A bioinspired three-dimensional graphene/chitosan hybrid scaffold for bone tissue engineering

Xue, Jingwen

2019

Xue, J. (2019). A bioinspired three-dimensional graphene/chitosan hybrid scaffold for bone tissue engineering. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/106428>

<https://doi.org/10.32657/10220/47958>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**A BIOINSPIRED THREE-DIMENSIONAL
GRAPHENE/CHITOSAN HYBRID SCAFFOLD FOR
BONE TISSUE ENGINEERING**

XUE JINGWEN

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2019

**A BIOINSPIRED THREE-DIMENSIONAL
GRAPHENE/CHITOSAN HYBRID SCAFFOLD FOR
BONE TISSUE ENGINEERING**

XUE JINGWEN

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of my original research work and has not been submitted for a higher degree to any other University or Institution.

March, 2019

.....

Date

.....

Xue Jingwen

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

March, 2019

.....

Date

.....
Prof Tan Thatt Yang Timothy

Authorship Attribution Statement

This thesis contains material from 1 papers in submission where I was the first author.

Chapter 4-6 is in submission as **Jingwen Xue**, Zhiping Zeng, Ziming He, Jing Liu, Cleo Choong, and Timothy Thatt Yang Tan. 2019. Bioinspired Novel 3D Electrochemically-Reduced Graphene Oxide/Chitosan Scaffold Coupling with Biphasic Electrical Stimulation on Promoting Osteoblast Functions.

The contributions of the co-authors are as follows:

- Prof. Timothy Tan provided the initial project direction and edited the manuscript drafts. Dr. Cleo choong also edited the manuscript drafts.
- I wrote and revised the manuscript drafts.
- I performed all the laboratory work at School of Chemical and Biomedical Engineering. I also analyzed the data.
- Dr. Zeng Zhiping assisted with the collection of Raman spectroscopy data.
- Dr. He Ziming and Dr. Liu Jing provided guidance in material fabrication.

March, 2019

.....

Date

.....

Xue Jingwen

Acknowledgements

I would like to express my deepest respect and most sincere gratitude to my supervisor, Assoc. Prof. Timothy Tan, and my co-supervisor, Dr. Cleo Choong, for their guidance and encouragement for all these years. All the work found in this dissertation is a direct result of their bright idea and inspirations. They also impart knowledge and experience to me and give me many good suggestions both in the research and for life.

Appreciation also goes out to all staff and colleagues in our groups for their kind help. Special thanks go to my seniors Dr. He Ziming and Dr. Liu Jing for their kind assistance and guidance on graphene work and Dr. Zhang Yan for her selfless help on my research work and life in Singapore.

Also, I would like to give my sincere thanks to my colleagues and friends, Dr. Khoo Siyun, Dr. Wei Wei, Dr. Wang Jun Kit, Dr. Chun Yongyao, Mr. Yu Zhongzheng and Dr. Zeng Zhiping for their great assistance and co-operation in my research and encouragement in my daily life.

Lastly but not least, I would like to thank my dearest parents and my dearest friend, Yu Hui, with all my heart and soul for their love and support.

Table of Contents

Acknowledgements	i
Table of Contents	iii
Table Captions	ix
Figure Captions.....	xi
Abbreviations	xv
Publications	xvii
Abstract.....	xix
Chapter 1 Introduction	1
1.1 Problem Statement	2
1.2 Objectives and Scope.....	7
1.3 Dissertation Overview	9
1.4 Findings and Outcomes/Originality	10
Chapter 2 Literature review.....	11
2.1 BTE Overview	12
2.1.1 Bone structure	13
2.1.2 Scaffolds.....	14
2.1.3 Bone cells	19
2.1.4 Signals	20
2.1.4.1 Roles of electrical signals in BTE	20
2.2 Graphene and its Derivatives	22
2.2.1 Introduction of graphene and its derivatives	22
2.2.1.1 Graphene oxide	23

2.2.1.2 RGO	24
2.2.1.3 Graphene composites in BTE.....	24
2.2.3 Biocompatibility and cytotoxicity of graphene and its derivatives	28
2.3 CHI.....	29
2.3.1 Introduction and properties of CHI	30
2.3.2 Techniques for fabricating 3D CHI scaffolds.....	31
2.3.3 CHI applications for tissue engineering	32
Chapter 3 Experimental Methodology	35
3.1 Materials.....	36
3.1.1 Graphene Oxide nanosheets	36
3.1.2 Chemical Reagents	36
3.1.3 Cell Culture Reagents.....	36
3.1.4 Cell Lines	36
3.2 Scaffold Fabrication	37
3.2.1 Fabrication of Three-dimensional (3D) RGO/CHI Scaffold.....	37
3.2.2 Fabrication of 3D CHI Scaffolds.....	38
3.2.3 Scaffold Rehydration.....	38
3.2.4 Sterilization and Preparation of Scaffold for Cellular Studies	39
3.3 Scaffold Properties Characterizations	39
3.3.1 Surface Characterizations.....	39
3.3.1.1 Atomic Force Microscopy Analysis.....	39
3.3.1.2 Field Emission Scanning Electron Microscopy (FESEM)	39
3.3.1.3 Fourier transform infrared spectroscopy (FTIR)	40
3.3.1.4 Raman spectroscopy.....	40
3.3.1.5 Energy dispersive X-ray spectrometer	40
3.3.1.6 BET	41
3.3.1.7 Zeta Potential Testing.....	41
3.3.1.8 X-ray diffraction analysis	41
3.3.2 Mechanical testing.....	41

3.3.3 Conductivity Study	42
3.3.4 Degradation Study	42
3.3.5 Bioactivity Study	43
3.3.6 Protein absorption.....	43
3.4 Cell Culture	44
3.5 <i>In vitro</i> Cellular behavior characterization	44
3.5.1 Cell attachment.....	44
3.5.2 Proliferation.....	45
3.5.3 Cell Viability	45
3.5.4 Alkaline Phosphatase (ALP) Assay.....	46
3.5.5 Mineral deposition.....	46
3.6 Electrical stimulation set-up	47
3.7 Statistical analysis	48
 Chapter 4 Development and Characterization of 3D Graphene/CHI Scaffolds ..	 49
4.1 Introduction	50
4.2 Experimental Methods	53
4.3 Results and Discussion	54
4.3.1 Fabrication and characterization of hybrid scaffolds	54
4.3.1.1 Characterization of RGO obtained by electrochemical reduction process in RGO/CHI scaffold.....	54
4.3.1.2 Structural characterization of scaffolds	56
4.3.2 Optimization of scaffold properties.....	58
4.3.2.1 Mechanical strength study	58
4.3.2.2 Electrochemical behavior of scaffolds	59
4.4 Summary	61
 Chapter 5 <i>In vitro</i> Biological Properties of Conductive 3D Graphene/CHI Scaffold.....	 63
5.1 Introduction	64

5.2 Experimental Methods	66
5.3 Results and Discussion	67
5.3.1 <i>In vitro</i> weight loss study	67
5.3.2 Bioactivity of scaffolds	71
5.3.2.1 Characterization of bone-like apatite layer.....	71
5.3.2.2 Possible mechanism for apatite formation on RGO/CHI scaffold.....	74
5.3.3 Biocompatibility of scaffolds	75
5.3.3.1 Cell attachment.....	75
5.3.3.2 Cell proliferation and viability	77
5.4 Summary	78
 Chapter 6 ES Study: Osteoblast Differentiation on Conductive 3D Graphene/CHI	
Scaffolds.....	81
6.1 Introduction	82
6.2 Experimental Methods	84
6.3 Results and Discussion	84
6.3.1 Optimization of parameters in stimulation to prevent cell death.....	84
6.3.1.1 Frequency	86
6.3.1.2 Seeding density	87
6.3.2 Effect of ES on osteoblast functions grown on hybrid substrates	89
6.3.2.1 Cell alignment	89
6.3.2.2 <i>In vitro</i> osteoblast study	91
6.4 Summary	95
 Chapter 7 Conclusions and Future Work.....	97
7.1 Overview of Research Findings	98
7.2 Implications of Research Findings	101
7.3 Future Work	106
7.3.1 3D scaffolds with controllable reduction states of RGO and their effects on cellular activities	106

7.3.2 Triggered drug release regulated by modulated ES via conductive scaffolds.....	107
7.3.3 Controllable drug release regulated by electrical stimulations.....	109
References.....	113

Table Captions

Table 5.1 Ca/P ratio of the particles on the surface of scaffolds..... **74**

Table 7.1 The zeta potentials of scaffolds with different GO concentrations..... **109**

Figure Captions

Figure 1.1 An overall schematic of this thesis. xix

Figure 2.1 The requirement for bone regeneration needs three essential elements: osteoconductive and osteoinductive matrix (scaffold), osteoconductive signals and cells that can respond to these signals to form the bone structure as well as an adequate blood supply.⁷⁶ 12

Figure 2.2 Bone structure from macro scale to nanoscale.⁷⁷ 13

Figure 2.3 Structure of one piece of graphene oxide (a), reduced graphene oxide (b).¹⁴⁷ 27

Figure 2.4 Structure of chitosan. 30

Figure 3.1 Schematic illustration of the fabrication procedure of GO/CHI and RGO/CHI scaffold with starting from GO and CHI mixture precursor solution, followed by ISISA method to fabricate the GO/CHI scaffolds. RGO/CHI scaffolds were produced by electro-reduction of GO/CHI scaffolds. 38

Figure 3.2 Schematic graph of the electrical stimulation set-up in the current thesis. 48

Figure 4.1 The schematic graph demonstrates that during ISISA method the unidirectional ice crystals could be formed and the polymer solutions are separated by the ice “walls” during the freezing process..... 52

Figure 4.2 Surface analysis results of (a) FTIR spectra of CHI scaffold, RGO/CHI scaffold, GO/CHI scaffold and GO nanosheets and (b) Raman spectra of CHI

scaffold, RGO/CHI scaffold and 0.3GO/CHI scaffold. The peaks in FTIR spectra indicated the changes in surface groups with the incorporation of GO into CHI scaffold and the reduction of GO to RGO. The increase in the intensity ratio of D band to G band from GO/CHI to RGO/CHI in Raman spectra manifested the successful reduction of GO to RGO by the electrochemical method. 55

Figure 4.3 FESEM pictures of the cross-section of the scaffold (a), CHI scaffold (b), 0.1RGO/CHI scaffold (c), 0.3RGO/CHI scaffold (d), 0.5RGO/CHI (e), 0.7RGO/CHI (f), layer surface of CHI scaffold (g), and layer surface of the RGO/CHI scaffold (h). The various structures of scaffolds (b-f) showed the comparison of the microstructure of the RGO/CHI scaffolds with various concentrations of RGO nanosheets. A different roughness of layer surfaces could be observed from the comparison of CHI and RGO/CHI scaffold. 57

Figure 4.4 Mechanical properties including Young's moduli and tensile strength of scaffolds. (* $p < 0.05$, $n = 10$ for each group)..... 59

Figure 4.5 The conductivity of the RGO/CHI scaffolds with various concentrations of RGO nanosheets (a) and the comparison of conductivities of CHI, 0.3GO/CHI, and 0.3RGO/CHI scaffolds (b). (* $p < 0.05$, $n = 5$ for each group)..... 60

Figure 5.1 (a) The weight loss profiles of as-fabricated CHI, 0.1RGO/CHI, 0.3RGO/CHI, 0.5RGO/CHI and 0.7RGO/CHI scaffolds for 7 days of lysozyme treatment (arrows pointed to the 0.3RGO/CHI and 0.7RGO/CHI scaffolds); and (b) comparison of weight loss for CHI, 0.1RGO/CHI, 0.3RGO/CHI, 0.5RGO/CHI and 0.7RGO/CHI scaffolds on Day 7 of the lysozyme treatment, corroborated by tensile strength data (mechanical property) and FESEM images (structural integrity) respectively. (* $p < 0.05$, $n = 3$ for each group)..... 69

Figure 5.2 FESEM images of the surface morphology of CHI and RGO/CHI

scaffolds after immersion in SBF for 0, 3 and 5 days..... 72

Figure 5.3 EDX spectra of the apatite formed on the surface of scaffolds and their Calcium/Phosphate ratio values as a function of immersion time. 73

Figure 5.4 The attachment of hFOB on the CHI and RGO/CHI scaffolds were quantified by the prestoblue assay. (* $p < 0.05$, $n = 6$ for each group)..... 76

Figure 5.5 The proliferation of hFOBs on the CHI and RGO/CHI scaffolds from Day 1 to Day 7 (a) was characterized by the prestoblue assay. The characterization of the viability of hFOB growing on the CHI and RGO/CHI scaffolds on Day 1 and Day 7 (b) was carried out by live/dead assay (green fluorescence = viable cells, red fluorescence = dead cells, scale bar = 100 μm). 78

Figure 6.1 The proliferation (%) of hFOBs on the RGO/CHI scaffolds after electrical stimulation at the current densities of 10, 20, 30, and 40 $\mu\text{A}/\text{cm}^3$ compared to the control group (without electrical stimulation) was characterized by the prestoblue assay. (* $p < 0.05$, $n = 3$ for each group)..... 85

Figure 6.2 The proliferation of hFOBs on the CHI (a), RGO/CHI (b) scaffolds after electrical stimulation at frequencies of 10, 20, and 30 Hz compared to the control group (without electrical stimulation) were characterized by prestoblue assay from Day1 to Day 7. (* $p < 0.05$, $n = 3$ for each group)..... 87

Figure 6.3 The proliferation of hFOBs on the CHI without electrical stimulation (a), RGO/CHI with electrical stimulation (b), and RGO/CHI without electrical stimulation (c) scaffolds with cell seeding density of 15k to 70k/ cm^2 were characterized by the prestoblue assay from Day1 to Day 7. (* $p < 0.05$, $n = 3$ for each group)..... 89

Figure 6.4 The morphology of hFOB cells on the unstimulated CHI scaffold, unstimulated RGO/CHI, and stimulated RGO/CHI scaffolds on Day2 to Day 6 was characterized by live/dead assay (green fluorescence =viable cells, red fluorescence = dead cells) Scale bar = 50µm.....	91
Figure 6.5 The effects of electrical stimulation on the proliferation and differentiation of hFOB cultured on various scaffolds after 1, 4, 7 days until 3 weeks of culture. 1) Comparison of hFOB cell proliferation on stimulated scaffolds with those on unstimulated RGO/CHI scaffold; 2) ALP activity of hFOB cells cultured on unstimulated CHI scaffold, unstimulated RGO/CHI scaffold and stimulated RGO/CHI scaffold; 3) calcium deposition by hFOB cultured on unstimulated (a) CHI scaffold, (b) unstimulated RGO/CHI scaffold (RGO/CHI-nES) and (c) stimulated RGO/CHI scaffold (RGO/CHI-ES) after 1, 2 and 3 weeks. (* p<0.05, n=6 for each group).....	94
Figure 7.1 The effect of GO concentrations on the loading efficiency of dextran of the GO/CHI scaffolds.	109

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AC	Alternating current
AFM	Atomic force microscopy
ALP	Alkaline phosphatase
BCA	Bicinchoninic acid
BET	Brunauer-emmett-teller
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
BTE	Bone tissue engineering
CHI	Chitosan
CNTs	Carbon nanotubes
CVD	Chemical vapor deposition
DC	Direct current
DOX	Doxorubicin
ECM	Extracellular matrices
EDX	Energy dispersive X-ray spectrometer
EG	Expandable graphite
EGFP	Enhanced green fluorescent protein
EIS	Electrochemical impedance spectra
FESEM	Field emission scanning electron microscopy
FET	Field-effect transistor
FGF	Fibroblast growth factor
FTIR	Fourier transform infrared spectroscopy
GICs	Graphite intercalation compounds
GO	Graphene oxide
H ₂ SO ₄	Sulfuric acid
HA	Hydroxyapatite
hFOBs	Human fetal osteoblasts

HMDS	Hexamethyldisilazane
hMSCs	Human mesenchymal stem cells
ISISA	Ice segregation induced self-assembly
MFC	Microbial fuel cell
MSCs	Mesenchymal stem cells
NaCl	Sodium chloride
NMP	N-methyl-pyrrolidone
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PEI	Polyethylenimine
PGA	Polyglycolic acid
PGA	Polyglycolic acid
PLA	Poly (lactic acid)
PLGA	Poly-dl-lactic-co-glycolic acid
PLGA	Poly-dl-lactic-co-glycolic acid
PLLA	Poly-l-lactic acid
PmPv	Poly (mphenylenevinylene-co-2,5-diethoxy-p-phenylenevinylene)
QDs	Quantum dots
RGO	Reduced graphene oxide
SBF	Simulated body fluid
SCE	Saturated calomel electrode
SDBS	Sodium dodecylbenzene sulfonate
SiC	Silicon carbide
ssDNA	Single-strand DNA
TCP	Tri-calcium phosphate
TGF- β	Transforming growth factor- β
TIPS	Thermally induced phase separation
VEGF	Vascular endothelial growth factor
XRD	X-ray diffraction
ΔG	Free energy change

Publications

[1] **Jingwen Xue**, Zhiping Zeng, Ziming He, Jing Liu, Cleo Choong, and Timothy Thatt Yang Tan, A Bioinspired Three-dimensional Conductive, Biodegradable and Bioactive Reduced Graphene Oxide/Chitosan Hybrid Scaffold for Bone Tissue Engineering. ACS Applied Materials & Interfaces. In submission

[2] **Jingwen Xue**, Cleo Choong, and Timothy Thatt Yang Tan Biomimetic synthesis and characterization of HA/reduced graphene oxide functionalized by chitosan biomineralization system. Acta Biomaterials. In submission

[3] Yan Zhang, Zhongzheng Yu, Jingqiu Li, Yanxiao Ao, **Jingwen Xue**, Zhiping Zeng, Xiangliang Yang, and Timothy Thatt Yang Tan. Ultrasmall-Superbright Neodymium Upconversion Nanoparticles via Energy Migration Manipulation and Lattice Modification: 808 nm-Activated Drug Release ACS Nano 2017, 11(3), 2846-2857

[4] Jing Liu, Ziming He, **Jingwen Xue** and Timothy Thatt Yang Tan. A metal-catalyst free, flexible and free-standing chitosan/vacuum-stripped graphene/polypyrrole three dimensional electrode interface for high performance dopamine sensing. J. Mater. Chem. B, 2014, 2, 2478-2482

Abstract

An important aspect of bone tissue engineering (BTE) is bone regeneration, an interdisciplinary process involving the use of cells, biomaterials, and therapeutic agents. Biomaterials, being an essential part of this process, still face challenges in mimicking the three-dimensional (3D) architecture of natural bone and inducing the functions of cells and tissues during bone tissue formation. Electrical stimulation (ES), as one of the signals governing the functions of cells and tissues, has been proved to induce the bone formation of damaged tissues *in vivo* with outstanding efficiency. However, the research on its potential to improve the performance of bone regeneration with tissue-engineered materials *in vitro* has been challenging due to the lack of conductive 3D scaffolds. It has been found that graphene with great conductivity could be hybridized to conductive 3D scaffolds composited with biocompatible chitosan (CHI). Thus, it could be hypothesized that assembling graphene and CHI into a 3D construct mimicking the environment of bone tissues and the conductive property for the functioning of ES could generate a novel conductive hybrid scaffold for bone tissue applications.

Figure 1.1 An overall schematic of this thesis.

Inspired by bone's internal structure and its ability to be electrically stimulated, a 3D graphene/CHI scaffold combined with ES for inducing osteoblast functions *in vitro* was investigated in this thesis. Figure 1 shows the overall schematics of this thesis. To achieve this overall objective, a 3D conductive graphene/CHI scaffold was synthesized after first acquiring a full understanding of its material properties. As current viable approaches of preparing graphene for biomedical applications are limited due to the toxic residuals or undesirable structures of the products, an environmentally friendly way to obtain graphene/CHI scaffold was developed in this study via the *in situ* electrochemical reduction of the graphene oxide (GO)/CHI scaffold to a reduced GO (RGO)/CHI scaffold. In addition, the effects of various RGO contents in the scaffold on THE morphology, mechanical strength, and conductivity of the scaffolds were explored in order to find the optimal content for later studies. Subsequently, to understand the performance of scaffolds in biological environments, the degradation against enzymes, bioactivity for inducing apatite formation, and capability of supporting human fetal osteoblast (hFOB) proliferation were measured. The scaffolds presented a tunable degradation rate by adjusting RGO contents, which enabled the scaffolds to provide space for new tissue formation in bone regeneration with controllable rates according to cell types. Attributed to the wrinkled layer surface of the RGO/CHI scaffold, it also presented higher bioactivity compared to CHI scaffolds for bone-like apatite formation. Similar behaviors of hFOB proliferation and viability were observed on the RGO/CHI scaffolds compared with the CHI scaffolds. This suggested the need of ES for further improvement.

The effects of scaffolds with ES on inducing hFOB functions were subsequently characterized. As there was no protocol in the literature, the parameters of ES were firstly optimized in terms of current density, frequency, and seeding density by comparing their proliferative effects on hFOBs. These optimal parameters were subsequently used to investigate the effects of RGO/CHI scaffolds with ES on hFOB functions, including cell alignment, proliferation, alkaline phosphatase (ALP) activity, and mineralization deposition. According to the results, electrical signals combined with RGO/CHI scaffolds not only affect the early stage of hFOB differentiation by inducing its proliferation and matrix organization but also

accelerate the progress of its mineralization stages by improving mineral deposition. Taken together, 3D conductive graphene/CHI scaffolds combined with ES could act as a promising platform by inducing the functions of osteoblasts *in vitro* for the development of BTE.

Chapter 1

Introduction

This chapter consists of the problem statement for the research project, objectives, and scope of the research, followed by an overview of the chapters in the dissertation. The summary of the findings and outcomes of the research is also described at the end of this chapter. This research project is mainly aimed at developing a three-dimensional (3D) conductive scaffold using graphene and chitosan (CHI) with properties suitable for bone tissue application. The scaffold could also be used for the development of platforms that induce bone formation via electrical stimulation (ES). Overall, this thesis explores the novel 3D graphene/CHI scaffold for its promising applications in bone tissue engineering (BTE).

1.1 Problem Statement

Bone is an essential organ in the human body that plays important roles in supporting muscular contraction and protecting internal organs. Moreover, bone is a reservoir that stores minerals and multiple progenitor cells that are necessary for the health of the body.¹ However, traumatic accidents, infections, and bone tumor resections can cause major bone defects requiring bone regeneration through bone graft treatment.² Spinal fusion and osteolysis adjacent to implants also need the regeneration of bone with bone grafting materials.³ Over 2 million bone grafts are required annually worldwide⁴ with an enormous cost of over US\$2.3 billion in 2015, which is estimated to increase by 56% by 2024.⁵ Thus, there is an urgent clinical need for efficient bone grafts.

Currently, autogenic and allogenic bone grafting is mainly used for treating bone defects. Bone autografting involves the use of the patient's own bone tissues, while allografting involves the use of bone tissues from the same species that are transplanted to the bone defect site.⁶ Bone autografting is the current "gold standard" for the treatment of bone defects and provides effective bone regeneration for small and non-load-bearing defects. However, it possesses the disadvantages of the limited amount that can be extracted, donor site morbidity, deep infection, and postoperative complications.⁷⁻⁹ Moreover, even though allografting can meet the requirement of a large quantity for large bone defects, its lack of osteoinductive factors would cause the problem of ineffective integration of the allograft with the host tissues.¹⁰⁻¹² Hence, there is an increasing need to develop a new effective treatment for bone regeneration.¹³

BTE provides a promising alternative to overcome the disadvantages of autogenic and allogenic bone grafts by utilizing synthetic substitutes or natural biomaterials. The ideal materials in BTE are effective substitutes with a structural organization functionally equivalent to the natural tissues.¹⁴ The development and maintenance of the structure and functionality are governed by various biophysical and biochemical stimuli in the human body.^{14,15} Hence, the stimuli signals play necessary roles in BTE to improve the quality of engineered substitutes and induce their osteoinductivity. Stimuli signals, including mechanical,¹⁶⁻¹⁸ chemical, topographic,¹⁹ and magnetic²⁰ stimuli, have exhibited their influence on inducing bone regeneration.^{21,22} In particular, ES has become an outstanding alternative stimuli process compared with other stimuli.^{14,23-25} By taking advantage of cell galvanotaxis, ES provides greater control over the division, proliferation, and differentiation of cells²⁶⁻³¹ than any other stimuli.¹⁴ Bioelectricity has been found to participate in the processes of wound regeneration,³² bone remodeling and healing,³³ and nerve injury repair.³⁴ Furthermore, the regulation effect of ES could help in building aligned and orientated constructs and lead to new possibilities in designing 3D tissue-engineered substitutes with higher complexity.³⁰ ES could also function efficiently by simply connecting tissues with the electrical source, which cuts down the total cost of the process by reducing the required level of other expensive techniques, such as the use of growth factors.²²

As a simple, flexible, efficient, and cheap process, ES has already been utilized in tissue engineering to improve the wound-healing treatment of skin tissues^{35,36} by enhancing cell migration and blood vessel growth, nervous system regeneration by increasing neurite length and blood vessel density,^{37,38} and the contractile and conductive properties of cardiac constructs.³⁹ The positive effect of ES on bone

formation has been successfully demonstrated in clinical studies on treating osteoarthritis and nonunion fractures.^{22,40} However, its potential applications on improving the performance of tissue-engineered materials and functioning synergistically with biomaterials in BTE *in vitro* are restricted due to the lack of suitable materials, which must be conductive as well as have a porous structure, tunable biodegradability,⁴¹ bioactivity,^{13,42,43} and the capacity to build 3D constructs. Current ES devices are mainly based on metallic, indium-tin oxide (ITO)-coated⁴⁴ or conductive polymer-coated³⁸ electrodes, which can neither transform ES signals onto cells cultured on scaffolds with complex geometry nor confine signals in a defined area.²⁶ Hence, a new scaffold with conductivity, a porous structure, biodegradability, bioactivity, and a 3D architecture needs to be developed.

Graphene, a carbon material with a unique 2D structure, has attracted a great deal of attention to utilizing its specific properties for biomedical applications.^{45–47} With its unique mechanical strength,^{48,49} it could work as a reinforcing agent for polymer films without offsetting their bioactivity.^{50,51} Furthermore, the high conductivity of graphene enables the delivery of electrical stimuli. Hence, a scaffold with graphene might be expected to possess multiple functionalities, including topographical, mechanical, and chemical enhancement, and it could provide a conductive platform for ES on cellular activities to accomplish a biomimetic environment superior to conventional materials for BTE. However, current porous 3D graphene architectures with graphene alone have great limitations in their applications in BTE. Their fabrication method with foam templates⁵² usually results in 3D constructs with random geometry,^{53,54} poor

mechanical strength due to weak bonding between graphene sheets,⁵⁵ and possible toxic residuals from the metal templates. Graphene is a novel and promising material for developing conductive substitutes, but to achieve the 3D and porous structure required by tissue engineering to mimic the extracellular matrix (ECM) for cell activities,^{56,57} the assistance of other suitable components and a green and non-toxic fabrication method are greatly needed.

