or organs affect millions of people, resulting in considerable losses in workforce and life quality. The treatment of major tissue injuries requires the development of advanced medical techniques that enhance the natural repair processes of the human body. Novel biomaterials can modulate the repair of organs and tissues by providing a suitable environment for the recruitment, proliferation and differentiation of stem and progenitor cells, allowing the recovery of degenerated or otherwise nonfunctional tissues. Peptide amphiphiles (PAs) serve as model biomaterials due to their capacity for self-assembly, which allows peptide monomers to form complex networks that approximate the structure and function of the natural extracellular matrix. Peptide networks can be further modified by the attachment of various epitopes and functional groups, allowing these materials to present bioactive signals to surrounding cells. Glycosaminoglycans (GAGs) are negatively charged, unbranched polysaccharides that constitute a substantial part of the ECM in various tissues and play an important role in maintaining tissue integrity. Therefore, mimicking GAGs presents a suitable means for modulating cell behavior and especially lineage commitment in stem cells. In this work, I describe the design and synthesis of several bioactive, three dimensional (3D) GAG-mimetic peptide amphiphile hydrogels for *in vitro* stem cell differentiation and *in vivo* pancreatic islet transplantation. In Chapter 1, I detail the extracellular environment of tissues and the importance of GAGs in maintaining cell and tissue function. In Chapter 2, I describe the *in vitro* experiments involving the effects of sulfonation and the presence of glucose units on the differentiation of mesenchymal stem cells. In Chapter 3, I utilize a heparin-mimetic PA to increase the survival of pancreatic islets transplanted into

the rat omentum, and demonstrate that increased angiogenesis results in enhanced survival. Lastly, in Chapter 4, I summarize my results and describe the course of future experiments for the artificial regeneration of tissues through peptide amphiphile networks.

Keywords: Extracellular matrix, Peptide hydrogels, Glycosaminoglycans, Biomimetic, Mesenchymal stem cell, Differentiation, Islet transplantation, Angiogenesis

ÖZET

REJENERATİF TIP UYGULAMALARI İÇİN ÜÇ BOYUTLU GLİKOZAMİNOGLİKAN BENZERİ PEPTİT AMFİFİL HİDROJELLER

Yasin Tümtaş

Malzeme Bilimi ve Nanoteknoloji, Yüksek Lisans

Tez danışmanı: Yard. Doç. Dr. Ayşe Begüm Tekinay

Mayıs, 2015

Doku ya da organlarda meydana gelen hasarlar ve işlev bozuklukları dünya çapında milyonlarca insanı etkilemektedir. Rejeneratif tıp uygulamaları disiplinlerarası çalışmaları kullanarak vücudun bu hastalıklı veya hasara uğramış bölümlerini yenileyerek insan hayatını iyileştirme konusunda gelecek vaat etmektedir. Rejeneratif tıp çalışmalarında, biyomalzeme üretme ve kullanma üzerine kurulu doku mühendisliği yöntemlerinden yoğun bir şekilde istifade edilmektedir. Bu yüzden gelecekteki doku yenilenebilirliği çalışma ve uygulamalarda yeni biyomalzeme geliştirilmesi oldukça önemli bir gereksinim arz etmektedir. Peptit amfifiller, kendilerini oluşturan yapıtaşlarının sahip oldukları kendiliğinden bir araya gelebilme özellikleriyle oluşturdukları ağ yapısı sayesinde, bu tür çalışmalarda kullanılmak için ideal biyomalzemelerdir. Peptit amfifillerin kolayca üç boyutlu yapılar oluşturabilmeleri, hücrelerin normal biyolojik şartlarda yaşadıkları doku ortamına daha çok benzediği için *in vitro* ve *in vivo* çalışmalarda kullanılabilmesi açısından çok büyük bir olanak sağlamaktadır. Bu yapısal benzerlik, istenilen epitoplara veya işlevsel kimyasal grupların peptit amfifillerin yapı taşlarına eklenmesiyle birlikte hücrelerin içinde yaşadıkları hücrelerarası maddeye daha çok benzerlik göstermelerini sağlamaktadır. Hücrelerarası madde, hücrelerin ve dolayısıyla da dokuların yapılarını destekleyen bir iskele görevi üstlenmekle beraber, hücrelerin faaliyetlerini sürdürebilmeleri için de gereklidir. Glikozaminoglikanlar dallanmamış, tek polisakkarit zincirinden oluşan, eksi yüklü ve dokuların sağlıklı kalabilmeleri ve fonksiyon yürütebilmeleri için oldukça önemli hücrelerarası madde bileşenleridir. Glikozaminoglikanlardan esinlenerek tasarlanmış, bu şeker yapılarının benzeri biyomalzeme üretimi, hücrelerarası maddenin özelliklerini taşıyabileceği için doku

mühendisliğinde yararlanma konusunda oldukça mantıklı bir yaklaşımdır. Bu sebeple, bu tezde anlatılan çalışmalarda kök hücre başkalaşımı ve pankreatik adacık nakli çalışmalarında glikozaminoglikan benzeri 3 boyutlu hidrojel oluşturan peptit amfifilleri tasarlandı ve sentezledni. Birinci bölümde detaylı olarak rejeneratif tıptan, dokuların hücrelerarası madde içeriklerinden ve glikozaminoglikanların öneminden bahsedildi. İkinci bölümde de peptit fiberlere birleşik halde glikoz ve değişen konsantrasyonlarda sülfonat miktarının mezenkimal kök hücrelerin *in vitro* ortamda başkalaşmasına olan etkisi tanımlandı. Üçüncü bölümde, heparin benzeri peptit amfifil kullanarak, omentuma nakledilen pankreatik adacıkların hayatta kalma özelliklerinin artan anjiyogenezle birlikte iyileştiği gösterildi. Dördüncü bölümde ise son iki bölümdeki çalışmaların sonuçları özetlendi ve gelecekte yapılabilecek deneyler tartışıldı.

Anahtar kelimeler: Hücrelerarası madde, Peptit hidrojeller, Glikozaminoglikanlar, Biyomimetik, Mezenkimal kök hücre, Başkalaşım, Adacık nakli, Anjiyogenez

ACKNOWLEDGEMENT

First of all, I would like to express my appreciation to be part of the research groups of Nanobiotechnology and Biomimetic Materials Laboratories along with such nice, helpful and talented people. I consider myself so lucky to know them and I will never forget their support and friendship.

I would like to thank my advisor Prof. Ayşe Begüm Tekinay, who opened her laboratory to me for my master studies and led me to be more mature and experienced in my research career. I also would like to thank Prof. Mustafa Özgür Güler who let me to study with his research group and he provided such an environment in which I looked biological systems from different perspectives. They both supported and guided me throughout my master studies. Their contributions made these works possible.

I would like to express my most intimate thanks to my precious friend Öncay Yaşa. He has been always helpful, supportive and motivating. I cannot imagine these last three years without him. İ. Ceren Garip-Yaşa (my mhysa) and Elif Arslan totally deserve more than a thank and I am very grateful those great friends due to their presence. They never gave up on me, but they helped me for my adaptation to NBT-BML research groups. They tried their best to teach me and never lost their patience. I owe them because I learned many techniques and tools from them. I thank Berna Şentürk and Seda Koyuncu for cheering me up, supporting me in the way of pursuing my academic career and helping me to solve my problems. They were like my big and little sisters and pushed me further.

Regarding my project about *in vitro* stem cell differentiation in 3D gel system, I have benefited from the experiences of Elif Arslan and Melike Sever at the beginning of the project. I am thankful to have their help. In addition, Berna Şentürk, Seher Yaylacı (Üstün), Gülistan Tansık and Elif Ergül helped me in optimizations and differentiation analyses. Their friendship and consultation led me to complete my studies. Also, Melis Şardan Ekiz made possible my *in vitro* experiments by supplying me Glc-PA. I thank her a lot.

I would like to express my special thanks to Gözde Uzunallı and Öncay Yaşa for their efforts to complete *in vivo* experiments. I have learnt a lot from Gözde regarding histological procedures that helped me even in my *in vitro* study. I wouldn't have seen the end of the project without her before my graduation. I would like to thank to the Etlik group especially to İbrahim Ulusoy who is one of the most talented people that I have met. I owe the people who sacrificed many things to finish our projects, I thank them a lot.

I would like to express my most sincere thanks to Dr. Oya N. San Keskin who is a great fellow, teacher and eager scientist, and deserves the best. I am so lucky to meet her while I was so inexperienced and new in my first year of master studies. I have never forgotten her kindness.

My unforgettable companions, Melis Göktaş, Gülcihan Gülseren, Gülistan Tansık and Didem Mumcuoğlu have a special place in my heart. They are not only hardworking fellows, which inspired me a lot with their effort, but they are also such people who make their environment more friendly and joyful. Besides them, I never hesitated to ask questions to my friends Hakan Ceylan, Büşra Mammadov, Melike Sever, Göksu Çınar, M. Aref Khalily, and Reşad Mammadov, and they have always allocated their time to answer. I benefited their intellectuality and experiences. In addition to them, I would like to thank Zeynep Ergül Ülger for her technical help. Also, Suna Temiz has always motivated me with her cheerful laughs and she taught me lessons that are not given in the top ranking universities. I am so happy and grateful to know her.

I would like to thank Alper Devrim Özkan for reading my thesis and giving suggestions in such a limited time.

I would like to thank İkra Gizem Yıldız (my grasshopper), Merve Öz, Zeynep Orhan, Çağla Eren, Fatih Yergöz, İdil Uyan, Nurcan Haştar, Canelif Yılmaz, and İbrahim Çelik. They were with me day and night (especially night) while writing my thesis.

I am so grateful to Mevhibe Yakut Geçer, Nuray Gündüz, Ömer Faruk Sarıoğlu, Murat Kılınç, Hepsî Hari Susapto, Şehmus Tohumdeken, Özüm Şehnaz Günel, Egemen Deniz Eren, Aslı Çelebioğlu, Ashif Shaikh, Zeliha Soran Erdem, Ebuzer

Kalyoncu, Tolga Tarkan Ölmez, Ayşe Özdemir, Aygöl Zengin, Meryem Hatip, Seren Hamsici, Gökhan Günay, Ruslan Garifullin, Zeynep Aytaç and Ahmet Emin Topal, for creating such a warm working environment, and wish the best for them. My special thanks are to Zeynep Erdoğan, a skilled technician, for her technical help. It was wonderful to work with all of them. I also thank little İpek and Gülnâre whose presence was just enough for me to feel better.

Emre Evin, M. Fatih Diler, Kübra Narcı, Yusuf T. Tamer, Bora Ergin and Ulvi O. Karaca were with me throughout my master years, and I thank them a lot for their support and companionships.

I would like to thank UNAM for providing me such an opportunity and to thank TÜBİTAK (The Scientific and Technological Research Council of Turkey) for financial support, BİDEB 2210-E M.Sc. fellowship and grant 113T045.

Lastly, I would like to express my most sincere gratitude to my family who always supported and loved me without any expectation. I am proud of them. I am so grateful for my two *angels*, my *earth* and *sea*, and my *blood* and *flesh*. I hope I never let them down.

CONTENTS

ABSTRACT	iii
ÖZET	v
ACKNOWLEDGEMENT	vii
CONTENTS	x
LIST OF FIGURES	xiv
LIST OF TABLES	xviii
ABBREVIATIONS	xix
CHAPTER 1) INTRODUCTION	1
1.1. REGENERATIVE MEDICINE AND TISSUE ENGINEERING IN THE LIGHT OF THE STOLEN FIRE	2
1.2. TARGET TISSUES FOR REGENERATIVE MEDICINE	5
1.2.1. Adipose Tissue	5
1.2.2. Cartilage Tissue	7
1.2.3. Bone Tissue	8
1.2.4. Pancreas Tissue	10
1.3. CELL SOURCES USED FOR THE REGENERATION OF DAMAGED TISSUES	13
1.4. BIOMATERIALS USED IN TISSUE ENGINEERING STUDIES	14
1.5. CHEMICAL CUES USED TO INDUCE DIFFERENTIATION	17
1.6. ASSESSMENT OF DIFFERENTIATION	18
1.7. EXTRACELLULAR MATRIX: AN INSPIRATION FOR MIMESIS	23
1.8. CELL CULTURE APPROACHES	32
CHAPTER 2) MONITORING DIFFERENTIATION PROFILE OF RAT MESENCHYMAL STEM CELLS IN THREE DIMENTIONAL GLYCOSAMINOGLYCAN MIMETIC PEPTIDE AMPHIPHILE HYDROGEL SCAFFOLDS	36
2.1. INTRODUCTION	37
2.2. MATERIALS AND METHODS	39

2.2.1. Synthesis, Purification and Characterization of Peptide Amphiphile Molecules	40
2.2.1.1 Synthesis of Peptide Amphiphile Molecules by Solid Phase Peptide Synthesis	40
2.2.1.2 Purification of Peptide Amphiphile Molecules.....	41
2.2.1.3 Circular Dichroism.....	42
2.2.1.4. Scanning Electron Microscopy	42
2.2.1.5. Oscillatory Rheology	42
2.2.2. Peptide Amphiphile Nanofiber Formation.....	43
2.2.2.1. PA Nanofiber Formation in 2D Surface Coatings	43
2.2.2.2. 3D PA Gel Scaffold Formation.....	44
2.2.3. Cell Culture and Viability Analyses	45
2.2.3.1 Cell Maintenance	45
2.2.3.2. Cell Maintenance for 2D Cell Coating Experiments and Viability Analysis.....	45
2.2.3.3. Cell Maintenance for 3D Cell-Laden Gel Experiments and Viability Analyses	46
2.2.4. Gel Embedding and Cryosectioning	47
2.2.4.1. Fixation of the Gels and Preparation for Cryosectioning.....	47
2.2.4.2. Cryosectioning of the Gels with Cryostat	47
2.2.5. Differentiation Analyses	47
2.2.5.1. Alkaline Phosphatase Activity	47
2.2.5.2. Oil Red O	48
2.2.5.3. Safranin O	48
2.2.5.4. Alizarin Red S	49
2.2.5.5. Immunostaining of Aggrecan and Analysis of Stained Cells	49
2.2.5.6. Quantitative Reverse Transcription Polymerase Chain Reaction	50

2.2.6. Statistical & Computer Based Analyses	50
2.3. RESULTS	52
2.3.1. Synthesis of Peptide Amphiphiles	52
2.3.2. Physical Characterization of Peptide Amphiphiles.....	59
2.3.2.1. Secondary Structure Analysis	59
2.3.2.2. Mechanical Characterization.....	59
2.3.2.3. Self-Assembly Analysis	59
2.3.3. Toxicity and Cell Survival Analyses	63
2.3.3.1. Biocompatibility of Peptide Amphiphiles.....	63
2.3.3.2. Survival of Cells in 3D Gel Environment	66
2.3.3.3. Proliferation of Cells in 3D Gel Environment	69
2.3.3.4. Visualization of Cells inside Gels	70
2.3.4. Effect of Peptide Amphiphiles to Cell Differentiation	75
2.3.4.1. Cell Differentiation on 2D Peptide Amphiphile Coatings	77
2.3.4.2. Determining Differentiation of Cells Encapsulated in 3D Gel System to Osteogenic Lineage.....	80
2.3.4.2.1. Alkaline Phosphatase Activity	80
2.3.4.2.2. Gene Expression analyses	81
2.3.4.3. Determining Differentiation of Cells Encapsulated in 3D Gel System to Adipogenic Lineage	85
2.3.4.4. Determining Differentiation of Cells Encapsulated in 3D Gel System to Chondrogenic Lineage	89
2.3.4.4.1. Gene Expression Analyses	89
2.3.4.4.2. Immunohistochemical Staining.....	94
2.4. DISCUSSION	101
2.5. CONCLUSION	108

CHAPTER 3) IMPROVING PANCREATIC ISLET TRANSPLANTATION EFFICIENCY USING HEPARIN MIMETIC PEPTIDE AMPHIPHILE NANOFIBER GEL	110
3.1. INTRODUCTION	111
3.2. MATERIAL & METHODS.....	113
3.2.1. Materials.....	113
3.2.2. Synthesis and Purification of Peptide Amphiphile Molecules.....	113
3.2.3. Physical, Mechanical and Chemical Characterization of Self-assembled Nanofiber Network.....	115
3.2.3.1. Transmission Electron Microscopy (TEM)	115
3.2.3.2 Scanning Electron Microscopy (SEM)	115
3.2.3.1. Circular Dichroism (CD).....	115
3.2.4. Animals	116
3.2.5. Islet Isolation and Culture	116
3.2.6 Transplantation.....	117
3.2.7 Scaffold Function Assessment	118
3.2.8 Histological Analysis	118
3.2.9 Statistical Analysis	119
3.3. RESULTS	119
3.3.1. Heparin Mimetic PA Molecules Self-assemble into Nanofibers	119
3.3.2. Transplantation within Bioactive PA Scaffold Improves Glucose Responsiveness of the Transplanted Islets.....	124
3.3.3. PA Scaffold Supports Islet Integrity and Enhance Vascular Density of the Transplantation Site	127
3.4. DISCUSSION	134
3.5. CONCLUSION	137
CHAPTER 4) CONCLUSION AND FUTURE PERSPECTIVES	138
BIBLIOGRAPHY	142

LIST OF FIGURES

Figure 1.1 General strategy of tissue engineering approaches that use porous biomaterial scaffolds.	4
Figure 1.2 Physiology of white adipose tissue.	6
Figure 1.3 The cartilage extracellular matrix.	8
Figure 1.4 Hierarchical organization of bone from macro- to nanoscale.	10
Figure 1.5 Schematic representation of the exocrine and endocrine units of pancreas.	12
Figure 1.6 Schematic representation of the function of the extracellular matrix.	24
Figure 1.7 Disaccharide units of extracellular matrix glycosaminoglycans.	28
Figure 1.8 Space-filling model of the short chains of hyaluronan, dermatan sulfate and heparan sulfate glycosaminoglycans.	31
Figure 1.9 Dynamic self-assembly of peptide amphiphiles into nanofibrous structures.	35
Figure 2.1 Molecular structures of the peptide amphiphiles.	53
Figure 2.2 Liquid chromatography-mass spectrometry (LC-MS) analysis of K-PA.	54
Figure 2.3 Liquid chromatography-mass spectrometry (LC-MS) analysis of Glc-PA.	55
Figure 2.4 Liquid chromatography-mass spectrometry (LC-MS) analysis of E-PA.	56
Figure 2.5 Liquid chromatography-mass spectrometry (LC-MS) analysis of SO ₃ -PA.	57
Figure 2.6 Liquid chromatography-mass spectrometry (LC-MS) analysis of RGD-PA.	58
Figure 2.7 Photographs of cell-laden PA hydrogels.	60
Figure 2.8 Secondary structure analyses of peptide amphiphile mixtures by circular dichroism.	60
Figure 2.9 Storage and loss moduli of peptide amphiphile gel groups obtained by oscillatory rheology.	61
Figure 2.10 Scanning electron micrographs of the nanofibrous structure of peptide amphiphile gels.	62

Figure 2.11 Viability of rMSCs on 2D coatings of peptide amphiphile combinations and on TCP after 1, 7, 14 and 21days of incubation.	64
Figure 2.12 Quantified viability of rMSCs on 2D peptide amphiphile coatings after 24 hours.	65
Figure 2.13 Viability of rMSCs inside the 3D gels of peptide amphiphile mixtures after one day of incubation.	67
Figure 2.14 Viability of rMSCs inside the 3D gels of peptide amphiphile mixtures after one week of incubation..	68
Figure 2.15 Proliferation of rMSCs inside the peptide amphiphile gel mixtures in seven days via metabolic activity measurement (alamarBlue).	69
Figure 2.16 Scanning electron microscopy images of cells on and inside the GS gels after 21 days of incubation with different magnifications.	70
Figure 2.17 Scanning electron microscopy images of cells on and inside the GES gels after 21 days of incubation with different magnifications.	71
Figure 2.18 Scanning electron microscopy images of cells on and inside the GE gels after 21 days of incubation with different magnifications.	72
Figure 2.19 Scanning electron microscopy images of cells on and inside the KE gels after 21 days of incubation with different magnifications.	73
Figure 2.20 Scanning electron microscopy images of cells on glass surface after 21 days of incubation with different magnifications.	74
Figure 2.21 Cell morphologies inside the gels.	76
Figure 2.22 Rat MSC differentiation pattern on 2D peptide amphiphile coatings. ..	78
Figure 2.23 Stainings of empty (without cells) coatings.	79
Figure 2.24 Alkaline phosphatase (ALP) activity of the cells inside gels and on glass surface after 7, 14 and 21 days of incubation.	80
Figure 2.25 Differentiation pattern of rMSCs into osteogenic lineage. Expression levels of <i>Runx2</i> on day 3, day 7, day 14, and day 21.	82
Figure 2.26 Differentiation pattern of rMSCs into osteogenic lineage. Expression levels of <i>Col1a1</i> on day 3, day 7, day 14, and day 21.	83
Figure 2.27 Differentiation pattern of rMSCs into the adipogenic lineage. Expression levels of <i>Adipoq</i> on day 3, day 7, day 14, and day 21.	86

Figure 2.28 Differentiation pattern of rMSCs into the adipogenic lineage. Expression levels of <i>Fabp4</i> on day 3, day 7, day 14, and day 21.....	87
Figure 2.29 Differentiation pattern of rMSCs into the chondrogenic lineage. Expression levels of <i>Sox9</i> on day 3, day 7, day 14, and day 21.....	90
Figure 2.30 Differentiation pattern of rMSCs into the chondrogenic lineage. Expression levels of <i>Acan</i> on day 3, day 7, day 14, and day 21.	91
Figure 2.31 Differentiation pattern of rMSCs into the chondrogenic lineage. Expression levels of <i>Col2a1</i> on day 3, day 7, day 14, and day 21.....	92
Figure 2.32 Immunohistochemical staining of cryosectioned rat cartilage tissue with anti-aggrecan antibody.....	95
Figure 2.33 Immunohistochemical staining of cryosectioned GS gels with anti-aggrecan antibody.	96
Figure 2.34 Immunohistochemical staining of cryosectioned GES gels with anti-aggrecan antibody..	97
Figure 2.35 Immunohistochemical staining of cryosectioned GE gels with anti-aggrecan antibody.	98
Figure 2.36 Immunohistochemical staining of cryosectioned KE gels with anti-aggrecan antibody..	99
Figure 2.37 Quantification of positively stained cells with aggrecan antibody per gel area (mm ²).....	100
Figure 3.1 Molecular structures of the peptide amphiphiles used in the study.....	120
Figure 3.2 Liquid chromatography-mass spectrometry (LC-MS) analysis of K-PA.	121
Figure 3.3 Liquid chromatography-mass spectrometry (LC-MS) analysis of HM-PA.	122
Figure 3.4 Self-assembly of peptide amphiphiles and characterizaiton.....	123
Figure 3.5 Technique used to implant peptide amphiphile hydrogel (with or without islets) into the omentum of the diabetic rats	125
Figure 3.6 Weight of the animals and blood glucose levels of the groups throughout 28 days (Day 0; transplantation day). Intraperitoneal Glucose Tolerance Test (IPGTT) at the end of 28 days.....	126
Figure 3.7 Hematoxylin & Eosin staining of omenta undergone operation.....	128

Figure 3.8 Masson's trichrome staining of in omenta undergone operation.....	129
Figure 3.9 Immunohistochemical staining of insulin in omenta undergone operation.	130
Figure 3.10 Immunohistochemical staining of macrophages in omenta undergone operation.....	131
Figure 3.11 Immunohistochemical staining of von Willebrand in omenta undergone operation.....	132
Figure 3.12 Average blood vessel number in omentum of animals.....	133

LIST OF TABLES

Table 2.1 Content of experimental groups in terms of peptide amphiphile percentage.	44
Table 2.2 Genes used to determine the differentiation pattern of rMSCs, their forward and reverse primers and annealing temperatures.....	51
Table 2.3 Sequences of the peptide amphiphiles, their molecular weights, and their theoretical overall charges at neutral pH.....	52
Table 2.4 Degree of significant difference of osteogenic gene expressions between groups within different days.....	84
Table 2.5 Degree of significant difference of adipogenic gene expressions between groups within different days.....	88
Table 2.6 Degree of significant difference of chondrogenic gene expressions between groups within different days.	93

ABBREVIATIONS

<i>Acan</i>	Gene of Aggrecan
<i>Adipoq</i>	Gene of Adiponectin
AFM	Atomic force microscopy
ALP	Alkaline Phosphatase
ANOVA	Analysis of variance
ARS	Alizarin Red S
BAT	Brown adipose tissue
BMP-2	Bone morphogenic protein-2
BMSC	Bone mesenchymal stem cell
Boc	<i>Tert</i> -butoxycarbonyl
BSA	Bovine serum albumin
CD	Circular dichroism
cDNA	Complementary DNA
<i>Col1a1</i>	Gene of Collagen type 1
<i>Col2a1</i>	Gene of Collagen type 2
CS	Chondroitin sulfate
DAB	Diaminobenzidine
DAB	3,3'-diaminobenzidine
DCM	Dichloromethane
DIEA	<i>N,N</i> -diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
DMF	<i>N,N</i> -Dimethylformamide
DMSO	dimethyl sulfoxide
DS	Dermatan sulfate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
E-PA	Lauryl-VVAGE
ESC	embryonic stem cell
ESI	Electrospray ionization
<i>Fabp4</i>	Gene of Fatty acid binding protein-4

FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
Fmoc	9-Fluorenylmethoxycarbonyl
FN	Fibronectin
GAG	Glycosaminoglycan
<i>Gapdh</i>	Gene of Glyceraldehyde 3-phosphate dehydrogenase
GES	Glc-PA/E-PA/SO3-PA/RGD-PA mixture
GES	Glc-PA/E-PA/RGD-PA mixture
GF	Growth factor
Glc-PA	Lauryl-VVAGKS(Glucose)-NH ₂
GS	Glc-PA/SO3-PA/RGD-PA mixture
H&E	Hematoxylin and eosin staining
HA	Hyaluronan
HBSS	Hank's Balanced Salt Solution
HBTU	<i>N,N,N',N'</i> -Tetramethyl-O-(1 <i>H</i> -benzotriazole-1-yl) uronium hexafluorophosphate
HM-PA	Heparin mimetic peptide amphiphile
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
HS	Heparan sulfate
HUVEC	Human umbilical vein endothelial cell
IPGTT	Intraperitoneal glucose tolerance test
iPSC	Induced pluripotent stem cell
KE	K-PA/E-PA/RGD-PA mixture
K-PA	Lauryl-VVAGK- NH ₂
KS	Keratan sulfate
LC-MS	Liquid chromatography-Mass spectroscopy
O.C.T. (or OCT)	Optimum cutting temperature compound
OA	Osteoarthritis
ORO	Oil Red O
P/S	Penicillin/Streptomycin

PA	Peptide amphiphile
PBS	Phosphate-buffered saline
PEK	Protein Extraction Kit
PG	Proteoglycan
PIK	Protease Inhibitor Cocktail
qRT-PCR	Quantitative real-time polymerase chain reaction
Q-TOF	quadrupole time flight
RGD-PA	Lauryl-VVAGERGD
rMSC	Rat mesenchymal stem cell
<i>Runx2</i>	Gene of Runt-related transcription factor 2
SEM (Microscopy)	Scanning electron microscope
SEM (Statistics)	Standard error of the mean
SO	Safranin O
SO₃-PA	Lauryl-VVAGEK(<i>p</i> -sulfobenzoate)-NH ₂
<i>Sox9</i>	Gene of Transcription factor SRY-box 9
STZ	Streptozotocin
T1D	Type I diabetes mellitus
T2D	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TBS-T	TBS with 0.025% Triton X-100
TCP	Tissue culture plate/plastic
TEM	Transmission electron microscope
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TIS	Triisopropylsilane
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue

CHAPTER 1

INTRODUCTION

1.1. REGENERATIVE MEDICINE AND TISSUE ENGINEERING IN THE LIGHT OF THE STOLEN FIRE

Ancient Greek myths speak of a titan who had been punished for his kindness to the human race. According to the story, Prometheus stole a spark from Mount Olympus, against the will of the chief god Zeus, and introduced fire to the human beings he pitied. However, Zeus was infuriated by the titan's insolence (!), and "rewarded" him by chaining him to a rock on the Caucasus Mountains for eternal punishment: Each day, an eagle was sent to feast on Prometheus' liver, and the immortal titan would recover from his injury just in time for the next day's torture.

The story of Prometheus was interpreted in myriad different ways across the ages, but a modern biologist, more than anything, would be impressed by the tenacity of his liver. The liver is well-known for its ability to regenerate from injury. Although it cannot regenerate overnight, and its daily consumption by an eagle would probably be fatal, it can nonetheless restore itself to its former size even after losing two-thirds of its mass [1]. Liver tissue is responsible for mitigating the damage from food-borne toxins and maintaining the sugar metabolism of the body, and its regenerative processes presumably evolved to allow its continuous function in the presence of the severe metabolic stresses these tasks entail. However, other organs of the body do not display the liver's astonishing capacity for renewal.

Many organisms in the animal kingdom are capable of self-renewal to a certain extent. This ability decreases as the animal species becomes more complex in terms of tissue and cell specification. For example, some flatworms and echinoderms can produce new organisms from each part that they have been divided into. Amphibians, on the other hand, cannot form different organisms from each piece, but are able to regrow lost limbs. Mammals also have the capacity to regenerate certain organs following injury. Human children, for example, can repair damaged fingertips, although this process is faster and more complete during the early life [2].

Unfortunately, not all organs and tissues can completely regenerate in humans, and regenerative capacity decreases significantly with age. As such, millions are suffering from complications caused by various diseases and external injuries, which prevent tissues from sustaining their natural objectives. Slow rates of renewal also

lead to the degeneration of tissues; consequently, serious tissue damage or large losses of tissue mass cannot be repaired, except through tissue transplantation. However, the scarcity of organ and tissue donations, the possibility of immune rejection and the requirement for the patient to take immunosuppressive drugs has led researchers to develop new approaches for overcoming the limits of the human capacity for regeneration. Tissue engineering and regenerative medicine are among these methods.

William Haseltine was among the first to use the term ‘regenerative medicine’ in the early 2000s [3]; however, neither the definition nor the borders of regenerative medicine has been concrete since then. On the other hand, there are few elements that any definition of the field will invariably include. These are: (i) the involvement of interdisciplinary research and application, (ii) the aim to repair, replace or regenerate the cells, tissues or organs, (iii) the use of pre-existing and/or newly emerging technologies beyond traditional transplantation or replacement therapies, (iv) the requirement that the treatment induces and supports the body’s own regenerative potential, (v) the application of soluble molecules, gene therapy, stem/progenitor cell therapy, tissue engineering, and the reprogramming of cell fate and tissue type to accomplish this task [4].

Once upon a time, a scientist (then called a “natural philosopher”) would accumulate knowledge in different fields of science and studied multiple areas with little or no common ground. In modern science, however, collaborations between scientists from various disciplines are necessary to fully understand natural phenomena. Regenerative medicine is no exception, and requires the efforts of specialists from the fields of medicine, biology, chemistry, physics, engineering and materials science to yield successful applications.

The terms “tissue engineering” and “regenerative medicine” are often used interchangeably, although their meanings are not exactly the same. The field of tissue engineering has arisen as a result of advances in the field of biomaterials, and is now used to facilitate the practical applications of regenerative medicine. The idea behind tissue engineering is not limited to the introduction of cells into the injured or diseased area for the replacement of lost tissue; instead, its focus is on autologous

grafts and tailor-made therapies that involve the isolation of cells from the patient, expansion of these cells under *in vitro* conditions and seeding of expanded cells into bioactive scaffolds for differentiation or a second round of expansion through a well-defined set of physical and biochemical signals (Fig. 1.1) [5]. These natural or synthetic biomaterials serve as temporary support for cells and induce their proliferation, migration or differentiation to produce a functional organ.

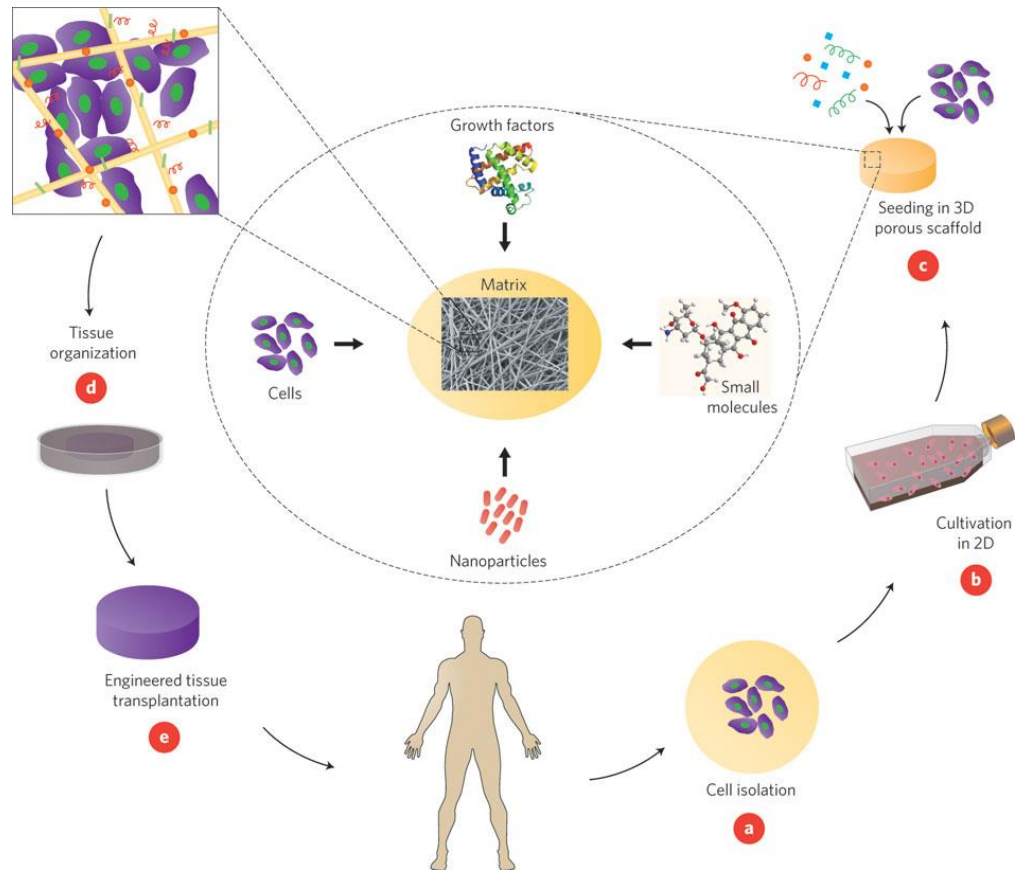


Figure 1.1 General strategy of tissue engineering approaches that use porous biomaterial scaffolds. Cells are first isolated (a) and, if necessary, cultivated (b) for further proliferation. The collected cells are seeded into scaffolds that present a specific set of signals (c), *e.g.* by releasing chemokines or therapeutic agents, or through their mechanical properties such as stiffness and porosity. In order to construct an optimal tissue environment, cells are incubated in bioreactors (d) and the functioning tissue is transplanted to the defective or damaged site (e) (Reproduced from Ref. [5] with permission from Nature Publishing Group.)

Various tissue types, such as liver [6], bone [7], bladder [8], arteries [9], heart [10], skin [11], cartilage [12] and lung [13] have been reconstructed by regenerative

medicine and tissue engineering techniques. The physical and chemical properties of the biomaterials used, the source of the cells that will be repopulating the damaged area, and chemical cues that direct these cells into the desired fates should be selected based on the tissue that needs to be regenerated. Therefore, the characteristics and general biology of the tissue of interest have a strong impact on the design of tissue-reconstructive materials. In addition, a thorough understanding of the tissue of interest is necessary to determine the effects of biomaterials on the behavior of cells under *in vitro* conditions (Chapter 2), as well as that of the overall tissue in *in vivo* applications (Chapter 3).

1.2. TARGET TISSUES FOR REGENERATIVE MEDICINE

Tissue engineering and regenerative medicine are not only based on the application of new techniques and biomaterials to medicine, but also include the experimental processes that must be performed prior to human trials. Therefore, in addition to the features of the materials used, the optimization of 2D or 3D *in vitro* culture conditions and the general biology of damaged or diseased tissues are of fundamental importance to the field.

1.2.1. Adipose Tissue

Adipose tissue, also known as body fat, is a loose connective tissue that insulates the body and stores excess energy to be utilized in times of scarcity. Adipose tissue was thought to be metabolically inert prior to the discovery of leptin; however, it is now recognized that fat tissue contributes to the body homeostasis by secreting various regulatory proteins. In addition to leptin, adipocytes produce adiponectin, resistin, visfatin, retinol binding protein 4, tumor-necrosis factor- α , interleukin-6, interleukin-1, CC-chemokine ligand 2 and plasminogen-activator inhibitor type 1, all of which play major roles in the maintenance of homeostasis, blood pressure, immune function, angiogenesis and the energy balance [14-16].

Mature adipocytes comprise one-third of adipose tissue. Other cell types present include small multipotent MSCs, regulatory T cells, endothelial precursor cells, fibroblasts, macrophages and preadipocytes (Fig. 1.2). Preadipocytes can be found in various developmental stages and are able to differentiate into adipocytes in the

presence of specific environmental factors, allowing adipose tissue to respond to external stimuli [17]. There are two kinds of adipose tissue, called white and brown adipose, although both types are largely similar in appearance. White adipose tissue (WAT) contains cells with one large lipid droplet that occupies the majority of the cell, while cells of the brown adipose tissue (BAT) display multilocular lipid droplets and large numbers of mitochondria. In addition, BAT is more vascularized and innervated when compared to WAT, which results in its distinctive brown color [17].

Although not vital for survival, adipose tissue defects and pathologies may cause soft tissue loss and result in aesthetic and psychological problems. In these cases, tissue engineering can be used to replace adipose in regions where tissue loss is severe. As with any other tissue, adequate cell sources, biomaterials and tissue-mimetic environments are required for the *in vitro* generation of adipose. In addition, immunological responses and the possibility of tissue morbidity following implantation should also be taken into consideration. Consequently, the development of tissue engineering techniques for the production of adipose tissue in minimally immunogenic scaffolds is an active area of research [14, 18].

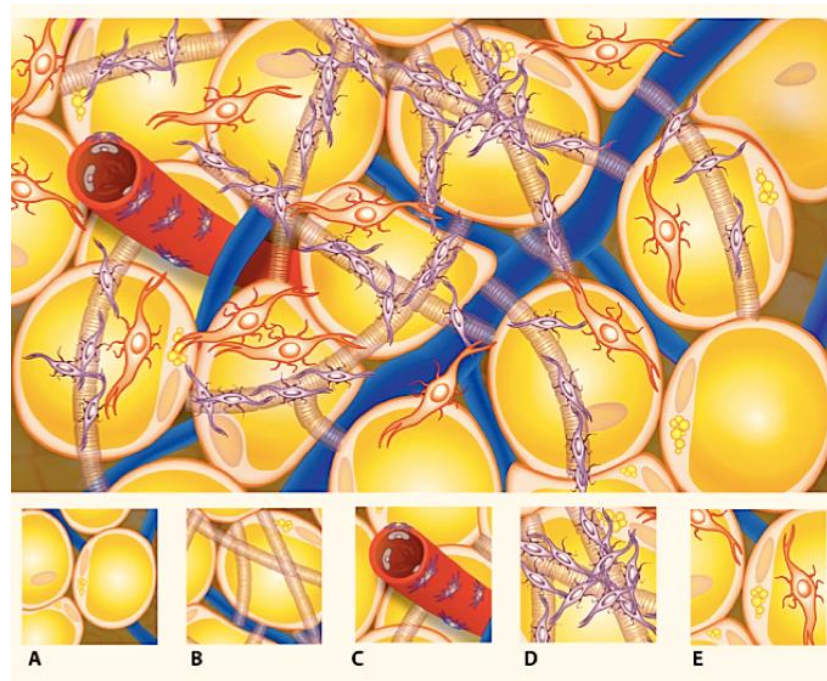


Figure 1.2 Physiology of white adipose tissue. A) Mature adipocytes, B) ECM of adipose tissue, C) blood vessels, D) adipose mesenchymal stem cells, and E) pre-adipocytes. Reproduced from Ref. [19].

1.2.2. Cartilage Tissue

One of the principal load-bearing tissues of the body, cartilage is distributed across multiple areas of the skeletal system. Cartilage tissue lacks blood vessels, nerves and lymph nodes; and is classified under three different types as hyaline cartilage, elastic cartilage, and fibrocartilage. Chondrocytes are the mature cells of cartilage tissue, and constitute 3-5% of the adult articular cartilage [20-24]. These cells are produced by the differentiation of mesenchymal stem cells adjacent to the perichondrium during embryonic development [25]. However, the load-bearing role of cartilage is mainly associated with its extracellular matrix components, rather than its cell population [26]. The cartilage ECM is also responsible for providing joint lubrication and articulation, and contains a complex mixture of proteins and proteoglycans to perform these functions [27]. Type II collagen is the primary collagen fibril found in ECM; other collagens present include types III, VI, IX, X, XI, XII and XIV [25]. Proteoglycans (mainly aggrecan), glycosaminoglycans (chondroitin sulfate and keratan sulfate), hyaluronan and the glycoprotein lubricin (PRG4) are other major components of the cartilage ECM [28]. Fig. 1.3 illustrates its main components [29].

Cartilage defects and diseases are among the major causes of locomotion disability. Osteoarthritis (OA) is one of the leading diseases of cartilage, and affects articular cartilage, the synovium and subchondral bone at molecular, cellular and tissue levels. Its symptoms include decreases in articular cartilage thickness, bone thickening, bone outgrowths on the joint margins and the modification of synovial fluid components [30]. Current treatments for OA are based on non-pharmacological therapies (*e.g.* weight reduction, physical therapy, disease education), drug treatments (*e.g.* pain relievers, chondro-protective molecules, growth factors) and surgical intervention (*e.g.* arthroplasty and osteotomy) [31]. Although these approaches favorably affect the management of OA, some are associated with severe drawbacks and no known treatment can completely reverse the effects of the disease. As such, tissue regeneration studies, which use a combination of cells, biomaterials and biochemical compounds to allow the repair and regeneration of damaged tissues, hold great promise for the development of future strategies for the treatment of OA and other cartilage-related defects.

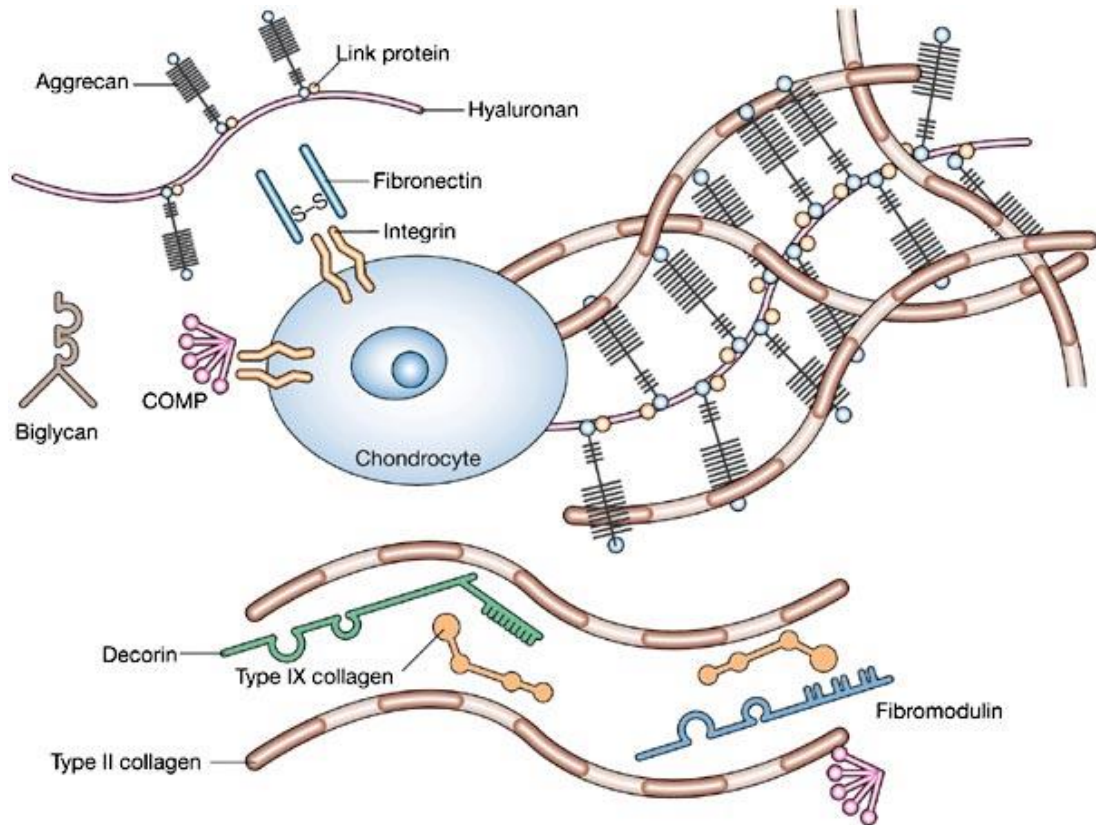


Figure 1.3 The cartilage extracellular matrix. Reproduced from Ref. [29] with permission from Nature Publishing Group.

1.2.3. Bone Tissue

Bone is a hierarchically organized, highly mineralized and dynamic tissue that relies on its organic content to regulate its inorganic matrix (Fig. 1.4). It provides skeletal support to the body, stores calcium and phosphate, and acts as a reservoir for the production of various cell types [32]. Hydroxyapatite crystals comprise the inorganic portion of bone and are incorporated in fibrous ECM proteins, which are mainly (90%) collagens (types I and V comprise 97% and 3% of the bone collagen content, respectively). The remaining 10% corresponds to non-collagenic proteins such as osteocalcin (2%), osteonectin (2%), bone sialoproteins (1.2%), proteoglycans (1%), osteopontin, fibronectin, growth factors and bone morphogenic proteins [33].

Osteoblasts, osteoclasts and osteocytes are the main cell types of bone tissue. They are naturally differentiated from bone mesenchymal stem cells (BMSC), except osteoclasts, which originate from hematopoietic stem cells. MSCs initially produce

primary cell types, osteoprogenitors, which then develop into early osteoblasts and mature osteoblasts. Osteoblasts are responsible for bone mineralization and regeneration, and secrete the collagens (principally type I) and non-collagenic proteins (*e.g.* osteocalcin and osteopontin) that form the necessary ECM for the subsequent deposition of hydroxyapatite. Some osteoblasts are embedded within the mineralized matrix they produce, and differentiate further into osteocytes in this state. Bone tissue is constantly produced and resorbed by osteoclasts and osteoblasts, respectively, and the hormonal regulation of these cells allows bone to respond to injuries and other environmental factors [32, 34-36].

Although bone tissue exhibits the capacity for self-renewal, it is slow to regenerate. As such, major injuries and bone diseases require the assistance of tissue engineering techniques to facilitate the rapid formation of healthy bone tissue. Half a million people annually receive bone defect repair operations in the United States alone, with a total cost of over \$2.5 billion dollars [37]. Due to the frequency and severity of major bone injuries, bone tissue engineering has become a highly advancing field in the recent years.

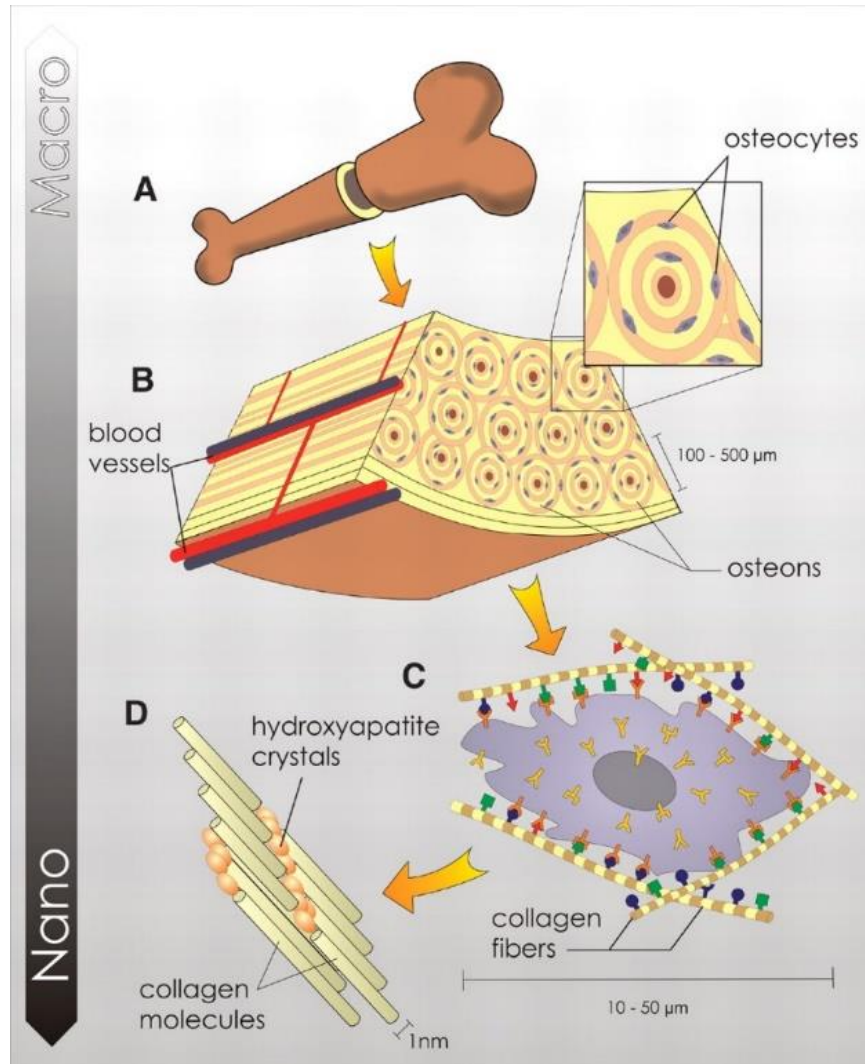


Figure 1.4 Hierarchical organization of bone from macro- to nanoscale. The highly calcified, mechanically strong structure of bone (A) is supported by cylindrical Haversian systems (osteons). Resident cells (B) interact with the bone ECM, the components of which form highly organized nanoarchitectures. Reproduced from Ref. [38].

1.2.4. Pancreas Tissue

Pancreas is a vital organ for digestion and glucose homeostasis, and the impairment of its function can result in serious diseases such as diabetes, pancreatitis and pancreatic cancer [39]. Its role is to produce a large variety of important exocrine and endocrine secretions (Fig. 1.5). Its exocrine function involves the secretion of digestive enzymes into the gastrointestinal system, while its endocrine function is to maintain the balance of the glucose metabolism through the secretion of hormones

such as insulin and glucagon into the bloodstream. These two functions are physiologically separated into distinct compartments in the pancreas. The exocrine enzymes, such as amylase, proteases and nucleases, are secreted by acinar cells and delivered to the intestine by duct cells. The endocrine function of the pancreas, in contrast, is performed by the islets (or islands) of Langerhans, which are responsible for hormone synthesis and release. Islets of Langerhans are made up of α , β , δ and PP cells, which secrete glucagon, insulin, somatostatin and pancreatic polypeptide, respectively [40]. Islet populations are dominated by insulin-producing β cells, the location of which differs depending on the species: In humans, they are located at the cores of the islets, while α , δ and PP (γ) cells (non- β cells) are found at their periphery [41].

Islets of Langerhans can be considered as micro-organs that occupy a unique niche within the pancreas; a healthy human contains about a million of islets [40]. Diabetes is the main disease of the islets, and has become a global epidemic in the past decades. Both types of diabetes mellitus involve β cell failure and a reduction in insulin secretion, which results in hyperglycemia (elevation in the blood glucose level) [42]. Type 2 diabetes mellitus (T2D) is mainly caused by continuous resistance to insulin followed by the impairment of β cell function due to high levels of insulin secretion, the heavy metabolic demands of which deplete the cell population. Beta cell mass reduction then leads to hyperglycemia in these patients, causing diabetes (although other factors are also involved in the presentation of the disease). T2D is further divided into subtypes depending on the immune response and inflammation patterns that the disease may present [42]. Type 1 diabetes mellitus (T1D), on the other hand, is caused by the direct autoimmune destruction of the insulin-producing β cells. This situation causes life-threatening ketoacidosis due to the hyperglycemia that results from hypoinsulinemia. This type of diabetes is also called juvenile diabetes, because its symptoms are observed starting from early ages [43].

While T2D is a more complex disease and consequently requires more advanced therapeutic approaches for treatment, T1D can be cured (or at least have its symptoms reversed) by the regular administration of insulin. While insulin injections are an option for disease management, this approach is limited in its effectiveness because of patient compliance issues. Pancreas transplantation is another approach

for treatment, but is seldom preferred due to the requirement for the patient to take immunosuppressive drugs. Hyperglycemia can be controlled to some extent through an adequate diet, regular physical exercise and insulin injections after meals, and the risk of infection following islet transplantation is much more severe than any decrease in life quality entailed by these tasks. As such, islet transplants are generally performed only after another transplantation procedure (*e.g.* of kidneys), such that the patient would be required to take immunosuppressive drugs in any case. The lack of donated organs and the sheer number of diabetic patients further limit the practical applications of pancreas (or islet) transplantation. An ideal solution for T1D, therefore, would be to instruct the immune system not to recognize the islets as foreign material, and subsequently transplant islets or β cells that are produced using the patient's own supply of cells.

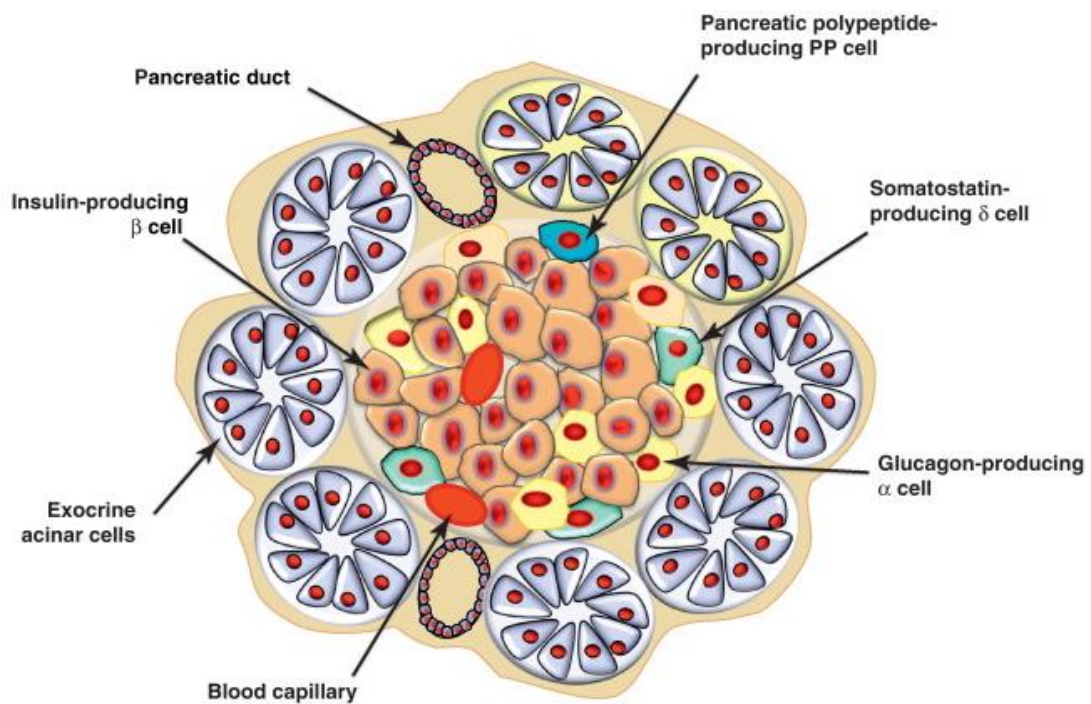


Figure 1.5 Schematic representation of the exocrine and endocrine units of pancreas. Acinar cells produce digestive enzymes, which are delivered to the small intestine by pancreatic ducts. The endocrine pancreas is responsible for the production of hormones involved in the energy metabolism, which are secreted directly into the bloodstream. The four specialized cell types, α , β , δ and PP cells, produce glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. Reproduced from Ref. [44] with permission from Elsevier.

