

INVESTIGATION OF USE OF BORON NITRIDE BASED NANOMATERIALS IN
TISSUE ENGINEERING SCAFFOLDS



by
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Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in
Biotechnology

Yeditepe University
2021

INVESTIGATION OF USE OF BORON NITRIDE BASED NANOMATERIALS IN
TISSUE ENGINEERING SCAFFOLDS

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Dedicated to my sweetheart son,

Furkan obandede

ACKNOWLEDGMENTS

It's hard not to get emotional while writing this thank you letter after a long and difficult journey. In this challenging but empowering journey, I have gained many valuable people who supported me, helped me improve and encouraged me to do my best to whom I can wholeheartedly thank.

First of all, I would like to express my sincere gratitude to my very valuable advisor, Prof. Mustafa Çulha, who gave me an opportunity to be a part of his laboratory. He always provided me with financial and moral support and always guided me with her precious knowledge and experiences.

I am grateful to my thesis monitoring committee members, Prof. Gamze Torun Köse and Prof. Bahattin Koç for their directive comments and suggestions during my PhD period.

I am grateful to Prof. Fikretin Şahin and Assistant Prof. Manolya Hatipoğlu for their support and help through hard times.

I am appreciative to Prof. Gamze Torun Köse's laboratory members for providing me cell line for my thesis work and for helping me understand some tissue engineering experiments.

A special thanks to my valuable friend Deniz Yılmaz not only for being very supportive and for helping me throughout my thesis but also for her friendship and encouragement. I would also like to thank my friend Ayşe Çinkılıç for her support, motivation, friendship and sincerity. Thank you very much my sweet friends, İpek Bedir, Berfin Uzunkaya and Fulya Şahin who warm my heart when I see their faces. Also, thank you very much dear İnci Kurt for the valuable experiences and knowledge you shared with me. I would also like to thank dear Hülya Yılmaz, who always supports me and helps me.

I would also like to thank my dear friends who are very important to me. I would like to thank to Hande Duru Köktaş for being supportive and motivating me and for the most sincere friendship. I would like to thank my dear friends Seda Keleştemur, Sevda Mert, Gamze Yeşilay and Melike Sarıçam for their help, guidance and constructive criticism,

Zeynep Işık for her warm friendship, Merve Ercan, Hamide Murtezaoğlu and Fatma Özen for their lovely friendship.

Additionally, I am grateful to Şaban Kalay and Kaan Kırbaş who were always there for me whenever I needed help and I am also thankful to Ertuğ Avcı for sharing his knowledge about the use of laboratory devices and Mine Altunbek and Pınar Akkuş Süt for their much appreciated help. I would like to thank Şahin Yılmaz for helping me for the ICP-MS analysis.

I would also like to thank mother-in-law, Keziban Çobandede, and father-in-law, Ömer Çobandede, for their help and support. I would like to thank my dear husband, Fatih Çobandede, for all your support and understanding me during my thesis process. Thank you very much for always listening to me and guiding me in the decisions I have made. And my dear son, whose love I can never describe, thank you so much for choosing me as your mother. You patiently accompanied me when I came to school to do my experiments. Thank you most sincerely my son, for the great understanding you have been able to do with your tiny heart. I hope I can achieve things that you can be proud of, but most importantly, I hope you will never stop loving me because I love you so much.

I would like to write my last paragraph to my biggest supporters in my life. Saniye Yılmaz and Muzaffer Yılmaz, my lovely mom and dad, I'm really proud of you. You have gone through such tiring ways to educate us and prepare us for life that I can never repay you whatever I'll do for you. Thank you so much for your endless love, unconditional support and being my mom and dad. Thank you very much for your support and love my lovely sister, Esra Turan, whose stance and effort towards life I admire.

ABSTRACT

INVESTIGATION OF USE OF BORON NITRIDE BASED NANOMATERIALS IN TISSUE ENGINEERING SCAFFOLDS

Tissue engineering, a promising and an interdisciplinary field of regenerative medicine, aims to develop solutions by mimicking tissue/organ using natural, synthetic or semi-synthetic materials within the required functionality for the recovery/regeneration of lost or damaged tissue or organs. The currently used scaffolds in tissue engineering have inadequacies to be applicable due to need of more physical, chemical and biological enhancements to provide better cell adhesion, penetration, growth and regeneration. At this point nanotechnology idea comes into stage by using nanomaterials that have significant contributions to tissue engineering applications as reinforcement material so far. Nanomaterials can be preferred to manufacture high-performance biomaterials with tailored physical, chemical and biological features due to their superior mechanical, physical and chemical as well as greater biocompatibility properties. In this study, first, a novel and non-toxic piezoelectric composite, which include both barium titanates (BaTiO_3) and boron nitride nanotubes (BNNTs) was aimed to develop to be used in biomedical applications. Then, three nanomaterials, barium titanates (BaTiO_3), BNNTs and the novel composite were used by embedding into poly(ϵ -caprolactone) (PCL) and chitosan polymers due to their high piezoelectric properties to introduce piezoelectric feature to the composite scaffolds. Electric-responsive Human Osteoblast cells (HOb) cells on scaffolds were stimulated by applying low-frequency ultrasound (US) stimulation during cell growth. Biocompatibility, cell adhesion, alkaline phosphatase activities and mineralization of HOb cells on piezo-composite scaffolds were investigated. Utilization of these piezo-composites scaffolds will be helpful for wireless tissue stimulation studies using piezoelectric phenomena in electrically sensitive cells or tissues to decrease the conventional invasiveness of in vivo electric stimulation.

ÖZET

BOR NİTRÜR TABANLI NANOMALZEMELERİN DOKU MÜHENDİSLİĞİ YAPI İSKELELERİNDE KULLANIMLARININ ARAŞTIRILMASI

Yenileyici tıbbın gelecek vaat eden ve disiplinler arası bir alanı olan doku mühendisliği, kaybolan veya hasar gören doku veya organların geri kazanılması/yenilenmesi için gereken işlevsellik dahilinde doğal, sentetik veya yarı sentetik malzemeler kullanarak doku/organı taklit ederek çözümler geliştirmeyi amaçlamaktadır. Doku mühendisliğinde halihazırda kullanılan iskeleler, hücre tutunması, penetrasyonu, büyümesi ve yenilenmesini sağlamak için daha fazla fiziksel, kimyasal ve biyolojik geliştirmeye ihtiyaç duyulduğundan, bazı yetersizliklere sahiptir. Bu noktada nanoteknoloji fikri, nanomalzemelerin takviye malzemesi olarak doku mühendisliği uygulamalarında kullanılmasıyla devreye girmektedir. Nanomalzemeler, üstün mekanik, kimyasal, fiziksel ve biyolojik özelliklerinin yanı sıra daha yüksek biyouyumlulukları nedeniyle özel fiziksel, kimyasal ve biyolojik özelliklere sahip yüksek performanslı biyomalzemeler üretmek için tercih edilebilir.

Bu çalışmada ilk olarak, biyomedikal uygulamalarda kullanılmak üzere hem baryum titanatlar ($BaTiO_3$) hem de bor nitrür nanotüpler (BNNT) içeren yeni ve toksik olmayan bir piezoelektrik kompozitin geliştirilmesi amaçlanmıştır. Daha sonra, $BaTiO_3$, BNNT'ler ve yeni kompozit olmak üzere üç farklı nanomateryal, yüksek piezoelektrik özelliklerinden dolayı poli (ϵ -kaprolakton) (PCL) ve kitosan polimerlerinin içine onlara piezoelektrik özelliği kazandırmak için gömülerek kullanılmıştır. Bu piezo-kompozit yapı iskelelerinin etkileri, elektriğe duyarlı İnsan Osteoblast hücreleri (HOb) üzerinde araştırılmıştır. Yapı iskeleleri üzerindeki HOb hücreleri, hücre büyümesi sırasında düşük frekanslı ultrasonik ses dalgaları ile uyarılmıştır. Piezo-kompozit yapı iskelelerinin biyouyumluluk, hücre tutunması, alkalik fosfataz aktiviteleri ve mineralizasyonu HOb hücreleri üzerinde incelenmiştir. Bu piezo-kompozit yapı iskeletlerinin kullanılması, in vivo elektrik stimülasyonunun geleneksel invazivliğini azaltarak elektriğe duyarlı hücrelerde veya dokularda piezoelektrik fenomenini kullanan kablosuz doku stimülasyon çalışmalarına oldukça katkı sağlayacaktır.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	v
ABSTRACT.....	vii
ÖZET	viii
LIST OF FIGURES	xiii
LIST OF TABLES.....	xx
LIST OF SYMBOLS/ABBREVIATIONS	xxi
1. INTRODUCTION	1
1.1. TISSUE ENGINEERING	1
1.1.1. Scaffolds as Key Aspect in Tissue Engineering.....	3
1.1.2. Bone Tissue Engineering (BTE).....	7
1.1.2.1. <i>Polymers Used as Scaffolds in Bone Tissue Engineering Applications</i>	10
1.2. NANOMATERIALS IN TISSUE ENGINEERING	14
1.2.1. Barium Titanate (BaTiO_3)	15
1.2.2. Boron Nitride Nanotubes (BNNTs).....	20
1.2.3. Piezoelectricity in Human Body	24
1.2.4. Piezoelectric Nanomaterials in Medical Applications.....	26
2. OBJECTIVE OF THE STUDY	30
3. MATERIALS AND METHODS.....	31
3.1. MATERIALS	31
3.1.1. Chemicals	31
3.1.2. Cell Lines.....	32
3.1.3. Cell Culture Reagents	32
3.1.4. Kits.....	32
3.2. METHODS	33
3.2.1. Boron Nitride Nanotubes (BNNTs) Synthesis	33

3.2.2. Composite (BaTiO ₃ / BNNT) Synthesis.....	33
3.2.3. Purification of BNNT and Composite	33
3.2.4. Characterization of the BaTiO ₃ , BNNT, and Composite	34
3.2.5. Cell Culture Experiments of BaTiO ₃ , BNNT, and Composite.....	35
3.2.5.1. Cytotoxicity Assay of BaTiO ₃ , BNNTs, and Composite	36
3.2.5.2. Cellular Uptake of BaTiO ₃ , BNNTs, and Composite.....	36
3.2.6. Preparation of Poly Caprolactone (PCL) and Chitosan Scaffolds.....	36
3.2.6.1. Preparation of Poly Caprolactone (PCL) Scaffold with Electrospinning.....	36
3.2.6.2. Preparation of Chitosan Scaffolds with Freeze Dryer	37
3.2.7. Characterization of PCL and Chitosan Scaffolds	37
3.2.7.1. Scanning Electron Microscopy (SEM) Analysis	37
3.2.7.2. Determination of BaTiO ₃ , BNNT, and Composite Distribution in PCL and Chitosan Scaffolds by Raman Spectroscopy.....	38
3.2.7.3. Mechanical Testing	39
3.2.7.4. Dynamic Contact Angle Measurements of PCL Scaffolds with Water	39
3.2.7.5. Determination of Equilibrium Water Content of Chitosan Scaffolds ..	39
3.2.8. In Vitro Cell Interaction Studies of PCL and Chitosan Scaffolds.....	40
3.2.8.1. Cell Proliferation Assay (WST-8)	41
3.2.8.2. Live/Dead Cell Viability Assays of Scaffolds.....	41
3.2.8.3. Scanning Electron Microscopy Images of Cell Seeded Scaffolds.....	41
3.2.8.4. Determination of Mineralization Using Von Kossa Staining	42
3.2.8.5. Alkaline Phosphatase (ALP) Activity.....	42
4. RESULTS AND DISCUSSION	44
4.1. CHARACTERIZATION OF BARIUM TITANATE (BaTiO ₃), BORON NITRIDE NANOTUBE (BNNT) AND COMPOSITE (BNNT/BaTiO ₃) STRUCTURES	44

4.1.1. Scanning Electron, Transmission Electron and Atomic Force Microscopy Analysis	44
4.1.1.1. <i>Characterization of BaTiO₃, by Scanning Electron and Atomic Force Microscopes</i>	44
4.1.1.2. <i>Characterization of BNNT by Scanning Electron, Transmission Electron and Atomic Force Microscopes</i>	45
4.1.1.3. <i>Characterization of Composite by Scanning Electron, Transmission Electron and Atomic Force Microscopes</i>	50
4.1.2. Characterization of BNNT and Composite with Inductively Coupled Plasma Mass Spectrometry (ICP-MS).....	53
4.1.3. Characterization of BaTiO ₃ , BNNT, and Composite by Fourier Transform Infrared Spectrophotometer (FTIR) and Raman Spectroscopy.....	54
4.1.4. Characterization of BaTiO ₃ , BNNT, and Composite by X-Ray Diffraction	56
4.1.5. Piezo Force Microscopy (PFM) Results of BaTiO ₃ , BNNT, and The Composite.....	57
4.2. IN VITRO CELL INTERACTIONS OF BaTiO ₃ , BNNTs, AND COMPOSITE .	61
4.2.1. WST-8 Cytotoxicity Assay of BaTiO ₃ , BNNTs, and Composite.....	61
4.2.2. Cellular Uptake of BaTiO ₃ , BNNTs and Composite by Flow Cytometry ..	62
4.3. CHARACTERIZATION OF POLY CAPROLACTONE (PCL) AND CHITOSAN SCAFFOLDS	63
4.3.1. Scanning Electron Microscopy (SEM) Analysis.....	63
4.3.1.1. <i>Scanning Electron Microscopy Analysis of Poly Caprolactone Scaffolds.....</i>	63
4.3.1.2. <i>Scanning Electron Microscopy Analysis of Chitosan Scaffolds</i>	65
4.3.2. Determination of BaTiO ₃ , BNNT, and Composite Distribution in PCL and Chitosan Scaffolds by Raman Spectroscopy.....	67
4.3.2.1. <i>BaTiO₃, BNNT, and Composite Distribution in PCL Scaffolds.....</i>	68
4.3.2.2. <i>BaTiO₃, BNNT, and Composite Distribution in Chitosan Scaffolds....</i>	71

4.3.3. Mechanical Testing.....	75
4.3.3.1. <i>Tensile Features of Poly Caprolactone Scaffolds</i>	76
4.3.3.2. <i>Compressive Features of Chitosan Scaffolds</i>	77
4.3.4. Water Contact Angle Analysis of Poly Caprolactone (PCL)	77
4.3.5. Equilibrium Water Content of Chitosan Scaffolds.....	79
4.4. IN VITRO DEGRADATION	80
4.4.1. In Vitro Degradation of PCL Scaffolds via Weight Loss.....	80
4.4.2. In vitro Degradation of Chitosan Scaffolds via Weight Loss.....	82
4.5. IN VITRO CELL INTERACTION STUDIES	84
4.5.1. WST-8 Cell Proliferation Assay of Poly Caprolactone (PCL) Scaffolds....	84
4.5.2. Live/Dead Assay.....	85
4.5.2.1. <i>Live/Dead Assay of PCL Scaffolds</i>	86
4.5.2.2. <i>Live/Dead Assay of Chitosan Scaffolds</i>	90
4.5.3. Scanning Electron Microscopy (SEM) Analysis.....	94
4.5.3.1. <i>Scanning Electron Microscopy (SEM) Analysis of PCL Scaffolds</i>	94
4.5.3.2. <i>Scanning Electron Microscopy (SEM) Analysis of Chitosan Scaffolds</i>	99
4.5.4. Alkaline Phosphatase (ALP) Activity Analysis	104
4.5.4.1. <i>Alkaline Phosphatase (ALP) Activity Analysis of PCL Scaffolds</i>	104
4.5.4.2. <i>Alkaline Phosphatase (ALP) Activity Analysis of Chitosan Scaffold</i> .	105
4.5.5. Determination of Mineralization by von Kossa Staining	106
4.5.5.1. <i>Determination of Mineralization on PCL Scaffolds by von Kossa</i> <i>Staining</i>	106
4.5.5.2. <i>Determination of Mineralization on Chitosan Scaffolds by von Kossa</i> <i>Staining</i>	109
5. CONCLUSIONS AND OUTLOOKS	111
REFERENCES	115

LIST OF FIGURES

Figure 1.1. Schematic representation of basic principles of tissue engineering	2
Figure 1.2. Schematic illustration of the basis for generation of 3D smart scaffolds for tissue regeneration [21].....	4
Figure 1.3. Confocal images of osteoblast cells (green) on porous collagen-GAG scaffold (red) [31].....	6
Figure 1.4. Bone structure and anatomy ("Bone cross-section" by Pbroks13 is licensed under CC BY 3.0).....	8
Figure 1.5. Bone tissue engineering strategies [43].....	9
Figure 1.6. Cross-linking reaction between genipin and chitosan [79]	12
Figure 1.7. Structures of BaTiO ₃ (a) cubic and (b) tetragonal [107]	15
Figure 1.8. Schematic illustration of phase transitions of BaTiO ₃ with temperature [108].	16
Figure 1.9. Crystal structure of BaTiO ₃ , (a) cubic phase and (b) tetragonal phase with Titanium (Ti) (blue sphere) cation displacement (Red sphere represents Oxygen (O), green sphere represents Barium (Ba)) [109].....	17
Figure 1.10. The orientation of BaTiO ₃ (a) before polarization, (b) post-polarization, (c) domain structure in BaTiO ₃ [109].....	18
Figure 1.11. Structural illustration of (a) a BN sheet and (b) a single-walled BN nanotube (The length between B and N atoms is 1.44 Å).....	20
Figure 1.12. Routes for the chemical functionalization of BNNTs [157]	22

Figure 1.13. Propagation of dipole vector after applied deformation (P and green arrows). (a) shows the respond when BNNTs are exposed to an axial stress, (b) shows the respond when BNNTs are exposed to torque with blue arrows that indicates the resultant deformation of BNNTs [172]	23
Figure 1.14. Illustration of human body location in which electrical and piezoelectric signals are relevant [187]	25
Figure 3.1. Schematic illustration of electrospinning setup.....	37
Figure 4.1. Characterization of BaTiO ₃ with (a) SEM and (b) AFM	44
Figure 4.2. The schematic illustration of BNNT synthesis steps.....	45
Figure 4.3. SEM images of BNNTs synthesized using precursors; (a) H ₃ BO ₃ +B ₂ O ₃ +Fe ₂ O ₃ , (b) H ₃ BO ₃ +B ₂ O ₃ +colemanite+Fe ₂ O ₃ , (c) colemanite+Fe ₂ O ₃ +Al ₂ O ₃ , (d) colemanite+Fe ₂ O ₃ +MgO, (e) colemanite+Fe ₂ O ₃ +ZnO (5h ball-milled), (f) colemanite+Fe ₂ O ₃ +ZnO (10 h ball-milled)	47
Figure 4.4. Characterization of BNNTs with (a, b) SEM, (c, d) TEM and (e) AFM	49
Figure 4.5. Schematic illustration of the composite synthesis steps and reaction conditions	50
Figure 4.6. SEM images of (a) 1 g colemanite+Fe ₂ O ₃ +100 mg BaCO ₃ +100 mg TiO ₂ , (b) 1 g colemanite+Fe ₂ O ₃ +250 mg BaCO ₃ +250 mg TiO ₂ , (c) 1 g colemanite+Fe ₂ O ₃ +500 mg BaCO ₃ +500 mg TiO ₂ , (d) 1 g colemanite+Fe ₂ O ₃ +1 g BaCO ₃ +1 g TiO ₂	51
Figure 4.7. Characterization of the composite with (a, b) SEM, (c, d) TEM and (e) AFM	52
Figure 4.8. Characterization of BaTiO ₃ with (a) FTIR and (b) Raman spectroscopy	54
Figure 4.9. Characterization of BNNTs (a) FTIR and (b) Raman spectroscopy	55

Figure 4.10. Characterization of the composite (a) FTIR and (b) Raman spectroscopy	56
Figure 4.11. Comparative X-Ray diffraction pattern of the BaTiO ₃ , BNNT, and composite	57
Figure 4.12. Piezoresponse force microscopy analysis of BaTiO ₃	59
Figure 4.13. Piezoresponse force microscopy analysis of BNNTs.....	60
Figure 4.14. Piezoresponse force microscopy analysis of the composite.....	60
Figure 4.15. WST-8 assay results of BaTiO ₃ , BNNTs, and composite on HOb cells. Statistically significant changes in comparison with negative control samples are signed with an asterisk (Student's t-test, (* $p \leq 0.05$)).....	61
Figure 4.16. Cellular uptake results of BaTiO ₃ , BNNT, and composite by HOb cells using flow cytometry. Statistically significant changes in comparison with control sample are signed with asterisks (Student's t-test, (** $p \leq 0.001$))	62
Figure 4.17. SEM images of PCL and BaTiO ₃ , composite and BNNT embedded PCL scaffolds	64
Figure 4.18. The mean fiber diameters of PCL, PCL-BaTiO ₃ , PCL-BNNT, and PCL-Composite scaffolds, which is calculated using SEM images.....	65
Figure 4.19. SEM images of a) chitosan and b) genipin crosslinked chitosan	66
Figure 4.20. SEM images of a) chitosan and b) chitosan-BaTiO ₃ , c) chitosan-BNNT and d) chitosan-Composite	66
Figure 4.21. Mean pore sizes of chitosan, chitosan-BaTiO ₃ , chitosan-BNNT, chitosan-Composite scaffolds, which is calculated using SEM images.....	67

Figure 4.22. Raman spectra of (a) PCL (50 μm objective lens, laser power=30 mW, exposure time= 10 seconds), (b) chitosan (50 μm objective lens, laser power=150 mW, exposure time= 2 seconds) scaffolds	68
Figure 4.23. 3D volume mapping at 308 cm^{-1} peak of BaTiO ₃ in PCL scaffolds	69
Figure 4.24. 3D volume mapping at 1366 cm^{-1} peak of BNNT in PCL scaffolds	70
Figure 4.25. 3D volume mapping at 1366 cm^{-1} peak of composite in PCL scaffolds	71
Figure 4.26. 3D volume mapping at 308 cm^{-1} peak of BaTiO ₃ in chitosan scaffolds	73
Figure 4.27. 3D volume mapping at 1366 cm^{-1} peak of BNNT in chitosan scaffolds	74
Figure 4.28. 3D volume mapping at 1366 cm^{-1} peak of composite in chitosan scaffolds ...	75
Figure 4.29. Tensile profile of electrospun PCL, PCL-BaTiO ₃ , PCL-BNNT, and PCL-Composite scaffolds.....	76
Figure 4.30. Stress-strain curves of chitosan, BaTiO ₃ , composite and BNNT embedded chitosan scaffolds.....	77
Figure 4.31. Water contact angle results of PCL and BaTiO ₃ , composite and BNNT embedded PCL scaffolds	78
Figure 4.32. Swelling ratio of chitosan, BaTiO ₃ , BNNT, and composite embedded chitosan scaffolds	80
Figure 4.33. Weight loss versus time graphs of PCL and BaTiO ₃ , BNNT, and composite embedded PCL scaffolds	81
Figure 4.34. Weight loss versus time graphs of US stimulated PCL and BaTiO ₃ , BNNT, and composite embedded PCL scaffolds	82

Figure 4.35. Weight loss versus time graphs of chitosan and BaTiO ₃ , BNNT, and composite embedded chitosan scaffolds.....	83
Figure 4.36. Weight loss versus time graphs of US stimulated chitosan and BaTiO ₃ , BNNT, and composite embedded chitosan scaffolds	83
Figure 4.37. Cell proliferation on PCL and BaTiO ₃ , BNNT, and composite embedded PCL scaffolds	85
Figure 4.38. Fluorescence images of Live/Dead assay of HOb cells on PCL scaffolds at 7 th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation	87
Figure 4.39. Fluorescence images of Live/Dead assay of HOb cells on PCL scaffolds at 14 th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation.....	88
Figure 4.40. Fluorescence images of Live/Dead assay of HOb cells on PCL scaffolds at 21 st day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation.....	89
Figure 4.41. Fluorescence images of Live/Dead assay of HOb cells on chitosan scaffolds at 7 th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation.....	91
Figure 4.42. Fluorescence images of Live/Dead assay of HOb cells on chitosan scaffolds at 14 th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation.....	92
Figure 4.43. Fluorescence images of Live/Dead assay of HOb cells on chitosan scaffolds at 21 st day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation.....	93

- Figure 4.44. Scanning electron microscopy images of HOb cells on PCL scaffolds at 7th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation 95
- Figure 4.45. Scanning electron microscopy images of HOb cells on PCL scaffolds at 14th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation 96
- Figure 4.46. Scanning electron microscopy images of HOb cells on PCL scaffolds at 21st day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation 97
- Figure 4.47. Scanning electron microscopy images of HOb cells on PCL scaffolds at 28th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation 98
- Figure 4.48. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 7th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation..... 100
- Figure 4.49. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 14th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation..... 101
- Figure 4.50. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 21st day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation..... 102
- Figure 4.51. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 28th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation. Closer images of the cells on the scaffolds are surrounded by red line 103

Figure 4.52. Alkaline phosphatase activity of HO _b cells throughout 28 days of incubation on PCL scaffolds.....	105
Figure 4.53. Alkaline phosphatase activity of HO _b cells throughout 28 days of incubation on chitosan scaffolds.....	106
Figure 4.54. Von Kossa staining images of PCL scaffolds at day 7.....	107
Figure 4.55. Von Kossa staining images of PCL scaffolds at day 14.....	108
Figure 4.56. Von Kossa staining images of PCL scaffolds at day 21.....	108
Figure 4.57. Von Kossa staining images of PCL scaffolds at day 28.....	108
Figure 4.58. Von Kossa staining images of chitosan scaffolds at day 7.....	109
Figure 4.59. Von Kossa staining images of chitosan scaffolds at day 14.....	110
Figure 4.60. Von Kossa staining images of chitosan scaffolds at day 21.....	110
Figure 4.61. Von Kossa staining images of chitosan scaffolds at day 28.....	110

LIST OF TABLES

Table 3.1. Preparation of standard dilutions	43
Table 4.1. ICP-MS elemental analysis results of colemanite, raw and pure products of BNNT and the composite	53
Table 4.2. Water contact angle values of PCL scaffolds	78



LIST OF SYMBOLS/ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
Å	Angstrom
AFM	Atomic force microscopy
Al ₂ O ₃	Aluminum dioxide
ALP	Alkaline phosphatase
APTES	(3-Aminopropyl)triethoxysilane
Ar	Argon
Au/Pd	Gold/Palladium
B	Boron
Ba	Barium
Ba ⁺²	Barium cation
BaCO ₃	Barium carbonate
BaTiO ₃	Barium titanate
Bi ⁺³	Bismuth cation
Bi ₄ Ti ₃ O ₁₂	Bismuth titanate
BMP2	Bone Morphogenetic Protein-2
BNNT	Boron nitride nanotubes
BTE	Bone tissue engineering
BTNPs	Barium titanate nanoparticles
C2C12	Myoblast cell line
Ca ⁺²	Calcium cation
CaF ₂	Calcium fluoride
cm ⁻¹	Wavenumber
CNT	Carbon nanotube
CVD	Chemical vapor deposition
d ₃₃	Piezoelectric constant
ddH ₂ O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

ECM	Extracellular matrix
eV	Electronvolt
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fe ₂ O ₃	Iron oxide
FTIR	Fourier transform infrared
GAG	Glycosaminoglycan
GMP	Good manufacturing practice
hBN	Hexagonal boron nitride
HCl	Hydrochloric acid
HF	Human fibroblast
HNO ₃	Nitric acid
HO _b	Human osteoblast cell line
HUVEC	Human umbilical vein endothelial cells
ICP-MS	Inductively coupled plasma mass spectroscopy
kHz	Kilohertz
MSCs	Mesenchymal stem cells
mW	Milliwatt
N	Nitrogen
N.A	Numerical aperture
N ₂	Molecular nitrogen
-NH ₂	Ammonia
NH ₃	Ammonia
-NH-CO-CH ₃	Acetylamino
NIH/3T3	Swiss albino mice cell line
nN	Nanonewton
O	Oxygen
O ⁻²	Oxygen anion
OCN	Osteocalcin
OPN	Osteopontin
Pb ⁺²	Lead cation
PbNb ₂ O ₆	Lead metaniobate
PbTiO ₃	Lead titanate

PC12	Neuronal cell line
PCL	Poly (ϵ -caprolactone)
PEG	Polyethylene glycol
Pen/Strep	Penicillin-Streptomycin
PFM	Piezoresponse force microscopy
PGA	Polyglycolide
PI	Propidium iodide
PLGA	Poly (d, l-lactic acid)
pNP	p-Nitrophenol
pNPP	p-nitrophenyl phosphate
pm/V	Picometer/volt
PMMA	Poly(methyl methacrylate)
PVDF	Poly(vinylidene fluoride)
PVDF-TrFE	Poly(vinylidene fluoride-trifluoroethylene)
px	Pixel
RGD	Arginine, glycine, aspartic acid
rpm	Revolutions per minute
Runx2	Runt-related transcription factor 2
SaOS-2	Human osteosarcoma cell line
SEM	Scanning electron microscopy
SH-SY5Y	Neuroblastoma cell line
SiC	Silicon carbide
SSC	Side-scattered light
Tc	Curie temperature
TEM	Transmission electron microscopy
Ti	Titanium
Ti ⁺⁴	Titanium cation
TiO ₂	Titanium dioxide
TPa	Terapascal
TrkA	Tropomyosin receptor kinase A
US	Ultrasound
V	Volt
w/v	Weight/volume

WST-8	Water-soluble tetrazolium-8
XRD	X-Ray diffraction
ZnO	Zinc oxide
ZnONWs	Zinc oxide nanowires



1. INTRODUCTION

1.1. TISSUE ENGINEERING

Tissue and organ damage or losses, as a result of illness, accident or aging, are one of the most commonly encountered health problems, which seriously affect patient's life quality. Transplantation, surgical therapy, devices, prosthesis and healing with drugs are the sought ways to treat. However, these curing options are not satisfying for full repairment and long-term recovery. A quarter of patients who needs organ transplants die while waiting for a suitable donor in the USA alone [1,2]. Request for organ and tissue transplants are unfortunately in excess and it seems that these demands will gradually increase. Even if they are transplanted, the organs or tissues need to be treated with immunosuppressants to prevent transplant rejection [3,4]. The cell transplantation has recently gained more importance as an alternative treatment than transplantation in the removal of organ dysfunction [5-7]. For the construction of an autologous implant, the donor is removed, harvested and separated into cells and a suitable substrate is used for cell attachment and culturing, which is then implanted at the interested part of the functioning tissue. While numerous cell populations can be proliferated in vitro via cell culture procedures, very few cells may be required for such implants to grow. Therefore, organ cells need particular locations which consist of a template for expansion since isolated cells are not capable of spontaneously creating new tissue by themselves. For this reason, the accomplishment of any cell transplantation treatment is based on the improvement of appropriate templates which is called as scaffolds in tissue engineering for both in vitro and in vivo tissue culture. In 1988, 'Tissue engineering' term has formally been introduced at a National Science Foundation workshop as 'the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function. Thus, a new research field, firstly introduced by Langer and Vacanti, has been created called tissue engineering [7,8]. This new field not only reduces the long waiting period for organ transplantation but also removes the risk of transplant rejection and the need for high-risk surgery.

Tissue engineering, a promising and an interdisciplinary field of regenerative medicine, aims to develop solutions by mimicking tissue/organ using natural, synthetic or semi-synthetic materials within the required functionality for the recovery/regeneration of lost or damaged tissue or organs. Tissue engineering idea includes isolation of cells from patient, expanding them in cell culture, seeding on a scaffold and then grafted functional cell-seeded scaffold back to patient as schemed in Figure 1.1. In this strategy, to accustom and manage mammalian cell growth and enhance tissue regeneration, highly porous scaffolds are required.

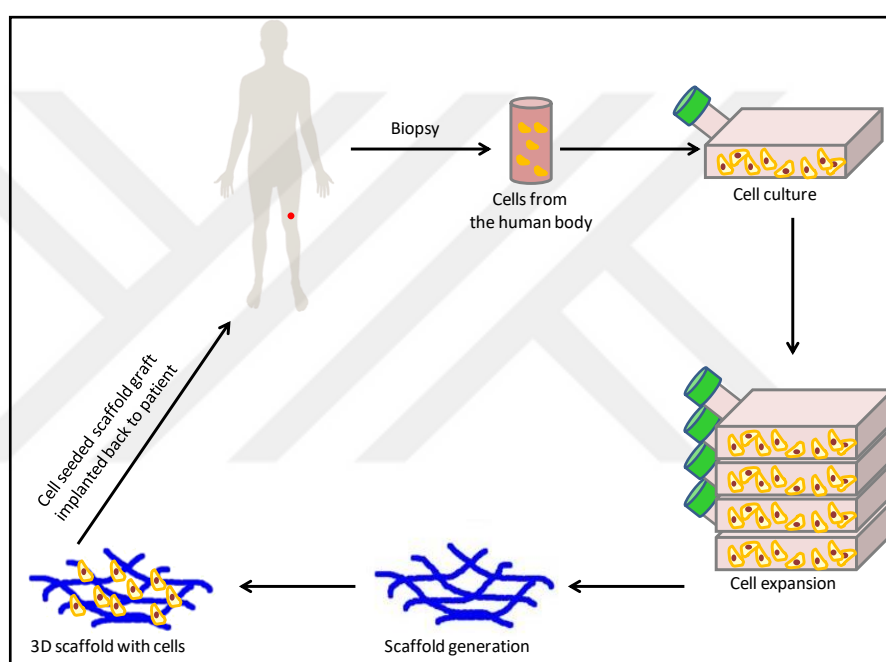


Figure 1.1. Schematic representation of basic principles of tissue engineering

Tissue constructions for the therapeutic purposes are already remarkably applied in hospitals. These revolutionary treatments in medicine include created skin [9], which is the first bio-artificially produced and applied on a burnt tissue in 1990, followed by bone [10-13], liver [5,6], pancreas, intestine, urothelium, esophagus [14], nerves [15], valve leaflet [16], cartilage [17], ligament and tendon [18] treatments. These scaffolds act as a mechanical template here to provide cells to adhere, proliferate and differentiate. Though, these currently used scaffolds in tissue engineering need to inadequacies to be applicable due to need of more mechanical strength, controllable degradation, sufficient biocompatibility and requirement of more interconnection channels to provide cell penetration into three dimensional matrices during growth [19].

1.1.1. Scaffolds as a Key Aspect in Tissue Engineering

The biomaterial porous scaffold is one of the most crucial fundamental in tissue engineering and regenerative medicine applications. They provide a controlled physical and chemical environment so that it directs cell behavior and function. Using different fabrication techniques many scaffolds can be designed with various biomaterials to be used in regeneration of different tissues and organs. The scaffolds can be created from natural (such as collagen) or synthetic materials and are designed as a tentative support to simulate the natural three-dimensional (3D) environment of ECM in order that cells can multiply and organize into tissues or organs by maintaining their specific configurations and morphologies [20]. Figure 1.2. highlights some of the basic requirements to take into consideration while constructing a well-designed 3D scaffold for tissue engineering applications [21].

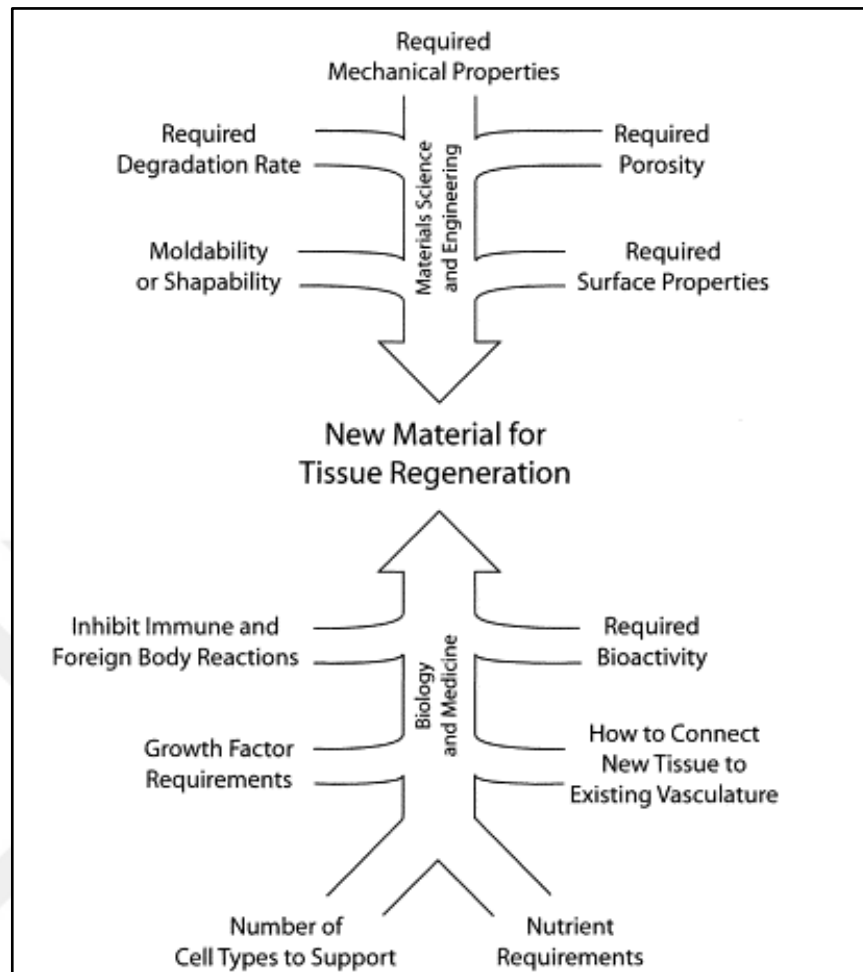


Figure 1.2. Schematic illustration of the basis for generation of 3D smart scaffolds for tissue regeneration [21]

The needs for scaffold preparation are quite sophisticated and precise to the configuration and task of the tissue of interest. Therefore these requirements for the scaffold manufacturing with desired characteristics are the needs to be considered.

After integration into body, scaffold has to show compatible property both with the tissue-specific cell type and local environment inside the human body [22]. Easy adhesion and migration through the scaffold surface, initiating normal functioning and proliferation on new matrix are the first criteria expected from a tissue scaffold. The scaffold must show a slight immune reaction after implantation to body to avoid an inflammatory response which may decrease healing or cause body rejection.

Another key feature of scaffolds is biodegradability. Tissue engineering aims to allow the body's own cells to replace the placed scaffold in time because scaffolds should not be considered as permanent implants. Thus, the scaffolds can be biodegraded for the purpose of allowing cells to generate their own ECM [23]. The decomposition products also should not be toxic and should remove without interfering with other organs.

As well as being biocompatible and biodegradable, scaffolds should sufficiently have mechanical strength to create the proper stress environment for neo-tissues, which are the tissues formed on a scaffold. The scaffolds must be strong enough to preserve their structure during implantation. The mechanical strength properties are of course crucial for all tissues, in cardiovascular and orthopedic applications it gains much more significance. Achieving sufficient mechanical properties in produced scaffolds are one of the major issues in bone and cartilage tissue engineering. For these tissues, the implanted scaffold should show adequate mechanical strength from implantation time to reshaping into interested tissue to start functioning [24]. Healing time and rate are also further challenge due to their dependence on age because healing progresses more slowly in elderly people than in young people. This is one of the factors that must be taken consideration while constructing of scaffolds for especially bone generation. However, to obtain mechanical properties similar to bone and cartilage are not always desired in vivo due to insufficient capacity for vascularization. Here, porosity factor becomes an important part of scaffold architecture to provide vascularization. The balance between mechanical properties and porosity is the key to generate a well-designed scaffold [25].

The structural design of scaffold has critical importance in tissue engineering applications. The pore size, geometry and permeability of scaffold are also significant for cell attachment and ingress of cells and gases, nutrients and wastes. Interconnected pore arrangement and high porosity structure affect the cell adhesion and penetration and also transfer of nutrients to cells and ECMs within the scaffold structure. The interconnected porous structure is also necessary to provide removal of wastes out of the scaffolds. It is important that the scaffold design should be capable of delivery of bio-signals such as drug molecules, growth factors, and cytokines to direct and control the biological responses of cells to specific tissue. Delivery of biological signals from porous scaffold is also important for supporting and enhancing tissue morphogenesis, viability and functionality [26-28].

Degradation products of the scaffolds should have ability to exit from body without any interaction with other organs and surrounded tissues. Due to the lack of vascularization and waste transfer from the constructed material, tissue engineering area have anxieties about the subject of scaffold degradation [29,30]. Pore size of the scaffold is another key aspect in tissue engineering. Cells first chemically attach on the scaffold surface with the help of biomolecules on the cell surfaces. The scaffolds constructed from natural materials such as collagen have these ligands in the form of Arg-Gly-Asp (RGD) [31] (Figure 1.3.). However, the scaffold constructed from synthetic materials requires these ligands in the scaffold during preparation period to enhance cell adhesion on to scaffold.