Polymers have attracted attention in BTE for their great design flexibility (i.e., they can be tailored to various specific structures⁵⁸) and controlled degradation properties.⁵⁶ Several synthetic polymers, such as polycaprolactone (PCL) and poly-L-lactic acid (PLLA), have been used to fabricate composites with graphene due to their easily tailored architecture and controlled degradation molecular design.⁵⁶ However, further chemical modifications are required, as they lack sites for cell adhesion and their degradation byproducts can trigger immune responses.⁵⁹ Bioactive natural polymers, including gelatin and alginate, have also been used for preparing composites with graphene oxide (GO) with properties to promote cellular activities, but the poor surface porosity of their resultant hydrogels due to the interfacial tension during solvent evaporation requires further optimization for applications in BTE.^{60,61} Scaffolds with gelatin as components were reported to possess more disordered pattern structures and greater damage to cells than those with CHI.⁶² The poor stability and quick degradation by enzymes are two other limitations of using gelatin in BTE.⁶³ The alginate hydrogels were reported to have difficulties in controlled degradation.⁶⁴ CHI, a natural polymer with minimal foreign-body responses and great biodegradability, has been widely used for BTE.^{51,65} Unlike other natural polymers derived from mammalian proteins at a

high cost, CHI is sourced from the natural and renewable shells of shrimp and crab.⁶⁶ Compared with synthetic polymers, CHI has a hydrophilic surface for cell adhesion and proliferation with non-toxic degradation products of glucosamine that can be metabolized or excreted from the body.⁶⁷ The cationic nature of CHI due to the free amine groups on its structure is of great interest for its applications in tissue engineering by attracting negatively charged biological macromolecules with electrostatic interactions. CHI has been found to support the attachment and proliferation of osteoblast cells and the formation of the mineralized bone matrix,⁶⁸ which makes it an especially attractive and promising component for bone scaffold design. Significantly, as one of the very few compounds with bioactive, biodegradable, and osteoconductive properties, CHI can be processed in various ways to form porous structures with controlled pore size orientations.⁶⁶ CHI was reported to form strong interactions with GO sheets via hydrogen bonding and electrostatic attractions, resulting in the uniform dispersion of GO sheets and the enhanced mechanical properties of the composites.^{69,70}

A reduced GO (RGO)/CHI conductive system could allow cells to be cultured directly on scaffolds that would subsequently be electrically regulated. The electrical currents and electrical potential gradients could be controlled precisely by adjusting the resistivity of the scaffolds. This scaffold could work as a bioactive and biodegradable substrate with multiple functions for cell and tissue growth *in vitro*. Furthermore, as with the substrates applied to BTE, this scaffold would also possess conductivity, a porous structure, biodegradability, and bioactivity.

Therefore, it was hypothesized that assembling graphene and CHI into a 3D structure could generate a novel conductive hybrid scaffold for bone tissue applications. Hence, the motivation of this PhD project is to develop a versatile and multifunctional 3D hybrid system mimicking the complex structure and functions of natural bone with osteoconductivity and osteoinductivity. In the described work, the material properties and biological functions of the system are investigated and optimized, and the bioactivity of the system after the optimization as well as its ability to deliver electrical stimuli to cells are subsequently evaluated. In addition, the cellular mechanism of electrical stimuli signals on osteoblast activities is explored utilizing this multifunctional system, which could provide opportunities for future BTE research to predict the formation of new tissues.

1.2 Objectives and Scope

In response to the shortcomings of existing BTE strategies applied to bone disease and damage, the design, synthesis, characterization, optimization, and utilization of multifunctional hybrid composites consisting of graphene are the overall objectives of this work. To improve bone regeneration, multifunctional 3D biomimetic graphene/CHI composites are designed with inspiration from components of collagen-apatite hybrids in natural bone tissue and characterized for their effects on osteoblast functions combined with ES.

The specific goals are as follows:

(a) Development and characterization of 3D conductive graphene/CHI composite scaffolds (Study 1)

The first aim was to develop a green method for fabricating RGO sheets and a suitable approach for synthesizing 3D porous structure scaffolds. The RGO sheets were obtained by *in situ* electrochemical reduction of the GO sheets in the precursor GO/CHI scaffold, which was prepared by the ice segregation induced self-assembly (ISISA) method. This method provided a porous hierarchical structure for the scaffold⁷¹ that would not be destroyed after reduction and could be maintained in the RGO/CHI scaffold. With the specific properties of graphene, the 3D multifunctional conductive scaffold was optimized by varying the RGO contents of the scaffold to meet the requirements of BTE in terms of the hierarchical topography and mechanical strength.

(b) Exploring the biological properties of the 3D conductive scaffold in terms of its biodegradability, bioactivity, and biocompatibility (Study 2)

The degradation behavior of a scaffold plays an essential role in the long-term performance of the implantation. Hence, the biodegradability of the RGO/CHI scaffold was investigated under accelerated conditions using high enzyme concentrations and was further compared with that of the CHI scaffold. To study the potential *in vivo* bioactive performance of the multifunctional 3D RGO/CHI scaffold, its ability to induce the *in situ* formation of bone-like apatite was evaluated by the simulated body fluid (SBF) approach because the utility of mineralization in SBF could be a useful marker for predicting *in vivo* bone bioactivity.⁷² To further explore the biocompatibility of the scaffold for the

subsequent cellular study of ES, the attachment, proliferation, and viability of the cells growing on the scaffolds were also observed.

(c) Study on the effect of electrical stimuli on osteoblast functions based on the current-conducting 3D graphene-based composite scaffolds (Study 3)

To explore the effects of electrical stimuli on the cellular activities toward bone tissue formation and its mechanism, the conductive composites with osteoblasts cultured *in vitro* were utilized as a multifunctional cell-material system. The mechanism of electrical stimuli on enhancing bone regeneration could improve the application of the *in vitro* preparation of artificial constructs for bone implantation.

1.3 Dissertation Overview

This thesis is organized as follows:

Chapter 1 briefly introduces the background, motivations, and objectives of this PhD research project.

Chapter 2 gives an overview of the graphene-based composites for biomedical applications, illustration of the principles for the current fabrication methods, and introduction of existing applications of graphene composites for bone regeneration in recent works.

Chapter 3 introduces the methods and experimental procedures in detail for the fabrication, characterization, and application of the 3D graphene-based scaffold described.

Chapter 4 reports the detailed fabrication method of the 3D graphene-based scaffold and investigation and optimization of the effect of graphene incorporation

on the composites. The mechanism by which graphene enhanced the properties of the scaffold is also proposed.

Chapter 5 reveals the bioactivity of the 3D graphene-based scaffold via biomineralization from the SBF approach and explores the characterization of the formed bone-like apatite layer. This chapter also presents the biocompatibility of the developed 3D graphene-based scaffold with different types of cells and its biodegradability under enzymes with accelerated conditions.

Chapter 6 studies the activity of osteoblasts under electrical stimuli *in vitro*.

Chapter 7 provides a general conclusion and recommendation of directions for future research.

1.4 Findings and Outcomes/Originality

The research carried out in this thesis has led to the following novel outcomes:

1. Novel 3D hierarchical structure of graphene/CHI scaffold fabricated by electrochemical reduction and ISISA method;
2. Advanced functions of graphene on tuning biodegradability, enhancing bioactivity, and improving biocompatible properties of its composites;
3. Regulation of osteoblast functions *in vitro* electrically achieved using RGO/CHI scaffold.

Chapter 2

Literature review

This chapter describes in detail the literature related to this thesis and provides the background, development, and current knowledge regarding the fields explored in this thesis. It provides an overview of the information about BTE and its essential components, specifically biomaterials in BTE. The part on biomaterials covers various topics, including the desired features for BTE, current biomaterials used in BTE, and their fabrication techniques. Subsequently, graphene and its composites are introduced followed by different approaches to obtain graphene and their biomedical applications.

2.1 BTE Overview

As current gold standard therapies involving harvesting “donor” bone from patients or other human beings and transplanting it into the defect site⁷³ have obvious shortcomings, such as the short supply and high risk of donor site morbidity,⁸ new strategies for bone regeneration are desperately needed. BTE is a developing and promising approach realized through the application of the design, fabrication, optimization, and growth of living tissues with cells and biomaterials in order to satisfy the clinical requirement.⁷⁴ Based on the goals of inducing new functional tissues rather than simply implanting new parts, BTE provides approaches to restore bone tissues by creating artificial scaffolds for supporting the regeneration of new tissues, delivering degradable scaffolds cultured with isolated cells from patients to the required site in the patient’s body to induce the formation of new tissue.⁷⁵ This is a system consisting of artificial material, cells, and signals, such as proteins and growth factors, to induce the formation and the functions of bone tissues (**Figure 2.1**).

Figure 2.1 The requirement for bone regeneration needs three essential elements: osteoconductive and osteoinductive matrix (scaffold), osteoconductive signals and cells that can respond to these signals to form the bone structure as well as an adequate blood supply.⁷⁶

2.1.1 Bone structure

Bone has a hierarchical structure (**Figure 2.2**) and a calcified compact layer on the outside (Figure 2.2a), which includes many cylindrical Haversian systems with a 10–30% porous cortical part and 30–90% porous cancellous part. The ECM of bone consists of an organic component (collagen), which is nonmineralized, and a mineralized inorganic component (hydroxyapatite, HA). The nanostructure of bone with flexible and tough collagen fibers enhanced by HA crystals is integrated to meet the requirement of high fracture toughness and compressive strength.

Figure 2.2 Bone structure from macro scale to nanoscale.⁷⁷

As a living tissue, to carry out its function, bone is continuously destroyed and its structure is constantly rebuilt by osteoclasts and osteoblasts, which is called bone remodeling.⁷⁸ However, bone tumor resections or incident injuries can cause large bone defects, which need therapy if they exceed a critical size (2.5 times the bone diameter). Bone diseases usually lead to the imbalance between bone resorption and formation, which means increases in osteoclast formation. For example,

osteoporosis results in osteoporotic fractures in the elderly; Paget's disease leads to bulky and weak bone structures, which are prone to bowing and fracture; and some types of cancer that affect bone tissue promote the formation of osteoclasts.⁷⁸

2.1.2 Scaffolds

As an important part of BTE, biomaterials working as scaffolds for the growth of cells and tissues with functions of carrying and delivering signals need to possess various properties.

2.1.2.1 Desired features of biomaterials for BTE

In order to mimic the complex structure and environment of natural bone, the key factors of ideal bone scaffolds are as follows:

1) Architecture. As bone tissue has a 3D configuration, a scaffold for BTE applications, therefore, also needs to provide a 3D structure with a specific environment and architecture for cells to grow in a 3D manner. Bone scaffolds should also have high porosity with an engineered pore size and interconnected pore structure for cell penetration, adequate nutrient diffusion, and waste product removal within the matrix; ECM formation by cells; and vascularization. Cells interact with scaffolds primarily through surface chemical groups on the material, while the density of groups is affected by the surface area of the scaffold interior structure, which is the available surface area within each pore for the adherence of cells.⁷⁹ Thus, to obtain a high surface area, the scaffold is required to have small enough pores to provide adequate chemical group density for efficient binding

with cells. However, there also exists a critical minimum size for the pores allowing the migration of cells into the scaffolds, neovascularization, and ECM production, which may depend on the cell and tissue type,⁵⁶ even though the pore size range required for bone tissue regeneration is still controversial.^{80,81} What is more, recent studies report that scaffolds with multiscale porous structures consisting of micro as well as macro porosities could perform better in tissue engineering than those with macroporous structures alone.⁸² However, the mechanical stability could be affected by the degree of porosity, so a balance between the mechanical properties and porosity is required according to the specific tissue requirements.

2) Mechanical properties. The high mechanical strength of a scaffold is needed not only for load bearing at the implantation site but also for allowing surgical handling during implantation. Fabricating scaffolds with sufficient mechanical strength is challenged by the requirements of both mechanical integrity to function thoroughly from the implantation time to the regeneration completion and different healing rates that vary across patients and tissue types.⁸³ Furthermore, many biomaterials utilized for BTE are reported to have problems in retaining porosity.⁸⁴ The balance between mechanical properties and architecture appropriate for allowing cellular activity and vascularization is a big challenge for new bone scaffolds.

3) Biodegradability. The ability to degrade at a controlled resorption rate to create space for cells to produce their own ECM and new bone tissue formation is another key factor for bone scaffolds.⁴¹ The degradability and resorption of the

scaffolds should be tunable at a suitable rate similar to the growth rate of newly formed tissues to provide necessary space for the new regeneration of the specific tissue.⁸⁵ The behavior of scaffold degradation should vary depending on applications to allow the scaffold to eventually be replaced by the body's own cells over time.

4) Surface properties. The chemical properties of the scaffold's surface significantly affect the attachment, proliferation, and migration of cells as well as the protein interactions with scaffolds.⁸⁶ The surface morphology of scaffolds can also influence the intracellular signaling. To integrate with the surrounding environment, namely to bond to living bone, the key requirement for the material is to be bioactive. That is, it must "form bone-like apatite on its surface when implanted in the living body," as stated by Kokubo and Takadama.⁷² It has been proved by bioactive materials such as glass-ceramic⁸⁷⁻⁸⁹ and sintered HA⁹⁰ found to bond to living bone with the formation of an apatite layer on the surface of the materials. It is speculated that the formed apatite layer is preferred by osteoblasts to attach, proliferate, and differentiate and forms a tight chemical bond between the material and surrounding living bones.^{72,88,91} The capability of forming an apatite layer by the biomaterials, therefore, is the fundamental requirement for materials to guide bone formation on their surfaces resulting in bone bonding.^{92,93}

5) Biocompatibility. This is one of the most important requirements for scaffolds in tissue engineering. To support the normal activities of cells, involving the molecular signaling systems, biocompatibility requires a smart bone scaffold to be osteoconductive and have the capability of osteoinductivity. Osteoconduction requires a scaffold to be able to allow and promote the bone cells' adherence and

proliferation on the surface and throughout the interior of the scaffold and formation of an ECM on the scaffolds, which, *in vitro*, is an ability to promote the attachment, migration, and proliferation of cells⁴² from the host into a 3D structure to form bone.¹³ Osteoinduction of a scaffold refers to the ability to promote the differentiation of mesenchymal stem cells, subsequently leading to the formation of mineralized tissue.⁴²

2.1.2.2 Biomaterials for BTE applications

Biological and materials scientists have been working on designing various types of scaffolds utilizing biomaterials, including natural and synthetic polymers and bioactive inorganic materials, such as ceramics, metals, and composites. The aim is to improve the biological response of biomaterial bone scaffolds to meet the clinical need.⁵⁶ Each of the individual biomaterials has specific advantages as well as drawbacks.

Natural polymers, including collagen, fibrin gels, silk, starch, various proteoglycans, alginate-based substrates, chitin/CHI, and hyaluronic acid derivatives, are bioactive, able to promote cell adhesion and growth, and have biodegradable properties. This allows host cells to produce their own ECMs for the replacement of degraded scaffolds. However, it is difficult to fabricate natural materials into scaffolds with homogeneous and reproducible structures as well as sufficient mechanical properties to match the load-bearing bone site. Synthetic polymers, like polystyrene, poly (lactic acid) (PLA), PLLA, polyglycolic acid (PGA), and poly-dl-lactic-co-glycolic acid (PLGA), overcome the fabrication problems with an easily tailored architecture and controlled degradation by

modulating polymer types.⁵⁶ Nevertheless, the synthetic materials are neither osteoinductive nor osteogenic without the supplement of growth factors or osteoblastic cells, so their bioactivities are reduced. Furthermore, the degradation products of some polymers, such as carbon dioxide⁹⁴ and carboxylic acids,⁹⁵ can change the local pH value, which leads to the possibility of tissue necrosis or immunogenicity.

With a typically high mechanical stiffness, ceramic scaffolds, including HA and tri-calcium phosphate (TCP), are widely utilized for bone regeneration applications with excellent biocompatibility. This is due to their similarity to natural bone in terms of chemical and structural properties. Calcium phosphates, such as HA and TCP, have been reported to enhance the attachment of osteoblasts and MSCs and their differentiation and proliferation by interacting with relevant cells,^{96,97} and they are used for bone defect filling and metallic implant coating.⁹⁴ Metals have contributed to major advances in biomedicine, particularly in orthopedic tissue replacement, with typical examples including cobalt-based alloys, titanium-based alloys, and stainless steels.⁹⁸ The brittleness and difficulties of shaping for implantation and controlling the degradation rate, however, have limited the clinical application of ceramic and metal materials.

The limitations of these individual biomaterials have driven researchers toward the development of composite scaffolds with more than one single phase of biomaterials. Inorganic–organic composites could mimic the components of natural bone as well as combine the tough and biodegradable properties of polymers with the mechanical strength of inorganic materials to produce scaffolds

with enhanced mechanical and degradation properties.⁵⁷ For example, the compressive yield strength of poly (lactide-*co*-glycolide) molded with HA could be improved by incorporating the inorganic part.⁹⁹ PLLA/apatite composites were also prepared by the group of Zhang via soaking the porous PLLA structure in SBF.¹⁰⁰ Due to the outstanding properties, CHI composites with other materials, such as HA, have been investigated,¹⁰¹ and the results have shown that the composites have biocompatibility as well as high osteoconductivity. The improved mechanical properties, cell attachment, and proliferation of the composites by the addition of HA compared with CHI scaffolds reveal the superiority of composites over single materials.

2.1.3 Bone cells

Despite the development of biomaterials with signals and biomolecules, the cells are also an essential part in the BTE process. Osteoblasts, as the first and most popular choice of cell source, have been preferred by researchers for their non-immunogenicity.¹⁰² However, the limitations including low efficiency and expansion rate *in vitro* have limited the cells available for seeding on scaffolds. The human fetal osteoblast (hFOB) cell line has thus been developed with a high capability of proliferation under specific conditions and osteoblastic differentiation, which could be used as a model cell source for BTE studies.¹⁰³ To support bone tissue formation and remodeling, the growth and differentiation of bone cells must be regulated throughout the process of regeneration and organism life. The osteoblast developmental sequence can be divided into three stages involving

proliferation, matrix maturation, and mineralization based on gene expression.¹⁰⁴

These three stages are functionally related with and regulated by each other.

2.1.4 Signals

The structure and functionality of tissues are regulated by various biochemical and biophysical signals^{14,15} in the human body. Biomolecules, such as growth factors, work as signaling molecules to stimulate bone tissue regeneration, osteogenesis, and ECM formation through differentiating the osteoprogenitor cells down specific lineages.^{82,105–109} Besides biomolecules, biophysical signals including mechanical,^{16–18} magnetic,²⁰ topographic,¹⁹ and electrical stimulation¹¹⁰ also significantly influence the induction of bone regeneration to improve the quality of engineered substitutes and induce their osteoinductivity.^{21,22}

2.1.4.1 Roles of electrical signals in BTE

Among the stimuli signals consisting of mechanical,^{16–18} magnetic,²⁰ and topographic,¹⁹ ES has been particularly known for its positive effects on bone fracture healing since the 1950s.^{144,145} The electric potentials *in vivo* are normally generated from piezoelectric functions or the flow of charged ionic energy through the constant pumping of ion channels across the cell membranes.^{111,112} Tissue-level electric potentials can be formed when cells couple into layers.^{34,112,113} These potentials have been found to exist in the tissues involving skin, lens, cornea, and bones^{31,34,112,114} with functions of regulating the cells' division²⁸ and their directional migration toward the injury.²⁷ The presence of these potentials in tissues plays an essential role during tissue regeneration, and it was reported that

with the disruption of electrical currents induced by these potentials, the reduction of regeneration would be observed.³⁴ Furthermore, this effect on cellular functions could override any other influence *in vivo*, including population pressure and chemical gradients.³⁴

However, when bone is fractured, these electric potentials change their distribution, in which case the ES from external devices compensates for the absent electrical signals.¹¹⁵ *In vitro* studies have demonstrated that various types of ES could either enhance the proliferation and growth of bone cells with the increase in the ECM synthesis or improve the differentiation of bone cells and bone mineralization. Experiments on animal models found that bone healing could be improved by ES with increased calcification and mechanical strength of bones in the treatment of nonunion fractures and osteoarthritis.^{168,169,171–174} Furthermore, the positive effects of electrical therapies have been demonstrated clinically in the treatment of fresh fractures, osteotomies, spine fusions, and nonunion fractures.^{40,175,176,180}

The exact mechanism of the enhancement effects caused by ES is still underexplored. It is proposed that there are two possible mechanisms depending on various types of electrical signals. The faradic products at the cathode^{182–186} created by the electrochemical reactions induced by direct currents (DCs) could change the oxygen concentration and pH of the environments,¹⁸⁷ which prevents resorption of bone and in turn increases bone formation. In another aspect, capacitive and inductive coupling signals could enhance the activated calmodulin levels by increasing the intracellular calcium stores.^{188,189} These activated levels

could promote osteoblast proliferation by upregulating their synthesis of nucleotides and various enzymatic proteins¹⁸⁸ and then promote tissue formation.

Current ES devices typically use metal electrodes¹¹⁶ to deliver the electrical signals. However, the limited extent to which electrical signals can be delivered still requires improvement.¹¹⁷ The new formation of bone usually occurs only near the tips of electrodes without covering the fractured tissues.¹¹⁸ Conductive scaffolds with bioactive and biocompatible capacities could act as potential substrates for well-controlled ES in the future.¹⁸¹

2.2 Graphene and its Derivatives

Two-dimensional (2D) graphene sheets have drawn attention since their first isolation in 2004.¹¹⁹ Graphene has extraordinary properties¹²⁰ such as a large specific surface area, good mechanical strength,¹²¹ exceptional electronic conductivity (mobility of charge carriers),¹²² thermal conductivity,¹²³ low cost, and relatively good biocompatibility.¹⁰ Thus, it has become one of the most fascinating materials for biological imaging and sensing, drug/gene delivery, antibacterial materials, and biocompatible scaffolds since the first study on the biomedical applications of graphene and its derivatives in 2008.¹²⁴

2.2.1 Introduction of graphene and its derivatives

Graphene has opened up a new field in science and provided fascinating potential for technological development since it was discovered in 2004.¹²⁵ Andre Geim and Konstantin Novoselov were awarded the Nobel Prize in Physics 2010 “for ground-breaking experiments regarding the 2D material graphene.” As a 2D nanomaterial

consisting of only one layer of carbon atoms, it is the parent of all other graphitic phases, such as fullerene, carbon nanotube (CNT), and graphite from 0D to 3D.¹²⁶ Although the rolling of graphene can yield CNT, the electronic and Raman spectra of graphene and CNT as well as their mechanical strength and electrical conductivity differ significantly.¹²⁷ The monolayer structure of graphene exhibits extraordinary electron/hole carrier mobility.^{128,129}

Graphene has been widely explored due to its remarkable features including high specific surface area of up to 2675 m²/g, chemical stability, excellent electrical properties with mobility of charge carriers (around 200000 cm² V⁻¹ s⁻¹),^{130,131} and high mechanical strength with high Young's modulus of around 1100 Gpa.¹³² With these fascinating properties and the development of synthesis approaches, the potential of graphene and its derivatives has been found in many applications, such as energy generation, conversion, and storage devices;¹³³ chemical and biological sensors;^{134,135} catalysis;¹³⁶ reinforced composites;¹³⁷ and biomedicine.^{138,139} The large surface area makes graphene an ideal substrate for the adsorption of certain biomolecules.

2.2.1.1 Graphene oxide

By oxidizing graphite into GO, the sp²-hybridized structure on the graphene sheets has been broken up, and oxygenated functional groups have been introduced on the GO surface. The negatively charged and hydrophilic surface due to the functional groups enables the stable and uniform dispersion of GO in aqueous media.¹¹⁹ Furthermore, the reactive functional groups such as COOH⁻ and OH⁻ on

the GO surface (**Figure 2.3a**) facilitate its conjugation with different systems, including materials like polymers, biomolecules, DNA, and nanoparticles. This imparts GO with multifunctionality for various biological and biomedical applications, such as drug delivery, enzyme immobilization, and biosensors.^{124,140}

2.2.1.2 RGO

Since single sheets of graphene were isolated in 2004 by the approach of mechanical exfoliation, researchers have shown great interest in graphene for its promising role as the most popular carbon allotrope for the next few years. As mentioned in Chapter 1, graphene can be synthesized from graphite by several methods, the most common of which is reducing GO powders or dispersions, i.e. RGO. RGO has structural defects and lower conductivity and worse mechanical strength than pure graphene sheets fabricated by mechanical exfoliation. However, the good scalability of RGO during the fabrication process has led to many RGO-related composites for biological applications.

2.2.1.3 Graphene composites in BTE

Recently, the biomedical applications of composites made with graphene and its derivatives have drawn increasing attention based on many research works. With their high elastic and flexible abilities, graphene composites could provide cures for many diseases. They were found to be adaptable to surfaces with flat or irregular shapes;¹⁰ to promote the sustained proliferation, proper adhesion, and differentiation of cells; as well as to work as the reinforcement for the composites' structures.

Graphene could improve the mechanical strength of composites. The interaction of graphene with other materials could enhance Young's modulus and hardness by the chemical bonds formed between the surface groups of components. The mechanical strength of hydrogels, including PVA, CHI, and gelatin, could be enhanced by incorporating GO and graphene.^{78,146,161} The elastic modulus and toughness of ceramic composites could also be improved with the addition of GO^{141,142} with enhanced cell proliferation. This positive effect of graphene on the osteogenic proliferation and differentiation of cells has also been reported in fibrous composites with PLLA¹⁴³ and PLGA.¹⁴⁴

By the incorporation with graphene and its derivatives, the nanotopography of polymer/graphene scaffolds can be changed in terms of roughness and surface area, which can affect the adhesion and differentiation of bone-related cells as well as the bioactivity of the composites. Furthermore, positive effects of graphene in PLLA composites on calcium deposition and collagen formation, which play essential roles in cell differentiation, were also reported.

The electrical properties of composites could be significantly improved by incorporating a graphene nanofiller due to its large surface area, its extremely high conductivity, its homogeneous dispersion in the matrix, and the interaction induced by the surface functional groups with other components. Graphene functionalized with various composites, including poly (vinyl chloride/vinyl acetate) copolymer, polystyrene, and polyethylene terephthalate composites,¹⁴⁵ exhibited enhanced electrical conductivity by the incorporation of graphene.

2.2.2 Methods of synthesizing graphene

The methods of synthesizing graphene sheets mainly include 1) mechanical exfoliation from graphite via Scotch tape, 2) chemical vapor deposition (CVD), 3) “unzipping” CNTs longitudinally, 4) epitaxial growth of graphene films, and 5) reduction of GO.¹⁴⁶ Limited by the small production yield of good-quality graphene sheets, the physical method of exfoliation via the Scotch-tape approach cannot be applied to fabricate large amounts. Even though high-quality graphene sheets can be produced more consistently through epitaxial growth, it requires high-vacuum conditions and an expensive fabrication system yet only generates small-area films. The longitudinal unzipping of CNTs can only produce graphene powder or small graphene monolayers in low quantities. CVD techniques have been found to be able to generate graphene monolayers with large surface areas, but the method has yet to be fully developed. Thus, the reduction of graphene derivatives becomes the primary strategy to produce large amounts of graphene sheets.¹¹⁹ This is because it is a low-cost chemical approach with high yield, and the starting material, GO (Figure 2.3a), is obtained from inexpensive graphite and can be dispersed stably in aqueous solution.¹⁴⁷ The graphene produced by the reduction of GO, i.e. RGO (Figure 2.3b), has poorer conductive and mechanical properties than the graphene sheets fabricated through mechanical exfoliation due to the defects remaining on the structure. However, the residual oxygen-containing functional groups and excellent scalability have enabled the fabrication of graphene-based composites with adequate properties.