1.3. CELL SOURCES USED FOR THE REGENERATION OF DAMAGED TISSUES

The ability of stem cells to differentiate into a multitude of mature cell types makes them popular agents for tissue engineering efforts. Stem cells can be derived from adult or embryonic sources. Adult stem cells are limited in their potential to differentiate and can usually produce only a small variety of cell types, while embryonic stem cells (ESCs) are pluripotent cells and can generate cell types belonging to the endoderm (liver, pancreas, gallbladder, digestive and respiratory tubes and their derivatives), mesoderm (urinary system, muscles, cartilages, bones, dermis and most of the circulatory system) or ectoderm (nervous system and epidermis) [45, 46]. Ethical concerns regarding the isolation of ESCs have also led to the use of induced-pluripotent stem cells (iPSCs) in tissue engineering. These cells display the ability to differentiate into all three germ layers, but low production efficiencies and the necessity of using viral vectors in their generation nonetheless limit their potential for clinical use. Although stem cells derived from somatic tissues have limited capacities for differentiation, they are also utilized for medical applications and have exhibited promising results in clinical trials. These cells can be isolated from somatic tissues such as bone marrow, fat tissue, dental pulp and umbilical cord blood [47]. In addition to stem cells, progenitor cells can also be utilized to form mature cell types and tissues; however, they are very restricted in their capability for differentiation.

The choice of cell source strongly affects lineage commitment and is vital for the success of adipose tissue engineering efforts, since not all cell types are able to differentiate into the adipogenic lineage. Mesenchymal stem cells obtained from bone marrow or body fat are generally used as a multipotent cell source, and can be induced to differentiate into adipocytes [48-56]. Embryonic stem cells [57] or pre-adipogenic cell lines [58] are also used for adipogenic differentiation; the mouse cell lines 3T3-L1 [57, 59-61], 3T3-F442A [17, 62] and Ob17 [17] are especially popular for *in vitro* adipose regeneration studies.

The low regenerative capacity of cartilage tissue has led to the investigation of many different cell types for their potential to induce its repair. Current treatment options

for the regeneration of cartilage tissue are autologous chondrocyte implantation (ACI), stem cell usage, and the utilization of scaffolds constructed from various biomaterials [31]. One of the main weaknesses of cartilage regeneration is the lack of an indigenous cell source: Chondrocytes originate from mesenchymal stem cells but, once fully matured, do not divide further under normal conditions [63]. Autologous chondrocytes can be used as a cell source, but their lack of numbers and limited *in vitro* self-renewal capacity prevents their use in tissue engineering approaches [64]. As such, alternative cell sources are typically used for the regeneration of cartilage; mesenchymal stem cells in particular are noted for their strong proliferative capacity and ability to commit to the chondrogenic lineage [65]. Mesenchymal stem cells can be obtained from various sources, including bone marrow, adipose tissue, synovium, muscle and the umbilical cord; which makes them very suitable candidates for cartilage tissue engineering applications [66-69]. In addition to mesenchyme-originated stem cells; embryonic stem cells, pre-chondrogenic cells (*e.g.* ATDC5) and iPSCs (induced pluripotent stem cells) have also been used in cartilage tissue engineering [30, 65].

An equally diverse number of cell types are used for *in vitro* osteogenesis studies. Cells that are able to differentiate into the osteogenic lineage are mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells (iPSCs), adipose-derived stem cells and stem cells from human exfoliated deciduous teeth (SHED) [21-23, 37, 70, 71]. These cells can be derived from adult tissues and are promising for future tissue engineering applications utilizing patients' own supply of cells. Osteosarcoma and pre-osteoblast cells are also used in osteogenesis studies due to their ease of maintenance and ability to differentiate into osteoblasts; MG-63 [24, 72], Saos-2 [73], U-2 OS [74], and MC3T3-E1 are among the more popular cell lines for this purpose [75-77]. C2C12 cells can also undergo osteogenic differentiation in the presence of BMP-2 [78].

1.4. BIOMATERIALS USED IN TISSUE ENGINEERING STUDIES

Scaffold material choice is as important as the cell source for the adequate repair of the tissue architecture, and numerous biomaterials have been used to support cell adhesion, viability, proliferation and differentiation for tissue engineering studies.

These materials are often engineered to be biocompatible, non-toxic, minimally immunogenic, highly porous, biodegradable and bioactive [79-82]. Natural products are intrinsically biocompatible and similar to the native tissue in their hydrophilic character and chemical properties, but they are also potentially immunogenic and may retain zoonoses due to inadequate purification methods [83-88]. Synthetic biomaterials, on the other hand, are chemically designed and engineered according to their purpose of application, and do not carry the risk of viral or bacterial infection [63]. Synthetic scaffolds are mechanically strong and stable in the body for long periods of time, and can be designed according to a specific application (*e.g.* to function optimally in a given tissue); however, they are also associated with certain drawbacks. The insolubility of some synthetic biomaterials, for example, is a major issue for their use in tissue regeneration, and results in insufficient cell adhesion and viability [89-96]. Toxic degradation products, harsh synthesis conditions and hazardous chemical compositions are other disadvantages of these materials [96]. As such, there is an active effort for the design and fabrication of more biocompatible and bioactive scaffolds.

Hybrid materials can be designed to combine the advantages of multiple material types, and nanofabrication and patterning techniques can likewise be utilized to increase the effectiveness of a material in promoting the adhesion, viability or differentiation of cells. Nanoscale surfaces can overcome limitations in cell adhesion by providing a larger surface area for cell and protein attachment, or by mimicking the structure of biologically important molecules [63]. Consequently, bioactive scaffolds can be fabricated to display native-like chemical compositions, surface topologies, biophysical features and immune-responses, and can be further functionalized by incorporating small biomolecules, such as growth factors, into their structure [97]. Among nanoscale ECM-like scaffolds, self-assembling supramolecular peptide amphiphiles are exceptional for their superior structural and functional characteristics [98]. Supramolecular peptide networks provide an adequate spatiotemporal environment for the growth and differentiation of cells through their porous structures, high surface-to-volume ratios and controlled assembly into well-defined structures of various sizes and structures; as such, they are commonly used for the engineering of a broad variety of tissues.

Many scaffold types have been used for adipogenic differentiation. Natural materials used for this purpose include hyaluronic acid, adipose-derived basement membrane [99], collagen [60, 100-104], Matrigel [105-107], fibrin [54, 108], gelatin [56, 108, 109], laminin [101], fibronectin [101], placental decellularized matrix (PDM) [110], silk [111] and alginate [112], while synthetic materials such as polycaprolactone, polyethylene glycol diacrylate (PEDGA), polyethylene terephthalate (PET), poly(lactic-co-glycolic acid) (PLGA), polytetrafluoroethylene (PTFE), polypropylene, polycaprolactone (PCL), poly(ethylene glycerol) (PEG), polyglycolic acid (PGA), poly-L-lysine, and polyacrylamide gels are also utilized for adipose tissue engineering research [49, 113-119].

Natural scaffolds used in cartilage regeneration studies include materials such as collagen, hyaluronic acid, chondroitin sulfate, fibrinogen, alginate and chitosan; and can be purified or derived from native extracellular matrices, plasma, algae or other biological sources [63]. Synthetic polyesters such as PLGA (poly[lactic-co-glycolic acid]) and PLA (poly lactic acid), polymers such as ϵ -caprolactone, polyethyleneoxide (PEO), PEG (polyethylene glycol), polyurethanes, poly (N-isopropyl acrylamide) and polyvinyl alcohol, and carbon fibers are also used in cartilage regeneration [63]. Supramolecular systems are also under investigation for their potential to induce cartilage regeneration [120]. These nanosystems have been shown to provide a suitable 3D environment for cells, possibly because of their intrinsic compatibility with biological systems. Peptide hydrogels in particular are applicable for the regeneration of a broad variety of tissues, and can also assist in cartilage tissue repair [120].

Many natural and synthetic scaffolds have been used for bone regeneration, including osteoinductive or immuno-modulatory hydrogels and hybrid biomaterials. It is well-documented that hydroxyapatite and hydroxyapatite-polymer composites trigger the osteogenic commitment of cells. Type I collagen, which forms the majority of the organic matrix of bone, is also used frequently, either by itself [22, 23] or in conjunction with ceramics [24]. Hyaluronic acid (HA) hydrogels [70], titanium-peptide amphiphile hybrids [77, 121], gelatin [122] and chitosan [72, 78, 123] are other materials used in the fabrication of bone-mimetic scaffolds. Synthetic materials such as polycaprolactone (PCL) [22, 71], poly(lactide-co-glycolide)

(PLGA) [124], poly(L-lactide) (PLLA) [124] and their polymer-polymer and polymer-ceramic composites are also frequently utilized in bone regeneration studies [37]. The immune system plays an important role in bone formation (osteogenesis), and the immune response is a major factor in determining implant stability and facilitating the formation of fibrous tissues that are later mineralized and converted to bone [21, 125, 126]. As such, immuno-modulatory scaffolds, which either minimize the immune response by using immune-compatible materials or increase it through the use of inflammatory cytokines, are able to enhance bone healing and cell differentiation [37]. The osteogenesis of bone mesenchymal cells, for example, was found to be enhanced in the presence of an extract of macrophage-conditioned beta tricalcium phosphate [21].

1.5. CHEMICAL CUES USED TO INDUCE DIFFERENTIATION

While bioactive scaffolds may be effective in promoting cellular differentiation by themselves, chemical ingredients can be incorporated into the culture medium to further assist this process. As such, the mechanical [53] or chemical [49] properties of the scaffolds are often enhanced through the use of chemicals that alter the lineage determination of cells. In addition to basal culture medium and serum; adipogenic differentiation is often enhanced through the use of dexamethasone [101, 111], 3-isobutyl-1-methylxanthine (IBMX) [110, 127], indomethacin [54, 59] and insulin [60, 61] at different concentrations and combinations, which are optimized to cell type used for differentiation [127]. Other factors used in adipogenic induction media include rosiglitazone [127], hydrocortisone [127], insulin-like growth factor I (IGF-I) [59], corticosterone [59], biotin [110, 127], pantothenate [118, 127], transferrin [110], triiodothyronine [110, 127], troglitazone [110, 127] and basic fibroblast growth factor (bFGF) [59].

Medium components required for chondrogenic differentiation are well-documented in the literature. The differentiation medium of choice is generally high-glucose DMEM supplemented with bone morphogenetic proteins (such as BMP-6), transforming growth factors (such as TGF β 1 and TGF β 3), dexamethasone, ascorbate-2-phosphate, proline, pyruvate, insulin, transferrin, selenious acid and bovine serum albumin [128]. Especially vital is the use of transforming growth

factor beta (TGF- β) superfamily proteins (TGF- β , and BMPs), which are master regulators that are heavily involved in embryonic development, postnatal tissue repair and the homeostasis of body [129]. The differentiation process is regulated by the interaction of these molecules with cell surface and cytosolic receptors via specific signaling pathways, including β -catenin signaling, Smad3 and chromatin remodeling [130] and Wnt signaling [129]. In addition, serum-free media can also be used for the induction of the chondrogenic phenotype [131].

A variety of medium components and physical environments have been investigated for their capacity to enhance bone differentiation [24, 72, 76, 132-154]. Bone morphogenetic protein (BMP), dexamethasone (dex), ascorbic acid and β -glycerol phosphate are commonly used supplements for bone regeneration, and have been shown to upregulate genes related to osteogenic differentiation [155]. BMP-2, a growth factor of the transforming growth factor- β (TGF- β) protein superfamily, is especially potent as an inducer of osteogenesis and functions under both *in vitro* and *in vivo* conditions [156]. In addition, it has been demonstrated that osteogenic differentiation can be induced in a continuous culture with dexamethasone, which stimulates alkaline phosphatase (ALP) activity and upregulates osteopontin, bone sialoprotein and osteocalcin expressions [156]. Dexamethasone is an unnatural corticosteroid used to mimic natural glucocorticoids such as testosterone, cortisol, vitamin D3 and retinoic acid [157]. It is included in many different types of differentiation media because of the relation between glucocorticoids and gene activator proteins. In absence of glucocorticoids, the latter are unable to bind to DNA and initiate gene expression [158]. Ascorbic acid also allows the expression of a stable osteoblast phenotype and speeds up osteoblast proliferation due to its role in collagen synthesis and alkaline phosphatase activity [159]. β -glycerol phosphate, while not a necessary factor for bone differentiation, is generally provided as a phosphate source for the production and subsequent deposition of hydroxyapatite.

1.6. ASSESSMENT OF DIFFERENTIATION

Although a correct combination of cell type, culture medium and differentiation-inducing factors is sufficient for lineage commitment, the quantification of the

differentiation process is vital for evaluating the effectiveness of a given biomaterial. Cell viability is the first parameter tested for any prospective biomaterial scaffold, and will often be followed by the evaluation of the differentiation and regeneration process. Adipogenic differentiation can be characterized by staining the accumulated lipids and observing the cells under light, fluorescent or confocal microscopy. As such, lipophilic dyes are often used to monitor these cells [14, 55]. Sudan Black B [51] and osmium tetroxide (OsO_4) [58] staining methods will show the oil droplets inside the cells. However, Oil Red O (ORO) [56, 100, 118, 160, 161] is the most frequently used method for the staining of lipids, although it is not suitable for experimental setups that include collagen scaffolds or paraffin embedding (collagen interferes with the staining and the solvents used in paraffin embedding dissolve intracellular lipids) [101]. Issues with paraffin-embedded samples can be circumvented by staining other parts of the cell, as the round, “empty” regions that remain in the stained sample will correspond to the dissolved lipid droplets [59].

The metabolic function of adipocytes is to serve as a deposit for excess glucose and fat, storing energy in the form of triglycerides and secreting hormones involved in the regulation of the energy homeostasis. The distinctive metabolic activity of adipocytes allows their identification through the measurement of fat-specific synthesis products, enzymatic activity and hormone secretions. Intracellular triglyceride amounts can be used to monitor the differentiation state of adipocytes [59]; while other methods based on glucose uptake [14, 55] and lipolytic cell responses [59, 118] can be likewise be used to quantify adipogenic commitment. Glycerol-3-phosphate dehydrogenase (GPDH), an enzyme that takes part in the lipid biosynthesis metabolism, is another marker for adipogenesis [52, 58, 161, 162]. In addition to cellular activity, fat-specific hormones can also be used to quantify adipogenic potential. Leptin [54, 56, 59, 118] and adiponectin [118] are among the hormones used for this purpose; their concentrations are typically measured by ELISA.

The detection of mRNA or protein expression is one of the most reliable ways of demonstrating lineage commitment and cellular differentiation (Table 1). Even the simplest of cellular actions occurs through a series of complex signaling networks; consequently, the regulation of a process as advanced as differentiation requires a

vast array of genetic feedback mechanisms. As such, many genes are up- or downregulated during adipogenesis [48]. Peroxisome proliferator-activated receptor γ (PPAR γ) [53, 54, 160] is a nuclear hormone receptor and is one of the most commonly used mRNA markers for early adipogenesis [56, 163]. Lipoprotein lipase (LPL) [51, 56, 111] and fatty acid binding protein-4 (FABP4) [14, 52, 54] are other early differentiation markers [52, 56]. Adipocyte P2 (aP2) [53, 54, 57, 111], adipocyte determination and differentiation factor 1 (ADD1) [160], fatty acid synthase (FAS) [160] and preadipocyte factor 1 (Pref-1) are also involved in adipocyte differentiation [164-166], while leptin [57, 59] and aP2 are among the late markers of adipogenesis [56]. CCAAT/enhancer-binding protein α or β (C/EBP α or β) [51, 53], adiponectin [51], acyl-CoA synthetase (ACS) [14, 100], adipsin [60, 100], facilitative glucose transporter-4 (GLUT4) [57, 100], β_3 -adrenoreceptor (β_3 AR) [59] and adipophilin [57] are used as other markers for the detection of adipogenic differentiation. In addition to RT-PCR, gene expression can be quantified at the protein level using blotting techniques. Expressions of PPAR γ [49, 59, 160] and CCAAT/enhancer-binding protein α (C/EBP α) [160] can be confirmed by both RT-PCR and blotting, and both proteins are heavily involved in adipogenesis (*e.g.* mice deficient in C/EBP α expression do not produce mature white or brown adipose tissue [167]). Other targets for blotting studies are adipokines (adiponectin [51], resistin, visfatin, retinol binding protein 4), inflammatory cytokines (tumor-necrosis factor- α , interleukin-6, interleukin-1, CC-chemokine ligand 2) and thrombosis-associated cytokines (plasminogen-activator inhibitor type 1) that are synthesized by adipocytes [14].

Primary differentiation analysis of chondrogenesis is performed through the morphology tracking of cells. This is mainly achieved by microscopy techniques, including optical, fluorescence, and electron microscopies [82]. Cells committed to the chondrogenic lineage are typically round in shape, and form cellular aggregates similar to the mesenchymal condensates that occur during native tissue development [30, 82]. The numbers and average areas of aggregates can be measured to quantify the morphological changes of differentiating cells [82]. Staining protocols can also be used to visualize cartilage-specific changes in cell components for the assessment of chondrogenic differentiation. Safranin-O and Alcian blue are the two major

cartilage-specific stains. Safranin-O is a cationic dye that stains sulfated glycosaminoglycans (GAGs), which are abundant in the cartilage extracellular matrix [82]. Likewise, Alcian blue stains neutral, sulfated and phosphate mucopolysaccharides and glycosaminoglycans; aggrecan is its main target in cartilage tissue [79, 82]. In addition, GAG concentrations can be quantified through spectrophotometric analysis via the DMMB (dimethylmethylene blue) assay [82, 128].

Proteins associated with chondrogenic commitment are another means of quantifying biomaterial-induced cellular differentiation. Collagen type II, aggrecan, COMP (cartilage oligomeric matrix protein) and Sox9 are the main proteins assayed for the evaluation of chondrogenic differentiation. The former three are found abundantly in the cartilage extracellular matrix, while the latter is a transcription factor involved in cartilage development [66]. Both mRNA expression and protein blots can be used for the quantification of these proteins [30, 68, 82, 168]. Immunocytochemistry analyses can also be performed to visualize their expression, secretion and localization [66, 68, 82, 168]. For example, Valonen *et al.* showed the collagen type II staining of hMSCs cultured in tightly-woven poly(epsilon-caprolactone) scaffolds for 21 days [168].

As osteogenic and chondrogenic differentiation pathways share a large number of features, bone-specific proteins such as collagen type I are also analyzed in cartilage regeneration studies to demonstrate that the cells are committed to chondrogenesis rather than osteogenesis. The expression of collagen type I is lower than that of type II in chondrogenic cells [66]. While RT-PCR and blotting are often used to quantify collagen levels, other instruments can also be used for the same purpose. Chonanant *et al.*, for example, used synchrotron radiation Fourier transform infrared (SR-FTIR) microspectroscopy to investigate the differentiation state of human mesenchymal stem cells (hMSCs), and quantified the changes in average spectra to demonstrate the upregulation of collagen and aggrecan [68].

Cartilage-specific ECM compositions are responsible for the unique structural, thermodynamic and mechanical properties of this tissue [169, 170]. As such, chondrogenic differentiation can be further characterized by mechanical

measurement methods, *e.g.* by the quantification of equilibrium modulus and dynamic stiffness through mechanical spectrometry [128], analysis of the dynamic oscillatory mechanical behavior of cartilage through atomic force microscopy (AFM) [171] or measurement of the molecular-level structural and mechanical properties of aggrecan through a combination of micro-contact printing and high-resolution force spectroscopy [172].

Many techniques are available for the visualization and quantification of osteogenic differentiation and biomineralization. Lineage-specific metabolic activity measurements, optical imaging, immunocytochemistry and PCR are the most commonly used methods for this purpose. Osteogenic differentiation and mineral deposition can be tested by alkaline phosphatase (ALP), Alizarin Red S and von Kossa stainings. These tests are used to visualize the deposition of hydroxyapatite, calcium and phosphates, respectively. The direct observation of calcium via EDX and electron microscopy is also feasible for 2D surfaces, although this method is unreliable in 3D culture environments.

Alkaline phosphatase is an important metalloenzyme that regulates phosphate homeostasis and bone mineralization; as such, it is often used as a marker for osteogenic differentiation [173, 174]. Its function is to hydrolyze phosphate residues from proteins, nucleotides, inorganic pyrophosphates and other phosphate sources. It plays different roles in tissues such as the intestine, placenta and liver, bone, and kidney (in which it is tissue-nonspecific and called TNAP) [175]. Both gene expression and enzyme activity analyses of ALP are performed for the evaluation of osteogenic differentiation. The enzyme activity assay (or “ALP assay”) is a quantitative method based on colorimetric changes resulting from the cleavage of a reference phosphate substrate [22-24, 72].

Another common technique uses Alizarin Red S to stain deposited calcium, the presence of which is indicated through an orange-red color [150]. Samples can subsequently be destained with 10% cetylpyridinium chloride solution in sodium phosphate buffer, and the solubilized dye can be measured by spectrophotometry to quantitatively evaluate calcium concentrations. Another staining technique, von Kossa staining [176], serves as an alternative to Alizarin Red S and is based on the

reaction of silver ions with phosphate. Silver appears black following exposure to UV light, although a brown-black color may be observed for lower concentrations. Since calcium forms aggregates with phosphate, silver staining of the latter can be used as an indirect indicator of the presence of hydroxyapatite. Although the variety of organic and inorganic components present in culture media makes it difficult to obtain accurate signals, techniques such as XRD and FTIR can also be used to quantify the peaks associated with the crystal structure or chemical bonds of hydroxyapatite [176].

Bone ECM is mainly composed of collagen types I and V, with relatively minor contribution from other proteins such as osteocalcin, osteopontin, bone sialoprotein, and osteonectin. The expression of these proteins is indicative of osteogenic differentiation; as such, quantitative analysis of the mRNA and protein levels of these factors is a reliable method for evaluating the course of osteogenesis. In addition, immunostaining of bone specific proteins is also used for the visualization of osteogenic commitment and differentiation.

1.7. EXTRACELLULAR MATRIX: AN INSPIRATION FOR MIMESIS

The extracellular matrix (ECM) can be defined as the non-cellular component of tissues, and is composed of a complex network of proteins and polysaccharides that are secreted by, and in turn encompass, the native cell population of the tissue [177]. ECM components are similar across a variety of taxa: From sponges to higher mammals, its components are shared by all metazoans. It exhibits different properties in different tissues, such as transparency in cornea, hardness in teeth and bones and elasticity in connective tissue. In addition to providing mechanical and architectural support, the ECM also plays a critical role in the development and maintenance of the body, and its defects and mutations result in serious disorders [178-182]. The composition of the ECM strongly affects the survival, migration, proliferation, shape, function and differentiation of the cells it contains (Fig. 1.6). ECM secretion is carried out by the local cells, which are generally fibroblasts, although their role can also be performed by specialized cells such as osteoblasts and chondroblasts. Cell attachment to ECM components is mediated by receptors on the cell surface, such as integrins, syndecans and discoidin domain receptors [183, 184]. The ECM is also a

dynamic structure that is continually synthesized, degraded and reorganized, which further adds to its complexity. Remodeling can be through enzymatic or non-enzymatic factors, as well as by post-translational modifications [185]. Moreover, by binding to growth factors (GF) and cell-surface receptors, the ECM indirectly regulates gene transcription through signal transduction. The ECM is mainly composed of two different macromolecules: (i) fibrous proteins and (ii) glycosaminoglycan (GAG) polysaccharide chains, which are usually attached to proteins and called proteoglycans.

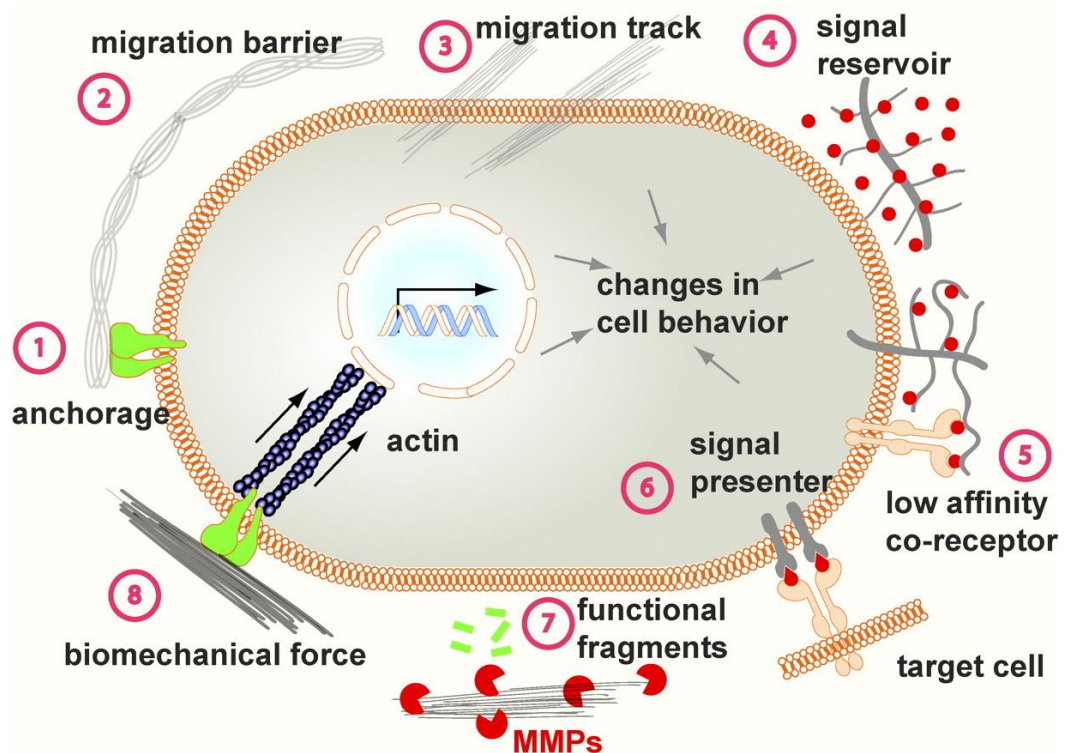


Figure 1.6 Schematic representation of the function of the extracellular matrix. The ECM may serve as an attachment point for the cells (which is important for asymmetric cell division and cell polarity), block their motility, or trigger cellular migration events (1, 2 and 3). The ECM also has the ability to bind different chemokines and store them to facilitate their slow release or create a chemical gradient (4). Some ECM components can act as signal co-receptors (5) or presenters (6). Endogenous domains of ECM proteins may also influence cell behavior (7). In addition, cells also sense the mechanical characteristics of their environment through ECM components, and adjust their behavior accordingly. Reproduced from Ref. [186].

Collagens are the most prevalent fibrous proteins of the ECM in multicellular animals. They are responsible for providing tensile strength to the ECM, regulating cell behavior, modulating chemotaxis and migration events, and assisting in tissue development. They constitute a quarter of the total protein mass of an organism, and are secreted largely by the connective tissue cells (and in lesser amounts by other cell types). In addition to synthesizing and secreting collagens, fibroblasts also organize the alignment of collagen fibrils by assembling them into sheets or cables [185]. Many collagen types co-occur in tissues, although one type usually dominates in any given tissue; such that collagen I is abundant in bone and collagen II is the principal component of cartilage [187]. Their structure is made up of protein fibrils (homotrimers or heterotrimers) called α chains, which assemble to form a triple superhelix. More than 40 alpha chains have been described in humans, and several other non-collagenous proteins bear collagen-like domains [188]. Glycine and proline are the predominant amino acids of collagen; Gly-X-Y (where X and Y can be any amino acid, but usually proline and hydroxyproline) is a common motif in their structure.

Collagens can be divided into subfamilies depending on the morphology of their self-assembled forms, they are known to produce fibrils, beaded filaments, anchoring fibrils, and networks [189]. In addition, certain collagens (fibril-associated collagens with interrupted triple helices, or FACITs) do not form fibrils themselves; and are instead associated with surface collagen fibrils. Their rod-like domains serves to attach them to collagen, and can also bind surface receptors, other proteins, GAGs, and nucleic acids [190]. Collagen fibers can also increase their tensile strength by forming bundles (as is found in tendons), the cross-linking of which occurs through lysine residues by the action of lysyl oxidases [191].

Laminins are glycoproteins composed of repeats and α -helical domains, and occur in the basal laminae of mesenchymal tissues [190]. Domains located on their surface allow their interaction with cells through receptors (such as integrins and dystroglycan) and ECM elements (such as entactins, perlecan and collagens) [192, 193]. Laminins are composed of three chains (α , β , and γ) that are attached to each other by their triple-helical coiled-coil domains, and can be cruciform (cross-shaped), Y-shaped (three arms) or rod-shaped (single arm). Five α -chains ($\alpha 1$ – $\alpha 5$), four β -

chains ($\beta 1$ – $\beta 4$) and three γ -chains ($\gamma 1$ – $\gamma 3$) have been described in the literature, and they form sixteen different heterotrimers [190]. Laminins are named by the identity of their chains (*e.g.* $\alpha 1\beta 1\gamma 1$ and $\alpha 3\beta 3\gamma 2$) [194]. Their primary function is to form bridges between molecules; however, laminin 111 has been demonstrated to form aggregates by itself.

Elastin is another ECM fibril that associates with collagen. As indicated by its name, elastin increases the elasticity of tissues, which is required for resisting repeated stretching motions. However, its recoil ability is limited by the other ECM molecules, such as collagen, to which elastin is tightly attached [195]. The precursor of elastin, tropoelastin, assembles into its mature, fibrillar form through the crosslinking of its lysine residues. [185]. The lysyl oxidase family is responsible for performing the crosslinking reaction. Elastin is also associated with glycoprotein fibrils, mainly fibrillins, which surround the macromolecule and support its integrity [195].

Fibronectin (FN) is a fibrous ECM component that is involved in the organization of the ECM and plays an important role in cell adhesion and function. Like elastin, it has the ability to stretch [196]. Interestingly, fibronectin undergoes conformational changes during stretching, which exposes the integrin-binding sites within its structure and thereby alters cellular behavior. As such, fibronectin can be considered to be an extracellular mechano-regulator [196]. It is an important ECM molecule for the migration of cells, especially during embryonic development, while its defects have been linked to cardiovascular diseases and tumor metastasis in adults [178, 197, 198]. Each subunit of FN is composed of three modules of repeating units, each with a distinct structure (type I, type II and type III). Around 20 isoforms of FN are known; these are produced by alternative splicing. These structures contain motifs that enable FN to bind cell surface receptors such as integrins and ECM components such as collagen and gelatin. Disulfide bridges between two subunits (type I and type II) stabilize the structure of FN, and antiparallel disulfide bonds at the C terminus allow the formation of FN dimers [199]. The integrin-binding capacity of FN dimers allows them to cluster around cell surface receptors, resulting in their activation. Integrin binding also leads to the formation of FN fibril structures. The RGD sequence (Arg-Gly-Asp) is found in FN and provides a signal for the cells to attach

by integrin $\alpha_v\beta_3$; this motif is commonly used in peptide-based tissue engineering studies [200].

Proteoglycans (PG) are proteins conjugated to the glycosaminoglycan polysaccharide chains found in the ECM. The primary functions of PGs are derived from the biochemical and hydrodynamic characteristics of GAGs. They bind to water to hydrate the tissue and allow it to resist compressive forces. GAGs are highly polar and unbranched polysaccharide chains formed by successive disaccharide units. Since one of the repeating units is always an amino sugar (N-acetylglucosamine or N-acetylgalactosamine), these molecules are called as GAGs (Fig. 1.7). In most of the cases, these amino sugars are also sulfated (Fig 1.8). The latter sugar unit is usually a uronic acid (glucuronic or iduronic). The sulfate and carboxylic acid groups cause GAG molecules to be highly negatively charged, which also makes the molecules highly hydrophilic. There are four major groups of GAGs: (i) hyaluronan, (ii) chondroitin sulfate and dermatan sulfate, (iii) heparan sulfate, and (iv) keratan sulfate [201].

Their hydrophilic structures enable those molecules to hold great amounts of water, occupy large amounts of volume compared to their mass, and form hydrogels even in low concentrations. Although their mass is less than 10% of all fibrous proteins, they occupy most of the ECM by volume due to their hydrogel-forming nature. Their negative charges also attract cations like Na^+ , which increase the osmotic pressure of GAG hydrogels and further increase their water absorption capacity. This property enables tissues containing high amount of GAGs to resist strong compressive stresses, such as those experienced by articular cartilage. Collagen fibrils, in contrast, are capable of withstanding strong stretching forces.

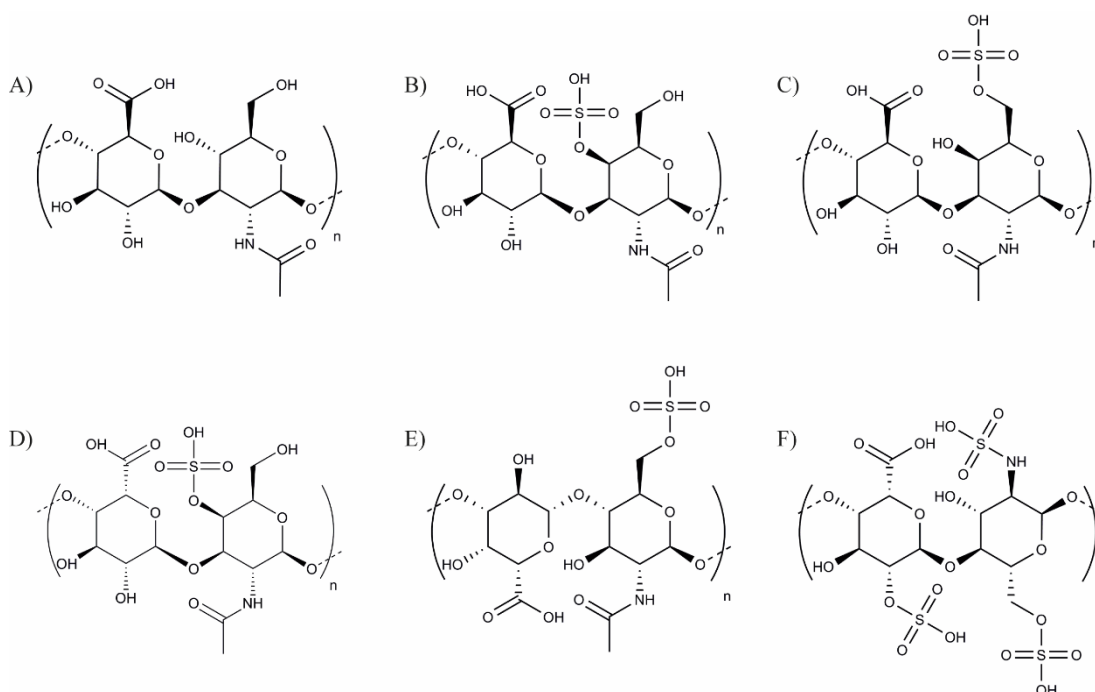


Figure 1.7 Disaccharide units of extracellular matrix glycosaminoglycans. **(A)** Hyaluronan, **(B)** chondroitin-4-sulfate, **(C)** chondroitin-6-sulfate, **(D)** dermatan sulfate, **(E)** keratan sulfate, and **(F)** heparan sulfate.

Hyaluronan, also called hyaluronate or hyaluronic acid (HA), is the simplest of GAGs and lacks sulfate groups. Hyaluronan is made up of alternating units of β -1,4-linked D-glucuronic acid- β -1,3-*N*-acetyl-D-glucosamine [202]. It may contain up to 25,000 disaccharide units in one strand. Hyaluronan is generally not linked to any other core protein and is made by a cell surface enzyme, unlike other GAG molecules, which are synthesized inside the cell and released by exocytosis. It is abundantly produced in embryonic development, during which it is responsible for filling extracellular matrices. It also plays a role in wound healing/remodeling and the initiation of the inflammation response [203].

Another glycosaminoglycan, chondroitin sulfate (CS) consists of disaccharide units of β -1,3-linked D-glucuronic acid- β -1,4-*N*-acetyl-D-galactosamine, and is a major component of cartilage and brain ECM [204]. In cartilage, it is found in aggrecan and allows the tissue to resist compression. In brain, it assists in neural plasticity, helps neural development and regeneration, and influences the processes of learning and memory [205]. The sulfation of this GAG molecule occurs on either the C-4 or C-6 position of the *N*-acetyl-D-galactosamine residue.

Another sulfated GAG, dermatan sulfate (DS), is composed of disaccharide units of β -1,3-linked L-iduronic acid- β -1,4-*N*-acetyl-D-galactosamine. Due to its resemblance to CS, dermatan sulfate is sometimes called chondroitin sulfate B. DS is found in the cornea, where it provides optical clarity by arranging fibril spacing; and in bone, where it regulates osteoclast behavior. It can also be used as an anticoagulant due to its ability to inhibit thrombin [206, 207]. The structure of DS is variable. It may consist of alternating units of α -D-glucosamine, uronic acid and either β -D-glucuronic acid or α -L-iduronic acid; which are linked by 1,4 glycosidic bonds. The α -D-glucosamine residue can be either N-sulfated or N-acetylated [208]. In addition to the cornea and bone, DS plays a variety of roles in other tissues: It assists in blood clotting, promotes angiogenesis, protects proteins from degeneration and binds growth factors [209, 210].

Keratan sulfate (KS) is composed of β -1,3-linked D-galactose- β -1,4-*N*-acetyl-D-glucosamine units, one or both of which are sulfated on the C-6 position [211]. It may be produced by various tissues at different developmental stages, and is more commonly found in cartilage, cornea and central nervous system. Due to its water-attracting nature, it helps resisting compression in cartilage, and regulates axonal growth following spinal cord injury [212, 213].

With the exception of hyaluronan, GAG molecules are often linked to proteoglycans. This association occurs in the Golgi apparatus and involves the attachment of a linker tetrasaccharide to the serine residue of the proteoglycan, followed by the elongation of the GAG by glycosyl transferases. These modifications are highly variable, and therefore, the number of potential proteoglycan forms is vast. Proteoglycans can be massive, as is the case for aggrecan (which is mostly found in cartilage), or comparatively smaller, as in decorin (which is secreted by fibroblasts and contains a single chain of GAG). The interactions of proteoglycans are responsible for creating very complex structures, such as the basal lamina, and play important roles in several tissues, *e.g.* in the glomeruli of kidneys.

In addition, proteoglycans are responsible for modulating biochemical signaling pathways by binding signaling molecules such as growth factors. Fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs), bone morphogenetic

proteins (BMPs) and transforming growth factors (TGFs), for example, are known to associate with proteoglycans (and even with GAGs), and this association is a topic of great interest for regenerative medicine [214]. These growth factors may bind various proteoglycans, such as perlecan, versican, aggrecan, decorin, biglycan, lumican, syndecans, glypicans, agrin, integrin binding sialoprotein, matrilins, fibrinogen, fibromodulin, fibrillins, fibulin, netrins, SPARC (osteonectin), secreted phosphoprotein (osteopontin), reelin, tenascins and vitronectin [190, 215]. In addition, proteoglycans also bind to proteolytic enzymes (proteases) and protease inhibitors, through which they modulate the degradation of ECM components [216].

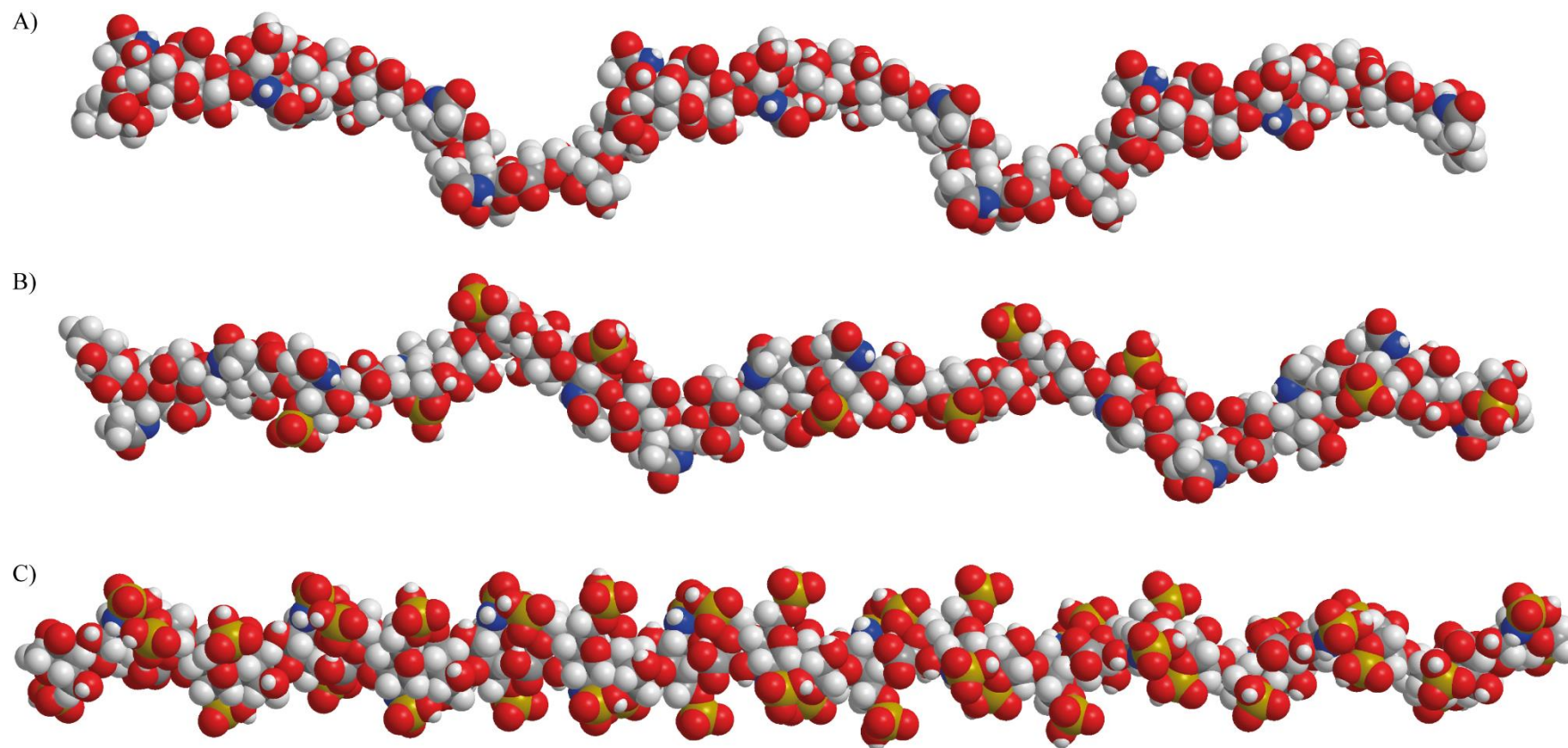


Figure 1.8 Space-filling model of the short chains of hyaluronan **(A)**, dermatan sulfate **(B)** and heparan sulfate **(C)** glycosaminoglycans. Hyaluronan has no sulfate residues, while dermatan sulfate and heparin are mildly and heavily sulfated. Sulfate residues make the molecule more negative, and are one of the main factors that influence the function of the molecule. (Atom colors: Grey: Carbon; White: Hydrogen; Red: Oxygen; Blue: Nitrogen; and Yellow: Sulfur)

1.8. CELL CULTURE APPROACHES

The complexity of living organisms is a big challenge for biological research, as a great number of variables may affect the outcome of any experiment. As such, cell culture systems are often used to eliminate these variables and establish a better control over the experimental procedure. Cell culturing is invaluable for basic research and also contributes significantly to the testing of potential applications in fields ranging from pathology to regenerative medicine. It is now possible to observe cancer cell behavior, measure the response to a drug, metabolite or any other chemical, study the molecular biology of cells and design biomaterials that can be used in tissue engineering or drug delivery systems in cell culture. However, a deeper understanding of cellular behavior on the surface of biomaterials or in scaffolds is still necessary, and the study of cells in artificial systems is an important topic in tissue engineering and regenerative medicine. 2D and 3D cell culture systems both have their distinct advantages and disadvantages, and the dimension and physical properties of the culture environment have a major effect on cell behavior. The viability, attachment, proliferation, differentiation, response to chemicals, and signaling mechanisms of cells in 2D systems are distinct from their 3D and *in vivo* counterparts, and it may be misleading to conclude that the former are representative of the latter. Advancements in 3D culturing systems, therefore, are one of the most rapidly growing research areas in life sciences. Compared to 2D systems, the properties of 3D cell culture systems are closer to *in vivo* conditions [217]. Since cells can sense chemical and physical cues around their environment, and are also able to modify and reorganize their surroundings, well-defined 3D scaffolds and conditions can enable isolated cells to maintain their natural state or differentiate into the desired cell type. 3D systems also allow researchers to co-culture different kinds of cells in one environment, in a manner similar to the niches that cells naturally occupy. Advancement in biomaterials, bio-printing, bioreactors and microfluidic systems also contribute substantially to 3D cell culture applications.

Materials utilized for constructing 3D systems vary greatly in origin, complexity and physical and chemical properties, and must be chosen based on the nature of study and cell type. 3D scaffolds can be produced by solvent casting and particulate

leaching (SCPL), emulsification/freeze-drying, gas foaming, nanofiber self-assembly, textile technologies (techniques to form nonwoven polymer meshes), electrospinning, computer aided design/manufacture technologies (3D structures produced by ink-jet printing of polymer powders), soft lithography, microfluidics, decellularized matrices and bioreactors [218].

3D systems can also be produced by the decellularization of a whole tissue or organ, which creates a natural matrix that provides an adequate chemical and physical environment for the growth and differentiation of seeded cells. Decellularized skin, small intestinal submucosa, urinary bladder, blood vessels, and heart valves are commonly used in tissue engineering research [219]. Pati *et al.* have also developed a system wherein the powders of adipose, cartilage and heart ECM are used to produce a “bio-ink” that can be 3D-printed into scaffolds and used to stimulate the growth and differentiation of stem cells, which is a testament to the capabilities of 3D bio-printing technology [220]. Instead of using whole tissue extracellular matrix (ECM), one type of protein or glycosaminoglycan can also be utilized for tissue engineering studies. Collagen, gelatin, fibronectin, laminin, vitronectin, elastin, and fibrin are the materials generally found in the natural ECM of mammalian cells, and are used for the construction of 3D scaffolds. Hyaluronan, alginate, agarose, chitin, chitosan, silk and cellulose are also naturally occurring polysaccharides that can be used to produce scaffolds in 3D culture studies. Due to their natural origin, cells can maintain their function or differentiate into the desired cell lineage without adverse effects in those materials. The spherical 3D co-culture of liver and pancreatic cells inside of a collagen-alginate composite material has also revealed that cells isolated from different tissues can be cultured together in natural scaffolds and continue their function after transplantation [221].

Although the intrinsic properties of natural materials are effective in altering cell behavior, their complex nature also limits the control of their chemical and physical properties. Synthetic materials, in contrast, can be produced with great control over stiffness, functional group concentration, porosity, degradability, epitope availability, release rate and various other characteristics. Furthermore, batch to batch variations and the risk of pathogen infection are diminished in synthetic materials. Synthetic scaffolds are generally produced from covalently cross-linked polymers or

supramolecules formed from monomers. Polystyrene, poly-L-lactic acid (PLLA), polyglycolic acid (PGA), poly(lactic-co-glycolic) acid (PLGA), poly(ethylene glycerol) (PEG), peptide amphiphiles and their polymer-polymer and polymer-ceramic combinations are commonly used synthetic materials [222, 223].

Peptide amphiphile systems are good candidates for the fabrication of 3D scaffold systems, as the spontaneous self-assembly of peptide monomers allows the formation of stable and well-ordered structures through non-covalent interactions (hydrogen bonding, hydrophobic and charge interaction, *etc.*) [5]. The self-assembly of peptide networks depends on their amphiphilic nature, and a typical peptide amphiphile molecule contains (i) a hydrophobic alkyl tail, which allows the molecules to aggregate due to hydrophobic collapse, (ii) a β -sheet forming unit composed of amino acids that allow the formation of well-ordered structures through hydrogen bonding and (iii) charged amino acids that increase peptide solubility and allow self-assembly to occur at a specific pH.

Their similarities with naturally occurring proteins allow the inclusion of protein epitopes (motifs) into the structure of peptide amphiphiles. The supramolecular structures produced by peptides will then display these epitopes on their surface, creating a nanofibrous 3D environment that provides an ECM-like architecture and presents bioactive signals necessary for cellular differentiation [224]. Peptide amphiphiles can easily be organized into hydrogel networks by changing the pH, adding an ionic solution or mixing two oppositely charged PAs (Fig. 1.9), which allows the production of scaffolds without resorting to potentially destructive methods such as UV exposure or enzyme or cross-linking agent treatment. In addition, cells or cell aggregates can be mixed into the peptide solutions prior to gel formation, ensuring that they will be distributed evenly across the scaffold. Zhang and coworkers, for example, showed that cardiomyocytes could be encapsulated into noodle-shaped strings made up of PA gels by drawing it into salty media [225]. These cardiomyocytes retained their function within the scaffold and were oriented in the direction of the peptide string, which is promising for the engineering of damaged muscles or nerves. The mechanical properties of PA gels also can be modified by combining PA molecules with other polymeric biomaterials. Goktas *et al.*, for example, showed that PA gels mixed with PEG can form gel systems with

adjustable stiffness depending on the concentration of PEG. These gels also supported the growth and stiffness-dependent differentiation of Saos-2 osteoprogenitors [226].

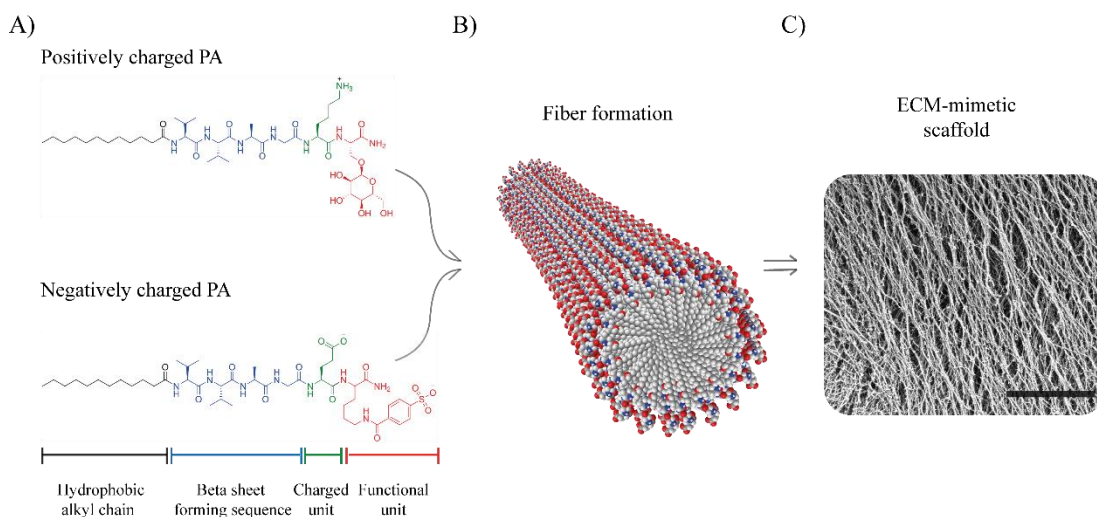


Figure 1.9 Dynamic self-assembly of peptide amphiphiles into nanofibrous structures. **A)** Units of peptide amphiphile molecules: the hydrophobic fatty acid tail and β -sheet forming amino acid sequence facilitate nanofiber formation, the charged unit allows the pH-mediated control of solubility and self-assembly, and the bioactive unit bears functional moieties such as peptide epitopes, sugar units or chemical groups. **B)** Nanofiber formation by charge neutralization of positively and negatively charged peptide amphiphiles. **C)** Overall structure generated by the self-assembly of peptide amphiphile fibers (Scale bar is 4 μm).

CHAPTER 2

**MONITORING DIFFERENTIATION PROFILES OF RAT
MESENCHYMAL STEM CELLS IN THREE DIMENSIONAL
GLYCOSAMINOGLYCAN MIMETIC PEPTIDE AMPHIPHILE
HYDROGEL SCAFFOLDS**

2.1. INTRODUCTION

Regenerative medicine aims to generate living and functional tissues/organs to repair or regain their natural function which is lost due to aging, diseases, trauma or birth defects [227]. It is an interdisciplinary area that requires the cooperation of many research fields like developmental biology, materials science, chemistry and so forth [228]. Stem cells have been considered to possess the capacity to replace cells composing damaged or diseased tissues, and can be used to repair malfunctioning or completely nonfunctional tissues [229]. While induced pluripotent stem cells still need further improvements regarding their production efficiencies and necessity of use of feeder cells, and embryonic stem cells have the potential to form cancerous tissue phenotypes and are subject to fierce ethical debates about their use, mesenchymal stem cells obtained from patients' own body sources offer promising results in tissue engineering studies [230]. Synthetic materials have also been used in tissue engineering research and are superior to natural materials due to their more reliable features in terms of less batch to batch variation, less infection risk, and versatile and adjustable mechanical and chemical features.

Almost every cell in the body lives in a 3D microenvironment in which they physically interact with the extracellular matrix and each other. In studies utilizing cell culture methods, unless the surface of the tissue culture plate is coated with a material, cells are subjected to inert and rigid 2D plastic surfaces. Even if they are coated and their mechanical, chemical or biological cues are optimized, cells still only attach from one dimension and most of the cell surface is still exposed to liquid culture solution [231]. However, 3D systems offer more ECM-like architectures for tissue engineering approaches, and they are readily transplantable or transportable without any further treatment such as trypsinization to detach the cells from the surface [231]. Nanotechnological advancements pave the way for developing novel 3D systems by utilizing bottom up techniques like supramolecular structures such as hydrogels formed by peptide amphiphiles. Peptide amphiphiles serve as ideal platforms to study tissue engineering because of their simple self-assembly mechanism. Non-toxic methods such as mixing two oppositely charged PAs, changing the pH or adjusting the ionic strength are being used to produce gels made

up of PA molecules. They also enable 3D culture systems, which is an effective means of eliciting desired cell responses. Peptide amphiphiles have been used in different 3D *in vitro* and *in vivo* studies. Zhang and co-workers produced strings of peptide fibers in which they encapsulated different types of cells [225]. By incorporating polymers into the PA gels, Goktas *et al.* showed the mechanical characteristics, porosity and bioactivity of the gels can be altered to stimulate cells towards a desired outcome.