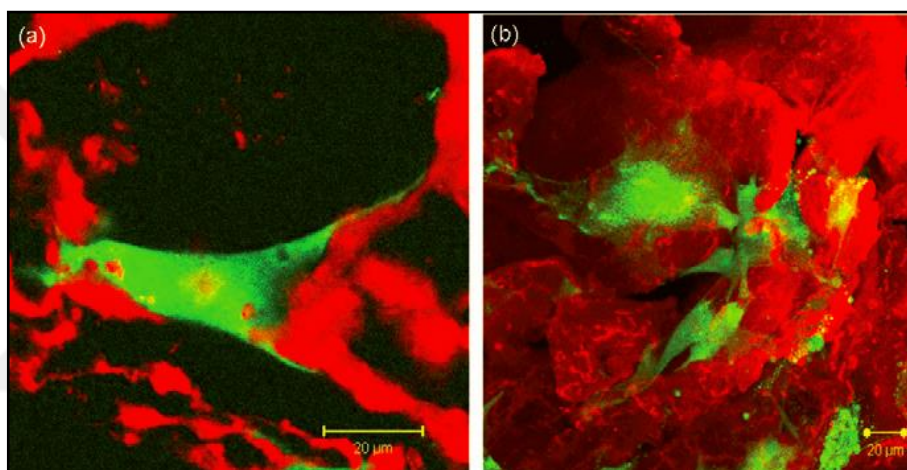


Figure 1.3. Confocal images of osteoblast cells (green) on porous collagen-GAG scaffold (red) [31]

Pores need to be large enough for successful cell adhesion to scaffold surface because number of ligand per a pore is affected by surface area of pores. The scaffolds therefore should have large enough mean pore sizes to provide efficient cell migration into the construction, but they should also have small enough mean pore sizes to obtain high binding capacity with critical cell numbers to ligands on a pore [31,32]. For any tissue engineered construction, pore sizes are one of the most critic topics in this field [33,34]. The cell type and scaffold material should be considered while deciding efficient pore sizes.

Finally, tissue engineering scaffolds that will be commercially available and be used in the clinic must be produced both at an affordable price and be scaled in desired amounts

simply [35]. The development of scalable production according to good manufacturing practice (GMP) standard is of great importance for the successful imposition of tissue engineering applications to the clinic [36].

1.1.2. Bone Tissue Engineering (BTE)

During life, bone is a dynamic and vascular tissue which is capable of variability such as regeneration, growth and shrinkage under the influence of metabolic factors and hormones. It is a highly complex tissue that provides mechanical support, acts as a mineral depot, supports muscle contractions that enables movement, carries weight and protects internal organs. Millions of patients are losing bone tissue every year due to surgery, disease or trauma. In the US alone, more than 20 million patients lose bone tissue owing to bone, cartilage and connective tissue defects and also diabetes. Approximately eight million surgical procedures are performed annually to treat bone loss and more than 7,000 people are waiting for transplant [37]. Autografts, a promising way for the treatment of bone defects, have a limited support and donor site morbidity is a serious trouble. Bone allografts are also another option, but in addition to being expensive, they carry the risk of negative host immune response [38].

Although bone is capable of regenerating itself to a certain extent without scarring when damaged, where the damage is severe, a bone tissue engineering approach is needed that provides an alternative to using autogenic and allogeneic resources to repair and regenerate bone. Bone tissue engineering is a promising field that is concerned to find rewarding solutions by encountering the limitations of bone grafts to enhance bone healing and regeneration thorough the use of biodegradable and biocompatible polymer scaffolds that mimic the extracellular matrix (ECM), acting as a temporary support on which cells attach and proliferate. Although several promising results upon pre-clinical studies with various animal models have been established, there are still ongoing troubles which are the need of interpretation into clinical settings, optimization of cell sources, deciding the type of biomaterial and in vitro preparation and transferring approach [39].

To accomplish bone repair and regeneration, understanding of bone structure and biology is crucial. Bone consists of compact cortical (80 percent) and cancellous or spongy trabecular (20 percent) tissues as represented in Figure 1.4. Cortical bone tissue which has

porosity about 5-10 percent forms the outer shell of bone with a dense structure. It is the primary component of the arm, leg bones and other long bones where extra strength and stiffness are needed. Trabecular bone tissue consisting of thin plates or trabeculae in a loose mesh structure with 50-90 percent porosity typically covers the inner region of the bones. Compared to cortical bone, trabecular bone tissue which is extremely vascularized has superior surface area but less dense and rigid. Trabecular tissue enclosed the bone marrow with elevated amount of hematopoietic and mesenchymal stem cells.

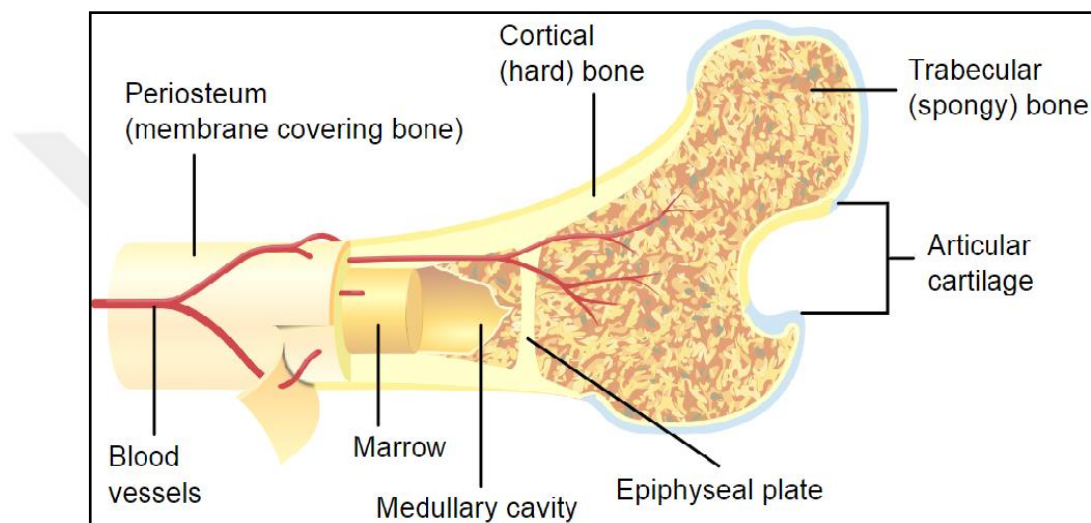


Figure 1.4. Bone structure and anatomy ("Bone cross-section" by Pbroks13 is licensed under CC BY 3.0)

Bone consists of mainly collagen fibers, small inorganic bone crystals and living bone cells arranged in a biomineral medium that formed from both organic (30 percent) and mostly inorganic (70 percent) portions [40]. 90 percent of this organic segment is composed of collagen and the other 10 percent segment includes lipids, proteoglycan molecules, non-collagenous proteins, osteopontin (OPN), osteocalcin (OCN) and other bone matrix proteins which have highly crucial role in tissue adhesion and mechanical strength [41].

The bone seems to be rebuilding in the desired shape, size and structure to supply the mechanical demands placed upon. Bone formation which is also named as osteogenesis includes differentiation of Mesenchymal stem cells (MSCs) into osteoblasts, production of extracellular matrix deposit called osteoid by osteoblasts, formation of osteocytes because of osteoblasts became trapped within the bone matrix, initiation of bone formation leading

by matrix mineralization and bone remodeling by the progressions of resorption and reformation [42].

Bone tissue engineering strategies include producing 3D materials such as polymers, ceramics or combinations using conventional technologies to assist and maintain the growth of new bone tissue and repair its functionality by the synergy of cells, signals and scaffolds (Figure 1.5.).

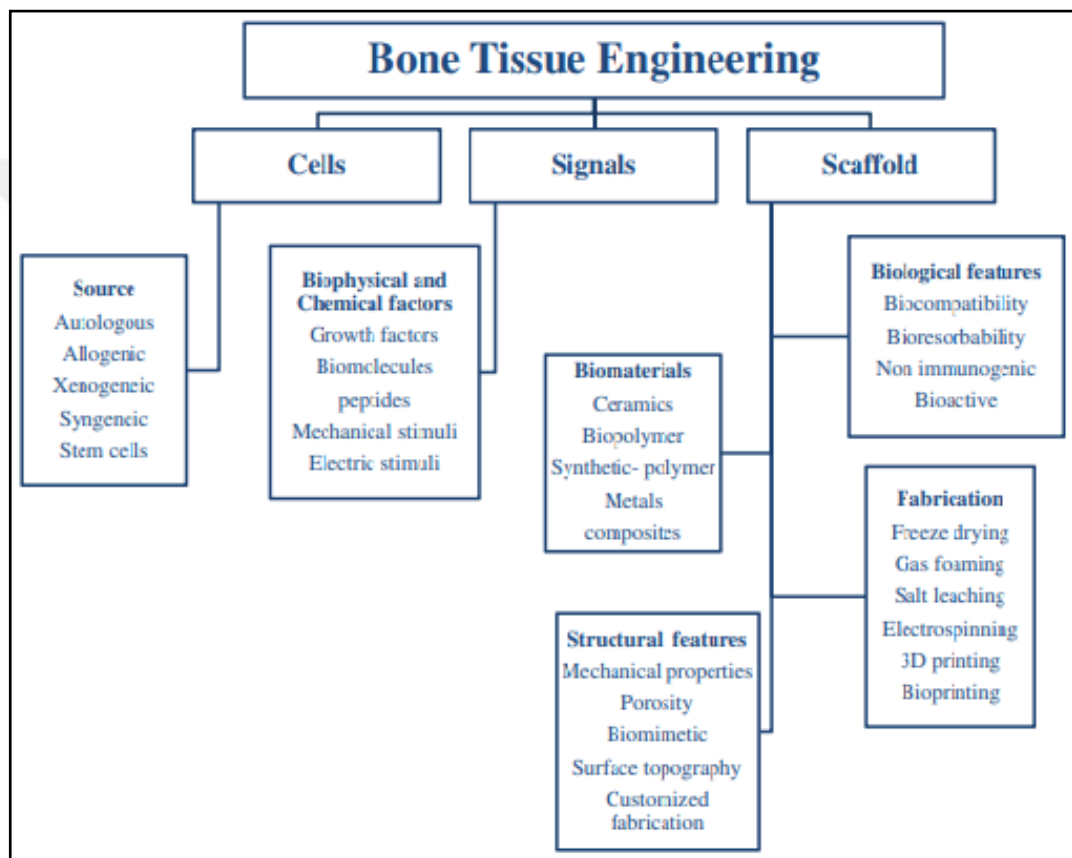


Figure 1.5. Bone tissue engineering strategies [43]

The choice of material design and development is directly related to the particular application area of the material. In bone tissue engineering approach, scaffolds play a crucial role in both enabling mechanical support to the skeleton and prospering integration of the bone-grafting material in the host tissue [44]. Since it will be the main support frame of bone grafts, scaffolds must have a rigid and flexible structure. Scaffolds must also be porous enough with fine pore interconnectivity to provide migration of cells and construction of 3D bone tissue in vitro and in vivo and to support flow for the transportation of nutrients and metabolic waste [45]. Cells already dedicated or

differentiated with osteoblastic lineage that can lead to the formation of new osteoblasts are preferred. These cells may thoroughly migrate within the scaffolds from damaged or neighboring native tissues and start to grow. Additionally, bone tissue engineering scaffolds must be osteoinductive, osteoconductive and talented for osteointegration.

1.1.2.1. Polymers Used as Scaffolds in Bone Tissue Engineering Applications

Bone tissue engineering scaffolds have been fabricated using biocompatible polymers which do not elicit an immunological reaction for body. The selected polymer should be degraded in a controlled manner while tissue regenerates and the degraded products should not be toxic and they must be straightforwardly excreted via metabolic pathways. Numerous kinds of polymeric supplies have been used in bone tissue engineering applications [41,46,47]. These polymers can be basically categorized as naturally and synthetically derived materials. Natural polymers, which also called as biopolymers have been commonly preferred due to their talent to network with cells by supporting cell adhesion and function. However, they have some troubles in controlling mechanical strength and degradability. Many of them also are not cost-effective therefore they are limited in supply. Natural polymers can be classified according to proteins (e.g. gelatin, collagen, silk fibroin), polysaccharides (e.g. alginate, chitosan, gellan gum and derivatives) and glycosaminoglycans (e.g. hyaluronic acid) [48].

Synthetic polymers differs in have both advantages and disadvantages as compared with natural polymers. It is easy to obtain in large-scale by controlling their strength and degradation rate. This advantage makes synthetic polymers useful materials for cell transplantation for tissue engineering. They have also several disadvantages such as causing immunologic reaction and not being able to incorporate with host tissues. Polyglycolic acid (or polyglycolide (PGA)), polylactic acid (or polylactide (PLA)), polylactide-co-glycolide (PLGA), poly (d,l-lactic acid), polyethylene glycol (PEG), and poly(ϵ -caprolactone) (PCL) are the synthetic polymers that are commonly preferred. Synthetic polymers can be also combined with natural polymers for better cell adhesion and growth.

Poly ϵ -Caprolactone (PCL)

Poly (ϵ -caprolactone) PCL is biocompatible, biodegradable and aliphatic polyester with a melting temperature above body temperature (ranging between 59-64°C), which thus

results in high toughness and superior mechanical stiffness and flexibility [49]. Inclusion in US Food and Drug Administration (FDA)-approved products makes also PCL a trendy synthetic polymer in bone tissue regeneration applications [50-52]. It is slowly degraded during time (2-3 years) by microorganisms or hydrolysis of ester linkage under physiological conditions [53]. Owing to their highly hydrophobic nature needs additional alterations to promote cell adhesion and function. Varying functionalization strategies have been developed to alter the disadvantages including low bioactivity, hydrophobicity as well as inadequate mechanical properties of PCL [54,55].

There are many reinforcement agents such as hydroxyapatite (HA) [55], starch, cellulose, etc. [56], silk fibers [57] and carbon nanotubes (CNT) [58], which have been investigated by researchers on mechanical improvement of PCL scaffolds. In order to increase biocompatibility of PCL scaffolds Aluminum oxide (Al_2O_3) and Hydroxyapatite (HA) were added to obtain PCL nano-composite scaffolds [59,60]. Blended scaffolds such as, silk protein/PCL/Gt, PCL/silk fibroin, PCL/biphasic calcium phosphate and PCL/chitosan composite scaffolds advanced the cell growth considerably compared to pure PCL [61-63]. It has been reported that calcium silicate embedded PCL scaffolds increased the bioactivity, mechanical properties and osteogenicity [64]. Silica is a well-known material since it enhances the bone regeneration, increases bone mineral density and promotes osteoblast like behavior [65-67]. Polylactide-polycaprolactone copolymer was also reinforced with boron nitride nanotubes (BNNTs). PCL-BNNT composite scaffolds increased the osteoblast activity and Runx2 gene and key regulator responsible for osteoblast differentiation in comparison with only PCL matrix [68].

Many techniques have been used including 3D printing, porogen leaching, phase separation and freeze-drying to construct porous PCL scaffolds and electrospinning and melt electrospinning to construct fibrous PCL scaffolds [69-73]. Electrospinning is one of the most favorable techniques among them to fabricate ultra-fine and controllable fibrous 3D scaffolds. PCL/Gelatin nanofibrous scaffolds prepared using electrospinning technique showed important potential in bone regeneration [74]. Nanofibrous scaffolds prepared using PCL/PLA blends demonstrated improved osteogenic differentiation of stem cell by activating of BMP 2 signaling [75].

Chitosan

Chitosan is a linear amino polysaccharide obtained by deacetylation of chitin. Chitin, on the other hand, is a natural biopolymer that is present in large quantities in shellfish shells and is the second most plentiful in the world after cellulose. Chitin is a polymer of N-acetyl-D-glucosamine and chitosan is a copolymer of D-glucosamine and N-acetyl-D-glucosamine. Deacetylated chitin is called chitosan. Deacetylation process is the conversion of acetylamino ($-\text{NH}-\text{CO}-\text{CH}_3$) groups on the chitin to amino ($-\text{NH}_2$) groups. Chitosanases, which is absent in mammals, is responsible for the biodegradation of chitosan [76].

Due to being a crystalline polysaccharide, chitosan is generally insoluble in the presence of pH greater than 7. However, in lower pHs ($\text{pH} < 6$) the free amino groups on chitosan skeleton are protonated, which is resulted in dissolve of the molecule [77].

Chitosan polymer can be cross-linked covalently via cross-linker agents such as glutaraldehyde and genipin to increase degradation and mechanical stability, which is called as hydrogel. However, due to being 10,000 times less cytotoxic than glutaraldehyde, genipin has been a commonly preferred cross-linker agent recently [78]. Genipin reacts with reactive amine side groups of chitosan polymer chain by forming chemical bridges through in mild conditions (Figure 1.6.).

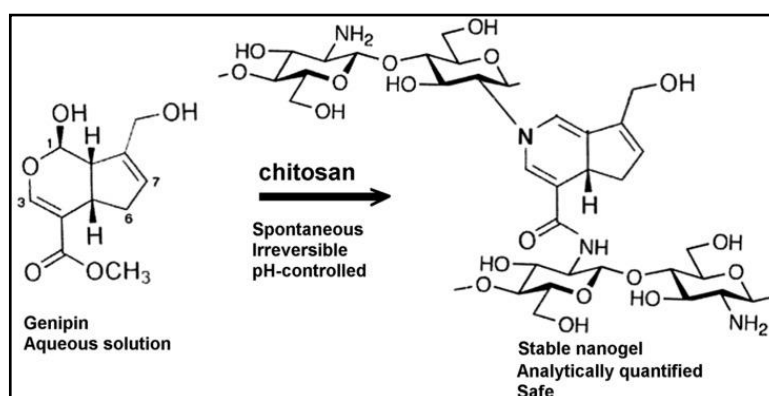


Figure 1.6. Cross-linking reaction between genipin and chitosan [79]

Chitosan exhibits highly bioactive, biodegradable, anti-bacterial and biocompatible features and its degradation products are harmless [80]. Besides the outstanding properties of chitosan, due to disadvantages such as uncontrolled degradation, risk of infection and

insufficient mechanical properties, chitosan only still does not meet tissue engineering requirements. Thus, researchers have been developed hybrid systems with superior properties to come through these challenges.

The polysaccharide backbone of chitosan is structurally like glycosaminoglycans, which are the main constituent of bone and cartilage extracellular matrix [81]. The manufacture of highly porous scaffolds with interconnected pores and their capability to increase osteoconductivity and bone formation both in vitro and in vivo are the further advantages of chitosan scaffolds for bone tissue engineering [81,82]. Although chitosan is a biocompatible substrate for cell proliferation and an effective template potentially used in repairing bone and chondral defects, improvement of their mechanical strength and biological property to make chitosan skeletons suitable for bone tissue engineering is needed.

Chitosan has been fabricated to form 3D porous scaffolds by using many materials including ceramics, polymers and nanomaterials to enhance its mechanical and biological properties in bone tissue engineering applications [83-85]. It has been reported in many studies that nanomaterial addition as reinforcement agent essentially improves the mechanical strength and biological features of chitosan scaffolds [86-90]. Chitosan scaffolds were prepared by adding BNNTs as reinforcement agent through the freeze-drying technique [90]. BNNTs were first hydroxylated for providing better distribution in chitosan matrix. BNNTs improved the mechanical stability, decreased the water absorption and biodegradation rate of chitosan scaffold. Better cell adhesion and proliferation were observed on hydroxylated BNNT embedded chitosan scaffold [90].

3D porous chitosan scaffolds can be commonly fabricated using phase separation and lyophilization, particulate leaching, gas foaming, freeze drying, rapid prototyping techniques [77,91-94]. It will be necessary to control the porosity, pore size, mechanical property and distribution of the pores in order to make suitable scaffolds for specific tissue regeneration. Multiscale porous scaffolds can also improve vascularization, as small and large pores are suitable for angiogenesis and bone growth, respectively.

To complete, although the design and implantation of accurately effective bone tissue engineered scaffolds remain a challenge, chitosan is a potent material that can be combined with biological factors and other materials to produce promising structures for bone.

1.2. NANOMATERIALS IN TISSUE ENGINEERING

Nanotechnology is a commonly used area for tissue engineering and regenerative medicine. Nanomaterials may help mimicking the tissue-specific bioenvironment by designing constructions with particular biochemical, mechanical, and electrical properties. Hence, tissue can be designed using these nanostructures by providing enhanced cell adhesion, proliferation, and differentiation. There is a proportional increase in the demand for innovative scaffolds with various properties, since the variety of tissues proposed to be built in engineering increases.

A porous scaffold that will serve as both substrate and support for tissue growth is needed in tissue engineering. The scaffold creates the three-dimensional composition necessary for the correct anatomical growth of cells. The nanostructures to be used in tissue engineering may have variable functionality depending on the scaffold design. For example, bone and cartilage tissues require enhanced mechanical properties while nerve tissue requires electrical conductivity. Biocompatibility in tissue engineering scaffolds is the main requirement to avoid inhibition of cell growth. Nanotechnology is a highly innovative approach to encounter these needs in scaffold fabrication.

Many nanomaterials have been used in tissue engineering scaffolds owing to their superior and diverse properties. For example, gold nanoparticles are mainly preferred due to surface conjugation and conductive properties [95]. The antimicrobial properties of metallic nanoparticles such as silver [96] and metal oxides [97,98], the fluorescent properties of quantum dots [99-101], the unique electrical and mechanical properties of carbon nanotubes (CNTs) [102,103], outstanding electrical conductivity, biocompatibility, surface area and thermal properties of graphene [104] have made them very functional in a great number of tissue engineering applications. Boron nitride based nanomaterials including hexagonal boron nitrides (hBNs) and boron nitride nanotubes (BNNTs) enhances the mechanical properties and thermal conductivity of tissue engineering scaffolds [105]. One of the most important features that make them attractive to be used in biomedical applications is that they are highly biocompatible [106].

1.2.1. Barium Titanate (BaTiO_3)

Barium titanate (BaTiO_3), an inorganic compound having the general formula ABO_3 called perovskites, was discovered in the 1940s. It is a well-known ferroelectric and dielectric material that exhibits photorefractive effect and piezoelectric properties, which allow it to be used in many technological applications. It has a cubic structure, where titanium cation (Ti^{+4}) is located in the center and surrounded with six oxygen (O) at the centre of all faces and eight barium cation (Ba^{+2}) on corners (Figure 1.7.). BaTiO_3 appears as white powder; however, if its size is getting larger, it appears transparent.

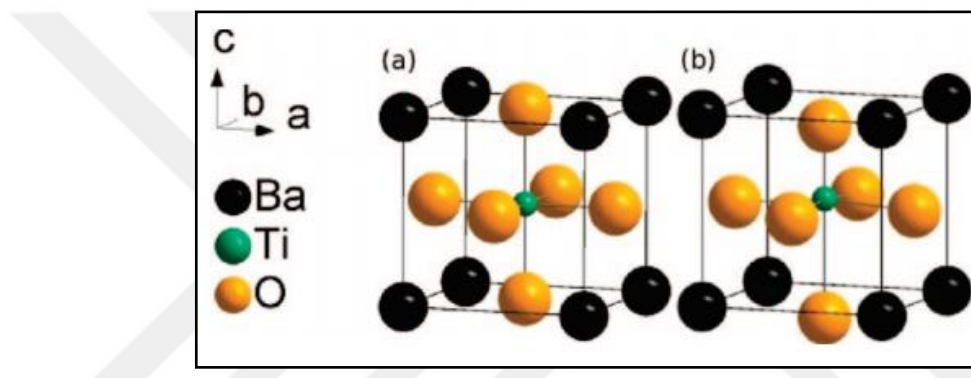


Figure 1.7. Structures of BaTiO_3 (a) cubic and (b) tetragonal [107]

The polymorphs of BaTiO_3 structure can vary with temperature. From low to high temperature there are four polymorphs including rhombohedral, orthorhombic, tetragonal and cubic crystal structure, respectively as illustrated in Figure 1.8. BaTiO_3 is polar, non-centrosymmetric, ferroelectric below curie temperature ($T_c = 120^\circ\text{C}$), the critical temperature at which a ferromagnetic material loses its permanent magnetism and becomes paramagnetic, whereas BaTiO_3 is non-polar, centrosymmetric and paraelectric above T_c . Except cubic crystalline phase, all phases demonstrate ferroelectric properties [108].

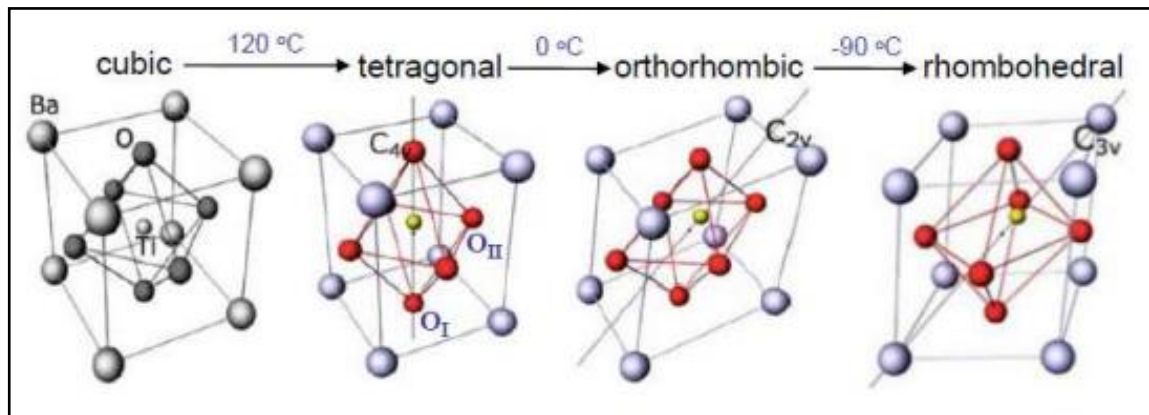


Figure 1.8. Schematic illustration of phase transitions of BaTiO₃ with temperature [108]

Piezoelectricity is the ability of a material to change electric field or electrical potential upon mechanical pressure to the material. The electrical polarization and electrical field formed in piezoelectric materials are vectorial and their values depend on the direction. Its dependence to this direction is determined by the crystal structure of the material and is a characteristic feature of the material. Therefore, the electrical polarization that a mechanical stress will create in the material will depend on the material and will take a characteristic value for each piezoelectric material according to its crystal structure.

The ferroelectric property observed in piezoelectric materials can be summarized as the ability of the crystal structure to be polarized instantaneously under a high electric field ($\sim 10^6$ V/m). Based on the periodic table, it can be said that two kinds of ion groups play an active role in this behavior [109]. In the first group, there is Ti^{4+} which forms an octahedral network with oxygen in the lattice and is located at the corners of the unit lattice structure. The Ti^{4+} ion can form TiO_6 complex with 6 O^{2-} neighbors. This octahedral-shaped complex undergoes some distortion to have lower symmetry in order to reduce the bond energy. This distortion enables the material to have a molecular dipole, thus showing ferroelectric and piezoelectric properties. In the case of BaTiO₃, displacement of Ti^{4+} ion away from the position of centrosymmetry within the oxygen octahedral is related to the ferroelectric situation of BaTiO₃ (Figure 1.9.).

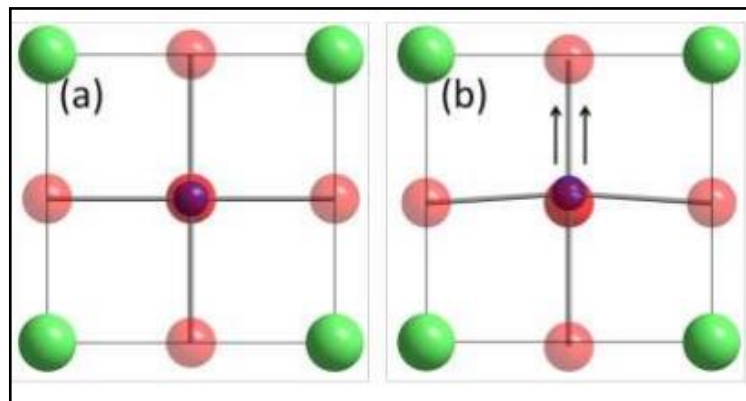


Figure 1.9. Crystal structure of BaTiO₃, (a) cubic phase and (b) tetragonal phase with Titanium (Ti) (blue sphere) cation displacement (Red sphere represents Oxygen (O), green sphere represents Barium (Ba)) [109]

The second group is formed by active ions such as Pb²⁺ and Bi³⁺ that can form pyramid coordination with oxygen. These structures are seen in oxide ferroelectric ceramics such as PbTiO₃, Bi₄Ti₃O₁₂ and PbNb₂O₆, which show high Curie temperatures.

Polycrystalline ferroelectric ceramics initially contain regions (domains) whose dipoles are polarized in random directions within each grain so that their total elastic energies are minimal. Due to this random arrangement, ferroelectric materials do not show piezoelectric properties. However, below curie temperatures, when exposed to a static electric field greater than a threshold value, in other words, when they are polarized, they can acquire a piezoelectric property. During polarization, the regions become anisotropic by rearranging in the direction of the electric field either by inversion or by gaining a certain angle. After the polarization process, some of the highly stressed regions may revert to their previous state, but in the vast majority of the polarization is permanent. Therefore, their pre-polarization properties are isotropic and in this case they do not show piezoelectric behavior. After polarization, with the applied electric field, the dipoles are directed towards an axis and a net electrical dipole moment occurs in the whole structure consisting of polycrystals. In this structure, there are regions (domains) formed by isolated dipoles directed in the same direction. The orientation in the internal structure of a ferroelectric material before and after polarization is shown in Figure 1.10.

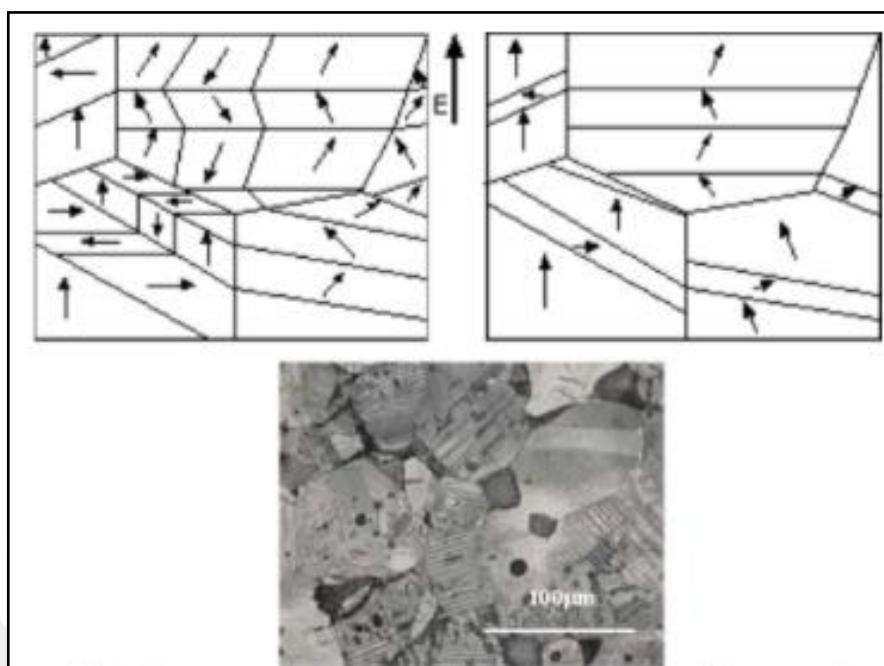


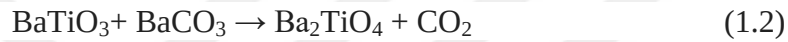
Figure 1.10. The orientation of BaTiO_3 (a) before polarization, (b) post-polarization, (c) domain structure in BaTiO_3 [109]

After the polarization process, the electrical voltage that is lower than the polarization voltage and given in the same direction creates an additional elongation in the direction of the polarization axis and shrinkage in the direction perpendicular to this axis, whereas the tension given in the opposite direction causes contraction in the direction of the polarization axis and a shortening in the direction perpendicular to this axis. Even if the elongation is compensated by shrinkage in the index or width, it may not be equal in ratio and therefore there may be a net change in volume. In both cases, when the tension is removed, the material returns to its polarized state.

There are several techniques for synthesizing of perovskite-type BaTiO_3 powders. One of them is solid state reactions, a traditional approach, which is performed at higher temperatures than 1000°C under controlled pH and pressure states [110,111]. Sol-gel [112,113], microwave [114], hydrothermal methods [115-117] and sputtering [118] are the other techniques that are offered for the BaTiO_3 synthesis. Soft chemistry methods for the development of one-dimensional nanowires [119,120], sonochemical routes to obtain size-tunable BaTiO_3 crystals [121] and biosynthesis methods for production of BaTiO_3 nanopowders [122] are the new techniques which have been introduced recently. BaTiO_3

has also been synthesized by solid-state reaction of BaCO_3 and TiO_2 as precursors after ball-milling process at above 1000°C [123]. Particle size was reported to decrease as the ball milling time increased. The piezoelectric constant d_{33} of BaTiO_3 was reported with the maximum value, 190 pC/N, when it fabricated by conventional sintering of BaCO_3 and TiO_2 [124].

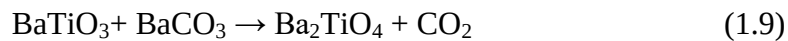
There are several mechanism assumptions for BaTiO_3 formation based on solid-state reactions between BaCO_3 and TiO_2 precursors at high temperatures. Pavlović et al. [125] was proposed the following reaction series:



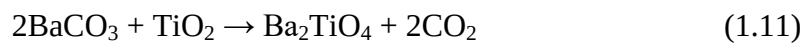
Beauger et al. [126] proposed two ideas, the first sequence was:



While the second one was:



Meanwhile Kong et al. [127] and Gallagher et al. [128] suggested following equations:



It has been showed from the above assumptions that different pathways may be possible to transform reactants to the final product, BaTiO_3 .

Due to its excellent ferroelectric, piezoelectric, photorefractive nature and exhibiting high density optical data storage makes it useful in many areas including sensors, lasers, optical computing, optical image processing, piezoelectric devices, pattern recognition, optoelectronic devices, pyroelectric sensors, dielectric amplifiers. In addition, BaTiO₃ has recently been an attractive material used in medical applications such as implants due to its piezoelectric property [129] and biocompatibility [130-132] by taking the advantage of providing wireless electric stimulation.

1.2.2. Boron Nitride Nanotubes (BNNTs)

After being discovered theoretically in 1994 [133,134], BNNTs have first been synthesized in 1995 by Chopra N.G. [135]. Although BNNTs are structural analog of carbon nanotubes (CNTs), they have considerably differed in their physical, chemical and electronic properties. BNNTs are electrical insulator with nearly 5.5 eV band gap independently from their chirality while the metallic or semiconducting CNTs changes with their chirality [136]. They may reach a Young modulus of 0.5-1.2 TPa, which make them very strong material mechanically [137,138]. BNNT consist of boron (B) and nitrogen (N) atoms in its structure as seen in Figure 1.11. and it is the cylindrical form of hexagonal boron nitride (hBN) with approximately 1-100 diameter and varying lengths. B-N bonds have asymmetric charge distribution in BNNTs compared to C-C bonds in CNTs [139]. The electron density on B atoms intensifies on to N atoms because nitrogen has higher electronegative character than boron. Therefore, the bond between B and N has slightly ionic nature leading to a gap between valance and conduction bands.

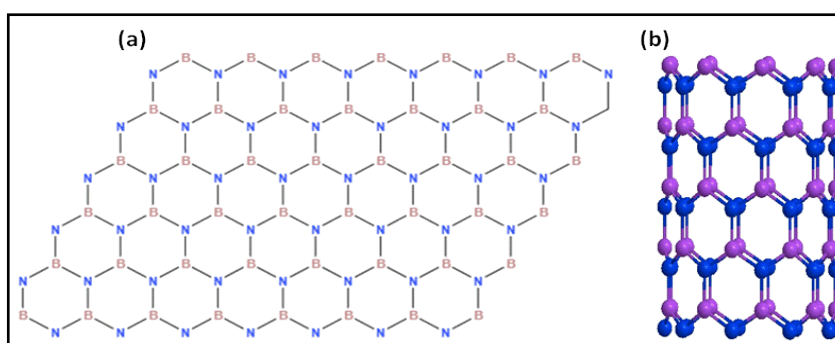


Figure 1.11. Structural illustration of (a) a BN sheet and (b) a single-walled BN nanotube
(The length between B and N atoms is 1.44 Å)

BNNTs have been synthesized using many techniques such as arc-discharge [135,140,141], chemical vapor deposition (CVD) [142-146], substitution reaction [147-151] and laser ablation [152-156]. Among them CVD is widely used technique in synthesizing BNNT. Cobalt, nickel, iron, magnesium and their derivatives are used as catalysts in this method. A boron-containing compound and a catalyst are reacted at temperatures ranging from 1000-1900°C in the presence of NH_3 , N_2 or Ar gas and BNNTs are obtained. Compared to other approaches, this technique is a key way to obtain high quality nanomaterial as it provides better controllability of growth parameters related to growth mechanism, experimental setup, precursors, catalysts and temperature [136].

BNNTs have more superior mechanical features and more stable to oxidation and heat compared to CNTs [157]. They have also high hydrogen storage capacity, radiation absorption and high thermal conductivity. Due to their highly biocompatible feature BNNTs are considered as special nanostructures in biomedical applications including tissue engineering [158,159], drug [160] and gene delivery [161]. Although their high hydrophobic nature attract attention to prepare super hydrophobic surfaces [162], it is not a desired feature in biomedical applications. BNNTs are generally preferred to be used in biomedical applications after covalent [163,164] or non-covalent [165] functionalization to provide water-dispersibility. Although BNNTs are highly resistant to harsh chemical conditions and are strengthening materials for covalent functionalization, the recent studies exhibited that this functionalization strategy is achievable [166]. Chemical functionalization is widely achieved through the $-\text{OH}$ and $-\text{NH}_2$ groups on B atom at the edges and defects on the tube structure as indicated on Figure 1.12. [167,168].

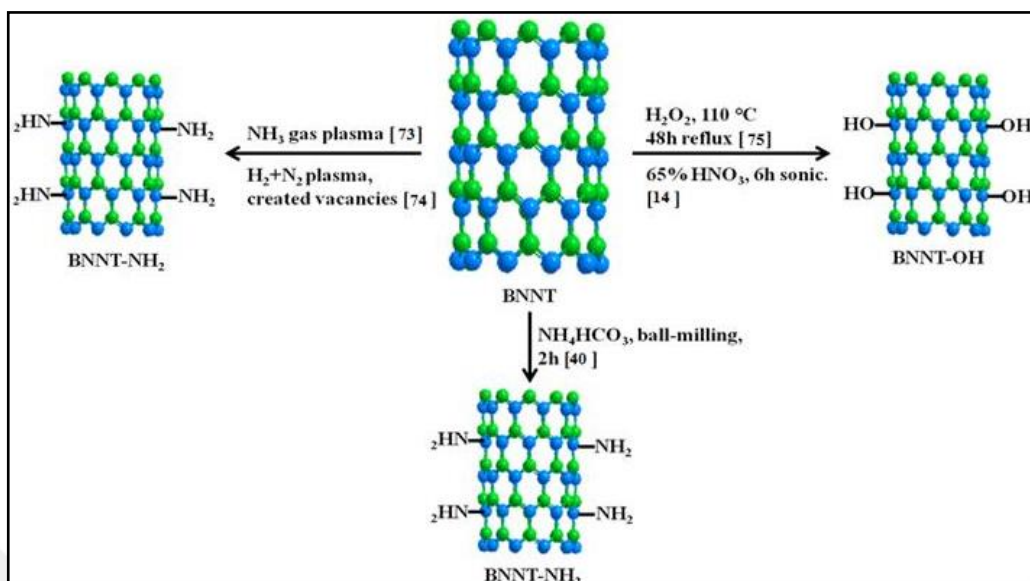


Figure 1.12. Routes for the chemical functionalization of BNNTs [157]

Preliminary functionalization of BNNTs with -NH₂ and -OH groups is to provide further functionalization via biologically relevant molecules including ferritin or fluorescent-tracking compounds to be used as intracellular nanovectors of bio-related molecules. Ciofani and his colleagues functionalized BNNTs with 3-aminopropyltriethoxysilane (APTES) which allowed further modification through functional amine groups on the surface of silica layer. Also, the silica coating covers the whole surface by intermolecular cross-linking of -Si to the stable Si-O-Si bonds [163]. Then, with a fluorescent dye, Oregon Green 488 carboxylic acid, succinimidyl ester was covalently bound to silica coated BNNTs. The labeled BNNTs with fluorescent dye were incubated with NIH/3T3 fibroblast cells to observe their cellular uptake. They found the labeled BNNTs in cytosol. Protein functionalized BNNTs can increase their applicability in nanomedicine field. The covalent attachment of human transferrin upon a carbamide bond on to BNNT was reported [169]. Primary human umbilical vein endothelial cells (HUVECs) were treated with the functionalized BNNTs to investigate their cellular uptake. The functionalization of BNNTs via targeting proteins could open intriguing opportunities for designing nanocarriers in nanomedicine applications.

Differently from CNTs, most of BN structures may exhibit spontaneous polarization field due to their non-centrosymmetric and polar nature. The piezoelectric properties of BNNTs are derived from the deformation of hBNs while rolling and the amount of piezoelectricity is based on their aspect ratio [170]. Mele and Král have investigated the piezoelectric

properties of BNNTs depending on polarizability under deformation using a Berry phase approach in 2003 [171]. They demonstrated that BNNTs produced a coupled electric dipole depending upon their chirality and type of mechanical stress. The two different structures of BNNTs, armchair and zigzag, show diverse answer when subjected to torsion as seen on Figure 1.13. [172]. While armchair give a coupled electric dipole respond, zigzag give respond to elongation. They also exhibited that the strength of the coupling changed with the chiral angle with a subjected torsion. Their piezoelectric prediction results revealed that BNNTs have better piezoelectric properties in comparison with piezoelectric polymers and wurtzite semiconductors [173].