Figure 2.3 Structure of one piece of graphene oxide (a), reduced graphene oxide (b).¹⁴⁷

Hummers and Offeman¹⁴⁸ developed a fast and safe approach for the fabrication of GO involving mixing concentrated sulfuric acid, sodium nitrate, and potassium permanganate at 45 °C for several hours to achieve a similar level of oxidation. Thus, considering the problems of toxic byproducts and inefficiency, Hummers' method is mainly used in this work to synthesize GO. Thermal and chemical approaches have been commonly used for GO reduction. A high heating temperature is usually required to exfoliate graphite oxide and decompose the oxygenated groups at elevated temperature¹⁴⁷ to achieve graphene in the thermal method. However, only small graphene sheets with wrinkled surfaces can be produced by this procedure since the decomposition process also removes atoms from graphene platelets, which cuts the sheet into small pieces. The treatment condition required for the thermal reduction is critical because the oxygen gas has to be excluded for the increasing etching of oxygen at high temperature. This method cannot be applied to composites with materials of low melting points. Unlike the condition required by thermal reduction, the chemical reduction can be operated at room temperature, which makes it an easier and cheaper way to

generate large amounts of graphene. Among the many methods of chemical reduction, the electrochemical procedure¹⁴⁹ draws our attention. This is because it is simply achieved through the electron exchange between electrodes and GO, which avoids the use of dangerous chemicals and eliminates byproducts. It requires no special chemical agent, can be carried out at room temperature, and causes no damage to the structure of composites.¹⁴⁷

2.2.3 Biocompatibility and cytotoxicity of graphene and its derivatives

Recent studies exploring the biocompatibility of graphene and its derivatives *in vitro* have shown that cell viability might be dose-dependent on the concentration of graphene and its derivatives in solutions.^{150–152} The accumulation of graphene sheets on the cell membrane due to high concentration led to high levels of oxidative stress.¹⁵³ The cytotoxic effect of GO was also found to be affected by the oxidation extent of GO,¹⁵⁴ which is due to the oxygen content and the number of carbonyl groups on GO fabricated by different oxidative treatments. The effects of graphene on cellular activities are also related to the dispersion of graphene and its derivatives. The particle size of graphene was shown to have a large impact on the toxicological response of red blood cells. That is, aggregated graphene sheets presented the lowest hemolytic activity, while GO with the smallest size caused the highest hemolytic activity.¹⁵¹ However, the compacted graphene sheets were found to damage human skin fibroblasts more than less densely packed ones.¹⁵¹ Reactive oxygen species (ROS) that mediate apoptosis through caspase-3 activation were believed to be relevant to the toxicity of aggregated graphene sheets, as the generation of ROS was observed in the cells when they were

exposed to the graphene sheets.^{151,155} The functionalization of graphene or GO with other materials including polyethylene glycol¹⁵⁶ and dextran¹⁵⁷ could decrease the strong hydrophobic interactions between graphene/GO and cells and reduce the level of ROS and improve their biocompatibility.¹⁵²

Similar effects of the oxidation and dispersion of graphene and its derivatives on their biocompatibility were observed in *in vivo* studies.¹⁵⁸ It was demonstrated that the oxidation of graphene was the major reason for its toxicity to the function of lungs and that unoxidized graphene well dispersed in Pluronic hydrogel could minimize this toxicity.¹⁵⁸ Studies in other living organisms indicated no significant abnormalities with the presence of GO¹⁵⁹ or functionalized graphene,¹⁶⁰ and no adult animal deaths were found.^{158,159,161} It should be noticed that the majority of recent studies exploring the toxicity and biocompatibility of graphene and its derivatives focused on directly exposing them to cells or organs.^{151,152,154,155,158,159,161} There is little knowledge on their toxic reaction when graphene and its derivatives are applied as scaffold materials for BTE,¹⁵³ which needs to be explored.

2.3 CHI

Among the natural polymers, CHI (**Figure 2.4**) has broad applicability and can be tailored to several tissue systems.¹⁶² This makes it a fascinating and suitable material for BTE¹⁶³ due to its unique properties, such as biocompatibility, biodegradability, nontoxicity, physiological inertness, and antibacterial nature.¹⁶⁴

Figure 2.4 Structure of chitosan.

2.3.1 Introduction and properties of CHI

CHI is a natural polymer derived from the shells of crabs and shrimps, which are renewable and natural sources. CHI possesses a structure similar to that of glycosaminoglycans (GAGs), which are one of the main components of ECMs playing an essential role in the interactions with cells. A large number of cytokines/growth factors could link to GAGs (with heparin and haparan sulfate mostly).¹⁶⁵ This endows CHI with great potential in tissue engineering applications as biomaterials. There are various types of reactive functional groups on the structure of CHI, including amino, primary, and secondary hydroxyl groups. These groups could allow functional modifications of CHI for plenty of applications. Furthermore, the unique cationic nature of CHI due to amino groups¹⁶⁶ could be attributed to electrostatic interactions with anionic GAGs and other negatively charged biomolecules, including lipids, proteins, and DNA. CHI, upon depolymerization, yields bioactive chito-oligosaccharides with superior anti-microbial properties, and its monomeric products (glucosamine) are metabolized or excreted from the body. Therefore, CHI is biodegradable and has excellent biocompatibility with almost all the tissues of the body.

2.3.2 Techniques for fabricating 3D CHI scaffolds

The most frequently utilized method for the fabrication of bulk CHI scaffolds is the phase separation and lyophilization technique. To utilize this technique, a CHI solution is prepared in dilute acetic acid, introduced into a mold of interest, and subjected to controlled freezing. Upon freezing, the ice crystals that form in the CHI solution are phase-separated from the CHI acetate salt. During the subsequent freeze-drying step, the ice crystals sublime to yield a porous structure of solid CHI material. Finally, the scaffold is neutralized and hydrated using sodium hydroxide and/or ethanol. Unidirectional thermal gradients can reduce pore interconnectivity due to parallel ice crystal growth, whereas radial thermal gradients are more likely to result in ice crystal branching leading to enhanced interconnectivity. However, radial thermal gradients, commonly associated with the surface cooling of a CHI solution in a glass mold, can lead to differences in ice nucleation conditions at the solution–mold interface and the formation of two levels of porosity within a scaffold.⁴¹ Care must be taken to maximize uniformity of the porous structure by maintaining uniform cooling throughout the CHI solution. Higher CHI concentrations correlate with smaller pores and increased pore wall thickness due to a higher CHI mass per unit volume. This, in turn, correlates with enhanced scaffold mechanical strength.⁵⁹ One major drawback of the phase separation technique is the formation of a surface skin if the porous scaffold matrix collapses at the scaffold–air interface due to interfacial tension during solvent evaporation.⁷⁰ This surface skin, with a non-porous structure, can block nutrient exchange and oxygen diffusion into the scaffold, which would be highly detrimental to tissue formation and ingrowth.

The particulate leaching method is often combined with phase separation and lyophilization to fabricate CHI scaffolds. Here, a sacrificial porogen is mixed with the CHI solution and then leached out into a solvent following the lyophilization step. Common porogens are generally inexpensive and easy to handle, but one drawback of particulate leaching is that it can be time consuming. In addition, there can be a lack of control of pore interconnectivity during processing because porogens are dispersed within the polymer phase. As another alternative to the use of a solid phase to induce porosity, gas foaming exploits the nucleation and growth of gas bubbles within a polymeric matrix to yield a porous structure.⁷⁰ Gas foaming does not necessitate the use of organic solvents, but pore formation and porosity depend on the rate of nucleation and gas diffusion, which can be difficult to control.⁸⁰ Overall, although the phase separation and lyophilization technique has its limitations, it is a relatively simple and efficient method of fabricating bulk CHI scaffolds.

2.3.3 CHI applications for tissue engineering

CHI displays significant osteoconductivity but minimal osteoinductive properties. It induces the proliferation of osteoblast cells and mesenchymal cells as well as *in vivo* neovascularization. CHI has been used as a scaffolding material in articular cartilage engineering^{7,21} due to its structural similarity with various GAGs found in articular cartilage. This is of high importance, because GAGs are considered to play a pivotal role in modulating chondrocyte morphology, differentiation, and function.

CHI has been extensively used in BTE since it was shown to promote growth and mineral-rich matrix deposition by osteoblasts in culture.⁴³ In addition, CHI is biocompatible (minimizes additional local inflammation) and biodegradable, and it can be molded into porous structures.⁴⁴ The ability of CS to bind growth factors is suitable for BTE.^{57–59} Due to its cationic nature and predictable degradation rate, CHI-based materials bind growth factors and release them in a controlled fashion. CHI has also been used to modify the surface properties of prosthetic materials for enhancing the attachment of osteoblasts.⁵⁸ CHI was reported to form strong interactions with GO sheets via hydrogen bonding and electrostatic attractions, which could result in the uniform dispersion of GO sheets and enhance the mechanical properties of the composites.^{69,70}

Chapter 3

Experimental Methodology

This chapter describes the detailed standardized experimental procedures used for the preparation, characterization, and analysis of scaffolds; cell characterization; and data analysis. The scaffolds with graphene and CHI were fabricated using the ISISA technique and characterized for their mechanical, surface, biodegradable, bioactive, and biocompatible properties. The effects of coupling scaffolds and ES on the cellular functions were determined by analyzing the cellular properties of hFOB cells including attachment, proliferation, viability, alignment, and differentiation.

3.1 Materials

3.1.1 Graphene Oxide nanosheets

Graphite oxide powder was fabricated by modified Hummer's method^{148, 167-168} using graphite (Aldrich, synthetic, <20 micro) as the starting material followed by sonicating under ambient condition and drying by lyophilization to obtain GO powder. Chitosan was bought from sigma with molecular weight of 50k-190k Da and deacetylated degree of 75-85%.

3.1.2 Chemical Reagents

All chemicals mentioned in the thesis were purchased from Sigma-Aldrich, USA, unless otherwise specified.

3.1.3 Cell Culture Reagents

All cell culture reagents mentioned in the thesis were bought from Life Technologies, USA unless otherwise specified.

3.1.4 Cell Lines

Human fetal osteoblasts (hFOB 1.19) (ATCC® CRL 11372™) were purchased from ATCC, USA.

3.2 Scaffold Fabrication

3.2.1 Fabrication of Three-dimensional (3D) RGO/CHI Scaffold

In current work, a simple two-step process for fabricating 3D porous RGO/CHI scaffolds with hierarchical structures was developed (**Figure 3.1**). This process involved 1) the preparation of GO/CHI scaffolds by an ice segregation induced self-assembly (ISISA) technique¹⁶⁹ using a mixture of GO powder and CHI solution precursors, and 2) direct *in situ* electrochemical reduction of GO/CHI to RGO/CHI.

A certain amount of synthesized GO powder (0.1, 0.3, 0.5, and 0.7 wt. %) was gradually added to a 3 ml CHI solution (2 wt. % in 0.2M acetic acid aqueous solution) and stirred overnight. The precursor solutions in the syringes were then dipped into liquid nitrogen bath at constant dipping rate (5mm/min)^{48, 169-170} and the frozen samples were then lyophilized using a Labconco FreeZone freeze dryer to obtain GO/CHI scaffolds. Finally, RGO/CHI scaffold was prepared by electrochemically reducing GO/CHI scaffold through applying a cathodic voltage of 10V in phosphate buffered saline (PBS) solution using a two-electrode system. The RGO/CHI scaffolds used for characterization studies were represented as CHI, 0.1RGO/CHI, 0.3RGO/CHI, 0.5RGO/CHI, and 0.7RGO/CHI, corresponding to the weight loadings of GO nanosheets in the precursor solutions, which were 0, 0.1, 0.3, 0.5, and 0.7 wt. % in suspension prior to freezing, and 0, 4.8, 13.0, 20.0, and 25.9 wt. % in monolith after freeze-drying, respectively. In other studies, the RGO/CHI scaffold stands for 0.3RGO/CHI scaffold unless mentioned. Scaffolds were finally equilibrated with PBS prior to mechanical testing and then oven dried ahead of other characterizations.

Figure 3.1 Schematic illustration of the fabrication procedure of GO/CHI and RGO/CHI scaffold with starting from GO and CHI mixture precursor solution, followed by ISISA method to fabricate the GO/CHI scaffolds. RGO/CHI scaffolds were produced by electro-reduction of GO/CHI scaffolds.

3.2.2 Fabrication of 3D CHI Scaffolds

For comparison, pristine CHI scaffold was prepared with pure CHI solution using the same ISISA method and freeze-dried to obtain scaffold.

3.2.3 Scaffold Rehydration

The scaffolds would rapidly dissolve if rehydrated in a neutral aqueous medium directly after lyophilization. To prevent the scaffold dissolution, the scaffolds were rehydrated in an ethanol series to reduce the soluble chitosan acetate existing in the scaffolds.¹⁷¹ Scaffolds were immersed in absolute ethanol for one hour, followed by 70% and 50% (v/v) ethanol for half an hour, respectively. Before

characterizations, scaffolds were finally equilibrated with PBS and then freeze-dried prior to characterizations.

3.2.4 Sterilization and Preparation of Scaffold for Cellular Studies

The scaffolds were sterilized by immersing in 70% ethanol overnight and then washed in sterile 1X PBS in sterile conditions prior to cell culture. All subsequent steps were carried out in a biosafety cabinet under sterile conditions.

3.3 Scaffold Properties Characterizations

3.3.1 Surface Characterizations

3.3.1.1 Atomic Force Microscopy Analysis

The roughness and topography of the surface of graphene oxide (GO) nanosheets were analyzed by atomic force microscopy (AFM, Asylum Research, MFP 3D) in an ambient air environment. The samples were analyzed using the tapping mode with the minimum amplitude set point at a scan rate of 0.8 Hz and a scan size of a square of area (1.1x1.1) μm^2 .

3.3.1.2 Field Emission Scanning Electron Microscopy (FESEM)

The morphologies of dried CHI, GO/CHI and RGO/CHI scaffolds were characterized by a JEOL JSM-6700F field emission scanning electron microscopy (FESEM). The samples were mounted onto the sample holders with carbon and copper tape followed by being coated with gold using an SPI sputter coater (SPI Supplies Inc, USA) before observing under the FESEM with an acceleration

voltage of 5 kV at various magnifications. The cell containing scaffolds were prepared by fixing in 2.5% glutaraldehyde for 1 h at 4 °C before dehydration in ethanol series (30%, 50%, 70%, 90% and 100%) for 5 min per grade. The samples were then soaked into 100% ethanol for 1 h and dried in hexamethyldisilazane (HMDS) for another 1 h. The samples were then prepared as mentioned above before they were viewed under FESEM.

3.3.1.3 Fourier transform infrared spectroscopy (FTIR)

The surface functional groups of the samples were investigated by Perkin Elmer Fourier transform infrared spectroscopy (FT-IR) with a universal ATR sampling accessory.

3.3.1.4 Raman spectroscopy

The spectrum was obtained with a Renishaw in Via Raman microscope at an excitation wavelength of 633 nm.

3.3.1.5 Energy dispersive X-ray spectrometer

The JEOL JSM-6700F field emission scanning electron microscopy (FESEM) was also used for EDX measurements to determine the elements on the surface of scaffolds in the bioactivity study.

3.3.1.6 BET

The specific surface area of CHI and RGO/CHI scaffolds was measured with a Quantachrome Adsorb-1 commercial pore and surface analyzer and calculated using the Brunauer-Emmett-Teller (BET) equation and Barrett-Joyner-Halenda (BJH) methods.

3.3.1.7 Zeta Potential Testing

Zeta potential of the precursor solutions and GO nanosheets were measured with a zeta-potential analyzer using Nano-ZS Zetasizer with DTS Nanosoftware.

3.3.1.8 X-ray diffraction analysis

The crystalline phases of the composites were verified and compared by X-ray diffraction (XRD) using a DMAX 2400 diffractometer at 40 kV. The diffraction angles were set between 5 and 40, incremented with a step size of 2 min^{-1} .

3.3.2 Mechanical testing

The mechanical strength of the scaffolds was measured using a Model 5567 universal mechanical testing machine based on ASTM D412 standard. All measurements were conducted at room temperature. The samples were fabricated with the same method as the cylindrical scaffolds with larger dimensions along the layer direction. During measurement, the scaffolds (n=10) were held between custom-made clamps positioned at a distance of 50 mm and pulled at a rate of 0.5 mm min^{-1} until failure with a load cell of 0.5 kN, during which the force and elongation were measured. The reported Young's modulus and tensile strength

reported here were all calculated from the 0-10% stress-strain curves obtained from the results. All failures were observed to occur at the middle region of the tested scaffolds.

3.3.3 Conductivity Study

The electrical conductivities of CHI, 0.3GO/CHI, and 0.3RGO/CHI scaffolds were measured by calculating the inverse of resistivity presented in equation (1), which was obtained by a standard four-probe method on a CMT series resistivity measurement system.

$$R_b = 2 \times s \left(\frac{V}{I} \right) \quad (1)$$

Where V was the voltage induced in the voltage probe, I was the current passed through the sample, and s was the probe spacing.

The electrochemical impedance spectra (EIS) of RGO/CHI and GO/CHI were performed by a Ecochemie PGSTAT30 Autolab system in a 10 mM K₃[Fe(CN)₆] + 1 M KCl solution using a three-electrode system, consisting of a working electrode (RGO/CHI or GO/CHI scaffold), a saturated calomel electrode (SCE) as the reference electrode, and a platinum foil counter electrode.⁴⁸ All tests were conducted over a frequency range from 0.01 Hz to 100 kHz at an open circuit potential, with a perturbation signal of 10 mV at room temperature.

3.3.4 Weight loss Study

In vitro weight loss study: Chitosan was slow in degradation *in vitro* but can be efficiently depolymerized enzymatically by lysozyme (Sigma).⁵¹ In this study, the

initial dry weight of the scaffolds with various RGO concentrations (0, 0.1, 0.3, 0.5, and 0.7 wt. % in suspension prior to freezing, and 0, 5, 15, 25, and 35 wt. % in monolith after freeze-drying) were recorded (W_i), followed by immersing them in 0.1 M phosphate buffered saline (PBS, pH=7.4) solution containing 1.5 mg/ml lysozyme at 37 °C. The scaffolds were taken out, freeze-dried and weighed again (W_f) every 2 days or 3 days. The extent of degradation was quantified by the change of sample weight over time. The weight change of the scaffolds was calculated as follow:

$$\text{Dry weight loss } R_l(\%) = \left(\frac{W_i - W_f}{W_i} \right) \times 100\%$$

3.3.5 Bioactivity Study

The effect of RGO on the biomineralization-mimicking crystallization of scaffolds was studied by incubating 0.3RGO/CHI, 0.3GO/CHI and 0.3CHI scaffolds in a highly saturated $1.5 \times$ SBF (Na^+ , 142.0 mM; K^+ , 5.0 mM; Mg^{2+} , 1.5 mM; Ca^{2+} , 3.75 mM; Cl^- , 148.8 mM; HCO_3^- , 4.2 mM; HPO_4^{2-} , 1.5 mM; SO_4^{2-} , 0.5 mM, pH = 7.46) solution at 37 °C for 3 and 5 days followed by a thorough washing with DI water and drying in oven. The $1.5 \times$ SBF solution was chosen in this study to speed up the mineralization process so as to avoid the influence of the degradation on the scaffolds.⁴³

3.3.6 Protein absorption

The scaffolds were cut to the same size as cell culture study and placed at the bottom of the wells of a 48-well plate. BSA was added to the plates and it was incubated at 37 °C for 6 h. The protein concentrations were measured using the

Pierce™ Bicinchoninic Acid (BCA) assay (Thermo scientific, USA), according to manufacturer's instructions.

3.4 Cell Culture

The hFOB cells were seeded onto the sterilized scaffolds at 5×10^4 cells ml⁻¹ in 24-well culture plates with 1 ml media in each well. Cells were cultured in Dulbecco's minimal essential medium/Ham's F12 (DMEM/F12-1:1 (w/w) mix) (Sigma, USA) cell culture medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA), 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO₂ for the first 7 days. After 7 days, the scaffolds with cells were all transferred to an incubator with a temperature of 39 °C to observe their subsequent behaviors. The attachment and proliferation of cells on scaffolds were studied by culturing cells on the scaffolds cutting to 0.8cm at length. The scaffolds were sterilized by soaking into 75% ethanol for overnight. The scaffolds were then washed with sterile phosphate buffered saline (PBS) for 6 times to remove all residues, followed put into 24 tissue culture plate (NUNC, Denmark) and washed with cell culture medium prior to seeding cells. Fresh media changes were carried out every two days until day 21.

3.5 *In vitro* Cellular behavior characterization

3.5.1 Cell attachment

The attachment of hFOB cells was characterized by PrestoBlue® assay (Life Technologies Corp, USA) 6h after cells were seeded onto the scaffolds following the previously established protocols.¹⁷² Shortly, the hFOB cells cultured in 24-well

plates were firstly incubated in the serum-free medium added with 10% PrestoBlue® reagent under 5% CO₂ environment at 37 °C. After 1h incubation, the medium was transferred carefully to a 96-well plate for fluorescence measurement at 560/590 nm via a microplate reader (Molecular Devices, USA). The number of cells corresponding to the particular relative fluorescence unit was then calculated from the standard curve subsequently, which was the plot of fluorescence vs. known cell numbers. New fresh medium was then added to the cultured cells, which would be incubated again. The attachment value was calculated with the cell numbers 6h after seeding normalized by the seeding numbers

3.5.2 Proliferation

The proliferation of hFOB cells cultured on the sterilized scaffolds was studied by the PrestoBlue® assay (Life Technologies Corp, USA) at various time points following previously established protocols.¹⁷² The detailed method was described in *Chapter 3, Section 3.5.1*.

3.5.3 Cell Viability

Cell viability was assessed using the LIVE/DEAD® viability/cytotoxicity kit (Life Technologies, USA) at different time points according to the given protocols by manufacturers.¹⁷³ Briefly, 4 mM Calcein AM and 2mM Ethidium homodimer-1 dyes were diluted 1:1000 by media and added to each scaffold followed by being left in the incubator for 1 h at 37°C in a humidified atmosphere of 5% CO₂. After taken out from the incubator, the scaffolds were viewed under the Zeiss Axio

Observer Z1 inverted fluorescence microscope fitted with a camera (Carl Zeiss, Germany).

3.5.4 Alkaline Phosphatase (ALP) Assay

The alkaline phosphatase (ALP) activity of cells cultured in scaffolds was analyzed. Cell scaffolds were kept frozen at -80°C before freeze-thawing in 1 ml of 0.2% Nonidet P-40 (NP-40) solution (Sigma, USA) for three times. 50 µl of each lysated cell solution was then added into a 96 well-plate before the addition of 100 µl of 4-methyl-umbelliferyl phosphate solution (Sigma, USA). The mixture was subsequently incubated at 37°C for 30 min and 50 µl of 0.6 M sodium carbonate solution (Sigma, USA) was added to stop the reaction and increase 4-methyl umbelliferone fluorescence. Fluorescence analysis was carried out at an excitation wavelength of 360 nm and an emission wavelength of 450 nm using the microplate reader. ALP content/activity was calculated against a standard curve plotted with known concentration of 4-methyl umbelliferone.

3.5.5 Mineral deposition

The mineral deposition was characterized by Alizarin Red S. The cells cultured on the scaffolds were washed with 1X PBS after removing culture medium. The scaffolds with cells were then added with 200 µl 10% acetic acid (Sigma) for 30 min at room temperature, transferred to a microcentrifuge tube and then vortex for 30 seconds. The scaffolds were then heated at 85 °C for 10 minutes followed by incubation on ice for 5 minutes. The slurry in the microcentrifuge tube was then

centrifuged at 10,000 RPM for 15 minutes and transferred 200 μ l of the supernatant to new tubes. 75 μ l of 10% ammonium hydroxide (Sigma) was then added to neutralize the acid and 50 μ l/well of the samples were then added to a 96-well plate with the same volume of Alizarin Red S solution prepared according to manufacturer protocol. The absorbance at 405 nm was measured with a plate reader and the quantity of mineral deposition was calculated according to the standard curve prepared ahead.

3.6 Electrical stimulation set-up

The system was set up as shown in **Figure 3.2**. Prior to electrical stimulation, the 24 tissue-plates used for stimulation devices were sterilized with 75% ethanol for 24 h and then washed with sterilized PBS for 6 times. The scaffolds were seeded with hFOB and pre-incubated for 2 days before put into the prepared stimulated well plates. The scaffolds were connected to an alternating current (AC) generator using stainless steel wires. An electrical source of the biphasic waveform with various currents (10 to 40 μ A/cm³) and frequencies (10 to 30 Hz) was applied to the experimental scaffolds for optimizations studies. The optimal seeding density of cells was also explored within the range of 15k to 70k cells/scaffold. All the optimization studies were characterized by observing the cell proliferation for 7 days. The detailed method was described in *Chapter 3, Section 3.5.2*. The cells were stimulated for 4 h¹⁷⁴⁻¹⁷⁵ at the 3rd, 6th, 10th, 13rd, 16th, 20th day of culturing for the optimization study and stimulated with the same pattern of exposing time until the 21st day for long-term cell function study. Cells were left to recover for another 24 h prior to proliferation measurement and staining. ES control group has the

same total cell culture time and conditions. At least four experiments were performed for each ES groups.

Figure 3.2 Schematic graph of the electrical stimulation set-up in the current thesis.

3.7 Statistical analysis

Statistical analysis was conducted by two-tailed Student's t-tests between scaffold groups with different contents of RGO. Differences were considered statistically significant when $p < 0.05$.

Chapter 4

Development and Characterization of 3D Graphene/CHI Scaffolds

This chapter describes Study 1, which contains the design, fabrication, and characterization of the 3D graphene-based composite. The fabrication approach of the scaffold consists of three steps, and the graphene in the composite was firstly synthesized via *in situ* electrochemical reductions of GO/CHI scaffolds. The mechanical strength of the scaffolds was then optimized by tuning the concentrations of RGO in the scaffolds. The conductivity of the scaffold, which would subsequently be used for ES, was also investigated.

4.1 Introduction

In recent decades, the design of versatile functional composites to simulate the complex structure and function of natural bone tissue for clinical applications has been one of the major research trends in BTE.^{56,57} The biomaterials, as one of the main factors of BTE, are required to mimic the natural architecture of bone with 3D structures as well as possess osteoinductivity to trigger the effects of cells and tissues. Inspired by natural bone tissues, the integration of biomaterials with inorganic components has opened new perspectives for developing bioactive scaffolds with the required structural and biological properties.¹⁷⁶ Hence, a scaffold consisting of graphene with unique conductivity and mechanical strength^{45–47} and CHI with biodegradability and biocompatibility^{51,65} might provide a promising alternative for BTE. Graphene has been used for bone tissue applications with promising biocompatibility on promoting the growth of bone cells.^{45–47,177} Additionally, the superior electrical conductivity of graphene could meet the requirement of ES, which plays an important role in inducing cellular proliferation¹⁷⁸ and bone healing.²⁴ With a high surface area and sufficient sp^2 domains allowing π - π interactions, graphene could be expected to interact with biological molecules. It could also enhance the mechanical and electrical properties of hybrid composites^{48,49} without compromising their bioactivity.^{50,51} A 3D GO/CHI scaffold has been reported to mediate the attachment and proliferation of osteoblasts,¹⁷⁹ but it requires additional reagents for linking GO and CHI.

A viable approach to obtaining high yields of graphene at a low cost is the reduction of exfoliated sheets of oxidized graphite through chemical^{127,180,181} or thermal treatments^{182,183} to obtain RGO. The chemical reduction needs additional

chemical agents,^{49,179,184} which is not favorable for tissue engineering, as the hazardous reducing agents¹⁸⁵ could contaminate the product.^{186,187} The thermal reduction could avoid the toxic chemicals. However, our group has found that the graphene obtained by thermal reduction could not completely combine into the CHI layers and formed microscale branches with sizes of 2–10 μm .⁷¹ These microscale irregularities are preferred by bacterial adhesion for microbial fuel cell (MFC) applications⁷¹ but might hamper cell spreading and decrease cell adhesion and proliferation in BTE.¹⁸⁸ Electrochemical reduction, using high potentials to overcome the energy barriers for reducing oxygen functionalities on the surface of graphene sheets,¹⁸⁶ provides an environmentally friendly, nondestructive, and efficient alternative to obtain RGO^{169,187} avoiding toxic chemicals and thermal treatment. Current methods of electrochemical reduction by depositing GO sheets onto the electrode surface to produce RGO film result in limited production yield.^{189,190} In this work, the plan was to use a 3D GO/CHI precursor scaffold as one of the electrodes and conduct an *in situ* electrochemical reduction without modifying the scaffold structure, which has not been reported before.