The extracellular matrix is composed of fibrillar and non-fibrillar proteins along with proteoglycans made up of proteins and glycosaminoglycans (GAGs). Since glycosaminoglycans play important roles in tissues, structures inspired by their architecture will yield more effective microenvironments for the maintenance, differentiation and implantation of cells. The isolation of natural pure GAGs without any contamination, however, is costly and any errors in the process can be severely hazardous; for example, heparin contamination caused the deaths of over a hundred people in 2007 [232]. Cheaper and contaminant-free production of biomaterials is therefore crucial for both tissue engineering and clinical studies, which can be accomplished by synthetic material fabrication. However, chemical characteristics of such GAG-mimetic biomaterials should be well defined. It is well-recognized that the sulfation ratios of GAGs determine the functionality of the polysaccharide in the native tissue [233]. Sulfation can determine cellular behavior by altering the mechanisms by which GAGs interact with different cell surface receptors, extracellular proteins, and growth factors. It has been shown that GAG binding to growth factors such as bone morphogenic factor-2 (BMP-2) is affected by the sulfation of the carbohydrate chain [234]. It was also shown that the incorporation of sulfonate group to a peptide amphiphile sequence allows the overall structure exhibit heparin-like properties in terms of growth factor binding [235], which can be utilized for angiogenesis, chondrogenesis and osteogenesis studies [82, 236, 237].

In this chapter, we describe peptide amphiphile hydrogels that mimic the extracellular matrix through their glycosaminoglycan-like properties. We designed a system containing sugar units that are presented on nanofiber surfaces alongside different concentrations of sulfonate group. Sulfonated (GAG-mimetic or heparin-mimetic) peptide amphiphiles have been used for many different purposes in our

group, and we have now incorporated a sugar unit, glucose, to one of the PA molecules to make the nanofiber system better resemble the GAG structure. Glucose-functionalized materials have been generally used in drug delivery studies [238-243] or other biotechnological research [244, 245]. Therefore, these materials need to be characterized for cellular response in cell culture experiments before their use in regenerative medicine studies. We monitored the differentiation pattern of rat mesenchymal stem cells in peptide amphiphile hydrogel systems in which sulfonate concentration is altered to mimic different types of sulfated or non-sulfated glycosaminoglycans. Investigation of these cellular responses allows the design of further *in vitro* and *in vivo* experiments for regenerative medicine.

2.2. MATERIALS AND METHODS

9-Fluorenylmethoxycarbonyl (Fmoc) and *tert*-butoxycarbonyl (Boc) protected amino acids except glycol-amino acid, [4-[α -(2',4'-dimethoxyphenyl) Fmoc-aminomethyl] phenoxy] acetamidonorleucyl-MBHA resin (Rink amide MBHA resin), Fmoc-Glu(OtBu)-Wang resin (100-200 mesh), Fmoc-Asp(OtBu)-Wang resin (100-200 mesh) and *N,N,N',N'*-Tetramethyl-O-(1*H*-benzotriazole-1-yl) uronium hexafluorophosphate (HBTU) were purchased from NovaBiochem, ABCR or Sigma-Aldrich. Fmoc-Ser[β -Glc(OAc)4]-OH was purchased from AAPPTec. (DIEA) and lauric acid were purchased from Merck. Piperidine, acetic anhydride, dichloromethane (DCM), dimethylformamide (DMF), trifluoroacetic acid (TFA) and triisopropylsilane (TIS) were purchased from Sigma-Aldrich. All other chemicals and materials used in this study were analytical grade and obtained from Invitrogen, Fisher, Merck, Alfa Aesar, and Sigma-Aldrich. Deionized water with a resistance of 18 M Ω .cm (Millipore Milli-Q) was used in all experiments. For cell culture experiments, I used Dulbecco's Modified Eagle Medium (DMEM), Penicillin/Streptomycin (P/S) antibiotic combination and Fetal Bovine Serum (FBS), which were purchased from Gibco, Life Technologies. Alamar Blue was purchased from Invitrogen. Safranin O, Oil Red O and Alizarin Red S were purchased from Sigma-Aldrich.

2.2.1. Synthesis, Purification and Characterization of Peptide Amphiphile Molecules

2.2.1.1 Synthesis of Peptide Amphiphile Molecules by Solid Phase Peptide Synthesis

Five peptide amphiphile molecules were synthesized by solid phase peptide synthesis method. Glc-PA [Lauryl-VVAGKS(Glucose)-NH₂], SO₃-PA [Lauryl-VVAGEK(*p*-sulfobenzoate)-NH₂] and K-PA [Lauryl-VVAGK-NH₂] were synthesized on Rink amide MBHA resin, while E-PA [Lauryl-VVAGE] and RGD-PA [Lauryl-VVAGERGD] were synthesized on Wang resins pre-loaded with Fmoc-Glu(OtBu) and Fmoc-Asp(OtBu), respectively. Prior to the synthesis, resins were swelled in DCM for 30 min, which was followed by washing with DMF. Fmoc protecting groups were removed prior to each coupling by incubating the resin in piperidine/DMF (20% v/v) solution for 20 min. Carboxylate group activation of 2 mole equivalents of each amino acid was performed by 1.95 mole equivalents of HBTU, and 3 mole equivalents of DIEA for 1 mole equivalent of functional sites on the solid resin, which were dissolved in 10 mL DMF and coupling time for each step was set to be 2.5 h for each cycle. After each reaction, resin-peptide molecules were washed three times with DMF, DCM and DMF, respectively. Completeness of each coupling was determined by Kaiser Test. Complete coupling was followed by 10% acetic anhydride/DMF solution treatment for 30 min to permanently acetylate the unreacted amine groups. When amino acid chain was completed, lauric acid addition was performed similarly to amino acid coupling, except that coupling time was raised up to 4 h. In order to synthesize SO₃-PA, sulfobenzoic acid was added to the side chain of lysine. Before this process, 4-methyltrityl (MTT) group, which provides side chain protection for lysine, was removed by a TFA/TIS/H₂O/DCM mixture (5:2.5:2.5:90 ratio) for 5 min.

Cleavage of protection groups and peptide amphiphile molecules from solid phase was carried out by TFA cleavage cocktail (95:2.5:2.5 TFA:TIS:ddH₂O) for 2 h. DCM and excess TFA were removed by rotary evaporation. The synthesized peptide amphiphile was then precipitated by overnight incubation in ice cold diethyl ether. The precipitate was collected by centrifuging the solution at 8000 rpm for 20 min. Supernatant (diethyl ether) was decanted without disturbing precipitated PAs and

remaining diethyl ether was evaporated. PA powder was then dissolved in ddH₂O, frozen at -80 °C overnight and lyophilized for 3 days.

After the synthesis procedure, peptide cleavage from the solid phase was carried out for 2 h at room temperature with 95% cleavage cocktail (95:2.5:2.5 TFA:TIS:ddH₂O) and excess TFA and DCM were subsequently removed with rotary evaporator. The remaining peptide amphiphile solution was then precipitated using overnight incubation in ice cold diethyl ether at -20 °C. The solution was then centrifuged at 8000 rpm for 15 min to completely precipitate peptide amphiphile molecules; diethyl ether was then decanted and the remainder in the flask was evaporated. Peptide amphiphile molecules were dissolved with ddH₂O. Finally, the peptide amphiphile solution was frozen at -80 °C for 4 h, lyophilized for 3 days and stored at -20 °C.

2.2.1.2 Purification of Peptide Amphiphile Molecules

In order to determine the purity of the synthesized peptide amphiphile molecules, an Agilent 6530 quadrupole time of flight (Q-TOF) mass spectrometer with electrospray ionization (ESI) source equipped with a reverse phase analytical high performance liquid chromatography was used. An Agilent Zorbax Extend-C18 (2.1 x 50 mm) column (basic condition) was used for the characterization of negatively charged PA molecules, while a Zorbax SB-C8 (4.6 x 100 mm) column (acidic condition) was used for positively charged PA molecules. An optimized gradient of water (0.1% formic acid or 0.1% NH₄OH) and acetonitrile (0.1% formic acid or 0.1% NH₄OH) was utilized as mobile phase during liquid chromatography.

A reverse phase preparative HPLC (Agilent 1200 series) equipped with either a Zorbax Extend-C18 (21.2 x 150 mm) column or a Zorbax SB-C8 (21.2 x 150 mm) column as a stationary phase was employed to purify negatively and positively charged PA molecules, respectively. An optimized gradient of water (0.1% acetonitrile or 0.1% NH₄OH) and acetonitrile (0.1% formic acid or 0.1% NH₄OH) was used as mobile phase during HPLC purification. Dialysis of K-PA dissolved in ddH₂O and placed into solution containing 0.1% HCl was enough for purification and removed the remaining residual TFA.

2.2.1.3 Circular Dichroism

Secondary structures of the nanofiber systems were investigated by circular dichroism analyses, which were performed by using a J-815 Jasco spectrophotometer in the far ultra-violet region. PA solutions and mixtures were prepared and used at a final concentration of 2×10^{-4} M for each analysis with quartz cuvettes bearing a path length of 1 mm. An average of three readings was performed between 190 nm and 300 nm by choosing a digital integration time of 1 s, a band width of 1 nm and with standard sensitivity. All spectra were smoothed by the Savitzky-Golay method with 9 convolution width. Obtained data were converted to molar ellipticity using the formula:

$$[\theta] \text{ (deg cm}^2 \text{ dmol}^{-1}\text{)} = \frac{\text{ellipticity (mdeg)} \times 10^6}{\text{pathlength (mm)} \times \text{concentration } (\mu\text{M})}$$

2.2.1.4. Scanning Electron Microscopy

Gels were prepared as described in section 2.2.2.2, but without cells. Gels that were incubated in cell maintenance conditions for 7 days were fixed with 2.5% gluteraldehyde and 2% osmium tetroxide solutions at room temperature for 1 h each. Samples were dehydrated by increasing concentrations of ethyl alcohol and dried with Tourismis Autosamdri®-815B critical point drier, which assists in the preservation of the structure of both cells and gel. Samples were coated with 10 nm Au/Pd and visualized using a FEI Quanta 200 FEG scanning electron microscope under high vacuum condition.

2.2.1.5. Oscillatory Rheology

An Anton Paar Physica RM301 Rheometer with a 25 mm parallel-plate configuration was used to determine the viscoelastic properties of peptide amphiphile gels. PA gels were prepared as described in section “2.2.2.2. 3D PA Gel Scaffold Formation” to form 250 μL total volume of gel that contains 8 mM of each constituent peptide amphiphile. Measurements were conducted at 25 $^{\circ}\text{C}$ with 500 μm of shear gap distance. Gelation kinetics of the groups of gels was characterized with time-dependent rheology until the system reached a plateau. The time-sweep test was employed within the linear viscoelastic range and with angular frequency (ω) and strain (γ) held constant at 10 rad s^{-1} and 0.01%, respectively.

2.2.2. Peptide Amphiphile Nanofiber Formation

Peptide amphiphiles form hydrogels through electrostatic interactions when mixed in ratios that neutralize their charges. Both 2D surface coatings and 3D hydrogels were produced by mixing GAG-mimetic PA molecules.

2.2.2.1. PA Nanofiber Formation in 2D Surface Coatings

Each working solution was prepared by dissolving peptide amphiphiles in double distilled water to a concentration of 2 mM, and pH of the solution was adjusted to 7-7.4 by adding 1 M of base (NaOH) or acid (HCl) depending on the pH of the solution. There were four experimental gel groups; all of which contained 10% RGD-PA (negatively charged), 50% positively charged PA (Glc-PA or K-PA) and 40% negatively charged PA combinations (only E-PA, only SO₃-PA or E/SO₃-PA mixture). The compositions of PAs in all groups can be found in Table 2.1. PA working solutions were sonicated at least for 30 min prior to mixing. Negatively charged PA solutions were first mixed with RGD-PA solution until the latter was diluted to 20% of the total concentration of the negative peptide solution. 50 μ L of the prepared solution was distributed to 96-well plates and 50 μ L of positively charged PA solution was added onto those wells. Solutions were carefully mixed and care was taken to ensure that all hydrogels were homogeneous. Plates were incubated for 1 h at 37 °C and then placed into laminar flow cabinet to dry overnight. The dried coatings were sterilized by exposing them to UV about 1 h before seeding cells onto them.

Experimental Groups	Content*				
	K-PA	Glc-PA	E-PA	SO ₃ -PA	RGD-PA
GS	-	50%	-	40%	10%
GES	-	50%	20%	20%	10%
GE	-	50%	40%	-	10%
KE	50%	-	40%	-	10%
Glass Surface[†]	Including no PA molecules				

* Each PA solution is 8 mM for constructing 3D hydrogel and 2 mM for coating 2D surface

† Tissue culture plate/plastic group for 2D experiments

Table 2.1 Contents of experimental groups in terms of peptide amphiphile percentage.

2.2.2.2. 3D PA Gel Scaffold Formation

Sterilized cover slips with 13 or 15 mm diameters were placed into 24-well plates based on the experimental setup. 13 mm diameter was preferred for experiments such as gel embedding and electron microscopy, which require the removal of the gel from well plates. Otherwise, 15 mm diameter was better choice to avoid the movement of gels inside the wells. Gels were prepared with similar technique as described in 2D coating part. Instead of using ddH₂O as a solvent, positively charged PA molecules were dissolved in 0.25 M sucrose solution whereas negatively charged molecules were dissolved in cell culture medium without phenol red. Peptides were sterilized by exposing the solutions to UV for 1 h and followed by sonication for at least 30 min. Each gel was produced by mixing 8 mM of 50 μ L working solution of both positively and negatively charged peptide amphiphiles. Firstly, positively charged PA solutions were dropped onto cover slides and negatively charged PA solutions in which cells were resuspended, were carefully mixed to ensure that solutions mixed homogenously and without disrupting the structure. 2.5×10^5 cells per gel proportion were provided at the beginning of each experiment and calculations were performed accordingly. After preparation of gels, plates were

placed into incubators for 1 h and 1 mL maintenance medium was added into each well.

2.2.3. Cell Culture and Viability Analyses

2.2.3.1 Cell Maintenance

Rat mesenchymal stem cells purchased from Invitrogen were used for performing the experiments. Cells were incubated at 37 °C in a humidified atmosphere supplied with 5% CO₂ and the medium utilized for cell maintenance was composed of low glucose DMEM supplemented with 10% (v/v) FBS, 1% (v/v) P/S and 1 mM L-glutamine. Cell culture was performed in 75, 162 or 225 cm² flasks depending of the needed cell number for the experiments, with seeding concentrations of around 3000 cells per cm². Cell media was replenished every 2 days and cells used in experiments were detached from flasks when they reached to ~90% confluency. In order to detach the cells, firstly, the medium was discarded and the region of the flask where cells were attached was washed with 1x PBS. Enough trypsin was added after discarding the PBS and samples were incubated at 37 °C for enough time to detach cells without overexposure. Then, trypsin was inactivated by adding medium equal to twice the volume of trypsin. The cell-containing medium was centrifuged, the supernatant discarded and the remaining pellet was dissolved with medium, serum or negatively charged PA solution. Each experiment was performed on cells at the seventh passage.

Cells were frozen in FBS containing 7.5% DMSO in cryovials containing around 1.5 x 10⁶ cells in 1 mL solution per vial. In order to thaw cells, cryovials were placed into 37 °C and as soon as the ice disappeared, the liquid in the vials were poured into maintenance medium to dilute DMSO. After centrifugation, cells were resuspended in medium and seeded into new flask or well plates in specified numbers.

2.2.3.2. Cell Maintenance for 2D Cell Coating Experiments and Viability Analysis

2D experiments were conducted after coating of 96-well plates as previously mentioned by seeding 5000 cells per well. Maintenance medium and incubation conditions were used as described in previous session. In none of the experiments, supplements, cytokines or minerals were utilized for the differentiation of rMSCs. Along with PA coated surface groups, there was a group in which cells were seeded

onto bare tissue culture plastic surface. Cell viabilities were monitored by incubating cells with 2 μ M Calcein-AM and 4 μ M ethidium homodimer in PBS for 40 min after discarding medium. No washing step was employed to avoid losing dead cells. Then, wells were visualized under fluorescence microscope and images were acquired. Other experiments based on stainings were performed on 2D surfaces in order to identify differentiation patterns and are explained in the section “2.2.5 differentiation analyses”.

2.2.3.3. Cell Maintenance for 3D Cell-Laden Gel Experiments and Viability Analyses

Gels loaded with cells were prepared as previously mentioned for 3D experiments. There was also a group that contained cells seeded onto cover glass surface as a control group. Cell culture maintenance medium was identical to that used in 2D cultures. No cell differentiation inducing supplement was added in order to ensure that all differentiation signal came from the gel. Every 2 days, 500 μ L medium was discarded and fresh medium was replenished till the day of planned experiments. Each week, the whole medium was replaced by fresh medium to keep the medium level steady and avoid volume errors caused by evaporation through prolonged cell incubation.

Viability of the cells was assessed by incubating cells with 2 μ M Calcein-AM and 4 μ M ethidium homodimer in PBS for 40 min after discarding medium. Then, wells were visualized under fluorescence microscope and images were taken. In addition, AlamarBlue® assay was applied at different days to show that cells were alive and proliferating. AlamarBlue® reagent was diluted 1:10 with normal maintenance medium and replaced with the medium in well plates. 500 μ L of the prepared solution was added into wells and incubated until the color of wells with cells turned to pink. Then, the fluorescence was monitored at 540 nm excitation wavelength and 590 nm emission wavelength by microplate reader (Molecular Devices Spectramax M5). In order to visualize the morphology and environment of the cells after 21 days of incubation, gels were fixed with 2.5% glutaraldehyde and 2% osmium tetroxide solutions at room temperature for 1 h each. In order to see cells inside, the gels were disturbed with a needle prior to fixation. Samples were dehydrated by increasing concentrations of ethanol replacements and dried by a Tourisim Autosamdri®-815B

critical point drier. Samples were then coated with 5 nm Au/Pd and analyzed by scanning electron microscopy (FEI Quanta 200 FEG).

2.2.4. Gel Embedding and Cryosectioning

2.2.4.1. Fixation of the Gels and Preparation for Cryosectioning

3D cell-laden gels were fixed with 4% paraformaldehyde in PBS for 10 min after discarding cell culture medium. The wells were washed with PBS for 10 min and 1 mL of 15% and 30% (w/v) sucrose in PBS solutions were replaced twice. The glass covers were taken out with tweezers and needles without disturbing the gels and the gels were placed into molds. The solution of O.C.T. was poured to cover whole surface of the gels and the molds were dipped into liquid nitrogen to freeze the gels immediately. The molds were placed into -80 °C freezers until cryosectioning.

2.2.4.2. Cryosectioning of the Gels with Cryostat

Gels were taken out and placed into a cryostat that was previously adjusted to -25 °C. After optimizing the orientation and blade angle, 10 µm sections were taken from positively charged microscope slides. Slides were air-dried for around 30 min and then placed into -20 °C freezer until staining.

2.2.5. Differentiation Analyses

2.2.5.1. Alkaline Phosphatase Activity

Alkaline phosphatase activity assay was applied to cells incubated for 7, 14 and 21 days. Experiments were performed on gels initially loaded with 2.5×10^5 of cells and glass surface seeded with the same number of cells. Their incubation and maintenance were performed as previously mentioned. The maintenance medium was discarded and gels and cells were washed prior to protein extraction. For protein extraction, wells were first filled with 500 µL PEK (Protein Extraction Kit)/PIK (Protease Inhibitor Cocktail) solution at a 95:5 ratio and incubated on a shaker for 30 min. The solution was collected to centrifuge tubes after making sure that gels and cells were lysed. After centrifugation at 14000 g at 4 °C for 10 min, the supernatant was transferred to other centrifuge tubes and these supernatant solutions were utilized for both protein concentration determination and alkaline phosphatase activity assays. Pierce® BCA protein assay (Thermo Scientific) was performed to quantify protein amounts obtained from the cells as described in manufacturer's

protocol. Absorbance was determined at 562 nm by using a micro plate reader (Molecular Devices Spectramax M5). In order to measure alkaline phosphatase activity, 50 μ L of protein sample obtained from the protein assay was incubated with 150 μ L of *p*-nitrophenol phosphate substrate in a 96-well plate for 30 min on a shaker. A standard curve was plotted by using serial dilutions of *p*-nitrophenol in 0.25 M NaOH. Finally, optical density was determined at 405 nm wavelength by using a microplate reader. The alkaline phosphatase activity values were normalized to total protein amounts.

2.2.5.2. Oil Red O

Oil Red O staining was performed on 2D gels to monitor adipogenic differentiation. Cells were seeded at a density of 5×10^3 cells per well in maintenance medium. Briefly, the culture medium was discarded and cells were washed with PBS and fixed by incubation with 4% paraformaldehyde in PBS for 10 min at room temperature. The fixative was then changed with fresh replacement and the plate was incubated for 1 h at RT. After discarding the fixative, wells were washed with ddH₂O and 60% (v/v) isopropanol in ddH₂O was added onto the cells for 5 min. Removal of diluted isopropanol was followed by air-drying of cells. Oil Red O working solution was added onto the air-dried cells for 10 min at room temperature and 4 times washing steps with ddH₂O were employed, immediately. Images were taken with an inverted light microscope.

2.2.5.3. Safranin O staining

Sulfated glycosaminoglycans secreted by cells were detected by Safranin O staining of 2D PA coated and uncoated 96-well plates. Cells were seeded at a density of 5×10^3 cells per well and incubated at the above-described maintenance conditions. Briefly, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After discarding the fixative and washing with PBS, the blocking procedure was performed. 1% (w/v) BSA in PBS was utilized for this purpose and samples were incubated for 30 min at room temperature. Then, cells were stained with 0.1% (w/v) Safranin O in 1% (v/v) acetic acid for 5 min at room temperature. Finally, wells were extensively washed 3-5 times with 0.1% (v/v) acetic acid in PBS until the washing buffer lost color in order to remove unbound dye.

Stained glycosaminoglycan depositions were observed under an inverted light microscope.

2.2.5.4. Alizarin Red S Staining

Alizarin Red S staining was performed in order to detect calcium deposition by cells. Cells seeded on 2D PA coating groups and TCP were fixed with ice-cold ethanol for 1 h. Then, the well plates were treated with 40 nM Alizarin Red S solution (pH 4.2) for 30 min. After discarding the dye and washing with ddH₂O until the washing water was colorless, images of the cells were acquired with an inverted light microscope.

2.2.5.5. Immunostaining of Aggrecan and Analysis of Stained Cells

Gel sections taken by cryostat were air-dried and washed with Tris-buffered saline (TBS) in order to remove O.C.T. Samples were then incubated with TBS with 0.025% Triton X-100 twice for 5 min for permeabilization. Afterwards, blocking solution containing 3.3% normal goat serum with 1% BSA in TBS was added onto the samples, which were incubated 2 h at room temperature in a humidified chamber to avoid drying. Primary anti-aggrecan antibody (Abcam, ab36861) was diluted to 1:500 in TBS with 1% BSA and added on the cleaned samples. Two hours at room temperature in a humidified chamber were enough for the binding of the primary antibody. Following incubation, samples were washed twice with 0.025% Triton X-100 in TBS for 5 min, and the secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Merck Millipore, AQ132P) which was diluted in TBS with 1% BSA at a ratio of 1:500 was added onto the slides. After 1 h of incubation at room temperature, slides were washed with TBS for 5 min once and 10 min twice. HRP activity was visualized by a chromogenic reaction with diaminobenzidine (DAB) substrate. All samples were dehydrated through gradually increasing concentrations of ethyl alcohol and finally with xylene. Samples were lastly mounted with an organic solvent based mounting medium and images were taken under light microscope. Analysis of aggrecan-immunostained cells were carried out by calculating the cell number per millimeter square of total gel area with ImageJ program.

2.2.5.6. Quantitative Reverse Transcription Polymerase Chain Reaction

Expressions of various genes related to differentiation in rMSCs were analyzed to determine the mesenchymal cell lineages selected by these cells through quantitative real-time polymerase chain reaction (qRT-PCR). TRIzol (Invitrogen) reagent was utilized for the isolation of total RNA from rat mesenchymal stem cells incubated for 3, 7, 14 and 21 days in 3D gels and glass surface. Extracted RNA samples were dissolved in ddH₂O and their purity and concentrations were assessed by Nanodrop 2000 (Thermo Scientific). Primer sequences were designed using Primer 3 software (Table 2.2). Reaction efficiencies of each primer were evaluated with standard curves by using 2-fold serial dilutions of total RNA. Complementary DNA synthesis from RNA and qRT-PCR were performed by using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen). The qRT-PCR reaction conditions were briefly as following: 55 °C for 5 min, 95 °C for 5 min for production of cDNA from mRNAs and 40 cycles of 95 °C for 15 sec, a specific annealing temperature for each primer (Table 2.2) for 30 sec, and 40 °C for 1 min, followed by a melting curve to confirm product specificity. Analyses of the expression data were completed by comparing primary gene expressions normalized to the expression of a housekeeping gene, *Gapdh*. A comparative Ct method was used to analyze the results. The expression levels of seven different genes were analyzed and quantified by qRT-PCR. These genes were glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), runt-related transcription factor 2 (*Runx2*), collagen 1 (*Col 1*), transcription factor SRY-box 9 (*Sox9*), aggrecan (*Acan*), collagen 2 (*Col 2*), adiponectin (*Adipoq*) and fatty acid binding protein-4 (*Fabp4*).

2.2.6. Statistical & Computer Based Analyses

All quantitative values are represented as means $\pm$ standard error of the mean (SEM), unless noted otherwise. All experiments were performed with at least three replicates for each group and with at least two independent repeats. One-way analysis of variance (ANOVA) with Tukey's post-test was used for statistical analysis and a *p* value of less than 0.05 was considered statistically significant, unless noted otherwise. All graphs were drawn in GraphPad Prism and figures were arranged in Adobe Illustrator. Cells were counted with ImageJ and molecular structures of the PAs were drawn by using ChemBioDraw and ChemBio3D.

Name of Gene (Abbreviation)	Primers: Forward and Reverse	Annealing Temperature (°C)
Adiponectin (<i>Adipoq</i>)	5'-AAGGGAGACGCAGGTGTTCTTGG-3' 5'-ATGGGAACATTGGGGACAGTGAC-3'	59
Aggrecan (<i>Acan</i>)	5'-GGTCACTGTTACCGCCACTT-3' 5'-CCCCTTCGATAGTCCTGTCA-3'	58
Collagen, type 1, alpha 1 (<i>Col1a1</i>)	5'- GAATATGTATCACCAGACGCAGA-3' 5'- GGACATCTGGGAAGCAAAGT-3'	59.5
Collagen, type 2, alpha 1 (<i>Col2a1</i>)	5'- CGAGGTGACAAAGGAGAAGC-3' 5'- TCCCGGTCTTCATGGGACTA-3'	58
Fatty acid binding protein 4 (<i>Fabp4</i>)	5'-CCCAGATGACAGGAAAGTGAAGAGC-3' 5'-CTTTCATGACACATTCCACCACCAG-3	59
Runt-related transcription factor 2 (<i>Runx2</i>)	5'-GGACGAGGCAAGAGTTTCACT-3' 5'-CCCTAAATCACTGAGGCGGT-3'	59.5
SRY (sex determining region Y)-box 9 (<i>Sox9</i>)	5'- CCACCCCGATTACAAGTACC-3' 5'- CAGCCACCTGGGACTCTAA-3	61.5
Glyceraldehyde- 3-phosphate dehydrogenase (<i>Gapdh</i>)	5'- GTGCCAGCCTCGTCTCATA-3' 5'- AACTTGCCGTGGGTAGAGTC-3'	58

Table 2.2 Genes used to determine the differentiation pattern of rMSCs, their forward and reverse primers and annealing temperatures.

2.3. RESULTS

2.3.1. Synthesis of Peptide Amphiphiles

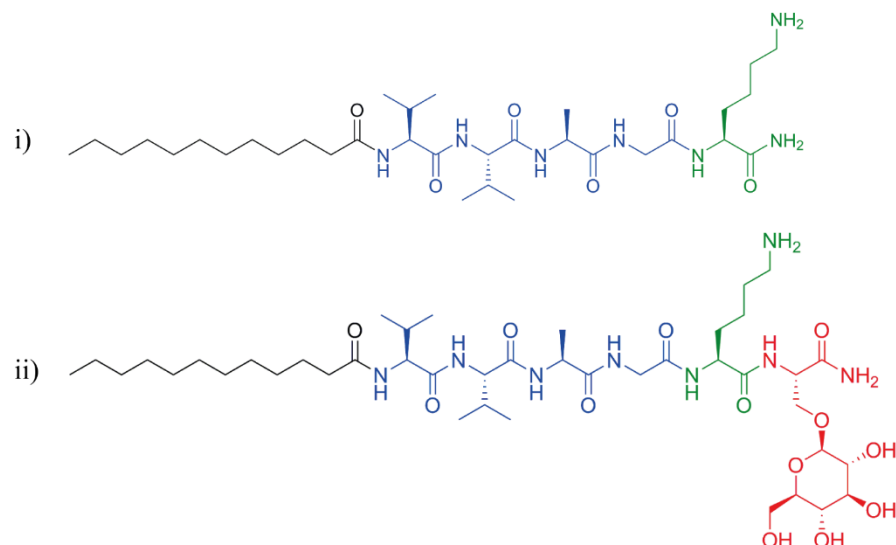
We have designed and synthesized five different peptide amphiphiles molecules with the molecular structures and sequences represented in Fig. 2.1 and Table 2.3. Molecular weights of the PA molecules were expected to be 902.57 for Glc-PA, 653.48 for K-PA, 966.51 for SO₃-PA, 655.42 for E-PA and 983.57 for RGD-PA. Synthesis of PA molecules was followed by determination of the efficiency of solid phase peptide synthesis, which was carried out by the verification of the purity and molecular weights of PAs with Q-TOF LC/MS. The mass spectra of the analyses verified the success in the synthesis. We experimentally found the molecular weight of the PA molecules as 654.50 for K-PA ([M+H]⁺), 903.58 for Glc-PA ([M+H]⁺), 965.47 for SO₃-PA ([M-H]⁻), 654.29 for E-PA ([M-H]⁻), and 982.54 for RGD-PA ([M-H]⁻). These measurements also identified the purity of PA molecules, which were further purified by dialysis or reverse phase HPLC. A second round of purity measurements was performed on PAs which was resulted in pure enough PA molecules to be used in the further characterization and cell culture experiments (Fig. 2.2- 2.6).

Peptide Amphiphile	Sequence	Molecular Weight	Net Charge
K-PA	Lauryl-VVAGK-Am	653.48	+1
Glc-PA	Lauryl-VVAGS(Glc)-Am	902.57	+1
E-PA	Lauryl-VVAGE	655.42	-2
SO ₃ -PA	Lauryl-VVAGEK(<i>p</i> -sulfobenzoate)-Am	966.51	-2
RGD-PA	Lauryl-VVAGERGD	983.57	-2

* Theoretical net charge at pH 7.4

Table 2.3 Sequences of the peptide amphiphiles, their molecular weights, and their theoretical overall charges at neutral pH.

A) Positively charged peptide amphiphiles



B) Negatively charged peptide amphiphiles

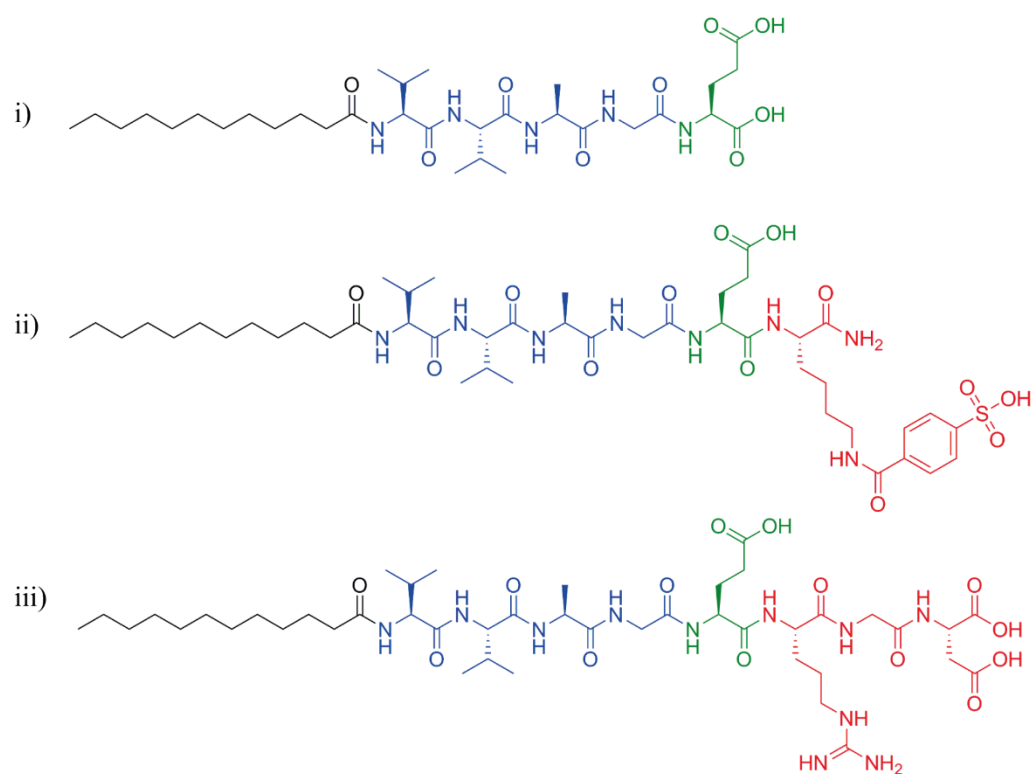


Figure 2.1 Molecular structures of the peptide amphiphiles. **(A)** Positively charged K-PA (i) and glucose bearing Glc-PA (ii). **(B)** Negatively charged E-PA (i), sulfonate bearing SO₃-PA (ii), and integrin binding unit bearing RGD-PA (iii). Fatty acid units are shown as black, beta sheet forming units are blue, charged amino acids are green, and bioactive units are illustrated as red.

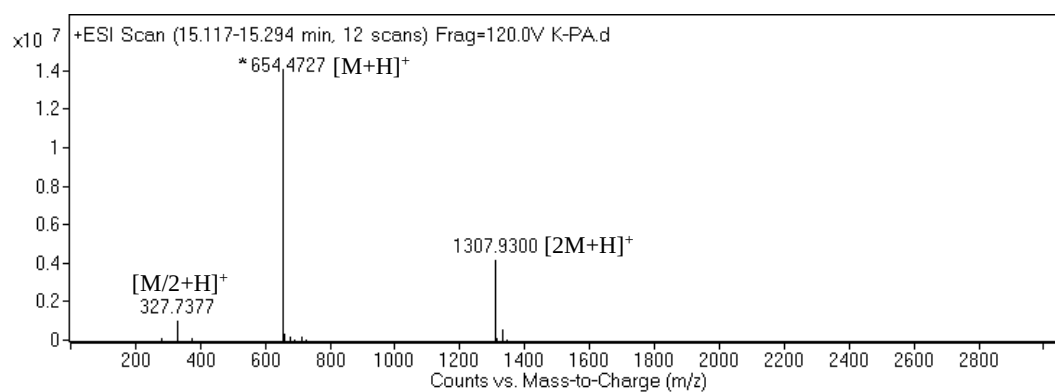
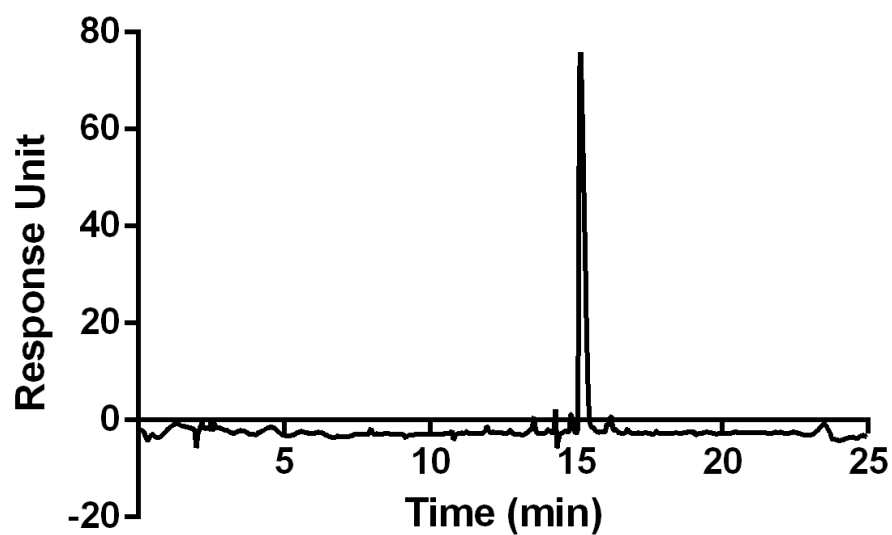


Figure 2.2 Liquid chromatography-mass spectrometry (LC-MS) analysis of K-PA. The purity of the crude product was analyzed according to the optical density at 220 nm.

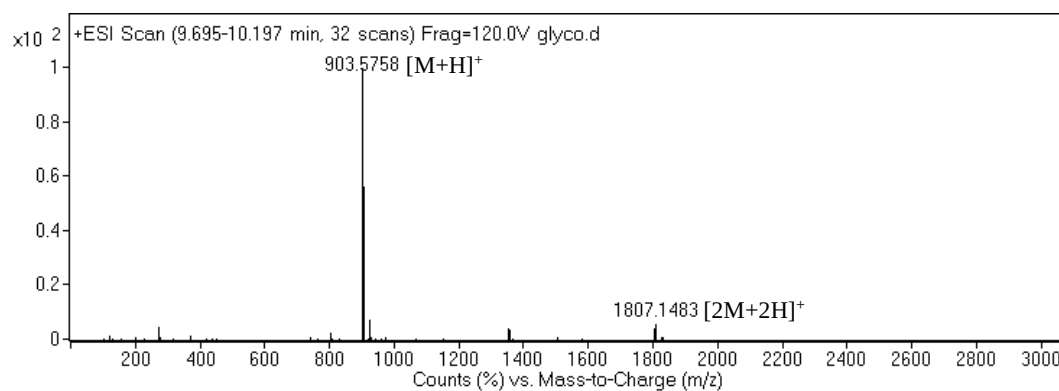
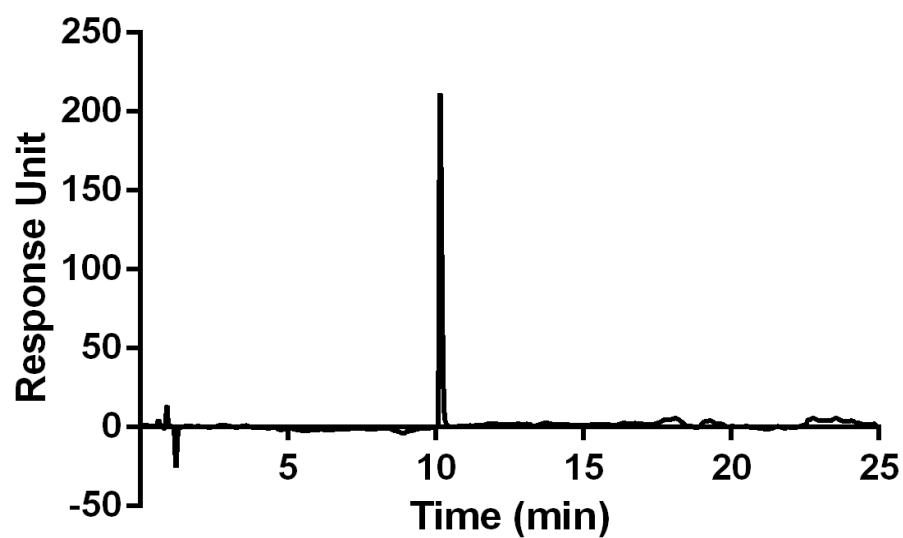


Figure 2.3 Liquid chromatography-mass spectrometry (LC-MS) analysis of Glc-PA. The purity of the crude product was analyzed according to the optical density at 220 nm.

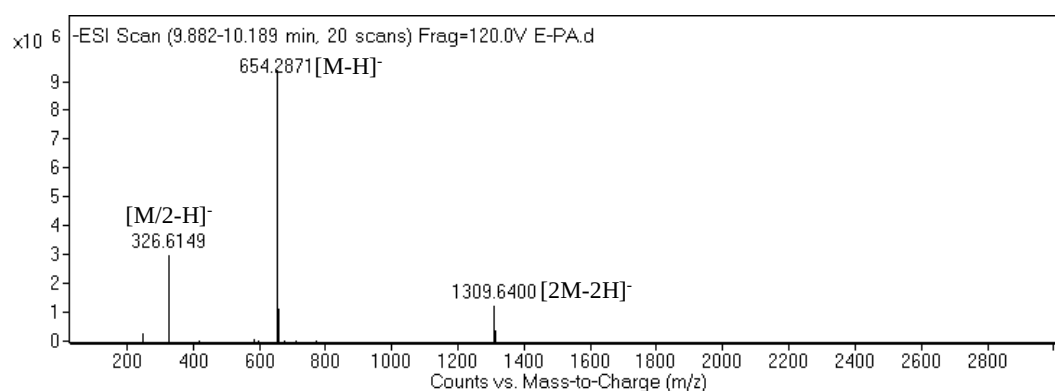
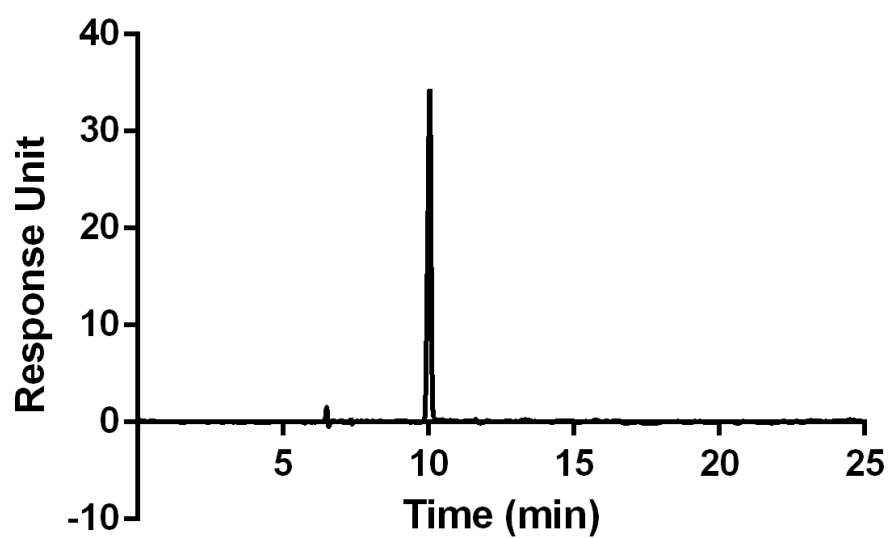


Figure 2.4 Liquid chromatography-mass spectrometry (LC-MS) analysis of E-PA. The purity of the crude product was analyzed according to the optical density at 220 nm.

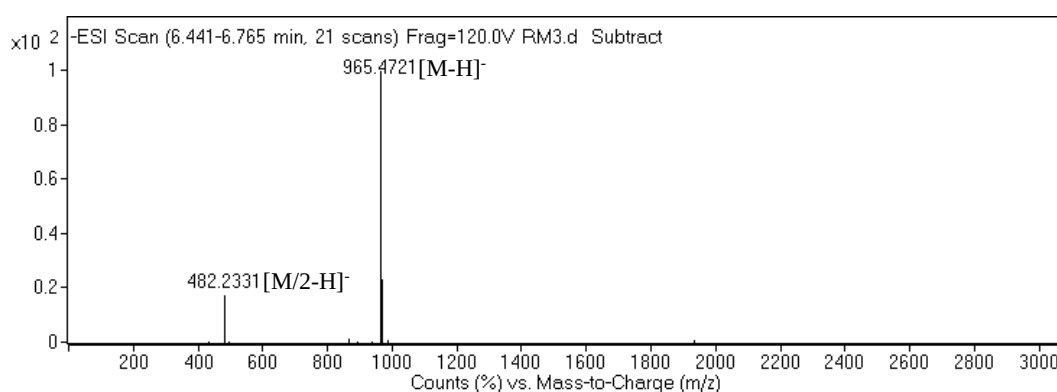
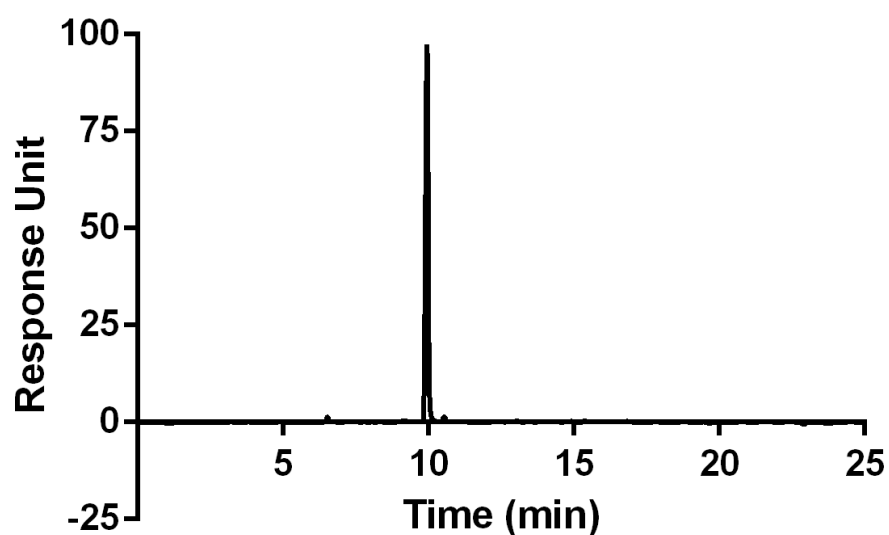


Figure 2.5 Liquid chromatography-mass spectrometry (LC-MS) analysis of $\text{SO}_3\text{-PA}$. The purity of the crude product was analyzed according to the optical density at 220 nm.

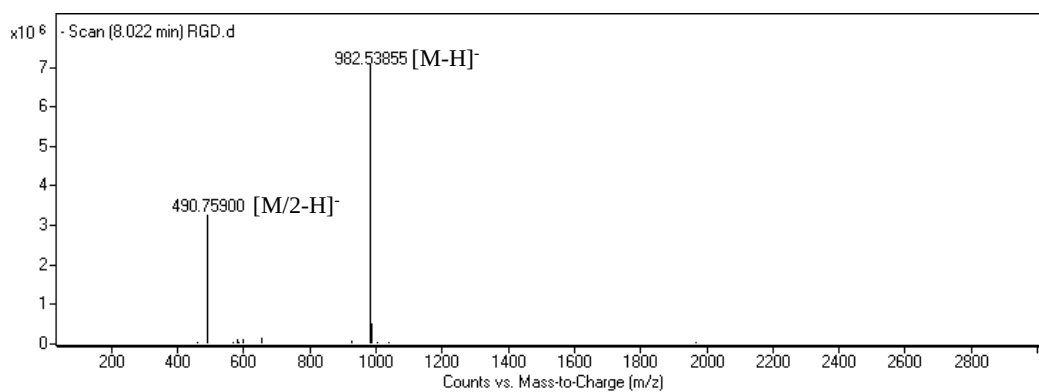
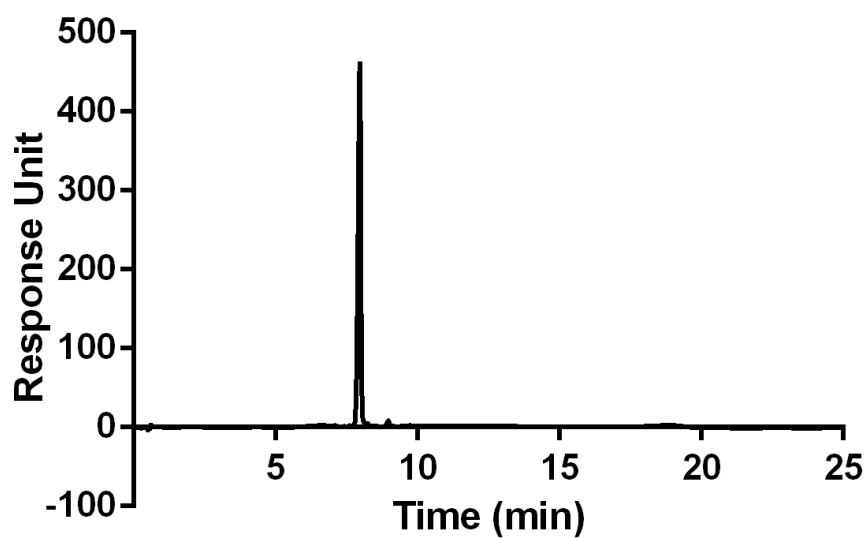


Figure 2.6 Liquid chromatography-mass spectrometry (LC-MS) analysis of RGD-PA. The purity of the crude product was analyzed according to the optical density at 220 nm.

2.3.2. Physical Characterization of Peptide Amphiphiles

Groups were constructed based on their sulfonate concentration, which were mixed with Glc-PA. There was a group that contains neither of glucose attached to the molecule and sulfonate, which is a type of control group. Table 2.1 shows the composition/preparation of the experimental groups. Mixing two oppositely charged PA solutions was enough to form gels whose physical characteristics were important for cell behavior (Fig. 2.7).

2.3.2.1. Secondary Structure Analysis

Circular dichroism results (Fig. 2.8) revealed that these molecules formed β -sheet structures due to the peak intensities around at 200 nm. A negative peak around 220 nm also confirms the presence of β -sheet for peptides composed of L-amino acids.

2.3.1.2. Mechanical Characterization

Mechanical properties, more particularly the gelation features of the peptide amphiphile mixtures, were assessed by oscillatory rheology measurements. Mechanical stiffnesses of the hydrogels were examined by keeping angular frequency (10 rad s^{-1}) constant in order to get average equilibrium moduli of the gels. For all of the combinations, storage modulus (G'), energy stored during deformation, was greater than loss modulus (G''), energy dissipated during deformation (Fig. 2.9). That confirmed the gel character of the resulting gel system. The KE group had less storage modulus value compared to other groups containing Glc-PA (Fig. 2.9).

2.3.1.3. Self-Assembly Analysis

In order to detect fiber formation within the gels, the samples were examined under electron microscope. Samples fixed with gluteraldehyde and osmium tetroxide were visualized under high vacuum and 5 kV energy after coating the samples with Au/Pd. Fig. 2.10 revealed the fibrous nature of the hydrogel samples.

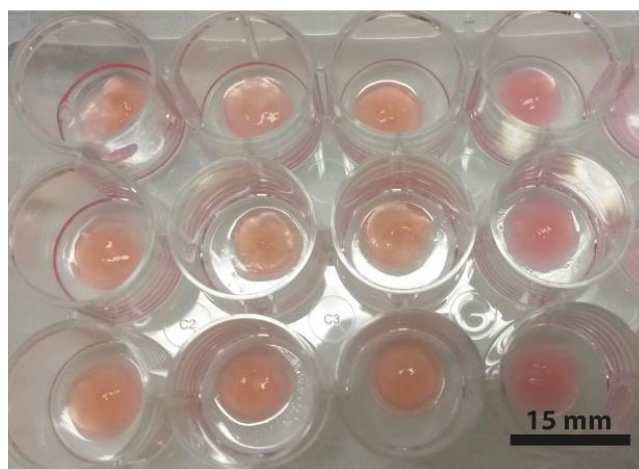


Figure 2.7 Photographs of cell-laden PA hydrogels.

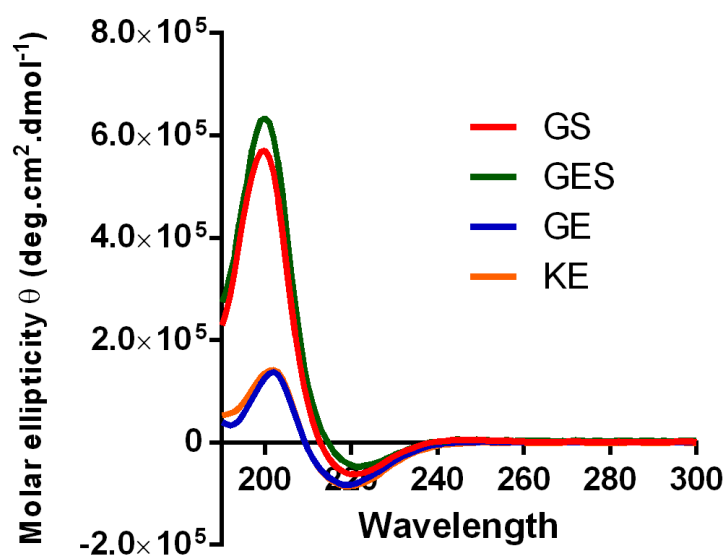


Figure 2.8 Secondary structure analyses of peptide amphiphile mixtures by circular dichroism. Peaks at around 200 nm and 220 nm reveal that the experimental groups contain β -sheet secondary structure.

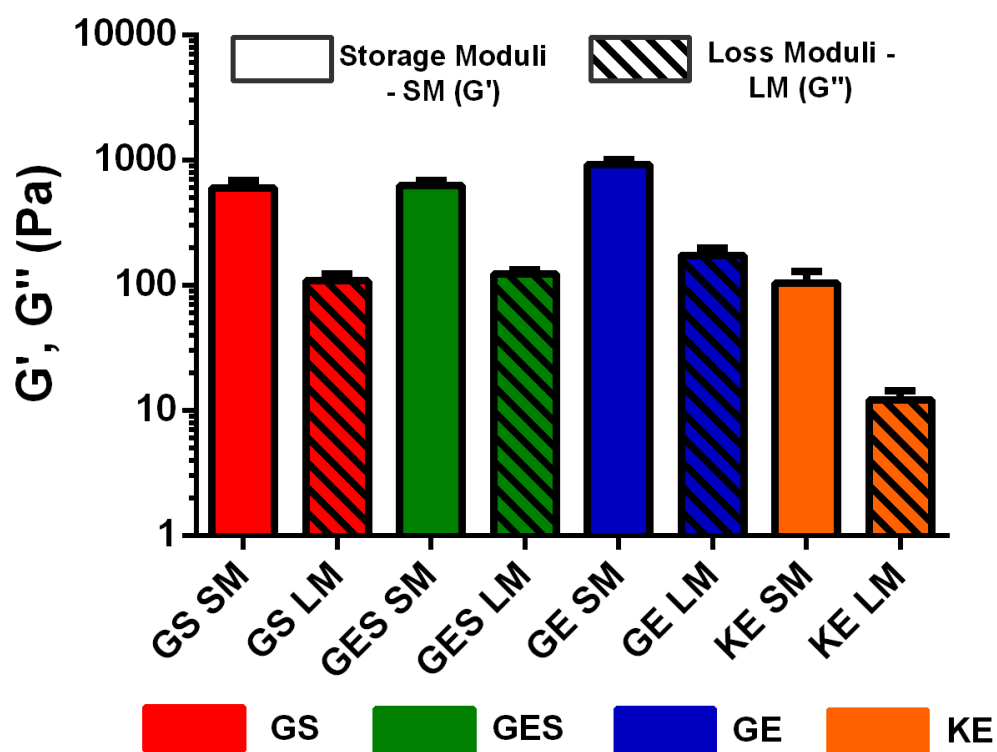


Figure 2.9 Storage and loss moduli of peptide amphiphile gel groups obtained by oscillatory rheology. Higher storage moduli than loss moduli is an indicator of hydrogel characteristics of the mixtures of peptide amphiphiles.