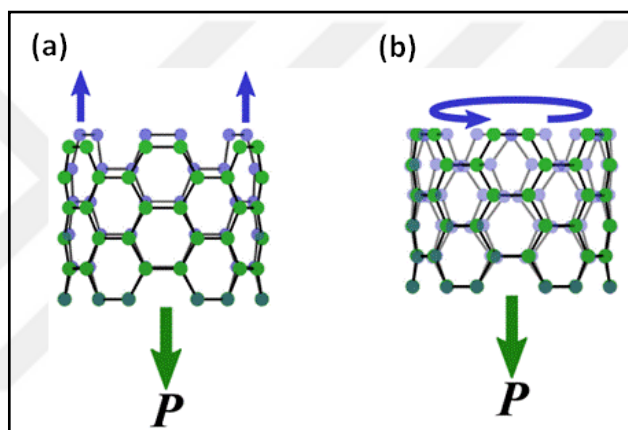


Figure 1.13. Propagation of dipole vector after applied deformation (P and green arrows). (a) shows the respond when BNNTs are exposed to an axial stress, (b) shows the respond when BNNTs are exposed to torque with blue arrows that indicates the resultant deformation of BNNTs [172]

The piezoelectric performance of multi-walled BNNTs has also been proved and besides the deformation-originating polarization has been confirmed in recent studies [174,175]. In addition, the extraordinary flexibility of BNNTs has been verified by studying cyclic mechanical deformations on BNNTs [176]. Depending on electro-structural elements, the piezoelectric coefficients of BNNTs have been examined and envisaged using a new continuum mechanics modality [177]. Compared to other approaches, this technique is better to obtain high quality nanomaterial as it provides better controllability of growth parameters related to growth mechanism, experimental setup, precursors, catalysts and temperature.

In tissue engineering applications, BNNTs are widely used for their excellent properties such as, mechanical strength, biocompatibility, non-toxicity and piezoelectricity. In general, BNNTs have been preferred as reinforcement in traditional scaffolds to enhance their mechanical strength. Lahiri *et al.* used BNNTs in hydroxyapatite (HA) by overcoming their poor fracture toughness/wear resistance to prepare orthopedic implant materials [158]. HA modified BNNTs showed enhanced elastic modulus and wear resistance. Additionally, the experiments indicated that they could be doubtlessly used in orthopedic implant field due to their high biocompatibility with osteoblasts. BNNTs were also used as reinforcement fillers to enhance mechanical strength of poly- ϵ -caprolactone (PCL) to investigate their effect in bone and cartilage tissue engineering [68]. The human osteoblast showed both optimal biocompatibility and effective cellular response on BNNT-embedded PCL scaffold.

1.2.3. Piezoelectricity in Human Body

In human body, numerous main functions in cells and organs are dominated by electric signals. In the 18th century, electrostatic charge is used to treat skin lesion [178]. It has been established that electric potentials stimulates the distinct effect on distinct cells and can direct the movement and migration of cell types including corneal, epidermal and epithelial [179,180]. Electric fields have also effect on modulation of vascular endothelial cells [181], on regeneration of nerve fibers [182] and also they are commonly used to heal ligament in orthopedic applications [183].

The piezoelectric properties of bone and other biologic substances have been shown with extensive studies. The piezoelectricity can be explained as a comprehensive feature of living tissue which is essential in several physiological phenomena [184]. Therefore, the piezoelectricity can be observed in many parts of the body including bone, cartilage, skin, dentin, collagen, tendon, ligament, deoxyribonucleic acids (DNA) and cell membranes Figure 1.14. [185,186].

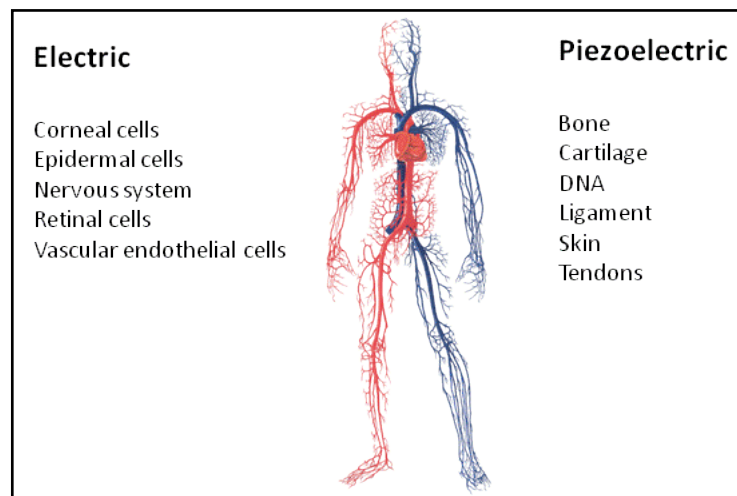


Figure 1.14. Illustration of human body location in which electrical and piezoelectric signals are relevant [187]

Piezoelectric effect is observed in tendons and ligaments due to their collagenous nature when exposed to a mechanical stress [188,189]. Both soft tissues including skin, callus, cartilage, tendons and hard tissues such as bone exhibit piezoelectricity and appear to be related to the presence of directed fibrous proteins [190]. Collagen, keratin, fibrin, elastin, reticulum or cellulose structures are the proteins that exist in all connective tissues and shows piezoelectric properties [184].

Owing to its piezoelectric character, bone is an active tissue that has easy adaptation and remodeling through complicated mechanisms with electromechanical operations. In 1955, the first piezoelectric effect of bone was investigated [191]. Following, the generation of electrical currents after mechanically evoking was verified [192,193]. These generated electric signals stimulate, promote and regulate bone growth and remodeling based on the Wolff's law [194]. At the cellular level, the osteocytes, which are types of bone cells that play a significant role in the development of bone structure and in involvement in bone mechanotransduction, have been described [195]. Bone generation includes the communication of bone cells such as osteoblast and osteoclast and also the effect of electric stimulation on bone regeneration has been investigated in vitro [196-199] and in vivo [200-203], demonstrating the enhancement and stimulation of osteogenic activities. Osteocytes can sense mechanical force through their processes in the canaliculi and then

produce several cytokines to regulate osteoclast-mediated bone resorption and osteoblast-mediated bone formation.

1.2.4. Piezoelectric Nanomaterials in Medical Applications

Piezoelectric nanotransducers have capability to generate electrical potential on their surfaces due to their piezoelectric virtue when exposed to mechanical force [204]. Their piezoelectric feature can be activated via varying types of mechanical stimulation such as vibration plates [205], sounds [206] and ultrasounds (USs) [204]. Among them, USs are the most preferable because they have ability to penetrate into biological tissues very deeply which may go up to tens of centimeters based on tissue impedance and US frequency [207]. They can efficiently be focused on where the piezoelectric nanomaterials are located in tissue through hyper-lenses to provide a remote stimulus with high spatial resolution [208].

Piezoelectric nanomaterials are favorable in medical applications especially for the stimulation of electrically responsive cells such as nervous, cardiomyocytes, skeletal myotubes and osteoblasts. Electrical stimulation on excitable cells enhances the tissue functionality and attracts in many medical treatments of common diseases such as cardiac arrhythmia, chronic pain, Parkinson's disease [209]. In traditional modality, wires need surgical operation to transport current to the relevant region in body, which may cause infections. Therefore, piezoelectric nanomaterials that can be called as nanotransducers have recently gained importance in biomedical applications for providing indirect electric stimulation. Moreover, the promising results obtained from in vivo experiments show the opportunity of using these materials in clinics.

The piezoelectric nanotransducers can be classified according to their chemical content such as boron nitride, barium titanate, zinc oxide, and their shape such as nanowires, nanotubes and nanospheres. Mechano-electrical transduction (piezoelectric coefficient, d), the biocompatibility level, purity of the material, and the availability (synthesizable or commercially available) criteria must be taken into consideration while preferring a nanomaterial for piezoelectric stimulation for the purpose of medical use [209]. BNNTs are a good candidate for piezoelectric stimulation due to their good piezoelectric feature and highly biocompatible nature [171]. Zinc oxide nanowires are known for their

piezoelectric properties (ZnONWs; d_{33} is about 10 pm/V) and they can simply be synthesized at large quantity and high purity [210]. However, their highly toxic nature limits their use in biomedical applications [211]. Barium titanate nanoparticles (BTNPs) are other type of nanotransducers with their outstanding piezoelectric features due to their perovskite structure (d_{33} is about 30 pm/V for tetrahedral configuration) [212]. Due to their high biocompatibility they can be also used at higher concentrations [213]. The piezoelectric BTNPs with tetrahedral configuration and non-piezoelectric BTNPs with cubic configuration are available at various sizes on the markets. Non-piezoelectric BTNPs can be used as negative controls [214].

Piezoelectric polymers including poly(vinylidene fluoride) (PVDF) and poly(vinylidene fluoride-trifluoroethylene) (PVDF-TrFE) attract attention in tissue engineering applications owing to their biocompatibility. Their different forms such as thin films, micro and nano fibers were accomplished in cell culture stimulation [205,215,216]. Moreover, these polymers are promising for further in vivo researches if their piezoelectric properties are enhanced with adding piezoelectric nanoparticles [213] or annealing [216].

BaTiO₃ nanoparticles for the first time were used to enhance piezoelectric feature of poly(vinylidene fluoride-trifluoroethylene, P(VDF-TrFE)) film for neuronal stimulation. The novel polymer/ceramic composite films supported the Ca⁺² transitions and cell differentiation. Genchi and his group have also reported several studies on the interaction of biologic environment with piezoelectric nanomaterials and on exploitation of their action via US stimulation. They found that PC12 neuron-like cells had 30 percent longer neuritis than the controls in the presence of US stimulated BNNT by the involvement of the TrkA receptor. They also evaluated that the effect of piezoelectric stimulation on SH-SY5Y neuron-like cells in the presence of piezoelectric BaTiO₃ nanoparticles [214]. In another study, the effect of BaTiO₃ and hydroxylated BaTiO₃ in chitosan matrix by fabricating biocompatible piezopolymers were investigated on biological responses of human fibroblasts (HF) cells [217]. Hydroxylation of BaTiO₃ nanoparticles provided well distribution in chitosan polymer, thus high biocompatibility with HF cells were observed after 24 h of contact. Chitosan-hydroxylated BaTiO₃ composite polymer had 11.29 pC/N piezoelectric coefficient, d_{33} , which was higher than d_{33} in human skin (between 0.02 and 0.19 pC/N). High d_{33} value of this composite piezopolymer was due to the synergistic effect of piezoelectric BaTiO₃, chitosan and flexible features of films [217]. BaTiO₃,

graphene and poly-methylmethacrylate (PMMA) bone cement were mixed and piezoelectric and mechanic features and biological activity were investigated [218]. The more graphene concentration increased, the higher piezoelectric coefficient value was obtained. The prepared bio-piezoelectric composite had no any toxic effect and improved the cell morphology and promoted osteoblasts cell proliferation. It was observed that the cell number increased with increasing piezoelectric coefficient [218]. 3D printing of barium titanate ceramics was used as scaffold in bone tissue engineering application due to their biocompatible and piezoelectric property. They found that this 3D printed BTNP ceramic might be a good candidate for better tissue growth for providing an electrical stimulus for future medical applications [219].

The use of BNNTs owing to their piezoelectric properties in medical applications is very few and fairly new but promising research area in the literature. In a study, the osteoblast maturation has been examined by treating HOB cells with piezoelectric BNNT under low frequency ultrasound (US) stimulation [220]. They found that BNNTs have ability to promote the late-stage bone protein expressions in vitro without needing any mineralizing culture supplements. The cells were treated with BNNTs upon US stimulation have the highest osteopontin and osteocalcin expressions and Ca^{+2} production. Following, they introduced BNNTs in to C2C12 myoblasts cells on 2D culture conditions and 3D microfiber scaffolds under mechanical stress, e.g. using low frequency US [221]. Connexin 43 (Cx43) expression, which is an important molecule in cell crosstalk and mechanotransduction, was increased when BNNTs were interacted with cells in 3D samples. Also, compared to 3D model, BNNTs and US showed an additive effect on myosin synthesis. BNNTs were also used to prepare a piezoelectric film with poly(vinylidenedifluoride-trifluoroethylene) (PVDF-TrFE)) and their effect was investigated on human (SaOS-2) cells [222]. They demonstrated that even 1 percent BNNT addition to the polymeric matrix improved the piezoelectric properties and Young's modulus of the film compared to only polymeric matrix. Given the standard deviation of the angle created by the BNNT axis relative to the direction of piezoelectric measurement, previous studies have suggested that the BNNT distribution may be sufficient to motivate the observed piezo response even for a non-piezoelectric polymer such as polyimide [223]. However, the physical mechanism involved remains unclear [223], and the fact that BNNT

piezoelectricity itself is still not fully understood makes it very difficult to put anything forward at this time [224].



2. OBJECTIVE OF THE STUDY

One of the unique purposes of this study is to develop a novel piezoelectric composite, which include both BaTiO_3 and BNNT, to be used in biomedical applications. The precursors which are needed for BNNT synthesis will be mixed with barium (Ba) and titanium (Ti) compounds and during the synthesis same conditions are aimed to be applied. With this strategy the formation of BNNT and BaTiO_3 products, two powerful nanomaterials rich in piezoelectric properties, are expected to be obtained in same synthesis batch at the end of reaction which is called “composite”. With that approach, it is aimed to support the piezoelectric properties of BNNTs with the formation of BaTiO_3 because BaTiO_3 are known as strong piezoelectric materials. In addition to the increased piezoelectric property, it is predicted that cell adhesion and proliferation will increase with the formation of BNNTs, which are known to be highly effective in cell growth. A novel piezoelectric composite is expected to be created for bone tissue engineering application in this study.

The another goal is that piezo-composite scaffolds using PCL and chitosan polymers by separately embedding three piezoelectric nanomaterials including BaTiO_3 nanoparticles, BNNTs and the composite are aimed to be fabricated and physical, chemical and biological properties of these scaffolds will be investigated. When these composite scaffolds are mechanically activated (with low-frequency ultrasound (US)), the produced wireless electric-like signals by piezo materials will be aimed to contribute cell therapy and regeneration in bone tissue engineering. These piezo-composite scaffolds will be able to act as active patches to generate responses in electrically sensitive cells or tissues via a wireless strategy to decrease the conventional invasiveness of in vivo electric stimulation.

By applying US stimulation during cell therapy, cell responses under either proliferative or differentiative conditions will be investigated. Utilization of these piezo-composite scaffolds will contribute to wireless tissue stimulation studies using piezoelectric phenomena.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

- Colemanite ($\text{Ca}_2\text{B}_6\text{O}_{11} \cdot 5(\text{H}_2\text{O})$) (gift from Eti Mine Works General Management (Turkey))
- Barium titanate (BaTiO_3) (Sigma-Aldrich, Germany)
- Zinc oxide (ZnO) (Sigma-Aldrich, Germany)
- Iron oxide (Fe_2O_3) (Sigma-Aldrich, Germany)
- Titanium dioxide (TiO_2) (Sigma-Aldrich, Germany)
- Barium carbonate (BaCO_3) (Sigma-Aldrich, Germany)
- Silicon carbide (SiC) plate (Civelek Porselen, Turkey)
- Nitric acid (HNO_3)
- Formic acid (HCOOH)
- Hydrochloric acid (HCl) (36.5-38.0 percent)
- Acetic acid (CH_3COOH) (anhydrous, ≥ 99.0 percent) (Sigma-Aldrich, Germany)
- Ethanol ($\text{C}_2\text{H}_5\text{OH}$) (anhydrous, ≥ 99.5 percent) (Sigma-Aldrich, Germany)
- Ammonia solution ($\text{NH}_{3(\text{aq})}$) (Sigma-Aldrich, Germany)
- Ammoniac (gas) (Habaş, Turkey)
- Poly- ϵ -caprolactone (PCL) ($\text{Mn}=80000$) (Sigma-Aldrich, Germany)
- Chitosan (Sigma-Aldrich, Germany)
- Genipin ($\text{C}_{11}\text{H}_{14}\text{O}_5$) (Sigma-Aldrich, Germany)
- Triton X-100 ($\text{C}_{14}\text{H}_{22}\text{O}(\text{C}_2\text{H}_4\text{O})_n$ ($n=9-10$)) (Sigma-Aldrich, Germany)
- Sodium azide (NaN_3) (Sigma-Aldrich, Germany)
- Sodium hydroxide (NaOH) (Sigma-Aldrich, Germany)
- Glutaraldehyde ($\text{C}_5\text{H}_8\text{O}_2$) (Sigma-Aldrich, Germany)

3.1.2. Cell Lines

- Human osteoblast cells (HObS) (Cell line no. 406-05a) (European Collection of Cell Cultures, UK).

3.1.3. Cell Culture Reagents

- Dulbecco's Modified Eagle Medium (1x DMEM, 1000 mg/L Glucose, with sodium pyruvate, without Phenol red) (Gibco, USA)
- Fetal bovine serum (FBS) (Gibco, USA)
- Penicillin-Streptomycin (Gibco, USA)
- L-glutamine (Gibco, USA)
- 1x Trypsin-EDTA (0.25 percent Trypsin- 0.02 percent EDTA) (Gibco, USA)
- HyClone™ phosphate buffered saline (10x PBS) (Gibco, USA)
- Colchicine (≥ 95 percent HPLC purified, powder) (Sigma-Aldrich, Germany)
- Dimethyl sulfoxide (anhydrous, ≥ 99.9 percent) (Sigma-Aldrich, Germany)
- Tissue culture flasks, well plates, falcon tubes, serological pipettes and cryotubes (TPP, Switzerland)

3.1.4. Kits

- WST-8 cell proliferation reagent (Roche Diagnostic GmbH, CELLPRO-RO, Germany).
- Alkaline phosphatase colorimetric assay kit (Abcam, UK)
- von Kossa staining kit (Abcam, UK)
- Calcein (Invitrogen, USA).
- Propidium iodide solution (10 mg/ml in water) was purchased from (Sigma-Aldrich, Germany)

3.2. METHODS

3.2.1. Boron Nitride Nanotubes (BNNTs) Synthesis

BNNTs were synthesized in large scale by developing a novel method. A 1 g colemanite, 80 mg iron (III) oxide (Fe_2O_3) and 40 mg zinc oxide (ZnO) were mixed and ball-milled with 10 stainless-steel balls during 10 h at 300 rpm. After ball-milling process was finished 2 mL of 13.8 M ammonia (NH_3) solution was added and ball-milled (Fritsch, Pulverisette 7 premium line) again for 3 min at 300 rpm. The mixture was then put on a silicon carbide (SiC) plate (3 cm x 2 cm) and the $\text{NH}_{3(\text{aq})}$ was evaporated at 150°C on a heater. The plate was placed in to the tubular furnace (Protherm, Furnaces PTF 14/50/450) and temperature was set up as 1280°C . The reaction was carried out in the presence of NH_3 gas for 2 h. NH_3 gas was closed at 570°C and when the furnace was completely cooled down the synthesized BNNTs were collected from the SiC plate surface.

3.2.2. Composite (BaTiO_3 / BNNT) Synthesis

A 1 g colemanite, 80 mg Fe_2O_3 and 500 mg barium carbonate (BaCO_3) and 500 mg titanium dioxide (TiO_2) were mixed and ball-milled with 10 stainless-steel balls during 15 hours at 300 rpm. After ball-milling (process was finished 2 mL of 13.8 M NH_3 solution was added and ball-milled again for 3 min at 300 rpm. The mixture was then put on a SiC plate (3 cm x 2 cm) and the $\text{NH}_{3(\text{aq})}$ was evaporated at 150°C on a heater. The plate was placed in to the tubular furnace and temperature was set up as 1300°C . The reaction was carried out in the presence of NH_3 gas for 3 h. NH_3 gas was closed at 975°C and when the furnace was completely cooled down the synthesized composite was collected from the SiC plate surface.

3.2.3. Purification of BNNT and Composite

The BNNT reaction product was taken up in 50 mL, 4 M HCl, stirred for 4 hours at 90°C and centrifuged at 4000 rpm for 20 min. The pellet was then dispersed and stirred in 50 mL, 1 M HNO_3 for 6 hours at 30°C and then centrifuged at 4000 rpm for 20 min. The

precipitated pellet was washed with deionized water until the acid was completely removed and dried overnight at 60°C in the vacuum oven.

The composite were mixed with 50 mL, 2 M formic acid and stirred during 8 h at 50°C [225]. The mixture centrifuged at 4000 rpm for 20 min and precipitated pellet was washed with deionized water until the acid was completely removed and dried overnight at 60°C in the vacuum oven.

3.2.3.1. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Analyses were performed using X Series 2 ICP-MS (Thermo Scientific) device and CETAC asx-520 autosampler. In the ICP-MS system, plasma power was set as 1350 W, plasma gas Ar, Nebulizer gas flow rate 0.95, grasping time 35 seconds and washing time 35 seconds. VHG Labs Z frequency 1007-100 multi-element standard stock solution containing (1000 µg / ml) B, Na, Mg, Al, Fe, Sr, Zn, Ca was used in the calibration. By using this stock solution, standards were prepared at concentrations of 0.1 µg/mL, 1 µg/mL, 10 µg/mL, and 100 µg/mL in 5 percent HNO₃. Calibration curves were created for each metal. 7-60 mg of the sample to be analyzed for ICP-MS was taken and boiled in 3 mL HNO₃: HCl (2:1 v/v) for 15 minutes and sonicated. The final volume is completed to 20 mL with pure water. After the samples were digested in this way, they were filtered through a 0.2 µm Millipore filter and ICP-MS analyses were performed (margins of error are given as ± standard deviation, n = 3).

3.2.4. Characterization of the BaTiO₃, BNNT, and Composite

3.2.4.1. Scanning Electron Microscopy (SEM)

Nanomaterials were pasted on two sided sticky carbon disc. Before measurement nanomaterials were coated with Au/Pt during 10 seconds via BALTEC SCD005 sputter and then SEM images were taken using Zeiss Evo40.

3.2.4.2. Transmission Electron Microscopy (TEM)

Nanomaterials were analyzed using FEI TALOS F200S TEM which can be operated in the range of 20-200 kV.

3.2.4.3. Atomic Force Microscopy (AFM)

Approximately 1 mg each of nanomaterials were dispersed in deionized water (ddH₂O) then dropped on a glass slide and AFM analysis were performed using Park System XE-100.

3.2.4.4. Fourier Transform Infrared Spectroscopy (FT-IR)

All nanomaterials were scanned 16 times with a resolution of 4 cm⁻¹, in the region of 400-4000 cm⁻¹ by using Thermo NICOLET IS50.

3.2.4.5. X-Ray Diffraction (XRD)

A PANalytical Empyrean XRD were used to characterize the nanomaterials in continuous mode with a scanning range of 10.0131° and 89.9891°.

3.2.4.6. Piezoresponse Force Microscopy (PFM)

Piezoelectric properties of BaTiO₃, BNNTs and the composite were analyzed using NX10 AFM system with a soft PPP-EFM cantilever coated with conductive PtIr. Spring constant (k) was 2.8 N/m, resonance frequency was 75 kHz and tip radius was under 10 nm. PFM was used in PinPoint mode (point-by-point force curves, PFM signal collected at lower turning point). Powdered samples were pasted on mica and then analyzed with a 5*5 μm scan size, 256*256 px resolution, 0.05-0.03 Hz scan rate and 76-60 nN nm setpoint. PFM excitation was 17 kHz, 5 V for BaTiO₃, 17 kHz, 8 V for BNNTs and 17 kHz, 5 V for the composite.

3.2.5. Cell Culture Experiments of BaTiO₃, BNNT, and Composite

Human osteoblast cell (HOb) line was used for the evaluation of cell proliferation and adhesion experiments on scaffolds. HOb cells were cultured in Low-Glucose DMEM medium without phenol red which contains 10 percent fetal bovine serum (FBS) and 1 percent Penicillin Streptomycin (PS) and 1 percent L-glutamine. Cells were incubated at 37°C with 5 percent CO₂ and 90 percent humidity.

3.2.5.1. Cytotoxicity Assay of BaTiO₃, BNNTs, and Composite

The cytotoxicity assay was performed using WST-8 colorimetric assay. 5×10^3 cells/well were seeded in 96 well plates. After 24 h incubation, cells were treated with BaTiO₃, BNNTs and composite at increasing concentrations (10-1000 $\mu\text{g/mL}$) for 24 h. After 24 h incubation, the nanomaterial containing medium was removed and cells were washed with phosphate buffer (PBS, pH 7.4) and then 100 μL , 5 percent of WST-8 solution was added to the each well and leaved for 2 h incubation at 37°C, the cell viability was then identified by measuring formazan salts at 450 nm via a micro plate reader (ELx808, BioTek, Winooski, VT, USA).

3.2.5.2. Cellular Uptake of BaTiO₃, BNNTs, and Composite

HOB cells were seeded in 24 well plates as 4×10^4 cells/well. After 24 h incubation, cells were treated with BaTiO₃, BNNTs and composite at increasing concentrations and incubated for 24 h. Cells were collected with 200 μL phenol red free trypsin. After centrifugation, cells were mixed with 200 μL PBS to analyze cellular internalization with flow cytometry (Guava® easyCyte Flow Cytometry System).

3.2.6. Preparation of Poly Caprolactone (PCL) and Chitosan Scaffolds

3.2.6.1. Preparation of Poly Caprolactone (PCL) Scaffold with Electrospinning

20 percent w/v PCL was dissolved in acetic acid by stirring on a magnetic stirrer. After PCL was completely dissolved, the viscous solution was put into 10 mL injector. Flow rate was 0.4 mL/h and applied voltage was 18 kV. Distance between collector and needle was 15 cm.

To prepare nanomaterial embedded PCL scaffolds, firstly, BaTiO₃, BNNT, and composite were separately dispersed in acetic acid at a concentration of 500 $\mu\text{g/mL}$ and sonicated during 10 minutes and then 20 percent w/v PCL was added in to solution as indicated above and same conditions were applied. The schematic representation of the electrospinning setup was given in Figure 3.1.

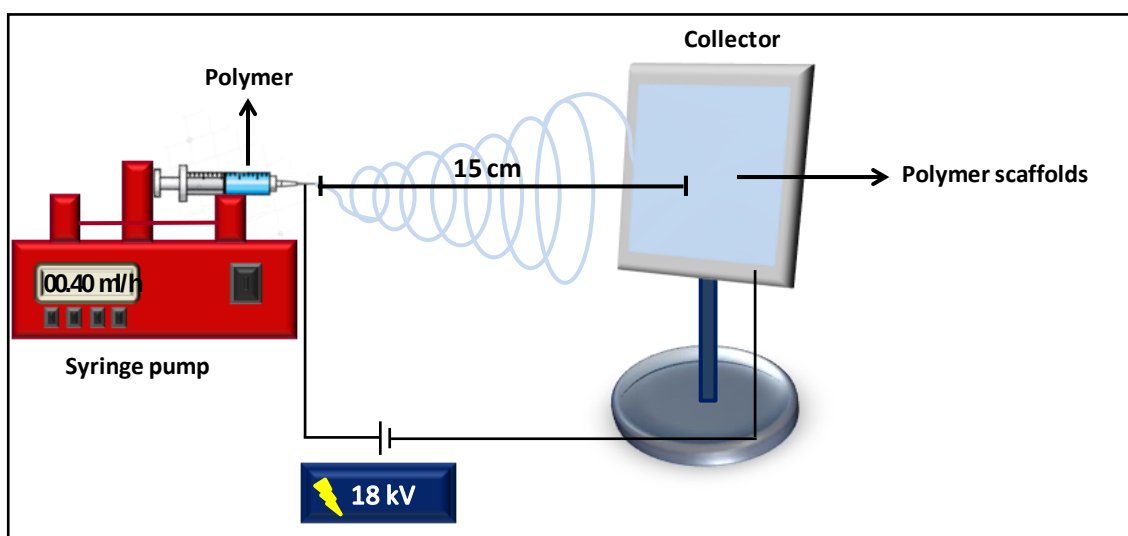


Figure 3.1. Schematic illustration of electrospinning setup

3.2.6.2. Preparation of Chitosan Scaffolds with Freeze Dryer

0,2 g chitosan were dissolved in 10 mL, 10 percent acetic acid at 40°C with 1000 rpm stirring rate. After chitosan was completely dissolved 200 μ L, 0,5 percent genipin solution in ethanol was added and waited for 3 h for crosslinking [79].

To prepare nanomaterial embedded chitosan scaffolds BaTiO₃, BNNT, and composite were firstly dispersed in 10 mL, 10 percent acetic acid solution during 10 minutes at a concentration of 500 μ g/mL and then 0.2 g chitosan were added to each with and stirred with 1000 rpm stirring rate. After chitosan was completely dissolved 200 μ L, 0,5 percent genipin solution in ethanol was added and waited for 3 h for crosslinking. 2 mL/well of chitosan or chitosan/nanomaterial mixture were put into 24 well plate. Samples were incubated at -80°C for 24 h and then lyophilized for 24 h.

3.2.7. Characterization of PCL and Chitosan Scaffolds

3.2.7.1. Scanning Electron Microscopy (SEM) Analysis

All scaffolds were coated with Au/Pd with a thickness of 15 nm before taking SEM images. At least thirty different fiber diameters for each PCL scaffolds and thirty different

pore sizes for each chitosan scaffolds were measured using their SEM images. Their average values were calculated and diameter and pore size bar graphs were plotted.

3.2.7.2. Determination of BaTiO₃, BNNT, and Composite Distribution in PCL and Chitosan Scaffolds by Raman Spectroscopy

PCL scaffolds within about 100 μm thickness were cut as 1 cm x 1 cm and chitosan scaffolds that have 1 cm diameter and nearly 500 μm heights were prepared. Each scaffold was pasted on a calcium fluoride (CaF₂) slide with two sided carbon sticker before measurement. A Renishaw inVia Reflex Raman spectrometer fitted with a high speed encoded stage (streamlineTM) and a Leica DM2500 upright microscope were used. Measurements were carried out using a photodiode laser beam (830 nm) with 1200 line/mm grating. 50x μm long distance objective lens was used to obtain optimal signal collection. Each objective lens differ from their collection competence and laser spot size at the focus point owing to their differences in numerical aperture (N.A) and chosen laser wavelength. Since, higher magnification objectives provide higher spatial resolution due to having higher numerical aperture that indicates the resolving power of lenses, which is 0.5 for 50x objective lens. The laser spot diameter of 50x μm objective lens was calculated using the $1.22 \lambda / \text{N.A}$ formula [226]. Laser spot diameter was calculated as 2 μm , which means that laser hit on a point of sample with about 2 μm area. Spectral data were collected from three different area of one type of scaffold with the size volume of 20 μm x 20 μm x 20 μm with a 2 μm step size. Laser power was beamed at 150 mW power with ten seconds exposure time for PCL scaffolds and two seconds for chitosan scaffolds. That means 1331 spectra were collected from one area of a sample to create a Raman image, which took about four hours and that step was repeated three times for a sample. Each spectrum was processed for baseline correction, cosmic ray removal and smoothing via Wire 4.2 software. Then, a Raman image was visualized at one characteristic peak of nanomaterials using 1331 processed spectra. 1331 spectra were then averaged. The representative average spectra of the scaffolds were obtained by averaging all 1331 spectra as one.

3.2.7.3. Mechanical Testing

Tensile Measurement of PCL Scaffolds

The tensile strength of PCL and BaTiO₃, BNNT, and composite embedded scaffolds were measured using Instron 3382 tensile tester (Instron Corporation, Canton, MA) at room temperature. The scaffolds were cut with the size of 2 cm x 1 cm (length x width) in rectangular shape before the measurement and each scaffold had 200 μ m thickness. The crosshead speed was 2.5 mm/min and load cell was 10 kN. Three samples were measured for each type of scaffolds and their average values were graphed.

Compressive Measurements of Chitosan Scaffolds

The compressive strength of chitosan and BaTiO₃, BNNT, and composite embedded chitosan scaffolds were determined using Instron 3382 tensile tester (Instron Corporation, Canton, MA) at room temperature. Cylindrical samples were prepared approximately within the size 9 mm x 14 mm (height x diameter) and were faced to compressive load with a 1 mm/min crosshead speed by using 100 N load cell. Three samples were tested for each types of scaffold.

3.2.7.4. Dynamic Contact Angle Measurements of PCL Scaffolds with Water

The dynamic water contact angle measurements were evaluated by using a Hamilton syringe and a KSV CAM 101 surface tension meter. As soon as a droplet of water was released onto the scaffolds, a camera at the opposite side of droplet took pictures at certain time intervals and recorded them. Contact angle of the droplet with the surface were then measured. For PCL scaffolds contact angle value at 60th second were used for comparison of hydrophobicity of the PCL scaffolds.

3.2.7.5. Determination of Equilibrium Water Content of Chitosan Scaffolds

Three samples from each scaffold were weighed into falcon tubes separately and recorded as W_{dry} after all scaffolds were dried at 37°C in a vacuum etuv. A 5mL of 1X PBS (pH=7.4) was introduced to the samples and allowed to swell during 24 hours at 37°C. Another set of samples were stimulated three times a day with a three-second with ultrasound (US) via a sonicator. As soon as the samples were removed from the PBS solution, excessive surface water was removed using a filter paper. Scaffolds were then

weighed and recorded as W_{wet} . Water content amounts and swelling ratio of the scaffolds were calculated using the following equations;

$$\text{Water content (percent)} = [(W_{\text{wet}} - W_{\text{dry}}) / W_{\text{wet}}] \times 100 \quad (2.1)$$

$$\text{Swelling Ratio (percent)} = [(W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}}] \times 100 \quad (2.2)$$

3.2.7.6. *In Vitro* Degradation

The examination of biodegradation rate of the PCL and chitosan scaffolds were accomplished by both weight and pH change measurements in PBS solution (pH= 7.4) at 37°C.

For the weight loss measurements, PCL and chitosan scaffolds were firstly weighed (W_0) and put into individual falcon tubes. Three samples were weighed from each type of scaffolds. A 5 mL of 0.9 percent containing 1X PBS solution (pH= 7.4) were added on to scaffolds and tubes were put into a shaking incubator at 37°C and 100 rpm. A group of scaffolds were also exposure to ultrasound (US) using a sonicator bath during nine seconds in one day. After 7, 15, 30, 45, 60, 75, 90 and 120 days of incubation, PBS solutions were removed and scaffolds were washed with ddH₂O three times. Scaffolds were dried in a vacuum etuv at 37°C during 24 h and then weighed (W_r). Weight loss percentages (W_L) of the scaffolds were calculated using the following equation [227-229];

$$W_L \text{ percent} = [(W_0 - W_r) / W_0] \times 100 \quad (2.3)$$

3.2.8. *In Vitro* Cell Interaction Studies of PCL and Chitosan Scaffolds

Human osteoblast cell (HOb) line was used for the evaluation of cell proliferation and adhesion experiments on scaffolds. HOb cells were cultured in Low-Glucose DMEM medium without phenol red which contains 10 percent fetal bovine serum (FBS) and 1 percent Penicillin Streptomycin (PS) and 1 percent L-glutamine. Cells were incubated at 37°C with 5 percent CO₂ and 90 percent humidity.

PCL (1x1 cm) and chitosan scaffolds were sterilized using 70 percent ethanol and incubated for 3 h at room temperature. Scaffolds were then washed three times with PBS solution by incubating for 5 minutes in every washing step. The scaffolds were then

incubated in cell culture medium for 24 h at 37°C. After medium was removed, 500 µL, 5×10^4 cells/well were seeded on scaffolds which were located in 24 well plates (low attachment) and incubated throughout 28 days at the CO₂ in a humidified incubator. The medium was refreshed in every three days. US stimulation was performed at 40 kHz frequency for 3 s three times a day for 3 seconds for one US stimulation during 21 days with 20 W output power.

3.2.8.1. Cell Proliferation Assay (WST-8)

WST-8 cell proliferation assay was performed for PCL scaffolds. At the end of incubation times (3, 7, 14 and 21 days) cell culture medium was aspirated and 500 µL, 5 percent of WST-8 solution in Low-Glucose DMEM was added to the each well and leaved for 2 h incubation at 37°C, the cell viability was then identified by measuring formazan salts at 450 nm via ELISA plate reader. To calculate alive cell numbers on each scaffold, a calibration curve was plotted as absorbance vs cell number.

3.2.8.2. Live/Dead Cell Viability Assays of Scaffolds

After determined time intervals (7, 14 and 21 days), cell culture medium was aspirated and cell seeded chitosan and PCL scaffolds were washed three times with 1X PBS. 0.2 µM calcein and 0.6 µM propidium iodide (PI) together were prepared in 1X PBS solution. The dye solution were then added on to cell seeded scaffolds and incubated for 20 min at 37°C. The dye solution was then aspirated and scaffolds were washed with 1X PBS for three times in order to remove residual dyes and waited in PBS solution until taking fluorescence image. The fluorescence images were obtained with a Zeiss AxioCam HR microscope.

3.2.8.3. Scanning Electron Microscopy Images of Cell Seeded Scaffolds

For the scanning electron microscopy (SEM) analysis of the scaffolds at the determined time intervals (7, 14, 21, 28 days), fixation process was applied using 2.5 percent glutaraldehyde solution. After waiting for 30 minutes at +4°C in glutaraldehyde solution, scaffolds were washed with 50 percent, 70 percent, 90 percent and 100 percent ethanol solution respectively for 5 minutes at room temperature. The scaffolds were then dried at 37°C for 3 hours and analyzed with SEM after coating with gold/platinum at 30 nm thickness.

3.2.8.4. Determination of Mineralization Using Von Kossa Staining

After determined time intervals, cell culture medium was removed and scaffolds were incubated in 5 percent silver nitrate solution for 60 minutes with exposure to ultraviolet light (Abcam Von Kossa Calcium Stain Kit). The scaffolds were then rinsed in several changes of distilled water and incubated in 5 percent sodium thiosulfate solution for 3 minutes to stop reaction. After incubation, scaffolds were rinsed with running tap water followed by 2 changes of distilled water. Then, the plates incubated in nuclear fast red solution for 5 minutes and washed with tap water followed by 2 changes of distilled water. Scaffolds were quickly dehydrated with absolute ethanol by changing 3 times. Finally, the digital images of scaffolds were taken for observation.

3.2.8.5. Alkaline Phosphatase (ALP) Activity

PCL and chitosan scaffolds were sterilized using 70 percent ethanol and incubated for 3h at room temperature. Scaffolds were then washed three times with PBS solution by incubating for 5 minutes in every washing step. The scaffolds were then incubated in cell culture medium for 24 h at 37°C. After medium was removed, 500 μ L, 5×10^4 cells/well were seeded on scaffolds which were located in 24 well plates (low attachment) and incubated for 7, 14, 21 and 28 days at the CO₂ in a humidified incubator. The medium was refreshed in every three days. Ultrasound stimulation (US) was applied 3 times a day for 3 seconds for one US stimulation.

Cell culture media were aspirated after determined time intervals (7, 14, 21, 28 days). Cell seeded scaffolds were washed twice with PBS. A 200 μ L Tris-Triton solution (0.1 percent Triton-X-100 in 0.1 M Tris buffer) were added into each wells and waited for cells to move from the surface and burst. Tris-Triton solutions were collected into eppendorfs (with scaffolds) and added another 200 μ L and then another 100 μ L of Tris-Triton solution. Scaffolds were crushed with the help of pipette tip. Samples were freezed at -80°C for 10 minutes and thawed +37°C for 10 minutes. This freeze-thaw cycle were repeated three times. Each sample was sonicated for 10 minutes with 30 second breaks at each minute and the centrifuged at 2000 rpm for 10 minutes. Supernatants were taken and kept them in ice-cold. The continuation of experiment was practiced using a colorimetric ALP assay kit (Abcam, United Kingdom) regarding to kit directions. Firstly, standard curve dilutions were prepared according to the instructions indicated in Table 3.1. 120 μ L

standard dilutions were transferred into 96 well plates from each dilution. Each dilution has adequate amount of standard for duplicate readings. 80 μ L were transferred to sample wells and also to background wells. 20 μ L stop solution were added to background control samples and mixed by pipetting up and down to terminate ALP activity in these samples. 50 μ L, 5 mM p-nitrophenyl phosphate (pNPP) solution were added to each sample wells and background sample controls. 10 μ L ALP enzyme solutions were added to standard well and mixed by pipetting up and down. The plate was incubated at 25°C for 60 minutes protected from light. pNPP substrate will convert to an equal amount of colored p-Nitrophenol (pNP) in the presence of ALP enzyme. After incubation, 20 μ L stop solution were added to sample wells and background control wells to stop reaction. Plate was gently shaken and measured at 405 nm via a micro plate reader.

Table 3.1. Preparation of standard dilutions

Standard	pNPP 1mM standard	Assay buffer (μ L)	Final volume standard in well (μ L)	End amount pNPP in well (nmol/well)
1	0	300	120	0
2	10	290	120	4
3	20	280	120	8
4	30	270	120	12
5	40	260	120	16
6	50	250	120	20

A standard curve is generated by plotting pNP (nmol) versus O.D. (405 nm). The trend line equation was calculated based on the standard curve data. ALP activity was calculated using the $ALP = B / (\Delta T \times V) \times D$ equation where B is the amount of pNP in sample well calculated from standard curve (μ mol), ΔT is reaction time (minutes), V is volume added into the reaction well (mL), D is sample dilution factor.

4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF BARIUM TITANATE (BaTiO_3), BORON NITRIDE NANOTUBE (BNNT) AND COMPOSITE (BNNT/ BaTiO_3) STRUCTURES

4.1.1. Scanning Electron, Transmission Electron and Atomic Force Microscopy Analysis

4.1.1.1. Characterization of BaTiO_3 , by Scanning Electron and Atomic Force Microscopes

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to determine size and shape characteristics of purchased BaTiO_3 . BaTiO_3 nanoparticles in the form of white powder were pasted on a carbon disc and analyzed with SEM after Au/Pt coating for 10 seconds. Size of BaTiO_3 nanoparticles indicated as 50 nm ranged from $1\text{ }\mu\text{m}$ to $200\text{ }\mu\text{m}$ as illustrated in SEM images in Figure 4.1a. This increase was due to tend to aggregate of BaTiO_3 nanoparticles when they were solid. However, after they were dispersed in deionized water by sonicating during 10 sec, they were dropped on a glass slide and analyzed with AFM. The size of BaTiO_3 nanoparticles were observed under 200 nm and their shapes were spherical as presented in AFM image (Figure 4.1b.).