The GO/CHI scaffold was fabricated by the ISISA method, which was a green bottom-up freeze-casting method involving unidirectional freezing to produce a self-supporting scaffold with controllable aligned and porous architectures.¹⁹¹ Current methods of engineering graphene into 3D porous macrostructures are based on the template-directed CVD method involving depositing graphene onto a 3D metal foam template, such as nickel.⁵² The product from this method, however, is not suitable for bone tissue applications because of its random geometry with little control over the pore size,^{53,54} poor mechanical strength due to the weak

bonding between the graphene sheets,⁵⁵ and possible residuals of nickel. The ISISA method uses the unidirectional ice crystals formed from aqueous dispersions of polymers¹⁹² as the templates. The formation of the ice crystals could cause the solute in the aqueous suspensions to be expelled to the boundaries of the adjacent ice “walls”¹⁹³ (**Figure 4.1**). With the subsequent freeze-drying process, empty areas where ice crystals originally resided form macroporous structures on the monolith. This method can produce self-supporting scaffolds with controllable aligned and porous architectures¹⁹¹ that could mimic the hierarchical architecture of natural tissue.¹⁹⁴

Figure 4.1 The schematic graph demonstrates that during ISISA method the unidirectional ice crystals could be formed and the polymer solutions are separated by the ice “walls” during the freezing process.

As such, we hypothesized that a composite, incorporating RGO nanosheets with CHI into a 3D structure, could be able to integrate multiple functionalities such as topographical, mechanical, chemical, and electrical enhancement in the same hybrid to accomplish a biomimetic environment superior to conventional bone scaffold materials. This chapter describes the fabrication of the graphene/CHI hybrid scaffold and its properties characterized and optimized for the subsequent *in vitro* ES of bone cells (*see Section 5.3.3*) in order to meet the requirements of BTE. Overall, the scaffold provides a promising alternative for bone tissue applications with great mechanical strength, tunable biodegradability, and high conductivity that is easy to fabricate by an environmentally friendly method.

The specific objectives of this chapter are as follows:

1. Fabricate a free-standing 3D scaffold with graphene and CHI;
2. Characterize and optimize the material properties of the scaffold (mechanical strength) for BTE by varying the graphene concentration in scaffolds;
3. Confirm the conductivity of the scaffold with the optimized concentration of graphene.

4.2 Experimental Methods

The GO/CHI and RGO/CHI scaffold were prepared as described in *Chapter 3, Section 3.2.1*. The confirmation of the reduction of GO/CHI to RGO/CHI was carried out and analyzed (*refer to Chapter 3, Section 3.3.1.3 and 3.3.1.4*). The morphologies of CHI, GO/CHI and RGO/CHI scaffolds were characterized (*refer to Chapter 3, Section 3.3.1.2*). The properties of the scaffolds were characterized in

term of their mechanical strength (*refer to Chapter 3, Section 3.3.2*), electrical property (*refer to Chapter 3, Section 3.3.3*).

4.3 Results and Discussion

4.3.1 Fabrication and characterization of hybrid scaffolds

4.3.1.1 Characterization of RGO obtained by electrochemical reduction process in RGO/CHI scaffold

The GO nanosheets were mixed into CHI solution to get a precursor GO/CHI suspension. The synthesized GO powder was analyzed by atomic force microscopy, which showed a thickness of 0.96–1.15 nm (data not shown) in the height profiles, indicating that single-layer GO nanosheets can be achieved under these conditions.¹⁹⁰ By immersing it in liquid nitrogen, the homogeneous precursor solution was unidirectionally frozen by the ISISA method^{148,169,170} and freeze-dried to obtain a sponge-like GO/CHI scaffold, as shown in Figure 3.1 (*see Section 3.2.1*). The final conductive RGO/CHI scaffold was prepared by electrochemically reducing the GO/CHI scaffold, and this reduction approach was characterized by Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy, which are shown in **Figure 4.2**.

The dominant surface groups of CHI could be characterized by the peaks for methyl, N-H stretching, and C=O stretching, observed at 1260 cm⁻¹, 1540 cm⁻¹, and 1634 cm⁻¹.^{195,196} The broad peak centered at 3320 cm⁻¹ corresponded to the stretching vibration of N-H from amino groups and O-H from hydroxyl. All the characteristic peaks of the CHI scaffold could be observed in the GO/CHI scaffold.

The existence of GO was evidenced by the peak at 1718 cm^{-1} corresponding to C=O in carboxylic acid and carbonyl moieties on the surface of GO sheets.^{50,197} As for the RGO/CHI sample, most peaks of the oxygenated groups observed in 0.3GO/CHI between 1300 cm^{-1} and 1800 cm^{-1} were attenuated,¹⁹⁷ indicating a successful reduction reaction of 0.3GO/CHI. The increased intensities of the peaks at 2960 cm^{-1} and 1260 cm^{-1} (assigned to the C–H stretching in alkyl groups on the graphene structure) indicated an increased amount of alkyl groups on the surface of RGO/CHI and the transformation of the oxygenated to hydrogenated groups during the electrochemical reduction.

Figure 4.2 Surface analysis results of (a) FTIR spectra of CHI scaffold, RGO/CHI scaffold, GO/CHI scaffold and GO nanosheets and (b) Raman spectra of CHI scaffold, RGO/CHI scaffold and 0.3GO/CHI scaffold. The peaks in FTIR spectra indicated the changes in surface groups with the incorporation of GO into CHI scaffold and the reduction of GO to RGO. The increase in the intensity ratio of D band to G band from GO/CHI to RGO/CHI in Raman spectra manifested the successful reduction of GO to RGO by the electrochemical method.

The Raman spectra of scaffolds displayed two peaks at 1580 cm^{-1} and 1350 cm^{-1} corresponding to the G and D bands, respectively. The G band was related to the in-plane vibration of the graphite lattice, and the D band corresponded to the defects in the structure and weak disorder caused by graphite edges.¹⁹⁸ The increase in the intensity ratio of G band to D band from 0.96 (GO/CHI) to 1.08 (RGO/CHI) might suggest that new graphitic domains were formed after reduction but they were smaller than those on the GO sheets, which caused the defects and partially disordered structure of the RGO sheets.

4.3.1.2 Structural characterization of scaffolds

After the electrochemical reduction process, the microscopic structure of the RGO/CHI scaffold was shown, as presented in **Figure 4.3**. The 0.1RGO/CHI and 0.3RGO/CHI scaffolds exhibited a porous structure with well-aligned layers. This provided the necessary space for cell adhesion and migration into the interior of the 3D structure.^{184,199,200} The 0.3RGO/CHI scaffold displayed a rough layer surface with a wrinkled texture compared with the 0.1RGO/CHI scaffold. This might have been due to the strong geometric constraints on the mobility of the CHI chains caused by increasing the number of RGO nanosheets.²⁰¹ There was no evidence of detached graphene nanoflakes, suggesting that the nanosheets were adsorbed strongly onto the CHI chains.⁶⁹ The rough topology of the 0.3RGO/CHI scaffold also provided an environment conducive to bone cell adhesion and proliferation.^{184,199,200} When the RGO concentration was increased, the RGO/CHI scaffolds presented fewer layered structures and 0.7RGO/CHI could not retain the

layered structure at all. This might have been caused by the higher viscosity of the composite,⁶⁹ which constrained the motion of CHI and RGO during the freezing approach and decreased the degree of unidirectional ice crystal formation.

Figure 4.3 FESEM pictures of the cross-section of the scaffold (a), CHI scaffold (b), 0.1RGO/CHI scaffold (c), 0.3RGO/CHI scaffold (d), 0.5RGO/CHI (e), 0.7RGO/CHI (f), layer surface of CHI scaffold (g), and layer surface of the RGO/CHI scaffold (h). The various structures of scaffolds (b-f) showed the comparison of the microstructure of the RGO/CHI scaffolds with various concentrations of RGO nanosheets. A different roughness of layer surfaces could be observed from the comparison of CHI and RGO/CHI scaffold.

4.3.2 Optimization of scaffold properties

4.3.2.1 Mechanical strength study

The effect of RGO concentrations on the mechanical strength of scaffolds was also investigated. **Figure 4.4** shows that scaffolds' Young's modulus and tensile strength were enhanced from 0.16 MPa and 0.59 MPa to 0.40 MPa and 1.18 MPa, respectively, with the increase of RGO concentration from 0 to 0.3 wt.% (in precursor solutions prior to freeze-drying). The enhancement effect of RGO was in agreement with graphene-enhanced polymer films,⁵⁰ which could be attributed to the nanoscale corrugated texture of the graphene nanosheets.⁴⁹ The incorporation of RGO also caused strong geometric constraints on the mobility of the CHI chains,²⁰¹ facilitating the stress transfer from CHI to graphene throughout the 3D network. In contrast, by increasing the RGO concentration further to 0.5 and 0.7 wt.% (in precursor solutions prior to freeze-drying), the scaffolds possessed lower strengths, which had a similar trend with the degree of aligned-layer structure in the morphology study. This might suggest that the layered structure could also enhance the mechanical strength of scaffolds.

Figure 4.4 Mechanical properties including Young's moduli and tensile strength of scaffolds. (* $p < 0.05$, $n=10$ for each group).

4.3.2.2 Electrochemical behavior of scaffolds

Even though pristine CHI is insulating, the electrical properties of the RGO/CHI scaffolds increased with the increasing concentration of RGO sheets in the scaffolds (**Figure 4.5a**) because of the excellent conductivity of graphene. The 0.1RGO/CHI (with an RGO concentration of just 4.8 wt.% in the monolith scaffold after freeze-drying) exhibited an improved conductivity to $7.0 \times 10^{-2} \text{ S m}^{-1}$, similar to the chemically RGO/CHI composite films reported by other groups.^{202,203} According to one report, there was a maximum conductivity in the RGO/CHI composite film with 6 wt.% RGO due to the aggregation of RGO sheets.²⁰² Conversely, the 0.3RGO/CHI scaffold in this work (with an RGO

concentration of 12 wt.% in the monolith scaffold after freeze-drying) presented even higher conductivity than 0.1RGO/CHI. This might suggest that the method used in this work to fabricate the RGO/CHI scaffold could result in the uniform dispersion of RGO sheets in the composite with higher concentrations of RGO than other methods. The well-dispersed RGO nanosheets throughout the scaffolds could form a 3D conductive network with more conductive pathways for electron transport than a 2D film structure. Further comparison with CHI and 0.3GO/CHI scaffolds (Figure 4.5b) also indicated an incredible increase in the conductivity of the 0.3RGO/CHI scaffold after the *in situ* electrochemical reduction method. As GO is intrinsically an insulator, unlike graphene, GO/CHI scaffolds showed significantly lower conductivity than RGO/CHI scaffolds. The conductivities of 0.1GO/CHI to 0.7GO/CHI were 1.31×10^{-4} , 1.65×10^{-4} , 3.2×10^{-4} , and $6.9 \times 10^{-2} \text{ S m}^{-1}$, respectively (data not shown).

Figure 4.5 The conductivity of the RGO/CHI scaffolds with various concentrations of RGO nanosheets (a) and the comparison of conductivities of CHI, 0.3GO/CHI, and 0.3RGO/CHI scaffolds (b). (* $p < 0.05$, $n = 5$ for each group).

4.4 Summary

In this chapter, a novel 3D conductive scaffold utilizing RGO was successfully fabricated via the ISISA method and the RGO in the composites was obtained by electrochemical reduction. It was found that these RGO/CHI scaffolds have hierarchical porous layered architectures, which could provide space for cellular penetration, the formation of vascularization, and adequate nutrient flow.²⁰⁴ The mechanical strength of the scaffolds was also improved due to the enhancement effect of RGO nanosheets. Moreover, the scaffolds were found to have high conductivity with the RGO sheet concentration of just 0.1 wt.% (in precursor solution prior to freeze-drying). By considering both the layered structure and mechanical strength of the scaffolds with various concentrations of RGO, it was concluded that 0.3 wt.% was the optimal concentration for RGO sheets in this 3D RGO/CHI scaffold. Thus, the 0.3RGO/CHI scaffold was prepared and characterized in preparation for subsequent studies.

Chapter 5

***In vitro* Biological Properties of Conductive 3D Graphene/CHI**

Scaffold

This chapter describes Study 2, which contains the characterization of the biological properties of the synthesized scaffolds to explore their potential applications for bone regeneration. The biodegradability of scaffolds was explored followed by their bioactivity by investigating their ability to induce the *in situ* formation of bone-like apatite. The RGO/CHI scaffolds were then used as a substrate for cellular studies with hFOB.

5.1 Introduction

Biodegradation is one of the crucial factors for scaffolds in BTE. In order to facilitate bone regeneration, the scaffolds should be able to degrade *in vivo* at a controllable rate and create space for the formation of new bone tissues.⁴¹ CHI, as a biopolymer, has the advantage of being biodegradable *in vivo* in the presence of lysozyme, which is the enzyme existing in human serum.²⁰⁵ It has been reported that GO/CHI composites with up to 3 wt.% GO could degrade at rates similar to or slightly lower than those of pristine CHI scaffolds.^{51,206} However, there are no reports on the effects of graphene with concentrations over 3 wt.% on the degradability of the composites. The structure of the scaffolds prepared by covalent linking⁵¹ was also found to be less porous after degradation than that of the pristine CHI scaffold, but the reason was not explained. Hence, it is necessary to investigate the performance of the RGO/CHI scaffolds with a large concentration range of graphene (4.8–25 wt.%) against enzymatic degradation in this work.

In addition, bioactivity, the ability of materials to bond to living bone rather than being isolated from the surrounding tissues when implanted, is also an essential requirement of BTE.⁷² To bond to living tissues, the materials need to possess a highly reactive surface available for the nucleation and growth of the apatite layer acting as the link between surrounding tissues and synthesized materials at the implantation interface.²⁰⁷ The apatite layer formed on the surface of scaffolds in the living body, which was found to have a composition and structure similar to that of natural bone,^{208,209} could support the bone cells' production of apatite and collagen *in vivo*.²¹⁰ It was found that the materials with this bone-bonding ability

tended to form a layer of bone-like apatite on their surface *in vitro* when contacted with SBF,⁹³ which has ion concentrations nearly equal to those of human blood plasma. Hence, the formation of this apatite layer in SBF could be an indicator of the bioactivity behavior of the synthesized scaffolds.

GO/CHI composites have been reported to support the deposition of HA particles on their surfaces when soaked in SBF solutions, which was suggested to be attributed to the strong electrostatic interactions of the surface functional groups with mineral ions.²¹¹ Nevertheless, in the same paper, the pristine GO film showed worse performance on inducing HA nucleation.²¹¹ This phenomenon was also observed in other reports, which found few minerals were formed on the GO substrate if it was not functionalized by other materials.²¹² This might suggest that GO alone has little effect on inducing apatite formation but that the wrinkled surface texture and sheet-like structure of the graphene sheets could crumple the surface of polymer matrices to induce the nucleation of apatite crystals by increasing the surface toughness of the composites.²¹³ This could also explain why the bioactivity of GO/polymer or graphene/polymer composites could be improved when GO or graphene nanosheets were incorporated into the hybrid acting as a reinforcement filler.^{213–217} The nanoscale-wrinkled texture might also enhance the adhesion and proliferation of osteoblasts.^{218,219} As mentioned in previous work (*see Section 4.3.1.2*), the surface of a synthesized RGO/CHI scaffold presents an obvious wrinkled texture. It can be expected that the 3D RGO/CHI scaffold could act as a bioactive and biocompatible platform for BTE applications.

The specific aims of this chapter are as follows:

1. Explore the biodegradation ability of the scaffolds with graphene incorporation;
2. Evaluate the bioactivity of the scaffolds with wrinkled layer surfaces by investigating their ability to induce the *in situ* formation of bone-like apatite with the biomineralization approach (SBF method);
3. Investigate the biocompatibility of the scaffold with wrinkled layer surfaces in terms of the cell attachment, viability, and proliferation of osteoblasts seeding on the scaffolds.

5.2 Experimental Methods

The RGO/CHI scaffolds with various concentrations of RGO nanosheets were characterized for their *in vitro* degradation ability (*refer to Chapter 3, Section 3.3.4*) and the dissolution of 0.3RGO/CHI scaffold was carried out in phosphate buffered saline (PBS) solution (*refer to Chapter 3, Section 3.3.4*). The bioactivity study of the scaffolds was carried out by biomineralization approach (*refer to Chapter 3, Section 3.3.5*). The morphology of the biomineralized scaffolds was viewed under FESEM (*refer to Chapter 3, Section 3.3.1.2*). The calcium/phosphate ratios of the biomineralized scaffolds were analyzed and calculated based on the results obtained from EDX (*refer to Chapter 3, Section 3.3.1.5*). Cellular studies including attachment, proliferation, viability, and morphology of the scaffolds were characterized (*refer to Chapter 3, Section 3.5*)

5.3 Results and Discussion

5.3.1 *In vitro* weight loss study

The biodegradation behavior of scaffold plays a vital role in the long-term performance of the scaffolds in tissue engineering. Chitosan can be degraded by lysozyme via enzymatic hydrolysis of its β -1, 4*N*-acetyl-glucosamine groups to aminosugars, which could then be incorporated into metabolic pathways of glycosaminoglycan (GAG) or glycoprotein, or excreted.¹⁻² Some contents of graphene are assumed to be extruded from the scaffolds with the hydrolysis of chitosan during the degradation process, which would also be biocompatible at doses up to 1mg/kg of body weight.³⁻⁴ It is essential to measure the weight loss of the scaffolds and study its structure integrity during their degradation process. The *in vitro* weight loss performances of the CHI and 0.1RGO/CHI-0.7RGO/CHI scaffolds were thus tested with lysozyme treatment (**Figure 5.1a**). Three stages of weight loss could be observed from the figure. The weight of all five types of scaffolds manifested no obvious decrease in day 1 (stage I), compared with a fast and rapid stage (stage II) occurred after day 3. Following stage II, a relatively slower rate of weight loss of these scaffolds could be observed after three days of lysozyme treatment. The initial slow weight loss for all groups was proposed to result from small amount of lysozyme binding sites. The lysozyme degraded chitosan by hydrolyzing the glycosidic linkages on the chitosan chains through the acetyl groups, necessary binding sites of lysozyme.⁵⁻⁶ The breakage of glycosidic bonds in a small number during the initial stage of chitosan degradation⁷ would not affect the whole structure of chitosan and scaffolds. When the amount of breakage of glycosidic bonds reached a critical point, the scaffold would be degraded in a

much faster rate, resulting in significant weight loss of all the scaffolds after day 1. Once the number of the binding sites on lysozyme were reduced along with the degradation progress,⁸ the weight loss rate of scaffolds slowed down for all groups after day 3. During stage III from day 3 to day 7, the weight loss of scaffolds decreased at different rates. CHI scaffolds lost 84 wt.% of their initial weight after one week compared to 68 wt.%, 73 wt.%, 83 wt.% and 96 wt.%, respectively for 0.3RGO/CHI, 0.1RGO/CHI, 0.5RGO/CHI and 0.7RGO/CHI scaffolds with the highest and lowest ones specifically pointed out in the figure with arrows. The weight of 0.1RGO/CHI and 0.3RGO/CHI scaffolds reached plateaus around day 7 with their weight loss varying no more than 2 wt.%. The 0.5RGO/CHI and 0.7RGO/CHI scaffolds continued to lose weight till day 7.

The weight losses of these scaffolds on day 7 were specifically plotted in Figure 5.1b for further discussion. Lower weight loss can be observed for 0.1RGO/CHI and 0.3RGO/CHI. On the contrary, the weight loss of 0.5RGO/CHI and 0.7RGO/CHI increased significantly with RGO concentrations. This phenomenon is consistent with the results of the morphology study (*Refer to Chapter 4, Section 4.3.1.2*) and the mechanical strength study (*Refer to Chapter 4, Section 4.3.2.1*). 0.3RGO/CHI scaffold with the most uniform hierarchical structure and highest mechanical strength presented the lowest weight loss, contrasted with that of 0.7RGO/CHI.

Figure 5.1 (a) The weight loss profiles of as-fabricated CHI, 0.1RGO/CHI, 0.3RGO/CHI, 0.5RGO/CHI and 0.7RGO/CHI scaffolds for 7 days of lysozyme treatment (arrows pointed to the 0.3RGO/CHI and 0.7RGO/CHI scaffolds); and (b) comparison of weight loss for CHI, 0.1RGO/CHI, 0.3RGO/CHI, 0.5RGO/CHI and 0.7RGO/CHI scaffolds on Day 7 of the lysozyme treatment, corroborated by tensile strength data (mechanical property) and FESEM images (structural integrity) respectively. (* $p < 0.05$, $n = 3$ for each group).

The lower weight loss of scaffolds (0.1RGO/CHI and 0.3RGO/CHI) can be explained by the presence of (small amount) graphene, which are impermeable to most molecules⁸⁻⁹ including the lysozyme. Furthermore, the hydrogen bonding, electrostatic interactions and π - π stacking between graphene sheets and chitosan¹⁰ can restrict the chain movement of macromolecules like chitosan.⁸ The cleaving and enzymatic degradation of chitosan would be slowed down in the 0.1RGO/CHI and 0.3RGO/CHI scaffolds. On the contrary, the high weight loss of 0.5RGO/CHI and 0.7RGO/CHI scaffolds could be attributed to the aggregation of graphene sheets in scaffolds as mentioned in the morphology study (*Refer to Chapter 4, Section 4.3.1.2*) and the mechanical strength study (*Refer to Chapter 4, Section 4.3.2.1*). The aggregation of graphene, of which amount above a threshold quantity, is hypothesized to disrupt the bonding sites between graphene and chitosan in scaffolds therefore lowering the structural strength of the scaffolds. It is worthy to notice that the initial weight ratios of chitosan in the as-fabricated dry scaffolds due to different RGO concentrations were also different (with values of 95.2 wt. %, 87 wt.%, 80 wt.% and 76.5 wt.%, respectively in 0.1RGO/CHI, 0.3RGO/CHI, 0.5RGO/CHI and 0.7RGO/CHI). The 0.7RGO/CHI scaffold with the lowest weight ratio of chitosan (76.5 wt.%) for supporting scaffold structure was suggested to have the worst structure stability. With such a large amount of weight loss during the lysozyme treatment, the 0.5RGO/CHI and 0.7RGO/CHI scaffolds would not be able to retain their structure and strength as well as other properties such as conductivities. Compared with these scaffolds, the 0.1RGO/CHI and 0.3RGO/CHI scaffolds would be more desirable in terms of mechanical and structural robustness, both properties crucial in bone tissue engineering scaffolds.¹¹ Last but not least, key consideration was given to high electrical conductivity of

scaffold to promote efficient electrical stimulations, the 0.3RGO/CHI scaffolds were chosen as the optimized scaffolds for the proceeding studies.

5.3.2 Bioactivity of scaffolds

5.3.2.1 Characterization of bone-like apatite layer

The bioactivity of scaffolds was investigated by immersion in SBF solution for up to five days at 37 °C. The surface morphologies of the apatite clusters formed on the layer surfaces of RGO/CHI and CHI scaffolds as a function of time were observed under the Field Emission Scanning Electron Microscopy (FESEM) (**Figure 5.2**).

Figure 5.2 FESEM images of the surface morphology of CHI and RGO/CHI scaffolds after immersion in SBF for 0, 3 and 5 days.

As mentioned in the previous work (*see Section 4.3.1.2*), the layer surface of RGO/CHI before immersion in SBF exhibited a wrinkled texture due to the incorporation of RGO nanosheets. After three days, a very fine dispersion of spherical mineral precipitates could be found on the layer surface of the RGO/CHI scaffold, while only a few particles were sparsely distributed on the pristine CHI scaffold. The larger amount of particles formed on the RGO/CHI surface than on the CHI scaffold at the beginning of immersion suggests that RGO/CHI could provide more nucleation sites for apatite formation than the pristine CHI scaffold. It was evident from the previous FTIR characterization (*see Section 4.3.1.1*) that the RGO/CHI scaffold had fewer surface functional groups than the pristine CHI

scaffold, implying that the surface functional groups had little effect on the nucleation of minerals. On the other hand, the wrinkled surface texture due to graphene incorporation and the defects of the graphene sheets could enhance the apatite nucleation.²¹³ Hence, the wrinkled layer surface of RGO/CHI might be the main reason for the enhancement of the nucleation of mineral particles formed on the scaffold surface.

Figure 5.3 EDX spectra of the apatite formed on the surface of scaffolds and their Calcium/Phosphate ratio values as a function of immersion time.

With a longer immersion time (five days), the particles on the surfaces of RGO/CHI became denser and larger, while far fewer presented on the pristine CHI. Further analysis by Energy dispersive X-ray spectrometer (EDX) (**Figure 5.3**) confirmed the presence of calcium and phosphate in the minerals deposited on the

surface of RGO/CHI. It also demonstrated that the Ca/P ratio of the particles on the RGO/CHI scaffold was ~ 1.65 (**Table 5.1**). This was quite close to the stoichiometric ratio of biological HA ($\text{Ca/P} = 1.67$), strongly indicating that the RGO/CHI scaffold possessed the ability of apatite formation.

Table 5.1 Ca/P ratio of the particles on the surface of scaffolds.

Ca/P	Day 0	Day 3	Day 5
CHI	0	2.76 ± 0.07	2.82 ± 0.03
0.3RGO/CHI	0	1.58 ± 0.04	1.65 ± 0.06

5.3.2.2 Possible mechanism for apatite formation on RGO/CHI scaffold

It was hypothesized that the formation of the apatite particles on the RGO/CHI surface might depend on the synergistic effects of the morphological functions of the wrinkled (rough) surface and noncovalent interactions such as electrostatic forces induced by the materials. In general, rough surfaces have been shown to promote calcium phosphate nucleation and growth by providing more active nucleation sites for apatite formation.²²³ As shown in Figure 5.3, the surface of the RGO/CHI scaffold had a wrinkled topography due to the incorporation of graphene nanosheets into the scaffolds, which was evident from the smooth surface of the CHI scaffold. The space between the convex and concave areas on the rough surface acted as a trap of the Ca^{2+} , PO_4^{3-} and OH^- ions with a higher local supersaturation, which strengthened their interactions for the critical nucleus formation²²⁴ and packed them into a compact structure.²²⁵

Furthermore, with the reduction process, RGO/CHI possessed less oxygenated groups compared with CHI (*see Section 4.3.1.1*). The oxygenated groups introduce the electronegative charge to the surface, which can induce electrostatic repulsion to the negatively charged pre-nucleation clusters of the apatite minerals.⁴² Consequently, the result of co-functions of wrinkled geometry and surface properties of the hybrid scaffolds induced by the corrugated RGO sheets could provide RGO/CHI with a high capacity to induce apatite formation. This was also expected to result in a positive effect on the cellular activities on RGO/CHI.

5.3.3 Biocompatibility of scaffolds

5.3.3.1 Cell attachment

As one of the essential processes for the subsequent proliferation and differentiation of bone tissue formation, cell adhesion on the CHI and RGO/CHI scaffolds was quantified, as shown in **Figure 5.4**. In comparison to CHI, the results of cell attachment rates revealed that hFOB adhered more to 0.3RGO/CHI than the other two scaffolds 6 h after inoculation. It has been demonstrated that initial osteoblast adhesion can be enhanced on materials with rough surfaces,²²⁶ which could encourage the entrapment of fibrin protein.²²⁷ The wrinkled GO film was also proved to induce the attachment of fibroblasts.²²⁸ As discussed in the FESEM images (*see Section 4.3.2.2*), the layer surface of RGO/CHI is much rougher than that of CHI because of the incorporation of RGO nanosheets. The wrinkled layer surface of RGO/CHI scaffolds (*see Section 5.3.2.1*) should contribute to the enhanced attachment of osteoblasts to the scaffolds.

Figure 5.4 The attachment of hFOB on the CHI and RGO/CHI scaffolds were quantified by the prestobblue assay. (* $p < 0.05$, $n = 6$ for each group).