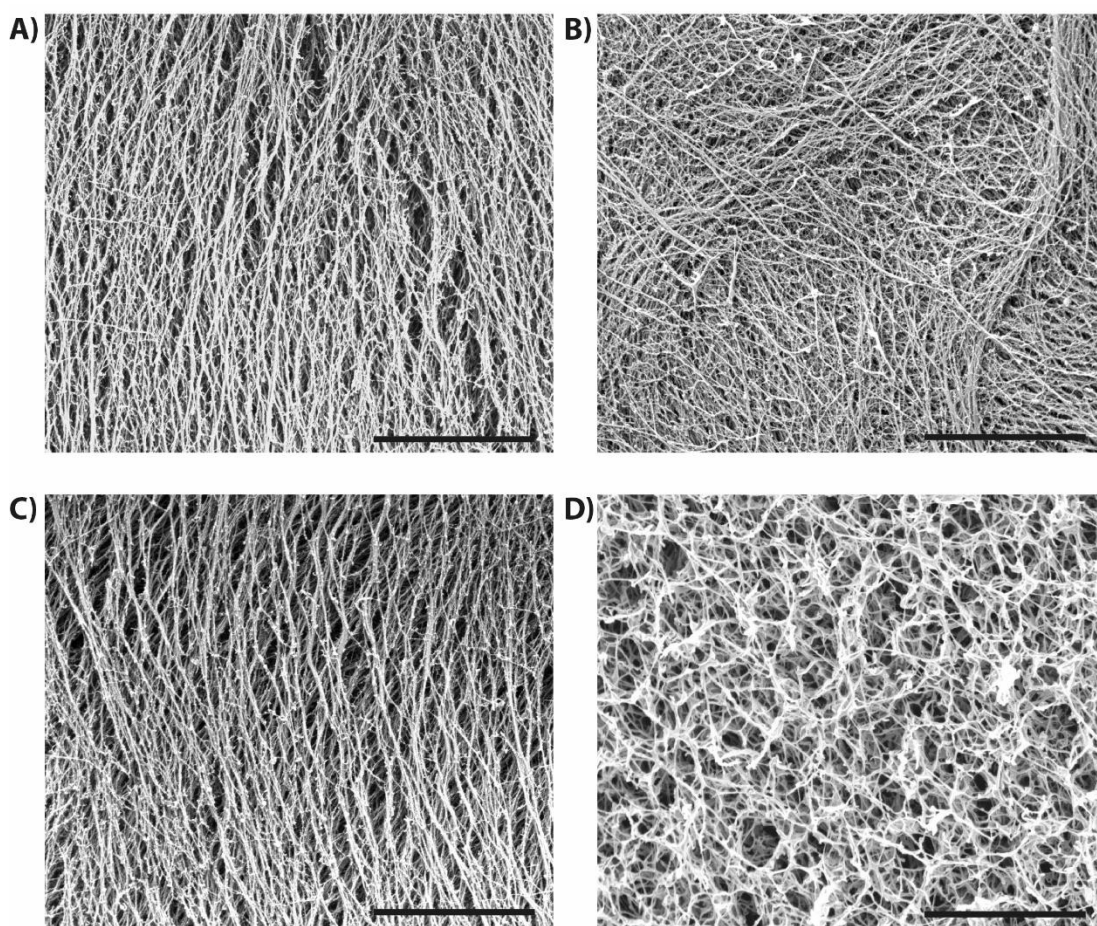


Figure 2.10 Scanning electron micrographs of the nanofibrous structure of peptide amphiphile gels. **(A)** GS, **(B)** GES, **(C)** GE, and **(D)** KE gel groups. Gels form and preserve their nanofibrous architecture even after seven days of incubation in cell culture medium. Scale bars are 4 μm .

2.3.3. Toxicity and Cell Survival Analyses

Biocompatibility of the PA molecules was assessed through cell viability assays and imaging.

2.3.3.1. Biocompatibility of Peptide Amphiphiles

Before starting the 3D experiments, the possible toxicity of PA molecules was predicted by seeding rat mesenchymal stem cells onto the 2D coatings. No apparent toxicity was observed in any of the groups tested (Fig. 2.11). Live/dead assay was implemented to cell cultures incubated for one day, one week, two weeks and three weeks. Green staining revealed live cells as a consequence of metabolized Calcein AM dye; whereas red dots are the nuclei of dead and dying cells stained with ethidium homodimer. This dye leaks into cells that are dying and have lost their cellular integrity. Quantitative analysis of cells' viability after one day of culturing was quite comparable with the cells seeded onto TCP, meaning no toxicity to rMSCs (Fig. 2.12). Within two weeks, however, cells started to die or formed clusters which detached from both PA coatings and TCP surface (Fig. 2.11). After the detachment of primary colonies, remaining cells started to invade the abandoned empty regions of experimental groups and TCP.

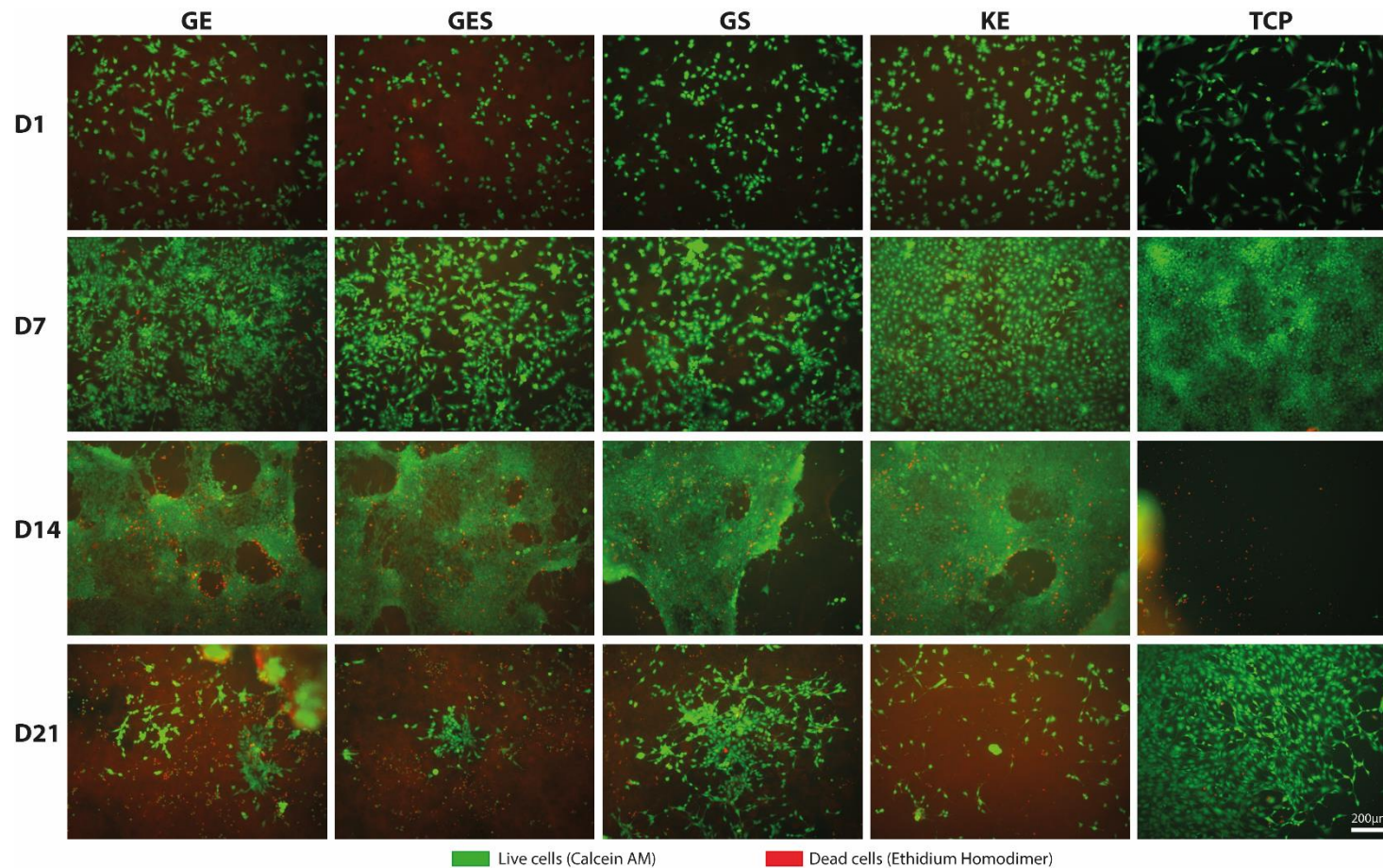


Figure 2.11 Viability of rMSCs on 2D coatings of peptide amphiphile combinations and on TCP after 1, 7, 14 and 21 days of incubation. In the second week, cells started to detach from the surfaces, and re-invade in the third week. Live cells are green and dead ones are stained as red dots. Scale bar is 200 μ m and valid for all pictures.

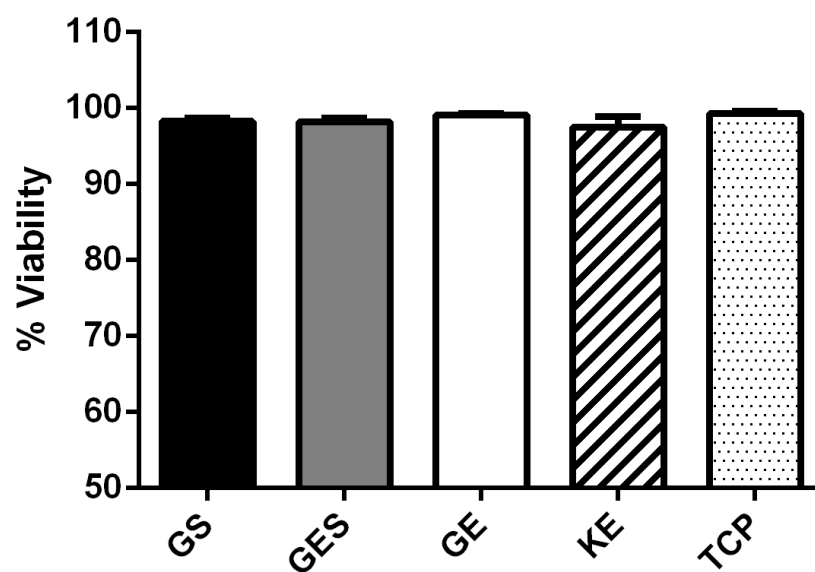


Figure 2.12 Quantified viability of rMSCs on 2D peptide amphiphile coatings after 24 h. There is no difference from TCP group meaning that the peptide amphiphile combinations do not show toxic effect to the cells.

2.3.3.2. Survival of Cells in 3D Gel Environment

Viability of cells was assessed indirectly via live/dead assay (Fig. 13) after 1 and 7 days of incubation. In samples incubated for one day, except the group KE, which was designed to mimic the nanofibrous nature of ECM without functional groups, all other groups showed undetectable cell toxicity. In KE group, there were plenty of red dots which were indicative of the nuclei of dead cells. The samples in which cells were incubated for one week revealed that cells began to form aggregates (Fig. 14). In KE group, however, cells were found both individually with longer extensions and in the form of aggregates. PA gels were also absorbing the ethidium homodimer, which interfered at the red emission region.

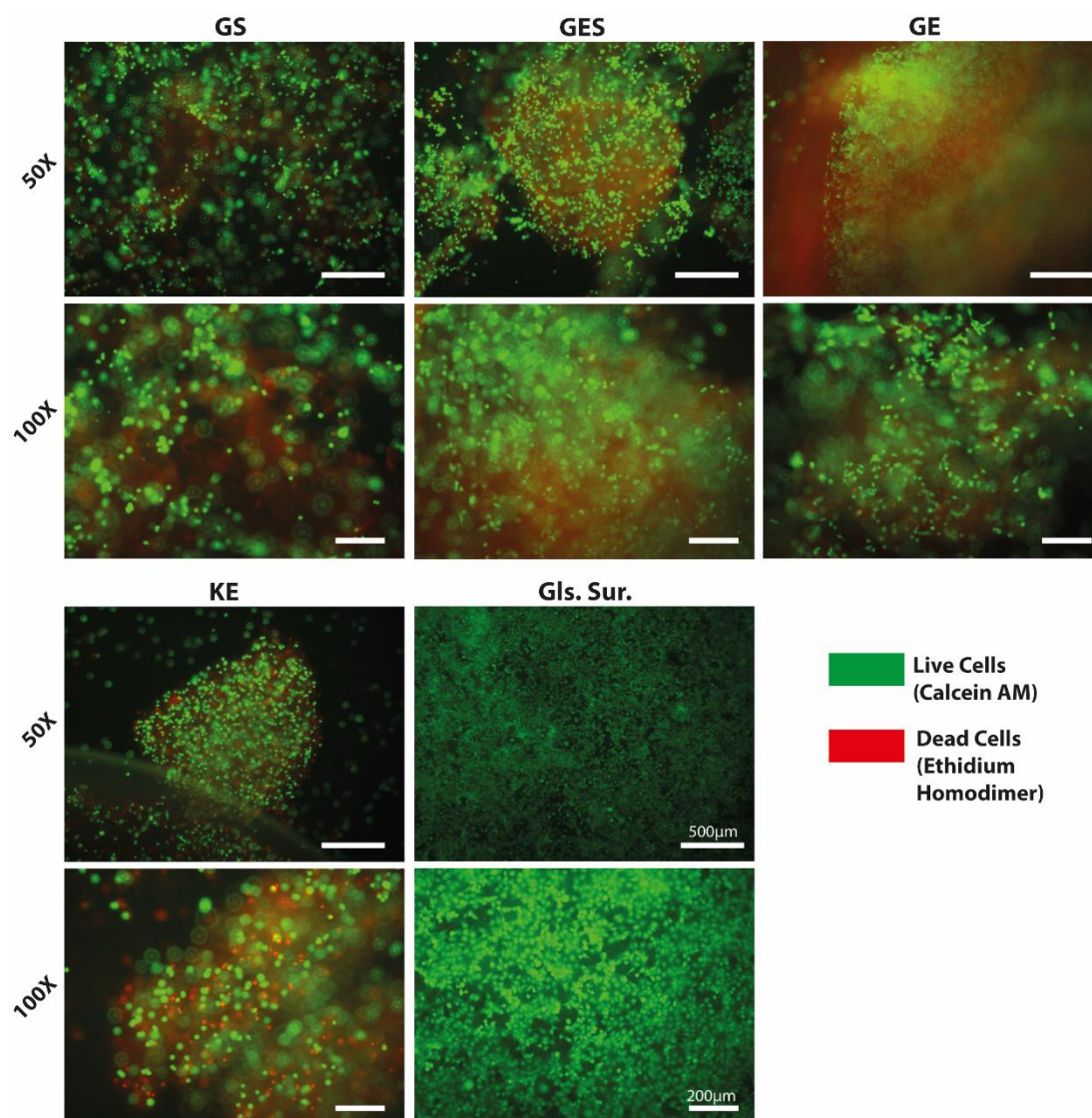


Figure 2.13 Viability of rMSCs inside the 3D gels of peptide amphiphile mixtures after one day of incubation. Except KE group, there are very few dead cells in all other groups. Red dots represent nuclei of dead cells and green color shows live cells. Scale bar for 50 times magnified images is 500 μm , and 100 times magnified images is 200 μm .

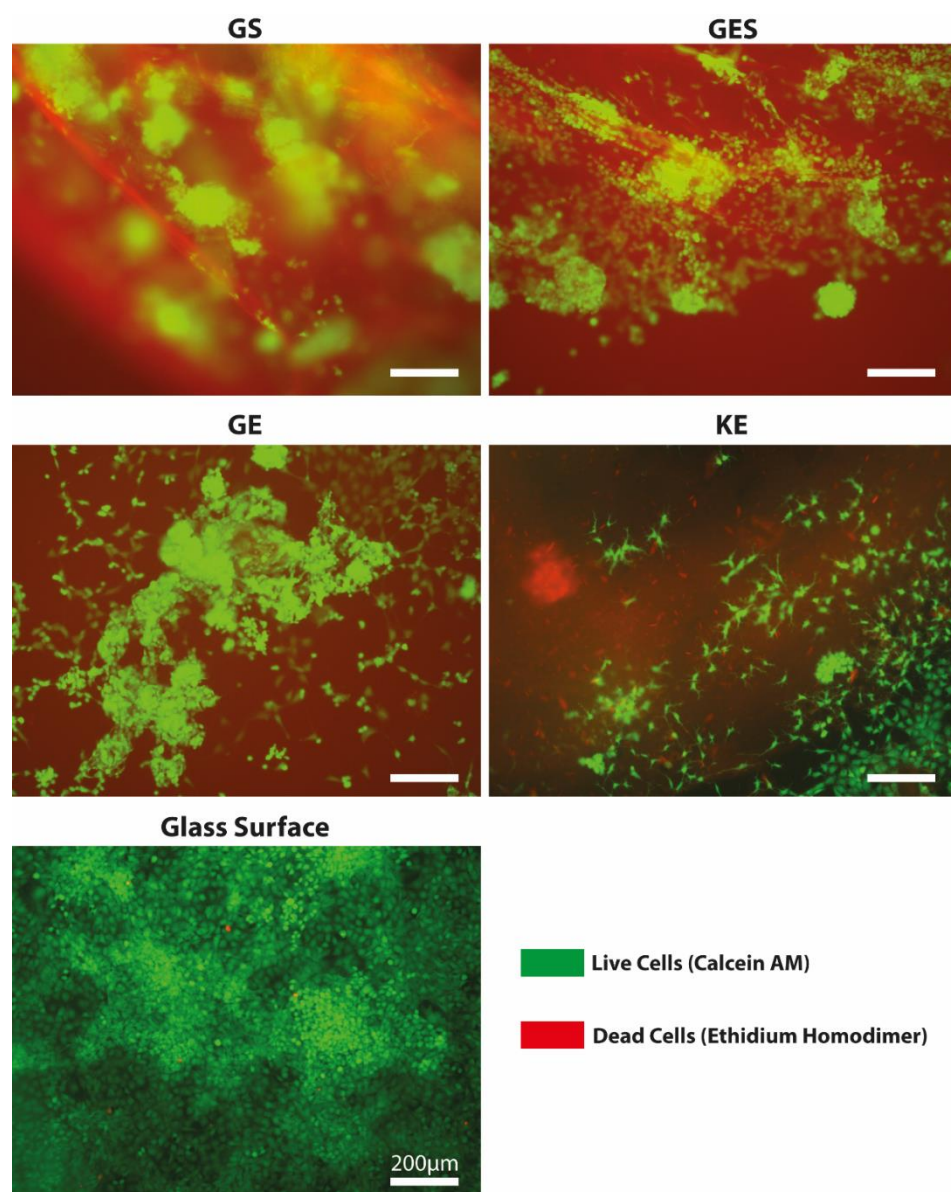


Figure 2.14 Viability of rMSCs inside the 3D gels of peptide amphiphile mixtures after one week of incubation. Except KE group, there were many cell aggregates in all other groups. Red dots represent nuclei of dead cells and green color shows live cells. Scale bars are 200 μm.

2.3.3.3. Proliferation of Cells in 3D Gel Environment

In order to observe whether the cells inside the gels were able to proliferate, we utilized AlamarBlue assay. This assay is exploited to show metabolic activity of the cells, which is assumed to correlate with cell numbers. The first indicator of the cell proliferation was that AlamarBlue reagent was reduced faster in each measurement time point and we stopped the experiment within a shorter time point (Fig. 2.15). When we assumed the reduction of the reagent was linear and calculated the reduction amount for 3 h of incubation (Fig. 2.15), the differences between the days were obvious. Except between D1 and D3 measurements of KE group, all groups had at least $p < 0.05$ value between days and the differences were significant.

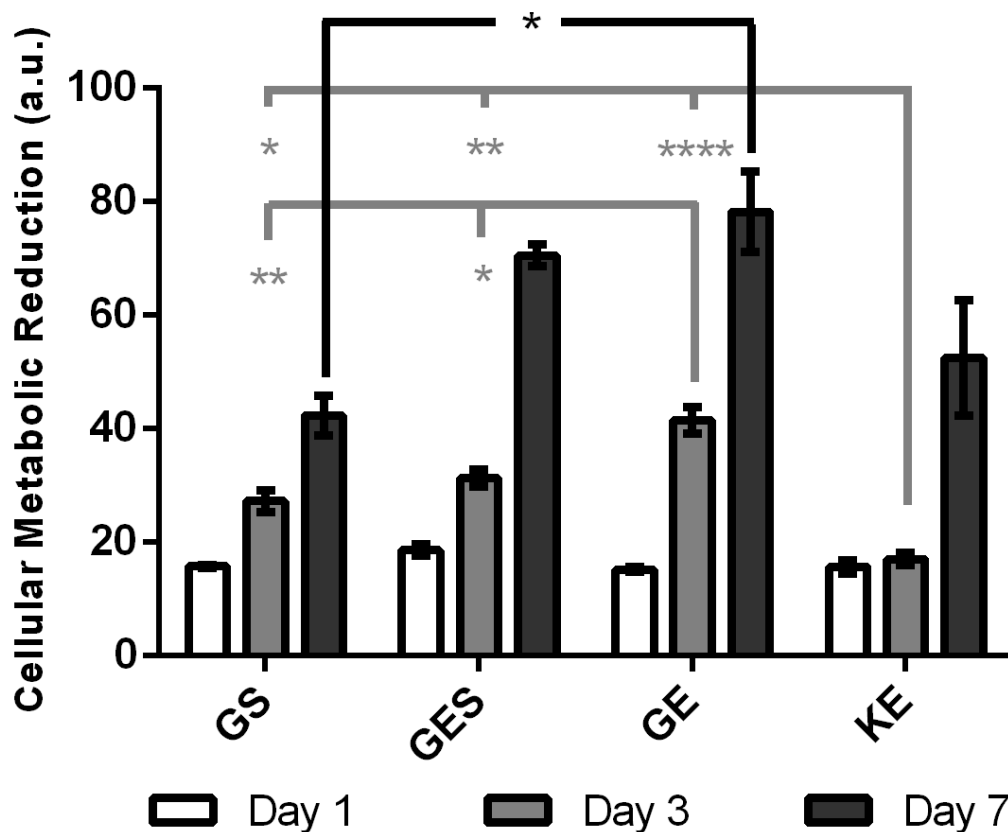


Figure 2.15 Proliferation of rMSCs inside the peptide amphiphile gel mixtures in 7 days via metabolic activity measurement (AlamarBlue).

2.3.3.4. Visualization of Cells inside Gels

Observing cells or even cell clusters within gels was not possible via regular microscopy (data not shown). Thus, we performed SEM in order to see cells inside the gels. Since scanning electron microscopy enables us to monitor only the surface of the samples, we disturbed some part of the gels with needle prior to the fixation procedure. Therefore, we were able to see cells that were embedded within gels, in addition to cells that began to invade and proliferate on the surface of the 3D gels. The following figures show SEM images of cells: GS (Fig. 2.16), GES (Fig. 2.17), GE (Fig. 2.18), KE (Fig. 2.19), and glass surface (Fig. 2.20). The samples were visualized after 21 days of incubation.

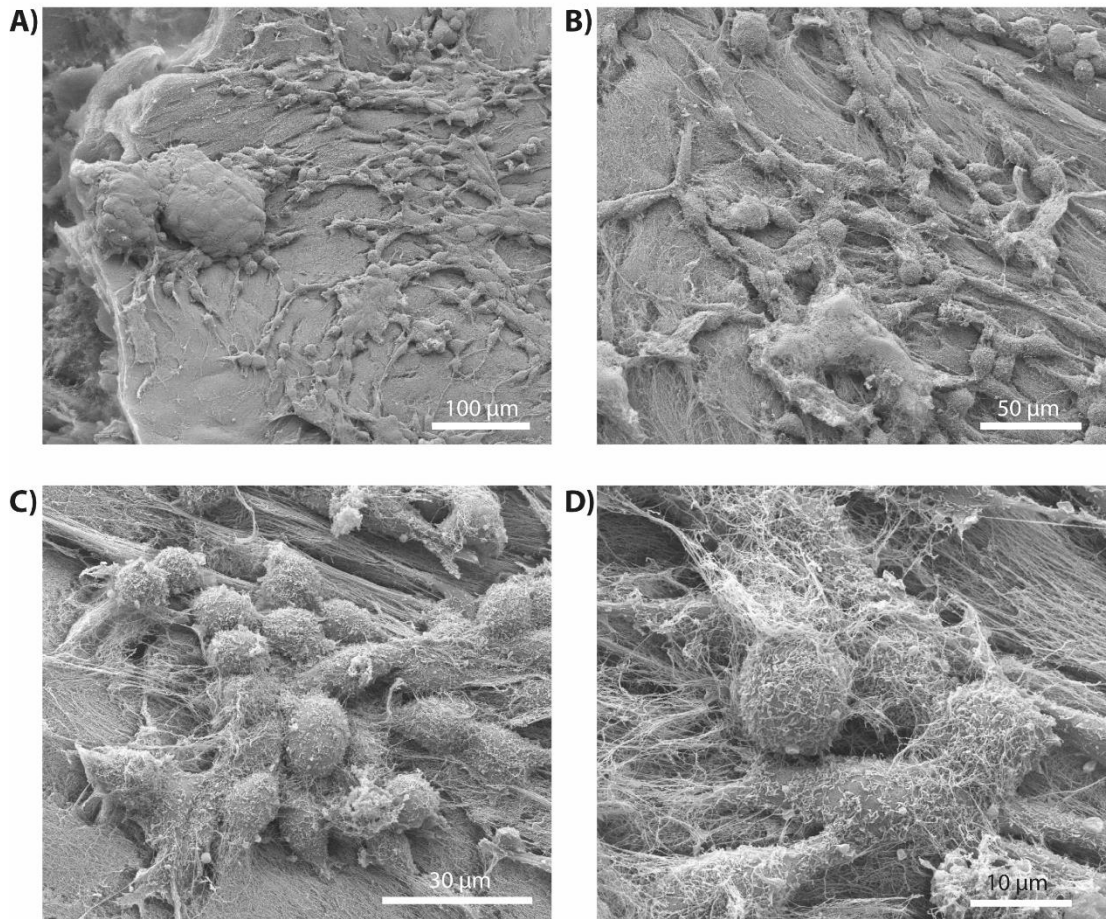


Figure 2.16 Scanning electron microscopy images of cells on and inside the GS gels after 21 days of incubation with different magnifications (A-D).

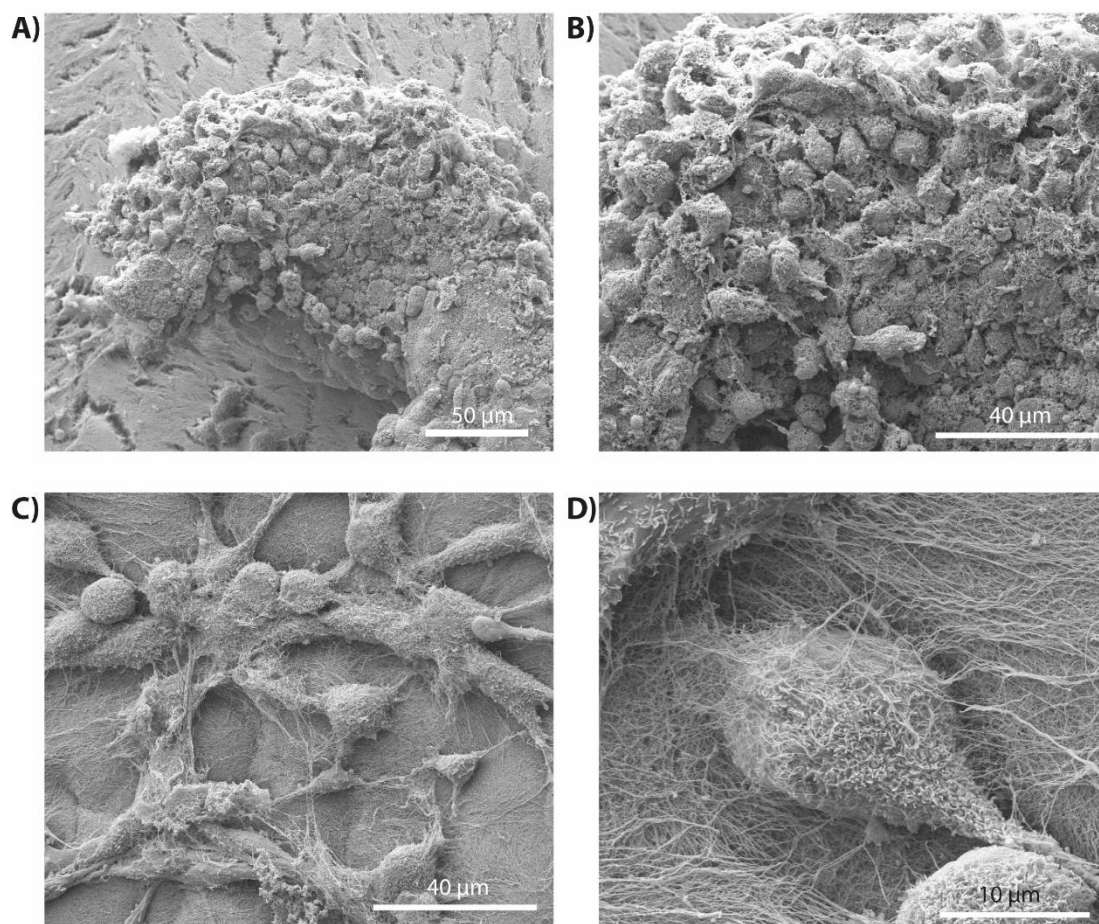


Figure 2.17 Scanning electron microscopy images of cells on and inside the GES gels after 21 days of incubation with different magnifications **(A-D)**.

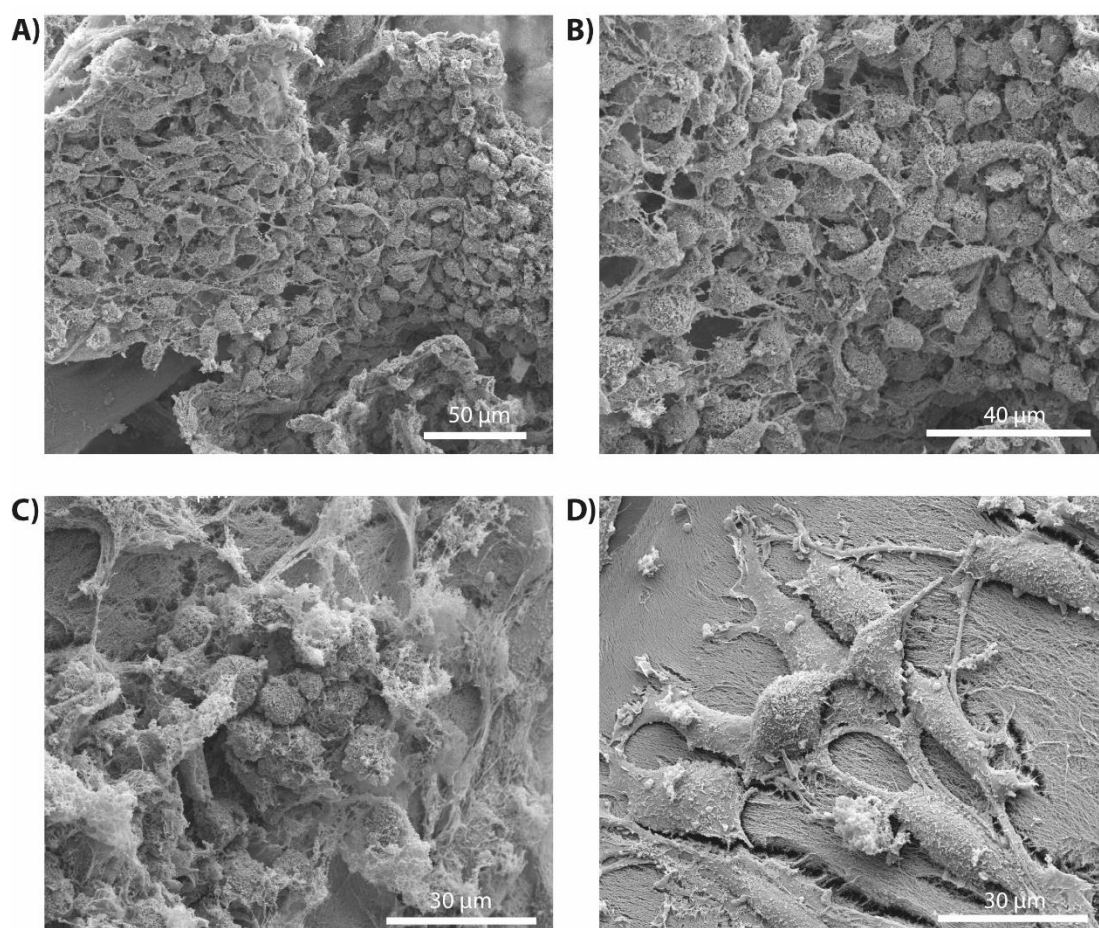


Figure 2.18 Scanning electron microscopy images of cells on and inside the GE gels after 21 days of incubation with different magnifications **(A-D)**.

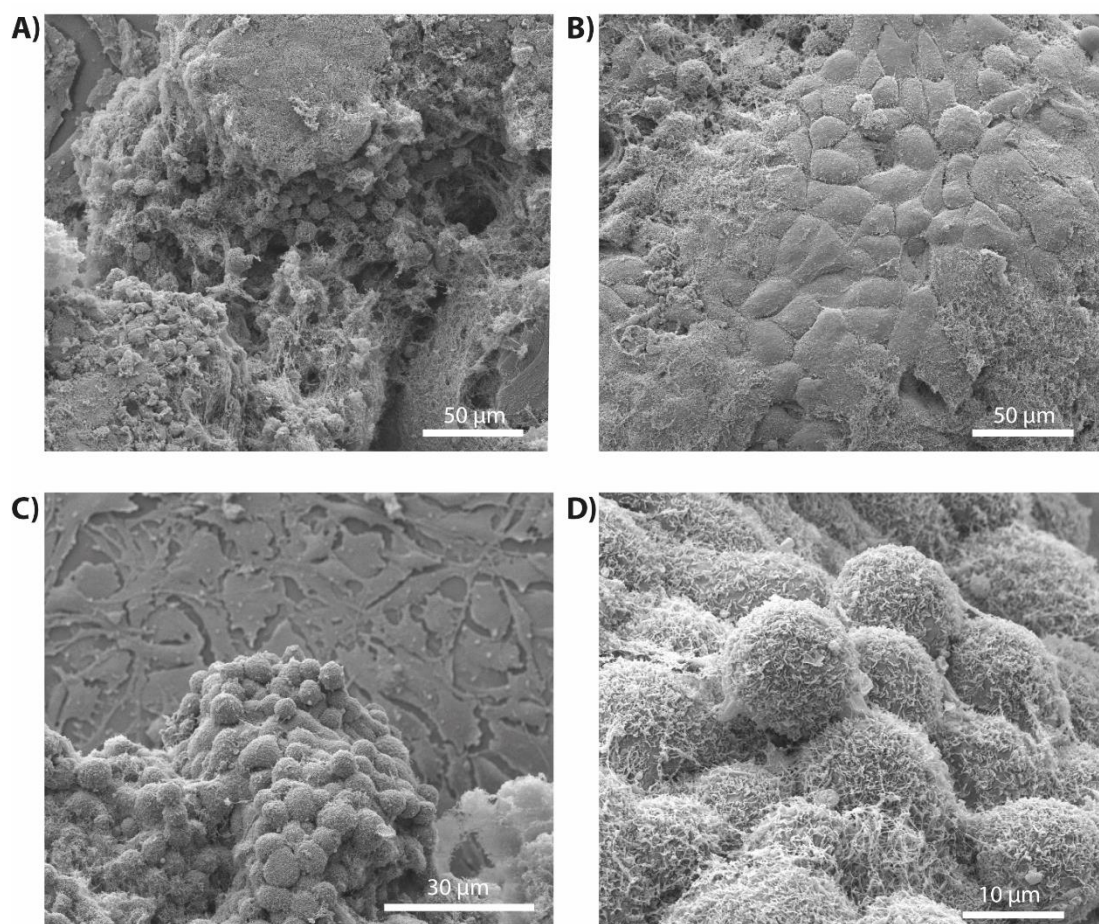


Figure 2.19 Scanning electron microscopy images of cells on and inside the KE gels after 21 days of incubation with different magnifications **(A-D)**.

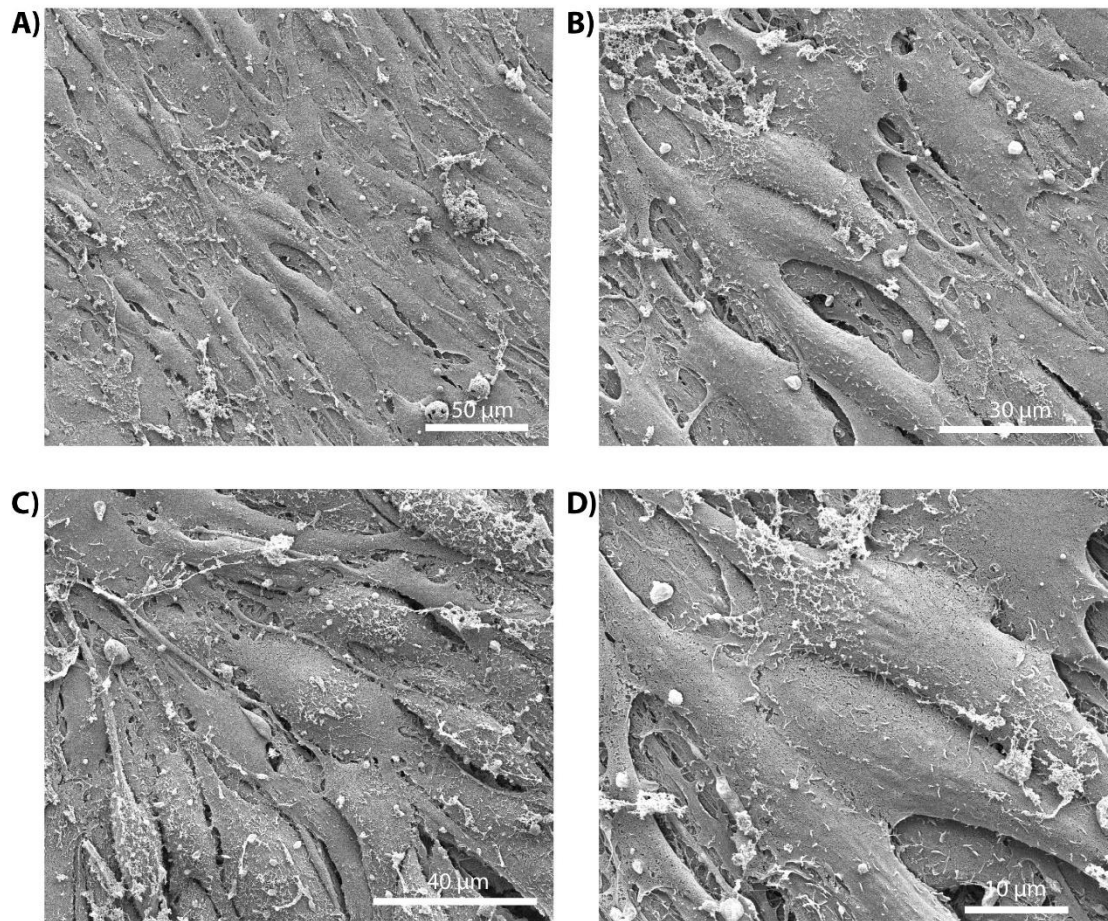


Figure 2.20 Scanning electron microscopy images of cells on glass surface after 21 days of incubation with different magnifications (A-D).

2.3.4. Effect of Peptide Amphiphiles on Cell Differentiation

Differentiation analyses of cells grown in a 3D environment are challenging due to the difficulties in using common staining procedures to detect differentiation cues. The scaffold tends to absorb the dyes, proteins, enzymes and chemicals, which results in false positive or negative results. Therefore, we tried to avoid staining techniques in 3D experiments and instead utilized gene expression analyses and/or immunostaining methods after sectioning the gels. All experimental groups were compared between themselves and to the gene expression levels of rat mesenchymal stem cells harvested at day 0 without exposure to peptide hydrogels (flask culture group). These analyses were carried out for each incubation day and specific gene among the corresponding days and gene, not between days or genes. The asterisks on the graphics of gene expression analyses only show difference between flask culture and experimental groups to avoid the crowding of symbols to indicate significant differences between each group. For staining, gels were cryosectioned after embedding in O.C.T. and checked to determine whether cells could be observed with histological and fluorescence staining techniques. In Fig. 2.21, individual cells and aggregates were stained with fast green, which is darker inside the cells and lighter on the peptide hydrogel. Cell nuclei were stained blackish blue with hematoxylin (Fig. 2.21A). Moreover, sectioned samples were stained by using phalloidin, a fluorescent dye attaching to actin filaments, in order to show that cells could also be visualized by fluorescence (Fig. 2.21B).

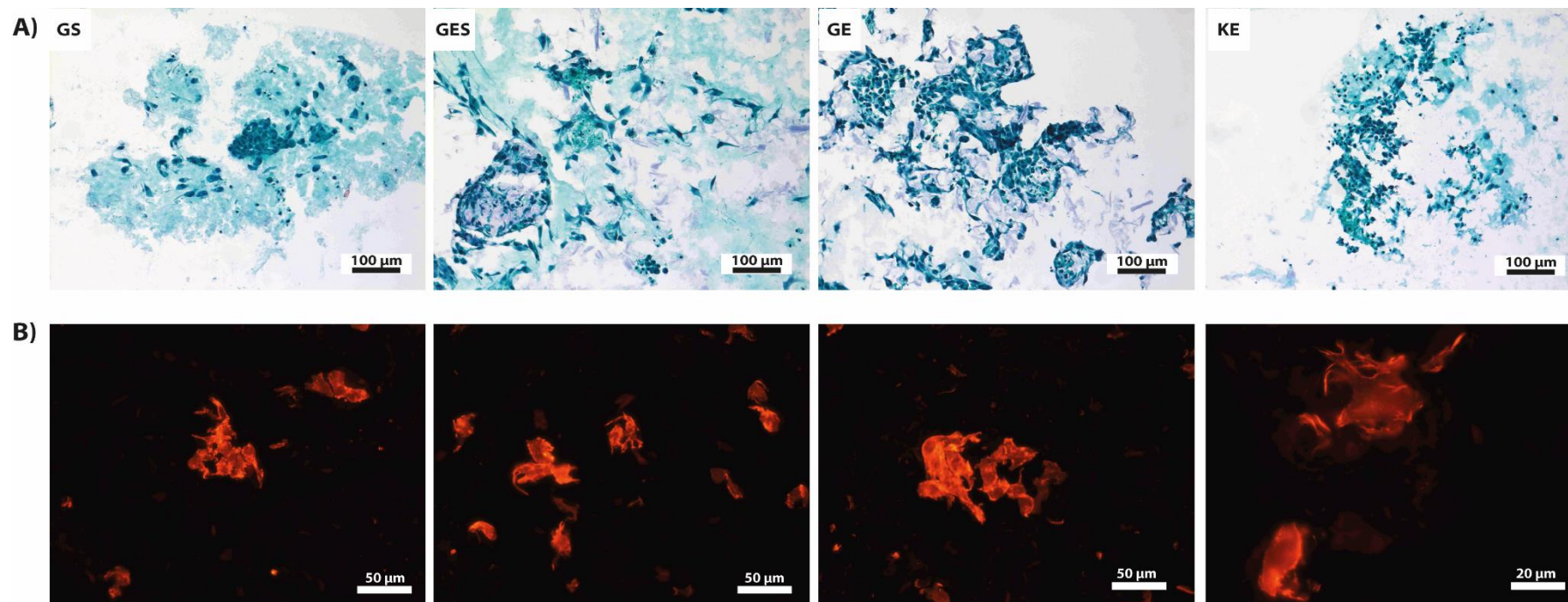


Figure 2.21 Cell morphologies inside the gels. **(A)** Cryosectioned slides were stained with histological dyes, hematoxylin and fast green, and imaged under microscope. Scale bars are 100 μm . **(B)** Phalloidin stained cells after cryosectioning were visualized by fluorescence microscopy.

2.3.4.1. Cell Differentiation on 2D Peptide Amphiphile Coatings

In order to pre-examine the differentiation pattern of rat mesenchymal stem cells, 2D experiments were employed to cells seeded onto PA coatings, which had the same content with the 3D systems. Common staining procedures, including Alizarin Red S, Safranin O and Oil Red O dyes, were utilized to observe cues of osteogenesis, chondrogenesis and adipogenesis, respectively. There was no distinct red color of Alizarin Red S, which results from the binding of the reagent to deposited calcium (Fig. 2.22). The pinkish color of Safranin O staining was a sign of sulfated glycosaminoglycan synthesis; however, the coatings without cells on them also absorbed Safranin O and exhibited some pink coloration (Fig. 2.23). Therefore, we were unsure about the specificity of the staining. Representative images of Oil Red O staining displayed more intense oil droplets in the KE and GS groups, and less positive staining was observed when sulfonate concentration gradually diminished (Fig. 2.22).

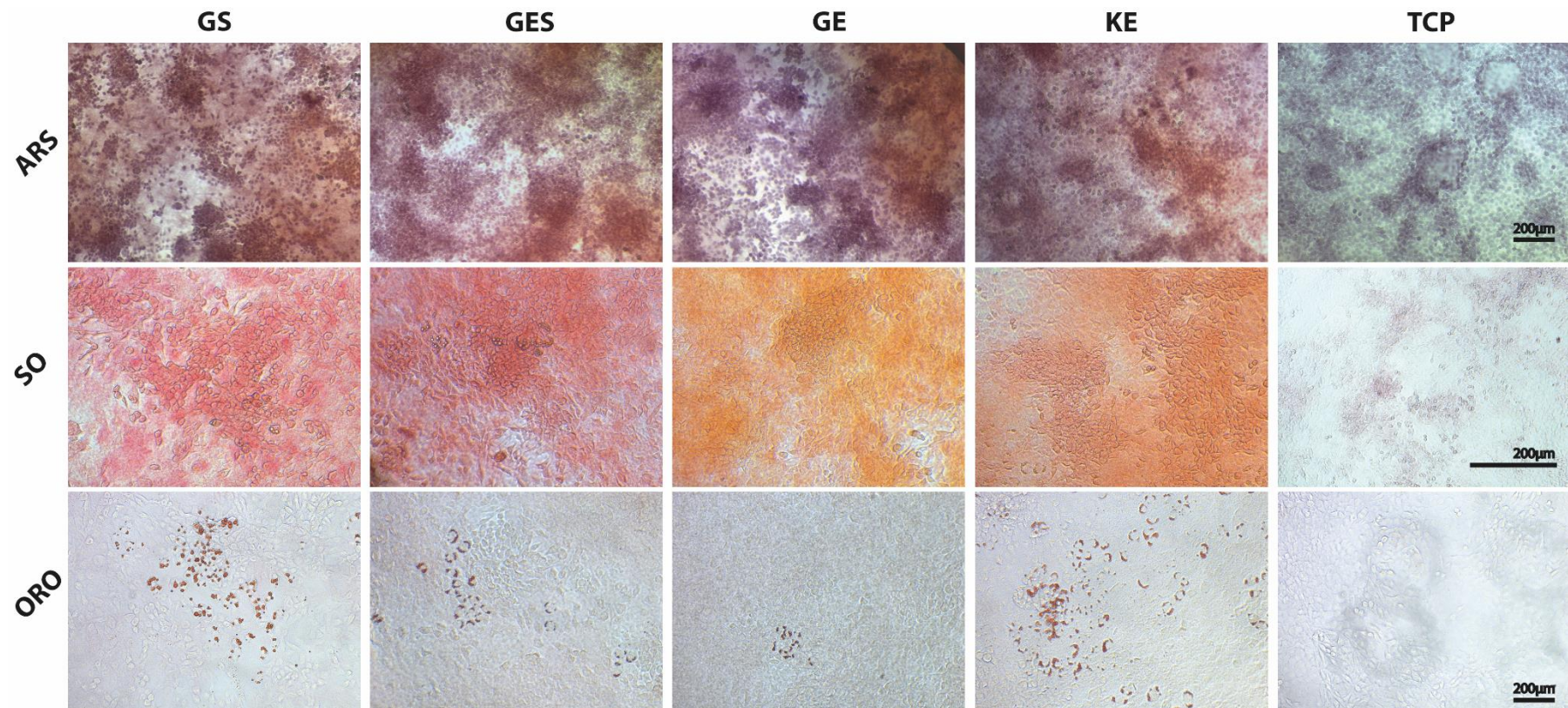


Figure 2.22 Rat MSC differentiation pattern on 2D peptide amphiphile coatings. All groups were stained with Alizarin Red S, Safranin O and Oil Red O after seven days of incubation. Pictures on the same row were imaged with the same magnification and scale bars are 200 μm .

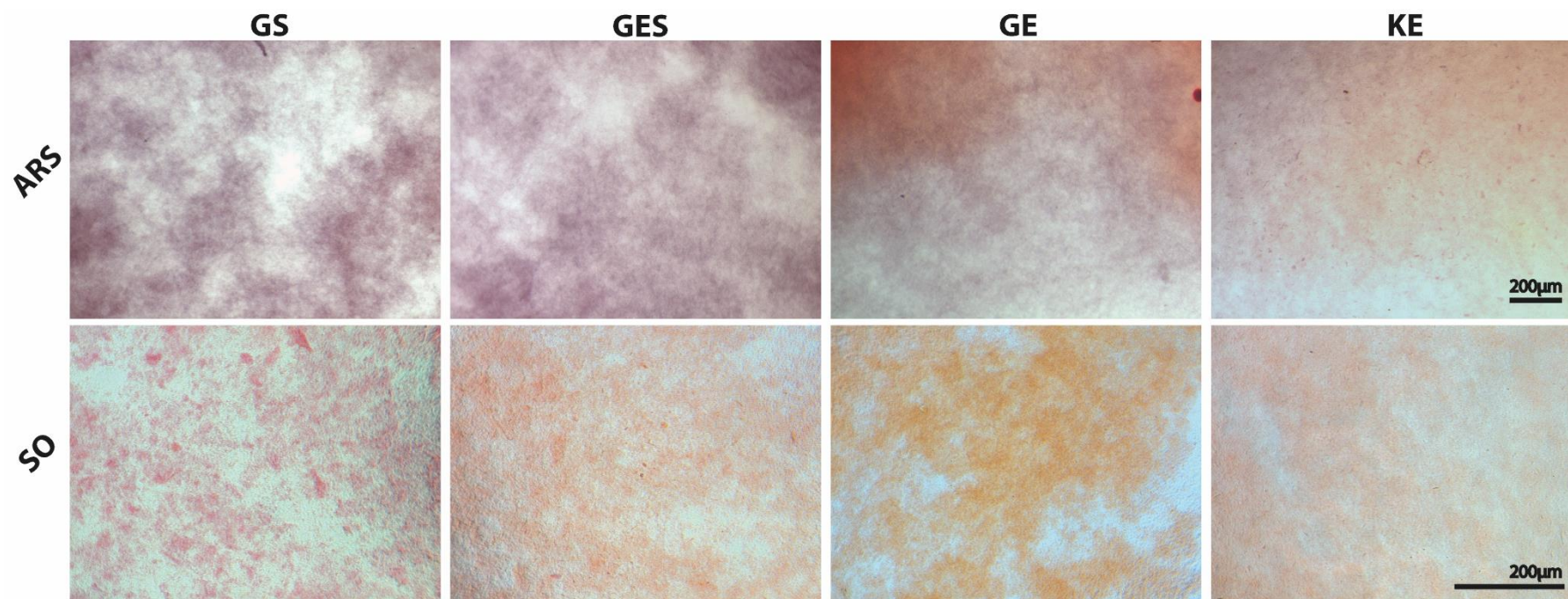


Figure 2.23 Stainings of empty (without cells) coatings. Alizarin Red S and Safranin O stainings of 2D peptide amphiphile coatings revealed that dyes can bind to PA scaffolds. Pictures on the same row were imaged with the same magnification and scale bars are 200 μm.

2.3.4.2. Determining Differentiation of Cells Encapsulated in 3D Gel System to Osteogenic Lineage

2.3.4.2.1. Alkaline Phosphatase Activity

High alkaline phosphatase (ALP) activity is a clue that cells are differentiating through osteogenic differentiation. Therefore, we employed alkaline phosphatase activity assay to cells incubated for one, two and three weeks inside gels and on glass surface as a control group (Fig. 2.24). A significant difference ($p < 0.05$) was observed between the ALP activities of KE and glass surface groups on day 14, but there were no significant differences between other groups or days, although an increase was observed on the day 14 for the ALP activity of each gel group.

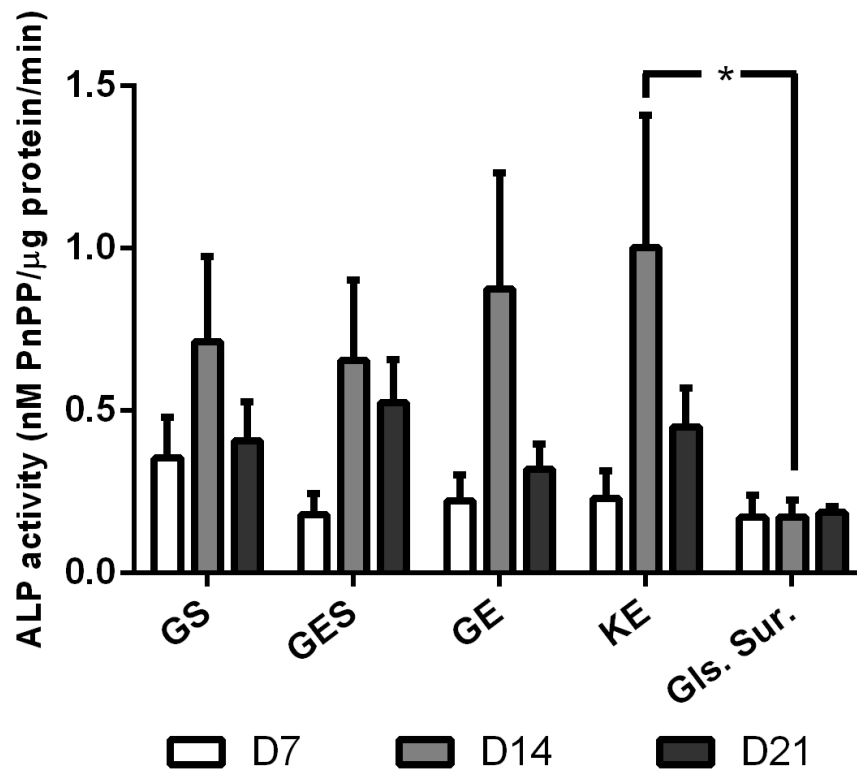


Figure 2.24 Alkaline phosphatase (ALP) activity of the cells inside gels and on glass surface after 7, 14 and 21 days of incubation.