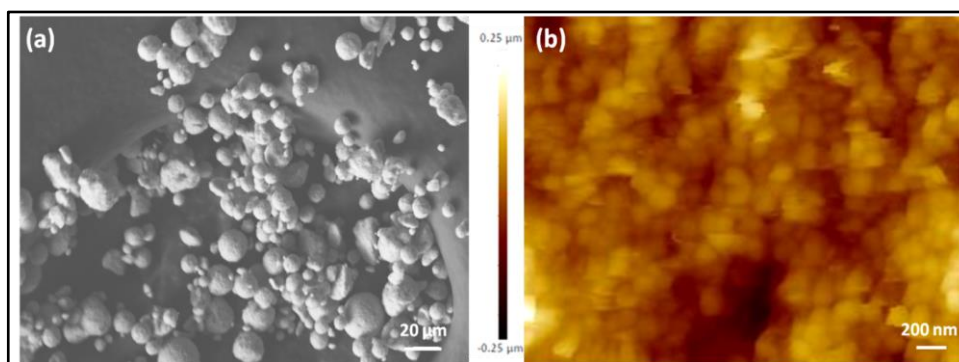


Figure 4.1. Characterization of BaTiO_3 with (a) SEM and (b) AFM

4.1.1.2. Characterization of BNNT by Scanning Electron, Transmission Electron and Atomic Force Microscopes

The BNNTs were synthesized using chemical vapor deposition (CVD) technique as illustrated in Figure 4.2. After 10 h ball milling process, the precursor on silicon carbide plate (SiC) was placed into tubular furnace and BNNTs were synthesized at 1280°C for 2 h in the presence of $\text{NH}_3(\text{g})$. The reaction was terminated at 570°C to avoid the movement of formed BNNT on the SiC surface with NH_3 gas. The white powder was collected and BNNTs were purified using acid solution as indicated method part 3.2.3.

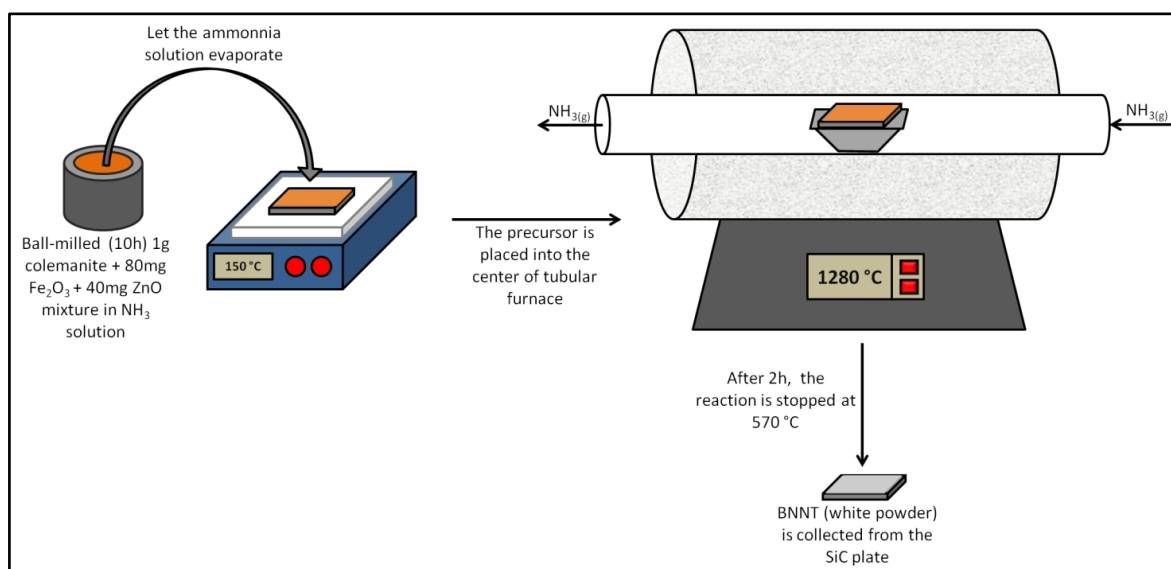


Figure 4.2. The schematic illustration of BNNT synthesis steps

One of the most desired goals in BNNT synthesis is to obtain them in high yield at one-synthesis. For that purpose different kinds of catalysts, reaction temperature and duration, ball-milling process before the synthesis are the parameters that are needed to be optimized for high yield BNNT synthesis. Most of these conditions were optimized and evaluated in our previous study [146]. However, the yield was not high enough even at these optimum conditions. In our published study, to obtain high yield BNNTs, colemanite ($2\text{CaO} \cdot 3\text{B}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) used as boron source, and Fe_2O_3 as catalyst were mixed at 12:1 (w/w) rate and were synthesized at 1280°C for 2 h in the presence of NH_3 gas. In addition to these optimization results, it had been focused on boron sources and catalyst types in this study. First, BNNTs were synthesized using $\text{H}_3\text{BO}_3 + \text{B}_2\text{O}_3$, $\text{H}_3\text{BO}_3 + \text{B}_2\text{O}_3 + \text{colemanite}$ precursors

in the presence of Fe_2O_3 catalyst at 1280°C in the presence of NH_3 gas for 2 h. When $\text{H}_3\text{BO}_3+\text{B}_2\text{O}_3$ were used as boron source, BNNT formation was observed but the yield was very low as shown in Figure 4.3a. However, $\text{H}_3\text{BO}_3+\text{B}_2\text{O}_3$ +colemanite precursors with Fe_2O_3 catalyst increased the rate of BNNT formation as seen in Figure 4.3b. It has been demonstrated that metal oxides and other impurities in colemanite enhanced the formation of BNNTs. Though, the diameter of BNNTs was very high ranging from 300 nm-1.5 μm . The effect of Fe_2O_3 , Al_2O_3 and ZnO catalysts on BNNT formation were evaluated separately when colemanite was used as boron source in our previous study. Fe_2O_3 was found the most effective catalyst compared to their BNNT formation yields. In this study, colemanite and two catalysts mixtures which were colemanite+ $(\text{Fe}_2\text{O}_3+\text{Al}_2\text{O}_3)$, colemanite+ $(\text{Fe}_2\text{O}_3+\text{MgO})$ and colemanite+ $(\text{Fe}_2\text{O}_3+\text{ZnO})$ were suggested to use as precursors for BNNT synthesis. The SEM images showed that when two catalysts were used, the BNNTs formation was increased (Figure 4.3c., 4.3d., 4.3e.). However, the best yield of BNNT was obtained when $\text{Fe}_2\text{O}_3+\text{ZnO}$ catalysts were used and also the size of the BNNTs was homogenous and smaller compared to the BNNTs when $\text{Fe}_2\text{O}_3+\text{Al}_2\text{O}_3$ and $\text{Fe}_2\text{O}_3+\text{MgO}$ were used (Figure 4.3e.). While the shorter ball milling time (5 h) caused the diameter ranging from 500 nm – 1 μm (Figure 4.3e.), the longer ball milling time (10 h) decrease the diameter of BNNTs under 500 nm (Figure 4.3f.). It had been reported that ball milling process enhanced the yield of BNNT formation [230]. Ball milling process increased the surface area of boron source and catalyst and, the boron source, catalyst and nitrogen source interacted as much as possible during the reaction.

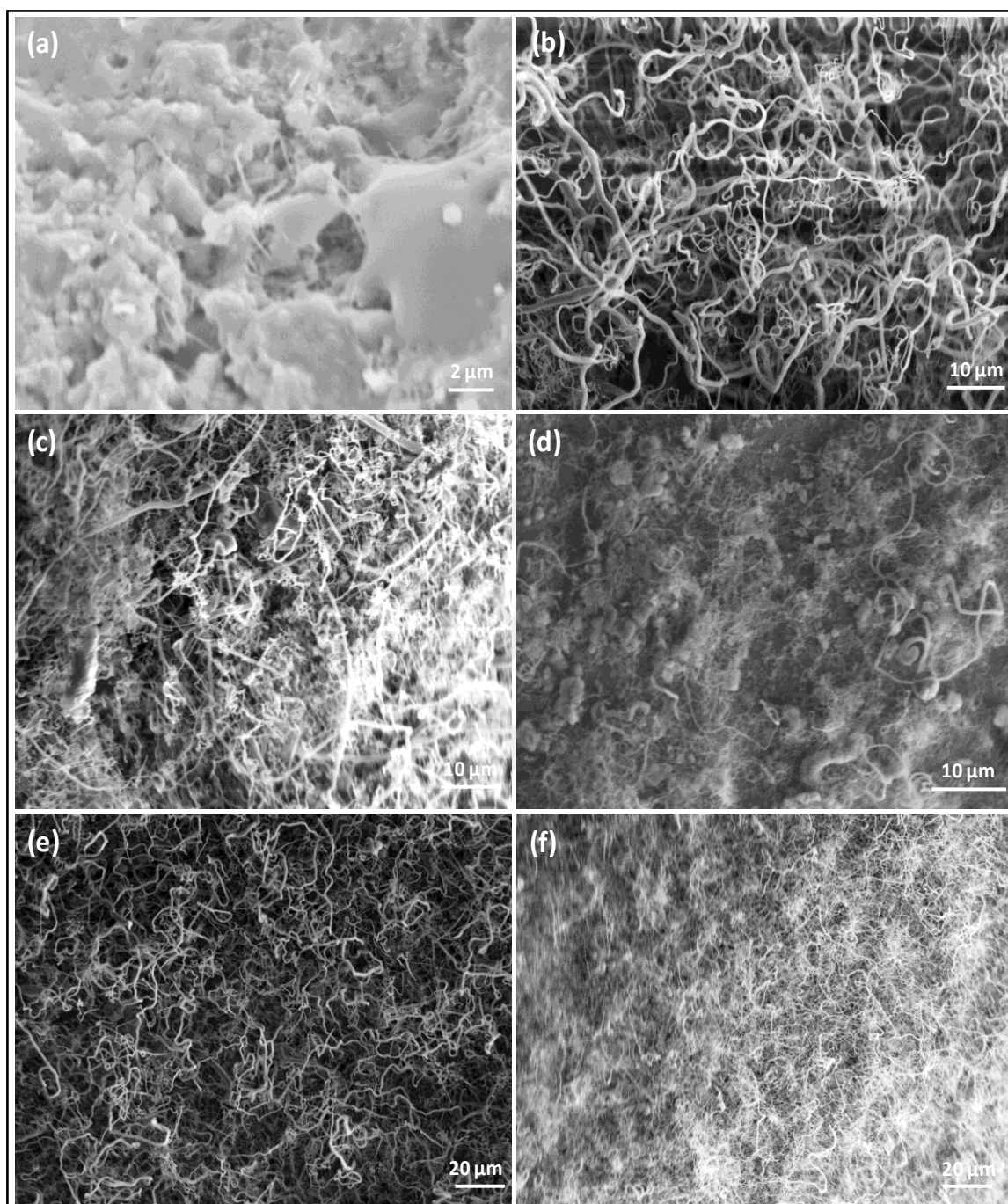
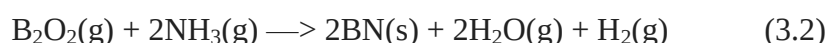
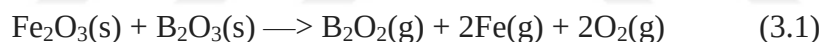


Figure 4.3. SEM images of BNNTs synthesized using precursors; (a) $\text{H}_3\text{BO}_3 + \text{B}_2\text{O}_3 + \text{Fe}_2\text{O}_3$, (b) $\text{H}_3\text{BO}_3 + \text{B}_2\text{O}_3 + \text{colemantite} + \text{Fe}_2\text{O}_3$, (c) $\text{colemantite} + \text{Fe}_2\text{O}_3 + \text{Al}_2\text{O}_3$, (d) $\text{colemantite} + \text{Fe}_2\text{O}_3 + \text{MgO}$, (e) $\text{colemantite} + \text{Fe}_2\text{O}_3 + \text{ZnO}$ (5h ball-milled), (f) $\text{colemantite} + \text{Fe}_2\text{O}_3 + \text{ZnO}$ (10 h ball-milled)

The formation of BNNT was observed with SEM and the sizes of BNNTs were observed under 200 nm and the sizes of all BNNTs were seen nearly homogeneous in SEM images

as illustrated in Figure 4.4a. and 4.4b. BNNTs were in multi-walled form and had cylindrical shape as seen in TEM image in Figure 4.4c. and 4.4d. However, the resolution of TEM was not enough to see how many walls a BNNT had and to measure the distance between the walls. The diameter of BNNTs was approximately measured as 50-100 nm. The growth mechanism of BNNTs might be similar to carbon nanotubes (CNTs) growth model but with a different catalytic route. Catalytic CVD growth on transition metal supported powder such as titanium oxide (TiO₂), aluminum oxide (Al₂O₃), silicon dioxide (SiO₂) and magnesium oxide (MgO) is regarded as an efficient approach to obtain high quality CNTs [231-234]. The catalytic CVD technique is an effective way to achieve high-yield and large-scale BNNT synthesis, as with CNTs [235]. Chemical reaction of the BNNT formation in the presence of catalyst and ammonia gas was represented in equation 3.1. and 3.2. In the first reaction solid catalyst and boron source evaporated when the temperature increases. BN nuclei germinate on the surface of the Fe catalyst at B and N supersaturation as indicated in equation 3.2. Open-ended BNNTs are elongated by continuous B and N support on the catalyst surface and BNNT formation is completed.



According to the mechanism which was proposed for CVD synthesis in this study, firstly a certain size of metallic Fe and Zn catalyst is formed from Fe₂O₃ and ZnO. This initial formation step of the metallic catalyst is the most important step in BNNT synthesis. After the metallic Fe/Zn catalyst is formed, the second step is the formation of the B-N starting complex on the catalyst surface. NH₃ gas which can be broken down radically at 1280 °C, and also B is required in the formation of this starting complex. BN nuclei germinate on the surface of the Fe/Zn catalyst at B and N super-saturation. Open-ended BNNTs are elongated with continuous B and N support on the catalyst surface as seen in bottom of AFM image in Figure 4.4e. and BNNT formation has been completed. AFM image of BNNTs, also informed us about the length of BNNTs, which was above 2 μm.

In this synthesis method, 300 mg BNNTs could be obtained from 1 g colemanite. Theoretically, 372 mg boron nitride (BN) is obtained from 1 g colemanite. Therefore, the

yield of formed BNNTs was found as 80.6 percent. Obtaining of high-yield BNNTs may attributed to catalyst hybrid powders as also reported in the literature [235].

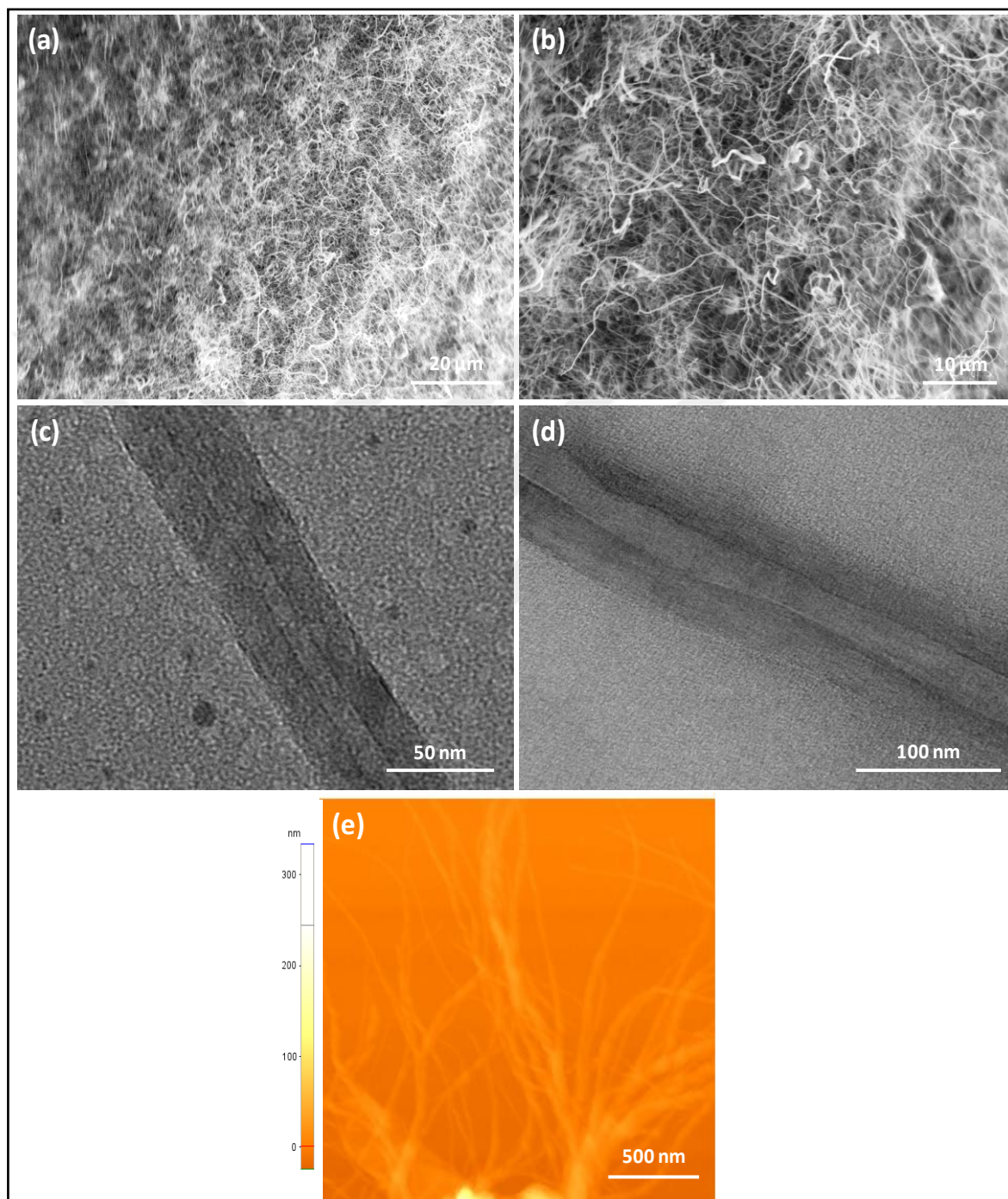


Figure 4.4. Characterization of BNNTs with (a, b) SEM, (c, d) TEM and (e) AFM

4.1.1.3. Characterization of Composite by Scanning Electron, Transmission Electron and Atomic Force Microscopes

One of the objectives of this thesis study was to develop a composite that have higher piezoelectric properties than only BaTiO_3 and BNNT. This idea was developed based on the ability to synthesize these two nanomaterials under similar conditions such as ball milling process before synthesis and annealing at high temperatures [146,225]. These two materials, BNNT and BaTiO_3 which are rich in piezoelectric properties were successfully synthesized on the same synthesis SiC plate with a CVD technique, which was the name called as “composite”. A certain amount of precursors which were needed for BNNT synthesis, colemanite and Fe_2O_3 catalyst, were mixed with barium carbonate (BaCO_3) and titanium dioxide (TiO_2) compounds and synthesized with a CVD technique as depicted in Figure 4.5. After 15 h ball milling process to reduce the size of the precursors and to increase their specific surface area and stored energy, the mixture in NH_3 solution was put on a SiC plate and then placed in to the center of tubular furnace. The reaction was carried out at 1300°C for 3h in the presence of NH_3 gas. The NH_3 gas flow was then terminated at 975°C to increase BaTiO_3 formations and the composite was collected from the SiC plate.

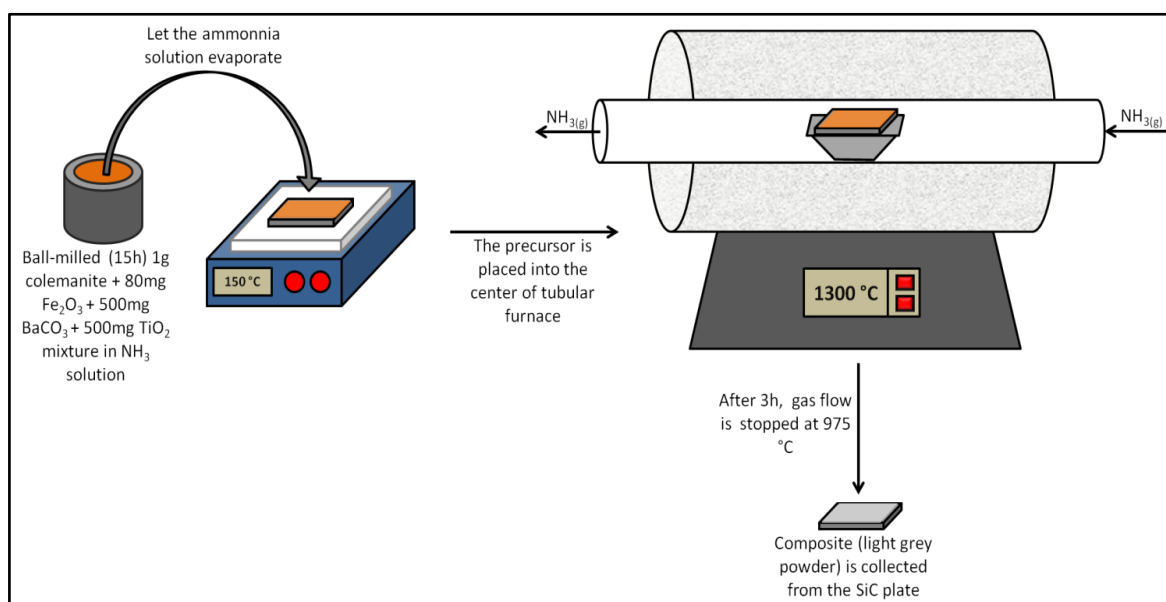


Figure 4.5. Schematic illustration of the composite synthesis steps and reaction conditions

The formation of the composite was first examined using SEM (Figure 4.6.). When the amount of colemanite and catalyst were same in the previous BNNT synthesis study (1 g colemanite : 80 mg Fe_2O_3) and the varying amounts of BaCO_3 and TiO_2 were used. While the amount of BaCO_3 and TiO_2 is 100 mg, BaTiO_3 like structures were mostly observed, however, there was not observed any BNNT formation (Figure 4.6a.). However, the amount of BaCO_3 and TiO_2 was increased up to 250 mg, BNNT structures were started to observe (Figure 4.6b.). When the amount of BaCO_3 and TiO_2 was used as half amount of colemanite, much more BNNT structures were obtained (Figure 4.6c.). BaTiO_3 formations were also slightly observed at the bottom of BNNT structures. The increasing amount of BaCO_3 and TiO_2 probably lead to an increase in BNNT formations due to their catalyst effect in BNNT growth. However, when BaCO_3 and TiO_2 amounts were equal to colemanite, BaTiO_3 structures were only observed (Figure 4.6d.). The rate of formed BaTiO_3 and BNNT in the composite will possibly affect the piezoelectric properties of the material. The composite which were synthesized from 1 g colemanite, 80 mg Fe_2O_3 , 500 mg BaCO_3 and 500 mg TiO_2 were decided as the most appropriate precursor rate based on their SEM results.

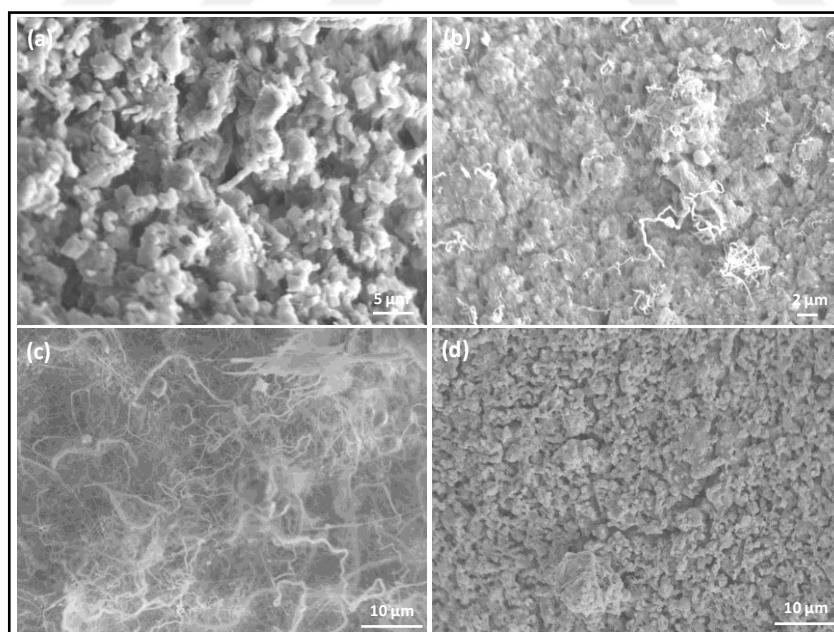


Figure 4.6. SEM images of (a) 1 g colemanite+ Fe_2O_3 +100 mg BaCO_3 +100 mg TiO_2 , (b) 1 g colemanite+ Fe_2O_3 +250 mg BaCO_3 +250 mg TiO_2 , (c) 1 g colemanite+ Fe_2O_3 +500 mg BaCO_3 +500 mg TiO_2 , (d) 1 g colemanite+ Fe_2O_3 +1 g BaCO_3 +1 g TiO_2

Although BNNTs were dominant in SEM images of the composite as seen in Figure 4.7a. and 4.7b., BaTiO₃, BNNTs and also hBN structures were clearly observed in TEM images (Figure 4.7c., 4.7d.). The shapes of BaTiO₃ were observed in cubic structure in TEM images. In SEM images taken from solid sample of composite, the BaTiO₃ structures were not clearly seen due to intense amount of BNNT formation on the surface. The composite was dispersed in ddH₂O and dropped on a glass slide, dried. By this way, BaTiO₃ structures could be examined using AFM (Figure 4.7e.)

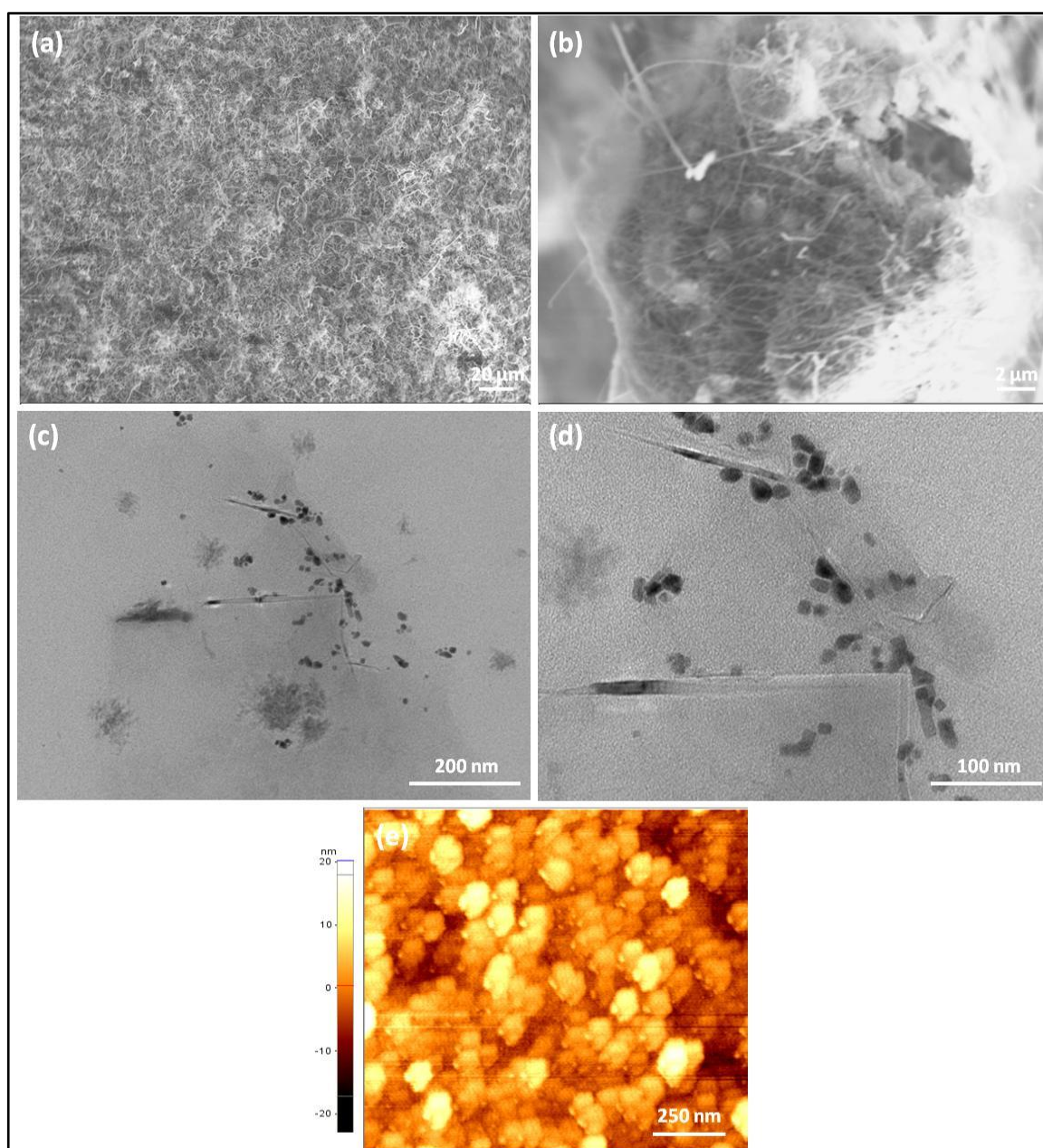


Figure 4.7. Characterization of the composite with (a, b) SEM, (c, d) TEM and (e) AFM

4.1.2. Characterization of BNNT and Composite With Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

It was necessary to remove the impurities and the components formed in the synthesized BNNTs and composite at high temperature by washing with acid solutions. According to the ICP-MS results, element concentrations in colemanite are higher than in all other samples except Fe and Zn. Fe and Zn were added additionally as catalyst for the growth of raw BNNT and composite (Table 4.1). Raw BNNT were washed with H_2SO_4 and HNO_3 while raw composite were washed with formic acid. Because BaTiO_3 nanoparticles in composite are soluble in strong acids, thus for the purification of composite, diluted formic acid were preferred. Na, Mg, Al, Ca, Fe and Sr impurities in raw composite could be substantially decreased after washing with diluted formic acid. Washing of the composite with formic acid also caused the removal of carbonate residues. Na, Mg, Al, Ca, Fe, Zn and Sr impurities in raw BNNT were also significantly decreased after H_2SO_4 and HNO_3 washing.

Table 4.1. ICP-MS elemental analysis results of colemanite, raw and pure products of BNNT and the composite

	Weighed amount (mg)	B^{10} (ppm)	Na^{23} (ppm)	Mg^{24} (ppm)	Al^{27} (ppm)
Colemanite	50.4	118958±5008	215.8±1.8	12944±385	404.1±0.9
Raw BNNT	31	46529±2104	172.5±29.5	9810.7±290.6	237.5±0.9
Pure BNNT	15	672.1±11.3	17.7±0.7	15.7±0.1	5.64±0.2
Raw composite	14	22926±1024	63.1±4.1	3563.7±51.8	155.8±3.4
Pure composite	51	165.5±2.5	9.68±0.3	102.9±1.9	1.26±0.01
	Weighed amount (mg)	Ca^{44} (ppm)	Fe^{57} (ppm)	Zn^{68} (ppm)	Sr^{88} (ppm)
Colemanite	50.4	110393±1150	1179.7±22.2	0	7947±114
Raw BNNT	31	96076±1637	73992±1615	7905	5969±10.9
Pure BNNT	15	123.6±1.7	17.7±0.3	0	8.07±0.4
Raw composite	14	40331±212	17525±823	0	3880±119
Pure composite	51	30.5±0.1	961.7±14.6	0	1.57±0.01

4.1.3. Characterization of BaTiO₃, BNNT, and Composite by Fourier Transform Infrared Spectrophotometer (FTIR) and Raman Spectroscopy

4.1.3.1. Characterization of BaTiO₃ by FTIR and Raman Spectroscopy

FTIR and Raman spectra of BaTiO₃ nanoparticles are represented in Figure 4.8a. and Figure 4.8b., respectively. The characteristic band around 486 cm⁻¹ was corresponded to Ti-O vibration mode and the peak around 1420 cm⁻¹ indicates the characteristic band of crystalline Ba-Ti-O [236].

Raman spectrum of BaTiO₃ which has been plotted in the wavenumber ranging from 100 cm⁻¹ to 1500 cm⁻¹ was shown in Figure 4.8b. Tetragonal phase of BaTiO₃ has Raman active vibration modes while cubic phase of BaTiO₃ does not have Raman active modes [237]. The sharp peak at around 185 cm⁻¹ indicates A₁(TO), a broad peak at about 248 cm⁻¹ is contributed to A₁(TO). A sharp peak at around 305 cm⁻¹, which is characteristic of tetragonal BaTiO₃ phase, is attributed to B₁, E(TO+LO). Sharpness of the peak at 305 cm⁻¹ is decreased in the case of cubic phase [238]. The asymmetric peak at 515 cm⁻¹ corresponds to [A₁(TO), E(TO)] and the peak at around 714 cm⁻¹ indicated [A₁(LO), E(LO)] [239].

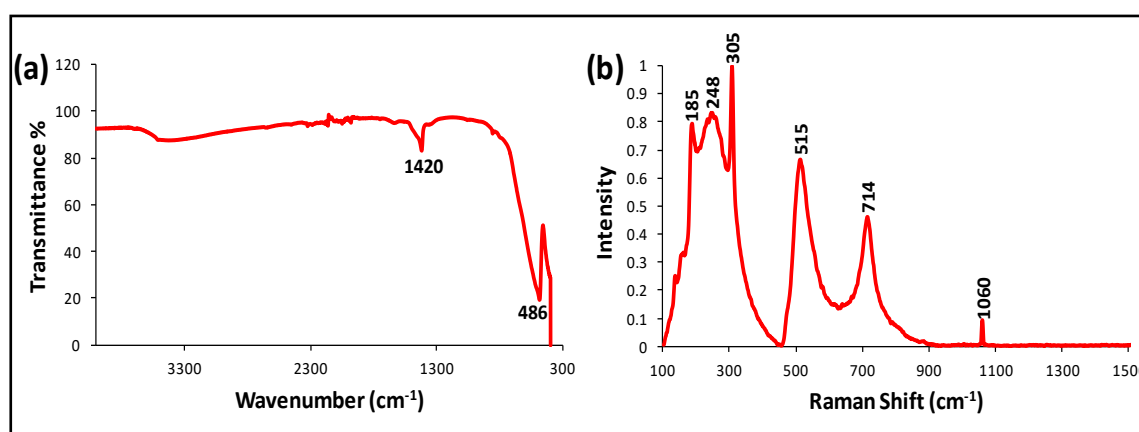


Figure 4.8. Characterization of BaTiO₃ with (a) FTIR and (b) Raman spectroscopy

4.1.3.2. Characterization of BNNT by FTIR and Raman Spectroscopy

The structural analysis of BNNTs was investigated using FTIR and Raman spectroscopy as shown in Figure 4.9. In the FTIR spectrum in Figure 4.9a., B-N-B planar binding (in-plane

binding) peak at 1327 cm^{-1} and secondary absorption peak belonging to h-BN at 758 cm^{-1} were observed, which they are characteristic peaks for BNNTs.

BNNT typically gives a sharp peak at 1366 cm^{-1} when using an 830 nm argon (Ar) laser. This peak shows the E_{2g} in-plane model of the h-BN network and it is caused by the atomic vibration between the B and N atoms on the same plane.

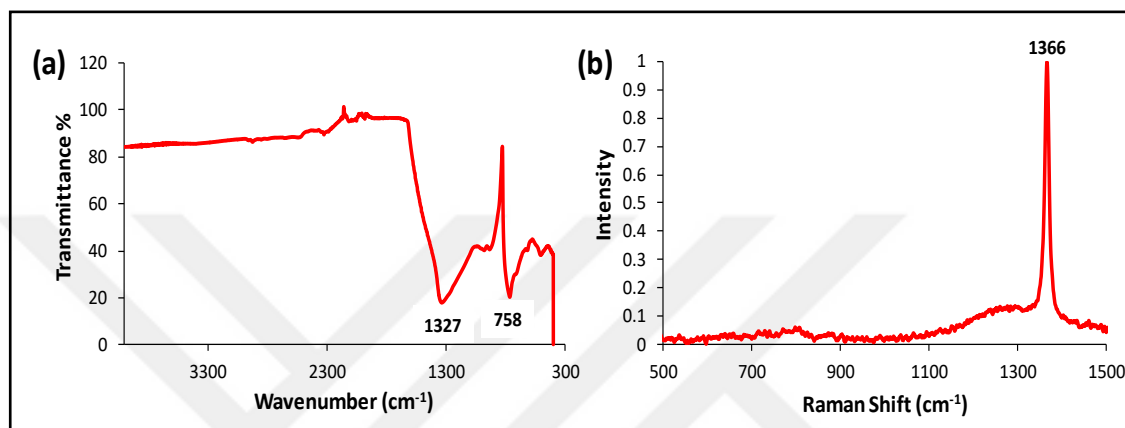


Figure 4.9. Characterization of BNNTs (a) FTIR and (b) Raman spectroscopy

4.1.3.3. Characterization of The Composite by FTIR and Raman Spectroscopy

The structural analysis of the composite was identified by FTIR and Raman spectroscopy as depicted in Figure 4.10. In FTIR spectrum of the composite, the peaks corresponded to characteristic peaks of BNNT were observed at 1347 and 756 cm^{-1} (Figure 4.10a.). A certain shifts were observed in FTIR spectrum of the composite compared to the FTIR spectrum of BNNTs, which may be due to the BaTiO_3 and other molecule formations on the same synthesis plate. Characteristic peaks of BaTiO_3 were not clearly observed due to suppression by broad and shallow peaks of BNNTs.

Molecular structure of the composite was also analyzed by Raman spectroscopy (Figure 4.10b.). The peak of BNNT typically gives a sharp peak at 1367 cm^{-1} when the 830 nm Ar laser is used. This peak shows the E_{2g} plane model of the h-BN network and originates from the atomic vibration between the B and N atoms on the same plane. The peaks corresponding to BaTiO_3 were observed at 714 cm^{-1} while tetragonal BaTiO_3 have four characteristic Raman peaks at about 261 , 304 , 517 and 715 cm^{-1} in the literature [240]. It was also observed the peaks at 248 , 282 , 405 and 564 cm^{-1} . These peaks could be

attributed to the characteristic peaks of BaTiO_3 . However, there are some shifts at that wavenumbers compared to the peak values of BaTiO_3 remarked in the literature. This shift in the Raman spectrum of composite as indicated in Figure 4.10b. could be due to the synthesis of BaTiO_3 in the presence of colemanite, Fe_2O_3 and NH_3 gas to obtain composite. The impurities might also have caused this situation. Additionally, Raman spectrum (Figure 4.10b.) and XRD spectrum (Figure 4.11.) of the composite demonstrated that there were other molecules except BaTiO_3 and BNNT in the synthesized composite.

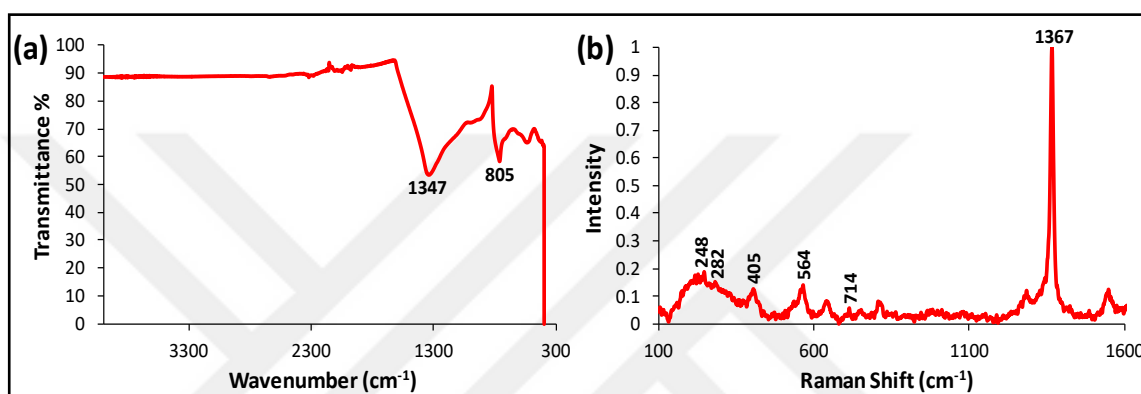


Figure 4.10. Characterization of the composite (a) FTIR and (b) Raman spectroscopy

4.1.4. Characterization of BaTiO_3 , BNNT, and Composite by X-Ray Diffraction

Figure 4.11. shows the comparative XRD results of BaTiO_3 , BNNT, and composite. In the BaTiO_3 spectrum, the peaks at $2\theta=22.208^\circ$ (100), 32.17° (110), 38.898° (111), 45.590° (200), 50.812° (210), 56.127° (211), 65.779° (220), 70.323° (300), 74.788° (310) and 79.011° (311) affirm the generation of perovskite BaTiO_3 [241]. The single and dominant hBN phase at $2\theta=27.03^\circ$ (002), 42.68° (100) and 50.02° (102) confirmed the formation of BNNTs [146].