Besides the topographical cues, the chemistry of the scaffold surface might also play a critical role in cell adhesion and protein adsorption.²²⁹ By incorporating graphene nanosheets into the scaffolds, the π - π stacking induced by molecular structures of graphene could enhance the interactions with proteins²³⁰ compared with pristine CHI scaffolds. After the electrochemical reduction of GO to RGO, there were fewer oxygenated groups and more hydrogenated groups in RGO/CHI as discussed in the FTIR spectra (*see Section 4.3.1.1*). It has been reported that RGO with moderate oxygenous content—which had a mixture of electrostatic forces, hydrogen bonding, and hydrophobic interactions—showed better behavior in the adsorption of cell attachment proteins.²³¹ The adsorption levels of proteins onto the surface of materials²³² could mediate cell interactions with biomaterial

surfaces including cell adhesion^{233–235} to influence the initial cellular behaviors.²³⁶ Therefore, the mixture of the structure and chemistry of the scaffold surface resulted in its good cell attachment performance.

5.3.3.2 Cell proliferation and viability

The number of hFOBs grown on scaffolds after cell attachment was further investigated. This presented a higher level of cell proliferation on 0.3RGO/CHI in comparison to CHI throughout the first to fifth days after seeding (**Figure 5.5**), which was consistent with the cell adhesion on these scaffolds. It could be observed that after seven days of culture, the cell numbers on RGO/CHI did not differ significantly from those of CHI scaffolds. This indicated that there might be few positive effects of the rough surface on the proliferation of osteoblasts, which had also been found by other groups for titanium substrates.²³⁷ The viability of hFOBs was also observed by live/dead viability staining, as demonstrated in Figure 5.6. The viability of hFOBs on 0.3RGO/CHI and CHI scaffolds after seven days of culture was consistent with the proliferation study. The number of live cells grown on 0.3RGO/CHI was similar to that grown on CHI and 0.3GO/CHI as expected.

Figure 5.5 The proliferation of hFOBs on the CHI and RGO/CHI scaffolds from Day 1 to Day 7 (a) was characterized by the prestobule assay. The characterization of the viability of hFOB growing on the CHI and RGO/CHI scaffolds on Day 1 and Day 7 (b) was carried out by live/dead assay (green fluorescence = viable cells, red fluorescence = dead cells, scale bar = 100 μ m).

5.4 Summary

The scaffold was biodegradable and had better stability in terms of degradation than the CHI scaffold. However, the RGO/CHI scaffold could maintain its structure in the normal dissolution test, which indicated that it could support long-

term cell culture. The hybrid RGO/CHI scaffold also presented better bioactivity for inducing bone-like apatite formation than CHI. The good proliferation and viability of osteoblasts could also be observed on the RGO/CHI, which was similar to CHI. The biocompatibility of the hybrid scaffold showed great potential for supporting the growth of hFOB.

Chapter 6

ES Study: Osteoblast Differentiation on Conductive 3D

Graphene/CHI Scaffolds

This chapter presents the effects of ES on the functions of hFOB cells, including cell alignment, proliferation, alkaline phosphatase (ALP) activity, and mineralization deposition. The optimal parameters used for experiments of ES, including current density, frequency, and seeding density, were firstly explored by measuring their proliferative effects on the behaviors of hFOB cells. The ES was then delivered through the conductive RGO/CHI scaffold utilizing the optimal parameters and characterized for its effects on hFOB functions.

6.1 Introduction

Since the 1950s,²³¹ the stimulation of bone growth by electric currents has been explored by many researchers^{23, 232-237} based on Yasuda's hypothesis. According to the hypothesis, the electrical activity found in bone in response to mechanical loading could be the mediator of its remodeling and the ES alone could also trigger this remodeling. The mechanical loading of bones induces the flow of extracellular fluid, and such fluid flow can form streaming potentials.²³⁸ These electric potentials, based on Wolff's law and Basset's theory,²³⁹⁻²⁴⁰ subsequently accelerate the migration of ions and simultaneously affect "cellular activity, collagen alignment and apatite formation."²⁴¹⁻²⁴²

The electric potentials generated from piezoelectric functions or the flow of charged ionic energy play an essential role in bone remodeling when bone is under mechanical stress. However, when bone is fractured, they change their distribution, in which case the ES from external devices compensates for the absent electrical signals.¹¹⁰ External ES has been found to promote bone healing experimentally²⁴³ and clinically by enhancing the proliferation of bone cells,²⁴ encouraging ECM deposition,²⁴⁴⁻²⁴⁶ and inducing the expression of growth factors.²⁴⁵⁻²⁴⁶

Several commonly used approaches to ES are employed for BTE applications, including DC stimulation, electromagnetic field, and capacitance coupling. However, DC ES is not suitable for *in vitro* cell culture due to its accumulation of charged proteins or chemical compounds on the electrodes during the stimulation process, which can decrease the efficiency of the stimulation.³¹ Moreover, the limited bone formation localized only near the electrode tips cannot lead to the

recovery of bone tissue damaged to a great extent.²⁴⁷ The electromagnetic field and capacitive coupling approaches, while suitable for *in vitro* cell culture, cannot be confined within the required area where new tissues are supposed to regenerate due to their spatially non-specific property *in vivo*.²⁴⁸ This leads to the indiscriminate effect of electrical signals on the normal tissues and a high risk of promoting tumor progression in the unexpected regions.²⁵

The 3D conductive RGO/CHI scaffold prepared in this thesis could solve these problems by confining the ES within the area of the specific scaffold and exposing the electrical signal only to the cells cultured on the scaffold when connected to an electrical source. Furthermore, biphasic electrical pulses were used in this work as the stimulation method due to its advantages of less faradic byproducts, lower accumulation of charged proteins, and fewer pH changes²⁵ with positive evidence on promoting osteoblast proliferation.^{25, 249}

Due to the good conductivity of the scaffold (*see Section 4.3.5*), the RGO/CHI scaffold could be expected to possess efficient responsiveness to the ES. The scaffolds' high biocompatibility for hFOB cells (*see Section 5.3.3*) also demonstrated their potential for applications on efficient responsiveness to ES. Therefore, the coupling of the RGO/CHI scaffold and ES was further studied in this work to maximize the functions of bone cells during osteogenesis.

The specific aims of this chapter are as follows:

1. Optimize the stimulation parameters to explore the optimal factors of the ES working on RGO/CHI scaffolds;

2. Investigate the effects of ES on osteoblasts cultured on the scaffold to demonstrate its potential in BTE.

6.2 Experimental Methods

The optimization of parameters for electrical stimulation on RGO/CHI scaffolds was characterized in terms of current density, frequency and seeding density (*refer to Chapter 3, Section 3.6*). The proliferation of hFOB cells on the scaffolds was investigated by PrestoBlue® assay (*refer to Chapter 3, Section 3.5.2*). The morphology of cells on its alignments was observed by fluorescence microscope (*refer to Chapter 3, Section 3.5*). The ALP activity of hFOB cells was quantified and mineral deposition on the scaffolds was also characterized (*refer to Chapter 3, Section 3.5.4 and 3.5.5*).

6.3 Results and Discussion

6.3.1 Optimization of parameters in stimulation to prevent cell death

Various parameters might affect cell functions in the stimulation process, including current density, frequency, and seeding density. Optimal parameters should be applied to cells for efficient stimulation. The physiological current of natural bone is around 10 μA ,²⁵⁰ and previous studies have shown that applying ES with a current of over 20 μA causes necrosis and ES failure.²⁵¹⁻²⁵²

Figure 6.1 The proliferation (%) of hFOB cells on the RGO/CHI scaffolds after electrical stimulation at the current densities of 10, 20, 30, and 40 $\mu\text{A}/\text{cm}^3$ compared to the control group (without electrical stimulation) was characterized by the prestobblue assay. (* $p < 0.05$, $n = 3$ for each group).

To further confirm the value of current suitable for the system in this work, a preliminary study was conducted on the effect of the current density on cell proliferation compared with the control group, which was not stimulated. The preliminary result in **Figure 6.1** showed that the proliferation of hFOB cells of groups with current densities of over 20 $\mu\text{A}/\text{cm}^3$ was inhibited compared with the group without ES. Only the proliferation of the group with the current density of 10 $\mu\text{A}/\text{cm}^3$ was not affected adversely by ES and increased by 65% with respect to that of the unstimulated group. This current density was consistent with the physiological current of bone tissues. Thus, it was chosen as the optimal current density for the subsequent study.

6.3.1.1 Frequency

To explore the effect of frequencies on hFOB3, the hFOB3 cultured on the conductive scaffolds were exposed to different kinds of stimulation, which varied in frequency with the same current density of $10 \mu\text{A}/\text{cm}^2$. In previous reports that explored the proliferative effect of ES on osteoblasts, frequencies ranging from 10Hz ²⁵³ to 20Hz ^{25, 249} had been used to successfully stimulate and enhance the functions of osteoblasts. However, due to the variety of devices and substrate materials, there were few works of literature or protocols exploring the optimal frequency for the functions of osteoblasts. Based on the values of frequency that had been investigated using various substrates by previous work,^{25, 237, 249, 253} the effect of frequencies from 10 to 30 Hz on the proliferation of hFOB3, thus, was explored in this study. As shown in **Figure 6.2b**, the proliferation of electrically stimulated hFOB3 on the RGO/CHI scaffolds increased significantly at Day 7 in the group at a frequency of 20 Hz compared with non-stimulated groups and groups with other frequencies. The result was consistent with the work reported by Batur Ercan and Thomas J. Webster,^{25, 249} who had shown that the proliferation of osteoblasts growing on titanium could be enhanced under ES at the same frequency. This also indicated that the effect of frequency on osteoblasts was independent of conductive substrate materials used for growth of cells. However, the cells on RGO/CHI scaffolds with 10 and 30 Hz failed to proliferate from the fourth day, which might suggest that inappropriate frequencies with not only higher but also lower than optimal values cause ES failure. Since the ES was started from the third day after seeding of cells, the cell numbers on the first day were similar without a significant statistically difference. The unhealthy growth of cells on CHI scaffolds under any kind of ES (Figure 6.2a) might have been due to

the insulation of CHI, emphasizing the importance of conductive scaffolds in the study of ES.

Figure 6.2 The proliferation of hFOB cells on the CHI (a), RGO/CHI (b) scaffolds after electrical stimulation at frequencies of 10, 20, and 30 Hz compared to the control group (without electrical stimulation) were characterized by prestobule assay from Day1 to Day 7. (* $p < 0.05$, $n = 3$ for each group).

6.3.1.2 Seeding density

Cell seeding density is well known to be an important factor for tissue functionality, but most of the current work focuses only on the effect of cell seeding density without external ES.²⁵⁴⁻²⁵⁵ Due to the coupling of novel scaffolds and ES in this thesis, we needed to investigate whether there would be an optimal value of seeding density for the proliferation of hFOB cells under ES in the current system. As various seeding densities had been used for seeding osteoblasts on biomaterials from 20 k to 62.5 k in the literature,²⁵⁶⁻²⁵⁷ four different seeding densities (15 k, 30 k, 50 k, and 70 k cells/scaffold with the scaffold volume of 1

cm³) were employed on the RGO/CHI and CHI scaffolds in this study. Among these sample groups, the RGO/CHI scaffolds with various seeding densities were stimulated electrically with the frequency of 20 Hz and the current density of 10 $\mu\text{A}/\text{cm}^3$ and compared with the unstimulated RGO/CHI scaffolds. The proliferation of hFOBs increased with the seeding density (**Figure 6.3a**) for the CHI (without ES) groups except for the groups with 70 k cells/scaffold, which had no statistically significant difference from the group with 50 k cells/scaffold. Similar results were observed in the RGO/CHI (with ES) groups from Day 4 in Figure 6.3b, indicating that at the seeding density of 50 k cells/scaffold, the proliferation rate of hFOBs had already reached its maximum on both CHI (without ES) and RGO/CHI (with ES) scaffolds. However, the control RGO/CHI group without ES (Figure 6.3c) showed a continuous increase in the proliferation of hFOBs with their seeding densities, indicating that without ES, it took more time for hFOBs to reach the maximum proliferation rate. Ishaug-Riley and Holy²⁵⁴⁻²⁵⁵ found that a lower cell seeding density delayed the process of proliferation, which was similar to what was found here in the groups with densities lower than 50 k cells/scaffold.

Figure 6.3 The proliferation of hFOBs on the CHI without electrical stimulation (a), RGO/CHI with electrical stimulation (b), and RGO/CHI without electrical stimulation (c) scaffolds with cell seeding density of 15k to 70k/cm² were characterized by the prestobule assay from Day1 to Day 7. (* $p < 0.05$, $n = 3$ for each group).

6.3.2 Effect of ES on osteoblast functions grown on hybrid substrates

6.3.2.1 Cell alignment

The morphologies of hFOBs grown on scaffolds were observed before and after one and two sessions of ES (Figure 6.4). It was found that osteoblasts on the RGO/CHI scaffold tended to grow in a certain direction after one session of ES

(ES+), which was perpendicular to the current flow direction (the direction of the arrows). However, this alignment of cells was not found in the groups without ES (CHI and RGO/CHI ES-). Even though the surface structure of substrates was reported to influence the orientation of osteoblasts,²⁵⁸ the layered structures of stimulated RGO/CHI and unstimulated RGO/CHI in this work had no significant difference, while only cells grown on RGO/CHI scaffolds with ES presented alignment. The notable alignment of hFOB cells on stimulated RGO/CHI scaffolds was more likely regulated by the introduction of ES on the conductive scaffolds. Similar effects of ES to promote the alignment of cells cultured on conductive substrates were also reported by other groups.²⁵⁹ Cell alignment was reported to have a significant correlation with cell elongation, improving DNA synthesis and cellular functions.²⁶⁰ The enhanced long-term cellular function of hFOB cells on the stimulated scaffolds, hence, could be expected.

Figure 6.4 The morphology of hFOB cells on the unstimulated CHI scaffold, unstimulated RGO/CHI, and stimulated RGO/CHI scaffolds on Day2 to Day 6 was characterized by live/dead assay (green fluorescence =viable cells, red fluorescence = dead cells) Scale bar = 50µm.

6.3.2.2 *In vitro* osteoblast study

In this work, the *in vitro* effect of ES on osteoblast cells on the scaffolds was evaluated. The cell experiments were divided into two groups including RGO/CHI-nES and RGO/CHI-ES. RGO/CHI-nES represented the group of osteoblasts cultured on the RGO/CHI scaffolds in the absence of ES and RGO/CHI-ES represented the group of osteoblast cells cultured on the RGO/CHI scaffolds with biphasic electrical pulse stimulation of 10 $\mu\text{A}/\text{cm}^2$ with a frequency of 20 Hz. In previous reported studies, the applied current ranging from 1.5 $\mu\text{A}/\text{cm}^2$ to 100 $\mu\text{A}/\text{cm}^2$ without scaffolds had been demonstrated to prove their

positive effects on proliferation of osteoblast cells.²⁶¹⁻²⁶³ In the preliminary studies for adequate current amplitude in this work (*refer to section 6.3.1*), the proliferations of osteoblast cells with the current densities of more than 10 $\mu\text{A}/\text{cm}^3$ were all reduced significantly, compared with cells without stimuli. All experiments were thus done under a current density of 10 $\mu\text{A}/\text{cm}^3$. The frequency of 20 Hz was chosen based on the preliminary study of frequency optimization (*refer to section 6.3.1.1*). The osteoblast cells grown on CHI scaffolds without ES (CHI-nES) were served as positive control.

With the ES applied on the third day after cells were seeded, the proliferation of osteoblast cells on scaffolds was measured on Days 1, 4 and 7 and shown in **Figure 6.5**. After exposed to ES, the amount of osteoblast cells in RGO/CHI-ES group increased from 82.4 ± 1.8 k to 248.6 ± 17 k cells within 7 days, which showed an increase of 1.3 fold compared with the proliferation of RGO/CHI-nES group (Figure 6.5b). Similar positive effects of ES on cell proliferation had been reported using monophasic alternating current and biphasic direct current without scaffolds involved.^{247, 263} It is not possible to directly compare the obtained results of present study with literature reports due to their differences in types of electrical stimuli. But despite of the differences in methodology, the proliferation of osteoblast cells cultured on 3D scaffolds in the present study had presented a consistent enhancement by ES with those reported in earlier literatures.^{14, 25, 249, 264} This enhancement of osteoblast proliferation stimulated is assumed to result from the activation of the calmodulin pathway by increasing the intracellular calcium concentration via voltage-gated calcium channel.^{247, 265}

Figure 6.5 The effects of electrical stimulation on the proliferation and differentiation of hFOB cultured on various scaffolds after 1, 4, 7 days until 3 weeks of culture. 1) Comparison of hFOB cell proliferation on stimulated scaffolds with those on unstimulated RGO/CHI scaffold; 2) ALP activity of hFOB cultured on unstimulated CHI scaffold, unstimulated RGO/CHI scaffold and stimulated RGO/CHI scaffold; 3) calcium deposition by hFOB cultured on unstimulated (a) CHI scaffold, (b) unstimulated RGO/CHI scaffold (RGO/CHI-nES) and (c) stimulated RGO/CHI scaffold (RGO/CHI-ES) after 1, 2 and 3 weeks. (* $p < 0.05$, $n = 6$ for each group).

After 7 days of culture, downregulation of the osteoblast cells proliferation could be observed on all groups with an increasing value of ALP activities from day 7 to day 14. ALP was measured as an indication of the differentiation of osteoblast cells, as its activity is the most representative early stage marker of *in vitro* osteoblast cells differentiation.²⁶⁴ ALP in RGO/CHI-ES group was expressed at a significant higher level compared with that of RGO/CHI-nES group in day 7. ALP levels increase with the maturation of osteoblast cells and the beginning of its matrix maturation period, and the highest value of ALP activity, i.e. the peak levels of ALP, indicated the differentiation of osteoblasts²⁶⁶. The peak value of ALP activity of RGO/CHI-ES group was observed around day 7, which was earlier than the RGO/CHI-nES group with its activity peaking in day 11. This suggested that ES could improve the differentiation of osteoblasts by accelerating its process of entering the maturation period via the 3D scaffold. The decrease of ALP activities after day 14 could be observed in all the groups, indicating the onset of deposition and mineralization of extracellular matrix,²⁶⁶ which resulted in the increase of calcium deposition (shown as Alizarin red S content) in all the group in day 21. As ALP is also proven to be involved in the ECM preparation for mineral deposition²⁶⁷⁻²⁶⁸, the earlier peak of ALP activities of RGO/CHI-ES group

is intended to explain the significant higher level of calcium deposition of this group than other control groups. Importantly, the present study showed that the enhancement of ES on the functions of osteoblast cells could be expanded from 2D surfaces^{25, 247, 257} to 3D scaffolds, which provided more alternatives for tissue engineering applications. The enhancement of calcium deposition by ES *in vitro* in the present study also proposed one possible explanation for the promoted bone formations and regenerations *in vivo* dealing with bone fractures treated with ES.²⁶⁹⁻²⁷¹ The apoptotic cells observed after day 7 in all groups suggested the number of surface osteoblast cells were regulated for further differentiation to osteocytes.^{266, 272}

6.4 Summary

In this chapter, the effects of ES on the functions of hFOBs, including cell alignment, proliferation, ALP activity, and mineralization deposition, were studied. Before these studies, to find the optimal parameters used for experiments of ES, the proliferative behaviors of different groups of cells on scaffolds stimulated with various current densities and frequencies were measured. An obvious enhancement of the proliferation was observed on the RGO/CHI scaffolds stimulated with the optimal current density of 10 $\mu\text{A}/\text{cm}^2$. A remarkable improvement of the hFOB proliferation was also found on the RGO/CHI scaffolds electrically stimulated with a frequency of 20 Hz. These results provided a protocol of ES on 3D conductive scaffolds for osteoblasts. By utilizing these optimal parameters, the ES could be delivered successfully through the conductive RGO/CHI scaffold and induce cell morphological changes to affect its matrix organization. The enhanced

proliferation of hFOB cells at the early stage of osteoblast differentiation had positive effects on the next stage of matrix maturation with higher ALP expression, which might have correlations with the mineralization stage with larger calcium depositions. Hence, coupling ES with the 3D conductive RGO/CHI scaffolds induced osteoblasts to develop into mineralization stages with potential for bone formation by improving mineral deposition.

Chapter 7

Conclusions and Future Work

This chapter summarizes all the work described in this thesis and presents the implications and impact of this thesis. Suggested future work is also described.

7.1 Overview of Research Findings

In this thesis, various studies were carried out to explore the following hypotheses:

(i) graphene can be used to develop a conductive 3D biomaterial scaffold for BTE applications and (ii) this scaffold could provide a conductive platform for ES to enhance bone regeneration. The 3D graphene structures obtained from previous methods, such as depositing graphene on a metal foam template, are not suitable for bone tissue applications due to their toxicity,⁵² pore size,⁵³⁻⁵⁴ and mechanical strength.⁵⁵ Thus, it is essential to introduce new methods and components to fabricate the 3D conductive scaffold.

Inspired by the integration of biomaterials with inorganic components from the structural composition of natural bone tissues,¹⁶² this thesis incorporated electrochemical RGO with CHI and fabricated the scaffold with the ISISA method to overcome the above-mentioned shortcomings. The main advantages of this fabrication approach are as follows. First, graphene formed strong interactions with CHI for enhancing the mechanical properties of the hybrid⁶⁹⁻⁷⁰ owing to its high surface area and sufficient sp^2 domains allowing π - π interactions. Second, the unidirectional ice crystals formed from aqueous dispersions¹⁷⁹ of graphene/polymer in the ISISA approach worked as templates without any risk of residuals from metal templates. Third, the solute in the aqueous suspensions was expelled to the boundaries of the adjacent ice “walls”¹⁸⁰ in the ISISA approach. This formed macroporous structures with controllable aligned and porous architectures¹⁷⁸ after removing ice crystals by the subsequent freeze-drying process. Fourth, electrochemical reduction¹⁷² provided an environmentally friendly, nondestructive, and efficient alternative to obtain RGO^{173, 175} avoiding toxic

chemicals or thermal treatment. Fifth, we used a 3D GO/CHI scaffold as one of the electrodes and conducted the electrochemical reduction *in situ* without modifying the macrostructure or pore size of scaffolds. Finally, unlike the current method of depositing GO sheets onto the electrode surface to produce RGO film with limited production yield,¹⁷⁶⁻¹⁷⁷ this *in situ* electrochemical reduction has wider applications and has not been reported before.

The main findings from this thesis are as follows:

1. The work explored in this thesis aimed to develop a novel 3D scaffold based on graphene for BTE applications. As such, the approaches for synthesizing the scaffold and its properties required for bone applications were investigated. Before the study of scaffold properties, the reduction of GO to RGO via *in situ* electrochemical reduction within the scaffold was successfully obtained and characterized. The morphology and mechanical strength of the RGO/CHI scaffold with different RGO contents (0.1–0.7%) were then observed and measured to explore the effects of RGO on scaffold properties. The RGO/CHI scaffold with 0.3% RGO was found to possess the most uniform hierarchical structure with RGO nanosheet interconnection and highest mechanical strength with moderate conductivity (*Chapter 4, Study 1*).
2. Considering the necessity of a controllable degradation rate of scaffolds for creating space for new bone formation during the bone regeneration process, the biodegradability and stability against enzyme degradation of the RGO/CHI scaffolds were investigated. A tunable degradation rate of the scaffolds in the

- enzyme environment was achieved by varying the RGO contents in the scaffolds. Due to the specific surface properties of graphene and uniform structure of scaffolds, the 0.3RGO/CHI scaffold revealed enhanced stability compared to CHI scaffolds. Despite degrading in the enzyme environment, the RGO/CHI scaffold maintained its structure in the normal dissolution test to provide long-term substrates for cell culture (*Chapter 5, Study 2*).
3. The hybrid 0.3RGO/CHI scaffold also presented much better bioactivity for inducing bone-like apatite formation on its surface than CHI scaffolds. The better bone-bonding performance of the RGO/CHI scaffold was a result of its wrinkled surface texture caused by the incorporation of graphene. This result suggests that graphene could enhance both the material and bioactive properties of the scaffolds that are required for BTE (*Chapter 5, Study 2*).
 4. The good proliferation and viability of hFOB3 cells was also observed on the 0.3RGO/CHI scaffolds, which was similar to CHI. The biocompatibility of the hybrid scaffold suggests great potential to support the growth of hFOB3 cells (*Chapter 5, Study 2*).
 5. The optimal parameters of ES for inducing the functions of hFOB3 cells on RGO/CHI scaffolds were obtained by measuring their proliferative effects on hFOB3 cells in terms of current density, frequency, and seeding density. The worse performance of hFOB3 cells on the electrically stimulated CHI scaffolds that were insulated compared to the unstimulated CHI scaffolds emphasized the

importance of conductivity for the scaffolds used in the ES system. These results also provide a protocol of ES on 3D conductive scaffolds for hFOBs.

6. The functions of hFOBs, including cell alignment, proliferation, ALP activity, and mineralization deposition, were shown to be enhanced when they were cultured on the electrically stimulated scaffolds. The electrical signals were delivered successfully through the conductive RGO/CHI scaffold and induced cell morphological changes to affect its matrix organization. The proliferation of hFOBs at the early stage of osteoblast differentiation was also enhanced. With ES, the scaffolds also induced osteoblasts to develop into mineralization stages with potential for bone formation by improving mineral deposition.

7.2 Implications of Research Findings

As a novel material, graphene has attracted a great deal of attention for its specific surface area, excellent mechanical strength, and high electron mobility.^{45-47, 273} Recent research has found that graphene has potential applications in BTE by promoting the cellular functions, including adhesion and proliferation, of bone-forming cells.^{46, 163} Additionally, the extraordinary electrical conductivity enables graphene to provide a conductive platform for therapies with ES, which is a newly developing cell stimuli signal.¹⁴ ES has been found to promote bone healing experimentally and clinically by enhancing the proliferation of bone cells,²⁴ encouraging ECM deposition,²⁴⁴⁻²⁴⁶ and inducing the expression of growth factors.²⁴⁵⁻²⁴⁶ Therefore, the coupling of 3D graphene scaffolds and ES is essential to maximize the functions of bone cells during osteogenesis. Despite the increasing

research interest in applying graphene to bone tissue regeneration, few reports have investigated the bone-related cell behaviors on 3D graphene scaffolds.^{165,274-275} The reduction approach to obtain RGO also needs to be improved.^{49, 165, 170-173} The work described in this thesis focused on creating a substrate for developing conductive platforms for BTE using novel 3D substrates obtained by a simple green fabrication approach.

Further characterization of the scaffolds with optimal RGO contents was carried out to explore their biological properties for meeting the requirements of bone tissue applications including biodegradability, structure stability, bioactivity, and biocompatibility. The scaffolds with RGO were all found to be degradable over time. The competition of graphene surface adsorption ability^{214, 276} and scaffold structure resulted in a critical value of RGO concentrations at which the scaffolds had the lowest rate of degradation and highest stability against enzymes (*Chapter 5, Figure 5.1*). This RGO content was consistent with that of scaffolds with the optimal mechanical strength and structure (*Chapter 4, Section 4.3.2*). Moreover, the scaffold with this RGO content was found to have high stability against normal dissolution in PBS solution (*Chapter 5, Figure 5.2*) so that it could work as long-term support for cell culture. The wrinkled surface of scaffold layers due to the incorporation of graphene significantly improved the capacity of scaffolds to induce apatite formation on their surfaces compared with CHI (*Chapter 5, Figure 5.3 & Figure 5.4*), which was found *in vivo* to link surrounding tissues with the scaffold surfaces as an implantation interface.¹⁹⁵ This ability was expected to endow RGO/CHI scaffolds with high bioactivity and positive effects on cellular behaviors,¹⁹⁸ including cell attachment, viability, and proliferation. The

enhancement of hFOB attachment compared with CHI scaffolds (*Chapter 5, Figure 5.5*) and similar performance on supporting cell proliferation with CHI scaffolds (*Chapter 5, Figure 5.6*) validated the high bioactivity of RGO/CHI scaffolds and presented their good biocompatibility for osteoblasts.