2.3.4.2.2. Gene Expression analyses

In order to monitor osteogenic differentiation pattern, we looked at the expression levels of two genes, *Runx2* and *Col1a1*, which are commonly checked to confirm osteogenic differentiation. Expression of *Runx2* gene was significantly higher ($p<0.01$) in the GES group than the GS and glass surface on day 3 (Table 2.4). On the day 21, all experimental groups' *Runx2* expression was diminished when compared to flask culture RNA expression ($p<0.01$, Table 2.4).

Collagen 1 gene expression was always lower in experimental groups than the flask culture group. The significance of this difference was between $p<0.05$ and $p<0.0001$ among groups when compared to flask culture gene expression on day 3 (Table 2.4). However, this difference became more drastic as the incubation time increased (Table 2.4, Fig. 2.25 and Fig. 2.26).

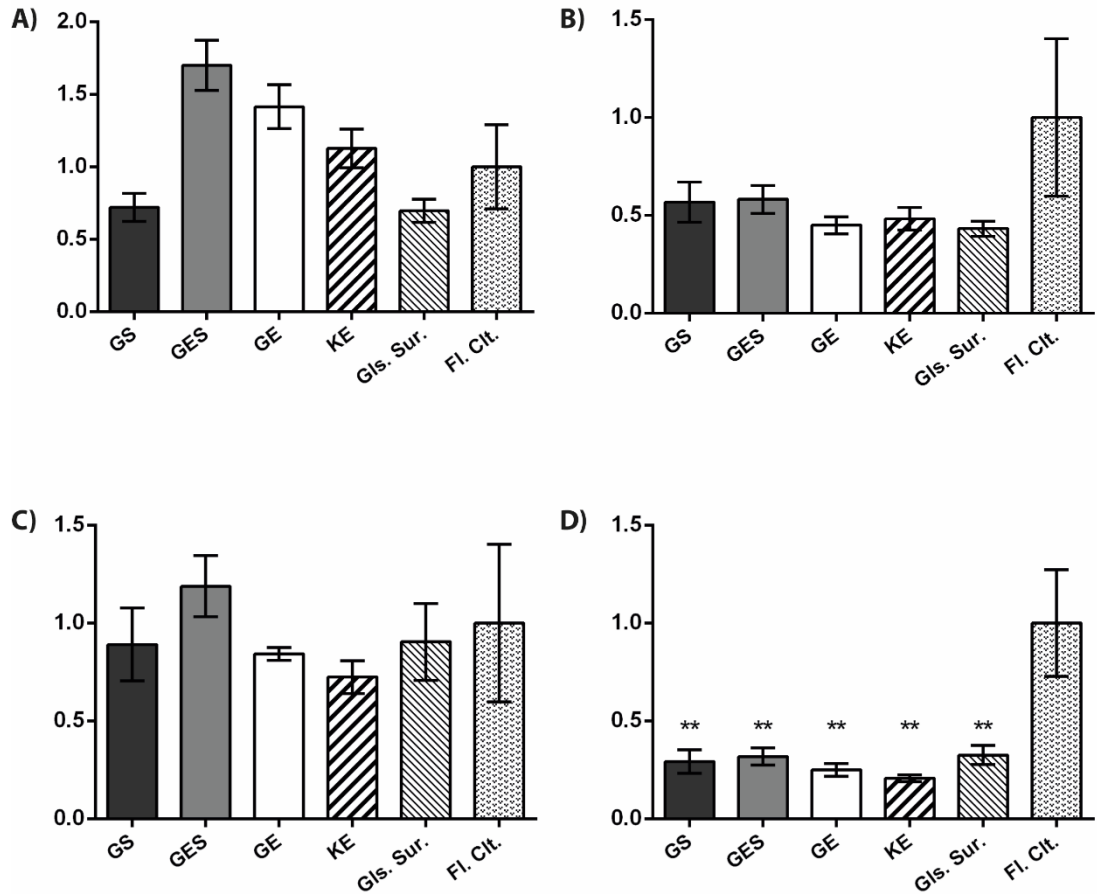


Figure 2.25 Differentiation patterns of rMSCs into osteogenic lineage. Expression levels of *Runx2* on day 3 (**A**), day 7 (**B**), day 14 (**C**), and day 21 (**D**). Expressions are normalized to GAPDH and scaled based on flask culture group. Each group was compared to other groups within the same day, and indicated as asterisk in the figure if there is a significant difference with flask culture group ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)). The comparison results of each group can be found in Table 2.4.

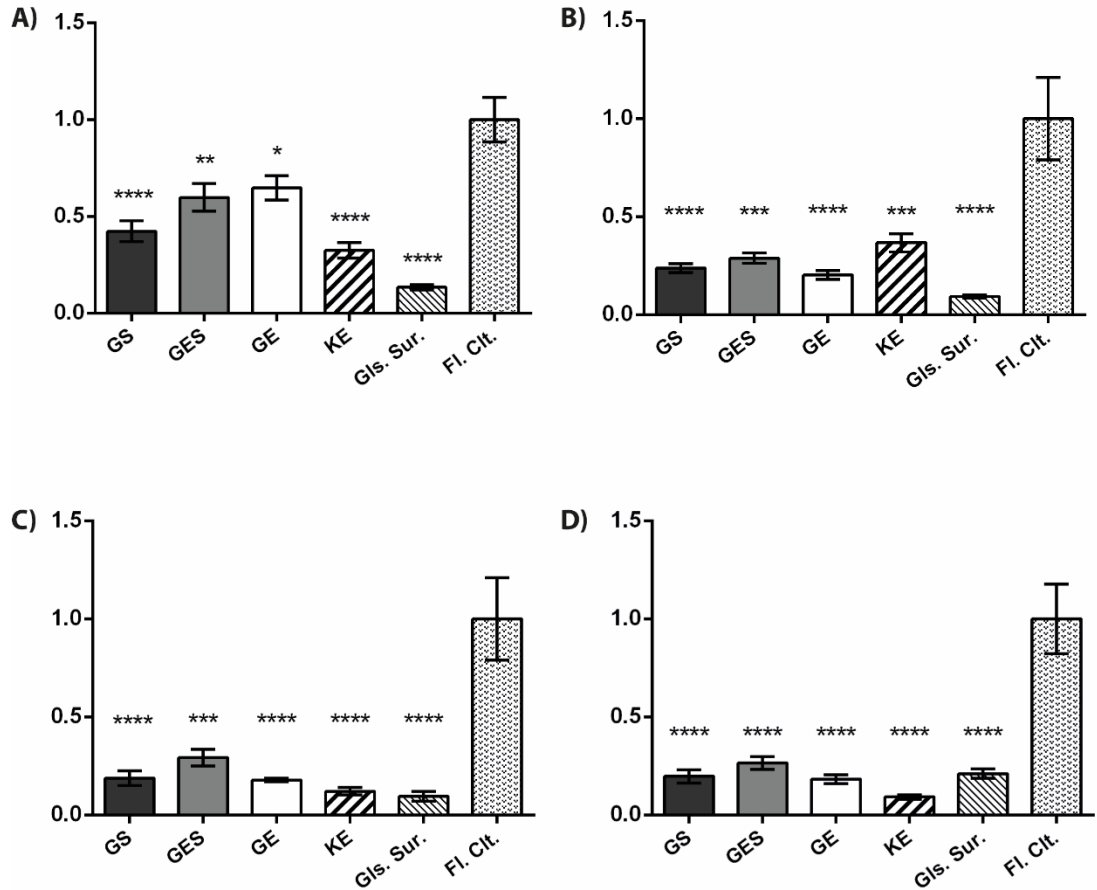


Figure 2.26 Differentiation patterns of rMSCs into osteogenic lineage. Expression levels of *Col1a1* on day 3 (A), day 7 (B), day 14 (C), and day 21 (D). Expressions are normalized to GAPDH and scaled based on flask culture group. Each group was compared to other groups within the same day, and indicated as asterisk in the figure if there is significant difference with flask culture group ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)). The comparison results of each group can be found in Table 2.4.

	GS over GES	GS over GE	GS over KE	GS over Gls. Sur.	GS over Fl. Clt.	GES over GE	GES over KE	GES over Gls. Sur.	GES over Fl. Clt.	GE over KE	GE over Gls. Sur.	GE over Fl. Clt.	KE over Gls. Sur.	KE over Fl. Clt.	Gls. Sur. over Fl. Clt.
<i>Runx2</i>															
D3	**							**							
D7															
D14															
D21					**				**			**		**	**
<i>Col1a1</i>															
D3	****							***	**	*	***	*		****	****
D7					****				***			****		***	****
D14					****				***			****			****
D21					****				****			****			****

Table 2.4 Degree of significant difference of osteogenic gene expressions between groups within different days. Brown and green colors represent significant increase and decrease, respectively, in groups over compared groups indicated upper row after one-way ANOVA analysis.

2.3.4.3. Determining Differentiation of Cells Encapsulated in 3D Gel System to Adipogenic Lineage

Adipogenic differentiation was examined by measuring the expression levels of *Adipoq* and *Fabp4* genes. On day 3, the expression of *Adipoq* appeared to be high in groups containing glucose residue and that was even more obvious for gels bearing the sulfonate group, when compared to glass surface and flask culture groups (Fig. 2.27, Table 2.5). On days 7 and 14, the expression of *Adipoq* was drastically increased in KE groups (with at least $p < 0.05$ significant difference compared to other groups). Its expression was also high in groups containing Glc-PA, but less than the KE group. On those days, the effect of sulfonate group was also observable, but the difference between this group and other groups was only significant on day 7 (Fig. 2.27). On day 21, expression levels decreased, but it could still be observed that Glc-PA mixed with sulfonate groups were expressing high amounts of *Adipoq* mRNA (at least $p < 0.05$, Table 2.5).

Fabp4 mRNA level was always higher in the gel groups compared to flask culture group up to day 21 (Fig. 28). We observed significant increase of the expression in the gel groups with at least $p < 0.05$ on day 3 (Table 2.5). In general, the similar pattern of expression of *Adipoq* was followed by *Fabp4* gene expression. KE group expressed high amounts of *Fabp4* on day 7 and 14 compared to other groups, except on day 14, where it was not significantly different than GS group. The decrease on the gene expression was prominent on day 21 with p values lower than 0.001 (Table 2.5, Fig. 2.28).

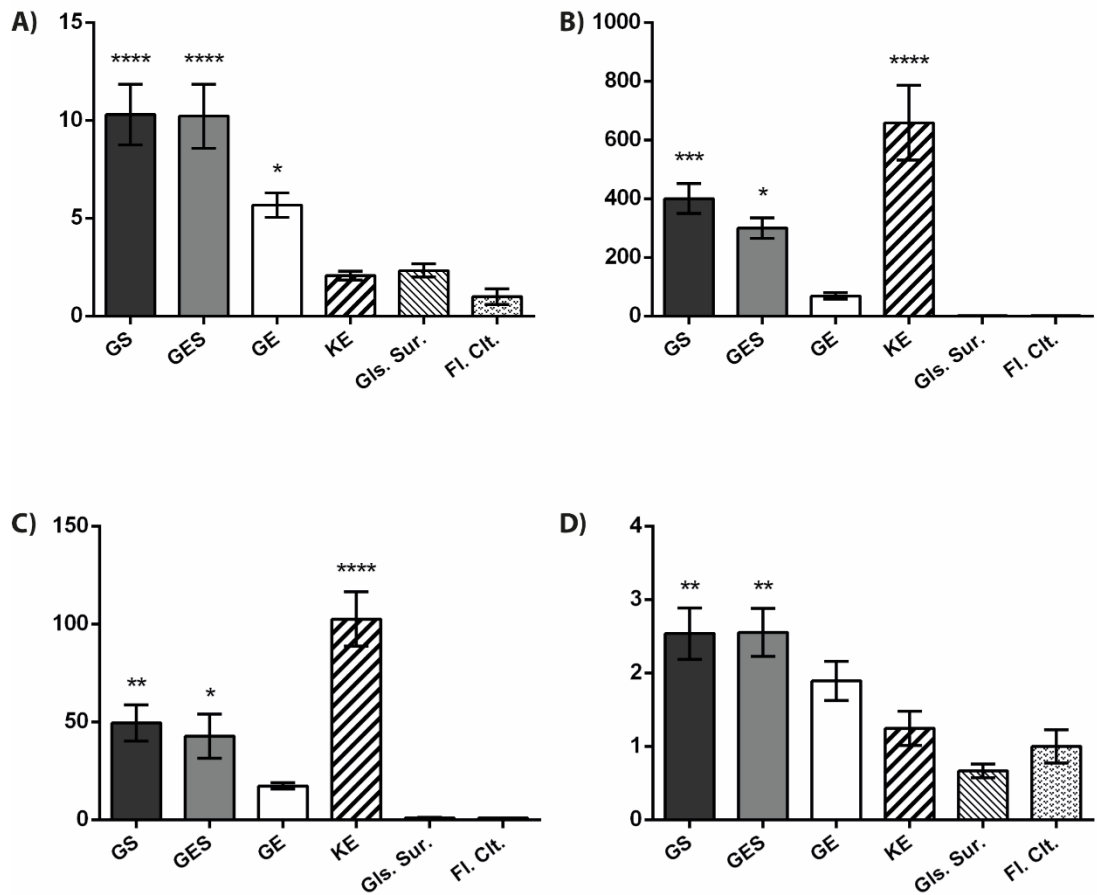


Figure 2.27 Differentiation patterns of rMSCs into the adipogenic lineage. Expression levels of *Adipoq* on day 3 (**A**), day 7 (**B**), day 14 (**C**), and day 21 (**D**). Expressions were normalized to GAPDH and scaled based on flask culture group. Each group was compared to other groups within the same day, and indicated as asterisk in the figure if there is significant difference with flask culture group ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)). The comparison results of each group can be found in Table 2.5.

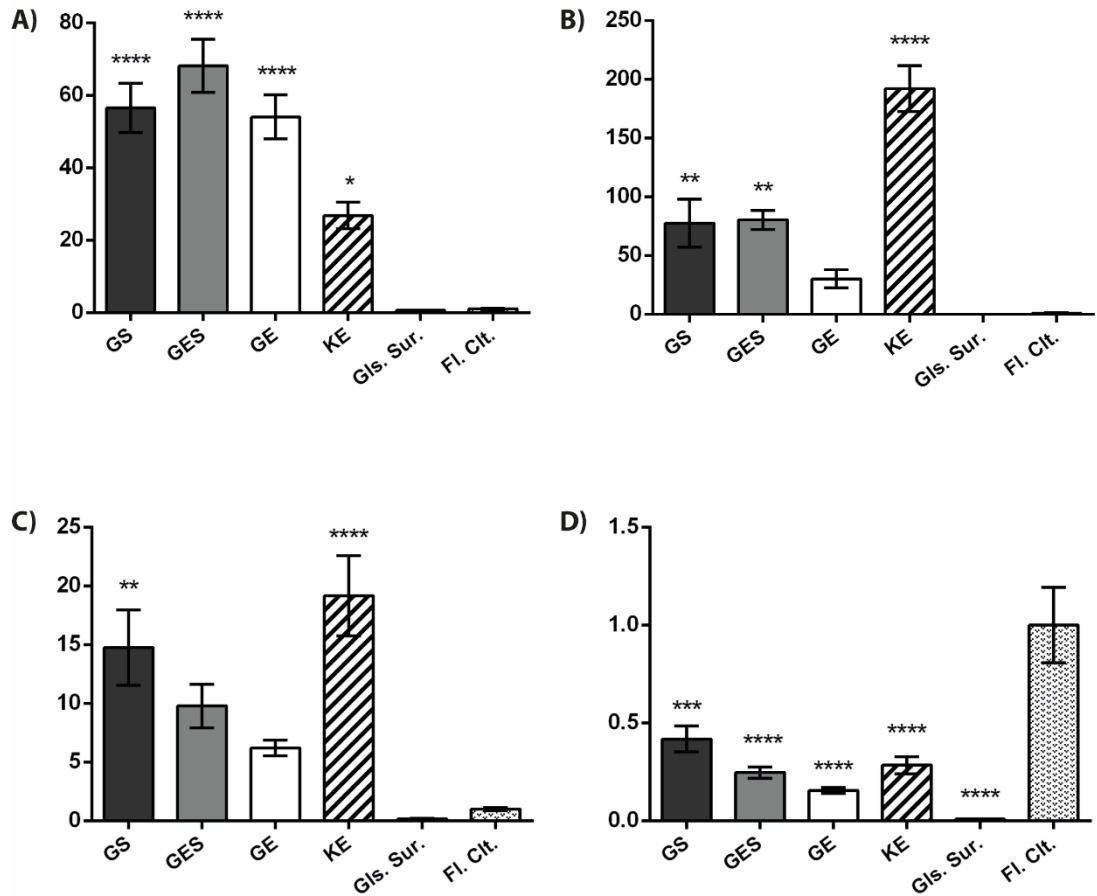


Figure 2.28 Differentiation patterns of rMSCs into the adipogenic lineage. Expression levels of *Fabp4* on day 3 (**A**), day 7 (**B**), day 14 (**C**), and day 21 (**D**). Expression levels were normalized to GAPDH and scaled based on flask culture group. Each group was compared to other groups within the same day, and indicated as asterisk in the figure if there is significant difference with flask culture group [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)]. The comparison results of each group can be found in Table 2.5.

	GS over GES	GS over GE	GS over KE	GS over Gls. Sur.	GS over Fl. Clt.	GES over GE	GES over KE	GES over Gls. Sur.	GES over Fl. Clt.	GE over KE	GE over Gls. Sur.	GE over Fl. Clt.	KE over Gls. Sur.	KE over Fl. Clt.	Gls. Sur. over Fl. Clt.
<i>Adipoq</i>															
D3		*	***	***	***	*	***	***	***			**			
D7		**	*	***	***		*	*	*	***			***	***	
D14			*	*	*		*	*	*	***			***	***	
D21			*	***	***		*	***	***		*				
<i>Fabp4</i>															
D3			*	***	***		***	***	***	*	***	***	*	*	
D7			***	*	*		***	*	*	***			***	***	
D14				***	*		*	*		*			***	***	
D21				*	***				***			***		***	***

Table 2.5 Degree of significant difference of adipogenic gene expressions between groups within different days. Brown and green colors represent significant increase and decrease, respectively, in groups over compared groups indicated upper row after one-way ANOVA analysis.

2.3.4.4. Determining Differentiation of Cells Encapsulated in 3D Gel System to Chondrogenic Lineage

2.3.4.4.1. Gene Expression Analyses

The genes *Sox9*, *Acan* and *Col2a1*, which play roles in cartilage tissue formation and maintenance, were examined for chondrogenic differentiation monitoring. We could observe effect of gels on days 3 and 7, with the general increase of *Sox9* expression (Fig. 2.29, Table 2.6). GE group was significantly different than both glass surface and flask culture groups ($p < 0.001$, Table 2.6) on day 3. Also, GS group had increased *Sox9* expression on day 3, with a p value of less than 0.05. We obtained a statistically meaningful difference on day 7 between GS and flask culture groups. On day 14, the expression of *Sox9* was elevated on Glc-PA containing groups compared to other groups, with p values below 0.05 (Table 2.6, Fig. 2.29). However, on day 21, *Sox9* expression profile changed and was lower compared to earlier days (Fig. 2.29).

Aggrecan (*Acan*) expression was interestingly higher in glass surface groups on days 3 and 7 (Table. 2.6). On day 3, its expression was higher in the glass surface group than the GS and flask culture groups; while the glass surface group expressed the highest amount of aggrecan on day 7. When the day 14 samples were examined, gel groups revealed increased aggrecan expression (Fig. 2.30) and sulfonate amount apparently had a positive effect on the expression of aggrecan. GS group was significantly different than all groups (at least $p < 0.05$), and GES group showed the same trend for groups which did not contain Glc-PA (Table 2.6). Expression of *Acan* was elevated when compared to the initial mRNA expression (flask culture) profile (Table 2.6). The day 21 samples had GS groups with higher *Acan* expression over experimental groups, but not over flask culture group. However, other groups were not significantly different than each other (Table 2.6).

Collagen 2 expressions on different days were similar to *Col 1* gene expression results with an exception on days 7 and 14 (Fig. 2.31). On days 3 and 21, all groups showed diminished gene expression; on the other hand, on days 7 and 14, the expressions were close to flask culture group. GE displayed lower expression (Table 2.6) on day 7, and KE and glass surface groups did so on day 14 (Table 2.6).

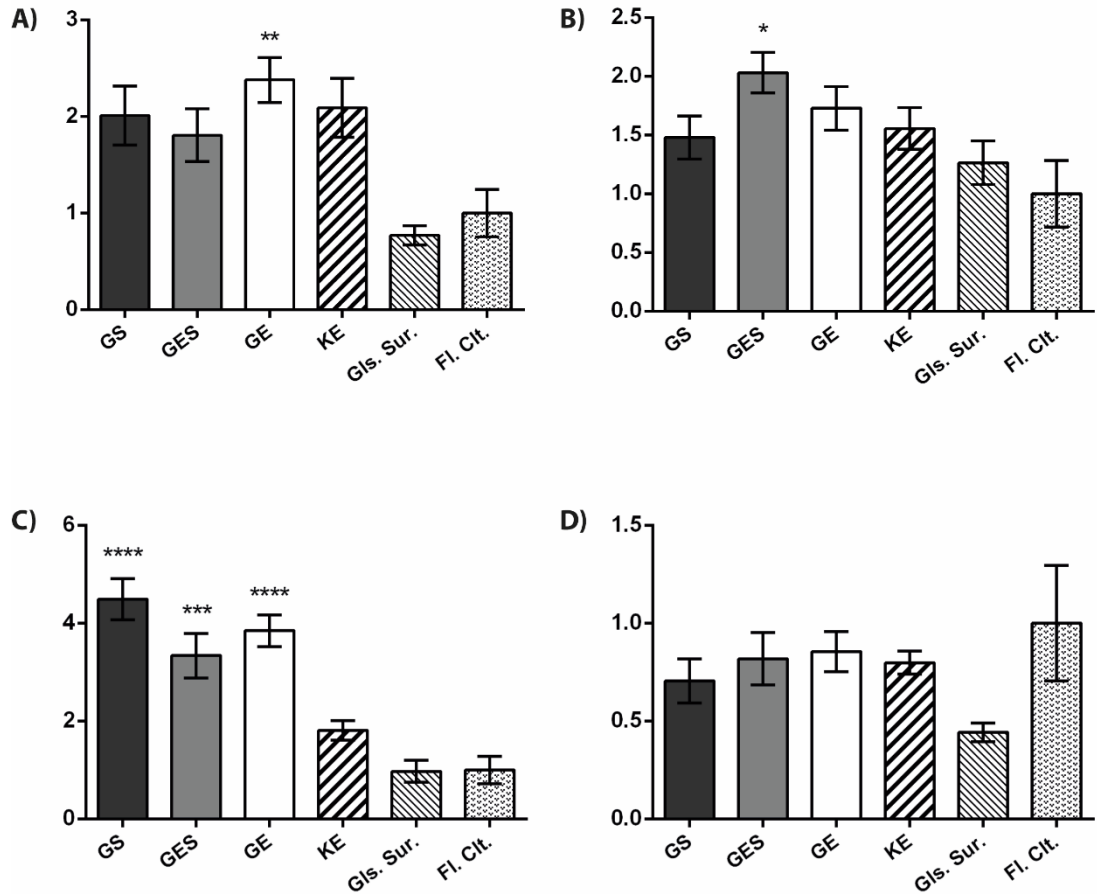


Figure 2.29 Differentiation patterns of rMSCs into the chondrogenic lineage. Expression levels of Sox9 on day 3 (**A**), day 7 (**B**), day 14 (**C**), and day 21 (**D**). Expressions are normalized to GAPDH and scaled based on flask culture group. Each group was compared to other groups within the same day, and indicated as asterisk in the figure if there is significant difference with flask culture group [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)]. The comparison results of each group can be found in Table 2.6.

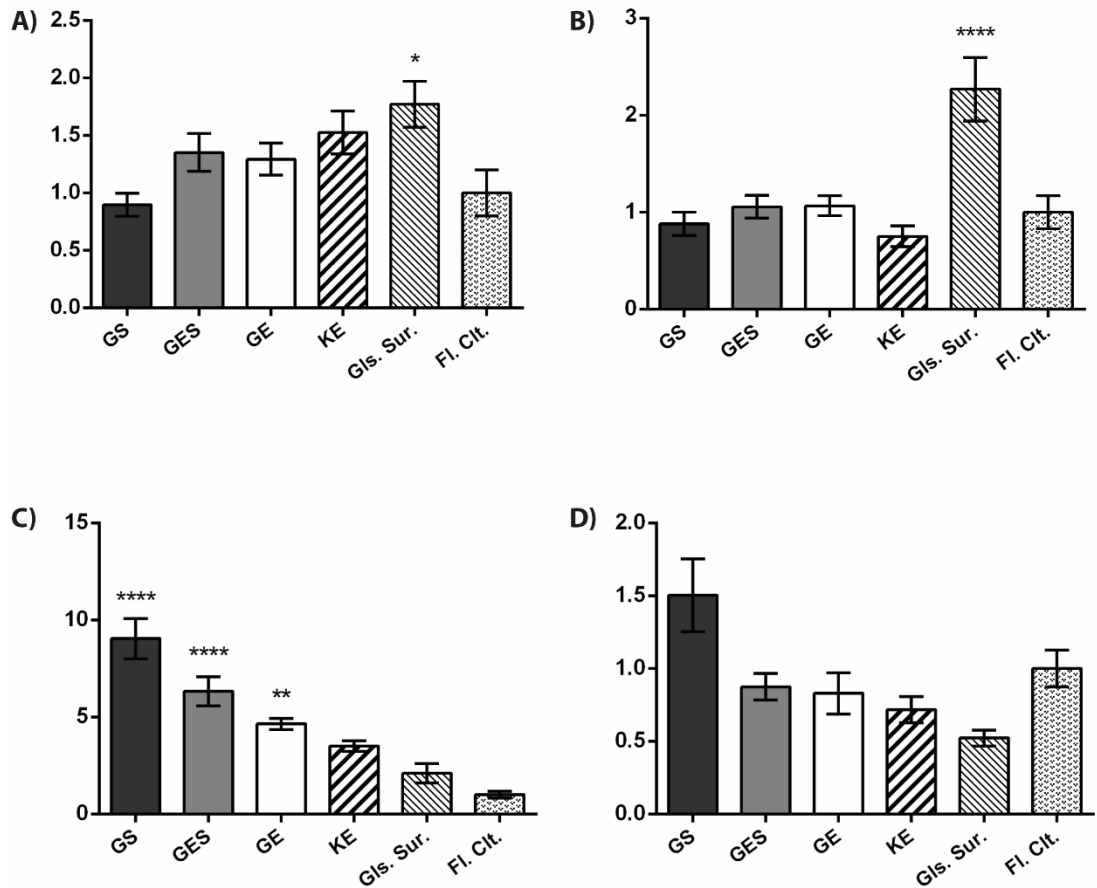


Figure 2.30 Differentiation patterns of rMSCs into the chondrogenic lineage. Expression levels of *Acan* on day 3 (**A**), day 7 (**B**), day 14 (**C**), and day 21 (**D**). Expressions are normalized to GAPDH and scaled based on flask culture group. Each group was compared to other groups within the same day, and indicated as asterisk in the figure if there is significant difference with flask culture group [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)]. The comparison results of each group can be found in Table 2.6.

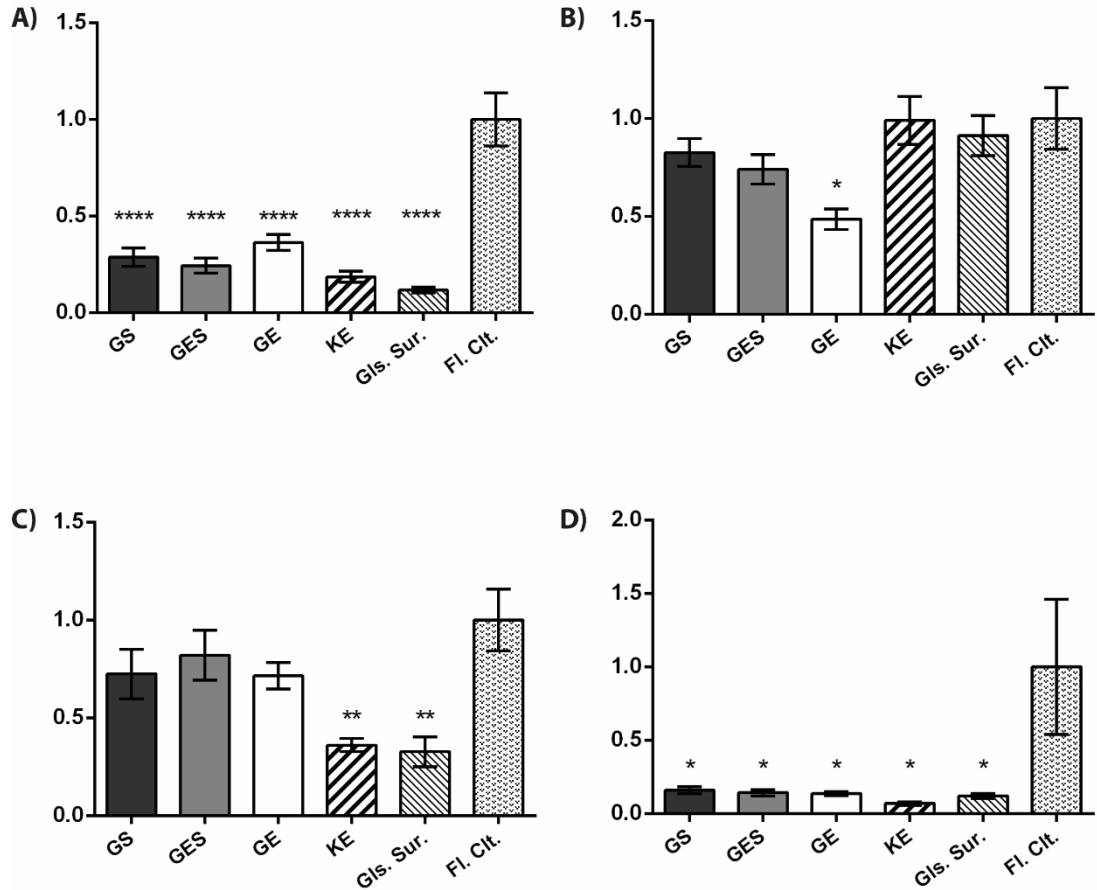


Figure 2.31 Differentiation patterns of rMSCs into the chondrogenic lineage. Expression levels of *Col2a1* on day 3 (**A**), day 7 (**B**), day 14 (**C**), and day 21 (**D**). Expressions are normalized to GAPDH and scaled based on flask culture group. Each group was compared to other groups within the same day, and indicated as asterisk in the figure if there is significant difference with flask culture group [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)]. The comparison results of each group can be found in Table 2.6.

	GS over GES	GS over GE	GS over KE	GS over Gls. Sur.	GS over Fl. Clt.	GES over GE	GES over KE	GES over Gls. Sur.	GES over Fl. Clt.	GE over KE	GE over Gls. Sur.	GE over Fl. Clt.	KE over Gls. Sur.	KE over Fl. Clt.	Gls. Sur. over Fl. Clt.
<i>Sox9</i>															
D3				*							**	**	*		
D7									*						
D14			****	****	****		*	***	***	**	****	****			
D21															
<i>Acan</i>															
D3															*
D7				****				***			****		***		****
D14	*	**	****	****	****		*	***	****			**			
D21	*	*	**	***											
<i>Col 2</i>															
D3					****				****			****		****	****
D7										*		*			
D14								*						**	**
D21					*				*			*		*	*

Table 2.6 Degree of significant difference of chondrogenic gene expressions between groups within different days. Brown and green colors represent significant increase and decrease, respectively, in groups over compared groups indicated upper row after one-way ANOVA analysis.

2.3.4.4.2. Immunohistochemical Staining

Aggrecan was stained by using immunoperoxidation. Firstly, specificities of the primary and secondary antibodies were tested by using rat cartilage tissue sections. As seen in the Fig. 2.32, anti-aggrecan antibody specifically bound to chondrocytes and their surroundings, and secondary antibody did not bind nonspecifically to the tissue and instead bound strongly to the primary antibody. Then, we employed a similar immunohistochemical staining procedure on cryosectioned samples. Figures 2.33, 2.34, 2.35, and 2.36 suggest that all groups contain at least some aggrecan-expressing cells. In order to quantify the cells expressing aggrecan, whole images of gels were taken under microscope and the dark brown cells were counted. The amount of stained cells was then divided by the area of gel to calculate the number of aggrecan-expressing cells per millimeter square (Fig. 37). When the means of each group were compared, the group containing glucose and sulfonate functional groups was found to bear more aggrecan expressing cells, the density of which was significantly higher than the KE group.

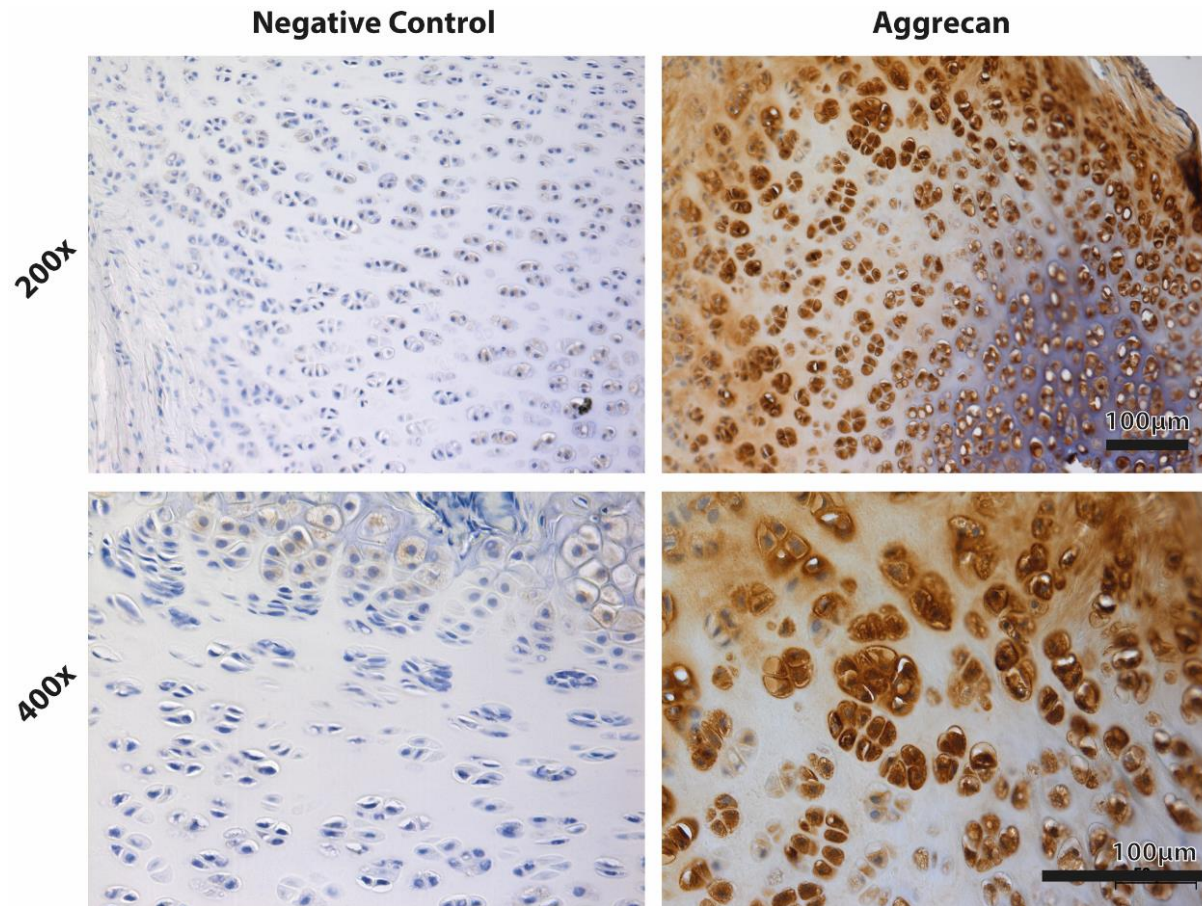


Figure 2.32 Immunohistochemical staining of cryosectioned rat cartilage tissue with anti-aggrecan antibody. Without the antibody, there is no staining (negative control). Brown coloration shows the presence of aggrecan (around chondrocytes). Images in the same lines are taken with the same magnification and scale bars are 100 μm .

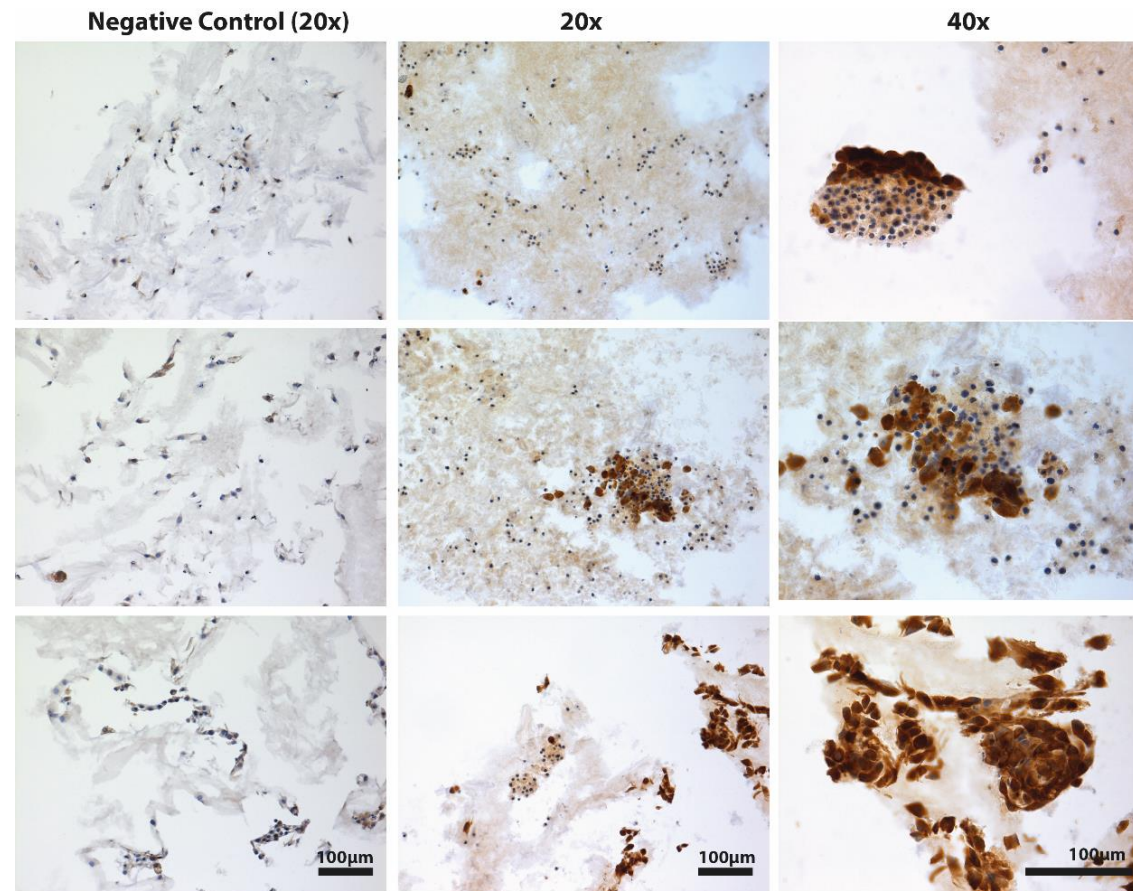


Figure 2.33 Immunohistochemical staining of cryosectioned GS gels with anti-aggrecan antibody. Without the antibody, there was no staining (negative control). Brown coloration shows the presence of aggrecan. Images in the same column are taken with the same magnification and scale bars are 100 µm.

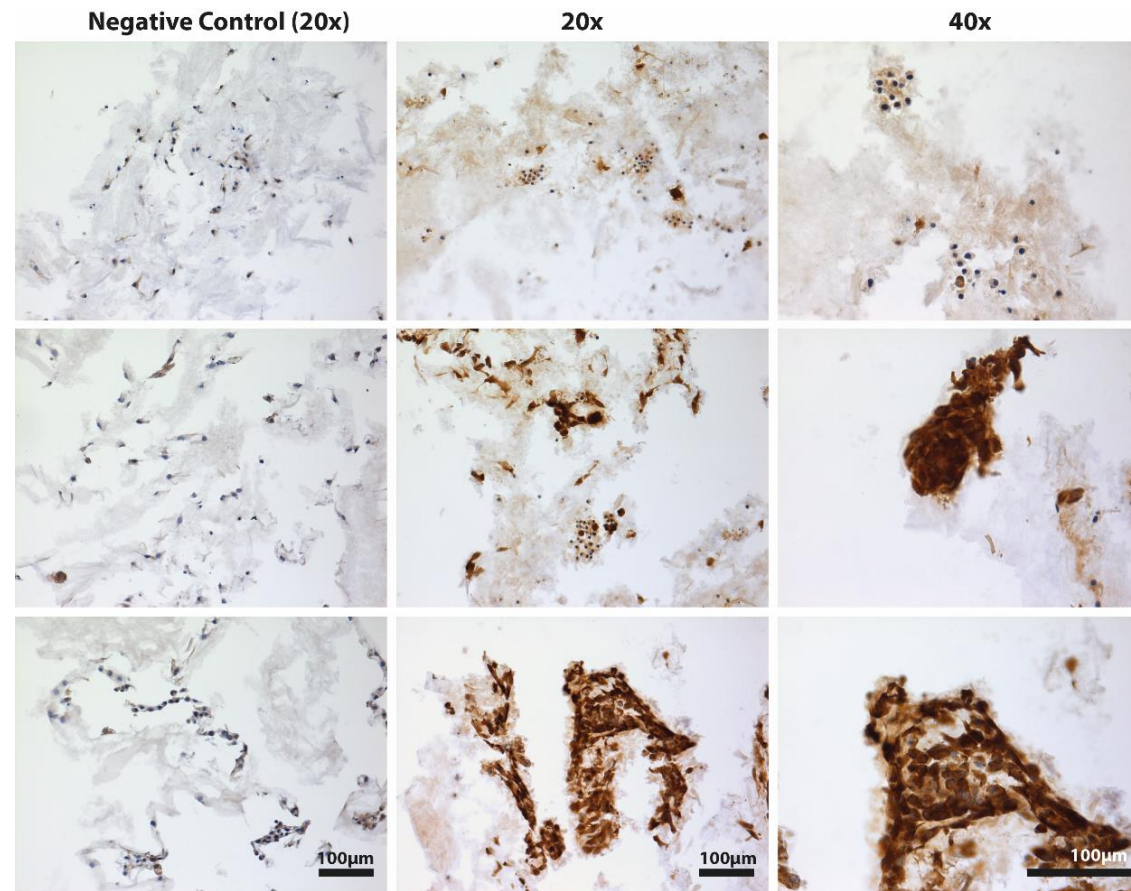


Figure 2.34 Immunohistochemical staining of cryosectioned GES gels with anti-aggrecan antibody. Without the antibody, there was no staining (negative control). Brown coloration shows the presence of aggrecan. Images in the same column are taken with the same magnification and scale bars are 100 µm.

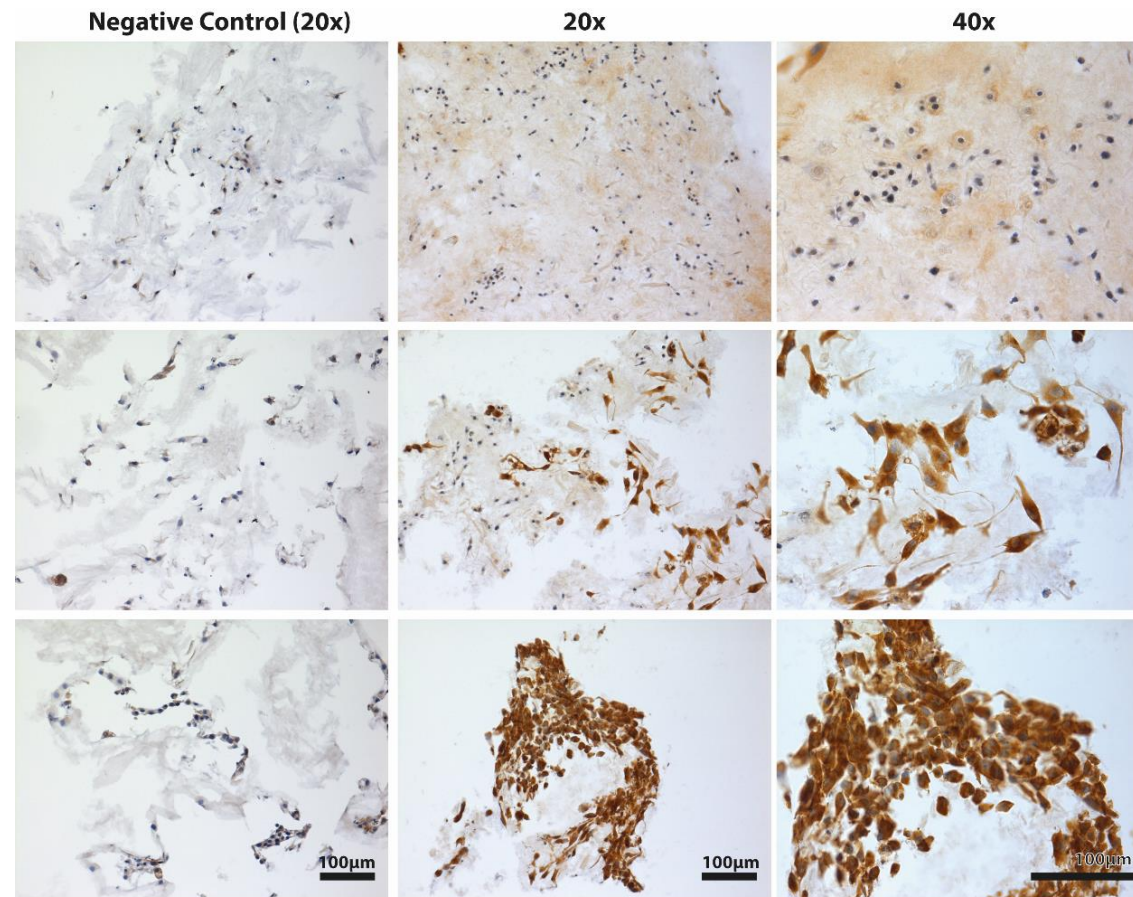


Figure 2.35 Immunohistochemical staining of cryosectioned GE gels with anti-aggrecan antibody. Without the antibody, there was no staining (negative control). Brown coloration shows the presence of aggrecan. Images in the same column are taken with the same magnification and scale bars are 100 µm.

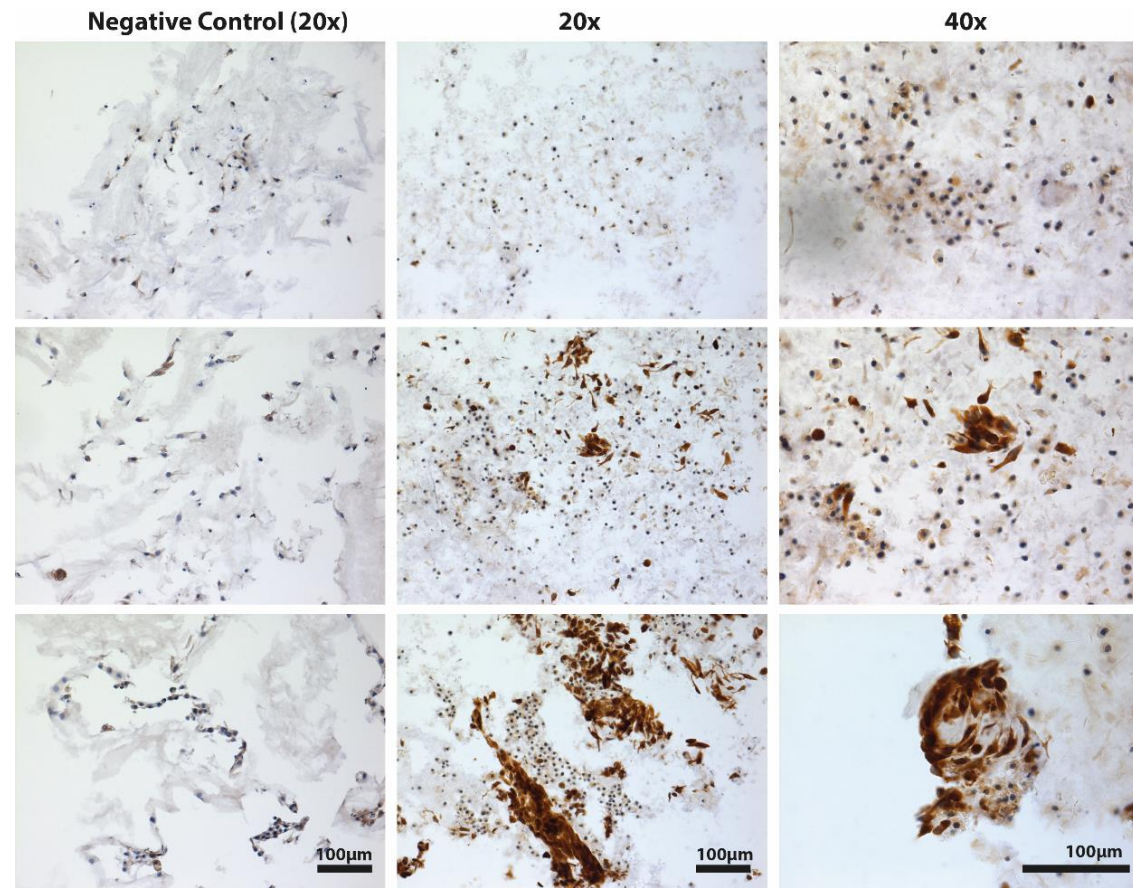


Figure 2.36 Immunohistochemical staining of cryosectioned KE gels with anti-aggrecan antibody. Without the antibody, there was no staining (negative control). Brown coloration shows the presence of aggrecan. Images in the same column are taken with the same magnification and scale bars are 100 µm.

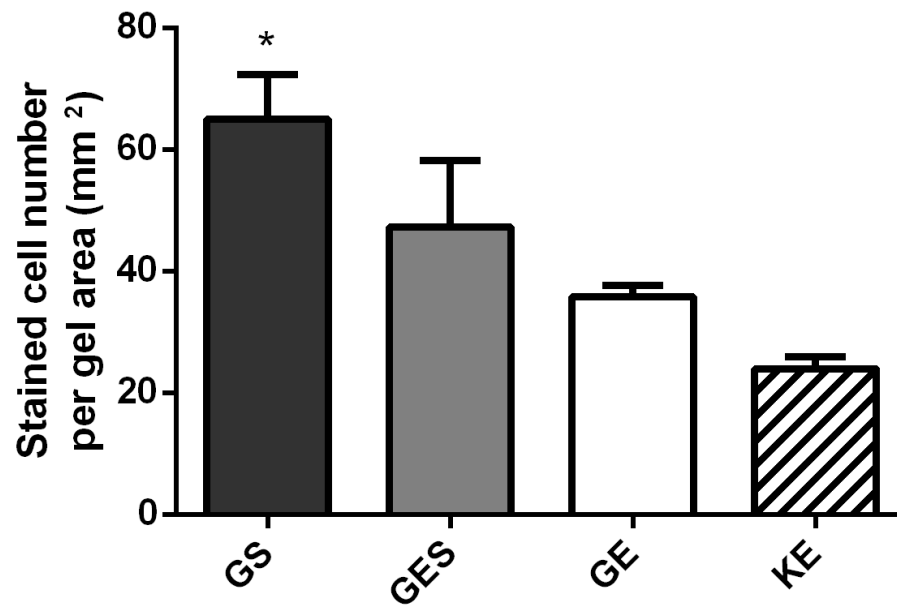


Figure 2.37 Quantification of positively stained cells with aggrecan antibody per gel area (mm²). Sulfonate amount gradually increased the cells expressing aggrecan.

2.4. DISCUSSION

Capable of self-assembling into fibrous structures through intermolecular interactions, peptide amphiphiles are powerful molecules for tissue engineering efforts due to their biocompatible and biodegradable nature, as well as their capacity for incorporating small, bioactive functional units into their structure. The alkyl chain attached to the protein unit experiences hydrophobic collapse in water to form micelle-like structures such as nanofibers if the fatty acids are combined with β -sheet forming amino acid sequences. These amino acids are aligned with the help of hydrogen bonding, creating a well-ordered structure. This architecture can be utilized to mimic the natural microenvironment of cells by adding bioactive protein epitopes to the peptide sequence. In this study, we used this property of peptide amphiphiles to design an ECM-mimetic environment through the self-assembly of supramolecular structures resembling glycosaminoglycans found in natural tissues. Glycosaminoglycans are composed of repeating disaccharide units with different chemical groups, the function of which can be altered by modulating their rate of sulfation. As such, we designed a PA molecule decorated by glucose residues (Glc-PA), and mixed it with another peptide molecule which contains sulfonate group (SO_3 -PA) to produce a structure analogous to a sulfated glycosaminoglycan chain. We decreased the concentration of sulfonate groups in the mixture by replacing SO_3 -PA with a PA lacking the sulfonate group (E-PA). Since the self-assembly of the PA molecules was designed to occur by mixing two oppositely charged molecules, we synthesized a glucose-bearing molecule with a positive amino acid (Lys) and other peptides with negatively charged glutamic acids. In addition, SO_3 -PA already has an intrinsic negative charge from its sulfonate moiety. In order to see the effect of the glucose residue attached to the PA molecule, we also used K-PA as a control that is structurally similar to Glc-PA but does not contain the glucose residue. Since the experimental design of our system is based on a 3D hydrogel system, we also incorporated a PA molecule containing an integrin binding sequence, RGD, which is known to trigger the adhesion of many cell types, including mesenchymal stem cells [246]. It is also a negatively charged peptide similar to E-PA, and has been used as to compose 10% of the total peptide concentration. Thus the experimental groups **GS** (50% Glc-PA, 40% SO_3 -PA, 10% RGD-PA), **GES** (50% Glc-PA, 20% SO_3 -PA,

20% E-PA, 10% RGD-PA), **GE** (50% Glc-PA, 40% E-PA, 10% RGD-PA) and **KE** (50% K-PA, 40% E-PA, 10% RGD-PA) were formed along with a control group in which rMSCs were incubated on the surface of glass coverslips. Molecular structures of the designed peptide amphiphiles and their mixtures can be found in Fig. 2.1 and Table 2.3, respectively.