The XRD patterns of the composite exhibited a set of diffraction peaks observed at $2\theta=22.39^\circ$, 31.72° , 39.09° , 56.62° , 65.53° , 66.53° , 73.96° and 78.74° , which were corresponded to a tetragonal structure of BaTiO_3 . The peaks at $2\theta=27.03^\circ$, 44.71° , 59.14° and 82.32° confirm the BNNT formation. In addition, the other diffraction peaks observed in the XRD spectrum of composite proposed barium titanium bis borate ($\text{B}_2\text{Ba}_6\text{TiO}_6$) and titanium nitrogen boron ($\text{Ti}_4\text{N}_3\text{B}_2$) structures. These side formations were envisaged. However, the formation of the desired structures rich in piezoelectric property, BaTiO_3 and

BNNT, in composite structure was the main goal for the continuation of the experiments in this study.

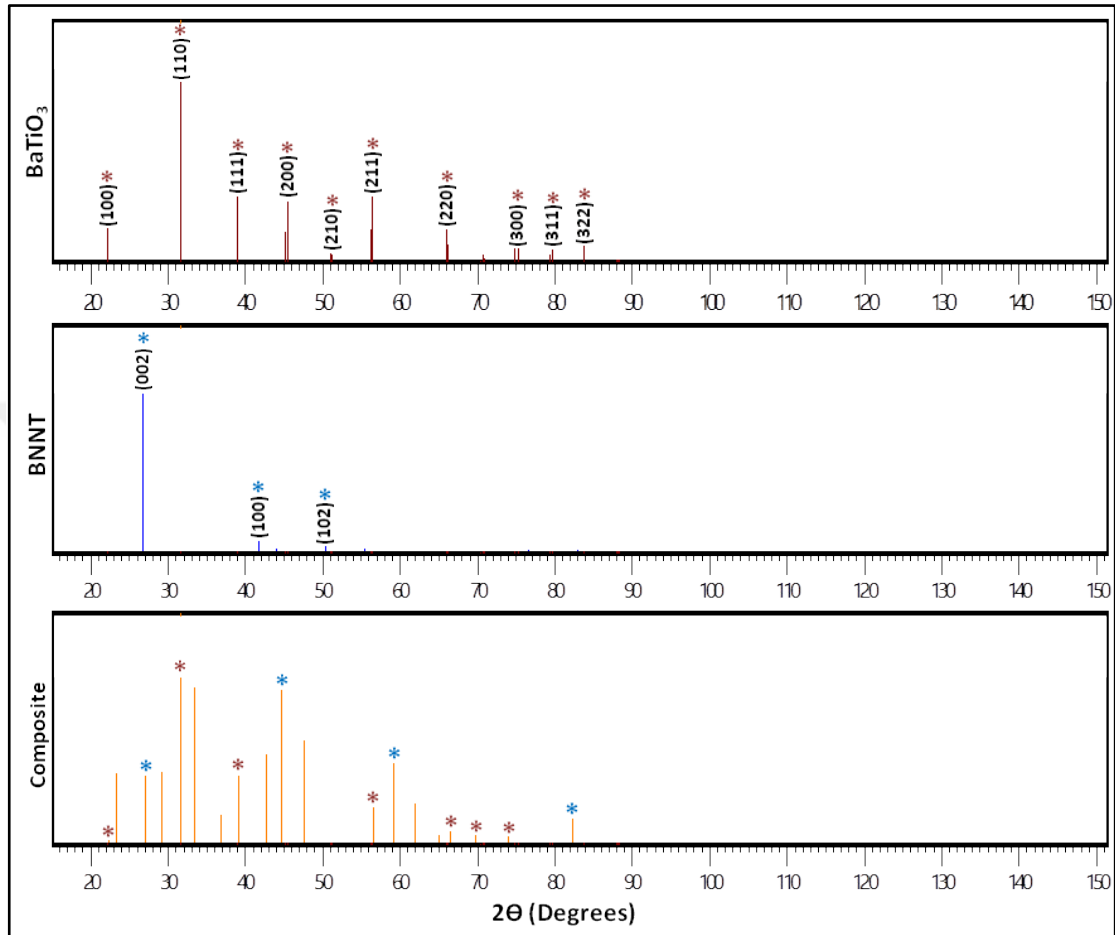


Figure 4.11. Comparative X-Ray diffraction pattern of the BaTiO₃, BNNT, and composite

4.1.5. Piezo Force Microscopy (PFM) Results of BaTiO₃, BNNT, and The Composite

Materials that have piezoelectric properties produce electric signals when exposed to mechanical pressure. This condition is called direct effect. In contrast to this situation, indirect effect is called. In indirect effect, the piezoelectric material shows dimensional extension or shortening against an applied electric field.

In the structure of solids that are not in the electric field, the numbers of positive and negative charges are equal, in other words, there is an electrically neutral situation, namely balance. In theory, this balance is expected to be disrupted by a dimensional change of shape in the structure and to form electrical charges on the material surfaces. Crystal

symmetry is the most important factor that enables the formation of these electrical charges.

The response of piezoelectric materials to the mechanical effect depends on the angular values between the "crystal orientation" determined by the arrangement of the atoms in the crystal structures and the direction of the elongation caused by the applied mechanical stress. The electrical polarization and electrical field formed in piezoelectric materials are vectorial magnitudes and their values are directional. Its adherence to this direction is determined by the crystal structure of the material and is a characteristic feature of the material. Therefore, the electrical polarization of a mechanical stress in the material will depend on the material and will take a characteristic value for each piezoelectric material based on its crystal structure.

Piezoresponse force microscopy (PFM), a variant of Atomic force microscopy (AFM), diagnoses piezoelectric and ferroelectric properties of materials by generating contrast. To produce a reverse piezoelectric field in the sample an alternating current (AC) voltage is applied between sample surface and atomic force microscopy (AFM) tip. The sample enlarges or contracts owing to this applied voltage and causing the PFM cantilever to deflect. A standard photodiode detector monitors this cantilever deviation which is then decrypted by a lock-in-amplifier. PFM amplitude and PFM phase of the cantilever oscillation are recorded. While the PFM amplitude signal enables knowledge about the strength of the signal which means the piezoelectric tensor of the material, the PFM phase signal provides information about the direction of polarization.

Figure 4.12. includes PFM amplitude and phase images and their distribution graphs of BaTiO₃. Clear PFM amplitude contrast was observed and was above noise level. Contrast differences means that grains have different piezoresponses. Mean PFM amplitude distribution was measured as 3.42 mV. PFM phase was quite weak that meant mono-domain surface or domains below resolution limit. Mean PFM phase contrast was 89°.

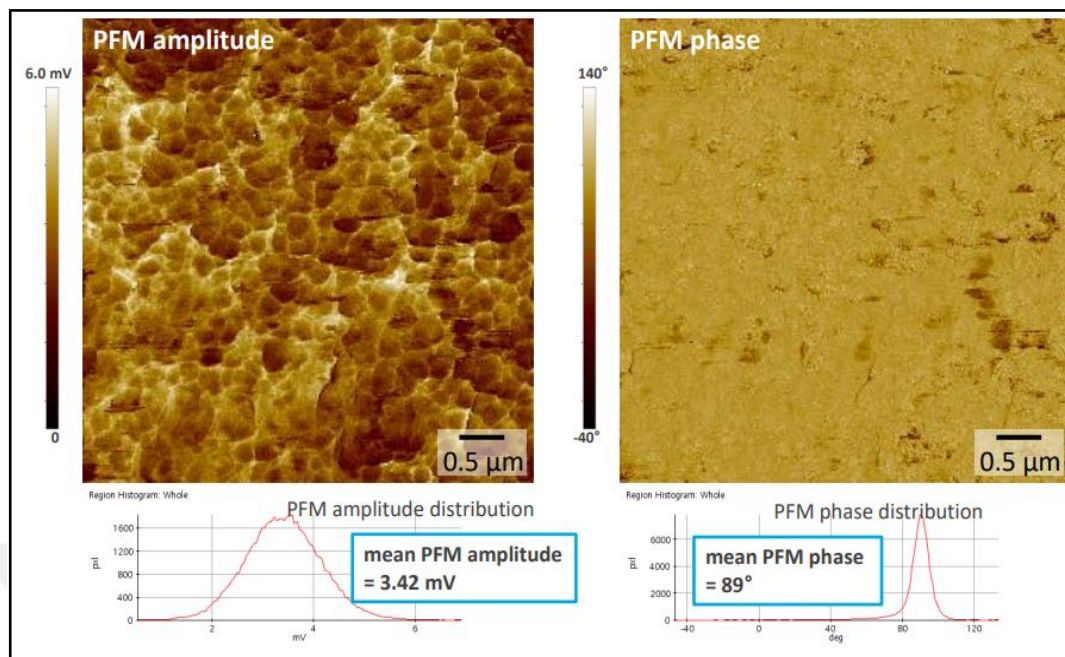


Figure 4.12. Piezoresponse force microscopy analysis of BaTiO₃

PFM results belonging to BNNTs were given in Figure 4.13. PFM amplitude contrast was above noise level and certain areas had higher PFM amplitude. Mean PFM amplitude distribution was indicated as 0.29 mV. PFM phase contrast of BNNTs was high and almost 180° which is meaning high spontaneous electrical polarization. BNNTs exhibit spontaneous polarization field due to their non-centrosymmetric and polar nature.

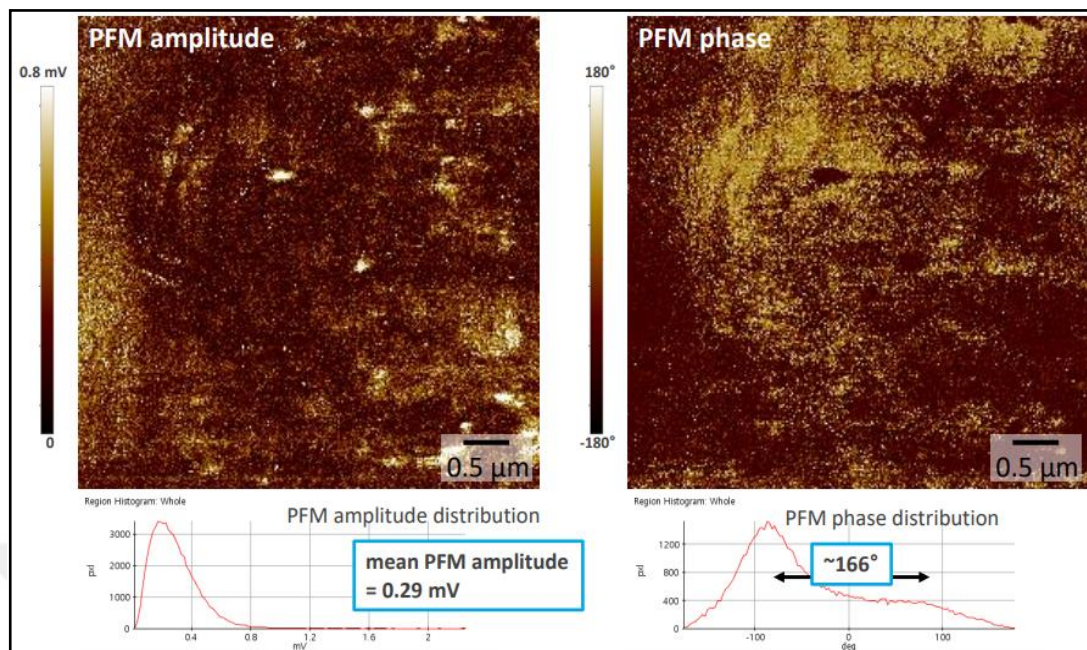


Figure 4.13. Piezoresponse force microscopy analysis of BNNTs

Figure 4.14. illustrates the PFM results of the composite material. Compared to the results of BNNTs, the composite had higher distinct PFM amplitude contrast measured as 0.83 mV. Differences in piezoresponse were observed as expected since the composite is mainly composed of BNNTs and BaTiO₃. PFM phase contrast was weak, which might mean mono domain surface or domains might be below resolution limit.

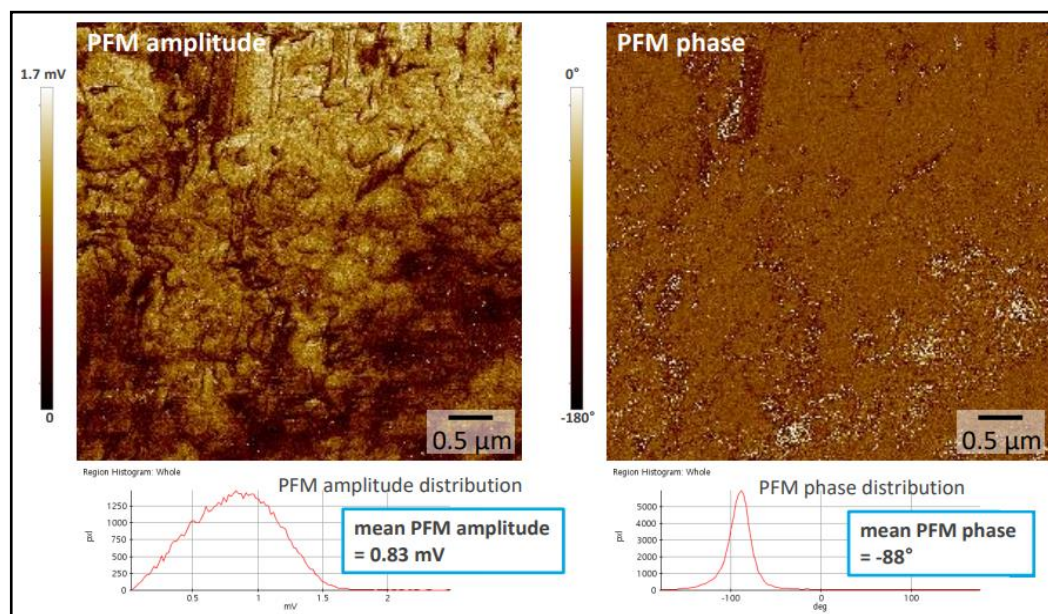


Figure 4.14. Piezoresponse force microscopy analysis of the composite

4.2. IN VITRO CELL INTERACTIONS OF BaTiO_3 , BNNTs AND COMPOSITE

4.2.1. WST-8 Cytotoxicity Assay of BaTiO_3 , BNNTs and Composite

The effect of BaTiO_3 , BNNTs and composite on Human osteoblast (HOb) cells were performed using WST-8 colorimetric assay. In the test, HOb cells were exposed to BaTiO_3 , BNNT, and composite at increasing concentrations for 24 h and the effect of nanomaterial on cell viability was investigated (Figure 4.15.). Any significant toxic effect was not observed at increasing BaTiO_3 and composite concentrations while BNNTs reduced the cell viability at increasing concentrations. However, BNNTs were able to reduce cell viability by up to 73 percent at maximum concentration, which is acceptable to be used in oncoming experiments. Considering the effects of nanomaterials on cell viability and the WST-8 results, the maximum dose to be used in scaffold construction was determined as 500 $\mu\text{g/ml}$.

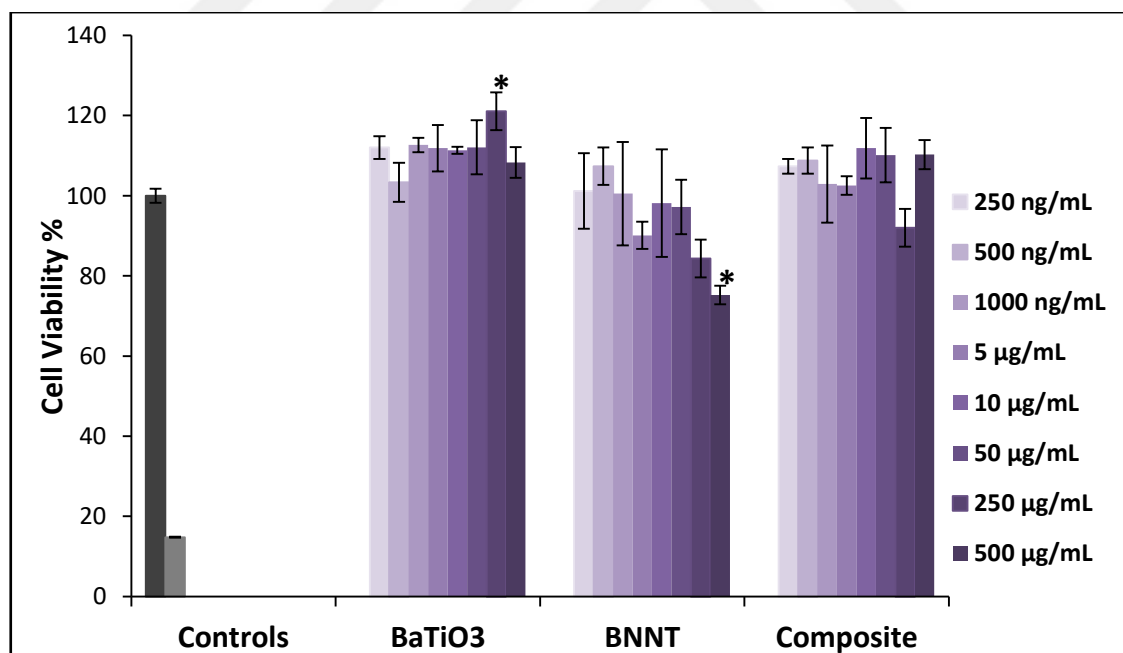


Figure 4.15. WST-8 assay results of BaTiO_3 , BNNTs and composite on HOb cells. Statistically significant changes in comparison with negative control samples are signed with an asterisk (Student's t-test, (* $p \leq 0.05$))

4.2.2. Cellular Uptake of BaTiO₃, BNNTs and Composite by Flow Cytometry

Cellular uptake of BaTiO₃, BNNT, and composite were evaluated on HOb cells with flow cytometry. The light scatter is measured by two optical detectors; one measures the scatter along path of the laser (forward scatter (FSC)) while other measures the scatter at a ninety degree angle relative to the laser (side scatter (SSC)). The FSC is generally used to identify cells based on their overall size whereas SSC is used to be informed about internal complexity of the cell. SSC is influenced by the internal granularity and complexity of the cell, thus cellular uptake of BaTiO₃, BNNT, and composite can be determined by measuring increment of SSC intensities due to causing granulation in cell. Figure 4.16. shows the SSC shift caused by BaTiO₃, BNNT, and composite in HOb cells. SSC shift, in other words uptake of BaTiO₃, BNNT, and composite significantly increased at elevating concentrations. High level uptake of BaTiO₃, BNNT, and composite can be associated with their hydrophobic character. Hydrophilic materials demonstrate higher efficiency with respect to hydrophobic materials [242]. Hydrophobic BaTiO₃, BNNT, and composite tended to interact with medium proteins in cell culture media, which enhances their cellular uptake by increasing their hydrophilicity.

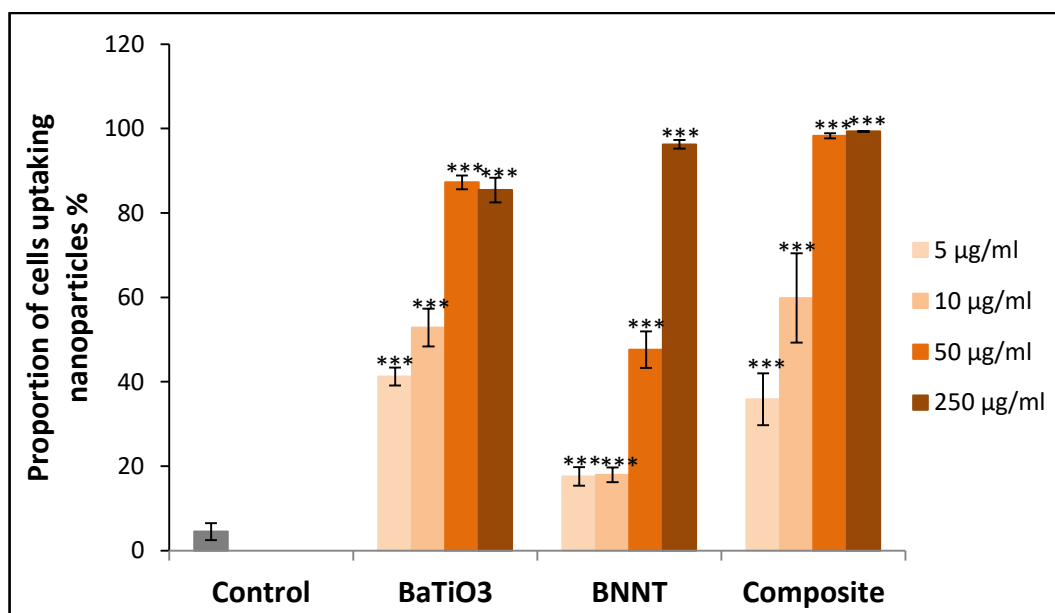


Figure 4.16. Cellular uptake results of BaTiO₃, BNNT, and composite by HOb cells using flow cytometry. Statistically significant changes in comparison with control sample are signed with asterisks (Student's t-test, (***) $p \leq 0.001$)

4.3. CHARACTERIZATION OF POLY CAPROLACTONE (PCL) AND CHITOSAN SCAFFOLDS

4.3.1. Scanning Electron Microscopy (SEM) Analysis

4.3.1.1. Scanning Electron Microscopy Analysis of Poly Caprolactone Scaffolds

Electrospun PCL based tissue engineering scaffolds are commonly preferred in many tissue engineering applications such as bone [243], skin [244], neural [245] and cardiovascular [246] tissue regeneration. However, their highly hydrophobic and biologically inert nature prevents good cell adhesion and proliferation, which therefore needs external agents. BaTiO₃, BNNT, and composite were used as reinforcement materials to contribute physical and biological enhancement to PCL scaffolds. PCL scaffolds and BaTiO₃, BNNT, and composite embedded PCL scaffolds prepared by electrospinning technique were optimized using SEM. There are many parameters such as solvent, viscosity of the solution, voltage, needle-target distance and flow rate etc. to construct a scaffold with interconnected pores, uniform and beadless fibers. In order to minimize the optimization parameters to be tested, the suggestions in the literature were used. Volatile solvents such as chloroform, methanol, dichloromethane, dimethylformamide and tetrahydrofuran, which they are highly toxic, are commonly used to obtain PCL scaffolds without beads [247]. However, they create dangerous situations for the applicator and also the environment. Their residues cannot be removed easily with water, which is very curial before biologic applications. Acetic acid is an alternative solvent which can reduce the risks [248]. Acetic acid was the chosen solvent for the construction of PCL scaffolds. Experimental conditions were explained in details in part 3.2.6.1. The PCL scaffolds could be obtained without any bead formation as given in Figure 4.17. BaTiO₃, BNNT, and composite added scaffolds were also prepared with the same conditions, however, bead like fiber formations were barely observed due to insoluble nanomaterial (Figure 4.17.). Nanomaterials could be aggregated in PCL solution leading to bigger bead like formations. The critical purpose of electrospinning is to achieve uniform, bead-free and interconnected fibers. Beaded fiber structures prevent reproducibility of scaffold construction. However, beads also might be good for cell growth as it provides a wide area for cell attachment.

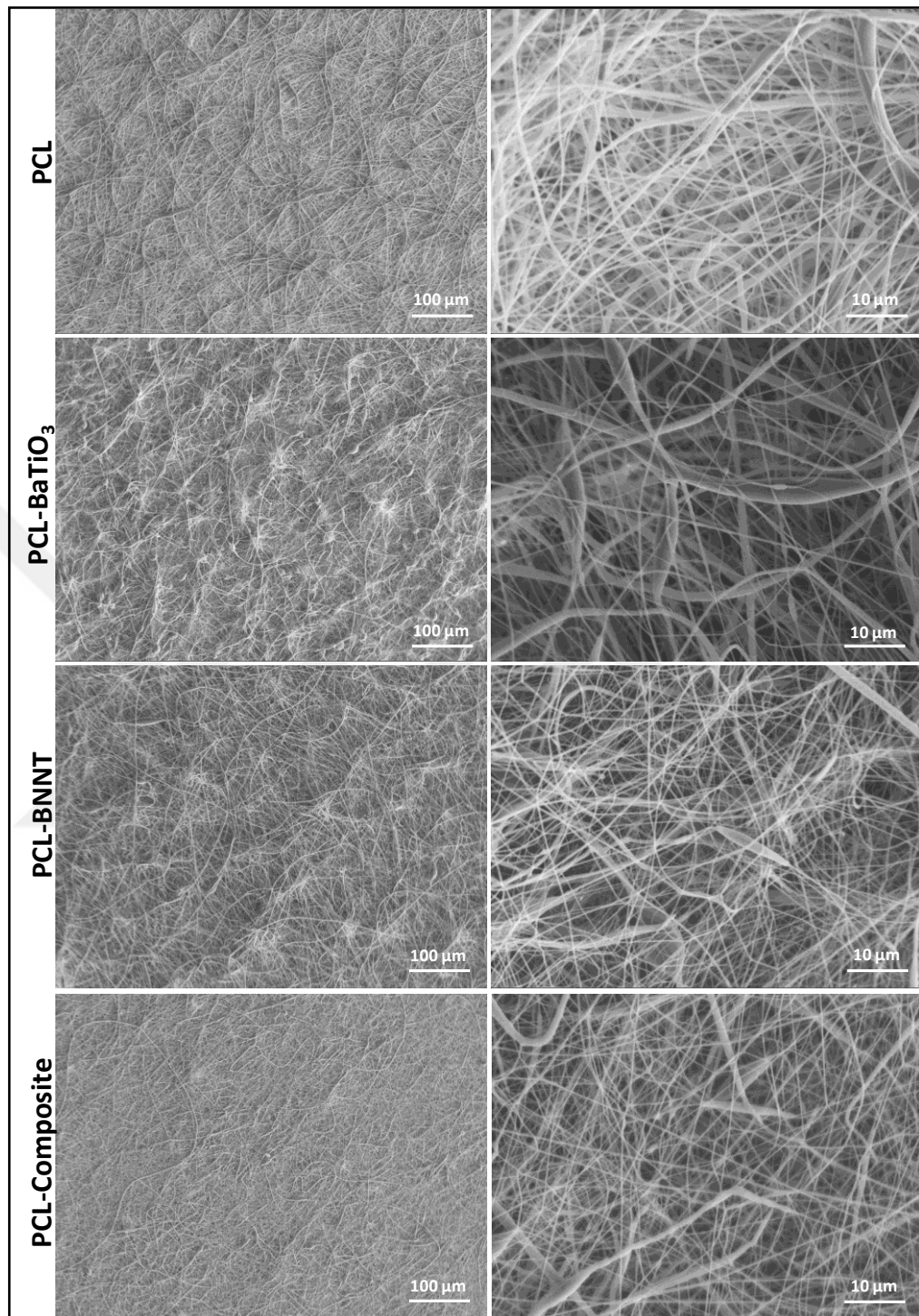


Figure 4.17. SEM images of PCL and BaTiO₃, composite and BNNT embedded PCL scaffolds

The average fiber diameter of scaffolds was calculated from SEM images by measuring the diameter of thirty fibers and the results were graphed as illustrated in Figure 4.18. The average diameter of PCL, BNNT and composite embedded PCL scaffolds was under 1 μm

and they had almost similar sizes. The average diameter of PCL, PCL-BNNT, and PCL-Composite were calculated as $410.83 \text{ nm} \pm 303.89$, $451.27 \text{ nm} \pm 352.06$ and $440.758 \text{ nm} \pm 187.185$, respectively. BaTiO₃ embedded PCL scaffolds had higher fiber diameter compared to the others, which is calculated as $1529.54 \text{ nm} \pm 423.35$. The mean diameter differences of BaTiO₃, BNNT, and composite embedded PCL scaffolds can be explained with their distribution profiles in PCL polymer. BNNT and composite seems to have better distribution compared to BaTiO₃. BNNT and composite are more hydrophobic and have higher efficacy to be distributed in highly hydrophobic PCL.

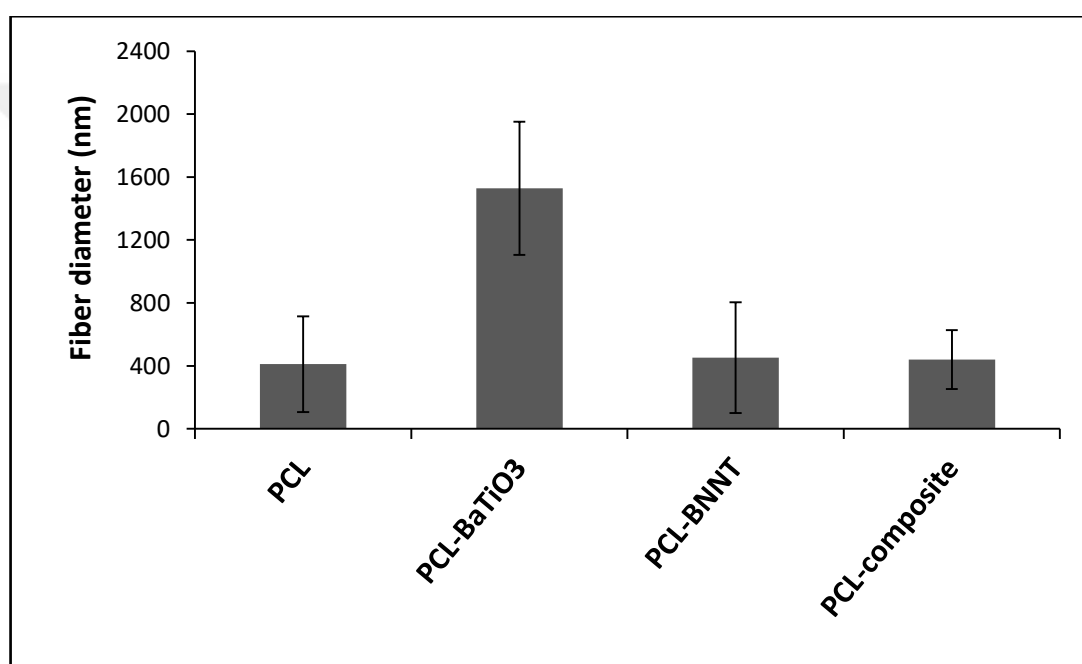


Figure 4.18. The mean fiber diameters of PCL, PCL-BaTiO₃, PCL-BNNT, and PCL-Composite scaffolds, which is calculated using SEM images

4.3.1.2. Scanning Electron Microscopy Analysis of Chitosan Scaffolds

Chitosan scaffolds were fabricated using freeze drying technique, which allows highly interconnected porous matrices as demonstrated with SEM images in Figure 4.19. Genipin was preferred to crosslink chitosan due to their biocompatibility, safety and stability instead of glutaraldehyde and other crosslinkers [249]. The pore size of only chitosan scaffold was between 20 μm and 80 μm (Figure 4.19a.), whereas the pore size of genipin-crosslinked chitosan scaffold ranged from 40 μm to 100 μm (Figure 4.19b.).

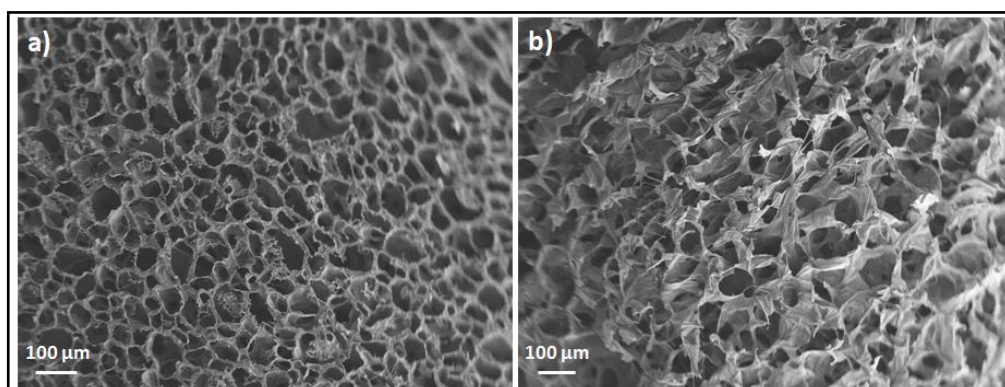


Figure 4.19. SEM images of a) chitosan and b) genipin crosslinked chitosan

Chitosan based scaffolds had very porous structure as seen in SEM images in Figure 4.20. When BaTiO_3 (Figure 4.20b.), BNNT (Figure 4.20c.) and composite (Figure 4.20d.) were embedded in chitosan, pore sizes increased compared to the only chitosan scaffolds (Figure 4.20a). Additionally, interconnected pores were observed in all types of chitosan scaffolds which are crucial for cell attachment and proliferation.

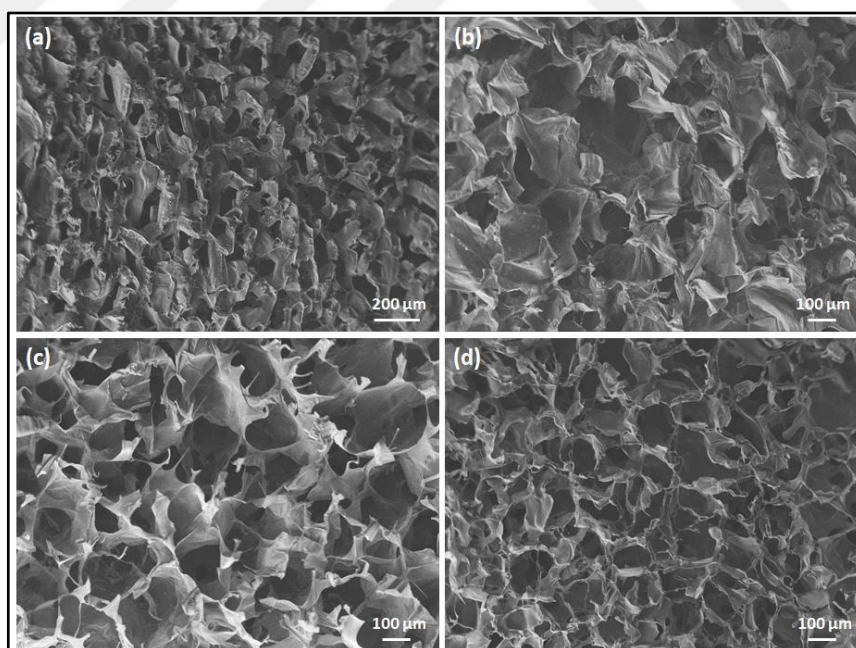


Figure 4.20. SEM images of a) chitosan and b) chitosan- BaTiO_3 , c) chitosan-BNNT and d) chitosan-Composite

Chitosan-BaTiO₃, chitosan-BNNT, and chitosan-Composite had similar mean pore sizes which are approximately between 100 and 120 μm while only chitosan scaffold had nearly 60-100 μm mean pore sizes as seen in Figure 4.21.

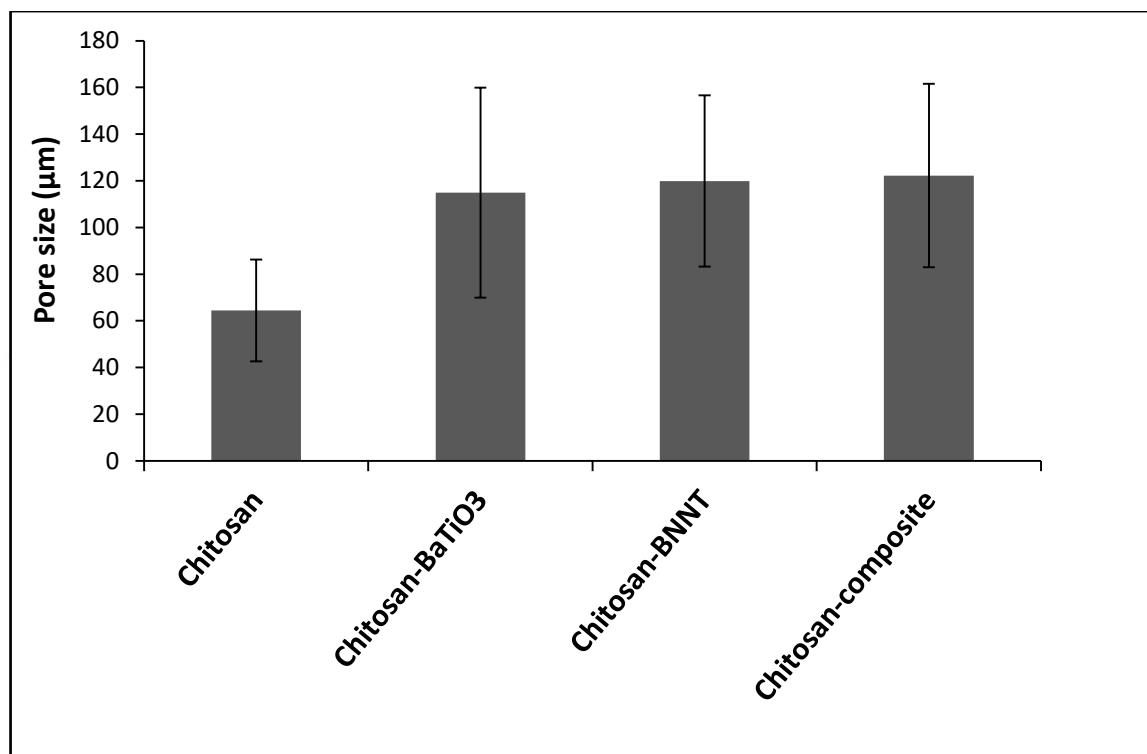


Figure 4.21. Mean pore sizes of chitosan, chitosan-BaTiO₃, chitosan-BNNT, chitosan-Composite scaffolds, which is calculated using SEM images

4.3.2. Determination of BaTiO₃, BNNT, and Composite Distribution in PCL and Chitosan Scaffolds by Raman Spectroscopy

Raman spectroscopy which is a vibrational spectroscopy technique based on inelastic scattering of light provides structural and chemical information about materials. This information can be visually exhibited via 1D, 2D or 3D Raman imaging (also known as mapping) by collecting spectral data from an array of points “on” or “in” a sample. A spectral hypercube is collected with Raman mapping instead of a simple intensity image, which means that a Raman spectrum is collected from each position “on” or “in” sample and saved as a single file. Then, each spectrum is analyzed and all of analyzed spectra generate a Raman image. It can be represented a number of parameters from the complex analysis of the whole spectrum or intensity of a particular Raman band.

In this study, determination of BaTiO₃, BNNT, and composite distribution in PCL and chitosan scaffolds was achieved by generating 3D Raman mapping. The 3D images were created at their characteristic peaks of each nanomaterial. The characteristic peaks of each nanomaterial were selected according to those that do not overlap with the characteristic peaks of the polymers. Figure 4.22. shows the Raman spectra of PCL and chitosan scaffolds and characteristic peak of BaTiO₃ at 308 cm⁻¹ and characteristic peak of BNNT at 1366 cm⁻¹ were marked. There were not any observed peaks corresponding to polymeric scaffolds at that defined points.

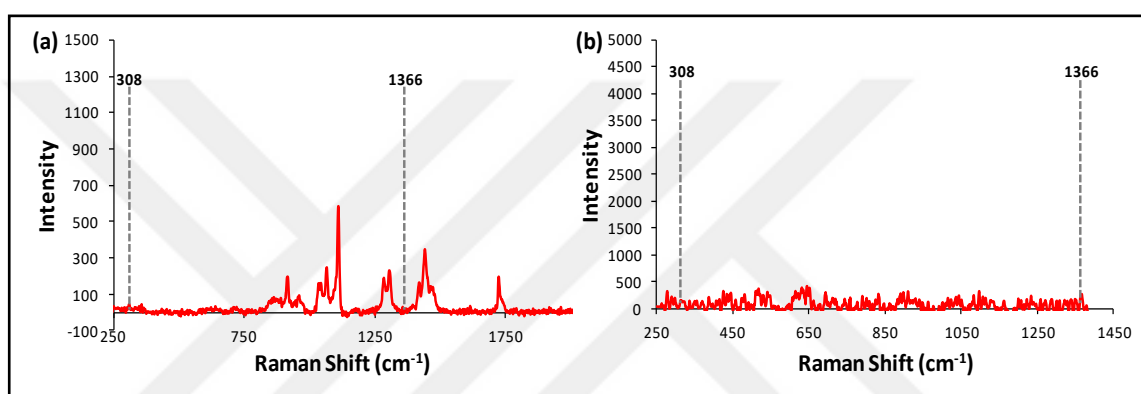


Figure 4.22. Raman spectra of (a) PCL (50 μ m objective lens, laser power=30 mW, exposure time= 10 seconds), (b) chitosan (50 μ m objective lens, laser power=150 mW, exposure time= 2 seconds) scaffolds

4.3.2.1. BaTiO₃, BNNT, and Composite Distribution in PCL Scaffolds

Figure 4.23., Figure 4.24. and Figure 4.25. illustrate the 3D Raman images and spectra of BaTiO₃, BNNT, and composite embedded PCL scaffolds mapped from three different areas on a sample at 308 cm⁻¹ for BaTiO₃ and at 1366 cm⁻¹ for BNNT and composite. Each average spectrum of 1331 spectra was represented opposite each image. Peak intensities were scaled with a color bar and brightest green color represent that the peak intensity highest at that area. The obtained peak intensities were generally very low in all types of scaffolds because the concentrations of nanomaterials, BaTiO₃, BNNT, and composite, used in scaffolds was very low which was determined as 500 μ g/mL according to its WST-8 results. In addition, during mapping, the focus of the objectives might deteriorate, resulting in reductions in peak intensities.

The bright green color decreases in some places because of the uneven distribution of the hydrophobic nanomaterial in highly hydrophobic PCL polymer. Preparation of PCL scaffolds with electrospinning technique might be also caused some aggregations.