In the last part of this thesis, the effects of coupling biphasic ES and RGO/CHI scaffolds on the functions of hFOBs were explored. With advantages of less faradic byproducts, lower accumulation of charged proteins, fewer pH changes,²⁵ and positive evidence on promoting osteoblast proliferation,^{25, 249} biphasic current stimulation could overcome the problems caused by DC.^{31, 247} By applying ES to cells through conductive scaffolds, the stimulated area could be refined within the location where new tissues were expected to regenerate. The risks of tumor progression in the unexpected regions could also be decreased, which could not be achieved by electromagnetic field and capacitive coupling approaches.²⁴⁸ To efficiently deliver the electrical signals, various parameters need to be optimized. The preliminary study on the effect of current density showed that hFOB proliferation on the conductive scaffolds could only be enhanced with a low current and that a high current would significantly inhibit the growth of hFOBs (*Chapter 6, Figure 6.1*). The optimal value of current density was similar to the physiological current of natural bone tissues.²⁵⁰ This suggested that the conductive scaffolds provided a simulated environment similar to that provided by natural tissues for ES. The frequency suitable for electrically stimulating hFOBs on RGO/CHI scaffolds was also defined by exploring the cell proliferation under stimulations with a range of frequencies. Unlike the current density, only the moderate value of frequency provided a result comparable with that of the non-

stimulated group (*Chapter 6, Figure 6.2*). Moreover, it was independent of the material type of conductive substrate since the same value of frequency was also found to enhance osteoblast proliferation on other types of materials.^{25, 249} Based on the results of current density and frequency, the most efficient seeding density of hFOB cells for proliferation under ES was obtained through characterizing the proliferation of hFOB cells. The scaffolds with a seeding density of 50 k cells/cm³ were found to possess the peak value of enhancing the rate of hFOB proliferation (*Chapter 6, Figure 6.3*), which could not be improved further by increasing the density.

Based on the optimal parameters of the ES and due to the good conductivity of the scaffolds, efficient responsiveness of the hFOB cells to the ES could be measured and cell behaviors at different stages of differentiation could be investigated in terms of proliferation, morphology, ALP activity, and mineralization deposition. After two sessions of ES, hFOB cells on the stimulated RGO/CHI scaffolds presented obvious morphologies of alignment (*Chapter 6, Figure 6.4*). This was suggested to have a significant correlation with cell elongation, improving DNA synthesis and cellular functions.²⁶⁰ This indicated that ES could improve cell functions (not only their proliferation but also their morphologies) independent of the substrate materials (*Chapter 6, Section 6.3.2.1*). This enhanced proliferation of hFOB cells at the early stage of osteoblast differentiation also contributed to the matrix maturation stage of hFOB cells differentiation with higher ALP expression at its beginning (*Chapter 6, Figure 6.5*), which subsequently induced the mineralization on the matrix with larger calcium depositions. Hence, coupling ES with the 3D conductive RGO/CHI

scaffolds induced osteoblasts to develop into mineralization stages with potential for bone formation by improving mineral deposition (*Chapter 6, Figure 6.5*).

This scaffold could be applied as highly effective assistance to bone regeneration in clinical cases of small defects with restricted areas or after tumor resections. First, this scaffold provides a 3D macrostructure that meets possible clinical requirements. The 3D structure is significant in tissue engineering, as cells in cell culture cannot self-assemble into 3D organs or organ-like structures in normal conditions. 3D scaffolds could guide the conversion from cell suspension into a useful 3D organ structure. The free-standing structure of this scaffold also enables the fabrication of different shapes in small sizes in clinical applications. Secondly, this scaffold provides a conductive platform for optimal ES treatments. Current ES devices are mainly based on metallic, ITO-coated,⁴⁴ or conductive polymer-coated³⁸ electrodes, which can neither transform ES signals onto cells cultured on scaffolds with complex geometry nor confine signals in a defined area.²⁶ The RGO/CHI conductive system could allow cells to be cultured directly on the scaffolds that would subsequently be electrically regulated. The electrical currents and electrical potential gradients can be controlled precisely by adjusting the resistivity of the scaffolds. By applying ES to cells through conductive scaffolds, the stimulated area could be refined within the location where new tissues are expected to regenerate. The risk of tumor progression in the unexpected regions could also be decreased, which could not be achieved by other ES methods, such as electromagnetic field and capacitive coupling approaches.²⁴⁸ This scaffold can potentially work as a multifunctional bioactive and biodegradable platform for cell and tissue growth in clinical applications.

This knowledge could be further utilized for designing, developing, and improving graphene-based 3D conductive substrates for BTE with applications related with the field of ES.

7.3 Future Work

7.3.1 3D scaffolds with controllable reduction states of RGO and their effects on cellular activities

The surface of biomaterials plays an important role in biological and biomedical applications²⁷⁷⁻²⁷⁸ and is believed to have a critical influence on the biocompatibility of substrates.²⁷⁹ Cell functions are influenced by the adsorption of protein molecules²⁸⁰⁻²⁸¹ and the hydrophilicity/hydrophobicity,²⁸² bio-recognition,²⁸¹ and micro/nano-patterning of biomaterials,²⁸³ which are all mediated by their surface properties. There are no clear rules for predicting cell behaviors based on only the surface properties of certain materials.²⁷⁷ Hence, to explore cell interactions with RGO further, engineering of the RGO surface properties is essential for future work.

The reduction states of RGO may affect these properties significantly. This is because the reduction progress of RGO changes its oxygen content, surface charge, and functional groups, which are all key factors for its surface properties.²⁸⁴ The adhesion and proliferation of osteoblasts cultured on thermally RGO films could be influenced by tuning the reduction states of GO due to various surface chemistry of graphene, including surface oxygen content and adsorption ability for ECM proteins.²²⁴ However, most of the literature prepared RGO with various

reduction states only by the thermal reduction approach, which involves tuning the reduction states by adjusting the reaction time.^{224, 285} Nevertheless, thermal reduction requires extra tests to determine the reduction degrees of the RGO, such as its conductivity, impedance, and capacitance-voltage (CV) curve. The chemical reduction of GO, based on a chemical reaction in the liquid phase, is hardly able to provide RGO with controllable sheets due to the aggregation tendency of GO in aqueous solutions²⁸⁶ and uncontrollable reactions. Based on the results of this thesis, *in situ* electrochemical reduction might provide an alternative for tuning the reduction states of RGO by controlling the parameters of electrochemical reduction and measuring its degrees of reduction in real time by characterizing its electrical properties in the meantime. Therefore, by using *in situ* electrochemical reduction to prepare RGO with various reduction states, the effects of the reduction states on cell behaviors could be investigated.

7.3.2 Triggered drug release regulated by modulated ES via conductive scaffolds

In this thesis, 3D scaffolds based on graphene have been demonstrated to work as promising substrates for supporting the healthy proliferation and viability of hFOBs (*Section 5.3.3.2*). Besides osteoblasts, the adhesion, growth, and proliferation of human mesenchymal stem cells (hMSCs) could be promoted on the graphene films.¹⁶³ Graphene has also found its position as a suitable substrate for the growth of other types of cells, such as mammalian NIH 3T3 fibroblast cells⁴⁵ and chondrogenic cells, by enhancing their cellular functions, including gene transfection and expression. Recently, MSCs cultured on graphene-coated substrates were found to differentiate into osteocytes in a controlled manner at an

accelerated rate.²⁸⁷ It was also reported that the differentiation of MSCs is induced toward osteogenesis by the substrates of graphene-polydimethylsiloxane (PDMS). This was suggested to act as a preconcentration platform for the osteogenic inducers, with the high loading capacity of β -glycerolphosphate and dexamethasone.¹⁰ These results suggested that graphene not only has distinct properties as a substrate for the proliferation and differentiation of different types of cells including stem cells but also has the potential to work as a substrate capable of the high loading of biomolecules.

GO has been explored by many researchers for its high loading capacity of biomolecules. Bone morphogenetic protein-2 (BMP-2), one of the most potent growth factors for the osteoinductivity of bone regeneration, has been reported to be delivered by GO by electrostatic interactions and π - π stacking.²⁸⁸ Anticancer drugs, such as Doxorubicin (DOX), were found to be loaded with high capacity on GO/polymer compositions.²⁸⁹ Graphene has a high specific surface area, unique structure with π - π stacking, and electrostatic interactions with other molecules caused by its surface charges. Hence, it could also be expected to work as an efficient platform for absorbing biomolecules including genes, growth factors, antibodies, and anticancer drugs. The various functional groups on the surface of graphene, including amino, hydroxyl, and carboxyl groups, could provide active sites for diverse linkers with other molecules. Moreover, treating or reducing the risk of tumors could be made more efficient by using graphene for absorbing targeting molecules for cancers and anticancer drugs.²⁹⁰

Figure 7.1 The effect of GO concentrations on the loading efficiency of dextran of the GO/CHI scaffolds.

A preliminary study was conducted to investigate the loading efficiency of the scaffolds, and the results are shown in Figure 7.1. It was found that scaffolds with GO indicated similar and higher efficiency compared with CHI scaffolds. This is consistent with the surface charge of these scaffolds, as shown in the zeta potential results (**Table 7.1**).

Table 7.1 The zeta potentials of scaffolds with different GO concentrations.

Sample	CHI	0.1GO/CHI	0.3GO/CHI	0.5GO/CHI	0.7GO/CHI	GO
Zeta potential (mV)	60.9	55.4	60.95	59.4	36.1	-26.7

7.3.3 Controllable drug release regulated by electrical stimulations

The delivery of various drugs provides important therapeutic solutions for clinical medicines by improving the efficacy and convenience²⁹¹⁻²⁹² and reducing

enzymatic degradation.²⁹³ Graphene, with its large specific surface area and 2D structure, has been used as a drug carrier with two available faces and edges unlike other types of carriers.²⁹⁴ The sufficient functional groups on its surface for binding also make it a promising drug carrier substrate.^{289, 295}

However, the large amount of biomolecules within specific areas could cause side-effects, toxicity,²⁹⁶ or tumor growth.²⁹⁷ Controllable drug delivery, thus, has attracted much interest, and the constant, controllable delivery of the required dosage of drugs to the target at a specific site is currently one of the biggest challenges for the development of drug therapies. As mentioned in this thesis, GO can be electrochemically reduced to RGO (*Section 4.3.1.1*), which changes its surface properties including functional groups (*Chapter 4, Figure 4.2*) and conductivity (*Chapter 4, Figure 4.4*). The molecules binding on the surface of GO via electrostatic interaction, hydrogen bonding, or π - π stacking,^{281, 288, 284, 298} are thus controllably released by the change of its surface properties induced by electrochemical reduction.

Moreover, it has been reported that the reduction/oxidation of multilayer RGO film could be reversed by reversal of the bias in air when low maximum bias values are used (< 2 V in the literature).²⁹⁹ However, the oxidation of graphene film could only be observed for biases over 0.5 V, which might have been due to the different activation energies required by reduction and oxidation (reduction $<$ oxidation). The mechanism of the reversible reduction of GO was suspected to be related with the energy provided by the electrical currents. GO flakes are unstable in ambient atmosphere and will slowly decompose to partially RGO at room temperature. The

negative charge could effectively increase the free energy change (ΔG) of the decomposition reaction, which could enhance the rate of the reaction, while the oxidation would be spontaneous under positive charge when sufficient binding energy of oxygen is provided. The reduction of GO and oxidization of RGO occur under different voltages,³⁰⁰ making it possible to switch the release of drugs on and off. Therefore, the response of GO to the electrochemical oxidation and reduction could change its conductivity, surface charge, and surface functional groups. The reversible oxidation/reduction of GO could enable the scaffold to be utilized for controllable loading and the release of drugs with unlimited times.

References

1. Salgado, A. J.; Coutinho, O. P.; Reis, R. L., Bone tissue engineering: state of the art and future trends. *Macromol Biosci* **2004**, *4* (8), 743-765.
2. O'Keefe, R. J.; Mao, J., Bone tissue engineering and regeneration: from discovery to the clinic—an overview. *Tissue Engineering Part B: Reviews* **2011**, *17* (6), 389-392.
3. Garc á-Gareta, E.; Coathup, M. J.; Blunn, G. W., Osteoinduction of bone grafting materials for bone repair and regeneration. *Bone* **2015**, *81*, 112-121.
4. Gupta, A.; Kukkar, N.; Sharif, K.; Main, B. J.; Albers, C. E.; El-Amin III, S. F., Bone graft substitutes for spine fusion: A brief review. *World journal of orthopedics* **2015**, *6* (6), 449.
5. Murugan, R.; Ramakrishna, S. Development of nanocomposites for bone grafting. 2005.
6. Burg, K. J. L.; Porter, S.; Kellam, J. F., Biomaterial developments for bone tissue engineering. *Biomaterials* **2000**, *21* (23), 2347-2359.
7. Porter, J. R.; Ruckh, T. T.; Popat, K. C., Bone tissue engineering: a review in bone biomimetics and drug delivery strategies. *Biotechnol Progr* **2009**, *25* (6), 1539-1560.
8. Silber, J. S.; Anderson, D. G.; Daffner, S. D.; Brislin, B. T.; Leland, J. M.; Hilibrand, A. S.; Vaccaro, A. R.; Albert, T. J., Donor site morbidity after anterior iliac crest bone harvest for single-level anterior cervical discectomy and fusion. *Spine* **2003**, *28* (2), 134-139.
9. Healy, K. E.; Guldberg, R. E., Bone tissue engineering. *Journal of musculoskeletal & neuronal interactions* **2007**, *7* (4), 328-30.
10. Zhang, Y.; Nayak, T. R.; Hong, H.; Cai, W., Graphene: a versatile nanoplatform for biomedical applications. *Nanoscale* **2012**, *4* (13), 3833-3842.
11. Nishida, J.; Shimamura, T., Methods of reconstruction for bone defect after tumor excision: a review of alternatives. *Medical Science Monitor* **2008**, *14* (8), RA107-RA113.
12. Hou, C.-H.; Yang, R.-S.; Hou, S.-M., Hospital-based allogenic bone bank—10-year experience. *Journal of Hospital Infection* **2005**, *59* (1), 41-45.
13. Burg, K. J.; Porter, S.; Kellam, J. F., Biomaterial developments for bone tissue engineering. *Biomaterials* **2000**, *21* (23), 2347-59.
14. Balint, R.; Cassidy, N. J.; Cartmell, S. H., Electrical Stimulation: A Novel Tool for Tissue Engineering. *Tissue Eng Part B-Re* **2013**, *19* (1), 48-57.
15. Petite, H.; Viateau, V.; Bensaid, W.; Meunier, A.; de Pollak, C.; Bourguignon, M.; Oudina, K.; Sedel, L.; Guillemain, G., Tissue-engineered bone regeneration. *Nature biotechnology* **2000**, *18* (9), 959.
16. Boonen, K. J.; Langelaan, M. L.; Polak, R. B.; van der Schaft, D. W.; Baaijens, F. P.; Post, M. J., Effects of a combined mechanical stimulation protocol: Value for skeletal muscle tissue engineering. *Journal of biomechanics* **2010**, *43* (8), 1514-1521.
17. Fox, S.; Chambers, T.; Chow, J., Nitric oxide is an early mediator of the increase in bone formation by mechanical stimulation. *American Journal of Physiology-Endocrinology And Metabolism* **1996**, *270* (6), E955-E960.
18. Robling, A. G.; Niziolek, P. J.; Baldridge, L. A.; Condon, K. W.; Allen, M. R.; Alam, I.; Mantila, S. M.; Gluhak-Heinrich, J.; Bellido, T. M.; Harris, S. E.,

Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *Journal of Biological Chemistry* **2008**, 283 (9), 5866-5875.

19. Au, H. T. H.; Cheng, I.; Chowdhury, M. F.; Radisic, M., Interactive effects of surface topography and pulsatile electrical field stimulation on orientation and elongation of fibroblasts and cardiomyocytes. *Biomaterials* **2007**, 28 (29), 4277-4293.

20. Kotani, H.; Kawaguchi, H.; Shimoaka, T.; Iwasaka, M.; Ueno, S.; Ozawa, H.; Nakamura, K.; Hoshi, K., Strong static magnetic field stimulates bone formation to a definite orientation in vitro and in vivo. *Journal of Bone and Mineral Research* **2002**, 17 (10), 1814-1821.

21. Gauvin, R.; Parenteau-Bareil, R.; Larouche, D.; Marcoux, H.; Bisson, F.; Bonnet, A.; Auger, F. A.; Bolduc, S.; Germain, L., Dynamic mechanical stimulations induce anisotropy and improve the tensile properties of engineered tissues produced without exogenous scaffolding. *Acta biomaterialia* **2011**, 7 (9), 3294-3301.

22. Balint, R.; Cassidy, N. J.; Cartmell, S. H., Electrical stimulation: a novel tool for tissue engineering. *Tissue Engineering Part B: Reviews* **2012**, 19 (1), 48-57.

23. Lavine, L.; Grodzinsky, A., Electrical stimulation of repair of bone. *JBJS* **1987**, 69 (4), 626-630.

24. Ciombor, D. M.; Aaron, R. K., The Role of Electrical Stimulation in Bone Repair. *Foot and Ankle Clinics* **2005**, 10 (4), 579-593.

25. Ercan, B.; Webster, T. J., The effect of biphasic electrical stimulation on osteoblast function at anodized nanotubular titanium surfaces. *Biomaterials* **2010**, 31 (13), 3684-3693.

26. Shi, G.; Zhang, Z.; Rouabhia, M., The regulation of cell functions electrically using biodegradable polypyrrole–polylactide conductors. *Biomaterials* **2008**, 29 (28), 3792-3798.

27. Wang, E.; Zhao, M.; Forrester, J. V.; McCaig, C. D., Bi-directional migration of lens epithelial cells in a physiological electrical field. *Experimental eye research* **2003**, 76 (1), 29-37.

28. Song, B.; Zhao, M.; Forrester, J. V.; McCaig, C. D., Electrical cues regulate the orientation and frequency of cell division and the rate of wound healing in vivo. *Proceedings of the National Academy of Sciences* **2002**, 99 (21), 13577-13582.

29. Sun, S.; Titushkin, I.; Cho, M., Regulation of mesenchymal stem cell adhesion and orientation in 3D collagen scaffold by electrical stimulus. *Bioelectrochemistry* **2006**, 69 (2), 133-141.

30. Hammerick, K. E.; Longaker, M. T.; Prinz, F. B., In vitro effects of direct current electric fields on adipose-derived stromal cells. *Biochemical and biophysical research communications* **2010**, 397 (1), 12-17.

31. Kim, I. S.; Song, J. K.; Song, Y. M.; Cho, T. H.; Lee, T. H.; Lim, S. S.; Kim, S. J.; Hwang, S. J., Novel effect of biphasic electric current on in vitro osteogenesis and cytokine production in human mesenchymal stromal cells. *Tissue Engineering Part A* **2009**, 15 (9), 2411-2422.

32. Illingworth, C. M.; Barker, A., Measurement of electrical currents emerging during the regeneration of amputated finger tips in children. *Clinical Physics and Physiological Measurement* **1980**, 1 (1), 87.

33. MacGinitie, L. A.; Wu, D.; Cochran, G., Streaming potentials in healing, remodeling, and intact cortical bone. *Journal of Bone and Mineral Research* **1993**, 8 (11), 1323-1335.
34. McCaig, C. D.; Rajniecek, A. M.; Song, B.; Zhao, M., Controlling cell behavior electrically: current views and future potential. *Physiological reviews* **2005**, 85 (3), 943-978.
35. Gardner, S. E.; Frantz, R. A.; Schmidt, F. L., Effect of electrical stimulation on chronic wound healing: a meta- analysis. *Wound Repair and Regeneration* **1999**, 7 (6), 495-503.
36. Houghton, P. E.; Campbell, K. E.; Fraser, C. H.; Harris, C.; Keast, D. H.; Potter, P. J.; Hayes, K. C.; Woodbury, M. G., Electrical stimulation therapy increases rate of healing of pressure ulcers in community-dwelling people with spinal cord injury. *Archives of physical medicine and rehabilitation* **2010**, 91 (5), 669-678.
37. Mendonça, A. C.; Barbieri, C. H.; Mazzer, N., Directly applied low intensity direct electric current enhances peripheral nerve regeneration in rats. *Journal of Neuroscience Methods* **2003**, 129 (2), 183-190.
38. Schmidt, C. E.; Shastri, V. R.; Vacanti, J. P.; Langer, R., Stimulation of neurite outgrowth using an electrically conducting polymer. *Proceedings of the National Academy of Sciences* **1997**, 94 (17), 8948-8953.
39. Tandon, N.; Cannizzaro, C.; Chao, P.-H. G.; Maidhof, R.; Marsano, A.; Au, H. T. H.; Radisic, M.; Vunjak-Novakovic, G., Electrical stimulation systems for cardiac tissue engineering. *Nature Protocols* **2009**, 4, 155.
40. Brighton, C. T.; Black, J.; Friedenberg, Z.; Esterhai, J.; Day, L.; Connolly, J., A multicenter study of the treatment of non-union with constant direct current. *J Bone Joint Surg Am* **1981**, 63 (1), 2-13.
41. Williams, D. F., On the mechanisms of biocompatibility. *Biomaterials* **2008**, 29 (20), 2941-2953.
42. Brown, J. L.; Kumbar, S. G.; Laurencin, C. T., Chapter II.6.7 - Bone Tissue Engineering. In *Biomaterials Science (Third Edition)*, Ratner, B. D.; Hoffman, A. S.; Schoen, F. J.; Lemons, J. E., Eds. Academic Press: 2013; pp 1194-1214.
43. Kretlow, J. D.; Mikos, A. G., Review: mineralization of synthetic polymer scaffolds for bone tissue engineering. *Tissue engineering* **2007**, 13 (5), 927-38.
44. Yaoita, M.; Ikariyama, Y.; Aizawa, M., Electrical effects on the proliferation of living HeLa cells cultured on optically transparent electrode surface. *Journal of biotechnology* **1990**, 14 (3-4), 321-332.
45. Ryoo, S. R.; Kim, Y. K.; Kim, M. H.; Min, D. H., Behaviors of NIH-3T3 fibroblasts on graphene/carbon nanotubes: proliferation, focal adhesion, and gene transfection studies. *Acs Nano* **2010**, 4 (11), 6587-98.
46. Nayak, T. R.; Andersen, H.; Makam, V. S.; Khaw, C.; Bae, S.; Xu, X.; Ee, P. L.; Ahn, J. H.; Hong, B. H.; Pastorin, G.; Ozyilmaz, B., Graphene for controlled and accelerated osteogenic differentiation of human mesenchymal stem cells. *Acs Nano* **2011**, 5 (6), 4670-8.
47. Lee, W. C.; Lim, C. H.; Shi, H.; Tang, L. A.; Wang, Y.; Lim, C. T.; Loh, K. P., Origin of enhanced stem cell growth and differentiation on graphene and graphene oxide. *Acs Nano* **2011**, 5 (9), 7334-41.
48. He, Z. M.; Liu, J.; Qiao, Y.; Li, C. M.; Tan, T. T. Y., Architecture Engineering of Hierarchically Porous Chitosan/Vacuum-Stripped Graphene Scaffold as Bioanode for High Performance Microbial Fuel Cell. *Nano Lett* **2012**, 12 (9), 4738-4741.

49. Wang, X.; Bai, H.; Yao, Z.; Liu, A.; Shi, G., Electrically conductive and mechanically strong biomimetic chitosan/reduced graphene oxide composite films. *Journal of Materials Chemistry* **2010**, 20 (41), 9032.
50. Fan, H.; Wang, L.; Zhao, K.; Li, N.; Shi, Z.; Ge, Z.; Jin, Z., Fabrication, mechanical properties, and biocompatibility of graphene-reinforced chitosan composites. *Biomacromolecules* **2010**, 11 (9), 2345-51.
51. Depan, D.; Girase, B.; Shah, J. S.; Misra, R. D., Structure-process-property relationship of the polar graphene oxide-mediated cellular response and stimulated growth of osteoblasts on hybrid chitosan network structure nanocomposite scaffolds. *Acta biomaterialia* **2011**, 7 (9), 3432-45.
52. Chen, Z.; Ren, W.; Gao, L.; Liu, B.; Pei, S.; Cheng, H.-M., Three-dimensional flexible and conductive interconnected graphene networks grown by chemical vapour deposition. *Nature materials* **2011**, 10 (6), 424-428.
53. Cao, X.; Shi, Y.; Shi, W.; Lu, G.; Huang, X.; Yan, Q.; Zhang, Q.; Zhang, H., Preparation of Novel 3D Graphene Networks for Supercapacitor Applications. *Small* **2011**, 7 (22), 3163-3168.
54. Burckel, D. B.; Xiao, X.; Polsky, R., *3D graphene scaffolds produced by interferometric lithography*. 2015.
55. Chakravarty, D.; Tiwary, C. S.; Woellner, C. F.; Radhakrishnan, S.; Vinod, S.; Ozden, S.; da Silva Autreto, P. A.; Bhowmick, S.; Asif, S.; Mani, S. A.; Galvao, D. S.; Ajayan, P. M., 3D Porous Graphene by Low-Temperature Plasma Welding for Bone Implants. *Adv Mater* **2016**, 28 (40), 8959-8967.
56. O'Brien, F. J., Biomaterials & scaffolds for tissue engineering. *Materials Today* **2011**, 14 (3), 88-95.
57. Stevens, M., Biomaterials for bone tissue engineering. *Materials Today* **2008**, 11 (5), 18-25.
58. Winey, K. I.; Vaia, R. A. E., *MRS Bull.* **2007**, 4, 314-319.
59. Liu, X.; Ma, P. X., Polymeric scaffolds for bone tissue engineering. *Annals of biomedical engineering* **2004**, 32 (3), 477-486.
60. Wan, C.; Frydrych, M.; Chen, B., Strong and bioactive gelatin–graphene oxide nanocomposites. *Soft Matter* **2011**, 7 (13), 6159-6166.
61. Ionita, M.; Pandele, M. A.; Iovu, H., Sodium alginate/graphene oxide composite films with enhanced thermal and mechanical properties. *Carbohydrate Polymers* **2013**, 94 (1), 339-344.
62. Donahue, H. J.; Li, Z.; Zhou, Z.; Yellowley, C. E., Differentiation of human fetal osteoblastic cells and gap junctional intercellular communication. *American Journal of Physiology-Cell Physiology* **2000**, 278 (2), C315-C322.
63. Nieto-Suárez, M.; López-Quintela, M. A.; Lazzari, M., Preparation and characterization of crosslinked chitosan/gelatin scaffolds by ice segregation induced self-assembly. *Carbohydrate Polymers* **2016**, 141, 175-183.
64. Drury, J. L.; Mooney, D. J., Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **2003**, 24 (24), 4337-4351.
65. Levengood, S. L.; Zhang, M., Chitosan-based scaffolds for bone tissue engineering. *Journal of materials chemistry. B, Materials for biology and medicine* **2014**, 2 (21), 3161-3184.
66. Hu, Q.; Li, B.; Wang, M.; Shen, J., Preparation and characterization of biodegradable chitosan/hydroxyapatite nanocomposite rods via in situ hybridization: a potential material as internal fixation of bone fracture. *Biomaterials* **2004**, 25 (5), 779-785.