We produced PA molecules by solid phase peptide synthesis method with the expected molecular weights, which was a confirmation of their successful production (Mass spectrums, Fig. 2.3-2.6). All the negatively charged PAs and Glc-PA were purified to eliminate the residues of their synthesis, whereas the dialysis of K-PA was enough for its purification (Elution graphics, Fig. 2.3-2.6). After the purification step, PAs were solubilized as described for characterizations and 2D and 3D *in vitro* experiments. 3D gels were formed by mixing 8 mM of each PA solution to form the gels shown in Fig. 2.7. Obtained hydrogels were strong enough to last for three weeks under *in vitro* conditions. It has been already known that these gels are formed by the factors mentioned above, and we confirmed that with circular dichroism analysis (Fig. 2.8). Charge neutralization-dependent gel formation was also supported by the secondary structure of β -sheets that form the nanofibers. A positive peak at around 200 nm and a negative peak at around 220 nm in the CD spectrum are signs of β -sheet formation in peptides containing L-amino acids [247]. We then investigated gel characteristics of the PA mixtures. All groups except KE had similar stiffnesses (Fig. 2.9). Since Glc-PA, an ingredient of some gel groups, had glucose unit on the outer surface, the hydroxyl groups flanking the molecule might provide strength to the supramolecular structure via hydrogen bonding. The formed gels were also analyzed by electron microscopy (Fig. 2.10) that clearly revealed their fibrous structure. As seen in Fig. 2.10D, KE group has a softer scaffold. This had been shown by oscillatory rheology characterization, and SEM images supported that KE group had softer characteristics among other experimental groups. Since we were investigating the effect of the concentration of chemical groups on the bioactivity of PA molecules, we did not increase the concentration of the PA solution for this group, which might have produced a stiffer gel with higher concentrations of K-PA and E-PA.

In order to assess whether the combinations of PA molecules are toxic to the cells, we firstly seeded rMSCs onto 2D surfaces coated by our designed combinations (Fig. 2.11). When we performed live/dead assay on the cultures, we observed that the PA combinations were not toxic to cells. The cells seeded onto PA coatings were as viable as the cells seeded onto TCP surface due to the biocompatible nature of our designed PA molecules (Fig 2.12). However, as an obstacle of *in vitro* experiments, viability of cells decreased gradually along with detachment of cells from the surfaces as days passed (Fig. 2.11). PA coated surfaces delayed the detachment, but did not totally prevent it from happening. Afterwards, viability of cells inside the 3D gels was visualized with live/dead assay, yet this time, we were not able to count live and dead cells due to focusing and opacity problems (Fig. 2.13). Nevertheless, except KE group, we did not observe frequent red dots inside the gels, instead, there were lots of green (alive) cells after one day. Peptide amphiphile gels tended to hold ethidium homodimer, and that caused a faint reddish background that nonetheless does not indicate the presence of dead cells. We observed that, over time, cells started to proliferate and form aggregates within the gels (Fig. 2.14). The proliferation of the cells was quantified through the indirect measurement of their metabolic activity. Their redox ability was measured by using AlamarBlue reagent, and assumed to be proportional to cell number. Measurements after 1, 3 and 7 days of incubation in gel culture revealed that metabolic activity, and therefore the cell numbers, was increasing throughout that period (Fig 2.15). Only the KE group showed small increases up to day 3, probably due to the inability of the cells to adjust to the 3D medium. However, then, the cells multiplied in the gel and showed increase in metabolic activity (Fig. 2.15).

More interestingly, when we checked the cell-laden gels following 21 days of incubation by SEM, we observed that cells were present both on the surface and inside of the gels (Fig. 2.16-19). In order to observe cells embedded into the gels, we had to disrupt the gels, therefore, the exposed cells might not fully reflect their natural morphology within the gels, as their interaction with peptide fibers and each other is limited. However, we could observe that the cells on the surface of gels nonetheless had morphologies different from that present on cells adhering to glass surfaces (Fig. 2.20). Compared to totally extended cells throughout the 2D surface,

cells inside gel systems resembled cells inside tissues to a greater extent: they were more round and spatially interacted with other cells. Moreover, the embedded cells were examined histologically and fluorescently to see their morphology (Fig. 2.21). Although the sections were not durable on the slides as normal animal tissue sections, their dense ECMs components can be observed which helped the adhesion of sectioned cell samples. As such, the sectioning of the gels was a good method to visualize and even immunochemically stain cells resident in hydrogel systems (Fig. 2.21B).

In order to analyze differentiation preference of cells in our designed gel systems, we firstly tried histological stainings, but failed to show marks of differentiation due to interference by the PA network. PA hydrogels absorbed the dyes and prevented the visualization of specific signs. Therefore, before starting other molecular techniques such as mRNA level measurement of differentiation specific genes, we tried to observe the pattern on 2D coatings (Fig. 2.22). Alizarin Red S, Oil Red O and Safranin O are the dyes heavily used for *in vitro* experiments to show calcium, sulfated glycosaminoglycan and triglyceride deposition, respectively, which are signs of osteogenesis, adipogenesis and chondrogenesis. Alizarin Red S selectively binds to deposited calcium on the matrix and imparts a red color where calcium is present. In the 2D culture, we did not visualize any red color formation which was a sign that rMSCs may not have committed to the osteogenic lineage (Fig. 2.22). Safranin O binds to sulfated glycosaminoglycans secreted to the environment and imparts a pink color. The cells were stained to a certain extent, but its specificity remained unclear due to the unspecific staining in the blank coatings, which had not been seeded by rMSCs (Fig. 2.23). Oil Red O staining was a straightforward method which directly stained neutral lipid droplets stored by adipogenic cells (Fig. 2.22). We were able to see the effect of the sulfonate functional group on rMSCs in samples stained with Oil Red O. The number of cells positively stained by Oil Red O gradually elevated by increases in sulfonate concentration, although cells with oil droplets were also observed in KE group as much as in the GS group.

Alkaline phosphatase is an enzyme that is required to derive inorganic phosphate from organic phosphate sources, and serves as an indirect indicator of osteogenic differentiation. In all gel groups, there was an increase in the activity of ALP,

particularly on day 14, but due to large variations between repeats, this difference was not enough to be significant, except for day 14 results as compared to its corresponding TCP group (Fig. 2.24). Although ALP activity is an indicator of osteogenic differentiation, it was also shown in 3T3-L1 pre-adipocytes, which are cells capable of further differentiating into mature adipocytes, and in bone marrow cells differentiating into adipocytes [248, 249]. Moreover, chondrocytes inside the cartilage (hypertrophic chondrocytes) are closer to the bone tissue in their metabolism, and also exhibit ALP activity [250]. Therefore, ALP activity in itself is not an indicator of osteogenic differentiation, instead it might be a sign of that the rMSCs are losing their multipotency and differentiating into one or more cell lines.

Since it is known that MSCs isolated from bone marrow display the tendency to become osteoblasts, we firstly checked the expression levels of genes related to the osteogenic lineage. *Runx2* is considered to be one of the master regulators of osteogenesis [251]. Its expression level increases throughout osteo-chondral differentiation and leads to the generation of osteoblasts and osteocytes. Its expression induces the maturation of hypertrophic chondrocytes, which then produce new bone on growth zones. Knockout mice that are deficient for Cbfa-1 (*Runx2*^{-/-}) lack differentiated osteoblasts, and the lack of mature bones causes their death in a very short time after birth [252]. Collagen 1 synthesis is also facilitated in cells undergoing osteogenesis, and serves a nucleation platform on which hydroxyapatite crystals grow [253]. Therefore, its presence is crucial for mature bone formation. We have checked their expression profile after the incubation of cells in PA hydrogels for 3, 7, 14 and 21 days. As seen in Fig. 2.25 and 2.26, there was no increase of *Runx2* or collagen I expression in cells loaded into the PA gels. It should be kept in mind that there was no induction medium (containing special growth factors) or ingredients (e.g. additional phosphate source) to induce differentiation of the rMSCs. Therefore, cues influencing the cells were the stiffness of the 3D environment (mechanical properties) and functional groups on the PA gel scaffold. Therefore, from this result, we may conclude that the stiffness of the gel might not be high enough for osteoblastic differentiation. As it is known from previous studies in the literature, mesenchymal stem cells can undergo osteogenesis on/in stiff materials

[254]. Our hydrogel groups are relatively softer materials (unless mixed with other materials), and that might have inhibited the osteogenic differentiation.

Transcription factor PPAR γ is regarded as the master regulator of adipogenesis and is induced before the transcriptional activation of many adipogenic genes [251, 255]. *Fabp4* (Fatty acid binding protein-4, also known as aP2) is expressed during the terminal differentiation of adipose cells, but in a PPAR γ -dependent manner [256]. Adiponectin is secreted by adipose tissue to regulate energy metabolism in the body [257]. Although many other cell types have the capacity to store lipid as droplets in their cytoplasm, mature adipocytes have an especially large reservoir that appears as a single lipid droplet. Thus, while adipocytes share the expression of many genes with other cell types, the upregulation of these genes can be considered to be specific for adipogenic cells. Adipose generation could clearly be observed in our hydrogel systems, and the number of rMSCs committing to the adipogenic lineage was higher for groups that incorporate sulfonates and Glc-PA (Fig. 2.27 and 2.28). Ono *et al.* showed that sulfated dextran accelerated the adipogenesis of 3T3-L1 cells [258]. They also mentioned that heparin, the most sulfated GAG, also causes a similar effect on these cells. Natural adipose tissue is also composed of GAG molecules like perlecan (known as basement membrane-specific heparan sulfate proteoglycan), and many other proteoglycans that are functionalized through the binding of sulfated GAGs [259]. Therefore, our results are consistent with the behavior of cells inside the gels and in the natural sulfate-rich environment of the adipose cells. However, the gene expression levels on day 7 and 14 revealed that the KE group was differentiating into adipocytes more than other groups. Considering its viscoelastic properties, we may suspect that it displays a softer structure and therefore supports adipogenic differentiation through its mechanical properties (Fig. 2.9), although 2D experiments suggest the reverse. On 2D cultures, coatings should have had similar stiffnesses due to the TCP surface present underneath, and still, we observed lots of adipocytes in KE group (Fig. 2.22). This was a surprising result for us, because the KE group has been used for many studies as a control group, and there was no observation for its enhancement of adipocyte differentiation [260]. The only difference from the other studies was that we had also used RGD-PA in our mixture. Therefore, there should be an influence of the RGD-sequence on adipogenic

differentiation in addition to the biochemical or biomechanical properties of the KE group. Thus, we might also deduce that adipogenic differentiation might be induced or accelerated in other groups by the addition of RGD-PA. The RGD sequence is primarily found in fibronectin, though it also occurs in other ECM proteins such as collagen type I and entactin [261], which are found in ECM of adipose tissue [259]. Although, it probably did not cause adipogenesis by itself, rMSCs may prefer to commit to adipogenic lineage under these circumstances.

Sox9 is a transcriptional factor playing a key role in early chondrogenesis and assists the translation of proteins related to cartilage differentiation, such as aggrecan and collagen type II [262, 263]. It is one of the master regulators of chondrogenesis and is upregulated to express genes related to cartilage development. Sox9^{+/-} mice die in perinatal period, and humans with malfunctions in this gene have severe skeletal dysmorphologies [262]. Aggrecan is a core protein that recruits the attachment of sulfated GAG chains (mostly chondroitin sulfate), and often bears long hyaluronan polysaccharide chains [264]. It is abundant in the cartilage tissue and is responsible for the stress bearing capacity of the joints by attracting cations and water to the tissue. The expression levels of genes corresponding to these two proteins, which are required to commit the cells to the chondrogenic lineage, were upregulated in some groups (Fig. 2.29 and 2.30). Sox9 was upregulated in all groups at as early as the third day of incubation, but then was selectively increased in Glc-PA containing groups (Fig 2.29). *Acan*, on the other hand, had an elevated expression on day 14 (Fig. 2.29 C). These expression profiles can be interpreted to determine the chondroblastic commitment of rMSCs on peptide hydrogels, and were further confirmed by the immunocytochemical staining of aggrecan (Fig. 2.32-2.36). Firstly, the specificity of the primary and secondary antibodies was tested by staining rat cartilage tissue (Fig. 2.32). We observed brown coloration in and around the chondrocytes, suggesting that the staining was successful. Experimental groups also showed aggrecan expression in some of the cells. Both individual cells and aggregates were stained by aggrecan, suggesting that cells within and outside aggregations may be at different stages of chondrogenic differentiation. When we quantified the positively stained cell numbers per area on day 14 samples, we observed a very similar pattern of *Acan* expression and aggrecan positive cells (Fig.

2.30-C and Fig. 2.37). Glc-PA containing groups had an increased number of aggrecan expressing cells, which was further elevated by addition of SO₃-PA (Fig. 2.37). Since cartilage has high amounts of sulfated GAGs (which is also the detection principle of Safranin O staining), higher concentrations of sulfonate groups within the PA gels may enhance the chondrogenic differentiation. However, the expression levels of collagen type II, which is a late chondrogenic marker and the abundant collagen type in mature cartilage tissue, were not upregulated in PA hydrogels (Fig. 2.31). We also did not observe the staining of collagen II in sectioned samples (data not shown). Therefore, we concluded that even though the addition of glucose and sulfonate groups may enhance the chondrogenesis of rMSCs, the process is incomplete and their maturation and further differentiation into chondrocytes might require other factors. Fibronectin is an ECM molecule found in the early cartilage tissue, and it is degraded when the cartilage tissue is mature [265, 266]. Higher expression of fibronectin is also characteristic of osteoarthritis [267]. Although, Hwang *et al.* demonstrated that chondrogenesis is possible in PEG scaffolds modified with the RGD sequence, they managed this in presence of chondrogenic growth factor, TGFβ1, but not in its absence [268]. Therefore, the incorporation of RGD-PA might prevent further chondrogenic maturation.

2.5. CONCLUSION

Synthetic scaffolds are gaining importance in tissue engineering studies and their characterizations regarding the stimulation of cellular responses is quite essential before their implementation in any *in vivo* application. Spatial feature requirements should be met in such systems in order to elicit the desired responses from cells, which can be provided by 3D systems. Peptide amphiphile hydrogel systems are ideal platforms for such studies due to their bioactive, biocompatible, and biodegradable intrinsic properties, which arise from their peptidic nature and can be altered by changing their amino acid sequence or attaching functional groups. They can be forced to assume hydrogel characteristics if the ionic strength or the pH of the environment is changed, or if they are mixed with an oppositely charged PA solution. Mimicking the extracellular matrix, the natural micro-environment of cells, is another strategy to stimulate cells into behaving as desired. However, none of the

synthetic materials can even vaguely approach the complexity of the ECM in any tissue. Therefore, the designed scaffolds should be monitored carefully in terms of cellular responses. Stem cell differentiation is one of the most studied areas of tissue engineering and allows the use of patient-derived or exogenous cells to regenerate diseased or injured tissue areas. Here, we report the rMSC differentiation profile in 3D environment-mimicking glycosaminoglycan hydrogels in the context of sulfate concentration, which is different in each natural GAG. The continuous repeats of glucose, hydroxyl, carboxyl and sulfonate groups on the fibrous structures formed by PA self-assembly is thought to mimic GAG chains found in proteoglycans. As hyaluronan is non-sulfated, chondroitin sulfate is mildly sulfated and heparan sulfate is highly sulfated, we designed the groups to present a broad variety of sulfonate group concentrations. We did not utilize any differentiation-inducing ingredients and the hydrogels largely exhibited similar mechanical properties; therefore, the differentiation was thought to be due to functional groups presented on PA molecules. In summary, we demonstrated that our gel system did not cause osteogenic differentiation, while increased concentrations of sulfonates resulted in both chondrogenic and adipogenic cell differentiation. Moreover, glucose-decorated PA-containing groups showed increased chondrogenesis but less adipogenic gene expression compared to groups lacking these moieties. These results can facilitate the design of scaffolds for further experiments that will be used in *in vitro* and *in vivo* experiments in future studies.

CHAPTER 3

IMPROVING PANCREATIC ISLET TRANSPLANTATION EFFICIENCY USING HEPARIN MIMETIC PEPTIDE AMPHIPHILE NANOFIBER GEL

This chapter of thesis is partially published in the following article [269]; “Uzunalli, G., **Tumtas, Y.**, Delibasi, T., Yasa, O., Mercan, S., Guler, M.O., and Tekinay, A.B., *Improving Pancreatic Islet in Vitro Functionality and Transplantation Efficiency by Using Heparin Mimetic Peptide Nanofiber Gels*. Acta Biomaterialia, 2015.”

3.1. INTRODUCTION

Chronic diseases cause suffering of millions of people all over the world and diabetes mellitus is one of the most prevalent illnesses among them. In the United States, it causes more than 200,000 deaths each year [270] and is associated with serious problems in cardiovascular, renal, ophthalmic, neurological, cerebrovascular, and peripheral vascular diseases [271-276].

Type 1 diabetes (T1D) is a chronic autoimmune disease caused by the destruction or damage of the insulin producing β -cells in the islets of Langerhans. Resulted insulin deficiency gives rise to hyperglycemia that can lead to acido-ketosis and to even death [277]. Regular insulin injection has been the standard therapeutic approach to overcome complications caused by T1D. However, due the fluctuation of the blood glucose levels of the effected people before and after the meals, insulin therapy alone is not an ideal way of dealing with this disease, as acute morbidity and mortality as well as a series of chronic complications were observed after insulin treatment [278, 279]. Although enhancements for insulin delivery has been suggested to provide strict control on blood glucose level, there are several limitations including poor patient compliance, risk of hypoglycemia and obstacles caused by using devices [279]. Pancreatic islet transplantation has been performed as a treatment for T1D to improve glycemic control in patients [280]. This method is found to be as effective as whole pancreas transplantation and less invasive [279]. Although it is a promising method, there are several limitations such as insufficient number of donors and decreased islet viability during and after isolation [281]. However, stem cell technology has been studying on producing functional insulin secreting β cells, and some has succeeded. Pagliuca et al. managed to decrease blood glucose levels of mice by artificially produced β cells, which was a promising development for patients [282].

Pancreatic islets are composed of clusters of different types of cells including β cells, and in the core of this structure, cells die due to decreased oxygen and nutrient transport. Pancreatic islets have extensive intra-islet vasculature to overcome these problems in natural tissue [283]. This vasculature, however, is damaged during the isolation procedure, and the damaged microvasculature only regenerates after 10 to

14 days following transplantation [284-287]. However, low diffusion of nutrients and oxygen leads to deterioration of cells [288] including β -cells and leads to hypoxia and death [289].

Using alternative transplantation sites, supportive scaffolds and minimizing the immunological responses are being investigated in order to increase the islet viability and consequently long term normoglycemia after transplantation. Until now, several alternative transplantation sites such as liver through the portal venous circulation [290], spleen, kidney [291], testis [292], anterior chamber of the eye [293], and greater omentum [294] have been tested to find an effective and less invasive surgical procedure for patient safety. Omentum has several advantages over other transplantation sites: (i) large transplantation area, (ii) less direct contact with blood that prevents instant immunological response and higher blood pressure, and (iii) not being a vital organ. On the other hand, it requires more islet numbers for transplantation and enhanced blood vessel formation for transplantation efficiency [295].

During islet isolation procedure, enzyme treatment disrupts islet-matrix interactions, which results in ECM degradation and apoptosis [296, 297]. Supporting the islets in *in vitro* conditions with matrices that contain ECM components such as collagen and fibrin increases viability of the islets [298-300], which shows the importance of scaffolds in providing an ECM-mimetic environment for islets.

In this study, peptide amphiphile (PA) nanofibers were used as a bioactive scaffold for islet transplantation. The PA molecules self-assemble into nanofibers due to intermolecular hydrogen bonding, electrostatic interactions between charged amino acids, and hydrophobic interactions [301, 302]. PA nanofibers form a 3D network in aqueous environment, which resemble natural ECM and this network can be enriched with specific bioactive sequences to modulate cell-material interactions. PA nanofibers have previously been used as functional scaffolds for bone [303, 304], neural [305], and cornea [306] regeneration and can also be used as drug delivery agents [307].

Here, heparin mimetic peptide amphiphile (HM-PA) nanofibers were used as a new therapeutic approach for type 1 diabetes to enhance the function of pancreatic islets

and to improve the islet transplantation efficiency. The HM-PA nanofibers induce capillary-like structure formation of endothelial cells *in vitro* and the PA scaffold applied with vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2), induce angiogenesis, in cornea *in vivo* [236]. The HM-PA provides an ECM like environment to enhance angiogenesis, which in turn enhances islet viability and functionality *in vivo*.

3.2. MATERIAL & METHODS

3.2.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc) and other protected amino acids, lauric acid, [4-[α -(2',4'-dimethoxyphenyl) Fmoc-amino methyl] phenoxy] acetamidonorleucyl-MBHA resin (Rink amide MBHA resin), *N,N,N',N'*-Tetramethyl-O-(1*H*-benzotriazole-1-yl) uronium hexafluorophosphate (HBTU), and diisopropylethylamine (DIEA) were purchased from Merck. ABCR Collagenase V and Streptozotocin (STZ) were purchased from Sigma-Aldrich. Piperidine, acetic anhydride, dichloromethane (DCM), dimethylformamide (DMF), trifluoroacetic acid (TFA) and triisopropylsilane (TIS) were purchased from Sigma-Aldrich. RPMI 1640, Hanks Balanced Salt Solution (HBSS), Fetal Bovine Serum (FBS), penicillin/streptomycin/ amphotericin B and L-glutamine were purchased from Lonza. Biocoll 1100 and Biocoll 1077 were purchased from Biochrom. All chemicals were used as provided.

3.2.2. Synthesis and Purification of Peptide Amphiphile Molecules

Peptide amphiphile molecules were synthesized by solid-phase Fmoc peptide synthesis method. Heparin mimetic peptide amphiphile (HM-PA) (Lauryl-VVAGEGD(K-pbs)S-Am) and K-PA (Lauryl-VVAGK-Am) were constructed on Rink Amide MBHA resin via solid phase peptide synthesis method. Coupling was performed with 1 equivalent of MBHA resin, 2 equivalent of Fmoc protected group amino acid, 1.95 equivalent of HBTU and 3 equivalent of DIEA for 2 h. Fmoc removal was performed with 20% piperidine/ DMF solution for 20 min. 10% acetic anhydride/DMF was used as blocking agent for the remaining free amine groups after coupling. The resin was washed three times with DMF, DCM and DMF,

respectively. In order to synthesize HM-PA, sulfobenzoic acid was added to the side chain of lysine. Before this process, 4-methyltrityl (MTT) group, which provides side chain protection for lysine, was removed by a TFA/TIS/H₂O/DCM mixture (5:2.5:2.5:90 ratio) for 5 min. Then, peptide cleavage was performed with 95:2.5:2.5 TFA: TIS: H₂O for 2 h at room temperature. After cleavage reaction, PA molecules were collected in a clean round bottom flask and DCM wash was performed several times. Excess TFA was removed by rotary-evaporator. After evaporation, ice-cold diethyl ether was added into the PA solution to precipitate synthesized molecules. The precipitate was collected by centrifuging the solution at 8000 rpm for 20 min. Supernatant (diethyl ether) was decanted without disturbing precipitated PAs and remaining diethyl ether was evaporated. Then, PA powder was dissolved in ddH₂O, put into -80 °C freezer overnight and lyophilized for 3 days. In order to determine the purity of synthesized peptide amphiphile molecules, Agilent 6530 quadrupole time of flight (Q-TOF) mass spectrometer with electrospray ionization (ESI) source equipped with a reverse phase analytical high performance liquid chromatography was used. For the characterization of negatively charged PA molecules Agilent Zorbax Extend-C18 (2.1 x 50 mm) column (basic condition) and for positively charged PA molecules Zorbax SB-C8 (4.6 x 100 mm) column (acidic condition) were used. An optimized gradient of water (0.1% formic acid or 0.1% NH₄OH) and acetonitrile (0.1% formic acid or 0.1% NH₄OH) was utilized as mobile phase during liquid chromatography. A reverse phase preparative HPLC (Agilent 1200 series) equipped with either Zorbax Extend-C18 (21.2 x 150 mm) column or Zorbax SB-C8 (21.2 x 150 mm) column as a stationary phase was employed to purify negatively and positively charged PA molecules, respectively. An optimized gradient of water (0.1% acetonitrile or 0.1% NH₄OH) and acetonitrile (0.1% formic acid or 0.1% NH₄OH) were used as a mobile phase during HPLC purification. Dialysis of K-PA dissolved in ddH₂O and placed into solution containing 0.1% HCl was enough for purification and that removed remaining residual TFA.

3.2.3. Physical, Mechanical and Chemical Characterization of Self-assembled Nanofiber Network

3.2.3.1. Transmission Electron Microscopy (TEM)

The nanostructures formed by self-assembly of PA molecules were visualized by transmission electron microscope (TEM). 1% (w/v) PA gel was prepared by combining two oppositely charged PAs, K-PA and HM-PA, which led to formation of dense hydrogel. After 5 min of gel formation, the gel was diluted 100 times by adding double distilled water. 5 μ L of the diluted PA solution was taken and dropped onto a Lacey carbon-coated 300-mesh copper grid. Solution was removed by a tissue paper after 5 min of incubation, and the sample was stained with 2 wt% uranyl acetate. After complete desiccation at room temperature, images were taken by a FEI Tecnai G2 F30 transmission electron microscope.

3.2.3.2 Scanning Electron Microscopy (SEM)

The architecture composed of nanofibers of PA molecules was examined with scanning electron microscope (SEM). 1% w/v PA solutions were mixed with the volume ratio of 1:1 to induce gel formation by charge neutralized self-assembly process. Gels were incubated for 15 min at room temperature to make sure fibers were formed. Later, dehydration was carried out by gradually increasing the concentration of ethanol from 20% to 100%. In order to avoid collapse of the structure of scaffold, samples were dried in a critical point dryer (Tousimis, Autosamdri-815B, Series C critical point dryer). Before SEM imaging, samples were coated with 8 nm of Au/Pd. FEI Quanta 200 FEG scanning electron microscope was utilized under high vacuum to monitor the surface formed networks.

3.2.3.1. Circular Dichroism (CD)

Secondary structure analysis was employed by using JASCO J815 circular dichroism spectropolarimeter before and after mixing PA molecules with each other. They were dissolved in ddH₂O at a concentration of 0.01% w/v and pH was adjusted around 7.4. The CD spectra of HM-PA, K-PA and HM-PA/K-PA were obtained from scanning at 190 nm to 300 nm using a digital integration time of 1 s, a band width of 1 nm and with standard sensitivity.

3.2.4. Animals

3 months old male Wistar rats were utilized for all *in vivo* experiments, for as both syngeneic islet donors and islet recipients. Rats were maintained *ad libitum* in a 12 h-12 h light-dark cycle. However, the night before the transplantation, their food was removed. All procedures were approved by Institutional Animal Care and Use Committee of Diskapi Yildirim Beyazit Training and Research Hospital for all *in vivo* experiments.

3.2.5. Islet Isolation and Culture

The pancreatic islets to be transplanted were isolated based on the methods modified from previous studies [308-310]. In summary, rats were anesthetized by an intraperitoneal injection of ketamycin (80 mg/kg) and xylazine (20 mg/kg). Then, midline laparotomy was performed, and the common bile duct was cannulated. 1 mg/mL concentration of collagenase Type V enzyme solution was prepared in cold Hanks' Balanced Salt Solution. Following the injection of 7 mL of cold collagenase (Type V, 1mg/mL) into the duct with a 22G cannula, the distended pancreas was removed and subjected to stationary digestion for 18 minutes at 37 °C. At the end of incubation time, cold HBSS supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin-Streptomycin-Amphotericin B was added for inhibition of enzyme activity and washing. After digested pancreas was centrifuged for 3 min at 1300 rpm with slow brake, the supernatant was removed gently. The pellet suspension was filtered by a steel sieve of 450 µm to remove islets from undigested tissue. Subsequently, the islets were purified by using Biocoll 1100 (1100 g/mL), Biocoll 1077 (1077 g/mL) and RPMI 1640 to create a discontinuous gradient and were centrifuged for 20 min at 2400 rpm with slow brake. After purification, the islets were washed with RPMI 1640 supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin-amphotericin B. Islets were counted and picked up under optical microscope via handpick method. Islet cells were cultured with 10% FBS, 1% penicillin/streptomycin/amphotericin B and 1% L-glutamine supplemented RPMI 1640 at 37 °C under the humidified atmosphere containing 5% CO₂ incubator.

3.2.6 Transplantation

In order to generate diabetic rat models, 220 mg/kg STZ in citrate buffer (0.1 M, pH 4.5) was intraperitoneally injected to the rats 10 days before transplantation. Blood glucose levels of the rats have been monitored using On Call Advanced Blood Glucose Monitoring System (Acon Laboratories Inc., CA USA). Blood glucose levels of rats were measured at day 5 and 3 before transplantation, and only animals with blood glucose level of greater than 300 mg/dL at both days were used for transplantation experiments. Approximately, 2000 islets were transplanted into the omentum of each rat. Isolated islets were collected with minimal volume of medium, and then mixed with HM-PA. Gelation was initiated with addition of K-PA. The final concentrations of PA solutions in scaffold were 1% w/v HM-PA and 1% w/v K-PA at 1:1 volume ratio.

Intraperitoneal injections of ketamycin (80 mg/kg) and xylazine (10 mg/kg) were used as anesthetic agents. The abdominal region of rats was shaved and sterilized with antiseptic solution (BATTICON solution, Adeka, Turkey). The greater omentum was reached by lower abdominal incision and it was spread onto a wet sterile gauze pad. Islets with PA gel were placed onto the omentum, and omentum was sewed with 3.0 PGLA (90% glycolide and 10% L-lactide) surgical sutures as a pouch model (Fig. 3.5). Animals were subcutaneously administered with 1000 mg/kg cefazolin (Mustafa Nevzat, Turkey) after the procedure to minimize infection risk. Recipient rats were grouped as (i) control (untreated with STZ, healthy, n=7), (ii) sham (n=7), (iii) PA (n=7), (iv) islet (n=7), (v) PA+islet groups (n=7).

PA gels without islets were transplanted to serve as negative control (PA group) for blood glucose measurements, but it was an experimental group for blood vessel number quantification. A sham group, comprising animals that have undergone surgery but did not receive PA or islet transplantation, was also included in the study in order to observe true effect of islets and PA gel. Healthy rats were used to compare both blood glucose levels and blood vessel numbers. Animals in the islet group were transplanted with only islets without PA gel. Rats were maintained *ad libitum* after the operation with regular controls during the experimental processes.

3.2.7 Scaffold Function Assessment

Blood glucose levels and weights of the animals were measured and recorded after transplantation around three times a week for 28 days. The graft was considered to be functional only if the blood glucose levels of the recipients were less than 300 mg/dL. Intraperitoneal glucose tolerance test (IPGTT) was carried out after 28 days of transplantation to examine the response to excess glucose. After fasting overnight, rats were anesthetized with intraperitoneal injection of anesthetic agents: ketamycin (80 mg/kg) and xylazine (10 mg/kg), and glucose solution with concentration of 2 g/kg was also intraperitoneally injected following sleep of animals. Blood glucose levels were measured from the tail vein before injection, and after 5, 10, 15, 30, 60, 90 and 120 minutes of injection.

3.2.8 Histological Analysis

At the end of the IPGTT, rats were transcardially perfused with saline and formalin, respectively. Omentum and pancreas tissues were collected put into tubes containing enough amount of formalin to fix tissues. Tissues fixed for about 18 h were then gradually dehydrated with ethanol exchange, and at the end, they were incubated in xylene. Afterwards, tissues were embedded into paraffin. Sectioning was performed by using Leica microtome to get 5 μ m thick samples on positively charged slides. Samples were deparaffinized in xylene and rehydrated in serial ethanol series for hematoxylin & eosin (H&E) staining and Masson's trichrome staining according to the standard protocol. For immunohistochemistry experiments, sections were stained with anti-insulin (1:500; Sigma I2018, clone K36AC10), anti-macrophages/monocytes (1:300; Millipore, MAB1435, clone ED-1) and anti-von Willebrand Factor (1:200; Abcam, ab6994) antibodies. After primary antibody staining, horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:500; Millipore) was utilized. 3,3'-diaminobenzidine (DAB) reagent was used to give coloration, and thus, show protein presence. All samples were mounted onto glass slides using xylene based mounting medium. Images were acquired with Zeiss Axio Scope A1 microscope. For blood vessel number quantitation in the omenta, 5 sectioned samples with interval of 100 μ m, from each rat were stained, and 10 images were taken with 200x magnification.

3.2.9 Statistical Analysis

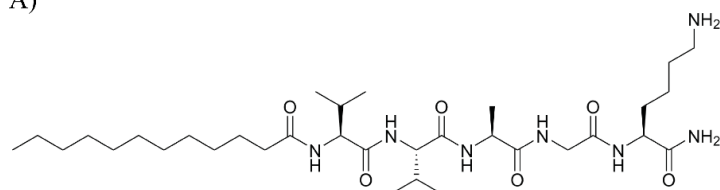
Statistical analysis was performed using one-way ANOVA with Bonferonni multiple comparison test. The level of significance was set at $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Error bars indicate standard error of mean.

3.3. RESULTS

3.3.1. Heparin Mimetic PA Molecules Self-assemble into Nanofibers

The HM-PA was previously designed with bioactive chemical groups to mimic heparin glycosaminoglycan (Fig. 3.1B) [236]. In order to trigger the self-assembly by charge neutralization, positively charged K-PA (Fig. 3.1A) was used to mix with HM-PA to form hydrogel system. All peptide molecules were characterized by liquid chromatography–mass spectrometry (LC–MS) and purified by preparative HPLC, which showed the success of syntheses (Fig. 3.2 and 3.3). As it was previously discussed, our designed peptide amphiphiles can generate nanofibrous structures via hydrophobic collapse, β sheet formation by the help of VVAG motif, and charge neutralization. This secondary structure was analyzed by circular dichroism (CD) spectroscopy. CD results showed that β -sheet structure dominates HM-PA/K-PA mixture with a negative band at 218 nm and a positive band at 195 nm (Fig. 3.4A). K-PA also showed smaller peaks at the mentioned points, but not HM-PA alone. That might be due to low dissociation ability of K-PA at neutral PA. TEM images of PA molecules revealed that nanofibers actually formed with 20–30 nm in diameter (Fig. 3.4B). SEM image revealed formation of a porous network with nanofibrous structure after combining HM-PA and K-PA (Fig. 3.4C).

A)



B)

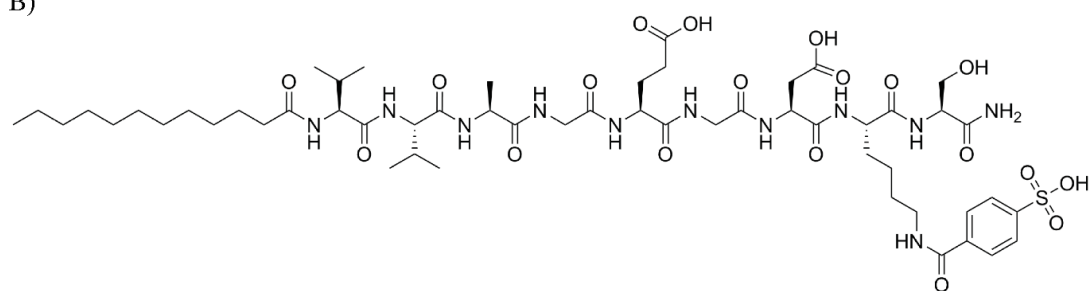
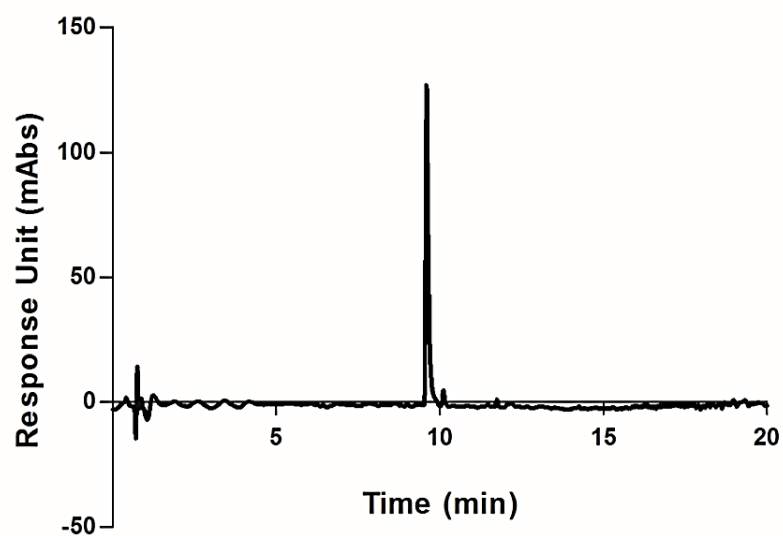


Figure 3.1 Molecular structures of the peptide amphiphiles used in the study. Positively charged K-PA (**A**) was used to induce self-assembly by mixing with negatively charged heparin mimetic peptide amphiphile (**B**).

A)



B)

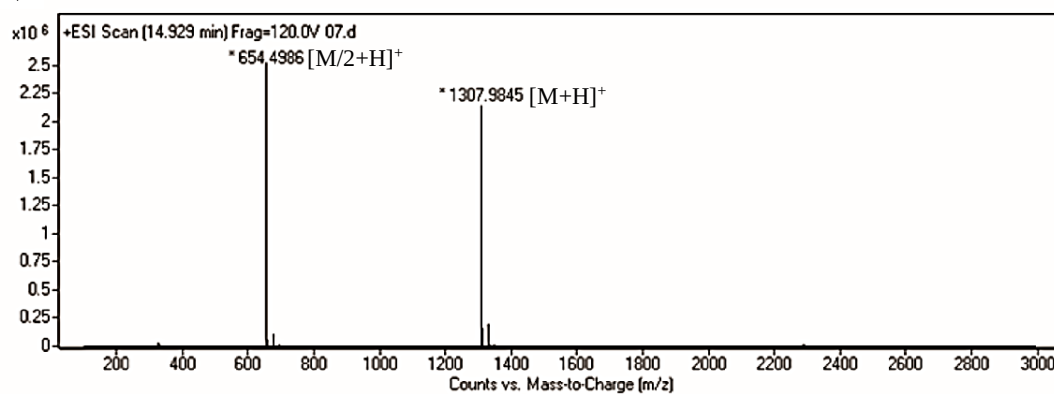
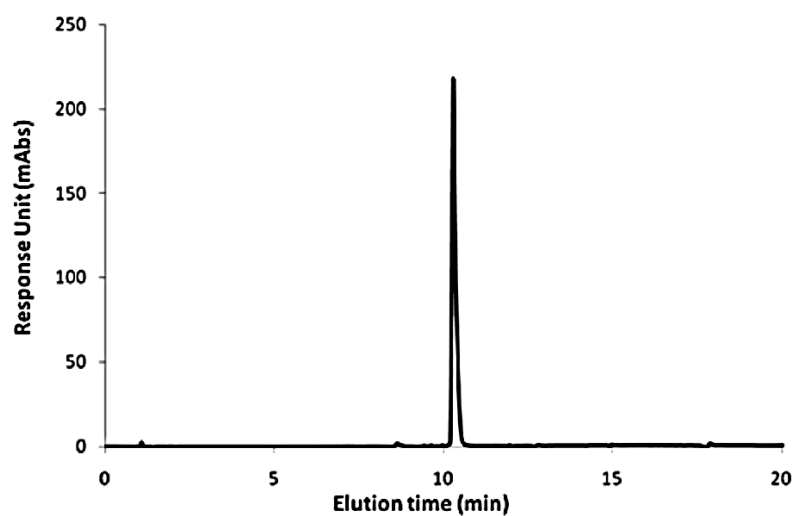


Figure 3.2 Liquid chromatography-mass spectrometry (LC-MS) analysis of K-PA. The purity of the crude product was analyzed according to the optical density at 220 nm.

A)



B)

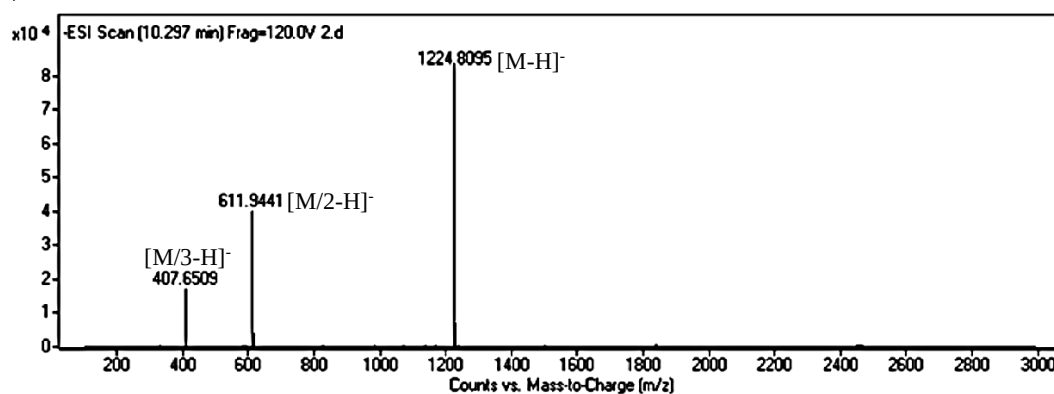


Figure 3.3 Liquid chromatography-mass spectrometry (LC-MS) analysis of HM-PA. The purity of the crude product was analyzed according to the optical density at 220 nm.

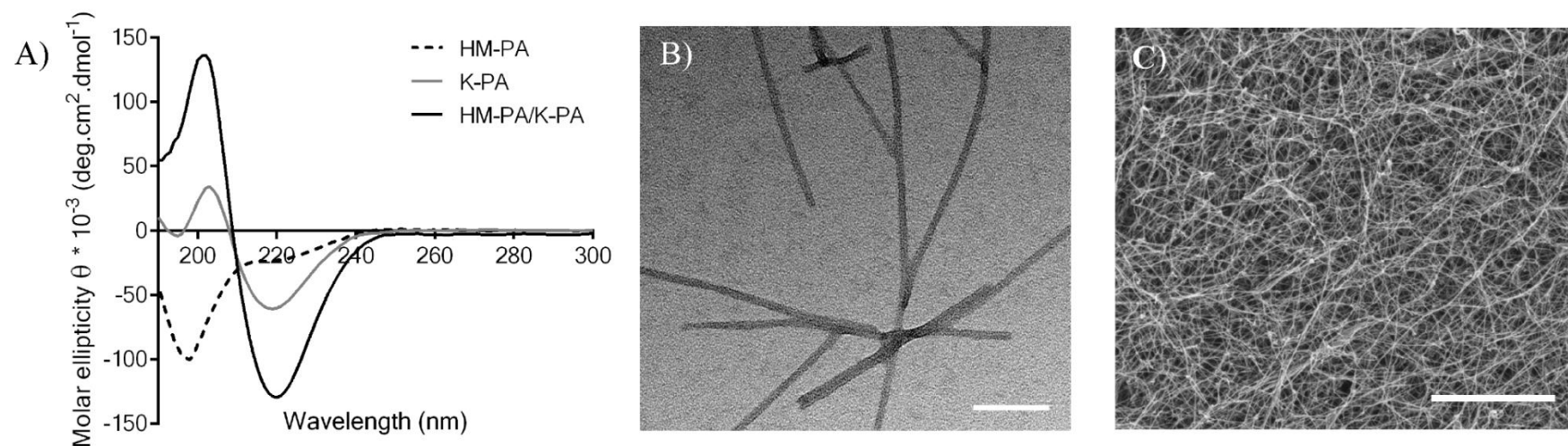


Figure 3.4 Self-assembly of peptide amphiphiles. **A)** Spectra of circular dichroism analyses of the PA molecules and PA hydrogel. The mixture of HM-PA and K-PA showed characteristic β sheet formation **(A)**. **B-C)** Electron microscopy results of individual fibers (TEM) and surface of gel (SEM). Scale bars are 100 nm **(B)** and 4 μ m **(C)**.

3.3.2. Transplantation within Bioactive PA Scaffold Improves Glucose Responsiveness of the Transplanted Islets

All the surgeries were conducted under general anesthesia. For transplantation, the omentum was reached through the abdominal cavity with a small incision. Omentum pouch model was employed to make proper environment for islet transplantation site. Islets with or without PA gel scaffold were placed onto it, and finally they were sewed together as a pouch (Fig. 3.5). Pouch was also produced by putting only PA gel or nothing. No complications were observed during and after the surgery. Weights of the animals did not alter significantly throughout the 28 days of experiment among all groups (Fig. 3.6A). Blood glucose levels of healthy rats, and the ones undergone surgery (n=7 for each group) are shown in Fig. 3.6B. Two recipient animals maintained normal glucose level and 5 out of 7 had blood glucose levels of less than 270 mg/dL over the course of 28 days, when isolated pancreatic islet were transplanted with HM-PA gel. “Only islets” group showed higher blood glucose levels when compared to “islets with PA scaffold” group. In order to examine the glucose responsiveness of animals, we carried out intraperitoneal glucose tolerance test (Fig. 3.6C). Contrary to the expectation, the blood glucose level increased up to 90 min after glucose loading. Although blood glucose levels typically start decreasing within 30 min in IPGT tests, This was probably caused by the administration of general anesthetics ketamycin and xylazine, which are known to interfere with IPGTT results [311].

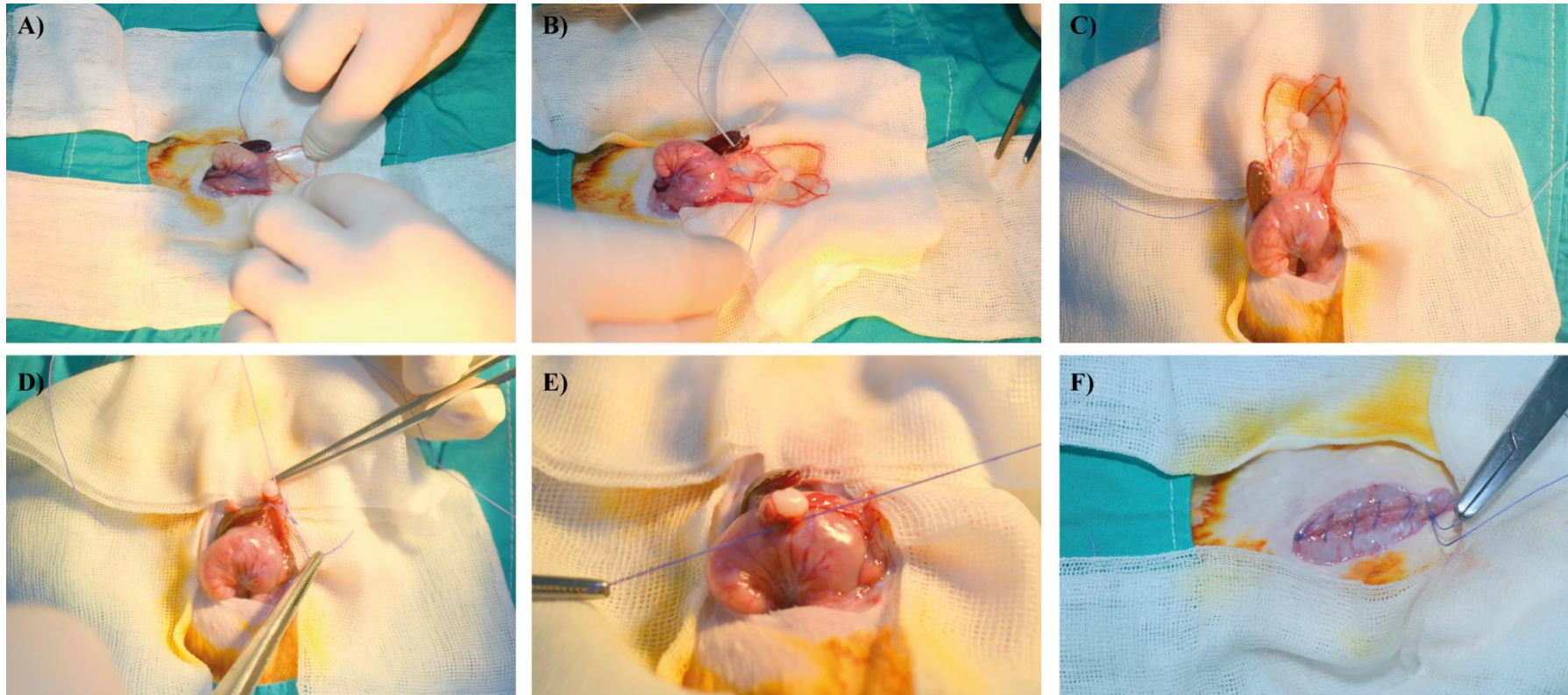


Figure 3.5 Technique used to implant peptide amphiphile hydrogel (with or without islets) into the omentum of the diabetic rats. The greater omentum was spread onto sterile gauze pad and cargo (unless it was sham) containing one of PA gel, islet or their combinations was placed onto it (**A-C**). Omentum was sewed like a pouch without disturbing the transplanted cargo (**D-E**). Omentum and stomach were placed back into abdomen and abdomen was sewed (**F**).

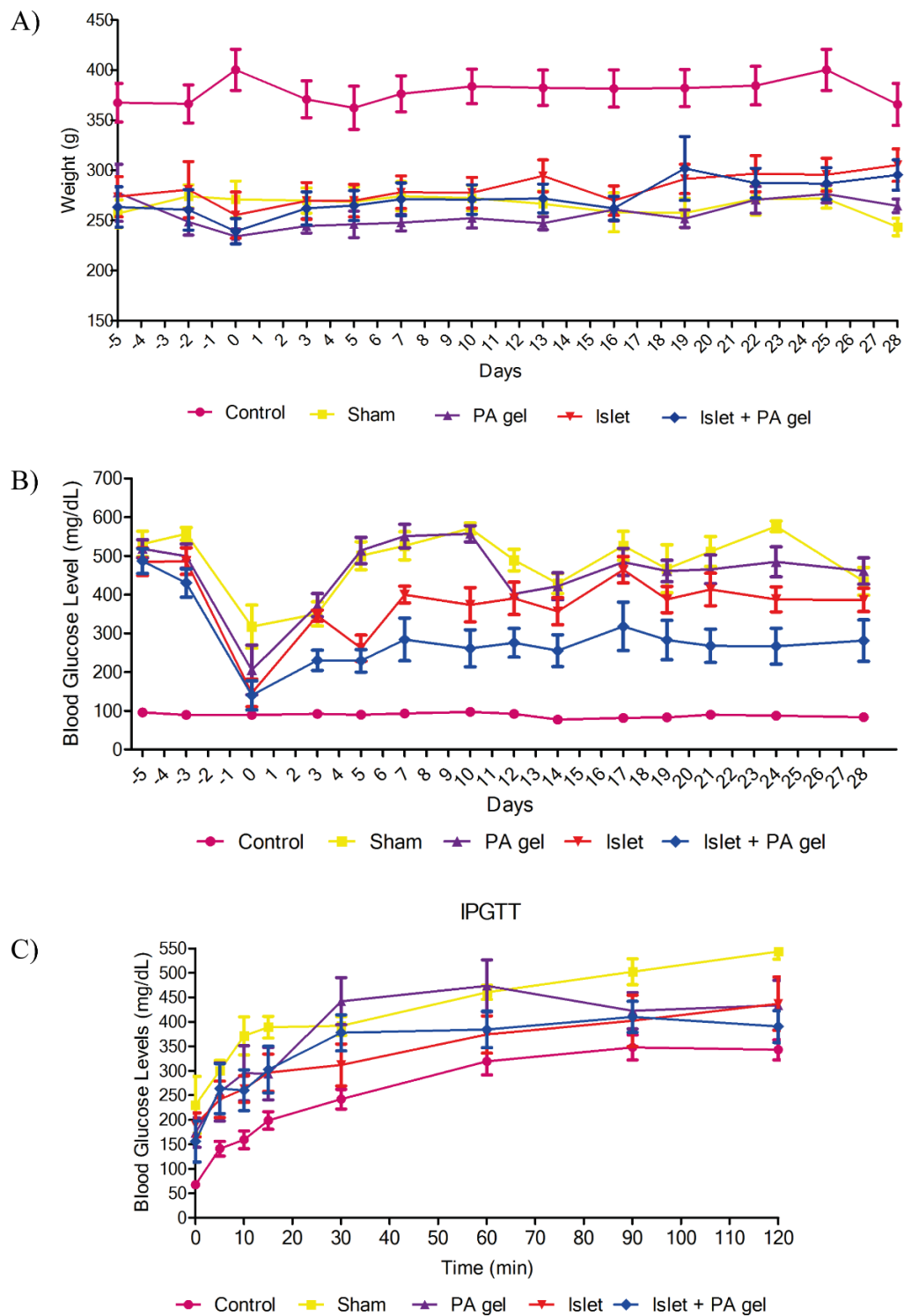


Figure 3.6 Weight of the animals **(A)** and blood glucose levels of the groups **(B)** throughout 28 days (Day 0; transplantation day). Intraperitoneal Glucose Tolerance Test (IPGTT) at the end of 28 days **(C)**.

3.3.3. PA Scaffold Supports Islet Integrity and Enhance Vascular Density of the Transplantation Site

In order to monitor morphology of the transplanted islets on day 28 post-transplantation, hematoxylin & eosin and Masson's trichrome stainings were carried out on omentum sections of animals that have undergone operation. Hematoxylin & eosin staining revealed that "peptide gel only" transplanted group showed regular omentum morphology and some peptide gel was still observable in the omentum (Fig. 3.7) although most of the gel was degraded by the day 28. The transplanted islets were observed to be in the group that islets were transplanted with PA gel. Moreover, compared to only islet group, islets transplanted in PA gel were more intact and had normal morphology (Fig. 3.7-3.8). This result shows that PA gel helped islets to maintain their integrity for up to 28 days. Masson's trichrome staining suggests that collagen is present in the islets belonging to "only PA", "only islet" and "islets in PA gel" groups, indicating fibroblast action around the transplantation site (Fig. 3.8). Immunohistochemical staining showed that viable islets at the transplantation site were positive for insulin, indicating that they were still functioning on day 28 of post-transplantation (Fig. 3.9). Immunological response was assessed with macrophage staining (Fig. 3.10). In order to observe blood vessels in omenta, anti-von Willebrand Factor staining was performed, as a vascular endothelial cell marker (Fig. 3.11). We also assessed the quantified vascularization by counting each blood vessel number (Fig.3.12). This assessment revealed that "only PA" and "islets in PA gel" groups had significantly more intraomental vessel number than other groups. Additionally, there was a significant difference between sham group and groups containing PA ($p < 0.001$), which demonstrate that PA itself promotes vascularization. The absence of statistical difference between "sham" and "only islet" groups and presence of statistical significant difference between "only islet" and "islet with PA groups" ($p < 0.05$) proved the fact that increased vascularization was provided by HM-PA.