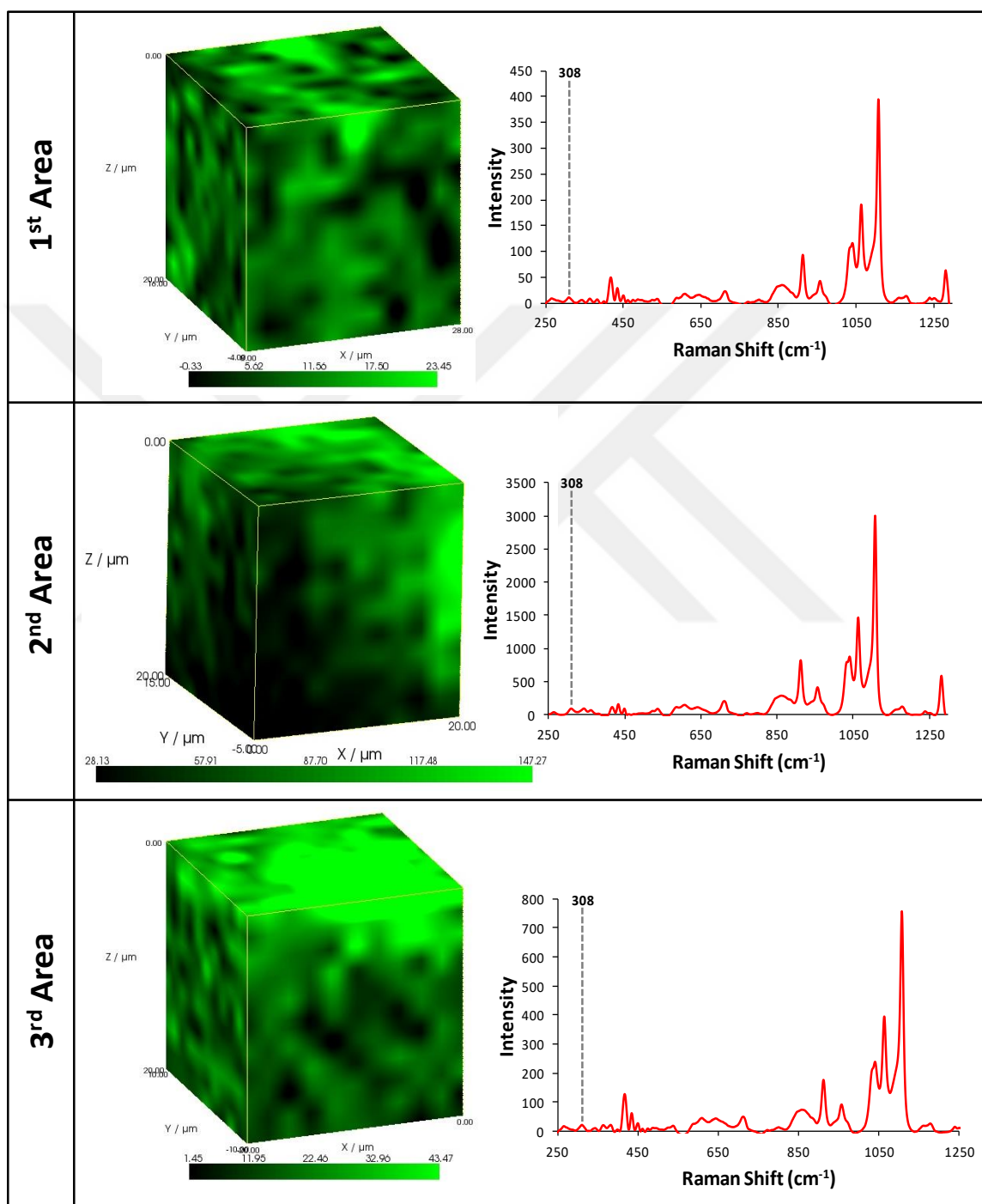


Figure 4.23. 3D volume mapping at 308 cm^{-1} peak of BaTiO_3 in PCL scaffolds

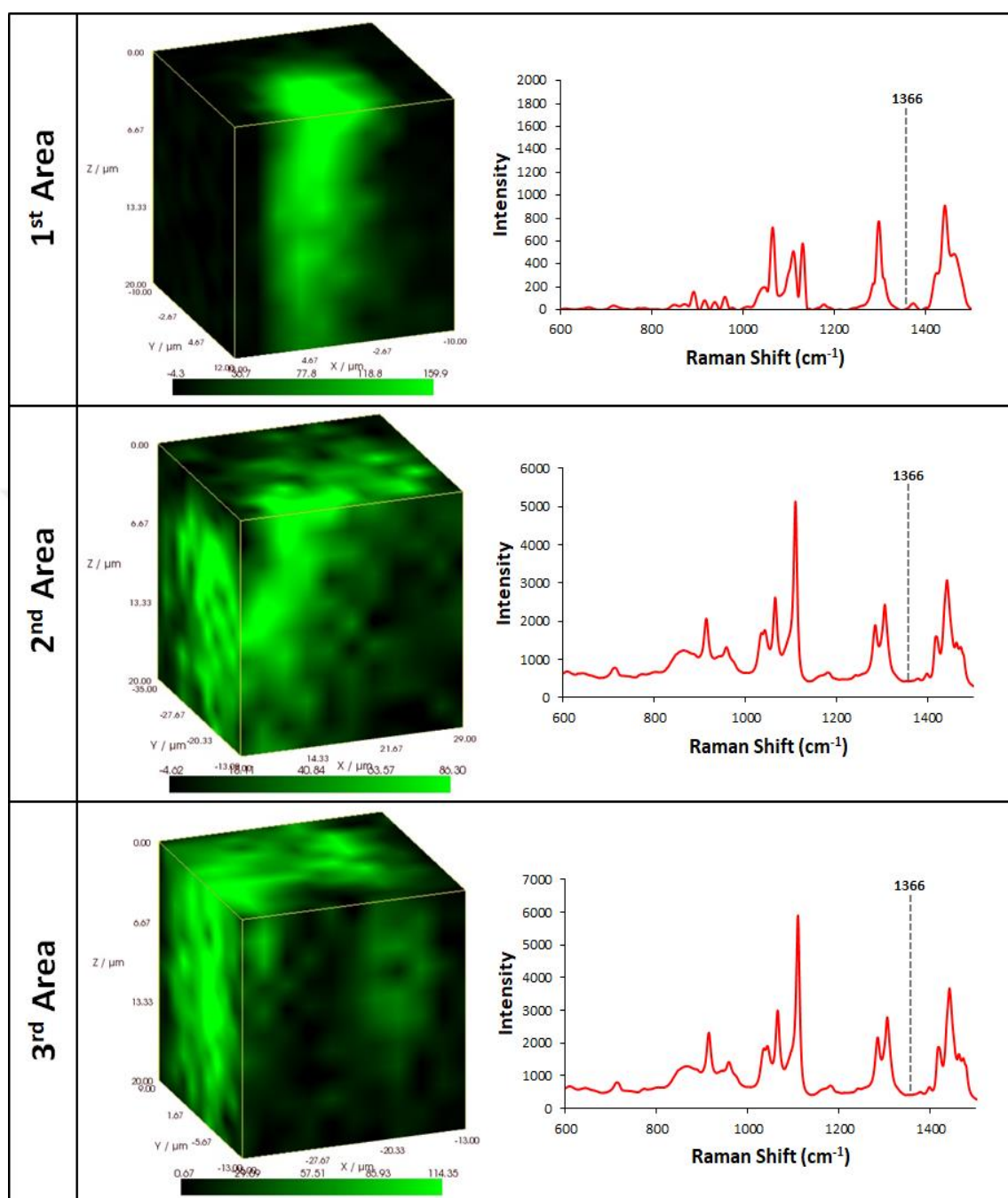


Figure 4.24. 3D volume mapping at 1366 cm^{-1} peak of BNNT in PCL scaffolds

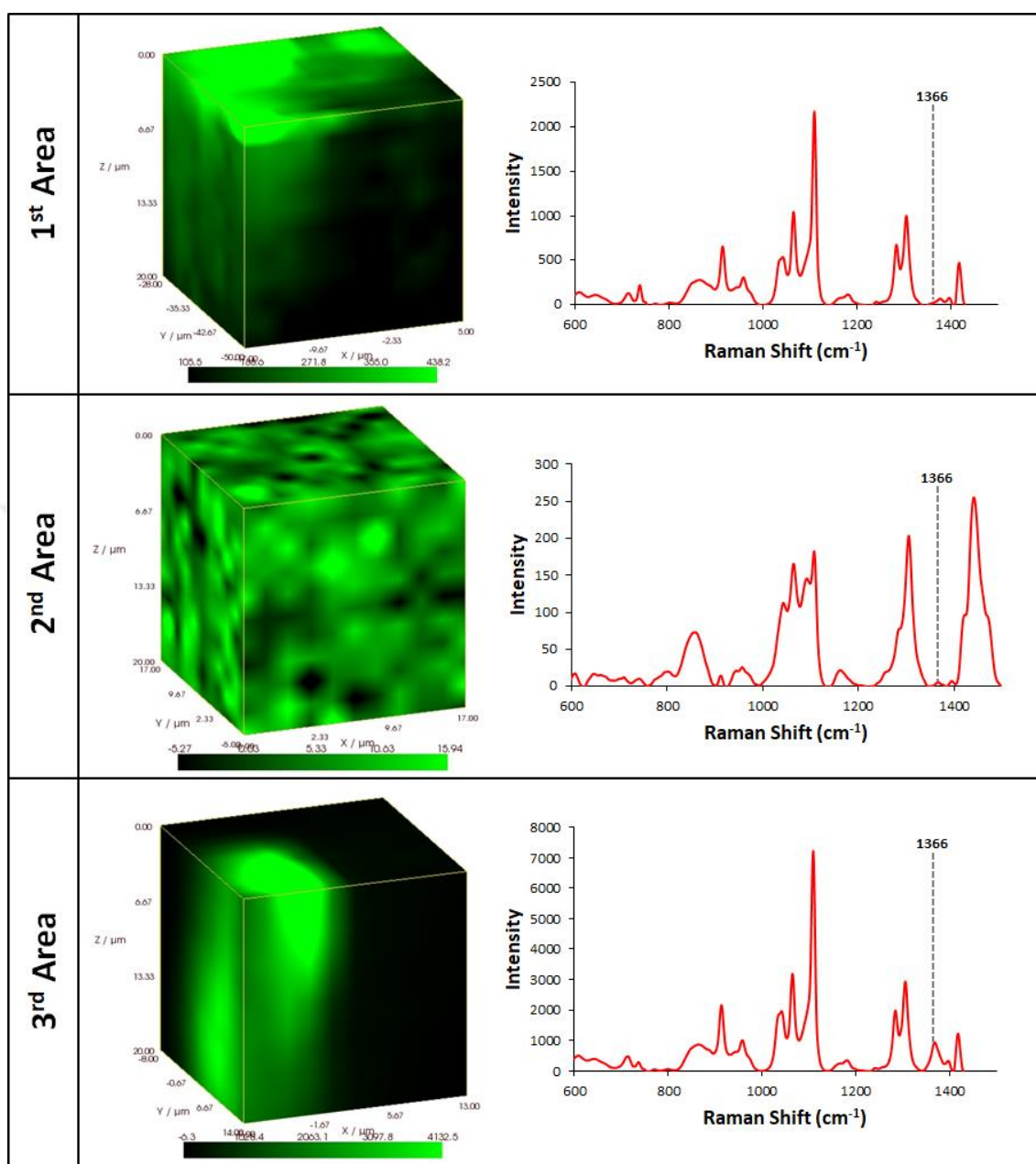


Figure 4.25. 3D volume mapping at 1366 cm^{-1} peak of composite in PCL scaffolds

4.3.2.2. *BaTiO₃, BNNT, and Composite Distribution in Chitosan Scaffolds*

Figure 4.26., Figure 4.27. and Figure 4.28. represents the 3D Raman images and spectra of BaTiO_3 , BNNT, and composite embedded chitosan scaffold mapped from three different areas on a sample at 308 cm^{-1} for BaTiO_3 and at 1366 cm^{-1} for BNNT and composite. Three different images showed almost same color profile. However, the peak intensities were very low, which is mainly related to the very low concentration of nanomaterials used. In addition, during mapping, the focus of the objectives might deteriorate due to the

3D form of the scaffolds, resulting in reductions in peak intensities. Bright green color accumulations were observed in some parts of the cube. These accumulations represent nanomaterial aggregations owing to exhibiting hydrophobic character in highly hydrophilic chitosan hydrogel.

Overall, it can be said that nanomaterials could be observed in almost every area in a cube; however, their intensity levels are variable due to aggregations owing to hydrophobic nature of both nanomaterials and polymer.



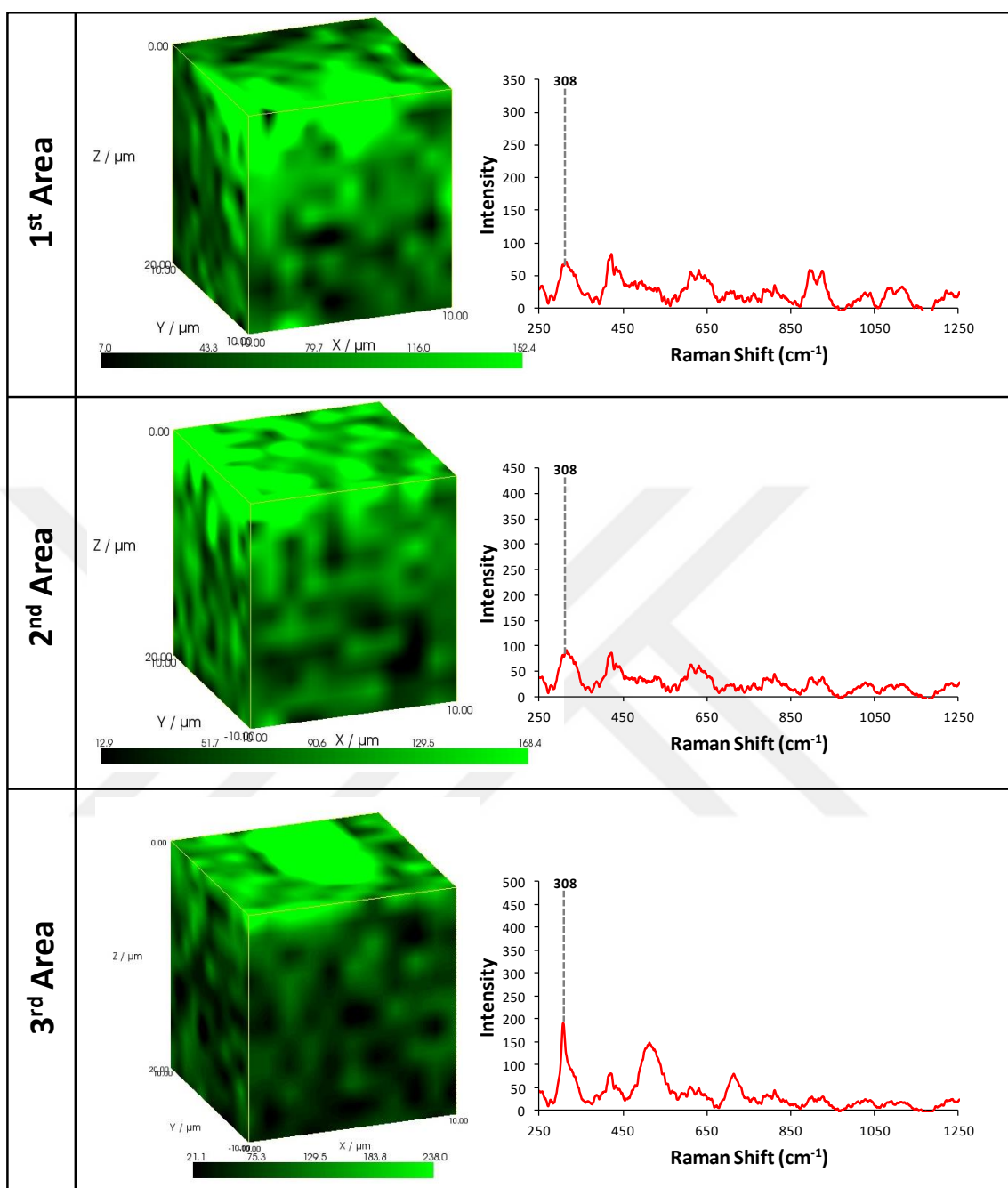


Figure 4.26. 3D volume mapping at 308 cm^{-1} peak of BaTiO_3 in chitosan scaffolds

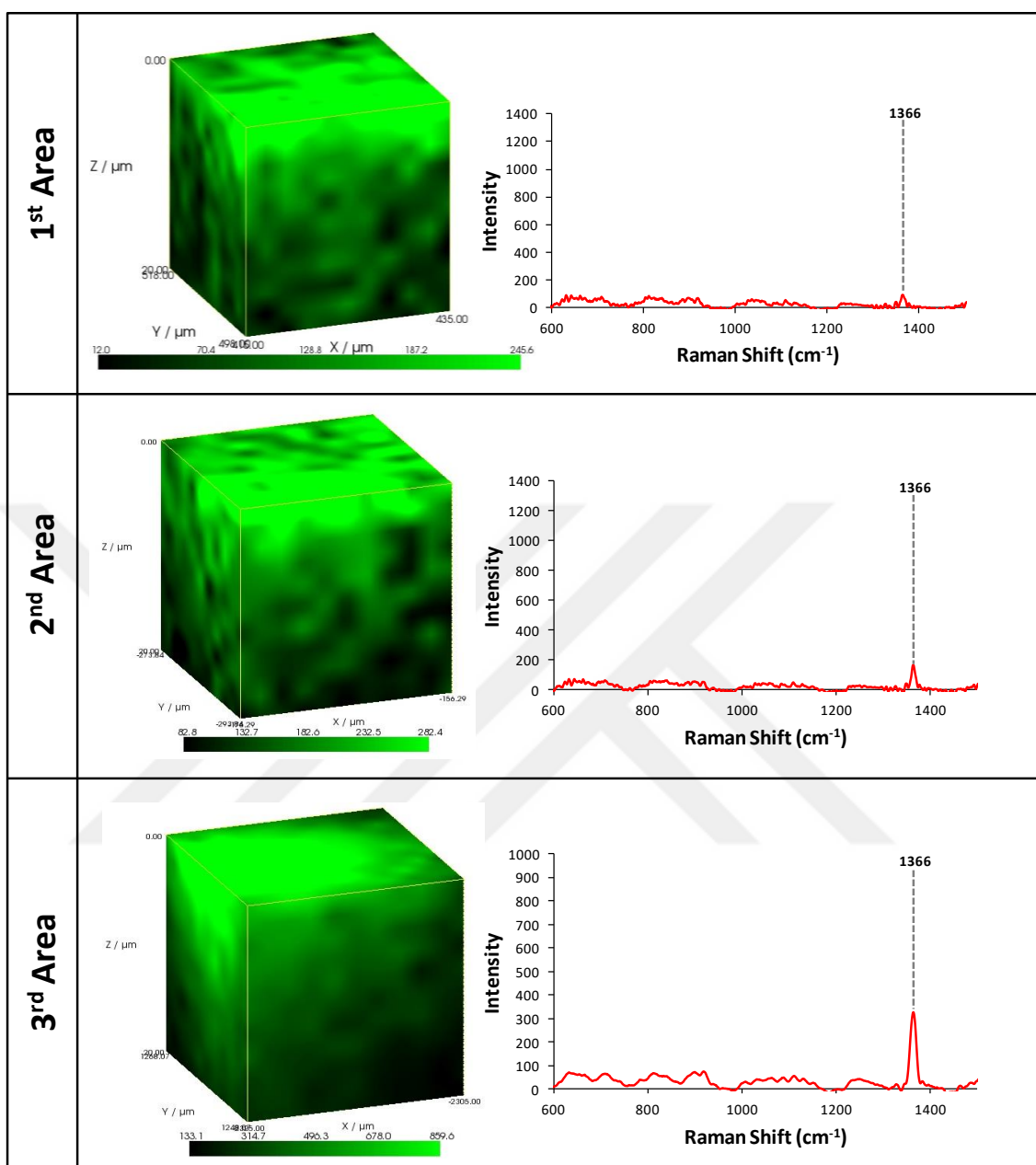


Figure 4.27. 3D volume mapping at 1366 cm^{-1} peak of BNNT in chitosan scaffolds

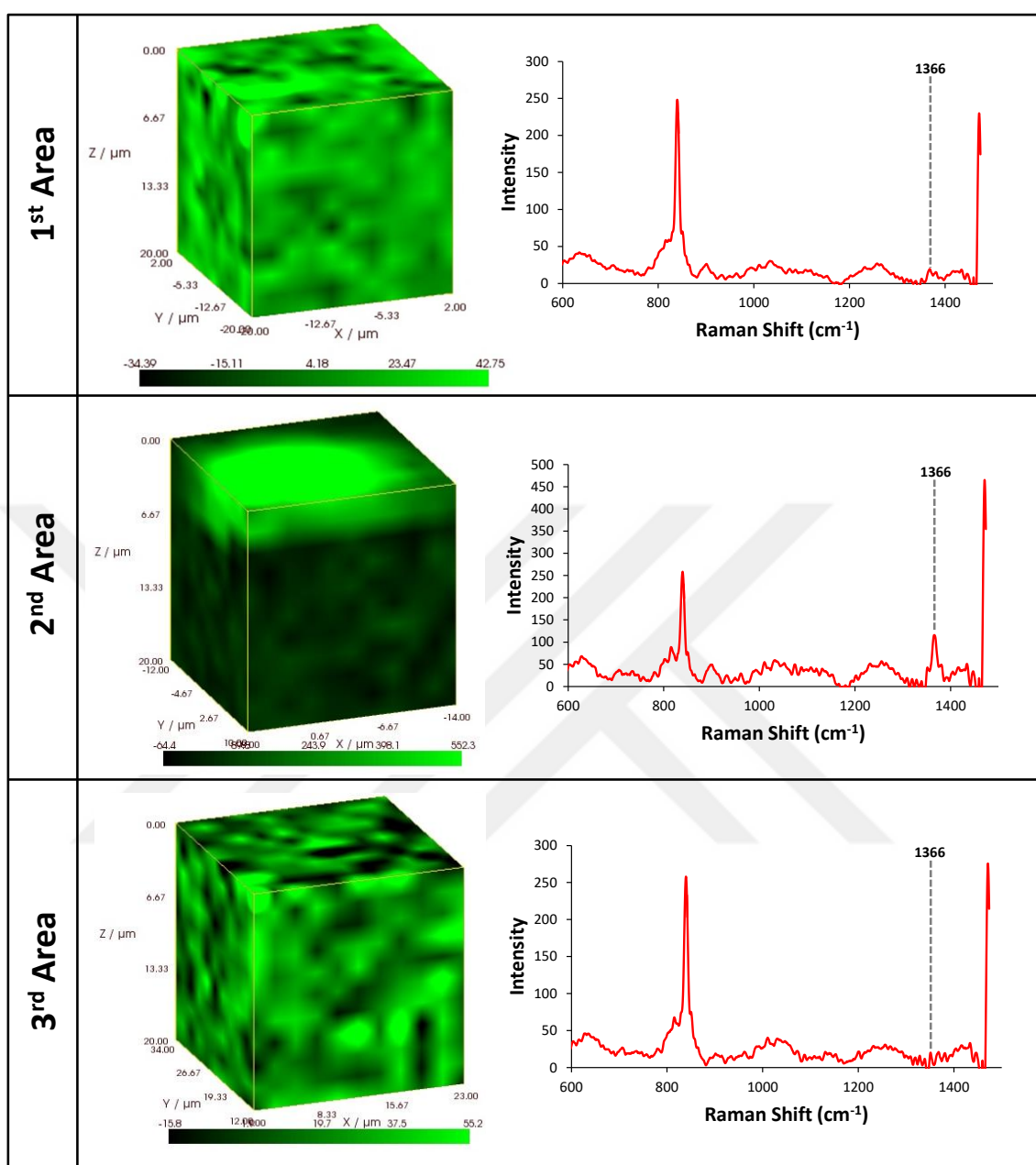


Figure 4.28. 3D volume mapping at 1366 cm^{-1} peak of composite in chitosan scaffolds

4.3.3. Mechanical Testing

Mechanical strength of polymeric scaffolds has significance for providing a sustainable platform when tissue regenerates. Toughness, shear modulus, tensile strength, compressive strength and fatigue strength are the significant mechanical properties of bone [250]. Tensile strength of PCL and BaTiO_3 , BNNT, and composite embedded PCL scaffolds and

compressive strength of chitosan and BaTiO₃, BNNT, and composite embedded scaffolds were evaluated.

4.3.3.1. Tensile Features of Poly Caprolactone Scaffolds

To evaluate mechanical properties of PCL and BaTiO₃, BNNT, and composite embedded scaffolds tensile strength measurements were accomplished. The stress-strain graphs of electrospun PCL scaffolds are given in Figure 4.29. BaTiO₃, BNNT, and composite enhanced the tensile strength of PCL scaffolds. Composite embedded PCL scaffolds had the highest tensile strength which is about 4.020 MPa and elongation at break 1.071. BNNT embedded PCL scaffolds had higher tensile strength (1.275 MPa) and elongation at break 0.740 compared to PCL and PCL-BaTiO₃. BNNT and composite showed higher elongation, which is attributed to the high flexibility of BNNTs [176]. PCL had higher extension at break 0.605 and less tensile strength at break 0.602 MPa in comparison with PCL-BaTiO₃. The results showed that BaTiO₃, BNNT, and composite enhanced the mechanical stability of PCL scaffolds.

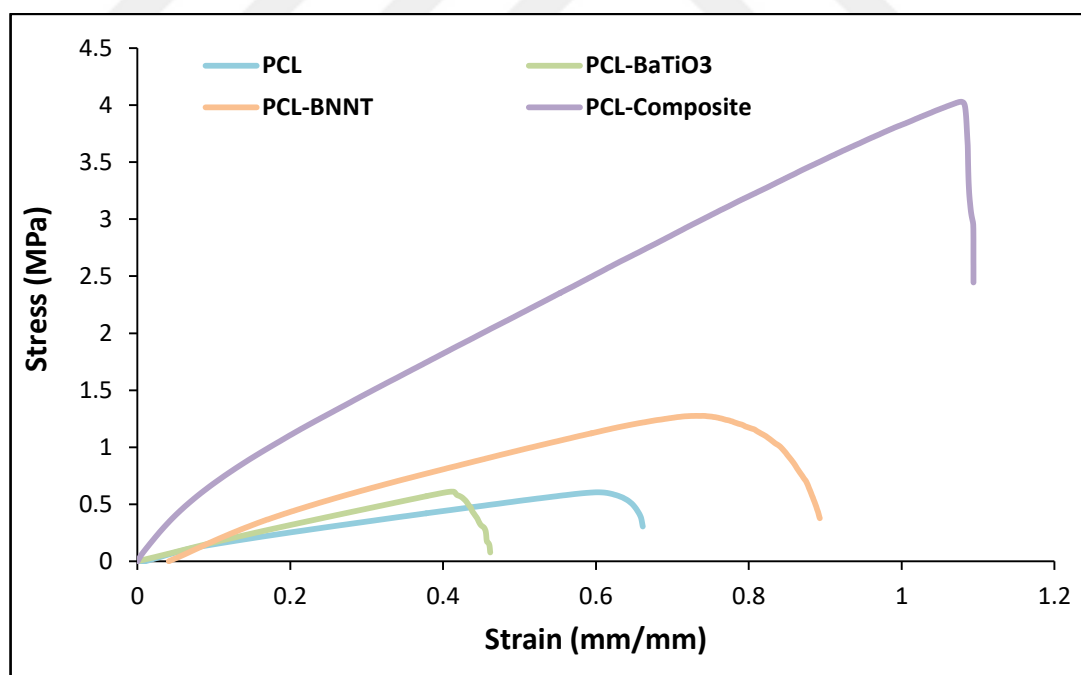


Figure 4.29. Tensile profile of electrospun PCL, PCL-BaTiO₃, PCL-BNNT, and PCL-Composite scaffolds

4.3.3.2. Compressive Features of Chitosan Scaffolds

Compressive strength of chitosan and BaTiO₃, BNNT, and composite embedded chitosan scaffolds were accomplished to evaluate mechanical strength of the chitosan derived scaffolds (Figure 4.30.). For chitosan-BaTiO₃, chitosan-BNNT and chitosan-Composite compressive stress were 0.137, 0.124 and 0.095 MPa, respectively while compressive stress of chitosan was 0.080 MPa. The use of BaTiO₃, BNNT, and composite as reinforcement agents in chitosan increased the compressive strength of chitosan. Although increasing pore size of scaffolds cause negative effect on their mechanical properties, when BaTiO₃, BNNT, and composite were used, both pore sizes and mechanical stability increased, which is desired for tissue engineering applications.

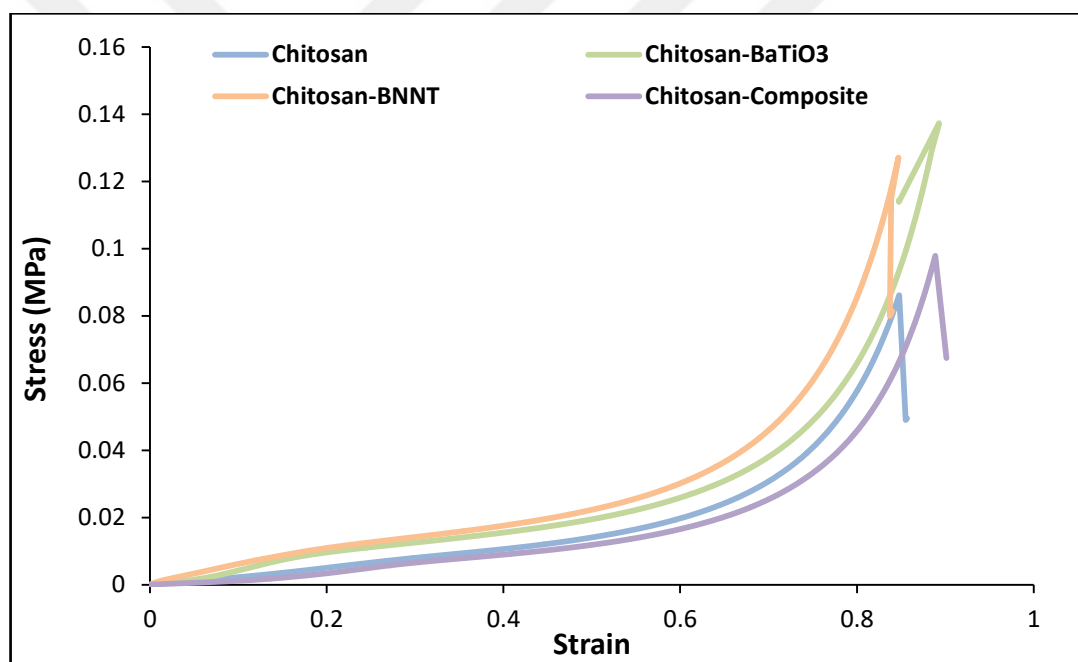


Figure 4.30. Stress-strain curves of chitosan, BaTiO₃, composite and BNNT embedded chitosan scaffolds

4.3.4. Water Contact Angle Analysis of Poly Caprolactone (PCL)

Hydrophobic features of scaffolds are highly important for better cell adhesion to provide adsorption of more pronounced proteins on the scaffold surface. Hydrophobic properties of PCL scaffolds were determined using water contact angle. The average dynamic contact angle with water values at the 60th second of measurements for PCL and BaTiO₃, BNNT,

and composite embedded PCL scaffolds are listed in Table 4.2. and also presented in column graphs in Figure 4.31. The used nanomaterials; BaTiO₃, BNNT, and composite decreased the hydrophobicity of PCL as summarized in Table 4.2. and Figure 4.31. Composite increased the wettability of PCL scaffolds more than BaTiO₃ and BNNT embedded scaffolds by decreasing the contact angle from 126,64±1,64 to 110,55±1,43.

Table 4.2. Water contact angle values of PCL scaffolds

	Contact Angle (deg)
PCL	126,64±1,64
PCL-BaTiO₃	111,33±2,75
PCL-BNNT	114,94±2,25
PCL-Composite	110,55±1,43

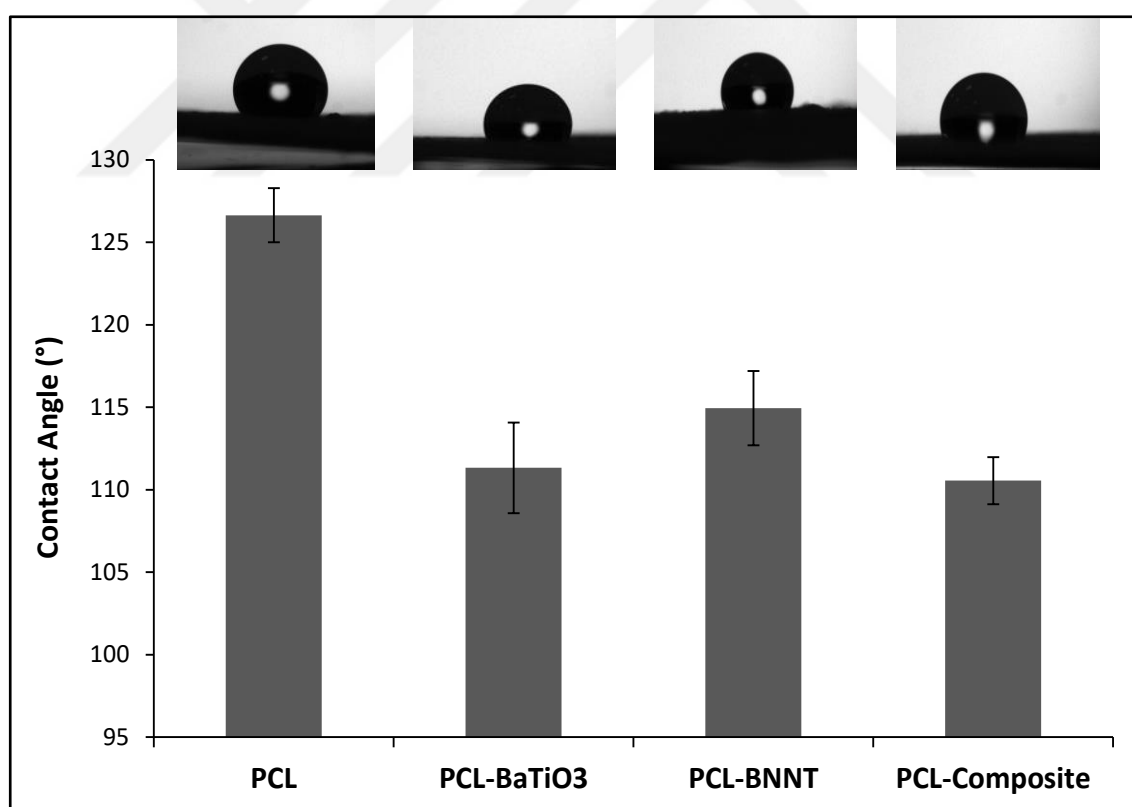


Figure 4.31. Water contact angle results of PCL and BaTiO₃, composite and BNNT embedded PCL scaffolds

4.3.5. Equilibrium Water Content of Chitosan Scaffolds

Swelling capacity of the scaffolds has importance due to providing the exchange of cell nutrients and metabolites. Swelling also increase the pore ratio of chitosan and BaTiO₃, BNNT, and composite embedded chitosan scaffolds with and without ultrasound (US) stimulation were graphed as illustrated in Figure 4.32. Owing to the high density of protonated amine groups, which represents a powerful affinity for water molecules chitosan scaffolds swell simply in aqueous solution. Therefore, chitosan scaffolds needs neutralization or crosslinking to reduce swelling. Swelling ratio of chitosan were 9.28 with US and 9.65 without US stimulation. BaTiO₃, BNNT, and composite addition decreased the swelling ratio of chitosan scaffold due to their highly hydrophobic nature compared to only chitosan scaffold. Crosslinking degree of chitosan scaffolds with genipin affects the swelling ratio however, the used amount of genipin in all kinds of scaffold types were same. Thus, hydrophobic nature of the used nanomaterials helps to exclude water molecules from the nanomaterial embedded composite scaffolds. The swelling ratio of chitosan-BaTiO₃, chitosan-BNNT and chitosan-Composite were respectively 8.20, 8.58 and 6.63 without US stimulation and 9.14, 9.84, 8.81 with US stimulation. Mechanical stimulation increased the swelling ratios of all kinds of scaffolds. It causes vibrational movements in the structure, thus, increase the mobility and migration of water molecules into the scaffold structure and cause more water adsorption.

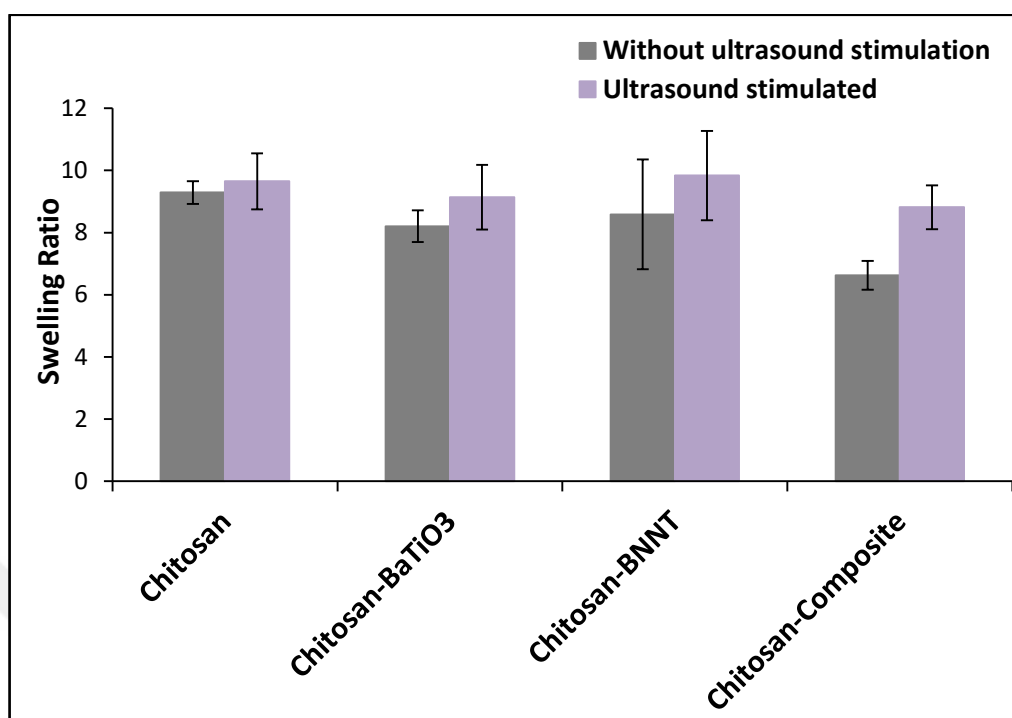


Figure 4.32. Swelling ratio of chitosan, BaTiO₃, BNNT, and composite embedded chitosan scaffolds

4.4. IN VITRO DEGRADATION

The biodegradation rate of scaffold materials should be matching the speed of new bone tissue formation. The scaffold degradation should be slow even after the tissue formation.

Biodegradation of PCL and chitosan derived scaffolds were followed in 0.9 percent NaN₃ containing 1X PBS buffer solution (pH=7.4) at 37°C via weight loss during 120 days with and without US stimulation.

4.4.1. In Vitro Degradation of PCL Scaffolds via Weight Loss

PCL is nonenzymatically degraded via random hydrolytic cleavage of ester groups, which resulted in decrease in molecular weight. The percentage weight loss versus time plots of PCL and BaTiO₃, BNNT, and composite embedded PCL scaffolds without US (Figure 4.33.) and with US (Figure 4.34.) were represented. Weight loss (percent) of only PCL scaffolds was 1.36 at the end of 120 days. The degradation rate of PCL scaffolds were

increased when BaTiO₃, BNNT, and composite nanomaterials were used. At the end of 120 days, weight loss (percent) of PCL-BaTiO₃, PCL-BNNT and PCL-Composite were 4.25, 5.37 and 4.25 respectively. These nanomaterials increased at the end of day 45, degradation rate was highest for PCL-BaTiO₃ and PCL-BNNT and then the degradation rates for both were slower after day 45. In the case of PCL-Composite, this similar situation was observed after the day of 60. This may be due to the release of nanomaterials adsorbed in the polymer up to the relevant day for all nanomaterials. In addition, BaTiO₃, BNNT, and composite increased the water affinity of PCL due to having less hydrophobic character compared to PCL.

US stimulation did not make a remarkable difference in degradation rate of PCL scaffolds compared to not US stimulated samples. At the end of 120 days, weight losses (percent) were determined for PCL, PCL-BaTiO₃, PCL-BNNT, and PCL-Composite as 1.56, 4.60, 4.03 and 5.61 respectively. BaTiO₃, BNNT, and composite increased the biodegradation rate of PCL scaffolds owing to increasing water affinity of PCL scaffolds resulted in more hydrolytic degradation of PCL.