67. LogithKumar, R.; KeshavNarayan, A.; Dhivya, S.; Chawla, A.; Saravanan, S.; Selvamurugan, N., A review of chitosan and its derivatives in bone tissue engineering. *Carbohydrate polymers* **2016**, *151*, 172-188.
68. Seol, Y.-J.; Lee, J.-Y.; Park, Y.-J.; Lee, Y.-M.; Rhyu, I.-C.; Lee, S.-J.; Han, S.-B.; Chung, C.-P., Chitosan sponges as tissue engineering scaffolds for bone formation. *Biotechnology letters* **2004**, *26* (13), 1037-1041.
69. Pan, Y.; Wu, T.; Bao, H.; Li, L., Green fabrication of chitosan films reinforced with parallel aligned graphene oxide. *Carbohydrate Polymers* **2011**, *83* (4), 1908-1915.
70. Yang, X.; Tu, Y.; Li, L.; Shang, S.; Tao, X. M., Well-dispersed chitosan/graphene oxide nanocomposites. *ACS applied materials & interfaces* **2010**, *2* (6), 1707-13.
71. He, Z.; Liu, J.; Qiao, Y.; Li, C. M.; Tan, T. T. Y., Architecture Engineering of Hierarchically Porous Chitosan/Vacuum-Stripped Graphene Scaffold as Bioanode for High Performance Microbial Fuel Cell. *Nano Lett* **2012**, *12* (9), 4738-4741.
72. Kokubo, T.; Takadama, H., How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* **2006**, *27* (15), 2907-2915.
73. Bauer, T. W.; Muschler, G. F., Bone Graft Materials: An Overview of the Basic Science. *Clinical orthopaedics and related research* **2000**, *371*, 10-27.
74. Lin, C. Y.; Schek, R. M.; Mistry, A. S.; Shi, X. F.; Mikos, A. G.; Krebsbach, P. H.; Hollister, S. J., Functional bone engineering using ex vivo gene therapy and topology-optimized, biodegradable polymer composite scaffolds. *Tissue engineering* **2005**, *11* (9-10), 1589-1598.
75. Shin, H.; Jo, S.; Mikos, A. G., Biomimetic materials for tissue engineering. *Biomaterials* **2003**, *24* (24), 4353-4364.
76. Saiz, E.; Zimmermann, E. A.; Lee, J. S.; Wegst, U. G.; Tomsia, A. P., Perspectives on the role of nanotechnology in bone tissue engineering. *Dental materials : official publication of the Academy of Dental Materials* **2013**, *29* (1), 103-15.
77. Bonzani, I. C.; George, J. H.; Stevens, M. M., Novel materials for bone and cartilage regeneration. *Current Opinion in Chemical Biology* **2006**, *10* (6), 568-575.
78. Logothetis, C. J.; Lin, S. H., Osteoblasts in prostate cancer metastasis to bone. *Nature reviews. Cancer* **2005**, *5* (1), 21-8.
79. Ma, P. X., Scaffolds for tissue fabrication. *Materials Today* **2004**, *7* (5), 30-40.
80. Boyan, B. D.; Hummert, T. W.; Dean, D. D.; Schwartz, Z., Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials* **1996**, *17* (2), 137-146.
81. Yoshikawa, T.; Ohgushi, H.; Tamai, S., Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. *J Biomed Mater Res* **1996**, *32* (3), 481-492.
82. Bose, S.; Roy, M.; Bandyopadhyay, A., Recent advances in bone tissue engineering scaffolds. *Trends in biotechnology* **2012**, *30* (10), 546-54.
83. Ramakrishna, S.; Mayer, J.; Wintermantel, E.; Leong, K. W., Biomedical applications of polymer-composite materials: a review. *Compos Sci Technol* **2001**, *61* (9), 1189-1224.
84. Meijer, G. J.; de Bruijn, J. D.; Koole, R.; van Blitterswijk, C. A., Cell-based bone tissue engineering. *Plos Med* **2007**, *4* (2), 260-264.

85. Langer, R.; Vacanti, J. P., TISSUE ENGINEERING. *Science* **1993**, 260 (5110), 920-926.
86. Healy, K. E.; Tsai, D.; Kim, J. E. In *Osteogenic cell attachment to degradable polymers*, MRS Proceedings, Cambridge Univ Press: 1991; p 109.
87. Ogino, M.; Ohuchi, F.; Hench, L. L., Compositional dependence of the formation of calcium phosphate films on bioglass. *J Biomed Mater Res* **1980**, 14 (1), 55-64.
88. Kitsugi, T.; Nakamura, T.; Yamamura, T.; Kokubu, T.; Shibuya, T.; Takagi, M., SEM- EPMA observation of three types of apatite- containing glass- ceramics implanted in bone: The variance of a Ca- P- rich layer. *J Biomed Mater Res* **1987**, 21 (10), 1255-1271.
89. Ohtsuki, C.; Kushitani, H.; Kokubo, T.; Kotani, S.; Yamamuro, T., Apatite formation on the surface of ceravital- type glass- ceramic in the body. *J Biomed Mater Res* **1991**, 25 (11), 1363-1370.
90. LeGeros, R.; Lin, S.; Rohanizadeh, R.; Mijares, D.; LeGeros, J., Biphasic calcium phosphate bioceramics: preparation, properties and applications. *Journal of materials science: Materials in Medicine* **2003**, 14 (3), 201-209.
91. Kokubo, T.; Ohtsuki, C.; Kotani, S.; Kitsugi, T.; Yamamuro, T. In *SURFACE STRUCTURE OF BIOACTIVE GLASS-CERAMIC A-W IMPLANTED INTO SHEEP AND HUMAN VERTEBRA*, Bioceramics. Proc. 2 nd Int. Symp. Ceramics in Medicine., 1989; pp 113-120.
92. Yuan, H.; de Groot, K., Calcium phosphate biomaterials: an overview. In *Learning from Nature How to Design New Implantable Biomaterials: From Biomineralization Fundamentals to Biomimetic Materials and Processing Routes*, Springer: 2004; pp 37-57.
93. Kokubo, T., Bioactive glass ceramics: properties and applications. *Biomaterials* **1991**, 12 (2), 155-163.
94. Liu, H.; Slamovich, E. B.; Webster, T. J., Less harmful acidic degradation of poly(lactico-glycolic acid) bone tissue engineering scaffolds through titania nanoparticle addition. *International journal of nanomedicine* **2006**, 1 (4), 541-5.
95. Sabir, M. I.; Xu, X. X.; Li, L., A review on biodegradable polymeric materials for bone tissue engineering applications. *J Mater Sci* **2009**, 44 (21), 5713-5724.
96. Sun, H. L.; Wu, C. T.; Dai, K. R.; Chang, J.; Tang, T. T., Proliferation and osteoblastic differentiation of human bone marrow-derived stromal cells on akermanite-bioactive ceramics. *Biomaterials* **2006**, 27 (33), 5651-5657.
97. Moreau, J. L.; Xu, H. H. K., Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate - Chitosan composite scaffold. *Biomaterials* **2009**, 30 (14), 2675-2682.
98. Yang, S. F.; Leong, K. F.; Du, Z. H.; Chua, C. K., The design of scaffolds for use in tissue engineering. Part 1. Traditional factors. *Tissue engineering* **2001**, 7 (6), 679-689.
99. Qi, X. P.; Ye, J. D.; Wang, Y. J., Improved injectability and in vitro degradation of a calcium phosphate cement containing poly(lactide-co-glycolide) microspheres. *Acta biomaterialia* **2008**, 4 (6), 1837-1845.
100. Zhang, R. Y.; Ma, P. X., Porous poly(L-lactic acid)/apatite composites created by biomimetic process. *Journal of biomedical materials research* **1999**, 45 (4), 285-293.
101. Pighinelli, L.; Kucharska, M., Chitosan-hydroxyapatite composites. *Carbohydr Polym* **2013**, 93 (1), 256-62.

102. Heath, C. A., Cells for tissue engineering. *Trends in biotechnology* **2000**, *18* (1), 17-19.
103. Harris, S. A.; Enger, R. J.; Riggs, L. B.; Spelsberg, T. C., Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. *Journal of Bone and Mineral Research* **1995**, *10* (2), 178-186.
104. Stein, G. S.; Lian, J. B.; Owen, T. A., Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *The FASEB journal* **1990**, *4* (13), 3111-3123.
105. Linkhart, T. A.; Mohan, S.; Baylink, D. J., Growth factors for bone growth and repair: IGF, TGF beta and BMP. *Bone* **1996**, *19* (1), S1-S12.
106. Janssens, K.; ten Dijke, P.; Janssens, S.; Van Hul, W., Transforming growth factor-beta1 to the bone. *Endocrine reviews* **2005**, *26* (6), 743-74.
107. Bikfalvi, A.; Klein, S.; Pintucci, G.; Rifkin, D. B., Biological roles of fibroblast growth factor-2. *Endocrine reviews* **1997**, *18* (1), 26-45.
108. Ferrara, N.; Gerber, H. P.; LeCouter, J., The biology of VEGF and its receptors. *Nat Med* **2003**, *9* (6), 669-676.
109. Li, J. F.; Hong, J. J.; Zheng, Q. X.; Guo, X. D.; Lan, S. H.; Cui, F. Z.; Pan, H. T.; Zou, Z. W.; Chen, C., Repair of Rat Cranial Bone Defects with nHAC/PLLA and BMP-2-Related Peptide or rhBMP-2. *J Orthop Res* **2011**, *29* (11), 1745-1752.
110. Brighton, C. T.; Wang, W.; Seldes, R.; Zhang, G.; Pollack, S. R., Signal transduction in electrically stimulated bone cells. *JBJS* **2001**, *83* (10), 1514-1523.
111. Sauer, H.; Bekhite, M. M.; Hescheler, J.; Wartenberg, M., Redox control of angiogenic factors and CD31-positive vessel-like structures in mouse embryonic stem cells after direct current electrical field stimulation. *Experimental cell research* **2005**, *304* (2), 380-90.
112. Nuccitelli, R., A role for endogenous electric fields in wound healing. *Current topics in developmental biology* **2003**, *58* (2), 1-26.
113. Zhao, M. In *Electrical fields in wound healing—an overriding signal that directs cell migration*, Seminars in cell & developmental biology, Elsevier: 2009; pp 674-682.
114. Huang, C. P.; Chen, X. M.; Chen, Z. Q., Osteocyte: the impresario in the electrical stimulation for bone fracture healing. *Medical hypotheses* **2008**, *70* (2), 287-290.
115. Hadjiargyrou, M.; McLeod, K.; Ryaby, J. P.; Rubin, C., Enhancement of fracture healing by low intensity ultrasound. *Clinical orthopaedics and related research* **1998**, *355*, S216-S229.
116. Black, J.; Baranowski Jr, T.; Brighton, C., Electrochemical aspects of dc stimulation of osteogenesis. *Bioelectrochemistry and Bioenergetics* **1984**, *12* (3-4), 323-327.
117. Bassett, C. A. L.; Pawluk, R. J.; BECKER, R. O., Effects of electric currents on bone in vivo. *Nature* **1964**, *204* (4959), 652.
118. Supronowicz, P.; Ajayan, P.; Ullmann, K.; Arulanandam, B.; Metzger, D.; Bizios, R., Novel current- conducting composite substrates for exposing osteoblasts to alternating current stimulation. *Journal of Biomedical Materials Research Part A* **2002**, *59* (3), 499-506.
119. Compton, O. C.; Nguyen, S. T., Graphene oxide, highly reduced graphene oxide, and graphene: versatile building blocks for carbon-based materials. *Small* **2010**, *6* (6), 711-23.

120. Geim, A. K.; Novoselov, K. S., The rise of graphene. *Nat Mater* **2007**, *6* (3), 183-191.
121. Lee, C.; Wei, X. D.; Kysar, J. W.; Hone, J., Measurement of the elastic properties and intrinsic strength of monolayer graphene. *Science* **2008**, *321* (5887), 385-388.
122. Bolotin, K. I.; Sikes, K. J.; Jiang, Z.; Klima, M.; Fudenberg, G.; Hone, J.; Kim, P.; Stormer, H. L., Ultrahigh electron mobility in suspended graphene. *Solid State Commun* **2008**, *146* (9-10), 351-355.
123. Balandin, A. A.; Ghosh, S.; Bao, W. Z.; Calizo, I.; Teweldebrhan, D.; Miao, F.; Lau, C. N., Superior thermal conductivity of single-layer graphene. *Nano Lett* **2008**, *8* (3), 902-907.
124. Shen, H.; Zhang, L.; Liu, M.; Zhang, Z., Biomedical applications of graphene. *Theranostics* **2012**, *2* (3), 283-94.
125. Novoselov, K. S.; Geim, A. K.; Morozov, S. V.; Jiang, D.; Zhang, Y.; Dubonos, S. V.; Grigorieva, I. V.; Firsov, A. A., Electric field effect in atomically thin carbon films. *Science* **2004**, *306* (5696), 666-9.
126. Berger, C.; Song, Z.; Li, T.; Li, X.; Ogbazghi, A. Y.; Feng, R.; Dai, Z.; Marchenkov, A. N.; Conrad, E. H.; First, P. N.; de Heer, W. A., Ultrathin Epitaxial Graphite: 2D Electron Gas Properties and a Route toward Graphene-based Nanoelectronics. *The Journal of Physical Chemistry B* **2004**, *108* (52), 19912-19916.
127. Stankovich, S.; Dikin, D. A.; Dommett, G. H. B.; Kohlhaas, K. M.; Zimney, E. J.; Stach, E. A.; Piner, R. D.; Nguyen, S. T.; Ruoff, R. S., Graphene-based composite materials. *Nature* **2006**, *442* (7100), 282-286.
128. Zhang, Y.; Tan, Y.-W.; Stormer, H. L.; Kim, P., Experimental observation of the quantum Hall effect and Berry's phase in graphene. *Nature* **2005**, *438* (7065), 201-204.
129. Novoselov, K.; Geim, A. K.; Morozov, S.; Jiang, D.; Katsnelson, M.; Grigorieva, I.; Dubonos, S.; Firsov, A., Two-dimensional gas of massless Dirac fermions in graphene. *Nature* **2005**, *438* (7065), 197-200.
130. Castro Neto, A. H.; Guinea, F.; Peres, N. M. R.; Novoselov, K. S.; Geim, A. K., The electronic properties of graphene. *Reviews of Modern Physics* **2009**, *81* (1), 109-162.
131. Sun, Y.; Wu, Q.; Shi, G., Graphene based new energy materials. *Energy & Environmental Science* **2011**, *4* (4), 1113-1132.
132. Lee, C.; Wei, X.; Kysar, J. W.; Hone, J., Measurement of the Elastic Properties and Intrinsic Strength of Monolayer Graphene. *Science* **2008**, *321* (5887), 385-388.
133. Stoller, M. D.; Park, S.; Zhu, Y.; An, J.; Ruoff, R. S., Graphene-based ultracapacitors. *Nano Lett* **2008**, *8* (10), 3498-502.
134. Lu, C. H.; Yang, H. H.; Zhu, C. L.; Chen, X.; Chen, G. N., A graphene platform for sensing biomolecules. *Angew Chem Int Ed Engl* **2009**, *48* (26), 4785-7.
135. Jiang, H. J., Chemical Preparation of Graphene-Based Nanomaterials and Their Applications in Chemical and Biological Sensors. *Small* **2011**, *7* (17), 2413-2427.
136. Qu, L.; Liu, Y.; Baek, J. B.; Dai, L., Nitrogen-doped graphene as efficient metal-free electrocatalyst for oxygen reduction in fuel cells. *Acs Nano* **2010**, *4* (3), 1321-6.

137. Young, R. J.; Kinloch, I. A.; Gong, L.; Novoselov, K. S., The mechanics of graphene nanocomposites: A review. *Composites Science and Technology* **2012**, 72 (12), 1459-1476.
138. Feng, L.; Liu, Z., Graphene in biomedicine: opportunities and challenges. *Nanomedicine (Lond)* **2011**, 6 (2), 317-24.
139. Chen, Y.; Tan, C.; Zhang, H.; Wang, L., Two-dimensional graphene analogues for biomedical applications. *Chemical Society reviews* **2015**, 44 (9), 2681-701.
140. Yu, G. H.; Hu, L. B.; Vosgueritchian, M.; Wang, H. L.; Xie, X.; McDonough, J. R.; Cui, X.; Cui, Y.; Bao, Z. N., Solution-Processed Graphene/MnO₂ Nanostructured Textiles for High-Performance Electrochemical Capacitors. *Nano Lett* **2011**, 11 (7), 2905-2911.
141. Li, M.; Wang, Y.; Liu, Q.; Li, Q.; Cheng, Y.; Zheng, Y.; Xi, T.; Wei, S., In situ synthesis and biocompatibility of nano hydroxyapatite on pristine and chitosan functionalized graphene oxide. *Journal of Materials Chemistry B* **2013**, 1 (4), 475-484.
142. Venkatesan, J.; Kim, S.-K., Nano-hydroxyapatite composite biomaterials for bone tissue engineering—a review. *Journal of biomedical nanotechnology* **2014**, 10 (10), 3124-3140.
143. Duan, S.; Yang, X.; Mei, F.; Tang, Y.; Li, X.; Shi, Y.; Mao, J.; Zhang, H.; Cai, Q., Enhanced osteogenic differentiation of mesenchymal stem cells on poly (l- lactide) nanofibrous scaffolds containing carbon nanomaterials. *Journal of Biomedical Materials Research Part A* **2015**, 103 (4), 1424-1435.
144. Shao, W.; He, J.; Sang, F.; Wang, Q.; Chen, L.; Cui, S.; Ding, B., Enhanced bone formation in electrospun poly (l-lactic-co-glycolic acid)–tussah silk fibroin ultrafine nanofiber scaffolds incorporated with graphene oxide. *Materials Science and Engineering: C* **2016**, 62, 823-834.
145. Huang, X.; Yin, Z. Y.; Wu, S. X.; Qi, X. Y.; He, Q. Y.; Zhang, Q. C.; Yan, Q. Y.; Boey, F.; Zhang, H., Graphene-Based Materials: Synthesis, Characterization, Properties, and Applications. *Small* **2011**, 7 (14), 1876-1902.
146. Li, D.; Kaner, R. B., Materials science - Graphene-based materials. *Science* **2008**, 320 (5880), 1170-1171.
147. Pei, S. F.; Cheng, H. M., The reduction of graphene oxide. *Carbon* **2012**, 50 (9), 3210-3228.
148. Hummer, W. K.; Smith, I. M., Staphylococcal Pneumonia - a Primary Disease. *J Lab Clin Med* **1958**, 52 (5), 824-824.
149. Dreyer, D. R.; Park, S.; Bielawski, C. W.; Ruoff, R. S., The chemistry of graphene oxide. *Chemical Society reviews* **2010**, 39 (1), 228-40.
150. Cai, X.; Tan, S.; Yu, A.; Zhang, J.; Liu, J.; Mai, W.; Jiang, Z., Sodium 1- Naphthalenesulfonate- Functionalized Reduced Graphene Oxide Stabilizes Silver Nanoparticles with Lower Cytotoxicity and Long- Term Antibacterial Activity. *Chemistry–An Asian Journal* **2012**, 7 (7), 1664-1670.
151. Liao, K.-H.; Lin, Y.-S.; Macosko, C. W.; Haynes, C. L., Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts. *ACS applied materials & interfaces* **2011**, 3 (7), 2607-2615.
152. Sasidharan, A.; Panchakarla, L.; Chandran, P.; Menon, D.; Nair, S.; Rao, C.; Koyakutty, M., Differential nano-bio interactions and toxicity effects of pristine versus functionalized graphene. *Nanoscale* **2011**, 3 (6), 2461-2464.
153. Gu, M.; Liu, Y.; Chen, T.; Du, F.; Zhao, X.; Xiong, C.; Zhou, Y., Is graphene a promising nano-material for promoting surface modification of

implants or scaffold materials in bone tissue engineering? *Tissue Engineering Part B: Reviews* **2014**, 20 (5), 477-491.

154. Chng, E. L. K.; Pumera, M., The toxicity of graphene oxides: dependence on the oxidative methods used. *Chemistry-A European Journal* **2013**, 19 (25), 8227-8235.

155. Zhang, Y.; Ali, S. F.; Dervishi, E.; Xu, Y.; Li, Z.; Casciano, D.; Biris, A. S., Cytotoxicity effects of graphene and single-wall carbon nanotubes in neural phaeochromocytoma-derived PC12 cells. *ACS nano* **2010**, 4 (6), 3181-3186.

156. Wojtoniszak, M.; Chen, X.; Kalenczuk, R. J.; Wajda, A.; Łapczuk, J.; Kurzewski, M.; Drozdziak, M.; Chu, P. K.; Borowiak-Palen, E., Synthesis, dispersion, and cytocompatibility of graphene oxide and reduced graphene oxide. *Colloids and Surfaces B: Biointerfaces* **2012**, 89, 79-85.

157. Zhang, S.; Yang, K.; Feng, L.; Liu, Z., In vitro and in vivo behaviors of dextran functionalized graphene. *Carbon* **2011**, 49 (12), 4040-4049.

158. Duch, M. C.; Budinger, G. S.; Liang, Y. T.; Soberanes, S.; Urich, D.; Chiarella, S. E.; Campochiaro, L. A.; Gonzalez, A.; Chandel, N. S.; Hersam, M. C., Minimizing oxidation and stable nanoscale dispersion improves the biocompatibility of graphene in the lung. *Nano letters* **2011**, 11 (12), 5201-5207.

159. Yan, L.; Wang, Y.; Xu, X.; Zeng, C.; Hou, J.; Lin, M.; Xu, J.; Sun, F.; Huang, X.; Dai, L., Can graphene oxide cause damage to eyesight? *Chemical research in toxicology* **2012**, 25 (6), 1265-1270.

160. Gollavelli, G.; Ling, Y.-C., Multi-functional graphene as an in vitro and in vivo imaging probe. *Biomaterials* **2012**, 33 (8), 2532-2545.

161. Yang, K.; Wan, J.; Zhang, S.; Zhang, Y.; Lee, S.-T.; Liu, Z., In vivo pharmacokinetics, long-term biodistribution, and toxicology of PEGylated graphene in mice. *ACS nano* **2010**, 5 (1), 516-522.

162. Madhally, S. V.; Matthew, H. W., Porous chitosan scaffolds for tissue engineering. *Biomaterials* **1999**, 20 (12), 1133-42.

163. Di Martino, A.; Sittering, M.; Risbud, M. V., Chitosan: A versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials* **2005**, 26 (30), 5983-5990.

164. Kim, I. Y.; Seo, S. J.; Moon, H. S.; Yoo, M. K.; Park, I. Y.; Kim, B. C.; Cho, C. S., Chitosan and its derivatives for tissue engineering applications. *Biotechnology advances* **2008**, 26 (1), 1-21.

165. Madhally, S. V.; Matthew, H. W., Porous chitosan scaffolds for tissue engineering. *Biomaterials* **1999**, 20 (12), 1133-1142.

166. Pavinatto, F. J.; Caseli, L.; Oliveira Jr, O. N., Chitosan in nanostructured thin films. *Biomacromolecules* **2010**, 11 (8), 1897-1908.

167. He, Z. M.; Guai, G. H.; Liu, J.; Guo, C. X.; Loo, J. S. C.; Li, C. M.; Tan, T. T. Y., Nanostructure control of graphene-composited TiO₂ by a one-step solvothermal approach for high performance dye-sensitized solar cells. *Nanoscale* **2011**, 3 (11), 4613-4616.

168. He, Z. M.; Phan, H.; Liu, J.; Nguyen, T. Q.; Tan, T. T. Y., Understanding TiO₂ Size-Dependent Electron Transport Properties of a Graphene-TiO₂ Photoanode in Dye-Sensitized Solar Cells Using Conducting Atomic Force Microscopy. *Adv Mater* **2013**, 25 (47), 6900-6904.

169. Zhou, M.; Wang, Y.; Zhai, Y.; Zhai, J.; Ren, W.; Wang, F.; Dong, S., Controlled synthesis of large-area and patterned electrochemically reduced graphene oxide films. *Chemistry* **2009**, 15 (25), 6116-20.

170. Liu, J.; He, Z. M.; Xue, J. W.; Tan, T. T. Y., A metal-catalyst free, flexible and free-standing chitosan/vacuum-stripped graphene/polypyrrole three dimensional electrode interface for high performance dopamine sensing. *J Mater Chem B* **2014**, 2 (17), 2478-2482.
171. Madhally, S. V.; Matthew, H. W. T., Porous chitosan scaffolds for tissue engineering. *Biomaterials* **1999**, 20 (12), 1133-1142.
172. Hoo, S. P.; Loh, Q. L.; Yue, Z.; Fu, J.; Tan, T. T. Y.; Choong, C.; Chan, P. P. Y., Preparation of a soft and interconnected macroporous hydroxypropyl cellulose methacrylate scaffold for adipose tissue engineering. *J Mater Chem B* **2013**, 1 (24), 3107-3117.
173. Yuan, S.; Xiong, G.; Wang, X.; Zhang, S.; Choong, C., Surface modification of polycaprolactone substrates using collagen-conjugated poly(methacrylic acid) brushes for the regulation of cell proliferation and endothelialisation. *Journal of Materials Chemistry* **2012**, 22 (26), 13039-13049.
174. Xie, J.; MacEwan, M. R.; Willerth, S. M.; Li, X.; Moran, D. W.; Sakiyama-Elbert, S. E.; Xia, Y., Conductive core-sheath nanofibers and their potential application in neural tissue engineering. *Advanced functional materials* **2009**, 19 (14), 2312-2318.
175. Huang, J.; Hu, X.; Lu, L.; Ye, Z.; Zhang, Q.; Luo, Z., Electrical regulation of Schwann cells using conductive polypyrrole/chitosan polymers. *Journal of Biomedical Materials Research Part A* **2010**, 93 (1), 164-174.
176. Rezwan, K.; Chen, Q. Z.; Blaker, J. J.; Boccaccini, A. R., Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials* **2006**, 27 (18), 3413-31.
177. Kalbacova, M.; Broz, A.; Kong, J.; Kalbac, M., Graphene substrates promote adherence of human osteoblasts and mesenchymal stromal cells. *Carbon* **2010**, 48 (15), 4323-4329.
178. Park, S. Y.; Park, J.; Sim, S. H.; Sung, M. G.; Kim, K. S.; Hong, B. H.; Hong, S., Enhanced Differentiation of Human Neural Stem Cells into Neurons on Graphene. *Adv Mater* **2011**, 23 (36), H263-+.
179. Depan, D.; Girase, B.; Shah, J. S.; Misra, R. D. K., Structure-process-property relationship of the polar graphene oxide-mediated cellular response and stimulated growth of osteoblasts on hybrid chitosan network structure nanocomposite scaffolds. *Acta biomaterialia* **2011**, 7 (9), 3432-3445.
180. Park, S.; Ruoff, R. S., Chemical methods for the production of graphenes. *Nature nanotechnology* **2010**, 5 (4), 217-24.
181. Tung, V. C.; Allen, M. J.; Yang, Y.; Kaner, R. B., High-throughput solution processing of large-scale graphene. *Nat Nano* **2009**, 4 (1), 25-29.
182. Schniepp, H. C.; Li, J. L.; Mcallister, M. J.; Sai, H.; Herrera-Alonso, M.; Adamson, D. H.; Prud'Homme, R. K.; Car, R.; Saville, D. A.; Aksay, I. A., Functionalized single graphene sheets derived from splitting graphite oxide. *Journal of Physical Chemistry B* **2006**, 110 (17), 8535-9.
183. McAllister, M. J.; Li, J.-L.; Adamson, D. H.; Schniepp, H. C.; Abdala, A. A.; Liu, J.; Herrera-Alonso, M.; Milius, D. L.; Car, R.; Prud'homme, R. K.; Aksay, I. A., Single Sheet Functionalized Graphene by Oxidation and Thermal Expansion of Graphite. *Chemistry of Materials* **2007**, 19 (18), 4396-4404.
184. Wan, Y. Q.; Wang, Y.; Liu, Z. M.; Qu, X.; Han, B. X.; Bei, J. Z.; Wang, S. G., Adhesion and proliferation of OCT-1 osteoblast-like cells on micro- and nano-scale topography structured pply(L-lactide). *Biomaterials* **2005**, 26 (21), 4453-4459.