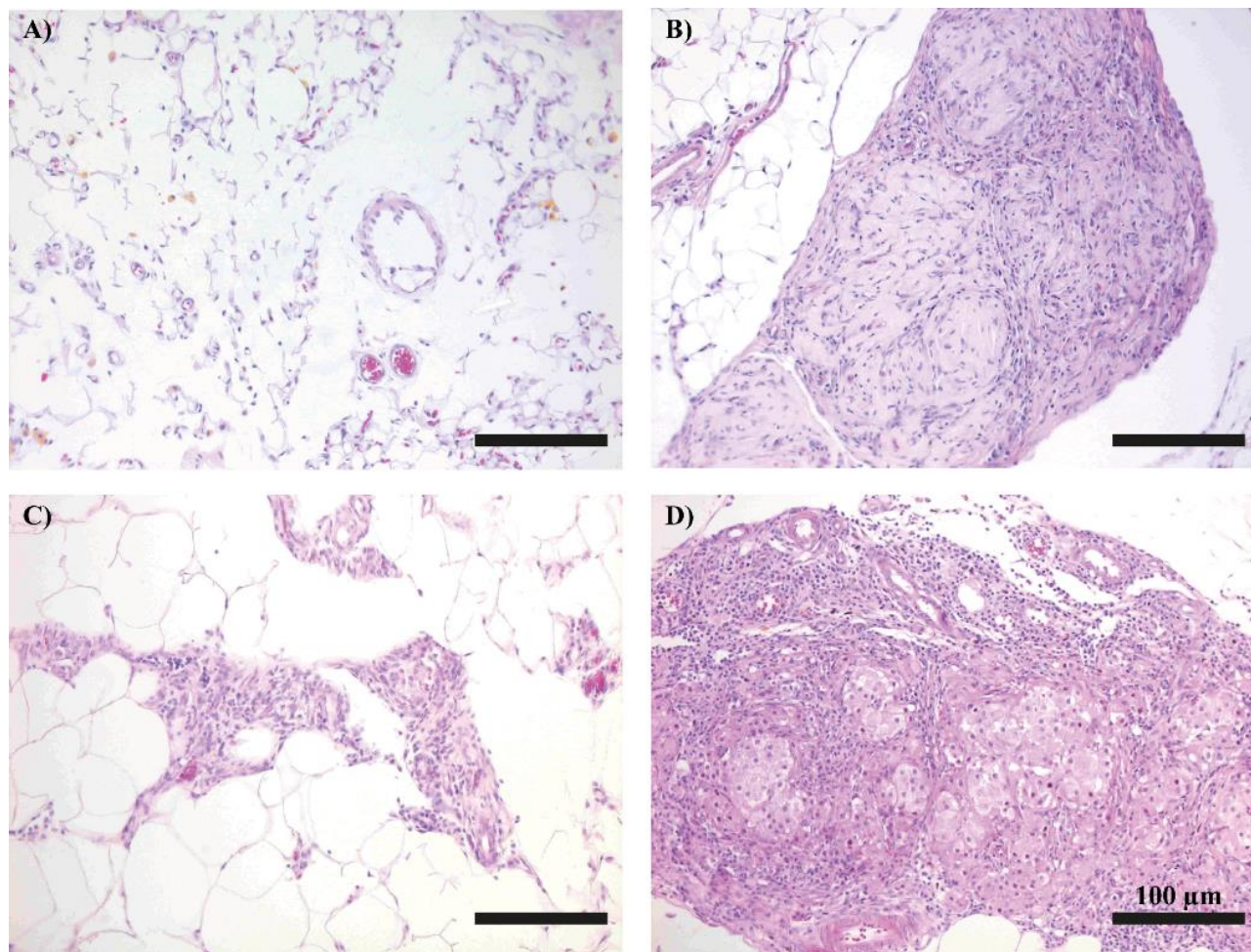


Figure 3.7 Hematoxylin & Eosin staining of omenta undergone operation: Sham **(A)**, PA hydrogel **(B)**, Islet **(C)**, Islet + PA hydrogel **(D)** groups. Scale bars are 100 μm .

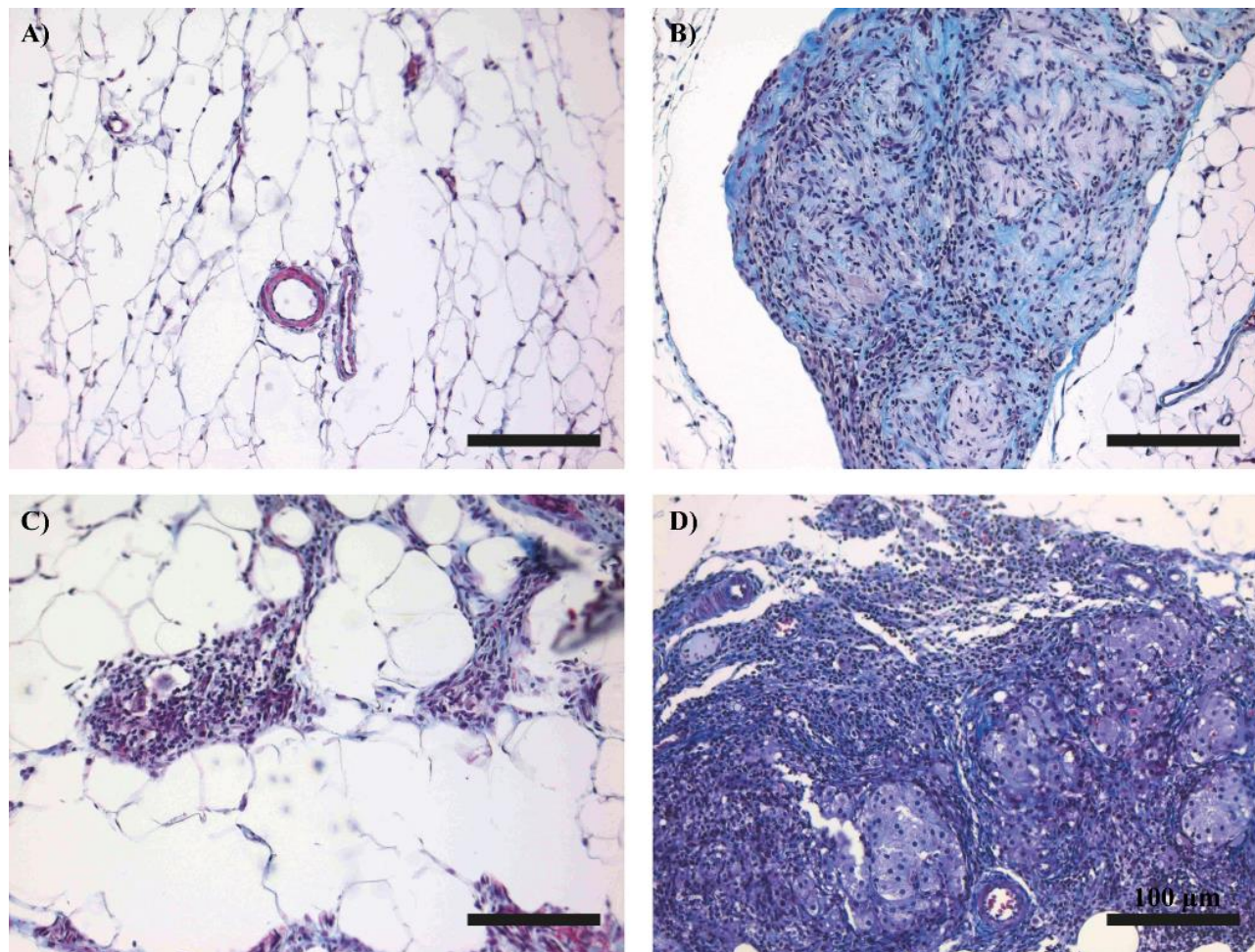


Figure 3.8 Masson's trichrome staining of in omenta undergone operation: Sham (A), PA hydrogel (B), Islet (C), Islet + PA hydrogel (D) groups. Blue color indicates collagen presence. Scale bars are 100 μm.

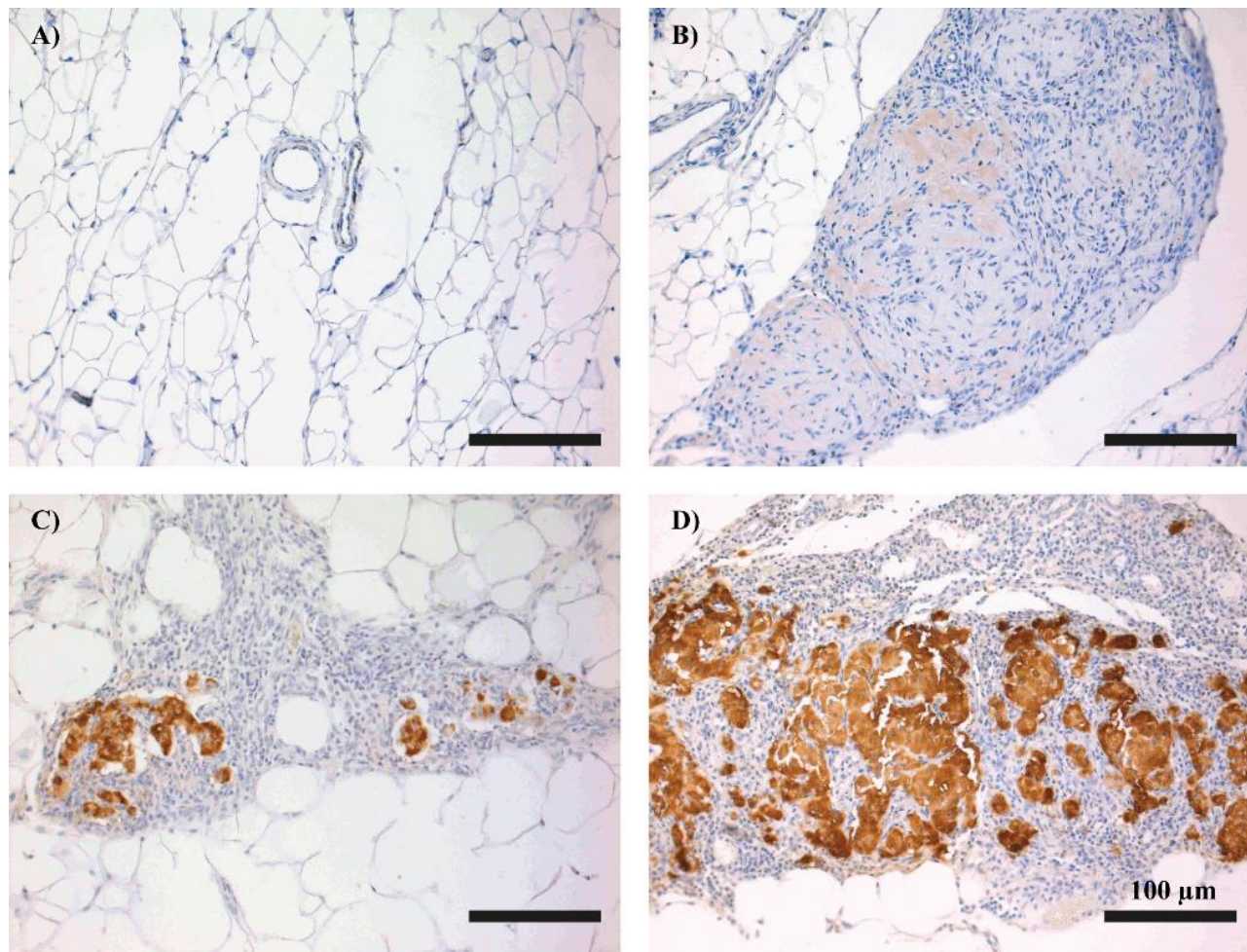


Figure 3.9 Immunohistochemical staining of insulin in omenta: Sham (A), PA hydrogel (B), Islet (C), Islet + PA hydrogel (D) groups. Brown color indicates presence of insulin. Scale bars are 100 μm .

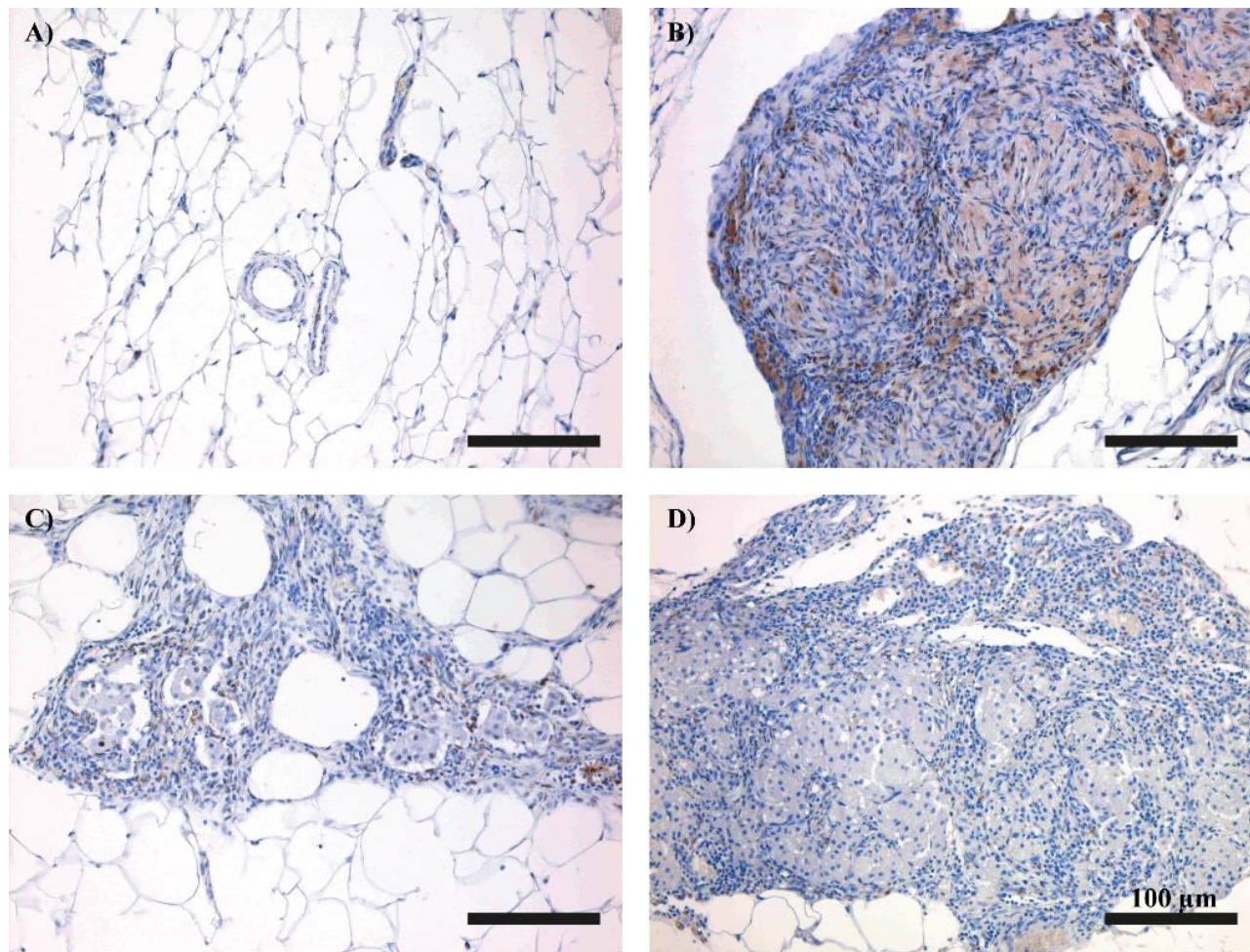


Figure 3.10 Immunohistochemical staining of macrophages in omenta: Sham **(A)**, PA hydrogel **(B)**, Islet **(C)**, Islet + PA hydrogel **(D)** groups. Brown color indicates presence of macrophages. Scale bars are 100 μm .

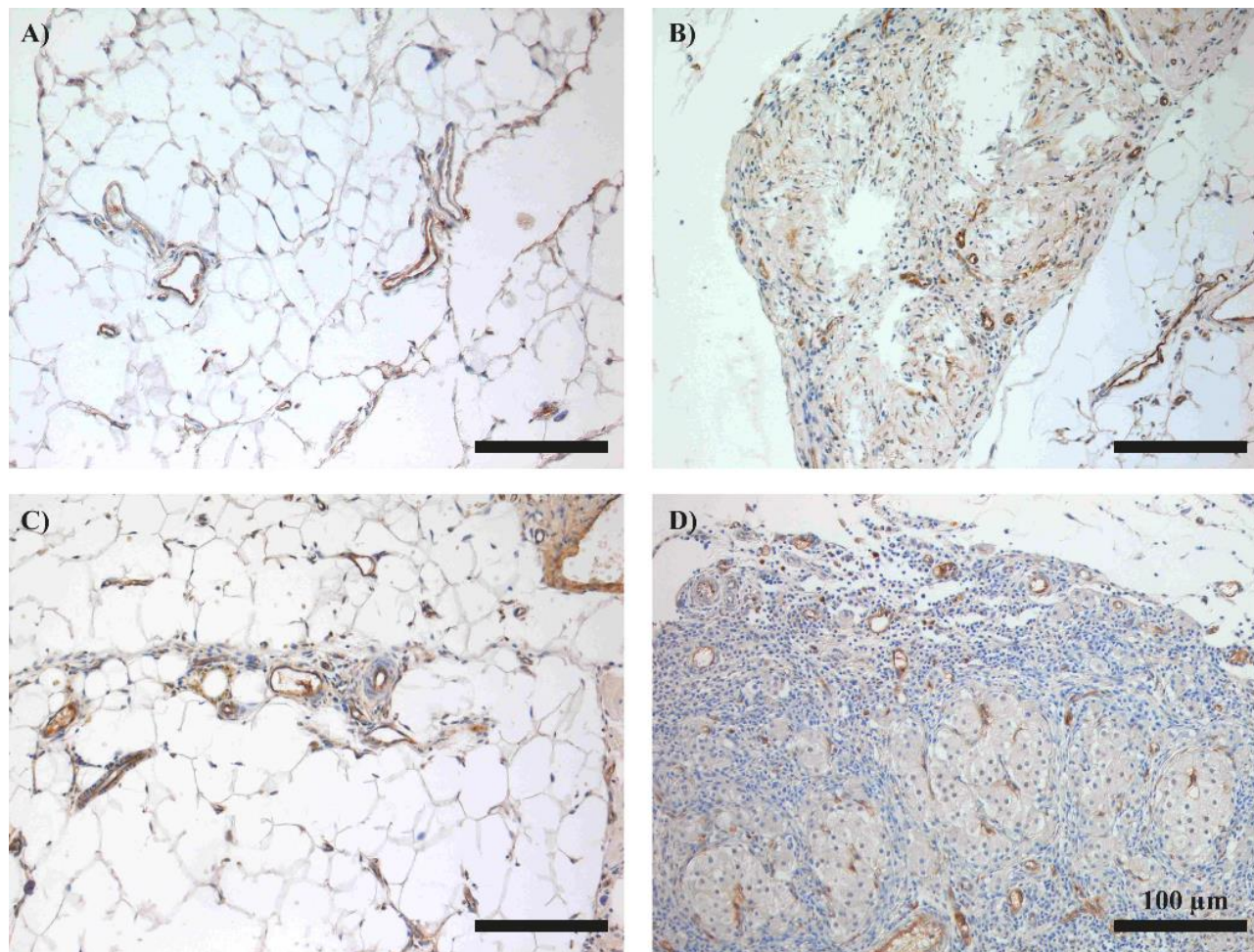


Figure 3.11 Immunohistochemical staining of von Willebrand in omenta: Sham **(A)**, PA hydrogel **(B)**, Islet **(C)**, Islet + PA hydrogel **(D)** groups. Brown color indicates presence of blood vessel. Scale bars are 100 μm .

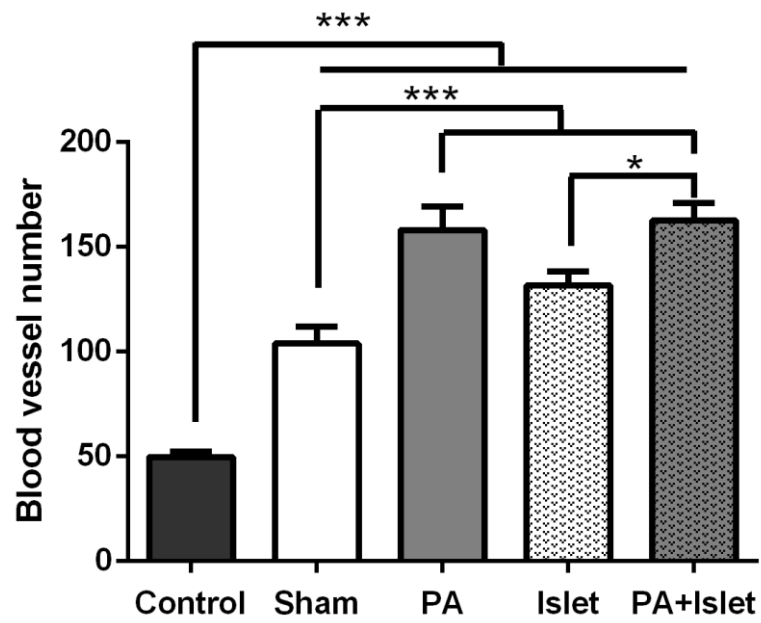


Figure 3.12 Average blood vessel number in omentum of animals. Except control group, other groups underwent omental pouch model operation.

3.4. DISCUSSION

Pancreatic islet transplantation is one of the most promising therapeutic methods for type I diabetes treatment, and has been practiced on humans since late 1970s. However, due to not well understood reasons, recipients cannot maintain euglycemia for long years [280]. Therefore researchers have been investigating various approaches to decrease failing attempts. Optimal transplantation sites, cell sources, and biomaterials to be transplanted with islets to extend islet survival have been studied [295, 312-314]. Since islets lose their vasculature during isolation process, angiogenesis is crucial for viability and functionality of islets after transplantation. In this study, we utilized a bioactive peptide nanofiber system for enhancing viability and functionality of islets *in vivo*. These nanofibers have been designed to form heparin mimetic structure through the self-assembly of PA molecules. Heparin is an important molecule capable of growth factor binding, which was previously shown to enhance angiogenesis *in vitro* and *in vivo* through its interactions with heparin-binding pro-angiogenic growth factors and their receptors [315]. Pancreatic islets are normally surrounded by ECM components that provide mechanical support and preserve cellular activities in the normal pancreas. Because it affects the metabolic activities and cellular signaling, morphology and integrity of the islets should be maintained after isolation *in vitro* conditions prior to the transplantation process [316]. Prior to the *in vivo* experiments, our colleagues have shown that when islets were incubated with HM-PA molecules in the solution, they retain their viability and glucose responsiveness *in vitro* [269].

However, vascular network integrity and cell-matrix interactions are diminished during isolation, resulting in a decrease in metabolic function [317]. Thus, scaffolds and materials are needed to enhance islet viability and function after the transplantation process. Designing materials to mimic the extracellular matrix is providing islets not only the required mechanical support, but also the porosity. The natural nanofibers comprising the ECM network are formed by glycosaminoglycans and other fibrous and non-fibrous protein to exchange nutrients and waste products [318]. It was previously shown that natural scaffolds such as collagen, fibrin or fibronectin increase the insulin secretion of islets [300, 319]. In addition, when

growth factor cocktails are added into the system, islets can maintain their spherical morphology and give highest insulin secretion [298]. Bennet *et al.* revealed a reduced damage in islets when cultured with heparin [320].

In this study, we utilized glycosaminoglycan mimetic peptide nanofiber scaffolds to provide the required support and retain islet shape without causing any toxic effect. The heparin mimetic peptide amphiphile (HM-PA) which was demonstrated to act like heparin by means of binding growth factors and to trigger new blood vessel formation [235, 236], was selected to enhance islet survival and function. Positively charged peptide amphiphile, K-PA, was synthesized to initiate nanofiber formation when mixed with negatively charged HM-PA via electrostatic interactions and hydrophobic collapse at physiological pH [321]. Secondary structure characterization along with electron microscopy results proved nanofiber formation via β -sheet formation (Fig. 3.4).

There were five groups in our *in vivo* experimental design. One of the groups was not treated with STZ, so that contained non-diabetic rats. This group was utilized to compare results of other groups in terms of blood glucose levels and blood vessel number in the omentum. Other groups included STZ-induced diabetic rats, and they were used for histological analyses as well as blood glucose levels and vascularization. Sham group (without PA and islet) was a control to see any effect of omental pouch model. The group transplanted with only HM-PA gel was used to observe the effects of the PA in histological analyses. The groups transplanted with islets with and without PA gel were actually the experimental groups that we compared with each other to determine the efficiency of the procedure. The omental pouch model (Fig. 3.5) was used for transplantation since transplantation of islets to the omental site both protects them from high blood pressure, prevents instant immune reaction and provides large transplantation volume [322, 323]. After transplantation, islets transplanted within HM-PA decreased blood glucose levels more than the “only islet” transplanted group (Fig. 3.6B). Furthermore, intraperitoneal blood glucose tolerance tests performed 28 days after transplantation revealed that “islets transplanted with PA gel” display a pattern similar to the healthy control group; however, this pattern was not observed in sham group and “islet only” group (Fig. 3.6C).

Morphological analyses of the integrity of islets by hematoxylin & eosin staining showed that “islets with PA gel” group preserved their original shape, while “only islet” group did not (Fig. 3.7). In healthy individuals, islets of Langerhans are surrounded by a collagen matrix [316, 324]. Masson’s trichrome staining revealed collagen deposited within groups implanted with PA gels regardless of the presence of islets (Fig. 3.8B and D). This biological activity, collagen deposition, might trigger formation of extracellular matrix for the islets similar to the natural microenvironment after transplantation, therefore, could enhance transplantation efficiency (Fig. 3.8). Correlation between morphological integrity and functionality of the transplanted islets was previously shown and that might have contributed to better glucose responsive of the “islets with PA gel” group [316]. Insulin positive cells were identified by immunohistochemistry (IHC) in islet grafts transplanted both in the presence and the absence of the PA gel, suggesting that the islets were functional *in vivo* under both conditions. However, the number of insulin-positive cells was strikingly enhanced in the presence of the PA gel (Fig. 3.9). This result supports the conclusion that preservation of islet integrity by the PA gel improves islet functionality (insulin production) *in vivo*.

Relatively fewer macrophages were present around islets in the PA gel compared to the numbers of macrophages around islets alone, as demonstrated by IHC (Fig. 3.10). Reduced macrophage infiltration after surgery might prevent excess cell death, help to maintain islet integrity and functionality, retain insulin secretory capacity, and lower blood glucose levels for a longer period. Implantation of the PA alone resulted in a more intense immune response (macrophage infiltration) than transplantation of islets in PA gel or transplantation of islets only. This might have resulted because of the release of various cytokines from injured islets that prevents macrophage recruitment to the transplantation site [325-329].

Anti-von Willebrand staining was used to demonstrate vascularization inside the islets and in the omentum when PA gel was applied (Fig. 3.11). After blood vessel counting and assessment for all animals in all groups, it was clarified that PA gel promotes vascularization *in vivo* (Fig. 3.12). The significant difference between vessel numbers in islets without PA and with PA may have been caused by the ability of HM-PA to bind to growth factors and concentrate them locally [235, 236].

In islet transplantation, number of blood vessels in transplantation site is an important issue. In order to increase transplantation efficiency, get a faster response to elevated glucose levels, and supply enough oxygen and nutrients to the islets, sufficient numbers of blood vessels are required in the transplantation sites. That provides an explanation for our *in vivo* vascularization result, comparing vessel numbers in the presence of the PA gel versus normal, non-transplanted controls, and sham groups. With the PA gel system, it was shown that vascularization in the omenta of animals can be increased; and when this system is used with islets in transplantation operation for T1D treatment, it increases the efficiency and stabilize blood glucose level more effectively compared to bare islet transplantation.

3.5. CONCLUSION

In order to increase the islet transplantation efficiency, it is important to maintain required environment for survival and function of islets *in vivo*. Herein, we presented a bioactive peptide amphiphile nanofiber gel system for pancreatic islet transplantation. Chemical and physiological characterization studies of PAs showed that they can be used as synthetic ECM mimicking monomer capable of forming nanofibrous supramolecular structures. Our results demonstrated that HM-PA gel provided a potent environment, diffusion and mechanical support for viability and function of islets. Furthermore, *in vivo* results indicated that islets transplanted with PA scaffolds controlled blood glucose levels more effectively than islets transplanted in the absence of the PA gel. Also, our histological analyses demonstrated that islets transplanted with the HM-PA gel were insulin positive for up to 28 days, and vessel numbers were increased in groups containing the HM-PA gel. These results may be explained by the affinity of HM-PA for growth factors (VEGF and FGF2) which are key elements of angiogenesis, which enhanced the survival of the transplanted islets.

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVES

Developments in medicine have extended the life expectancy of the human population and inadvertently led to a major increase in complications resulting from aging, such as organ failures and various degenerative disorders. Physical traumas by accidents or surgical practices also result in organ impairments, and further medical advancements are required for the repair of these injuries. Tissue engineering and regenerative medicine use a combination of materials science, molecular biology and clinical practice in order to combat these complications and enhance the quality of human life. These fields depend heavily on the development of new biomaterials for the repair of a specific organ or tissue. It is crucial, however, to monitor the influence of these novel materials on both intended and non-target tissues to minimize their negative impact on human patients. Therefore, *in vitro* and *in vivo* studies are of fundamental importance for the discipline of tissue engineering. Since almost all of the cells inside the body exist in a 3D environment covered by an extracellular matrix, the ideal way to cultivate cells for clinical use involves the utilization of 3D scaffolds. Although synthetic biomaterials are much less complex compared to the natural ECM of tissues, this lack of complexity also allows the modification of their mechanical, chemical and biological properties.

Here we have developed a bio-mimetic, ECM-like 3D hydrogel system that exhibits features similar to naturally occurring glycosaminoglycans. GAG-mimetic scaffolds were generated by designing peptide amphiphile molecules that can form supramolecular structures such as nanofibers to produce ECM-like architectures through the self-assembly process. This phenomenon occurs with the help of hydrophobic and charge interactions as well as hydrogen bonding, which are modulated through the bioactive moieties on PA molecules.

In the first study, we monitored the behavior of rat mesenchymal stem cells in terms of differentiation with respect to the presence of glucose-bearing units and changing concentrations of the sulfonate group in the absence of differentiation induction components. Since the function of GAG chains is known to be affected by the concentration of sulfate groups on their monomers, sulfate groups can be an important means of directing stem cell behavior in *in vitro* culture. Therefore, we expected the differentiation pattern of mesenchymal stem cells to be affected by the concentrations of functional sulfonate groups present on the peptide hydrogels. The

experimental groups were comprised of three glucose-decorated peptide amphiphile (Glc-PA) groups with no sulfonate bearing PAs (E-PA), with a sulfonate-containing PA (SO₃-PA), and their mixture (half concentration of sulfonate group). There was also a group containing neither of the functional groups. All groups also contained the RGD sequence to facilitate cell adhesion. When we checked the differentiation profile of rMSCs in 3D PA hydrogels, we observed that all gel groups showed some extent of adipogenic differentiation. However, Glc-PA presence decreased adipocyte generation. While glucose alone did not trigger adipogenic differentiation, increasing concentrations of sulfonates triggered increasingly stronger adipogenic responses and the fully sulfonated PA had a rate of adipogenic commitment comparable to group that lacked both glucose and sulfonates. Besides adipogenesis, the chondrogenic differentiation of rMSCs was also elevated with the addition of Glc-PA and increased sulfonate concentrations. There was no detectable osteogenic differentiation. For further studies, FACS analyses can be performed to reveal the percentage of cells that commit to each lineage. These conditions can be also altered in the future to intentionally direct differentiation by mixing these PA molecules with polymeric materials to adjust the mechanical characteristics of the resulting scaffold, which is known to determine differentiation. Moreover, different integrin binding units can be utilized rather than RGD, creating various differentiation profiles. While our study showed the presence of adipogenic differentiation, its type was not detected. Further investigations can focus on whether adipose cells differentiate into white or brown adipose cells. That can then be used for obesity research area. The findings also can be confirmed in *in vivo* experiments.

In the second study, we have tried to enhance the transplantation efficiency of pancreatic islets by using a glycosaminoglycan-mimetic peptide amphiphile, particularly a heparin-mimetic structure, by transplanting the islets within the gel. As millions of people suffer from type I diabetes mellitus, it is important to regularly supply them with the required insulin. That situation makes insulin injection treatment less desirable due to patient compliance issues. Pancreas transplantation is another method that provides control over the blood glucose level, although it requires immunosuppressive drugs and organ donation. In this scenario, islet transplantation promises better conditions for the management of type I diabetes.

Since isolated islets lose their vasculature and their survival is dependent on the influx of nutrients and oxygen, it is essential to form new vessels for their long term functionality after transplantation. The combination that we utilized has been previously shown to induce angiogenesis *in vitro* and *in vivo* via binding growth factors such as VEGF. Therefore, we expected to find more blood vessels around the transplantation site in the presence of bioactive peptide hydrogels. The site was selected as the omentum, which is a large tissue without a vital function, in order to transplant enough islets for decreasing blood glucose levels in STZ-induced rats. We first demonstrated that the bioactive peptide combinations did not cause any toxicity to isolated islets; on the contrary, the presence of PA molecules in the maintenance medium of incubated islets allowed them to preserve their functions. Then, we moved onto *in vivo* experiments to demonstrate that islets transplanted with heparin-mimetic peptide amphiphile gels decreased the blood glucose levels of diabetic rats more than islets transplanted without gels. When we analyzed the histological results, we found out that more blood vessels were formed in islets containing HM-PAs. Therefore, we concluded that blood vessel availability might increase islet survival within omental pouches. For further investigation, other transplantation sites can be exploited, like kidney capsule or immune reaction-free testes. Other approaches can involve the incorporation of chemokines to PA gels in order to reduce immune rejection, because the minimization of the immune response was not a major concern in our experiments. PA-supported islets have created lower blood glucose levels than the medically used islet transplantation procedure; as such, the system is promising for further studies and can be used for successful diabetes treatments where the islets are produced artificially from the cells of the patients themselves and transplanted to the body as an artificial tissue.

BIBLIOGRAPHY

1. Michalopoulos, G.K. and DeFrances, M.C., *Liver regeneration*. Science, 1997.
2. Muller, T.L., Ngo-Muller, V., Reginelli, A., Taylor, G., Anderson, R., and Muneoka, K. *Regeneration in higher vertebrates: limb buds and digit tips*. in *Regeneration in higher vertebrates: limb buds and digit tips*. 1999. Elsevier.
3. Petit-Zeman, S., *Regenerative Medicine*. Regenerative Medicine, 2001. **19**(3): p. 201-206.
4. Daar, A.S. and Greenwood, H.L., *A proposed definition of regenerative medicine*. Journal of Tissue Engineering and Regenerative Medicine, 2007. **1**(3): p. 179-184.
5. Dvir, T., Timko, B.P., Kohane, D.S., and Langer, R., *Nanotechnological strategies for engineering complex tissues*. Nature nanotechnology, 2011. **6**(1): p. 13-22.
6. Basak, E.U., Alejandro, S.-G., Hiroshi, Y., Maria-Louisa, I., Maria, A.G., Carley, S., et al., *Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix*. Nature Medicine, 2010. **16**(7): p. 814-820.
7. Grayson, W.L., Fröhlich, M., Yeager, K., Bhumiratana, S., Chan, M.E., Cannizzaro, C., Wan, L.Q., Liu, X.S., Guo, X.E., and Vunjak-Novakovic, G., *Engineering anatomically shaped human bone grafts*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(8): p. 3299-3304.
8. Sharma, A.K., Bury, M.I., and Fuller, N.J., *Cotransplantation with specific populations of spina bifida bone marrow stem/progenitor cells enhances urinary bladder regeneration*. Proceedings of the ..., 2013.
9. Liqiong, G., Akihito, M., Stephen, A.C., Christopher, K.B., and Laura, E.N., *Development of Decellularized Human Umbilical Arteries as Small-Diameter Vascular Grafts*. Tissue Engineering Part A, 2009. **15**(9): p. 2665-2676.
10. Harald, C.O., Thomas, S.M., Saik-Kia, G., Lauren, D.B., Stefan, M.K., Theoden, I.N., and Doris, A.T., *Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart*. Nature Medicine, 2008. **14**(2): p. 213-221.
11. Mansbridge, J.N., *Tissue-engineered skin substitutes in regenerative medicine*. Current opinion in biotechnology, 2009.
12. Jung, Y., Park, M.S., Lee, J.W., Kim, Y.H., Kim, S.H., and Kim, S.H., *Cartilage regeneration with highly-elastic three-dimensional scaffolds prepared from biodegradable poly (l-lactide-co-ε-caprolactone)*. Biomaterials, 2008.
13. Petersen, T.H., Calle, E.A., Zhao, L., Lee, E.J., Gui, L., Raredon, M.B., et al., *Tissue-engineered lungs for in vivo implantation*. Science (New York, N.Y.), 2010. **329**(5991): p. 538-541.
14. Choi, J., Gimble, J., Lee, K., Marra, K., Rubin, J., Yoo, J., Vunjak-Novakovic, G., and Kaplan, D., *Adipose tissue engineering for soft tissue regeneration*. Tissue engineering. Part B, Reviews, 2010. **16**(4): p. 413-426.

15. Rosen, E. and MacDougald, O., *Adipocyte differentiation from the inside out*. Nature reviews. Molecular cell biology, 2006. **7**(12): p. 885-896.
16. Lau, D., Dhillon, B., Yan, H., Szmitko, P., and Verma, S., *Adipokines: molecular links between obesity and atherosclerosis*. American journal of physiology. Heart and circulatory physiology, 2005. **288**(5): p. 41.
17. Moreno-Navarrete, J. and Fernández-Real, J., *Adipocyte Differentiation*. Adipose Tissue Biology, 2012.
18. Casadei, A., Epis, R., Ferroni, L., Tocco, I., Gardin, C., Bressan, E., Sivoletta, S., Vindigni, V., Pinton, P., Mucci, G., and Zavan, B., *Adipose tissue regeneration: a state of the art*. Journal of biomedicine & biotechnology, 2012. **2012**: p. 462543.
19. Peterson, N. *Healing from Within: Same-Day Adult Stem Cell Procedure*. 2014; Available from: <http://oregonregenerativemedicine.com/healing-joint-injuries-matter-weeks-day-adult-stem-cell-procedure/>.
20. Kim, H., Kim, J., Park, D., Park, K., Kang, S., Lee, J., Jeong, M., and Yoon, T., *Osteogenesis induced by a bone forming peptide from the prodomain region of BMP-7*. Biomaterials, 2012. **33**(29): p. 7057-7063.
21. Chen, Z., Wu, C., Gu, W., Klein, T., Crawford, R., and Xiao, Y., *Osteogenic differentiation of bone marrow MSCs by β -tricalcium phosphate stimulating macrophages via BMP2 signalling pathway*. Biomaterials, 2014. **35**(5): p. 1507-1518.
22. Liao, H.-T., Lee, M.-Y., Tsai, W.-W., Wang, H.-C., and Lu, W.-C., *Osteogenesis of adipose-derived stem cells on polycaprolactone- β -tricalcium phosphate scaffold fabricated via selective laser sintering and surface coating with collagen type I*. Journal of tissue engineering and regenerative medicine, 2013.
23. Chiu, L.-H., Lai, W.-F.T., Chang, S.-F., Wong, C.-C., Fan, C.-Y., Fang, C.-L., and Tsai, Y.-H., *The effect of type II collagen on MSC osteogenic differentiation and bone defect repair*. Biomaterials, 2014. **35**(9): p. 2680-2691.
24. Hsu, E., Ghodasra, J., Ashtekar, A., Nickoli, M., Lee, S., Stupp, S., and Hsu, W., *A comparative evaluation of factors influencing osteoinductivity among scaffolds designed for bone regeneration*. Tissue engineering. Part A, 2013. **19**(15-16): p. 1764-1772.
25. Han, L., Grodzinsky, A., and Ortiz, C., *Nanomechanics of the Cartilage Extracellular Matrix*. Annual review of materials research, 2011. **41**: p. 133-168.
26. Slomianka, L. *Blue Histology - Skeletal Tissues - Cartilage*. 2009 [cited 2014; Available from: <http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Cartilage/Cartil.htm#Hyaline>.
27. Taipaleenmaki, H., *Factors Regulating Chondrogenic Differentiation*, in *Medical Biochemistry and Genetics*. 2010, University of Turku: Turku, Finland
28. Eyre, D., *Collagen of articular cartilage*. Arthritis Res, 2002. **4**(1): p. 30-35.
29. Chen, F., Rousche, K., and Tuan, R., *Technology Insight: adult stem cells in cartilage regeneration and tissue engineering*. Nature clinical practice. Rheumatology, 2006. **2**(7).

30. Lin, Y., Luo, E., Chen, X., Liu, L., Qiao, J., Yan, Z., Li, Z., Tang, W., Zheng, X., and Tian, W., *Molecular and cellular characterization during chondrogenic differentiation of adipose tissue-derived stromal cells in vitro and cartilage formation in vivo*. Journal of Cellular and Molecular Medicine, 2005. **9**(4): p. 929-939.
31. Clouet, J., Vinatier, C., Merceron, C., Pot-vaucel, M., Maugars, Y., Weiss, P., Grimandi, G.I., and Guicheux, J.r.m., *From osteoarthritis treatments to future regenerative therapies for cartilage*. Drug discovery today, 2009. **14**(19-20): p. 913-925.
32. de Baat, P., Heijboer, M., and de Baat, C., *[Development, physiology, and cell activity of bone]*. Nederlands tijdschrift voor tandheelkunde, 2005. **112**(7): p. 258-263.
33. Anselme, K., *Osteoblast adhesion on biomaterials*. Biomaterials, 2000.
34. Long, F., *Building strong bones: molecular regulation of the osteoblast lineage*. Nature reviews. Molecular cell biology, 2012. **13**(1): p. 27-38.
35. Matsuo, K. and Irie, N., *Osteoclast-osteoblast communication*. Archives of biochemistry and biophysics, 2008. **473**(2): p. 201-209.
36. Peter, V.B., *Wnt signaling in bone*. BoneKEy-Osteovision, 2007. **4**.
37. Amini, A., Laurencin, C., and Nukavarapu, S., *Bone tissue engineering: recent advances and challenges*. Critical reviews in biomedical engineering, 2012. **40**(5): p. 363-408.
38. Stevens, M.M. and George, J.H., *Exploring and engineering the cell surface interface*. Science (New York, N.Y.), 2005. **310**(5751): p. 1135-1138.
39. St-Onge, L., Wehr, R., and Gruss, P., *Pancreas development and diabetes*. Curr Opin Genet Dev, 1999. **9**(3): p. 295-300.
40. *Islets of Langerhans*. 2015; Available from: <http://global.britannica.com/EBchecked/topic/329670/islets-of-Langerhans>.
41. Bosco, D., Armanet, M., Morel, P., Niclauss, N., and Sgroi, A., *Unique arrangement of α -and β -cells in human islets of Langerhans*. Diabetes, 2010.
42. Oliver-Krasinski, J.M. and Stoffers, D.A., *On the origin of the beta cell*. Genes Dev, 2008. **22**(15): p. 1998-2021.
43. Atkinson, M.A. and Eisenbarth, G.S., *Type 1 diabetes: new perspectives on disease pathogenesis and treatment*. The Lancet, 2001.
44. Efrat, S. and Russ, H.A., *Making beta cells from adult tissues*. Trends Endocrinol Metab, 2012. **23**(6): p. 278-85.
45. Gilbert, S.F., *Early mammalian development*. Early mammalian development, 2000.
46. Zorn, A.M. and Wells, J.M., *Vertebrate endoderm development and organ formation*. Annual review of cell and developmental biology, 2009. **25**: p. 221.
47. *Biomimetic Cell Culture Proteins as Extracellular Matrices for Stem Cell Differentiation*. Chem. Rev., 2012. **112**.
48. Hung, S.-C., Chang, C.-F., Ma, H.-L., Chen, T.-H., and Low-Tone Ho, L., *Gene expression profiles of early adipogenesis in human mesenchymal stem cells*. Gene, 2004. **340**(1): p. 141-150.
49. Benoit, D., Schwartz, M., Durney, A., and Anseth, K., *Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells*. Nature materials, 2008. **7**(10): p. 816-823.

50. Qian, S.-W., Li, X., Zhang, Y.-Y., Huang, H.-Y., Liu, Y., Sun, X., and Tang, Q.-Q., *Characterization of adipocyte differentiation from human mesenchymal stem cells in bone marrow*. BMC developmental biology, 2010. **10**: p. 47.
51. Yang, H., Park, J., Woo, D., Jeon, S., Do, H.-J., Lim, H.-Y., Kim, J.-H., and Park, K.-H., *C/EBP- α and C/EBP- β -mediated adipogenesis of human mesenchymal stem cells (hMSCs) using PLGA nanoparticles complexed with poly(ethyleneimine)*. Biomaterials, 2011. **32**(25): p. 5924-5933.
52. Hoshiba, T., Kawazoe, N., and Chen, G., *The balance of osteogenic and adipogenic differentiation in human mesenchymal stem cells by matrices that mimic stepwise tissue development*. Biomaterials, 2012. **33**(7): p. 2025-2031.
53. Young, D., Choi, Y., Engler, A., and Christman, K., *Stimulation of adipogenesis of adult adipose-derived stem cells using substrates that mimic the stiffness of adipose tissue*. Biomaterials, 2013. **34**(34): p. 8581-8588.
54. Peterbauer-Scherb, A., Danzer, M., Gabriel, C., van Griensven, M., Redl, H., and Wolbank, S., *In vitro adipogenesis of adipose-derived stem cells in 3D fibrin matrix of low component concentration*. Journal of tissue engineering and regenerative medicine, 2012. **6**(6): p. 434-442.
55. Gerlach, J., Lin, Y.-C., Brayfield, C., Minteer, D., Li, H., Rubin, J., and Marra, K., *Adipogenesis of human adipose-derived stem cells within three-dimensional hollow fiber-based bioreactors*. Tissue engineering. Part C, Methods, 2012. **18**(1): p. 54-61.
56. Chang, K.-H., Liao, H.-T., and Chen, J.-P., *Preparation and characterization of gelatin/hyaluronic acid cryogels for adipose tissue engineering: in vitro and in vivo studies*. Acta biomaterialia, 2013. **9**(11): p. 9012-9026.
57. Kang, X., Xie, Y., Powell, H., James Lee, L., Belury, M., Lannutti, J., and Kniss, D., *Adipogenesis of murine embryonic stem cells in a three-dimensional culture system using electrospun polymer scaffolds*. Biomaterials, 2007. **28**(3): p. 450-458.
58. Halbleib, M., Skurk, T., de Luca, C., von Heimburg, D., and Hauner, H., *Tissue engineering of white adipose tissue using hyaluronic acid-based scaffolds. I: in vitro differentiation of human adipocyte precursor cells on scaffolds*. Biomaterials, 2003. **24**(18): p. 3125-3132.
59. Fischbach, C., Seufert, J., Staiger, H., Hacker, M., Neubauer, M., Göpferich, A., and Blunk, T., *Three-dimensional in vitro model of adipogenesis: comparison of culture conditions*. Tissue engineering, 2004. **10**(1-2): p. 215-229.
60. Davidenko, N., Campbell, J., Thian, E., Watson, C., and Cameron, R., *Collagen-hyaluronic acid scaffolds for adipose tissue engineering*. Acta biomaterialia, 2010. **6**(10): p. 3957-3968.
61. Daquinag, A., Souza, G., and Kolonin, M., *Adipose tissue engineering in three-dimensional levitation tissue culture system based on magnetic nanoparticles*. Tissue engineering. Part C, Methods, 2013. **19**(5): p. 336-344.
62. Pairault, J. and Lasnier, F., *Control of the adipogenic differentiation of 3T3-F442A cells by retinoic acid, dexamethasone, and insulin: a topographic analysis*. Journal of cellular physiology, 1987. **132**(2): p. 279-286.

63. Tombuloglu, A., Tekinay, A.B., and Guler, M.O., *Materials for Articular Cartilage Regeneration*. Recent Patents on Biomedical Engineering, 2012. **5**(3): p. 187.
64. Muir, H., *The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules*. BioEssays : news and reviews in molecular, cellular and developmental biology, 1995. **17**(12): p. 1039-1048.
65. Keeney, M., Lai, J., and Yang, F., *Recent progress in cartilage tissue engineering*. Current opinion in biotechnology, 2011. **22**(5): p. 734-740.
66. Bosnakovski, D., Mizuno, M., Kim, G., Takagi, S., Okumura, M., and Fujinaga, T., *Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis*. Biotechnology and bioengineering, 2006. **93**(6): p. 1152-1163.
67. Beane, O. and Darling, E., *Isolation, characterization, and differentiation of stem cells for cartilage regeneration*. Annals of biomedical engineering, 2012. **40**(10): p. 2079-2097.
68. Chonanant, C., Jearanaikoon, N., Leelayuwat, C., Limpai boon, T., Tobin, M., Jearanaikoon, P., and Heraud, P., *Characterisation of chondrogenic differentiation of human mesenchymal stem cells using synchrotron FTIR microspectroscopy*. The Analyst, 2011. **136**(12): p. 2542-2551.
69. Jo, C., Ahn, H., Kim, H., Seong, S., and Lee, M., *Surface characterization and chondrogenic differentiation of mesenchymal stromal cells derived from synovium*. Cytotherapy, 2007. **9**(4): p. 316-327.
70. Khetan, S., Guvendiren, M., Legant, W., Cohen, D., Chen, C., and Burdick, J., *Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels*. Nature materials, 2013. **12**(5): p. 458-465.
71. Zou, L., Luo, Y., Chen, M., Wang, G., Ding, M., Petersen, C., et al., *A simple method for deriving functional MSCs and applied for osteogenesis in 3D scaffolds*. Scientific reports, 2013. **3**: p. 2243.
72. Kavya, K., Jayakumar, R., Nair, S., and Chennazhi, K., *Fabrication and characterization of chitosan/gelatin/nSiO₂ composite scaffold for bone tissue engineering*. International journal of biological macromolecules, 2013. **59**: p. 255-263.
73. Rezwan, K., Chen, Q., Blaker, J., and Boccaccini, A., *Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering*. Biomaterials, 2006. **27**(18): p. 3413-3431.
74. de Ruijter, J., ter Brugge, P., Dieudonné, S., van Vliet, S., Torensma, R., and Jansen, J., *Analysis of integrin expression in U2OS cells cultured on various calcium phosphate ceramic substrates*. Tissue engineering, 2001. **7**(3): p. 279-289.
75. Itoh, M., Shimazu, A., Hirata, I., Yoshida, Y., Shintani, H., and Okazaki, M., *Characterization of CO₃Ap-collagen sponges using X-ray high-resolution microtomography*. Biomaterials, 2004. **25**(13): p. 2577-2583.
76. Karageorgiou, V. and Kaplan, D., *Porosity of 3D biomaterial scaffolds and osteogenesis*. Biomaterials, 2005. **26**(27): p. 5474-5491.

77. Sargeant, T., Guler, M., Oppenheimer, S., Mata, A., Satcher, R., Dunand, D., and Stupp, S., *Hybrid bone implants: self-assembly of peptide amphiphile nanofibers within porous titanium*. Biomaterials, 2008. **29**(2): p. 161-171.
78. Guzmán, R., Nardecchia, S., Gutiérrez, M., Ferrer, M., Ramos, V., del Monte, F., Abarategi, A., and López-Lacomba, J., *Chitosan scaffolds containing calcium phosphate salts and rhBMP-2: in vitro and in vivo testing for bone tissue regeneration*. PloS one, 2014. **9**(2).
79. Yamashita, A., Nishikawa, S., and Rancourt, D., *Identification of five developmental processes during chondrogenic differentiation of embryonic stem cells*. PloS one, 2010. **5**(6).
80. Guzzo, R.M., Gibson, J., Xu, R.-H., Lee, F.Y., and Drissi, H., *Efficient differentiation of human iPSC-derived mesenchymal stem cells to chondroprogenitor cells*. Journal of Cellular Biochemistry, 2013. **114**(2): p. 480-490.
81. Diekman, B., Christoforou, N., Willard, V., Sun, H., Sanchez-Adams, J., Leong, K., and Guilak, F., *Cartilage tissue engineering using differentiated and purified induced pluripotent stem cells*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(47): p. 19172-19177.
82. Ustun, S., Tombuloglu, A., Kilinc, M., Guler, M., and Tekinay, A., *Growth and differentiation of prechondrogenic cells on bioactive self-assembled peptide nanofibers*. Biomacromolecules, 2013. **14**(1): p. 17-26.
83. Pieper, J.S., van der Kraan, P.M., Hafmans, T., Kamp, J., Buma, P., van Susante, J.L., van den Berg, W.B., Veerkamp, J.H., and van Kuppevelt, T.H., *Crosslinked type II collagen matrices: preparation, characterization, and potential for cartilage engineering*. Biomaterials, 2002. **23**(15): p. 3183-92.
84. Geistlich, P. and Schloesser, L., *Resorbable extracellular matrix containing collagen I and collagen II for reconstruction of cartilage*. 2013, Google Patents.
85. Liu, L. and Spiro, R.C., *Collagen-polysaccharide matrix for bone and cartilage repair*. 2003, Google Patents.
86. Wang, C.-T., Lin, J., Chang, C.-J., Lin, Y.-T., and Hou, S.-M., *Therapeutic Effects of Hyaluronic Acid on Osteoarthritis of the Knee A Meta-Analysis of Randomized Controlled Trials*. The Journal of Bone & Joint Surgery, 2004. **86**(3): p. 538-545.
87. Fussenegger, M., Meinhart, J., Hübbling, W., Kullich, W., Funk, S., and Bernatzky, G., *Stabilized Autologous Fibrin-Chondrocyte Constructs for Cartilage Repair in Vivo*. Annals of Plastic Surgery, 2003. **51**(5): p. 493-498.
88. Lahiji, A., Sohrabi, A., Hungerford, D.S., and Frondoza, C.G., *Chitosan supports the expression of extracellular matrix proteins in human osteoblasts and chondrocytes*. Journal of Biomedical Materials Research, 2000. **51**(4): p. 586-595.
89. Sha'ban, M., Kim, S.H., Idrus, R.B., and Khang, G., *Fibrin and poly(lactic-co-glycolic acid) hybrid scaffold promotes early chondrogenesis of articular chondrocytes: an in vitro study*. J Orthop Surg Res, 2008. **3**: p. 17.
90. Zhang, X., Li, Y., Chen, X., Wang, X., Xu, X., Liang, Q., Hu, J., and Jing, X., *Synthesis and characterization of the paclitaxel/MPEG-PLA block copolymer conjugate*. Biomaterials, 2005. **26**(14): p. 2121-8.