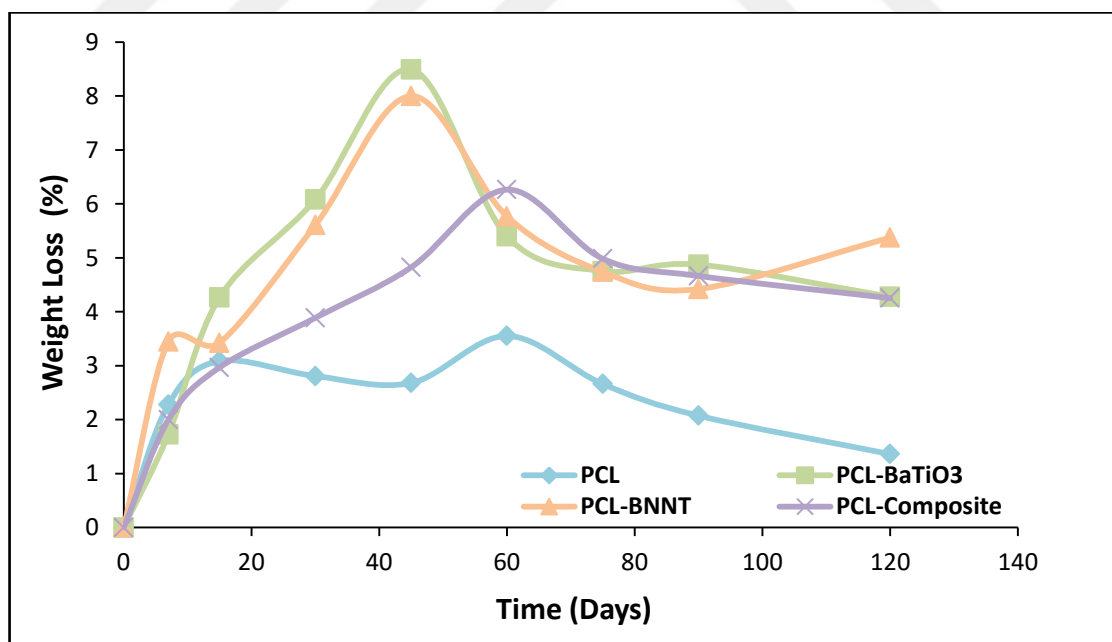


Figure 4.33. Weight loss versus time graphs of PCL and BaTiO₃, BNNT, and composite embedded PCL scaffolds

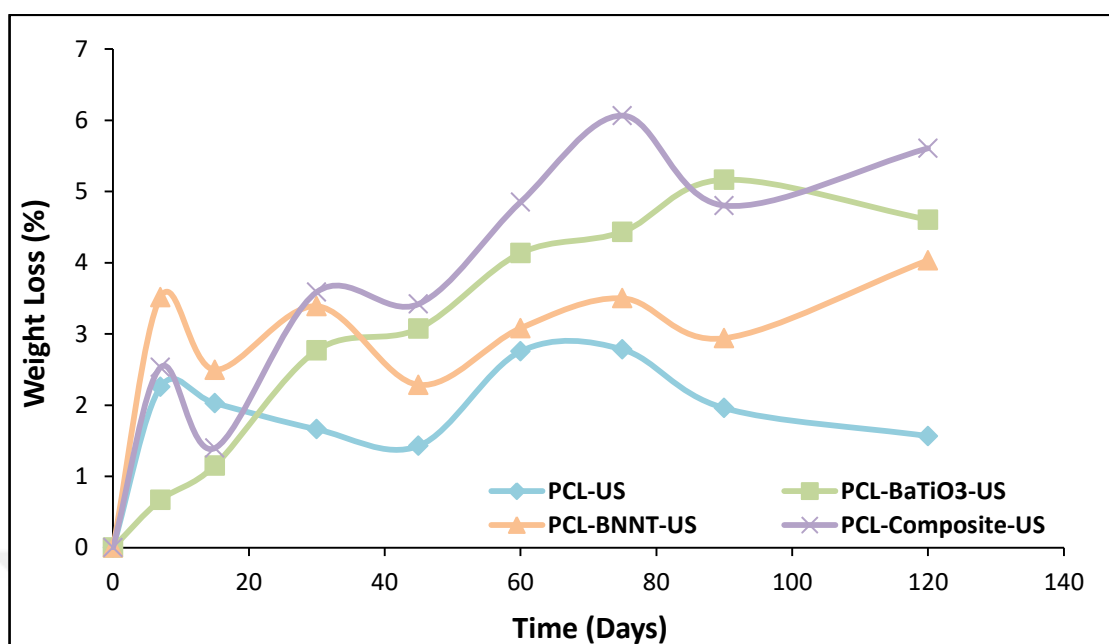


Figure 4.34. Weight loss versus time graphs of US stimulated PCL and BaTiO₃, BNNT, and composite embedded PCL scaffolds

4.4.2. In vitro Degradation of Chitosan Scaffolds via Weight Loss

The fast degradation of chitosan scaffolds is not a desired feature especially in bone tissue engineering applications. Thus, its degradation process can be delayed by crosslinking or by adding reinforcement agents.

Figure 4.35. shows the percentage weight loss of chitosan and BaTiO₃, BNNT, and composite embedded chitosan scaffolds at increasing times. BaTiO₃, BNNT, and composite decreased the degradation rate of chitosan scaffold compared to only chitosan scaffold in all time points. At the end of 120 days, weight loss (percent) of chitosan-BaTiO₃, chitosan-BNNT and chitosan-Composite were 36.17, 31.34, 28.63 and 28.61 respectively. Decreased swelling ratio due to the use of BaTiO₃, BNNT, and composite in chitosan may also positively affect the biodegradation.

The percentage weight loss versus time plots of US stimulated chitosan and BaTiO₃, BNNT, and composite embedded chitosan scaffolds were shown in Figure 4.36. US stimulation did not markedly change the degradation profile of chitosan scaffolds compared to the scaffolds without US stimulated. At the end of 120 days, weight loss

(percent) of chitosan-BaTiO₃, chitosan-BNNT and chitosan-Composite were 35.48, 31.13, 29.10, 29.09 respectively. BaTiO₃, BNNT, and composite decreased the water affinity, thus, decreased swelling ratio of chitosan scaffold, which exhibited decreased degradation profile.

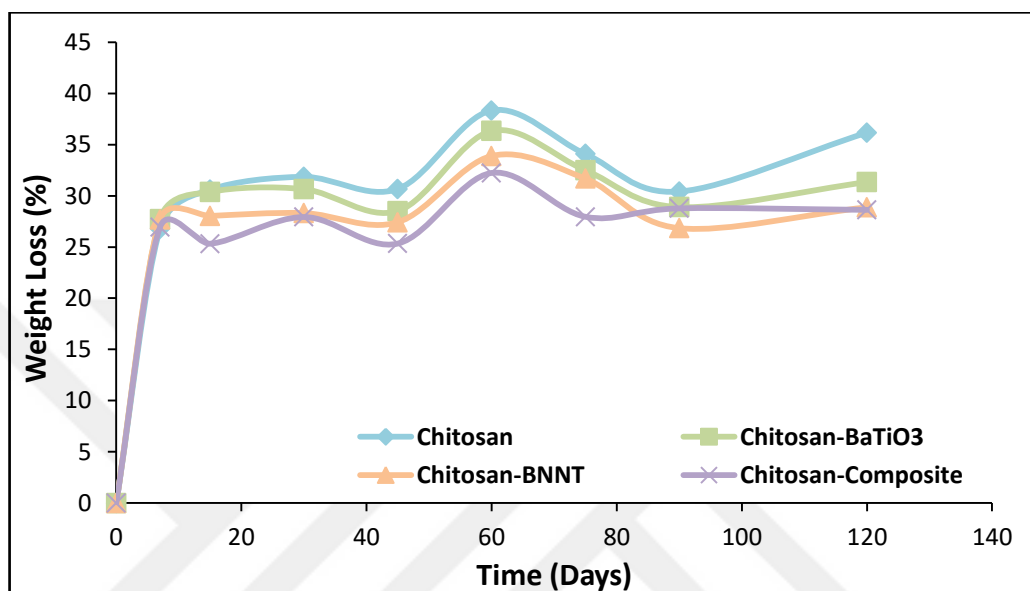


Figure 4.35. Weight loss versus time graphs of chitosan and BaTiO₃, BNNT, and composite embedded chitosan scaffolds

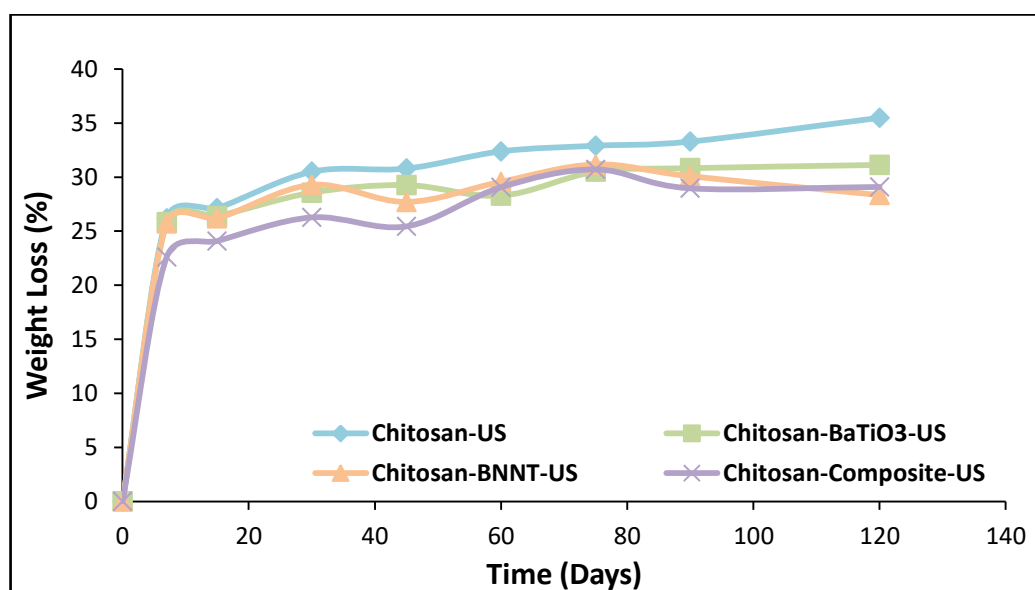


Figure 4.36. Weight loss versus time graphs of US stimulated chitosan and BaTiO₃, BNNT, and composite embedded chitosan scaffolds

4.5. IN VITRO CELL INTERACTION STUDIES

4.5.1. WST-8 Cell Proliferation Assay of Poly Caprolactone (PCL) Scaffolds

WST-8 (Water-Soluble Tetrazolium 8) cell proliferation assay is based on the colorimetric detection of the formazan salts, WST-8, cleaved by mitochondrial dehydrogenase enzymes in viable cells. Proliferation of HOb cells on PCL scaffolds was determined using WST-8 throughout 21 days (Figure 4.37.).

While cell number of US stimulated control group was significantly decreased after 3rd day, cell number of control group which was not US stimulated was decreased after 14th day as seen on Figure 4.37. US stimulation caused a serious cell death when any polymer substrate was not used. However, cell number of HOb cells seeded on PCL scaffolds increased throughout incubation time when both US stimulation were applied and not. Cell number on PCL-BaTiO₃ scaffolds was much more compared to cell number of US stimulated PCL-BaTiO₃ scaffolds. However, cell number on PCL-BNNT and PCL-Composite scaffolds increased when US stimulation were applied. Composite is mainly composed of BNNT and BaTiO₃ which they are known their superior piezoelectric properties. BaTiO₃ only did not provide a proper cell attachment on to PCL scaffolds while BaTiO₃ combined with BNNT, which is called composite, increased cell attachment and proliferation throughout increasing incubation times. US stimulated all scaffolds had higher cell number compared to not US stimulated scaffolds at day 14. Decrease in cell number on PCL scaffolds when US stimulation was applied at 21st day compared to the results of 14th day was due to over proliferation of HOb cells. Since they did not find any blank place to attach and they began to break off the scaffold's surface and die [251].

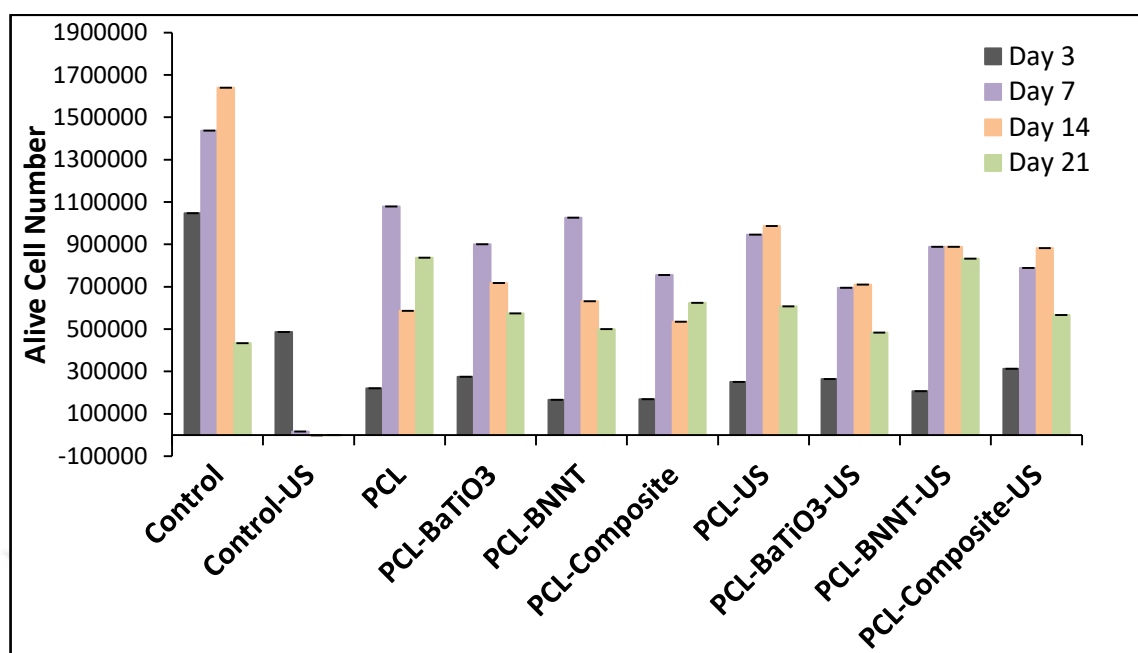


Figure 4.37. Cell proliferation on PCL and BaTiO₃, BNNT, and composite embedded PCL scaffolds

WST-8 was not a proper way to observe HOb proliferation on chitosan scaffolds. Chitosan adsorbed the dye in its structure, thus any color change would not be observed in the medium.

4.5.2. Live/Dead Assay

Live/Dead assay which is a very popular cell staining method was performed to evaluate proportion of live cells and dead cells on chitosan and PCL scaffolds after 7, 14 and 21 days of incubation. Live cells labeled with calcein generated green fluorescence afterward excitation of their cytoplasm and dead cells stained with propidium iodide (PI) which binds to their DNA and fluoresces red.

To compare cell proliferation on PCL and chitosan scaffolds, fluorescence images were analyzed with ImageJ program and relative cellular green fluorescence levels of the HOb cells were determined. Fluorescence intensity levels of cells gives information about their proliferation rates. Background intensity was subtracted not to affect intensity coming from the cell.

4.5.2.1. *Live/Dead Assay of PCL Scaffolds*

All designed PCL scaffolds were nontoxic and very good candidate for cell attachment and proliferation as understood from the excess amount of living cells in the Figures (Figure 4.38., Figure 4.39. and Figure 4.40.) compared to dead cells.

The used nanomaterials caused an increase in cell proliferation both when US stimulation was applied and not applied on the day 7. However, US stimulation provide higher cell viability rate as understood from mean fluorescence intensity levels in Figure 4.38. On all time intervals, even only PCL scaffolds enhanced the cell growth under US stimulation, which is related to the positive response coming from HOB cells to mechanic stimulation. However, it can be said that the composite embedded PCL scaffolds provided better cell growth under US stimulation with increased incubation times.

At the end of day 21, cell proliferation decreased on the surface of BaTiO₃, BNNT, and composite embedded PCL scaffolds compared to only PCL scaffold, since there is no empty place for the cells to adhere and grow, thus cells began to detach from the surface. The use of BaTiO₃, BNNT, and composite resulted in better cell attachment and growth as it reduced the hydrophobicity of PCL scaffolds.

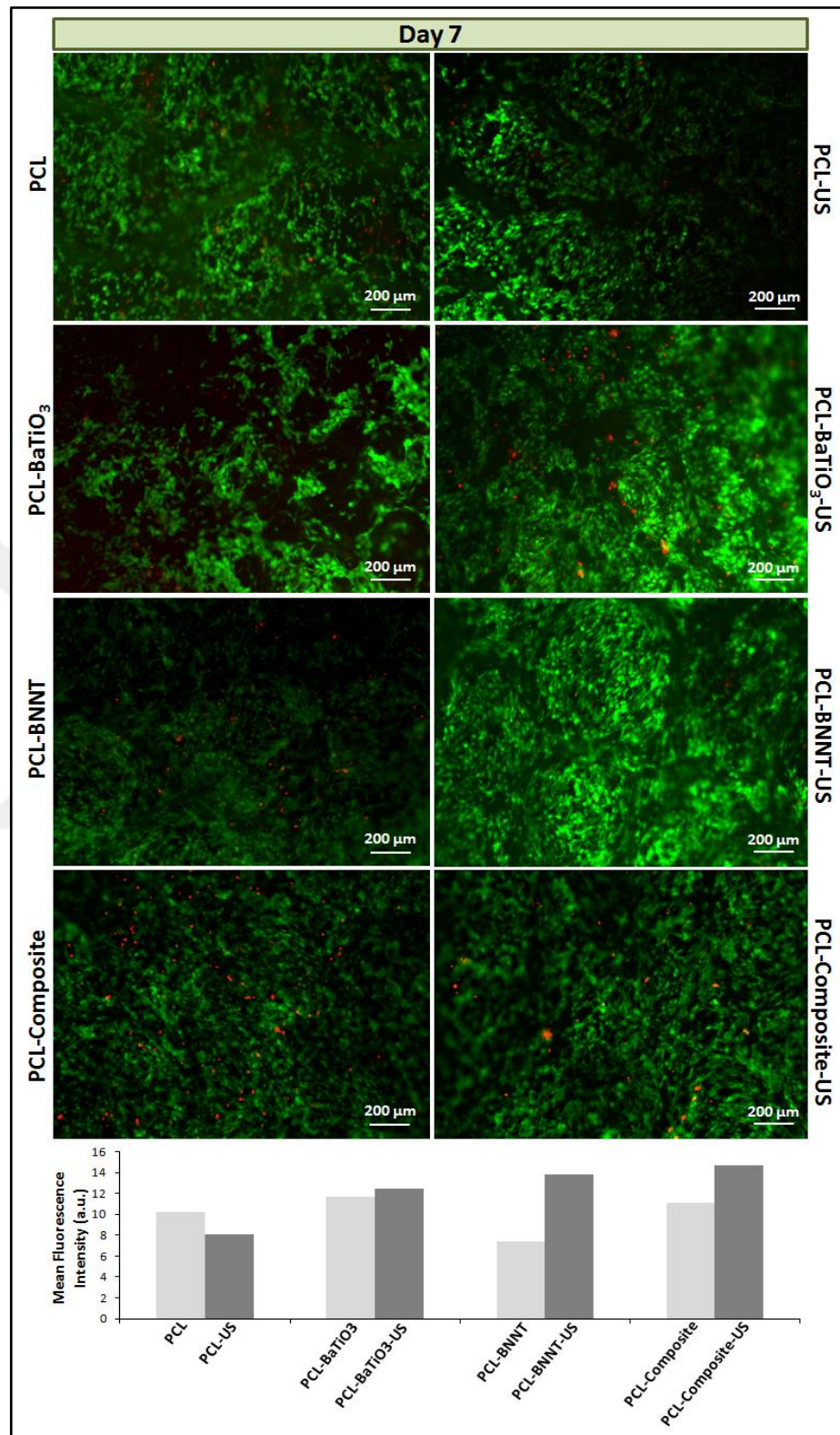


Figure 4.38. Fluorescence images of Live/Dead assay of HOb cells on PCL scaffolds at 7th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

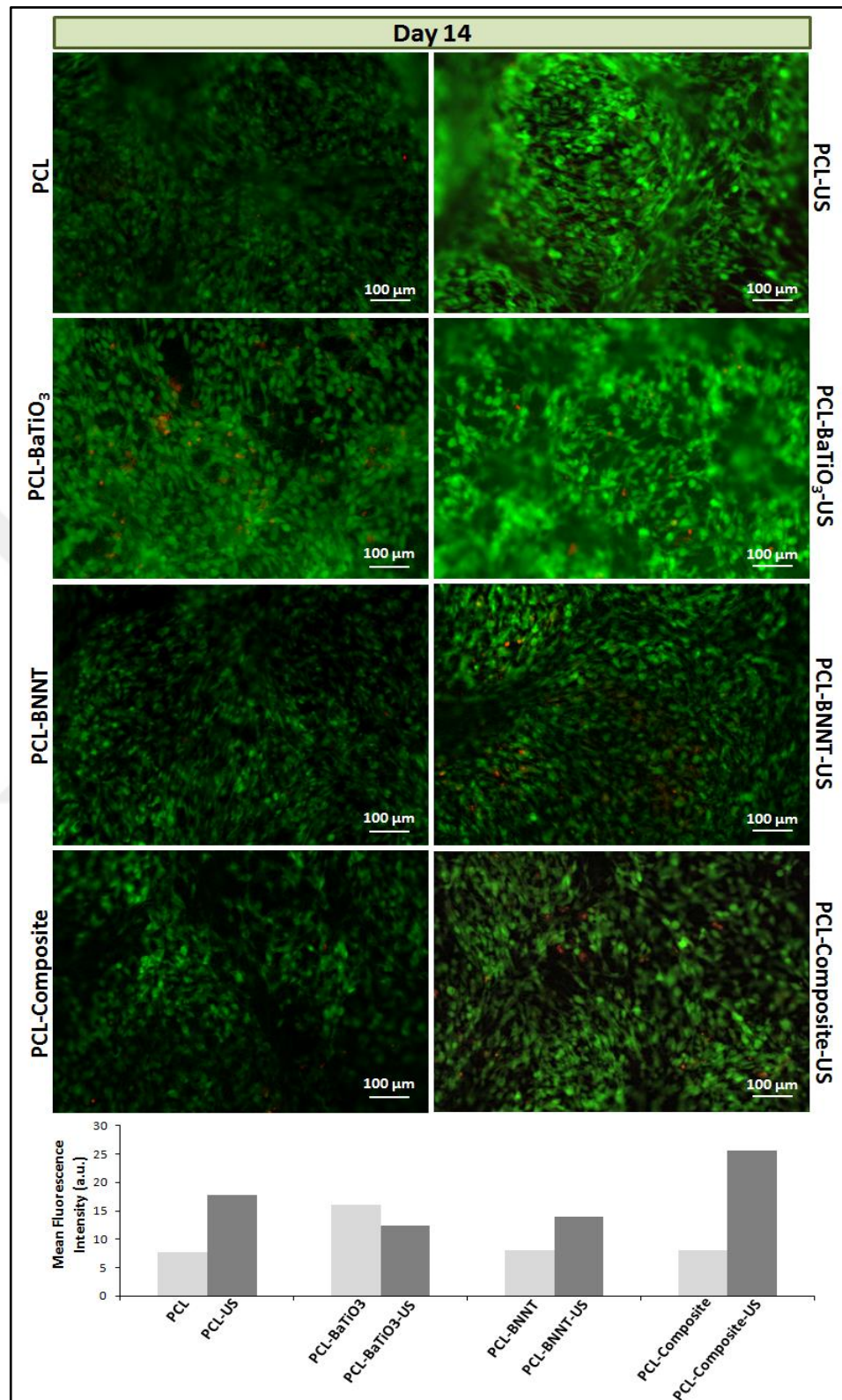


Figure 4.39. Fluorescence images of Live/Dead assay of HOb cells on PCL scaffolds at 14th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

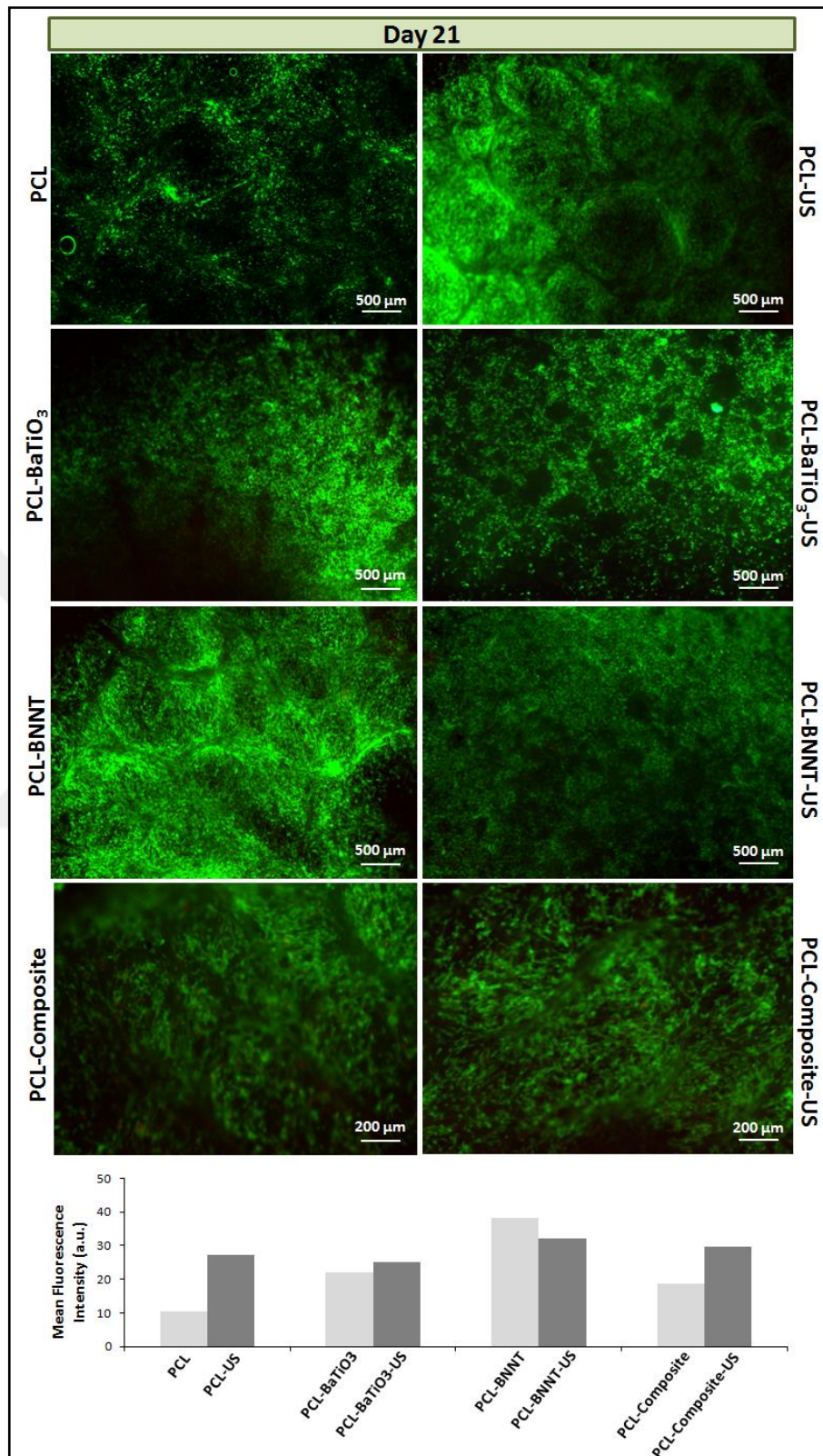


Figure 4.40. Fluorescence images of Live/Dead assay of HOb cells on PCL scaffolds at 21st day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

4.5.2.2. Live/Dead Assay of Chitosan Scaffolds

HOb cells on chitosan scaffolds tended to migrate through the scaffold as seen in Figure 4.41. and Figure 4.42. The migration of HOb cells on chitosan scaffolds can be explained by their larger pore sizes. In addition, cell migration can be promoted by mechanical property of matrix, which is known as mechanotaxis.

Green fluorescence mainly radiated under the surface of chitosan scaffold until day 14. However, after 21 days of incubation, seeded HOb cells were observed on the surface of designed chitosan scaffolds. Moreover, cell number on US stimulated BaTiO₃, BNNT, and composite embedded chitosan scaffolds increased compared to scaffolds not stimulated with US at the time points of 21st day as illustrated in fluorescence images and also mean fluorescence intensity graph in Figure 4.43. Mean fluorescence intensities were only determined for day 21 samples because on the other incubation times cells were generally migrated through the scaffolds and they were not on the surface. US stimulation exploded electric signals due to the piezoelectric features of BaTiO₃, BNNT, and composite in chitosan, which contributed cell attachment and growth to electric responsive HOb cells. At the end of day 21, cells were mostly observed on chitosan scaffolds when US stimulation was applied compared to not stimulated chitosan scaffold. This effect can be attributed to mechanotaxis of cells promoted by the US stimulation.

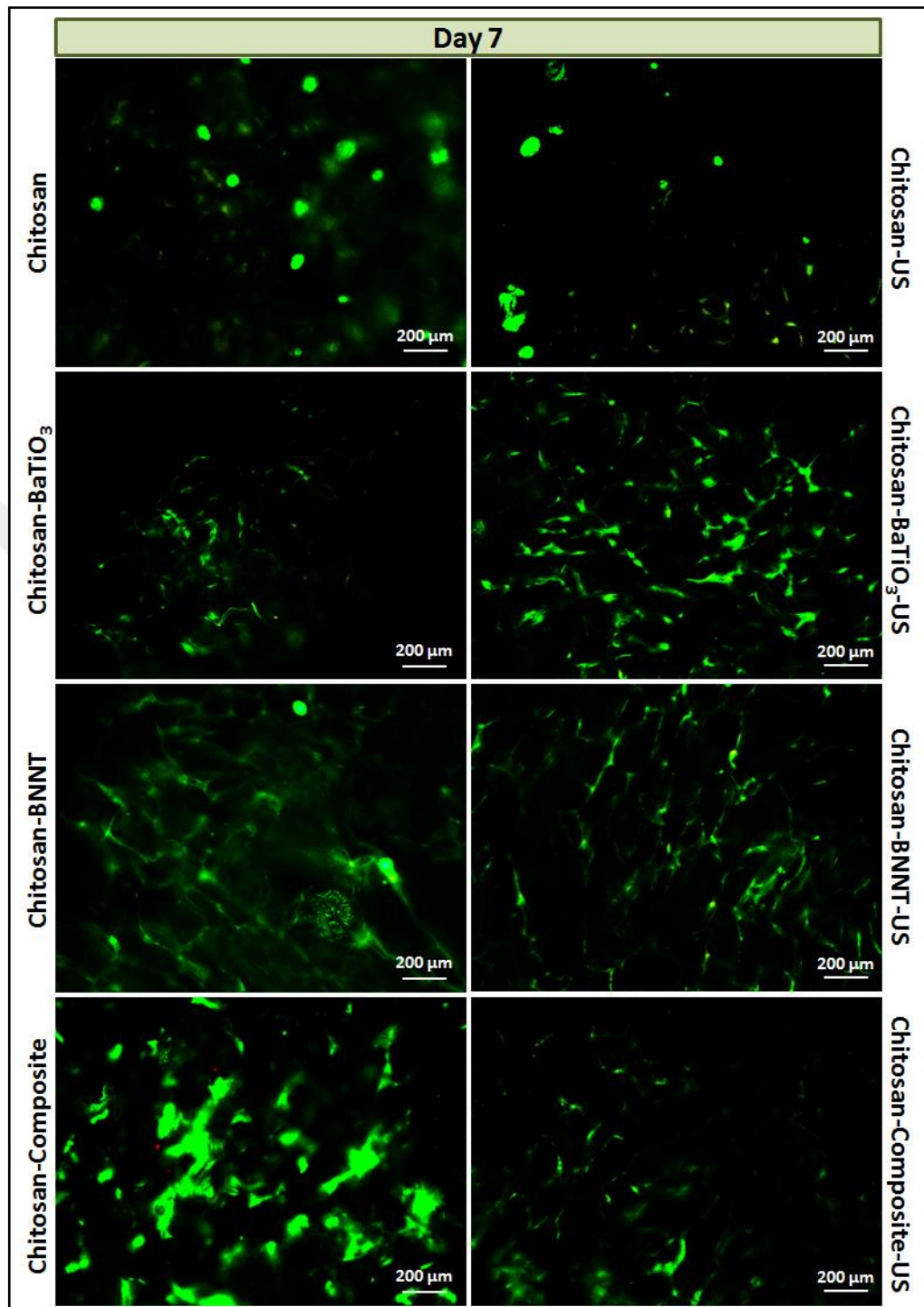


Figure 4.41. Fluorescence images of Live/Dead assay of HOb cells on chitosan scaffolds at 7th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

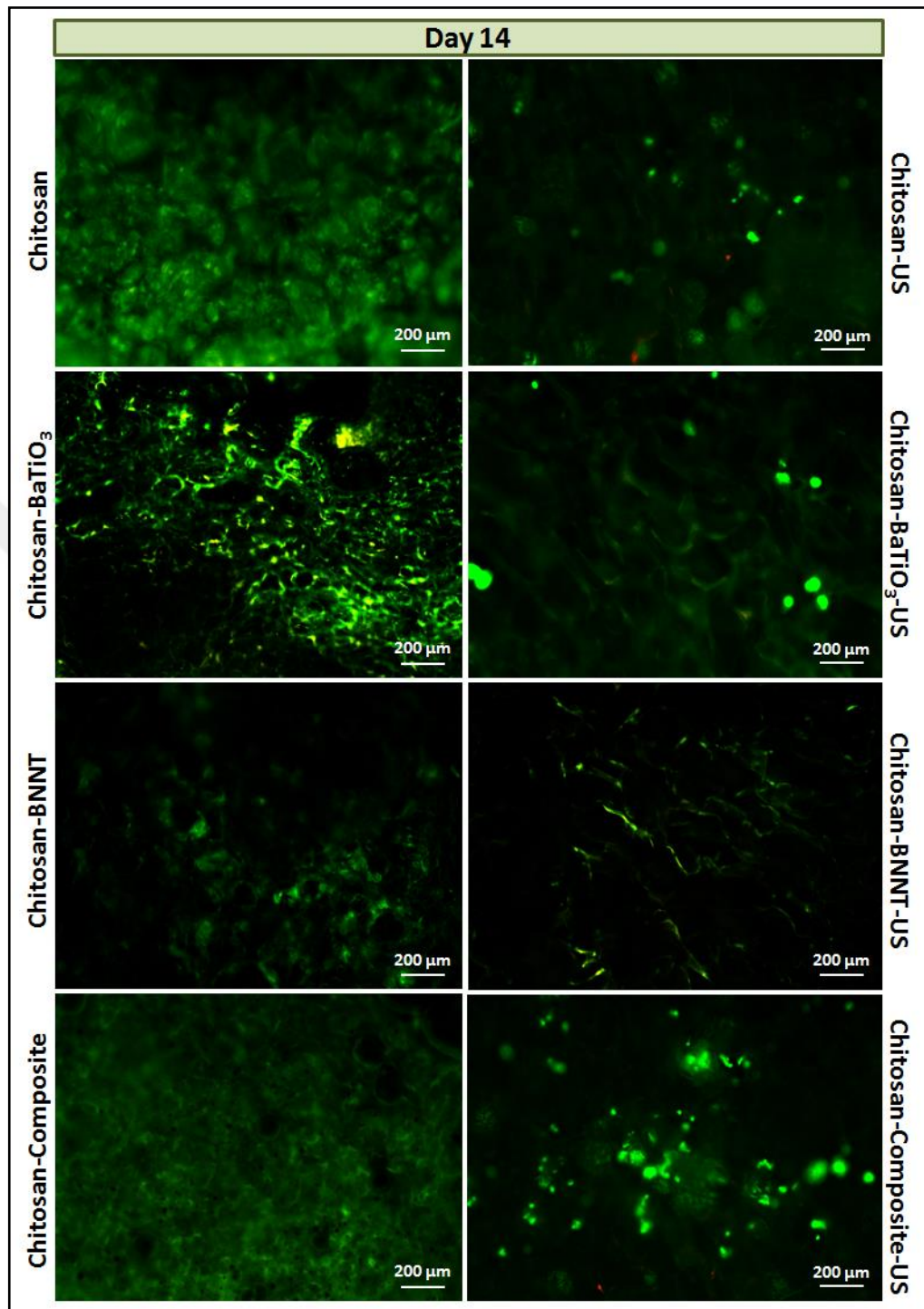


Figure 4.42. Fluorescence images of Live/Dead assay of HOb cells on chitosan scaffolds at 14th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

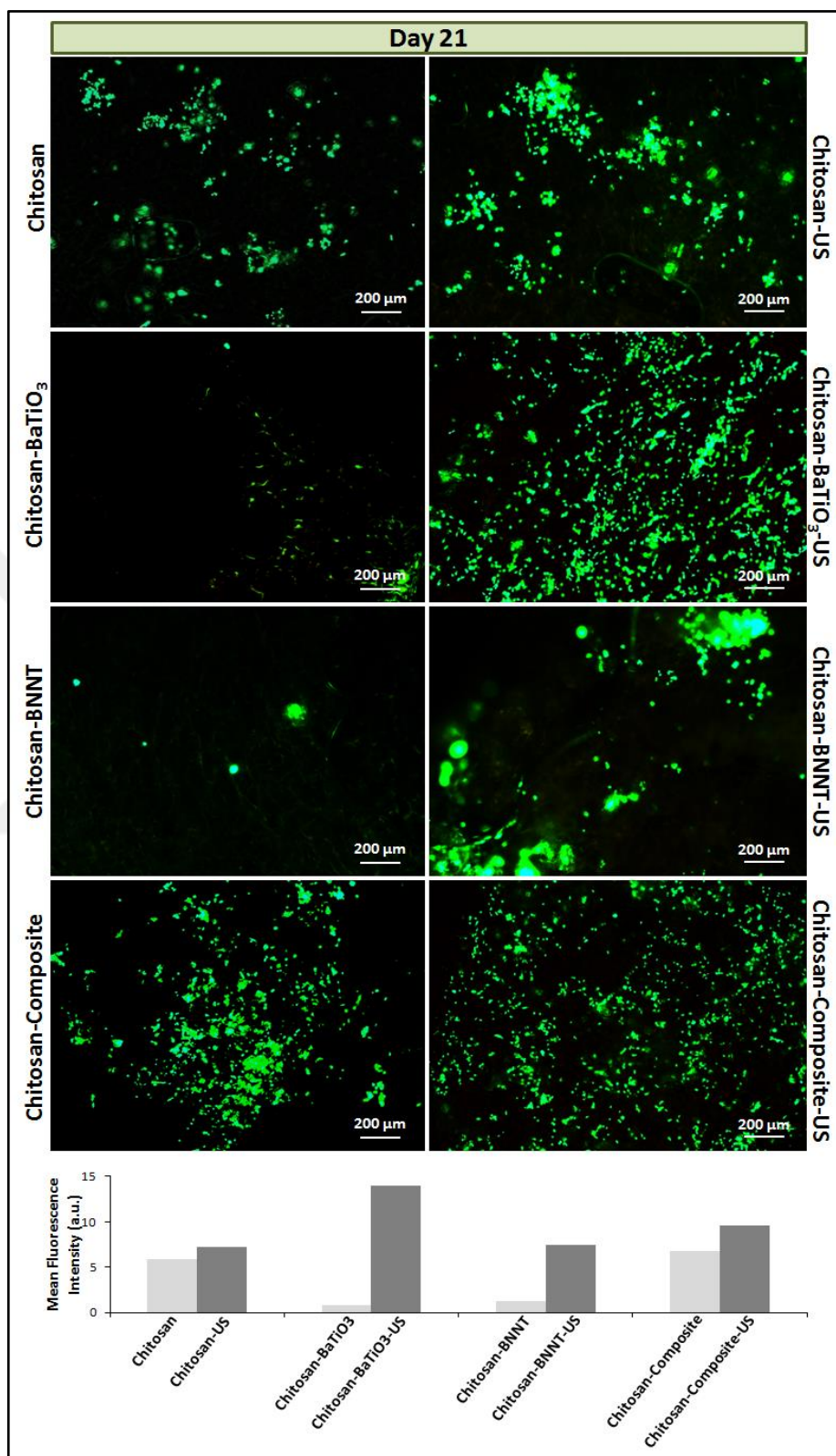


Figure 4.43. Fluorescence images of Live/Dead assay of HOb cells on chitosan scaffolds at 21st day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

4.5.3. Scanning Electron Microscopy (SEM) Analysis

In order to investigate morphology, attachment and proliferation characteristics of HOb cells on designed PCL and chitosan scaffolds, Scanning electron microscopy (SEM) were performed at the time points of 7th, 14th, 21st and 28th days as represented in from Figure 4.44. to Figure 4.47.

4.5.3.1. Scanning Electron Microscopy (SEM) Analysis of PCL Scaffolds

Cell proliferation and propagation were extensively increased on all designed PCL scaffolds as indicated from Figure 4.44. to Figure 4.47. HOb cells are adherent cells that grow on a scaffold. HOb cells are adherent cells that grow and proliferate rapidly on a surface they like. The observed morphologies of HOb cells were typically, which covered polymer surfaces in the form of layers. High amount of extracellular matrix were observed on all types of scaffolds. SEM images at day 7 showed that HOb cells on US stimulated, BaTiO₃, BNNT, and composite embedded PCL scaffolds proliferated and spreaded rapidly. In the following days, this high proliferation and spread were observed on all PCL scaffolds. Before day 28, all surfaces were confluent, while detachments were also observed.