185. Shao, Y. Y.; Wang, J.; Engelhard, M.; Wang, C. M.; Lin, Y. H., Facile and controllable electrochemical reduction of graphene oxide and its applications. *Journal of Materials Chemistry* **2010**, 20 (4), 743-748.
186. Guo, H.-L.; Wang, X.-F.; Qian, Q.-Y.; Wang, F.-B.; Xia, X.-H., A Green Approach to the Synthesis of Graphene Nanosheets. *Acs Nano* **2009**, 3 (9), 2653-2659.
187. Paredes, J. I.; Villar-Rodil, S.; Fernandez-Merino, M. J.; Guardia, L.; Martinez-Alonso, A.; Tascon, J. M. D., Environmentally friendly approaches toward the mass production of processable graphene from graphite oxide. *Journal of Materials Chemistry* **2011**, 21 (2), 298-306.
188. Vandrovicova, M.; Bacakova, L., Adhesion, growth and differentiation of osteoblasts on surface-modified materials developed for bone implants. *Physiol Res* **2011**, 60 (3), 403-17.
189. Kauppila, J.; Kunnas, P.; Damlin, P.; Viinikanoja, A.; Kvarnström, C., Electrochemical reduction of graphene oxide films in aqueous and organic solutions. *Electrochimica Acta* **2013**, 89, 84-89.
190. Eda, G.; Fanchini, G.; Chhowalla, M., Large-area ultrathin films of reduced graphene oxide as a transparent and flexible electronic material. *Nat Nano* **2008**, 3 (5), 270-274.
191. Qian, L.; Zhang, H., Controlled freezing and freeze drying: a versatile route for porous and micro- /nano- structured materials. *Journal of chemical technology and biotechnology* **2011**, 86 (2), 172-184.
192. Han, S.; Wu, D.; Li, S.; Zhang, F.; Feng, X., Porous Graphene Materials for Advanced Electrochemical Energy Storage and Conversion Devices. *Adv Mater* **2014**, 26 (6), 849-864.
193. Gutiérrez, M. C.; Ferrer, M. L.; del Monte, F., Ice-templated materials: sophisticated structures exhibiting enhanced functionalities obtained after unidirectional freezing and ice-segregation-induced self-assembly. *Chemistry of Materials* **2008**, 20 (3), 634-648.
194. Stevens, M. M., Biomaterials for bone tissue engineering. *Materials Today* **2008**, 11 (5), 18-25.
195. Pan, Y.; Bao, H.; Li, L., Noncovalently functionalized multiwalled carbon nanotubes by chitosan-grafted reduced graphene oxide and their synergistic reinforcing effects in chitosan films. *ACS applied materials & interfaces* **2011**, 3 (12), 4819-30.
196. Liu, L.; Li, C.; Bao, C. L.; Jia, Q.; Xiao, P. F.; Liu, X. T.; Zhang, Q. P., Preparation and characterization of chitosan/graphene oxide composites for the adsorption of Au(III) and Pd(II). *Talanta* **2012**, 93, 350-357.
197. Pan, Y. Z.; Bao, H. Q.; Li, L., Noncovalently Functionalized Multiwalled Carbon Nanotubes by Chitosan-Grafted Reduced Graphene Oxide and Their Synergistic Reinforcing Effects in Chitosan Films. *ACS applied materials & interfaces* **2011**, 3 (12), 4819-4830.
198. Eda, G.; Fanchini, G.; Chhowalla, M., Large-area ultrathin films of reduced graphene oxide as a transparent and flexible electronic material. *Nature nanotechnology* **2008**, 3 (5), 270-274.
199. Dowling, D. P.; Miller, I. S.; Ardhaoui, M.; Gallagher, W. M., Effect of Surface Wettability and Topography on the Adhesion of Osteosarcoma Cells on Plasma-modified Polystyrene. *J Biomater Appl* **2011**, 26 (3), 327-347.
200. Anselme, K., Osteoblast adhesion on biomaterials. *Biomaterials* **2000**, 21 (7), 667-81.

201. Rafiee, M. A.; Rafiee, J.; Wang, Z.; Song, H. H.; Yu, Z. Z.; Koratkar, N., Enhanced Mechanical Properties of Nanocomposites at Low Graphene Content. *Acs Nano* **2009**, *3* (12), 3884-3890.
202. Wang, X.; Bai, H.; Yao, Z.; Liu, A.; Shi, G., Electrically conductive and mechanically strong biomimetic chitosan/reduced graphene oxide composite films. *Journal of Materials Chemistry* **2010**, *20* (41), 9032-9036.
203. Sayyar, S.; Murray, E.; Thompson, B.; Chung, J.; Officer, D. L.; Gambhir, S.; Spinks, G. M.; Wallace, G. G., Processable conducting graphene/chitosan hydrogels for tissue engineering. *J Mater Chem B* **2015**, *3* (3), 481-490.
204. Hutmacher, D. W., Scaffolds in tissue engineering bone and cartilage. *Biomaterials* **2000**, *21* (24), 2529-2543.
205. Varum, K. M.; Myhr, M. M.; Hjerde, R. J.; Smidsrod, O., In vitro degradation rates of partially N-acetylated chitosans in human serum. *Carbohydr Res* **1997**, *299* (1-2), 99-101.
206. Justin, R.; Chen, B., Characterisation and drug release performance of biodegradable chitosan-graphene oxide nanocomposites. *Carbohydrate Polymers* **2014**, *103*, 70-80.
207. Jongwattanapisan, P.; Charoenphandhu, N.; Krishnamra, N.; Thongbunchoo, J.; Tang, I. M.; Hoonsawat, R.; Smith, S. M.; Pon-On, W., In vitro study of the SBF and osteoblast-like cells on hydroxyapatite/chitosan-silica nanocomposite. *Materials Science and Engineering: C* **2011**, *31* (2), 290-299.
208. Kokubo, T.; Ito, S.; Huang, Z.; Hayashi, T.; Sakka, S.; Kitsugi, T.; Yamamuro, T., Ca, P- rich layer formed on high- strength bioactive glass-ceramic A- W. *J Biomed Mater Res* **1990**, *24* (3), 331-343.
209. Kokubo, T.; Kushitani, H.; Sakka, S.; Kitsugi, T.; Yamamuro, T., Solutions able to reproduce in vivo surface- structure changes in bioactive glass- ceramic A- W3. *J Biomed Mater Res* **1990**, *24* (6), 721-734.
210. Neo, M.; Nakamura, T.; Ohtsuki, C.; Kokubo, T.; Yamamuro, T., Apatite formation on three kinds of bioactive material at an early stage in vivo: a comparative study by transmission electron microscopy. *Journal of Biomedical Materials Research Part A* **1993**, *27* (8), 999-1006.
211. Depan, D.; Pesacreta, T. C.; Misra, R. D. K., The synergistic effect of a hybrid graphene oxide-chitosan system and biomimetic mineralization on osteoblast functions. *Biomaterials Science* **2014**, *2* (2), 264-274.
212. Liu, H.; Cheng, J.; Chen, F.; Hou, F.; Bai, D.; Xi, P.; Zeng, Z., Biomimetic and cell-mediated mineralization of hydroxyapatite by carrageenan functionalized graphene oxide. *ACS applied materials & interfaces* **2014**, *6* (5), 3132-3140.
213. Zhang, L.; Liu, W.; Yue, C.; Zhang, T.; Li, P.; Xing, Z.; Chen, Y., A tough graphene nanosheet/hydroxyapatite composite with improved in vitro biocompatibility. *Carbon* **2013**, *61*, 105-115.
214. Wan, C.; Frydrych, M.; Chen, B., Strong and bioactive gelatin-graphene oxide nanocomposites. *Soft Matter* **2011**, *7* (13), 6159-6166.
215. Rajan Unnithan, A.; Ramachandra Kurup Sasikala, A.; Park, C. H.; Kim, C. S., A unique scaffold for bone tissue engineering: An osteogenic combination of graphene oxide-hyaluronic acid-chitosan with simvastatin. *Journal of Industrial and Engineering Chemistry* **2017**, *46*, 182-191.
216. Zanin, H.; Rodrigues, B. V. M.; Ribeiro Neto, W. A.; Bretas, R. E. S.; Da-Silva, N. S.; Marciano, F. R.; Oliveira Lobo, A., High loading of graphene oxide/multi-walled carbon nanotubes into PDLLA: A route towards the design of

- osteoconductive, bactericidal and non-immunogenic 3D porous scaffolds. *Materials Chemistry and Physics* **2016**, *177*, 56-66.
217. Došić, M.; Eraković, S.; Janković, A.; Vukašinović-Sekulić, M.; Matić, I. Z.; Stojanović, J.; Rhee, K. Y.; Mišković-Stanković, V.; Park, S.-J., In vitro investigation of electrophoretically deposited bioactive hydroxyapatite/chitosan coatings reinforced by graphene. *Journal of Industrial and Engineering Chemistry* **2017**, *47*, 336-347.
218. Ribeiro, N.; Sousa, S. R.; Monteiro, F. J., Influence of crystallite size of nanophased hydroxyapatite on fibronectin and osteonectin adsorption and on MC3T3-E1 osteoblast adhesion and morphology. *J Colloid Interface Sci* **2010**, *351* (2), 398-406.
219. Webster, T. J.; Waid, M. C.; McKenzie, J. L.; Price, R. L.; Ejiofor, J. U., Nano-biotechnology: carbon nanofibres as improved neural and orthopaedic implants. *Nanotechnology* **2003**, *15* (1), 48.
220. Hong, Y.; Song, H.; Gong, Y.; Mao, Z.; Gao, C.; Shen, J., Covalently crosslinked chitosan hydrogel: Properties of in vitro degradation and chondrocyte encapsulation. *Acta biomaterialia* **2007**, *3* (1), 23-31.
221. Yang, F.; Liu, Y.; Gao, L.; Sun, J., pH-Sensitive Highly Dispersed Reduced Graphene Oxide Solution Using Lysozyme via an in Situ Reduction Method. *The Journal of Physical Chemistry C* **2010**, *114* (50), 22085-22091.
222. Justin, R.; Chen, B. Q., Strong and conductive chitosan-reduced graphene oxide nanocomposites for transdermal drug delivery. *J Mater Chem B* **2014**, *2* (24), 3759-3770.
223. Barrere, F.; Snel, M. M.; van Blitterswijk, C. A.; de Groot, K.; Layrolle, P., Nano-scale study of the nucleation and growth of calcium phosphate coating on titanium implants. *Biomaterials* **2004**, *25* (14), 2901-2910.
224. Asanithi, P., Surface porosity and roughness of micrographite film for nucleation of hydroxyapatite. *Journal of biomedical materials research. Part A* **2014**, *102* (8), 2590-9.
225. Liu, Y. X.; Wang, X. J.; Lu, J.; Ching, C. B., Influence of the roughness, topography, and physicochemical properties of chemically modified surfaces on the heterogeneous nucleation of protein crystals. *The journal of physical chemistry. B* **2007**, *111* (50), 13971-8.
226. Zhao, L.; Mei, S.; Chu, P. K.; Zhang, Y.; Wu, Z., The influence of hierarchical hybrid micro/nano-textured titanium surface with titania nanotubes on osteoblast functions. *Biomaterials* **2010**, *31* (19), 5072-5082.
227. Le Guehennec, L.; Lopez-Heredia, M.-A.; Enkel, B.; Weiss, P.; Amourig, Y.; Layrolle, P., Osteoblastic cell behaviour on different titanium implant surfaces. *Acta biomaterialia* **2008**, *4* (3), 535-543.
228. Wang, Z.; Tonderys, D.; Leggett, S. E.; Williams, E. K.; Kiani, M. T.; Spitz Steinberg, R.; Qiu, Y.; Wong, I. Y.; Hurt, R. H., Wrinkled, wavelength-tunable graphene-based surface topographies for directing cell alignment and morphology. *Carbon* **2016**, *97*, 14-24.
229. Anselme, K., Osteoblast adhesion on biomaterials. *Biomaterials* **2000**, *21* (7), 667-681.
230. Liu, J. B.; Fu, S. H.; Yuan, B.; Li, Y. L.; Deng, Z. X., Toward a Universal "Adhesive Nanosheet" for the Assembly of Multiple Nanoparticles Based on a Protein-Induced Reduction/Decoration of Graphene Oxide. *Journal of the American Chemical Society* **2010**, *132* (21), 7279-+.

231. Shi, X. T.; Chang, H. X.; Chen, S.; Lai, C.; Khademhosseini, A.; Wu, H. K., Regulating Cellular Behavior on Few-Layer Reduced Graphene Oxide Films with Well-Controlled Reduction States. *Adv Funct Mater* **2012**, 22 (4), 751-759.
232. Clarke, B.; Kingshott, P.; Hou, X.; Rochev, Y.; Gorelov, A.; Carroll, W., Effect of nitinol wire surface properties on albumin adsorption. *Acta biomaterialia* **2007**, 3 (1), 103-11.
233. Wang, C. M.; Bai, J.; Gong, Y. H.; Zhang, F.; Shen, J. B.; Wang, D. A., Enhancing Cell Affinity of Nonadhesive Hydrogel Substrate: The Role of Silica Hybridization. *Biotechnol Progr* **2008**, 24 (5), 1142-1146.
234. Wang, D. A.; Varghese, S.; Sharma, B.; Strehin, I.; Fermanian, S.; Gorham, J.; Fairbrother, D. H.; Cascio, B.; Elisseeff, J. H., Multifunctional chondroitin sulphate for cartilage tissue-biomaterial integration. *Nature materials* **2007**, 6 (5), 385-92.
235. Yang, D.; Lu, X.; Hong, Y.; Xi, T.; Zhang, D., The molecular mechanism of mediation of adsorbed serum proteins to endothelial cells adhesion and growth on biomaterials. *Biomaterials* **2013**, 34 (23), 5747-58.
236. Zeng, H.; Chittur, K. K.; Lacefield, W. R., Analysis of bovine serum albumin adsorption on calcium phosphate and titanium surfaces. *Biomaterials* **1999**, 20 (4), 377-384.
237. Martin, J.; Schwartz, Z.; Hummert, T.; Schraub, D.; Simpson, J.; Lankford, J.; Dean, D.; Cochran, D.; Boyan, B., Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). *Journal of Biomedical Materials Research Part A* **1995**, 29 (3), 389-401.
238. Yasuda, Y., Dynamic callus and electric callus. *J. Bone. Jt. Surg.* **1955**, 37, 1292.
239. Xu, J.; Wang, W.; Clark, C. C.; Brighton, C. T., Signal transduction in electrically stimulated articular chondrocytes involves translocation of extracellular calcium through voltage-gated channels. *Osteoarthritis and Cartilage* **2009**, 17 (3), 397-405.
240. Nerubay, J.; Marganit, B.; Bubis, J.; Tadmor, A.; Katznelson, A., Stimulation of bone formation by electrical current on spinal fusion. *Spine* **1986**, 11 (2), 167-169.
241. Weinstein, A. M.; Kalwiter, J. J.; Cleveland, T. W.; Amoss, D. C., Electrical stimulation of bone growth into porous Al₂O₃. *Journal of Biomedical Materials Research Part A* **1976**, 10 (2), 231-247.
242. Kane, W. J., Direct current electrical bone growth stimulation for spinal fusion. *Spine* **1988**, 13 (3), 363-365.
243. BeDell, K. K.; Scremin, A. E.; Perell, K. L.; Kunkel, C. F., Effects of functional electrical stimulation-induced lower extremity cycling on bone density of spinal cord-injured patients¹. *American journal of physical medicine & rehabilitation* **1996**, 75 (1), 29-34.
244. Zhuang, H.; Wang, W.; Seldes, R. M.; Tahernia, A. D.; Fan, H.; Brighton, C. T., Electrical stimulation induces the level of TGF- β 1 mRNA in osteoblastic cells by a mechanism involving calcium/calmodulin pathway. *Biochemical and biophysical research communications* **1997**, 237 (2), 225-229.
245. You, J.; Reilly, G. C.; Zhen, X.; Yellowley, C. E.; Chen, Q.; Donahue, H. J.; Jacobs, C. R., Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. *Journal of Biological Chemistry* **2001**, 276 (16), 13365-13371.

246. Wolff, J., Das gesetz der transformation der knochen. *DMW-Deutsche Medizinische Wochenschrift* **1893**, 19 (47), 1222-1224.
247. Bassett, C., Electro-mechanical factors regulating bone architecture. In *Calcified tissues 1965*, Springer: 1966; pp 78-89.
248. Jahn, T. L., A Possible Mechanism for the Effect of Electrical Potentials on Apatite Formation in Bone*. *Clinical orthopaedics and related research* **1968**, 56, 261-274.
249. Jørgensen, T. E., The effect of electric current on the healing time of crural fractures. *Acta Orthopaedica Scandinavica* **1972**, 43 (5), 421-437.
250. Markel, D. C.; Horton, J., The role of electrical stimulation, bone morphogenic protein, and future directions in osteonecrosis of the femoral head. *Seminars in Arthroplasty* **2004**, 15 (3), 171-174.
251. Kim, I. S.; Song, J. K.; Song, Y. M.; Lee, T. H.; Cho, T. H.; Lim, S. S.; Pan, H.; Kim, S. J.; Hwang, S. J., Novel action of biphasic electric current in vitro osteogenesis of human bone marrow mesenchymal stromal cells coupled with VEGF production. *Bone* **2008**, 43, S43-S44.
252. Kim, I. S.; Song, J. K.; Song, Y. M.; Cho, T. H.; Lee, T. H.; Lim, S. S.; Kim, S. J.; Hwang, S. J., Novel effect of biphasic electric current on in vitro osteogenesis and cytokine production in human mesenchymal stromal cells. *Tissue engineering. Part A* **2009**, 15 (9), 2411-22.
253. Zhuang, H.; Wang, W.; Seldes, R. M.; Tahernia, A. D.; Fan, H.; Brighton, C. T., Electrical stimulation induces the level of TGF-beta1 mRNA in osteoblastic cells by a mechanism involving calcium/calmodulin pathway. *Biochemical and biophysical research communications* **1997**, 237 (2), 225-9.
254. Kim, I. S.; Song, J. K.; Zhang, Y. L.; Lee, T. H.; Cho, T. H.; Song, Y. M.; Kim, D. K.; Kim, S. J.; Hwang, S. J., Biphasic electric current stimulates proliferation and induces VEGF production in osteoblasts. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **2006**, 1763 (9), 907-916.
255. Meng, S.; Zhang, Z.; Rouabhia, M., Accelerated osteoblast mineralization on a conductive substrate by multiple electrical stimulation. *Journal of bone and mineral metabolism* **2011**, 29 (5), 535-544.
256. Ercan, B.; Webster, T. J., Greater osteoblast proliferation on anodized nanotubular titanium upon electrical stimulation. *International journal of nanomedicine* **2008**, 3 (4), 477.
257. Black, J., Electrical stimulation: its role in growth, repair and remodeling of the musculoskeletal system. **1986**.
258. Black, J.; Baranowski, T. J.; Brighton, C. T., Electrochemical aspects of d.c. stimulation of osteogenesis. *Bioelectrochemistry and Bioenergetics* **1984**, 12 (3), 323-327.
259. Friedenberg, Z.; Andrews, E.; Smolenski, B.; Pearl, B.; Brighton, C., Bone reaction to varying amounts of direct current. *Surgery, gynecology & obstetrics* **1970**, 131 (5), 894-899.
260. Supronowicz, P.; Ajayan, P.; Ullmann, K.; Arulanandam, B.; Metzger, D.; Bizios, R., Novel current- conducting composite substrates for exposing osteoblasts to alternating current stimulation. *Journal of biomedical materials research* **2002**, 59 (3), 499-506.
261. Holy, C. E.; Shoichet, M. S.; Davies, J. E., Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: investigating initial cell-seeding density and culture period. *Journal of biomedical materials research* **2000**, 51 (3), 376-382.

262. Ishaug-Riley, S. L.; Crane-Kruger, G. M.; Yaszemski, M. J.; Mikos, A. G., Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **1998**, *19* (15), 1405-1412.
263. Bodhak, S.; Bose, S.; Kinsel, W. C.; Bandyopadhyay, A., Investigation of in vitro bone cell adhesion and proliferation on Ti using direct current stimulation. *Materials Science and Engineering: C* **2012**, *32* (8), 2163-2168.
264. Meng, S.; Zhang, Z.; Rouabhia, M., Accelerated osteoblast mineralization on a conductive substrate by multiple electrical stimulation. *J Bone Miner Metab* **2011**, *29* (5), 535-44.
265. Pilia, M.; Guda, T.; Shiels, S. M.; Appleford, M. R., Influence of substrate curvature on osteoblast orientation and extracellular matrix deposition. *J Biol Eng* **2013**, *7* (1), 23.
266. Chen, M. C.; Sun, Y. C.; Chen, Y. H., Electrically conductive nanofibers with highly oriented structures and their potential application in skeletal muscle tissue engineering. *Acta biomaterialia* **2013**, *9* (3), 5562-5572.
267. Aubin, H.; Nichol, J. W.; Hutson, C. B.; Bae, H.; Sieminski, A. L.; Cropek, D. M.; Akhyari, P.; Khademhosseini, A., Directed 3D cell alignment and elongation in microengineered hydrogels. *Biomaterials* **2010**, *31* (27), 6941-6951.
268. Golub, E. E.; Boesze-Battaglia, K., The role of alkaline phosphatase in mineralization. *Current Opinion in Orthopaedics* **2007**, *18* (5), 444-448.
269. Owen, T. A.; Aronow, M.; Shalhoub, V.; Barone, L. M.; Wilming, L.; Tassinari, M. S.; Kennedy, M. B.; Pockwinse, S.; Lian, J. B.; Stein, G. S., Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *Journal of cellular physiology* **1990**, *143* (3), 420-430.
270. Hagiwara, T.; Bell, W. H., Effect of electrical stimulation on mandibular distraction osteogenesis. *Journal of Cranio-Maxillofacial Surgery* **2000**, *28* (1), 12-19.
271. Lee, Y.; Bae, S.; Jang, H.; Jang, S.; Zhu, S. E.; Sim, S. H.; Song, Y. I.; Hong, B. H.; Ahn, J. H., Wafer-Scale Synthesis and Transfer of Graphene Films. *Nano Lett* **2010**, *10* (2), 490-493.
272. Crowder, S. W.; Prasai, D.; Rath, R.; Balikov, D. A.; Bae, H.; Bolotin, K. I.; Sung, H.-J., Three-dimensional graphene foams promote osteogenic differentiation of human mesenchymal stem cells. *Nanoscale* **2013**, *5* (10), 4171-4176.
273. Song, F.; Jie, W.; Zhang, T.; Li, W.; Jiang, Y.; Wan, L.; Liu, W.; Li, X.; Liu, B., Room-temperature fabrication of a three-dimensional reduced-graphene oxide/polypyrrole/hydroxyapatite composite scaffold for bone tissue engineering. *RSC Advances* **2016**, *6* (95), 92804-92812.
274. Ma, Z.; Mao, Z.; Gao, C., Surface modification and property analysis of biomedical polymers used for tissue engineering. *Colloids and Surfaces B: Biointerfaces* **2007**, *60* (2), 137-157.
275. Castner, D. G.; Ratner, B. D., Biomedical surface science: Foundations to frontiers. *Surf Sci* **2002**, *500* (1), 28-60.
276. Ikada, Y., Surface modification of polymers for medical applications. *Biomaterials* **1994**, *15* (10), 725-736.
277. Elbert, D. L.; Hubbell, J. A., Surface treatments of polymers for biocompatibility. *Annual Review of Materials Science* **1996**, *26* (1), 365-394.
278. Kasemo, B., Biological surface science. *Surf Sci* **2002**, *500* (1), 656-677.

279. Vogler, E. A., Structure and reactivity of water at biomaterial surfaces. *Advances in colloid and interface science* **1998**, *74* (1), 69-117.
280. Stevens, M. M.; George, J. H., Exploring and engineering the cell surface interface. *Science* **2005**, *310* (5751), 1135-1138.
281. Chua, C. K.; Pumera, M., Chemical reduction of graphene oxide: a synthetic chemistry viewpoint. *Chemical Society reviews* **2014**, *43* (1), 291-312.
282. Wei, X.-Q.; Hao, L.-Y.; Shao, X.-R.; Zhang, Q.; Jia, X.-Q.; Zhang, Z.-R.; Lin, Y.-F.; Peng, Q., Insight into the interaction of graphene oxide with serum proteins and the impact of the degree of reduction and concentration. *ACS applied materials & interfaces* **2015**, *7* (24), 13367-13374.
283. Kim, Y.-K.; Kim, M.-H.; Min, D.-H., Biocompatible reduced graphene oxide prepared by using dextran as a multifunctional reducing agent. *Chemical communications* **2011**, *47* (11), 3195-3197.
284. Nayak, T. R.; Andersen, H.; Makam, V. S.; Khaw, C.; Bae, S.; Xu, X.; Ee, P.-L. R.; Ahn, J.-H.; Hong, B. H.; Pastorin, G.; Özyilmaz, B., Graphene for Controlled and Accelerated Osteogenic Differentiation of Human Mesenchymal Stem Cells. *ACS nano* **2011**, *5* (6), 4670-4678.
285. La, W.-G.; Jin, M.; Park, S.; Yoon, H.-H.; Jeong, G.-J.; Bhang, S. H.; Park, H.; Char, K.; Kim, B.-S., Delivery of bone morphogenetic protein-2 and substance P using graphene oxide for bone regeneration. *International journal of nanomedicine* **2014**, *9* (Suppl 1), 107.
286. Sun, X.; Liu, Z.; Welsher, K.; Robinson, J. T.; Goodwin, A.; Zaric, S.; Dai, H., Nano-graphene oxide for cellular imaging and drug delivery. *Nano research* **2008**, *1* (3), 203-212.
287. Bijju, V., Chemical modifications and bioconjugate reactions of nanomaterials for sensing, imaging, drug delivery and therapy. *Chemical Society reviews* **2014**, *43* (3), 744-764.
288. Liu, J.; Cui, L.; Losic, D., Graphene and graphene oxide as new nanocarriers for drug delivery applications. *Acta biomaterialia* **2013**, *9* (12), 9243-9257.
289. Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M., Polymeric systems for controlled drug release. *Chemical reviews* **1999**, *99* (11), 3181-3198.
290. Rösler, A.; Vandermeulen, G. W.; Klok, H.-A., Advanced drug delivery devices via self-assembly of amphiphilic block copolymers. *Adv Drug Deliver Rev* **2012**, *64*, 270-279.
291. Yang, X.; Zhang, X.; Liu, Z.; Ma, Y.; Huang, Y.; Chen, Y., High-Efficiency Loading and Controlled Release of Doxorubicin Hydrochloride on Graphene Oxide. *The Journal of Physical Chemistry C* **2008**, *112* (45), 17554-17558.
292. Liu, Z.; Robinson, J. T.; Sun, X.; Dai, H., PEGylated nanographene oxide for delivery of water-insoluble cancer drugs. *Journal of the American Chemical Society* **2008**, *130* (33), 10876-10877.
293. Cleland, J. L.; Langer, R., *Formulation and delivery of proteins and peptides*.
294. Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C., Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Molecular pharmaceutics* **2008**, *5* (4), 505-515.

295. Li, M.-j.; Liu, C.-m.; Xie, Y.-b.; Cao, H.-b.; Zhao, H.; Zhang, Y., The evolution of surface charge on graphene oxide during the reduction and its application in electroanalysis. *Carbon* **2014**, *66*, 302-311.
296. Ekiz, O. O.; Urel, M.; Guner, H.; Mizrak, A. K.; Dana, A., Reversible electrical reduction and oxidation of graphene oxide. *Acs Nano* **2011**, *5* (4), 2475-2482.
297. Zhuge, F.; Hu, B.; He, C.; Zhou, X.; Liu, Z.; Li, R.-W., Mechanism of nonvolatile resistive switching in graphene oxide thin films. *Carbon* **2011**, *49* (12), 3796-3802.