91. Vange, J., Schwach-Abdellaoui, K., Everland, H., Nielsen, P.S., Nielsen, B., Jespersen, L.K., and Nielsen, L.F., *Degradable hydrophilic block copolymers with improved biocompatibility for soft tissue regeneration*. 2007, Google Patents.
92. Elisseeff, J., McIntosh, W., Anseth, K., Riley, S., Ragan, P., and Langer, R., *Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks*. Journal of Biomedical Materials Research, 2000. **51**(2): p. 164-171.
93. Grad, S., Kupcsik, L., Gorna, K., Gogolewski, S., and Alini, M., *The use of biodegradable polyurethane scaffolds for cartilage tissue engineering: potential and limitations*. Biomaterials, 2003. **24**(28): p. 5163-5171.
94. Chia, S.L., Gorna, K., Gogolewski, S., and Alini, M., *Biodegradable elastomeric polyurethane membranes as chondrocyte carriers for cartilage repair*. Tissue Eng, 2006. **12**(7): p. 1945-53.
95. Kobayashi, M. and Oka, M., *Characterization of a polyvinyl alcohol-hydrogel artificial articular cartilage prepared by injection molding*. J Biomater Sci Polym Ed, 2004. **15**(6): p. 741-51.
96. Kus, W.M., Gorecki, A., Strzelczyk, P., and Swiader, P., *Carbon fiber scaffolds in the surgical treatment of cartilage lesions*. Ann Transplant, 1999. **4**(3-4): p. 101-2.
97. Woo, K., Chen, V., and Ma, P., *Nano-fibrous scaffolding architecture selectively enhances protein adsorption contributing to cell attachment*. Journal of biomedical materials research. Part A, 2003. **67**(2): p. 531-537.
98. Benders, K., van Weeren, P., Badylak, S., Saris, D.I., Dhert, W., and Malda, J., *Extracellular matrix scaffolds for cartilage and bone regeneration*. Trends in biotechnology, 2013. **31**(3): p. 169-176.
99. Uriel, S., Huang, J.-J., Moya, M., Francis, M., Wang, R., Chang, S.-Y., Cheng, M.-H., and Brey, E., *The role of adipose protein derived hydrogels in adipogenesis*. Biomaterials, 2008. **29**(27): p. 3712-3719.
100. Mauney, J., Volloch, V., and Kaplan, D., *Matrix-mediated retention of adipogenic differentiation potential by human adult bone marrow-derived mesenchymal stem cells during ex vivo expansion*. Biomaterials, 2005. **26**(31): p. 6167-6175.
101. Daya, S., Loughlin, A., and Macqueen, H., *Culture and differentiation of preadipocytes in two-dimensional and three-dimensional in vitro systems*. Differentiation; research in biological diversity, 2007. **75**(5): p. 360-370.
102. Cho, S.-W., Kim, S.-S., Rhie, J., Cho, H., Choi, C., and Kim, B.-S., *Engineering of volume-stable adipose tissues*. Biomaterials, 2005. **26**(17): p. 3577-3585.
103. Alhadlaq, A., Tang, M., and Mao, J., *Engineered adipose tissue from human mesenchymal stem cells maintains predefined shape and dimension: implications in soft tissue augmentation and reconstruction*. Tissue engineering, 2005. **11**(3-4): p. 556-566.
104. Fernandes, H., Mentink, A., Bank, R., Stoop, R., van Blitterswijk, C., and de Boer, J., *Endogenous collagen influences differentiation of human multipotent mesenchymal stromal cells*. Tissue engineering. Part A, 2010. **16**(5): p. 1693-1702.

105. von Heimburg, D., Zachariah, S., Heschel, I., Kühling, H., Schoof, H., Hafemann, B., and Pallua, N., *Human preadipocytes seeded on freeze-dried collagen scaffolds investigated in vitro and in vivo*. *Biomaterials*, 2001. **22**(5): p. 429-438.
106. Kawaguchi, N., Toriyama, K., Nicodemou-Lena, E., Inou, K., Torii, S., and Kitagawa, Y., *De novo adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor*. *Proceedings of the National Academy of Sciences*, 1998. **95**.
107. Abberton, K., Bortolotto, S., Woods, A., Findlay, M., Morrison, W., Thompson, E., and Messina, A., *Myogel, a novel, basement membrane-rich, extracellular matrix derived from skeletal muscle, is highly adipogenic in vivo and in vitro*. *Cells, tissues, organs*, 2008. **188**(4): p. 347-358.
108. Kimura, Y., Ozeki, M., Inamoto, T., and Tabata, Y., *Adipose tissue engineering based on human preadipocytes combined with gelatin microspheres containing basic fibroblast growth factor*. *Biomaterials*, 2003. **24**(14): p. 2513-2521.
109. Hiraoka, Y., Yamashiro, H., Yasuda, K., Kimura, Y., Inamoto, T., and Tabata, Y., *In situ regeneration of adipose tissue in rat fat pad by combining a collagen scaffold with gelatin microspheres containing basic fibroblast growth factor*. *Tissue engineering*, 2006. **12**(6): p. 1475-1487.
110. Flynn, L., Prestwich, G., Semple, J., and Woodhouse, K., *Adipose tissue engineering in vivo with adipose-derived stem cells on naturally derived scaffolds*. *Journal of biomedical materials research. Part A*, 2009. **89**(4): p. 929-941.
111. Mandal, B. and Kundu, S., *Osteogenic and adipogenic differentiation of rat bone marrow cells on non-mulberry and mulberry silk gland fibroin 3D scaffolds*. *Biomaterials*, 2009. **30**(28): p. 5019-5030.
112. Moya, M., Cheng, M.-H., Huang, J.-J., Francis-Sedlak, M., Kao, S.-W., Opara, E., and Brey, E., *The effect of FGF-1 loaded alginate microbeads on neovascularization and adipogenesis in a vascular pedicle model of adipose tissue engineering*. *Biomaterials*, 2010. **31**(10): p. 2816-2826.
113. Patel, P., Gobin, A., West, J., and Patrick, C., *Poly(ethylene glycol) hydrogel system supports preadipocyte viability, adhesion, and proliferation*. *Tissue engineering*, 2005. **11**(9-10): p. 1498-1505.
114. Patrick, C., *Adipose tissue engineering: the future of breast and soft tissue reconstruction following tumor resection*. *Seminars in surgical oncology*, 2000. **19**(3): p. 302-311.
115. Hemmrich, K., von Heimburg, D., Rendchen, R., Di Bartolo, C., Milella, E., and Pallua, N., *Implantation of preadipocyte-loaded hyaluronic acid-based scaffolds into nude mice to evaluate potential for soft tissue engineering*. *Biomaterials*, 2005. **26**(34): p. 7025-7037.
116. Mauney, J., Nguyen, T., Gillen, K., Kirker-Head, C., Gimble, J., and Kaplan, D., *Engineering adipose-like tissue in vitro and in vivo utilizing human bone marrow and adipose-derived mesenchymal stem cells with silk fibroin 3D scaffolds*. *Biomaterials*, 2007. **28**(35): p. 5280-5290.
117. Walton, R., Beahm, E., and Wu, L., *De novo adipose formation in a vascularized engineered construct*. *Microsurgery*, 2004. **24**(5): p. 378-384.

118. Stacey, D., Hanson, S., Lahvis, G., Gutowski, K., and Masters, K., *In vitro adipogenic differentiation of preadipocytes varies with differentiation stimulus, culture dimensionality, and scaffold composition*. Tissue engineering. Part A, 2009. **15**(11): p. 3389-3399.
119. Hemmrich, K. and von Heimburg, D., *Biomaterials for adipose tissue engineering*. Expert Rev Med Devices, 2006. **3**(5): p. 635-45.
120. Arslan, E., Garip, I., Gulseren, G., Tekinay, A., and Guler, M., *Bioactive supramolecular peptide nanofibers for regenerative medicine*. Advanced Healthcare Materials, 2014.
121. Sargeant, T., Oppenheimer, S., Dunand, D., and Stupp, S., *Titanium foam-bioactive nanofiber hybrids for bone regeneration*. Journal of tissue engineering and regenerative medicine, 2008. **2**(8): p. 455-462.
122. Kim, H.-W., Kim, H.-E., and Salih, V., *Stimulation of osteoblast responses to biomimetic nanocomposites of gelatin-hydroxyapatite for tissue engineering scaffolds*. Biomaterials, 2005. **26**(25): p. 5221-5230.
123. Zhang, Y., Ni, M., Zhang, M., and Ratner, B., *Calcium phosphate-chitosan composite scaffolds for bone tissue engineering*. Tissue engineering, 2003. **9**(2): p. 337-345.
124. Zhang, P., Hong, Z., Yu, T., Chen, X., and Jing, X., *In vivo mineralization and osteogenesis of nanocomposite scaffold of poly(lactide-co-glycolide) and hydroxyapatite surface-grafted with poly(L-lactide)*. Biomaterials, 2009. **30**(1): p. 58-70.
125. Franz, S., Rammelt, S., Scharnweber, D., and Simon, J., *Immune responses to implants - a review of the implications for the design of immunomodulatory biomaterials*. Biomaterials, 2011. **32**(28): p. 6692-6709.
126. Takayanagi, H., *Inflammatory bone destruction and osteoimmunology*. Journal of periodontal research, 2005. **40**(4): p. 287-293.
127. Scott, M., Nguyen, V., Levi, B., and James, A., *Current methods of adipogenic differentiation of mesenchymal stem cells*. Stem cells and development, 2011. **20**(10): p. 1793-1804.
128. Kisiday, J., Jin, M., Kurz, B., Hung, H., Semino, C., Zhang, S., and Grodzinsky, A., *Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair*. Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(15): p. 9996-10001.
129. Taipaleenmäki, H., *Factors Regulating Chondrogenic Differentiation*. Annales Universitatis Turkuensis D 938, 2010.
130. Furumatsu, T., Ozaki, T., and Asahara, H., *Smad3 activates the Sox9-dependent transcription on chromatin*. The International Journal of Biochemistry & Cell Biology, 2009. **41**(5): p. 1198-1204.
131. Ho, S., Yang, Z., Hui, H., Oh, K., Choo, B., and Lee, E., *A Serum Free Medium that Conserves The Chondrogenic Phenotype of In Vitro Expanded Chondrocytes*, in *13th International Conference on Biomedical Engineering*, C. Lim and J.H. Goh, Editors. 2009, Springer Berlin Heidelberg. p. 1255-1257.
132. Anderson, J., Vines, J., Patterson, J., Chen, H., Javed, A., and Jun, H.-W., *Osteogenic differentiation of human mesenchymal stem cells synergistically*

- enhanced by biomimetic peptide amphiphiles combined with conditioned medium. *Acta biomaterialia*, 2011. **7**(2): p. 675-682.
133. Baino, F. and Vitale-Brovarone, C., *Three-dimensional glass-derived scaffolds for bone tissue engineering: current trends and forecasts for the future*. *Journal of biomedical materials research. Part A*, 2011. **97**(4): p. 514-535.
 134. Barralet, J., Wang, L., Lawson, M., Triffitt, J., Cooper, P., and Shelton, R., *Comparison of bone marrow cell growth on 2D and 3D alginate hydrogels*. *Journal of materials science. Materials in medicine*, 2005. **16**(6): p. 515-519.
 135. Birmingham, E., Niebur, G., McHugh, P., and Shaw..., G., *Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche*. *Eur Cell ...*, 2012.
 136. Burg, K., Porter, S., and Kellam, J., *Biomaterial developments for bone tissue engineering*. *Biomaterials*, 2000.
 137. Fiedler, T., Salamon, A., Adam, S., Herzmann, N., Taubenheim, J., and Peters, K., *Impact of bacteria and bacterial components on osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells*. *Experimental cell research*, 2013.
 138. Guo, X., Park, H., Liu, G., Liu, W., Cao, Y., Tabata, Y., Kasper, F., and Mikos, A., *In vitro generation of an osteochondral construct using injectable hydrogel composites encapsulating rabbit marrow mesenchymal stem cells*. *Biomaterials*, 2009. **30**(14): p. 2741-2752.
 139. Handschel, J., Berr, K., Depprich, R., Kübler, N., Naujoks, C., Wiesmann, H.-P., Ommerborn, M., and Meyer, U., *Induction of osteogenic markers in differentially treated cultures of embryonic stem cells*. *Head & face medicine*, 2008. **4**: p. 10.
 140. Hee, C., Jonikas, M., and Nicoll, S., *Influence of three-dimensional scaffold on the expression of osteogenic differentiation markers by human dermal fibroblasts*. *Biomaterials*, 2006. **27**(6): p. 875-884.
 141. Holzwarth, J. and Ma, P., *Biomimetic nanofibrous scaffolds for bone tissue engineering*. *Biomaterials*, 2011. **32**(36): p. 9622-9629.
 142. Jones, E. and Yang, X., *Mesenchymal stem cells and bone regeneration: current status*. *Injury*, 2011. **42**(6): p. 562-568.
 143. Kruger, T., Miller, A., and Wang, J., *Collagen scaffolds in bone sialoprotein-mediated bone regeneration*. *TheScientificWorldJournal*, 2013. **2013**: p. 812718.
 144. Kyllönen, L., Haimi, S., Mannerström, B., Huhtala, H., Rajala, K., Skottman, H., Sándor, G., and Miettinen, S., *Effects of different serum conditions on osteogenic differentiation of human adipose stem cells in vitro*. *Stem cell research & therapy*, 2013. **4**(1): p. 17.
 145. Mallick, K., Winnett, J., van Grunsven, W., Lapworth, J., and Reilly, G., *Three-dimensional porous bioscaffolds for bone tissue regeneration: fabrication via adaptive foam reticulation and freeze casting techniques, characterization, and cell study*. *Journal of biomedical materials research. Part A*, 2012. **100**(11): p. 2948-2959.
 146. Mandal, B. and Kundu, S., *Non-mulberry silk gland fibroin protein 3-D scaffold for enhanced differentiation of human mesenchymal stem cells into osteocytes*. *Acta biomaterialia*, 2009. **5**(7): p. 2579-2590.

147. Mygind, T., Stiehler, M., Baatrup, A., Li, H., Zou, X., Flyvbjerg, A., Kassem, M., and Bünger, C., *Mesenchymal stem cell ingrowth and differentiation on coralline hydroxyapatite scaffolds*. *Biomaterials*, 2007. **28**(6): p. 1036-1047.
148. Niemeyer, P., Fechner, K., Milz, S., Richter, W., Suedkamp, N., Mehlhorn, A., Pearce, S., and Kasten, P., *Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma*. *Biomaterials*, 2010. **31**(13): p. 3572-3579.
149. Poldervaart, M., Wang, H., van der Stok, J., Weinans, H., Leeuwenburgh, S., Öner, F., Dhert, W., and Alblas, J., *Sustained release of BMP-2 in bioprinted alginate for osteogenicity in mice and rats*. *PLoS ONE*, 2013. **8**(8).
150. Shen, F., Werner, B., Liang, H., Shang, H., Yang, N., Li, X., Shimer, A., Balian, G., and Katz, A., *Implications of adipose-derived stromal cells in a 3D culture system for osteogenic differentiation: an in vitro and in vivo investigation*. *The spine journal : official journal of the North American Spine Society*, 2013. **13**(1): p. 32-43.
151. Sommar, P., Pettersson, S., Ness, C., Johnson, H., Kratz, G., and Junker, J., *Engineering three-dimensional cartilage- and bone-like tissues using human dermal fibroblasts and macroporous gelatine microcarriers*. *Journal of plastic, reconstructive & aesthetic surgery : JPRAS*, 2010. **63**(6): p. 1036-1046.
152. Sundelacruz, S. and Kaplan, D., *Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine*. *Seminars in cell & developmental biology*, 2009. **20**(6): p. 646-655.
153. Trojani, C., Weiss, P., Michiels, J.-F., Vinatier, C., Guicheux, J., Daculsi, G., Gaudray, P., Carle, G., and Rochet, N., *Three-dimensional culture and differentiation of human osteogenic cells in an injectable hydroxypropylmethylcellulose hydrogel*. *Biomaterials*, 2005. **26**(27): p. 5509-5517.
154. Vaccaro, A., *The role of the osteoconductive scaffold in synthetic bone graft*. *Orthopedics*, 2002.
155. Gandavarapu, N., Mariner, P., Schwartz, M., and Anseth, K., *Extracellular matrix protein adsorption to phosphate-functionalized gels from serum promotes osteogenic differentiation of human mesenchymal stem cells*. *Acta biomaterialia*, 2013. **9**(1): p. 4525-4534.
156. Rickard, D., Sullivan, T., Shenker, B., Leboy, P., and Kazhdan, I., *Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2*. *Developmental biology*, 1994. **161**(1): p. 218-228.
157. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P., *Molecular biology of the cell*. New York: Garland Science, 2008. **1**.
158. Nuttelman, C., Tripodi, M., and Anseth, K., *Dexamethasone-functionalized gels induce osteogenic differentiation of encapsulated hMSCs*. *Journal of biomedical materials research. Part A*, 2006. **76**(1): p. 183-195.
159. Hitomi, K., Torii, Y., and Tsukagoshi, N., *Increase in the Activity of Alkaline-Phosphatase by L-Ascorbic-Acid 2-Phosphate in a Human Osteoblast Cell-Line, Huo-3n1*. *Journal of Nutritional Science and Vitaminology*, 1992. **38**(6): p. 535-544.

160. Garcia Palacios, V., Morita, I., and Murota, S., *Expression of adipogenesis markers in a murine stromal cell line treated with 15-deoxy Delta(12,14)-prostaglandin J2, interleukin-11, 9-cis retinoic acid and vitamin K2*. Prostaglandins, leukotrienes, and essential fatty acids, 2001. **65**(4): p. 215-221.
161. Yu, C., Bianco, J., Brown, C., Fuetterer, L., Watkins, J., Samani, A., and Flynn, L., *Porous decellularized adipose tissue foams for soft tissue regeneration*. Biomaterials, 2013. **34**(13): p. 3290-3302.
162. Neubauer, M., Hacker, M., Bauer-Kreisel, P., Weiser, B., Fischbach, C., Schulz, M., Goepferich, A., and Blunk, T., *Adipose tissue engineering based on mesenchymal stem cells and basic fibroblast growth factor in vitro*. Tissue engineering, 2005. **11**(11-12): p. 1840-1851.
163. Barak, Y., Nelson, M., Ong, E., Jones, Y., Ruiz-Lozano, P., Chien, K., Koder, A., and Evans, R., *PPAR gamma is required for placental, cardiac, and adipose tissue development*. Molecular cell, 1999. **4**(4): p. 585-595.
164. Tontonoz, P., Kim, J., Graves, R., and Spiegelman, B., *ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation*. Molecular and cellular biology, 1993. **13**(8): p. 4753-4759.
165. Hunt, C., Ro, J., Dobson, D., Min, H., and Spiegelman, B., *Adipocyte P2 gene: developmental expression and homology of 5'-flanking sequences among fat cell-specific genes*. Proceedings of the National Academy of Sciences of the United States of America, 1986. **83**(11): p. 3786-3790.
166. Smas, C. and Sul, H., *Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation*. Cell, 1993. **73**(4): p. 725-734.
167. Wang, N., Finegold, M., Bradley, A., Ou, C., Abdelsayed, S., Wilde, M., Taylor, L., Wilson, D., and Darlington, G., *Impaired energy homeostasis in C/EBP alpha knockout mice*. Science (New York, N.Y.), 1995. **269**(5227): p. 1108-1112.
168. Valonen, P., Moutos, F., Kusanagi, A., Moretti, M., Diekman, B., Welter, J., Caplan, A., Guilak, F., and Freed, L., *In vitro generation of mechanically functional cartilage grafts based on adult human stem cells and 3D-woven poly(epsilon-caprolactone) scaffolds*. Biomaterials, 2010. **31**(8): p. 2193-2200.
169. Buschmann, M. and Grodzinsky, A., *A molecular model of proteoglycan-associated electrostatic forces in cartilage mechanics*. Journal of biomechanical engineering, 1995. **117**(2): p. 179-192.
170. Nap, R. and Szleifer, I., *Structure and interactions of aggrecans: statistical thermodynamic approach*. Biophysical journal, 2008. **95**(10): p. 4570-4583.
171. Lee, B., Han, L., Frank, E., Chubinskaya, S., Ortiz, C., and Grodzinsky, A., *Dynamic mechanical properties of the tissue-engineered matrix associated with individual chondrocytes*. Journal of biomechanics, 2010. **43**(3): p. 469-476.
172. Lee, H.Y., Kopesky, P., Plaas, A., Sandy, J., Kisiday, J., Frisbie, D., Grodzinsky, A., and Ortiz, C., *Adult bone marrow stromal cell-based tissue-engineered aggrecan exhibits ultrastructure and nanomechanical properties superior to native cartilage*. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society, 2010. **18**(11): p. 1477-1486.

173. Hakan, C., Samet, K., Ayse, B.T., and Mustafa, O.G., *Surface-adhesive and osteogenic self-assembled peptide nanofibers for bioinspired functionalization of titanium surfaces*. Soft Matter, 2012. **8**.
174. Kocabey, S., Ceylan, H., Tekinay, A., and Guler, M., *Glycosaminoglycan mimetic peptide nanofibers promote mineralization by osteogenic cells*. Acta biomaterialia, 2013. **9**(11): p. 9075-9085.
175. Ellis, E.G. and Kathleen, B.-B., *The role of alkaline phosphatase in mineralization*. Current Opinion in Orthopaedics, 2007. **18**.
176. *Calcification as an indicator of osteoinductive capacity of biomaterials in osteoblastic cell cultures*. Biomaterials, 2005. **26**(24): p. 4964-4974.
177. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. *Molecular Biology of the Cell*. 2002; 5th:[Available from: <http://www.ncbi.nlm.nih.gov/books/NBK21054/>].
178. Tsang, K.Y., Cheung, M.C., Chan, D., and Cheah, K.S., *The developmental roles of the extracellular matrix: beyond structure to regulation*. Cell and tissue research, 2010. **339**(1): p. 93-110.
179. Brown, N., *Extracellular matrix in development: Insights from mechanisms conserved between invertebrates and vertebrates*. Cold Spring Harbor Perspectives in Biology, 2011. **3**(12).
180. Kleinman, H., Philp, D., and Hoffman, M., *Role of the extracellular matrix in morphogenesis*. Current Opinion in Biotechnology, 2003. **14**(5).
181. Raghunath, J., Salacinski, H., Sales, K., Butler, P., and Seifalian, A., *Advancing cartilage tissue engineering: The application of stem cell technology*. Current Opinion in Biotechnology, 2005. **16**(5).
182. Amini, A., Laurencin, C., and Nukavarapu, S., *Bone tissue engineering: recent advances and challenges*. Critical reviews in biomedical engineering, 2012. **40**(5).
183. Larsen, M., Artym, V., Green, J., and Yamada, K., *The matrix reorganized: extracellular matrix remodeling and integrin signaling*. Current Opinion in Cell Biology, 2006. **18**(5).
184. Jacquemet, G., Humphries, M., and Caswell, P., *Role of adhesion receptor trafficking in 3D cell migration*. Current Opinion in Cell Biology, 2013. **25**(5).
185. Frantz, C., Stewart, K.M., and Weaver, V.M., *The extracellular matrix at a glance*. Journal of cell science, 2010. **123**(24): p. 4195-4200.
186. Lu, P., Weaver, V.M., and Werb, Z., *The extracellular matrix: a dynamic niche in cancer progression*. The Journal of Cell Biology, 2012. **196**(4): p. 395-406.
187. Gentili, C. and Cancedda, R., *Cartilage and bone extracellular matrix*. Current pharmaceutical design, 2009. **15**(12).
188. Ricard-Blum, S. and Ruggiero, F., *The collagen superfamily: from the extracellular matrix to the cell membrane*. Pathologie-biologie, 2005. **53**(7): p. 430-442.
189. Ricard-Blum, S., *The collagen family*. Cold Spring Harbor perspectives in biology, 2011. **3**(1).
190. Mouw, J., Ou, G., and Weaver, V., *Extracellular matrix assembly: a multiscale deconstruction*. Nature Reviews Molecular Cell Biology, 2014. **15**(12).

191. Myllyharju, J. and Kivirikko, K.I., *Collagens, modifying enzymes and their mutations in humans, flies and worms*. TRENDS in Genetics, 2004.
192. Miner, J.H., *Laminins and their roles in mammals*. Microsc Res Tech, 2008. **71**(5): p. 349-56.
193. Timpl, R. and Brown, J.C., *The laminins*. Matrix biology : journal of the International Society for Matrix Biology, 1994. **14**(4): p. 275-281.
194. Aumailley, M., Bruckner-Tuderman, L., Carter, W.G., Deutzmann, R., Edgar, D., Ekblom, P., et al., *A simplified laminin nomenclature*. Matrix biology : journal of the International Society for Matrix Biology, 2005. **24**(5): p. 326-332.
195. Wise, S.G. and Weiss, A.S., *Tropoelastin*. The international journal of biochemistry & cell biology, 2009. **41**(3): p. 494-497.
196. Smith, M.L., Gourdon, D., Little, W.C., Kubow, K.E., Eguiluz, R.A., Luna-Morris, S., and Vogel, V., *Force-induced unfolding of fibronectin in the extracellular matrix of living cells*. PLoS biology, 2007. **5**(10).
197. Rozario, T. and DeSimone, D.W., *The extracellular matrix in development and morphogenesis: a dynamic view*. Developmental biology, 2010. **341**(1): p. 126-140.
198. Lukashev, M. and Werb, Z., *ECM signalling: Orchestrating cell behaviour and misbehaviour*. Trends in Cell Biology, 1998. **8**(11).
199. Singh, P., Carraher, C., and Schwarzbauer, J.E., *Assembly of fibronectin extracellular matrix*. Annual review of cell and developmental biology, 2010. **26**: p. 397-419.
200. Arnaout, M.A. and Mahalingam, B., *Integrin structure, allostery, and bidirectional signaling*. Annu. Rev. Cell Dev. Biol., 2005.
201. Yanagishita, M., *Function of proteoglycans in the extracellular matrix*. Acta Pathol Jpn, 1993. **43**(6): p. 283-93.
202. Roden, L., *Structure and metabolism of connective tissue proteoglycans*. Springer, 1980: p. 267-371.
203. Jiang, D., Liang, J., and Noble, P., *Hyaluronan in tissue injury and repair*. Annual review of cell and developmental biology, 2007. **23**: p. 435-461.
204. Baeurle, S.A., Kiselev, M.G., Makarova, E.S., and Nogovitsin, E.A., *Effect of the counterion behavior on the frictional-compressive properties of chondroitin sulfate solutions*. Polymer, 2009. **50**.
205. Galtrey, C. and Fawcett, J., *The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system*. Brain research reviews, 2007. **54**(1): p. 1-18.
206. Shinmyouzu, K., Takahashi, T., Ariyoshi, W., Ichimiya, H., Kanzaki, S., and Nishihara, T., *Dermatan sulfate inhibits osteoclast formation by binding to receptor activator of NF-kappa B ligand*. Biochemical and Biophysical Research Communications, 2007. **354**(2): p. 447-452.
207. Vitale, C., Verdecchia, C., Bagnis, C., Ganzaroli, M., Giorcelli, G., and Marangella, M., *Effects of dermatan sulfate for anticoagulation in continuous renal replacement therapy*. Journal of nephrology, 2008. **21**(2): p. 205-212.
208. Dreyfuss, J., Regatieri, C., Jarrouge, T., Cavalheiro, R., Sampaio, L., and Nader, H., *Heparan sulfate proteoglycans: structure, protein interactions and cell signaling*. Anais da Academia Brasileira de Ciências, 2009. **81**(3): p. 409-429.

209. Jiao, X., Billings, P., O'Connell, M., Kaplan, F., Shore, E., and Glaser, D., *Heparan sulfate proteoglycans (HSPGs) modulate BMP2 osteogenic bioactivity in C2C12 cells*. The Journal of biological chemistry, 2007. **282**(2): p. 1080-1086.
210. Rabenstein, D., *Heparin and heparan sulfate: structure and function*. Natural product reports, 2002. **19**(3): p. 312-331.
211. Funderburgh, J., *Keratan sulfate: structure, biosynthesis, and function*. Glycobiology, 2000. **10**(10): p. 951-958.
212. Kitayama, K., Hayashida, Y., Nishida, K., and Akama, T., *Enzymes responsible for synthesis of corneal keratan sulfate glycosaminoglycans*. The Journal of biological chemistry, 2007. **282**(41): p. 30085-30096.
213. Jones, L. and Tuszynski, M., *Spinal cord injury elicits expression of keratan sulfate proteoglycans by macrophages, reactive microglia, and oligodendrocyte progenitors*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2002. **22**(11): p. 4611-4624.
214. Kim, S., Turnbull, J., and Guimond, S., *Extracellular matrix and cell signalling: The dynamic cooperation of integrin, proteoglycan and growth factor receptor*. Journal of Endocrinology, 2011. **209**(2).
215. Ferdous, Z. and Grande-Allen, K.J., *Utility and control of proteoglycans in tissue engineering*. Tissue engineering, 2007.
216. Shapiro, S., *Matrix metalloproteinase degradation of extracellular matrix: biological consequences*. Curr.Opin.Cell Biol., 1998. **10**.
217. *3D Cell Culture Systems: Advantages and Applications*. Journal of Cellular Physiology, 2015. **230**(1): p. 16-26.
218. *Tissue Engineering and Regenerative Medicine: History, Progress, and Challenges*. Chemical and Biomolecular Engineering, 2011. **2**(1): p. 403-430.
219. *Whole-Organ Tissue Engineering: Decellularization and Recellularization of Three-Dimensional Matrix Scaffolds*. Biomedical Engineering, 2011. **13**(1): p. 27-53.
220. Falguni, P., Jinah, J., Dong-Heon, H., Sung Won, K., Jong-Won, R., Jin-Hyung, S., Deok-Ho, K., and Dong-Woo, C., *Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink*. Nature Communications, 2014. **5**.
221. *3D co-culturing model of primary pancreatic islets and hepatocytes in hybrid spheroid to overcome pancreatic cell shortage*. Biomaterials, 2013. **34**(15): p. 3784-3794.
222. *Biomaterials in Tissue Engineering*. Nature Biotechnology, 1995. **13**(6): p. 565-576.
223. Higuchi, A., Ling, Q.-D.D., Ko, Y.-A.A., Chang, Y., and Umezawa, A., *Biomaterials for the feeder-free culture of human embryonic stem cells and induced pluripotent stem cells*. Chemical reviews, 2011. **111**(5): p. 3021-3035.
224. *Biomimetic self-assembling peptides as scaffolds for soft tissue engineering*. Nanomedicine (London, England), 2013. **8**(5): p. 823-847.
225. Shuming, Z., Megan, A.G., Alvaro, M., Liam, C.P., Ronit, B., Jason, R.M., Conrado, A., Monica Olvera de la, C., and Samuel, I.S., *A self-assembly pathway to aligned monodomain gels*. Nature Materials, 2010.

226. Goktas, M., Cinar, G., Orujalipoor, I., Ide, S., Tekinay, A., and Guler, M., *Self-Assembled Peptide Amphiphile Nanofibers and PEG Composite Hydrogels as Tunable ECM Mimetic Microenvironment*. Biomacromolecules, 2015.
227. Lane, S., Williams, D., and Watt, F., *Modulating the stem cell niche for tissue regeneration*. Nature biotechnology, 2014. **32**(8).
228. Boekhoven, J. and Stupp, S., *25th anniversary article: Supramolecular materials for regenerative medicine*. Advanced Materials, 2014. **26**(11).
229. Bressan, E., Carraro, A., Ferroni, L., Gardin, C., Sbricoli, L., Guazzo, R., Stellini, E., Roman, M., Pinton, P., Sivoletta, S., and Zavan, B., *Nanotechnology to drive stem cell commitment*. Nanomedicine (Lond), 2013. **8**(3): p. 469-86.
230. Higuchi, A., Ling, Q.-D., Hsu, S.-T., and Umezawa, A., *Biomimetic Cell Culture Proteins as Extracellular Matrices for Stem Cell Differentiation*. Chemical Reviews, 2012. **112**(8).
231. Zhang, S., *Designer self-assembling Peptide nanofiber scaffolds for study of 3-d cell biology and beyond*. Adv Cancer Res, 2008. **99**(07): p. 335-62.
232. Oh, Y.I., Sheng, G.J., Chang, S.-K.K., and Hsieh-Wilson, L.C., *Tailored glycopolymers as anticoagulant heparin mimetics*. Angewandte Chemie (International ed. in English), 2013. **52**(45): p. 11796-11799.
233. Scott, R. and Panitch, A., *Glycosaminoglycans in biomedicine*. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2013. **5**(4).
234. Hintze, V., Samsonov, S., Anselmi, M., Moeller, S., Becher, J., Schnabelrauch, M., Scharnweber, D., and Pisabarro, M., *Sulfated glycosaminoglycans exploit the conformational plasticity of bone morphogenetic protein-2 (BMP-2) and alter the interaction profile with its receptor*. Biomacromolecules, 2014. **15**(8).
235. Mammadov, R., Mammadov, B., Guler, M., and Tekinay, A., *Growth factor binding on heparin mimetic peptide nanofibers*. Biomacromolecules, 2012. **13**(10).
236. Mammadov, R., Mammadov, B., Toksoz, S., Aydin, B., Yagci, R., Tekinay, A., and Guler, M., *Heparin mimetic peptide nanofibers promote angiogenesis*. Biomacromolecules, 2011. **12**(10).
237. Kocabey, S., Ceylan, H., Tekinay, A., and Guler, M., *Glycosaminoglycan mimetic peptide nanofibers promote mineralization by osteogenic cells*. Acta Biomaterialia, 2013. **9**(11).
238. Strano, M., *Biomedical materials: Nanoscale radiosurgery*. Nature materials, 2010. **9**(6).
239. Bernardes, G., Kikkeri, R., Maglinao, M., Laurino, P., Collot, M., Hong, S., Lepenies, B., and Seeberger, P., *Design, synthesis and biological evaluation of carbohydrate-functionalized cyclodextrins and liposomes for hepatocyte-specific targeting*. Organic & biomolecular chemistry, 2010. **8**(21).
240. Li, J., Ma, F.-K., Dang, Q.-F., Liang, X.-G., and Chen, X.-G., *Glucose-conjugated chitosan nanoparticles for targeted drug delivery and their specific interaction with tumor cells*. Frontiers of Materials Science, 2014. **8**(4).

241. Shastri, P., Lamichhane, S., Arya, N., Ojha, N., and Kohler, E., *Glycosaminoglycan-functionalized poly-lactide-co-glycolide nanoparticles: synthesis, characterization, cytocompatibility, and cellular uptake*. International Journal of Nanomedicine, 2015.
242. Chiodo, F., Marradi, M., Calvo, J., Yuste, E., and Penadés, S., *Glycosystems in nanotechnology: Gold glyconanoparticles as carrier for anti-HIV prodrugs*. Beilstein Journal of Organic Chemistry, 2014. **10**.
243. Liu, J., Gray, W., Davis, M., and Luo, Y., *Peptide- and saccharide-conjugated dendrimers for targeted drug delivery: a concise review*. Interface Focus, 2012. **2**(3).
244. Li, X., Wu, P., Gao, G., and Cheng, S., *Carbohydrate-functionalized chitosan fiber for influenza virus capture*. Biomacromolecules, 2011. **12**(11).
245. Slaney, A., Wright, V., Meloncelli, P., Harris, K., West, L., Lowary, T., and Buriak, J., *Biocompatible carbohydrate-functionalized stainless steel surfaces: A new method for passivating biomedical implants*. ACS Applied Materials and Interfaces, 2011. **3**(5).
246. Kim, I.L., Khetan, S., Baker, B.M., Chen, C.S., and Burdick, J.A., *Fibrous hyaluronic acid hydrogels that direct MSC chondrogenesis through mechanical and adhesive cues*. Biomaterials, 2013. **34**(22): p. 5571-80.
247. Niece, K.L., Czeisler, C., Sahni, V., Tysseling-Mattiace, V., Pashuck, E.T., Kessler, J.A., and Stupp, S.I., *Modification of gelation kinetics in bioactive peptide amphiphiles*. Biomaterials, 2008. **29**(34): p. 4501-9.
248. Ali, A., Penny, C.B., Paiker, J.E., Niekerk, C., Smit, A., Ferris, W.F., and Crowther, N.J., *Alkaline phosphatase is involved in the control of adipogenesis in the murine preadipocyte cell line, 3T3-L1*. Clinica chimica acta, 2005. **354**(1): p. 101-109.
249. Bianco, P., Costantini, M., and Dearden, L.C., *Alkaline phosphatase positive precursors of adipocytes in the human bone marrow*. British journal of ..., 1988.
250. Miao, D. and Scutt, A., *Histochemical localization of alkaline phosphatase activity in decalcified bone and cartilage*. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 2002. **50**(3).
251. James, A.W., *Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation*. Scientifica (Cairo), 2013. **2013**: p. 684736.
252. Otto, F., Thornell, A.P., Crompton, T., Denzel, A., and Gilmour, K.C., *Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development*. Cell, 1997.
253. Traub, W., Arad, T., and Weiner, S., *Origin of mineral crystal growth in collagen fibrils*. Matrix, 1992. **12**(4): p. 251-5.
254. Even-Ram, S., Artym, V., and Yamada, K., *Matrix Control of Stem Cell Fate*. Cell, 2006. **126**(4).
255. Gregoire, F.M., Smas, C.M., and Sul, H.S., *Understanding adipocyte differentiation*. Physiological reviews, 1998.
256. Cristancho, A. and Lazar, M., *Forming functional fat: a growing understanding of adipocyte differentiation*. Nature Reviews Molecular Cell Biology, 2011. **12**(11).

257. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., and Waki, H., *Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase*. Nature medicine, 2002.
258. Ono, M. and Kitagawa, Y., *Acceleration of Adipose Conversion of 3T3-L1 Cells by Dextran Sulfate*. Bioscience, 1994.
259. Mariman, E. and Wang, P., *Adipocyte extracellular matrix composition, dynamics and role in obesity*. Cellular and molecular life sciences : CMLS, 2010. **67**(8).
260. Sever, M., Mammadov, B., Guler, M., and Tekinay, A., *Tenascin - C Mimetic Peptide Nano fi bers Direct Stem Cell Di ff erentiation to Osteogenic Lineage*. Tenascin - C Mimetic Peptide Nano fi bers Direct Stem Cell Di ff erentiation to Osteogenic Lineage, 2014.
261. Ruoslahti, E., *RGD and other recognition sequences for integrins*. Annual review of cell and developmental biology, 1996. **12**.
262. Kawakami, Y., Rodriguez-León, J., and Belmonte, J., *The role of TGFβs and Sox9 during limb chondrogenesis*. Current Opinion in Cell Biology, 2006. **18**(6).
263. Mackie, E., Ahmed, Y., Tatarczuch, L., Chen, K., and Mirams, M., *Endochondral ossification: How cartilage is converted into bone in the developing skeleton*. International Journal of Biochemistry and Cell Biology, 2008. **40**(1).
264. Hardingham, T., Beardmore-Gray, M., Dunham, D., and Ratcliffe, a., *Cartilage proteoglycans*. Ciba Foundation symposium, 1986. **124**.
265. Glant, T.T., Hadházy, C., Mikecz, K., and Sipos, A., *Appearance and persistence of fibronectin in cartilage*. Histochemistry, 1985. **82**(2): p. 149-158.
266. Kulyk, W.M., Upholt, W.B., and Kosher, R.A., *Fibronectin gene expression during limb cartilage differentiation*. Development (Cambridge, England), 1989. **106**(3): p. 449-455.
267. Chevalier, X., *Fibronectin, cartilage, and osteoarthritis*. Seminars in arthritis and rheumatism, 1993. **22**(5): p. 307-318.
268. Hwang, N.S., Varghese, S., Zhang, Z., and Elisseeff, J., *Chondrogenic differentiation of human embryonic stem cell-derived cells in arginine-glycine-aspartate-modified hydrogels*. Tissue Eng, 2006. **12**(9): p. 2695-706.
269. Uzunalli, G., Tumtas, Y., Delibasi, T., Yasa, O., Mercan, S., Guler, M.O., and Tekinay, A.B., *Improving Pancreatic Islet In Vitro Functionality and Transplantation Efficiency by Using Heparin Mimetic Peptide Nanofiber Gels*. Acta Biomaterialia, 2015.
270. Olefsky, J.M., *Prospects for research in diabetes mellitus*. JAMA, 2001. **285**(5): p. 628-32.
271. Barrett-Connor, E. and Khaw, K.T., *Diabetes mellitus: an independent risk factor for stroke?* Am J Epidemiol, 1988. **128**(1): p. 116-23.
272. Barrett-Connor, E. and Orchard, T.J., *Insulin-dependent diabetes mellitus and ischemic heart disease*. Diabetes Care, 1985. **8 Suppl 1**: p. 65-70.
273. Bild, D.E., Selby, J.V., Sinnock, P., Browner, W.S., Braveman, P., and Showstack, J.A., *Lower-extremity amputation in people with diabetes*. Epidemiology and prevention. Diabetes Care, 1989. **12**(1): p. 24-31.

274. Geiss, L.S., Herman, W.H., and Teutsch, S.M., *Diabetes and renal mortality in the United States*. Am J Public Health, 1985. **75**(11): p. 1325-6.
275. Moss, S.E., Klein, R., and Klein, B.E., *The incidence of vision loss in a diabetic population*. Ophthalmology, 1988. **95**(10): p. 1340-8.
276. Nathan, D.M., *Long-term complications of diabetes mellitus*. N Engl J Med, 1993. **328**(23): p. 1676-85.
277. LaPorte, R.E., Tajima, N., Akerblom, H.K., Berlin, N., Brosseau, J., Christy, M., Drash, A.L., Fishbein, H., Green, A., Hamman, R., and et al., *Geographic differences in the risk of insulin-dependent diabetes mellitus: the importance of registries*. Diabetes Care, 1985. **8 Suppl 1**: p. 101-7.
278. Bloomgarden, Z.T., *Diabetes complications*. Diabetes Care, 2004. **27**(6): p. 1506-14.
279. Narang, A.S. and Mahato, R.I., *Biological and biomaterial approaches for improved islet transplantation*. Pharmacol Rev, 2006. **58**(2): p. 194-243.
280. Rother, K. and Harlan, D., *Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus*. Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus., 2004. **114**.
281. Robertson, R.P., *Islet transplantation as a treatment for diabetes - a work in progress*. N Engl J Med, 2004. **350**(7): p. 694-705.
282. Pagliuca, F.W., Millman, J.R., Gurtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., and Melton, D.A., *Generation of functional human pancreatic beta cells in vitro*. Cell, 2014. **159**(2): p. 428-39.
283. Menger, M.D., Vajkoczy, P., Beger, C., and Messmer, K., *Orientation of microvascular blood flow in pancreatic islet isografts*. J Clin Invest, 1994. **93**(5): p. 2280-5.
284. Beger, C., Cirulli, V., Vajkoczy, P., Halban, P.A., and Menger, M.D., *Vascularization of purified pancreatic islet-like cell aggregates (pseudoislets) after syngeneic transplantation*. Diabetes, 1998. **47**(4): p. 559-65.
285. Furuya, H., Kimura, T., Murakami, M., Katayama, K., Hirose, K., and Yamaguchi, A., *Revascularization and function of pancreatic islet isografts in diabetic rats following transplantation*. Cell Transplant, 2003. **12**(5): p. 537-44.
286. Merchant, F.A., Diller, K.R., Aggarwal, S.J., and Bovik, A.C., *Angiogenesis in cultured and cryopreserved pancreatic islet grafts*. Transplantation, 1997. **63**(11): p. 1652-60.
287. Vajkoczy, P., Menger, M.D., Simpson, E., and Messmer, K., *Angiogenesis and vascularization of murine pancreatic islet isografts*. Transplantation, 1995. **60**(2): p. 123-7.
288. Mattsson, G., *The endothelial cells in islets of langerhans*. Ups J Med Sci, 2005. **110**(1): p. 1-15.
289. Vasir, B., Aiello, L.P., Yoon, K.H., Quickel, R.R., Bonner-Weir, S., and Weir, G.C., *Hypoxia induces vascular endothelial growth factor gene and protein expression in cultured rat islet cells*. Diabetes, 1998. **47**(12): p. 1894-903.
290. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E., and Ballinger, W.F., *Transplantation of isolated pancreatic islets into the portal vein of diabetic rats*. Nature, 1973. **244**(5416): p. 447.

291. Carlsson, P.O., Palm, F., Andersson, A., and Liss, P., *Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site*. Diabetes, 2001. **50**(3): p. 489-95.
292. Selawry, H.P. and Whittington, K., *Extended allograft survival of islets grafted into intra-abdominally placed testis*. Diabetes, 1984. **33**(4): p. 405-6.
293. Perez, V., Caicedo, A., Berman, D., Arrieta, E., Abdulreda, M., Rodriguez-Diaz, R., Pileggi, A., Hernandez, E., Dubovy, S., and Parel, J., *The anterior chamber of the eye as a clinical transplantation site for the treatment of diabetes: a study in a baboon model of diabetes*. Diabetologia, 2011. **54**(5): p. 1121-1126.
294. Kriz, J., Vilk, G., Mazzuca, D.M., Toleikis, P.M., Foster, P.J., and White, D.J., *A novel technique for the transplantation of pancreatic islets within a vascularized device into the greater omentum to achieve insulin independence*. Am J Surg, 2012. **203**(6): p. 793-7.
295. Merani, S., Toso, C., Emamaullee, J., and Shapiro, a., *Optimal implantation site for pancreatic islet transplantation*. Optimal implantation site for pancreatic islet transplantation, 2008. **95**.
296. Paraskevas, S., Maysinger, D., Wang, R., Duguid, T.P., and Rosenberg, L., *Cell loss in isolated human islets occurs by apoptosis*. Pancreas, 2000. **20**(3): p. 270-6.
297. Thomas, F., Wu, J., Contreras, J.L., Smyth, C., Bilbao, G., He, J., and Thomas, J., *A tripartite anoikis-like mechanism causes early isolated islet apoptosis*. Surgery, 2001. **130**(2): p. 333-8.
298. Lucas-Clerc, C., Massart, C., Champion, J.P., Launois, B., and Nicol, M., *Long-term culture of human pancreatic islets in an extracellular matrix: morphological and metabolic effects*. Mol Cell Endocrinol, 1993. **94**(1): p. 9-20.
299. Wang, R.N. and Rosenberg, L., *Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship*. Journal of Endocrinology, 1999. **163**(2): p. 181-190.
300. Beattie, G.M., Montgomery, A.M., Lopez, A.D., Hao, E., Perez, B., Just, M.L., Lakey, J.R., Hart, M.E., and Hayek, A., *A novel approach to increase human islet cell mass while preserving beta-cell function*. Diabetes, 2002. **51**(12): p. 3435-9.
301. Jiang, H.Z., Guler, M.O., and Stupp, S.I., *The internal structure of self-assembled peptide amphiphiles nanofibers*. Soft Matter, 2007. **3**(4): p. 454-462.
302. Stendahl, J.C., Rao, M.S., Guler, M.O., and Stupp, S.I., *Intermolecular forces in the self-assembly of peptide amphiphile nanofibers*. Advanced Functional Materials, 2006. **16**(4): p. 499-508.
303. Mata, A., Geng, Y., Henrikson, K.J., Aparicio, C., Stock, S.R., Satcher, R.L., and Stupp, S.I., *Bone regeneration mediated by biomimetic mineralization of a nanofiber matrix*. Biomaterials, 2010. **31**(23): p. 6004-6012.
304. Murphy, M.B., Blashki, D., Buchanan, R.M., Fan, D., De Rosa, E., Shah, R.N., Stupp, S.I., Weiner, B.K., Simmons, P.J., Ferrari, M., and Tasciotti, E., *Multi-composite bioactive osteogenic sponges featuring mesenchymal stem cells, platelet-rich plasma, nanoporous silicon enclosures, and Peptide*

- amphiphiles for rapid bone regeneration*. J Funct Biomater, 2011. **2**(2): p. 39-66.
305. Mammadov, B., Mammadov, R., Guler, M.O., and Tekinay, A.B., *Cooperative effect of heparan sulfate and laminin mimetic peptide nanofibers on the promotion of neurite outgrowth*. Acta biomaterialia, 2012. **8**(6): p. 2077-2086.
 306. Uzunalli, G., Soran, Z., Erkal, T.S., Dagdas, Y.S., Dinc, E., Hondur, A.M., Bilgihan, K., Aydin, B., Guler, M.O., and Tekinay, A.B., *Bioactive self-assembled peptide nanofibers for corneal stroma regeneration*. Acta biomaterialia, 2014. **10**(3): p. 1156-1166.
 307. Cui, H., Webber, M.J., and Stupp, S.I., *Self-assembly of peptide amphiphiles: From molecules to nanostructures to biomaterials*. Peptide Science, 2010. **94**(1): p. 1-18.
 308. Dagli Gul, A.S., Fadillioglu, E., Karabulut, I., Yesilyurt, A., and Delibasi, T., *The effects of oral carvacrol treatment against H₂O₂ induced injury on isolated pancreas islet cells of rats*. Islets, 2013. **5**(4): p. 149-55.
 309. Morgan, N.G., Diakogiannaki, E., and Russell, M.A., *The incubation and monitoring of cell viability in primary rat islets of Langerhans and pancreatic beta-cell lines*. Methods Mol Biol, 2009. **560**: p. 53-64.
 310. Carter, J.D., Dula, S.B., Corbin, K.L., Wu, R., and Nunemaker, C.S., *A practical guide to rodent islet isolation and assessment*. Biol Proced Online, 2009. **11**: p. 3-31.
 311. Ayala, J.E., Samuel, V.T., Morton, G.J., Obici, S., Croniger, C.M., Shulman, G.I., Wasserman, D.H., McGuinness, O.P., and Consortium, N.I.H.M.M.P.C., *Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice*. Disease models & mechanisms, 2010. **3**(9-10): p. 525-534.
 312. Borg, D. and Bonifacio, E., *The use of biomaterials in islet transplantation*. The use of biomaterials in islet transplantation, 2011. **11**.
 313. Brady, A.-c., Martino, M., Pedraza, E., Sukert, S., Pileggi, A., Ricordi, C., Hubbell, J., and Stabler, C., *Proangiogenic hydrogels within macroporous scaffolds enhance islet engraftment in an extrahepatic site*. Proangiogenic hydrogels within macroporous scaffolds enhance islet engraftment in an extrahepatic site., 2013. **19**.
 314. Kriz, J., Vilk, G., Mazzuca, D., Toleikis, P., Foster, P., and White, D., *A novel technique for the transplantation of pancreatic islets within a vascularized device into the greater omentum to achieve insulin independence*. A novel technique for the transplantation of pancreatic islets within a vascularized device into the greater omentum to achieve insulin independence, 2012. **203**.
 315. Rak, J. and Weitz, J.I., *Heparin and angiogenesis: size matters!* Arterioscler Thromb Vasc Biol, 2003. **23**(11): p. 1954-5.
 316. Stendahl, J.C., Kaufman, D.B., and Stupp, S.I., *Extracellular Matrix in Pancreatic Islets: Relevance to Scaffold Design and Transplantation*. Cell Transplantation, 2009. **18**(1): p. 1-12.
 317. Ilieva, A., Yuan, S., Wang, R.N., Agapitos, D., Hill, D.J., and Rosenberg, L., *Pancreatic islet cell survival following islet isolation: the role of cellular*

- interactions in the pancreas*. Journal of Endocrinology, 1999. **161**(3): p. 357-364.
318. Gelain, F., Bottai, D., Vescovi, A., and Zhang, S., *Designer self-assembling peptide nanofiber scaffolds for adult mouse neural stem cell 3-dimensional cultures*. PLoS One, 2006. **1**: p. e119.
 319. Wang, R.N. and Rosenberg, L., *Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship*. Journal of Endocrinology, 1999. **163**(2): p. 181-90.
 320. Bennet, W., Sundberg, B., Lundgren, T., Tibell, A., Groth, C.G., Richards, A., White, D.J., Elgue, G., Larsson, R., Nilsson, B., and Korsgren, O., *Damage to porcine islets of langerhans after exposure to human blood in vitro, or after intraportal transplantation to cynomolgus monkeys - Protective effects of sCR1 and heparin*. Transplantation, 2000. **69**(5): p. 711-719.
 321. Niece, K.L., Hartgerink, J.D., Donners, J.J., and Stupp, S.I., *Self-assembly combining two bioactive peptide-amphiphile molecules into nanofibers by electrostatic attraction*. J Am Chem Soc, 2003. **125**(24): p. 7146-7.
 322. Shah, S., Lowery, E., Braun, R.K., Martin, A., Huang, N., Medina, M., et al., *Cellular Basis of Tissue Regeneration by Omentum*. PLoS One, 2012. **7**(6).
 323. Merani, S., Toso, C., Emamaullee, J., and Shapiro, A.M., *Optimal implantation site for pancreatic islet transplantation*. Br J Surg, 2008. **95**(12): p. 1449-61.
 324. Daoud, J., Petropavlovskaja, M., Rosenberg, L., and Tabrizian, M., *The effect of extracellular matrix components on the preservation of human islet function in vitro*. Biomaterials, 2010. **31**(7): p. 1676-82.
 325. Chen, T., Yuan, J., Duncanson, S., Hibert, M.L., Kodish, B.C., Mylavaganam, G., et al., *Alginate Encapsulant Incorporating CXCL12 Supports Long-Term Allo- and Xenoislet Transplantation Without Systemic Immune Suppression*. Am J Transplant, 2015. **15**(3): p. 618-27.
 326. Lang, R., Rutschman, R.L., Greaves, D.R., and Murray, P.J., *Autocrine deactivation of macrophages in transgenic mice constitutively overexpressing IL-10 under control of the human CD68 promoter*. J Immunol, 2002. **168**(7): p. 3402-11.
 327. Meiron, M., Zohar, Y., Anunu, R., Wildbaum, G., and Karin, N., *CXCL12 (SDF-1alpha) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific regulatory T cells*. J Exp Med, 2008. **205**(11): p. 2643-55.
 328. Yano, T., Liu, Z., Donovan, J., Thomas, M.K., and Habener, J.F., *Stromal cell derived factor-1 (SDF-1)/CXCL12 attenuates diabetes in mice and promotes pancreatic beta-cell survival by activation of the prosurvival kinase Akt*. Diabetes, 2007. **56**(12): p. 2946-57.
 329. Liu, Z., Stanojevic, V., Avadhani, S., Yano, T., and Habener, J.F., *Stromal cell-derived factor-1 (SDF-1)/chemokine (C-X-C motif) receptor 4 (CXCR4) axis activation induces intra-islet glucagon-like peptide-1 (GLP-1) production and enhances beta cell survival*. Diabetologia, 2011. **54**(8): p. 2067-76.