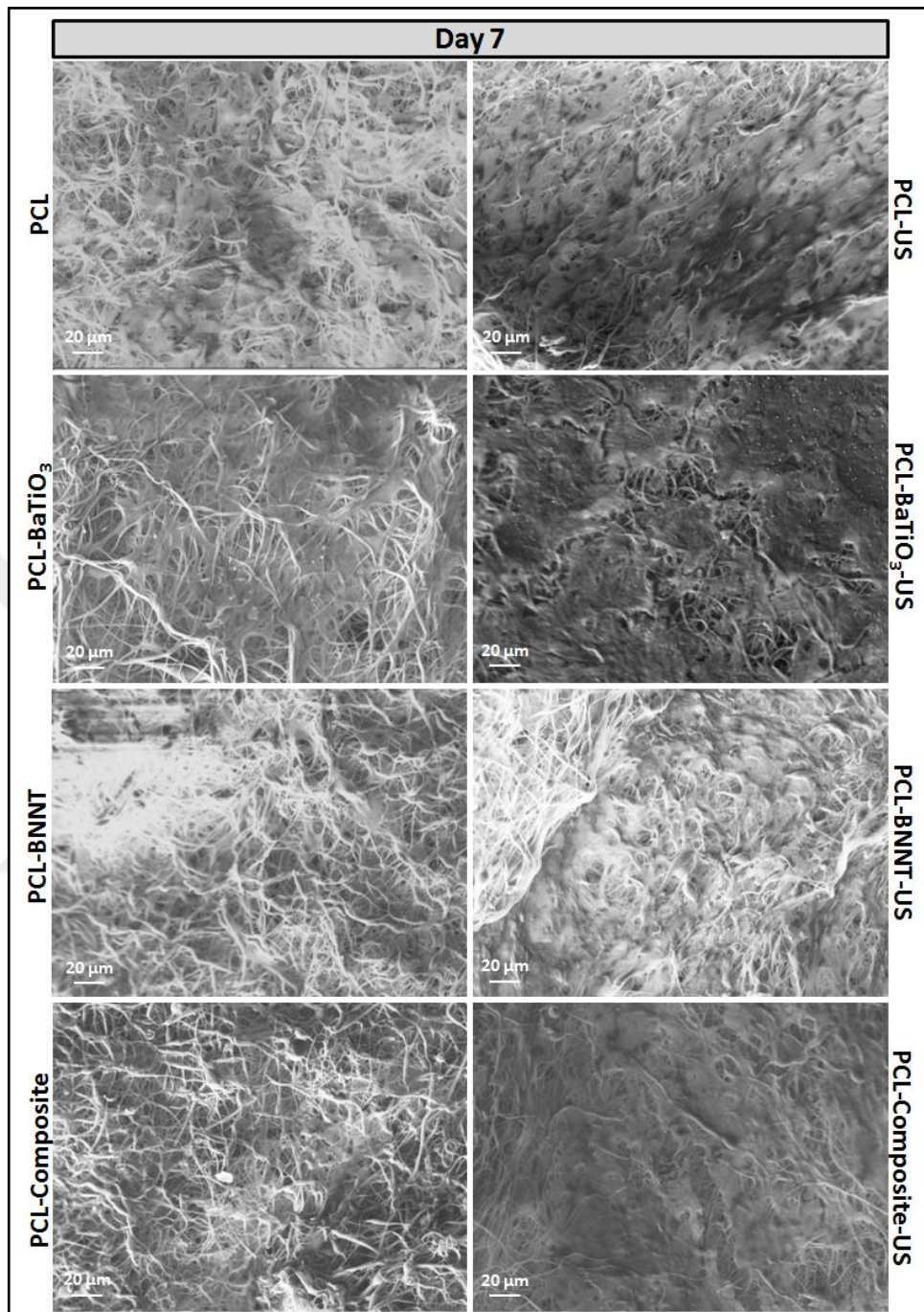


Figure 4.44. Scanning electron microscopy images of HOb cells on PCL scaffolds at 7th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation

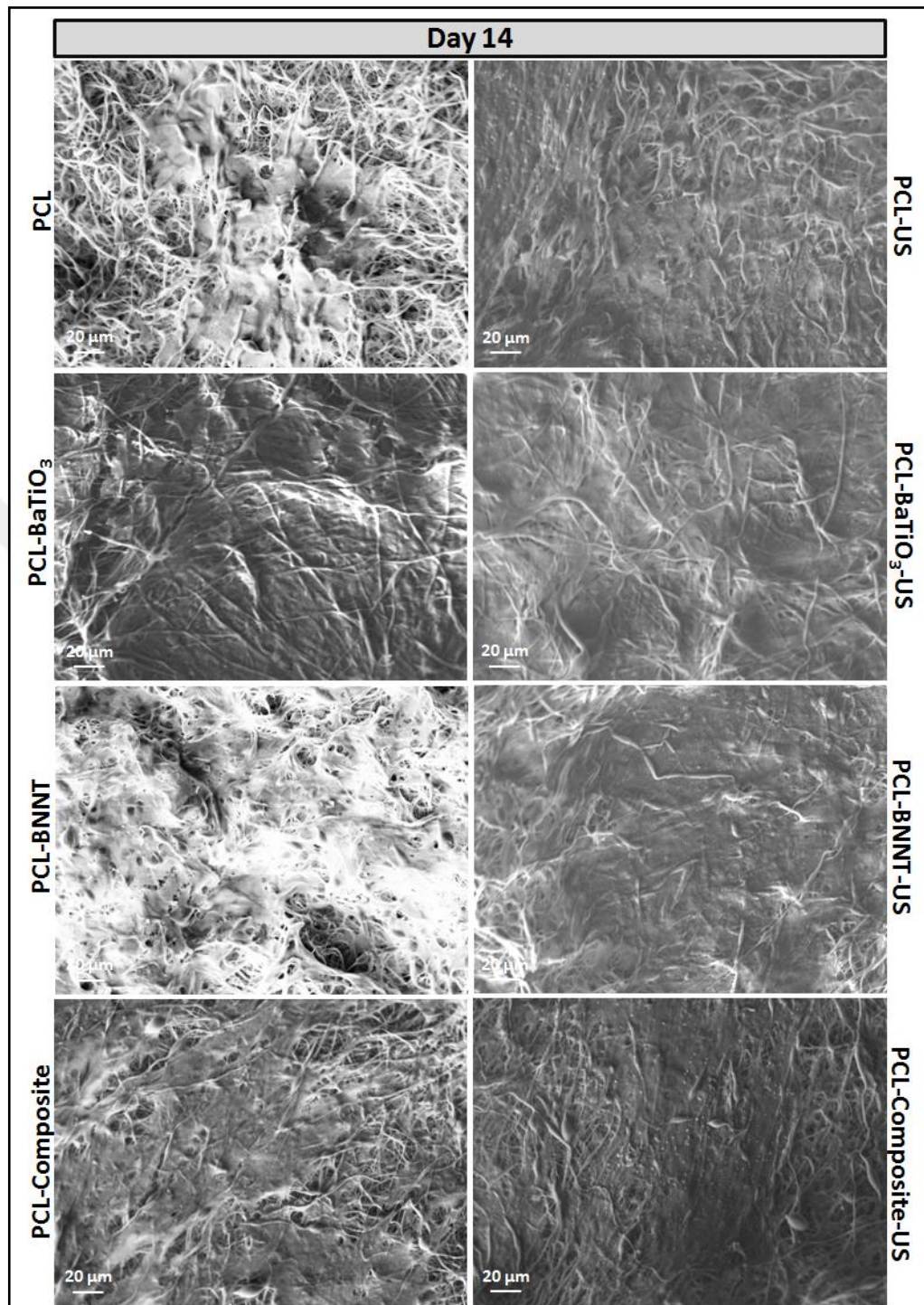


Figure 4.45. Scanning electron microscopy images of HOb cells on PCL scaffolds at 14th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation

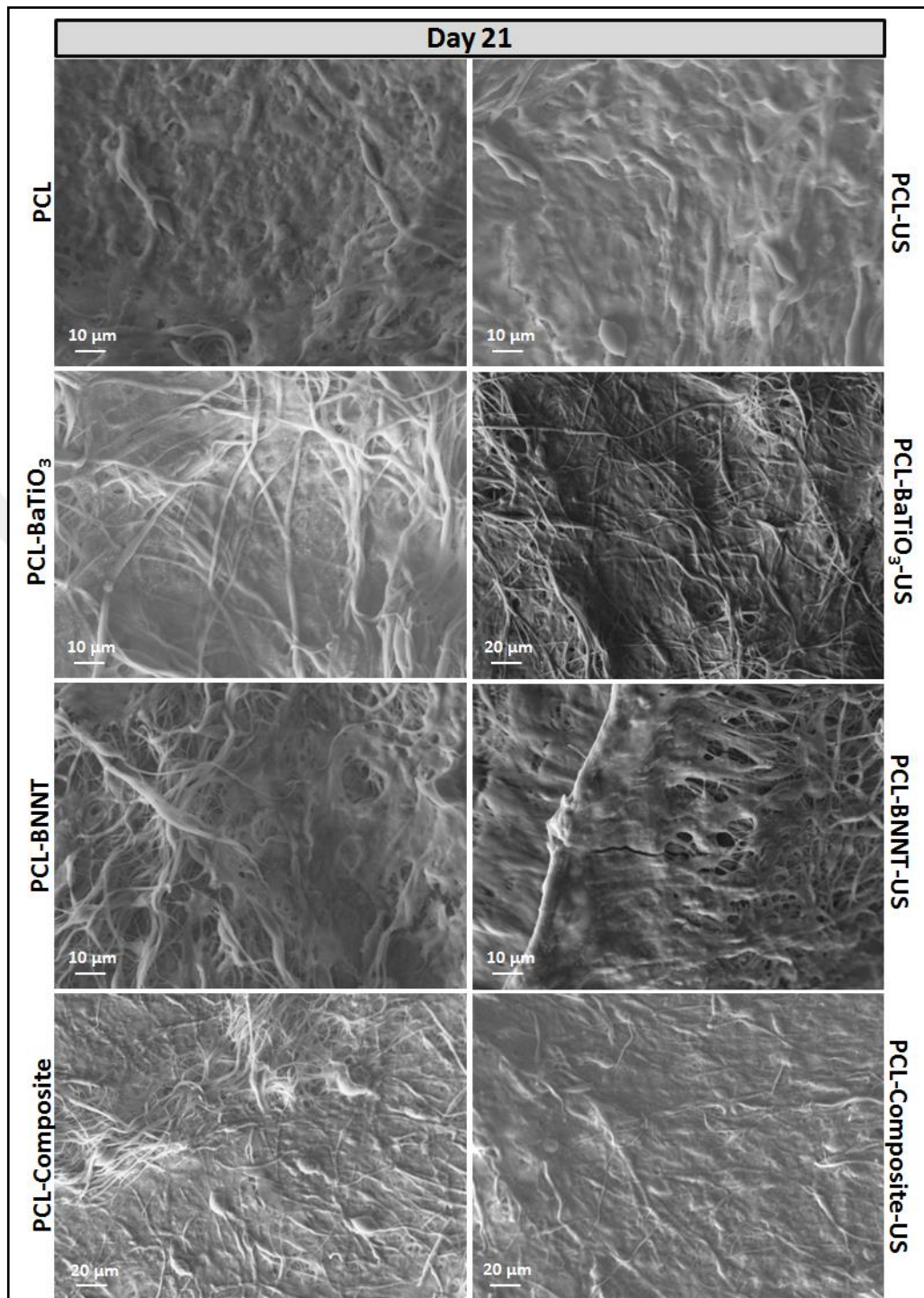


Figure 4.46. Scanning electron microscopy images of HOb cells on PCL scaffolds at 21st day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation

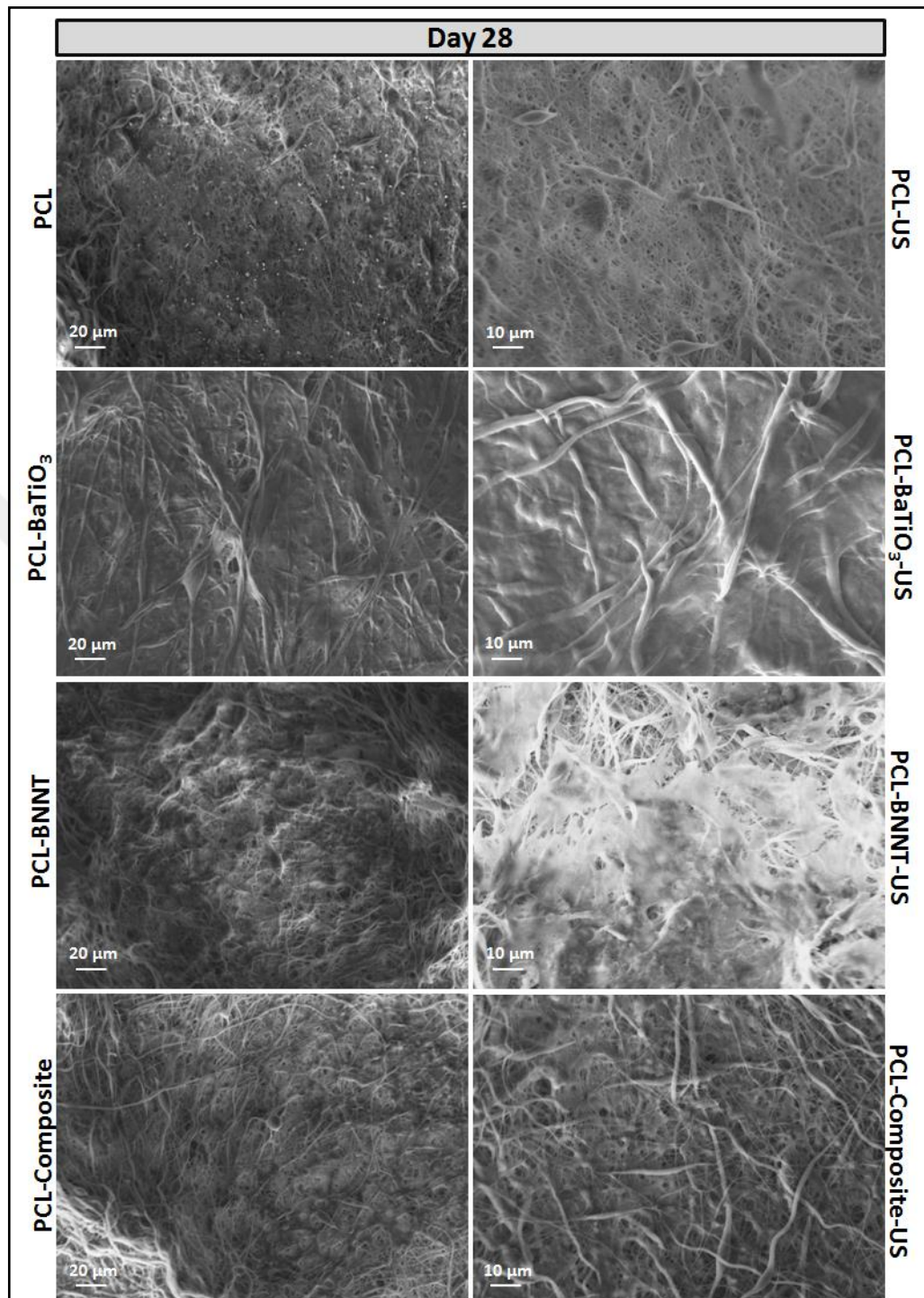


Figure 4.47. Scanning electron microscopy images of HOb cells on PCL scaffolds at 28th day. Left column represents HOb cells on PCL scaffolds without US stimulation and right column represents HOb cells on PCL scaffolds with US stimulation

4.5.3.2. Scanning Electron Microscopy (SEM) Analysis of Chitosan Scaffolds

Chitosan has been reported to increase mineralization in osteoblast cells by regulating related genes [252]. Differently from HOb cells on PCL scaffolds, increased bone mineralization were observed on chitosan scaffolds. Although chitosan triggers the formation of bone mineralization, the minerals formed on BaTiO₃, BNNT, and composite embedded chitosan scaffolds were considerably higher when US stimulation was applied. In addition, US stimulation increased the cell attachment and proliferation at the time intervals of 14th, 21st and 28th days. Cells were observed more clearly on chitosan scaffold at the end of day 28 correlated with the Live/Dead assay results.



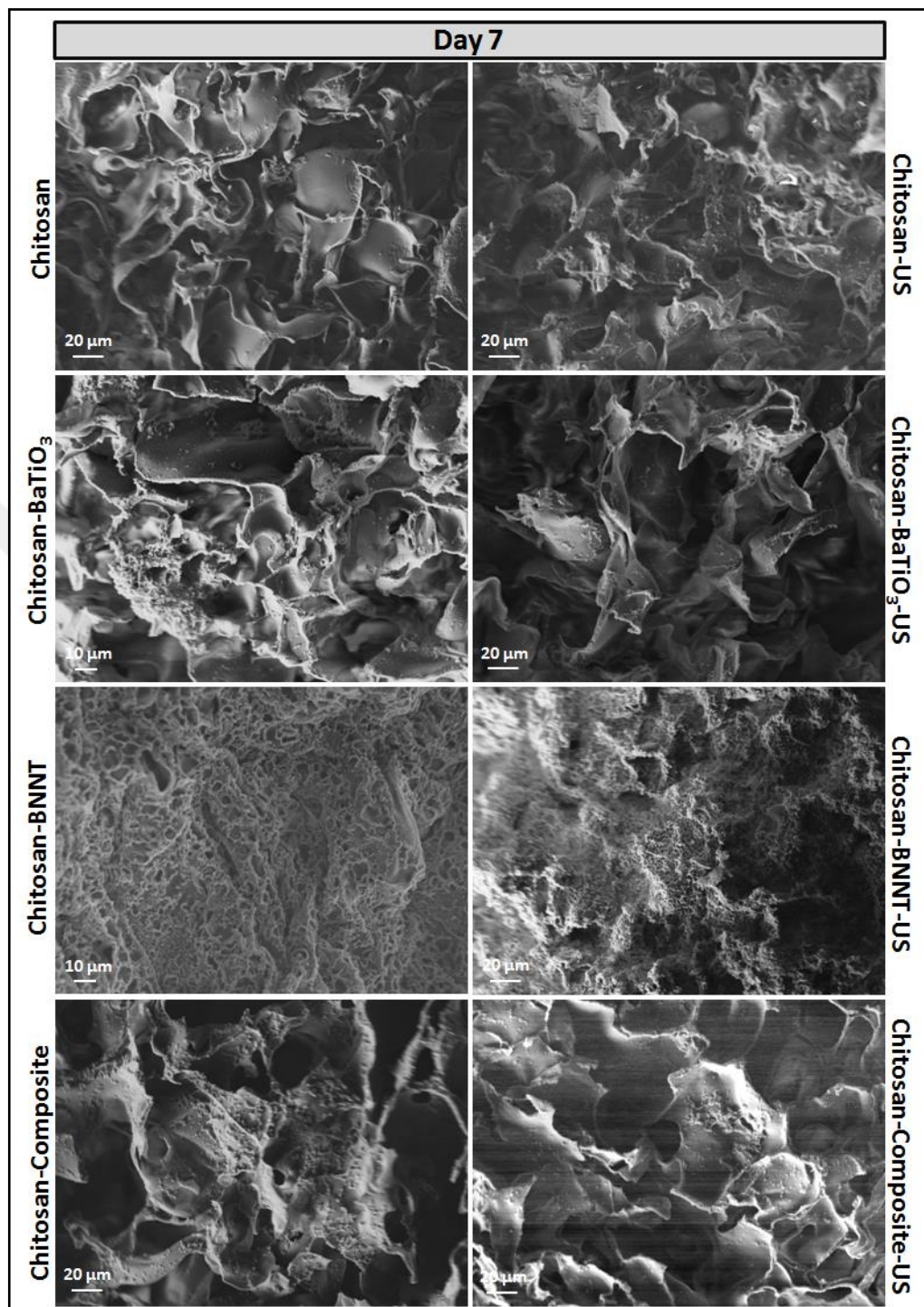


Figure 4.48. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 7th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

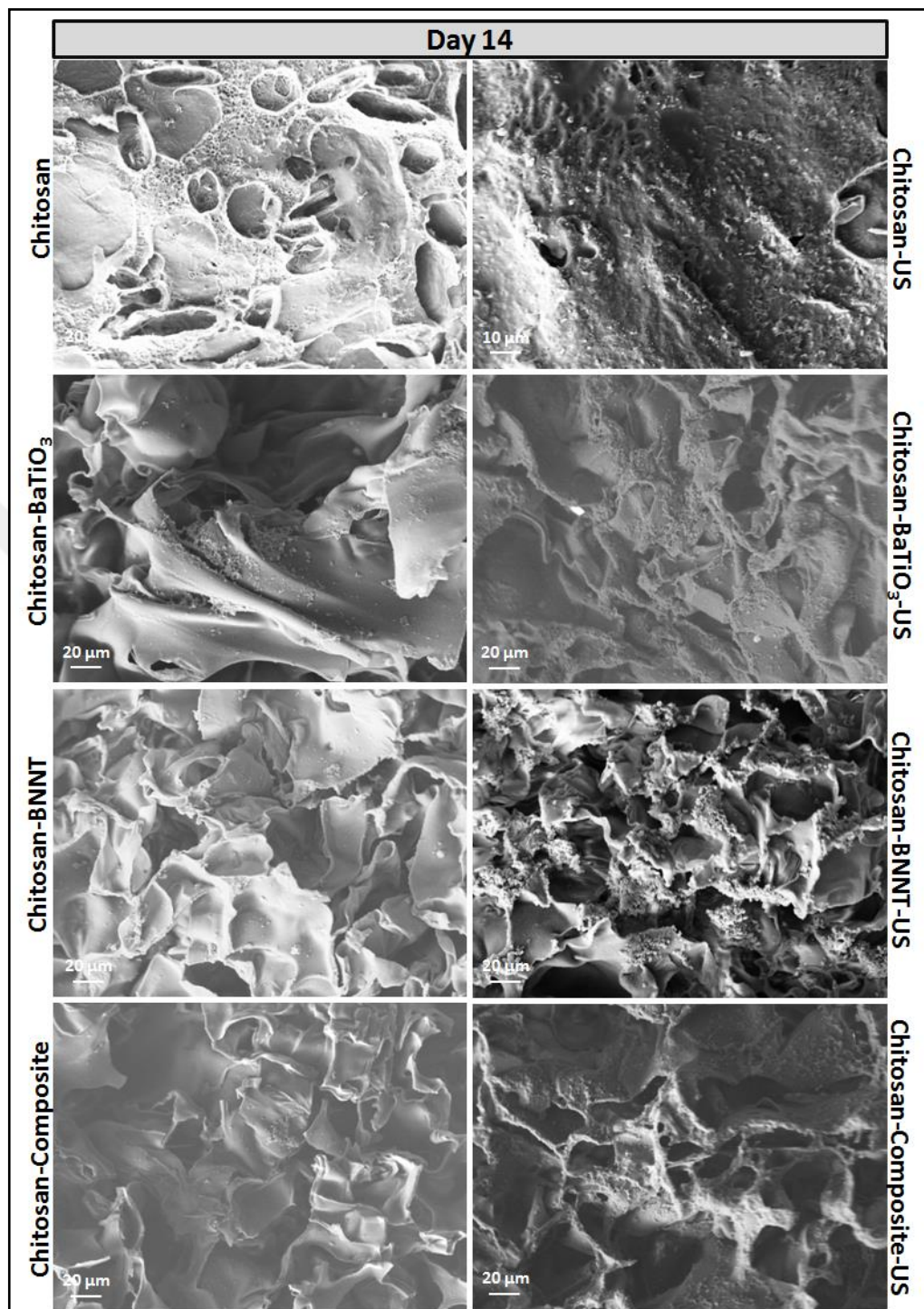


Figure 4.49. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 14th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

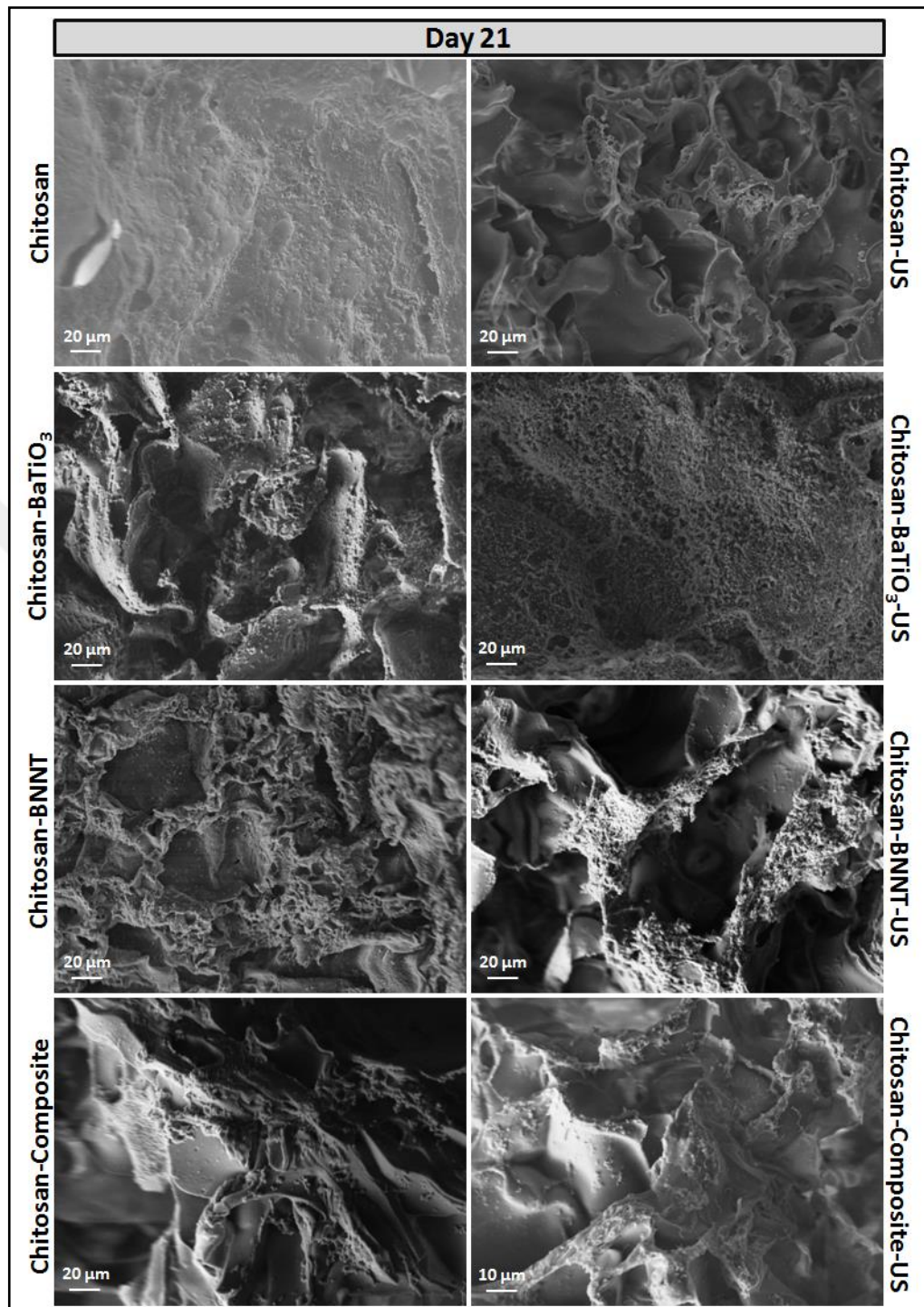


Figure 4.50. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 21st day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation

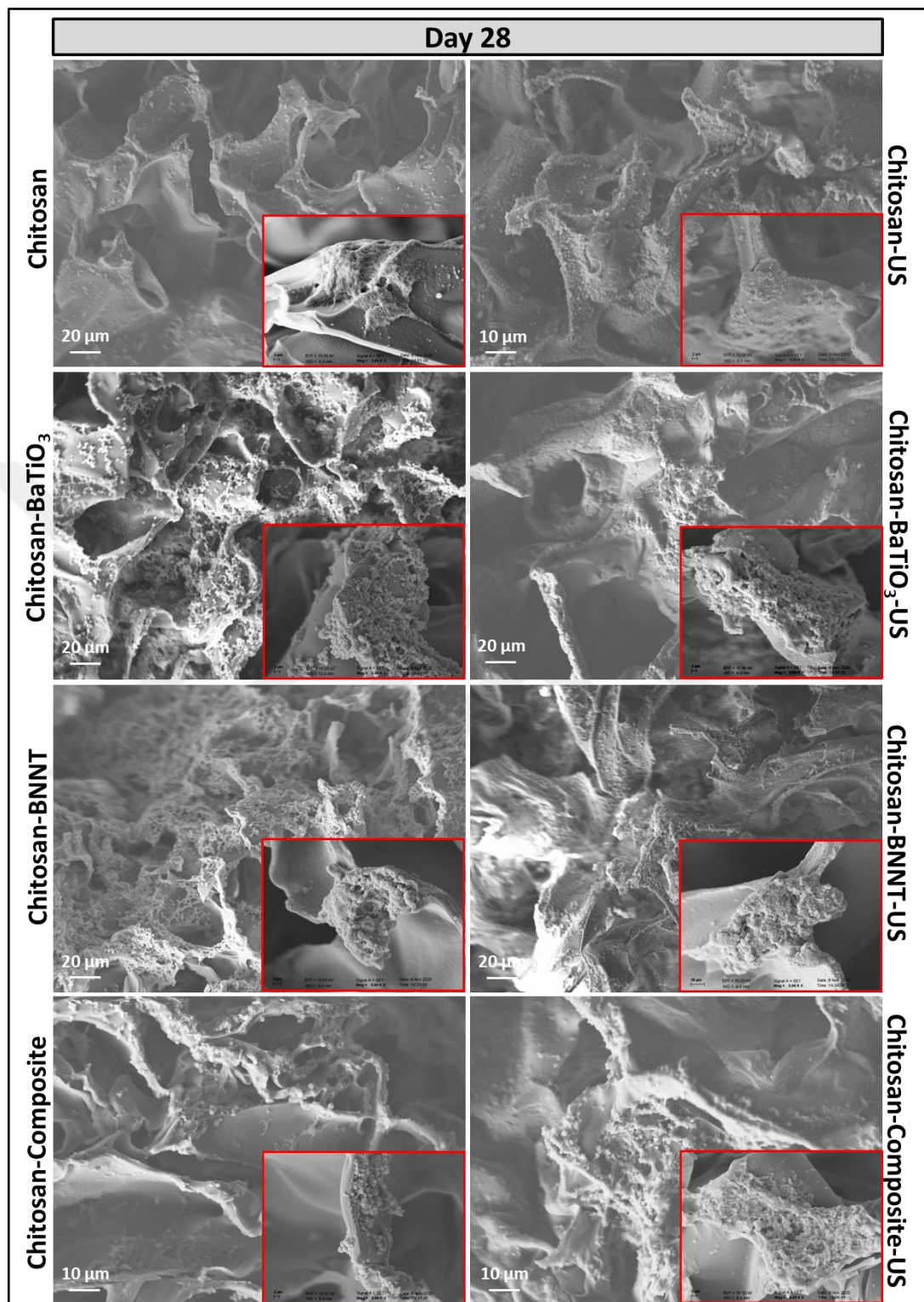


Figure 4.51. Scanning electron microscopy images of HOb cells on chitosan scaffolds at 28th day. Left column represents HOb cells on chitosan scaffolds without US stimulation and right column represents HOb cells on chitosan scaffolds with US stimulation. Closer images of the cells on the scaffolds are surrounded by red line

4.5.4. Alkaline Phosphatase (ALP) Activity Analysis

Alkaline phosphatase (ALP), found throughout the body is one kind of membrane-bound tetrameric enzyme which linked to glycosyl-phosphatidylinositol moieties located on outer surface of cell. ALP has a remarkable role in osteoid formation and mineralization of bone [253]. ALP hydrolyzes inorganic phosphate responsible which is a naturally occurring inhibitor responsible for mineralization. In addition, ALP is responsible for producing inorganic phosphate from pyrophosphate and organic phosphomonoesters for the production of hydroxyapatite [254].

4.5.4.1. Alkaline Phosphatase (ALP) Activity Analysis of PCL Scaffolds

Alkaline phosphates activities of PCL and BaTiO₃, BNNT, and composite embedded PCL scaffolds with and without ultrasound stimulated (US) were illustrated in Figure 4.52. Alkaline phosphatase is known as early marker in bone formation. Therefore, an increase until 7th day was observed in all types of scaffolds and a decrease from 7th day to 21st day and again an increase from 21st day to 28th day were clearly observed. This is related to the growth division cycle of osteoblast cells, which indicates that concentration change in ALP activity is cyclic [255]. Higher ALP activity marks the maturation of bone matrix.

In the case of scaffolds without US stimulation, an increase was observed in ALP concentration on BaTiO₃, BNNT, and composite embedded PCL scaffolds on day 7 compared to only PCL scaffold. At the end of day 14 and 21, ALP activity of cells on PCL-Composite was higher than PCL-BaTiO₃ and PCL-BNNT. ALP concentration was higher in PCL-Composite than PCL, PCL-BaTiO₃, and PCL-BNNT at 28th day.

PCL-Composite samples showed an increase in ALP concentration level when US stimulated at 28th day. Composite includes both BaTiO₃ and BNNT in it, thus, this result might be due to increased piezoelectric property which enhanced ALP concentration and supported osteogenesis.

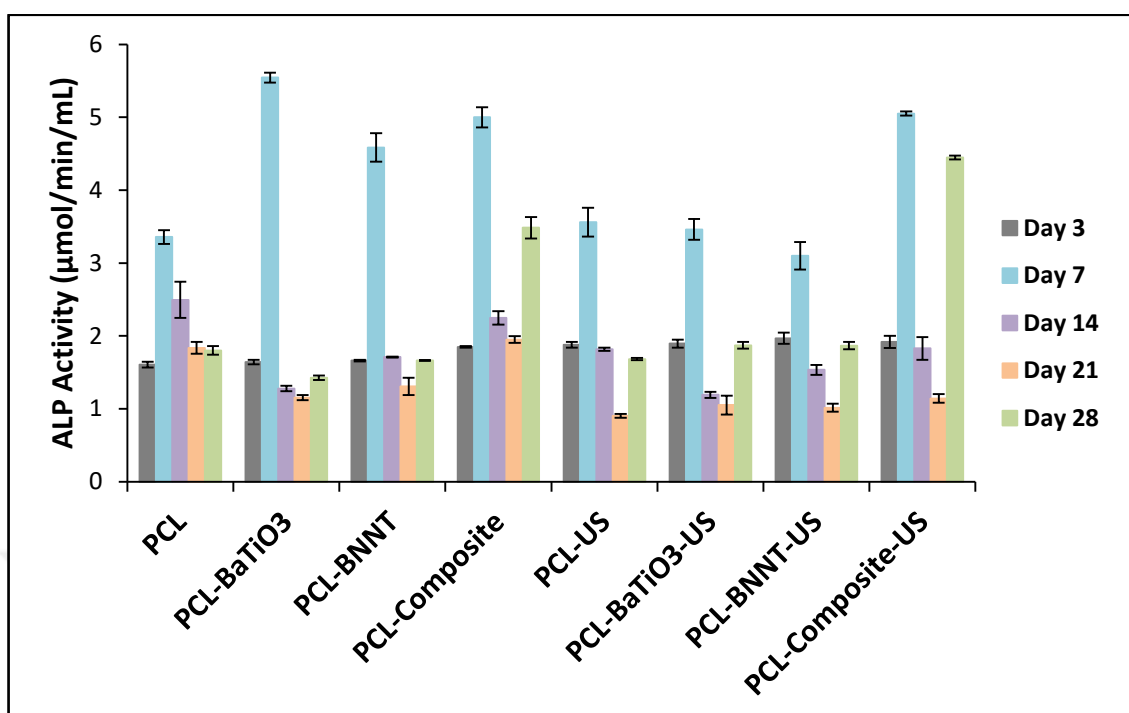


Figure 4.52. Alkaline phosphatase activity of HOb cells throughout 28 days of incubation on PCL scaffolds

4.5.4.2. Alkaline Phosphatase (ALP) Activity Analysis of Chitosan Scaffold

Alkaline phosphates activities of chitosan and BaTiO₃, BNNT, and composite embedded scaffolds and ultrasound stimulated (US) scaffolds were illustrated in Figure 4.53. ALP activity was top at day 3 and they all exhibited almost similar ALP activity. A faster decrease was then observed in only chitosan and chitosan-US scaffolds at day 7 compared to other types of scaffolds. ALP level of chitosan scaffold remained almost similar at day 14 and 21 compared to day 7. ALP activity was higher in all types of scaffolds compared to only chitosan scaffolds at day 7-28. HOb cells on only chitosan scaffolds responded to US stimulation considering their ALP activities throughout 28 days. This increase may be attributed to being mechanical stress-sensitive of HOb cells [256].

An increase in ALP concentration was observed when chitosan-BNNT and chitosan-Composite were stimulated with US compared to when they were not stimulated at day 21 and 28.

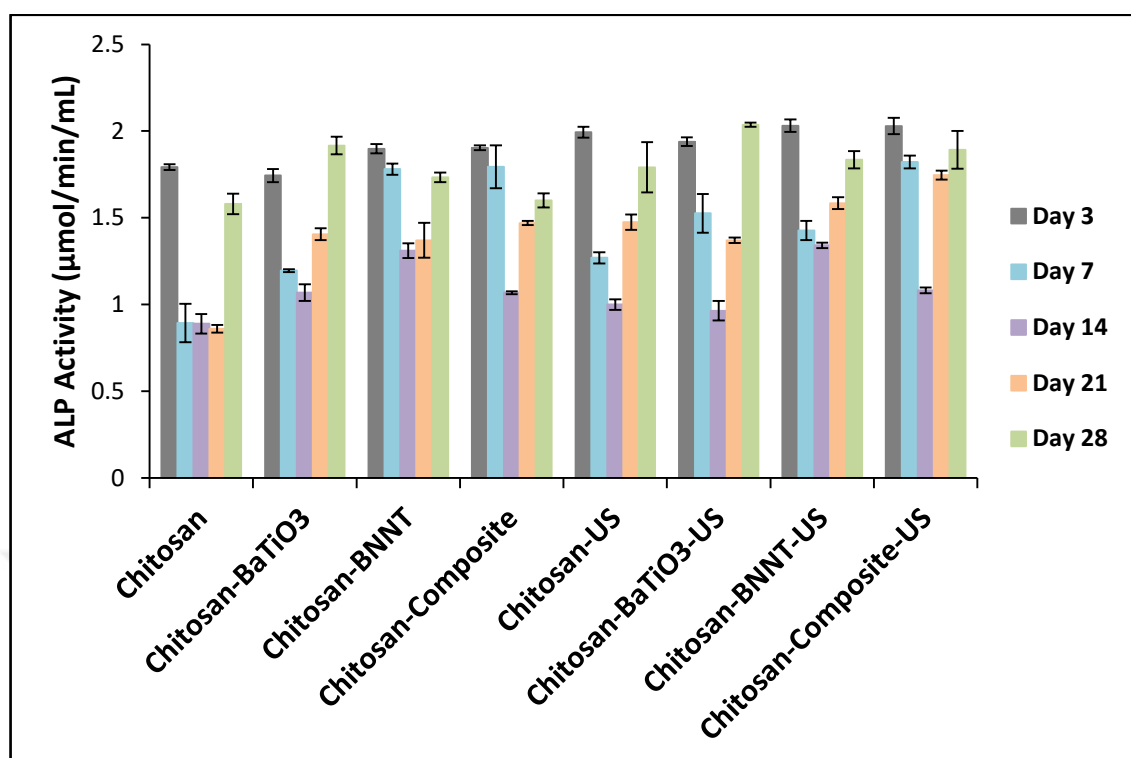


Figure 4.53. Alkaline phosphatase activity of HOb cells throughout 28 days of incubation on chitosan scaffolds

4.5.5. Determination of Mineralization by von Kossa Staining

Determination of calcium deposits on PCL and chitosan scaffolds were detected by von-Kossa staining. This method is based on a precipitation reaction between phosphate and silver ions in the presence of acidic material. Then, silver phosphate photochemically degraded to metallic silver under UV light. The darkness of the color in the scaffolds indicates the calcium deposits.

Mineral depositions on PCL and chitosan scaffolds and US stimulated PCL and chitosan scaffolds were observed by von Kossa staining. Digital images of all scaffolds were taken for observation.

4.5.5.1. Determination of Mineralization on PCL Scaffolds by von Kossa Staining

Front and back images of stained PCL scaffolds with and without US stimulation at day 7, 14, 21 and 28 are presented in Figure 4.54.-4.57. Calcium depositions seen as black accumulations were intensely observed on only PCL and PCL-BNNT scaffolds at day 7

and were the most when stimulated with US (Figure 4.54.). US stimulated HOOb cells on only PCL scaffold showed an increase in calcium depositions, which can be due to response to mechanical irradiation of mechanical-stress sensitive HOOb cells [256].

14th (Figure 4.55.) and 21st (Figure 4.56.) days showed an increase on PCL-BNNT and PCL-Composite and mineral deposits were much more when US applied to that scaffolds. The calcium deposition increase in PCL-BNNT and PCL-Composite at increasing days when US stimulated is correlated to ALP activity results. ALP plays an essential role in extracellular matrix mineralization by locally increasing the concentration of phosphate, which is called as mineralization promoter.

Black accumulations were less on BaTiO₃ embedded PCL scaffolds when applied US on all days. However, composite, composed of BNNT and BaTiO₃, showed more calcium deposition. This can be explained via BNNT formation in composite. The results showed that BNNT enhanced the mineralization.

At the end of 28 days of incubation calcium deposits were seen slightly, which is due to over proliferation of HOOb cells (Figure 4.57.). Cells began to detach from the surface that is why black mineral accumulations were significantly decreased.

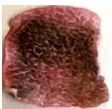
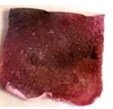
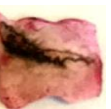
Day 7	PCL		PCL-BaTiO ₃		PCL-BNNT		PCL-Composite	
	Front	Back	Front	Back	Front	Back	Front	Back
Without US Stimulation								
US Stimulated								

Figure 4.54. Von Kossa staining images of PCL scaffolds at day 7

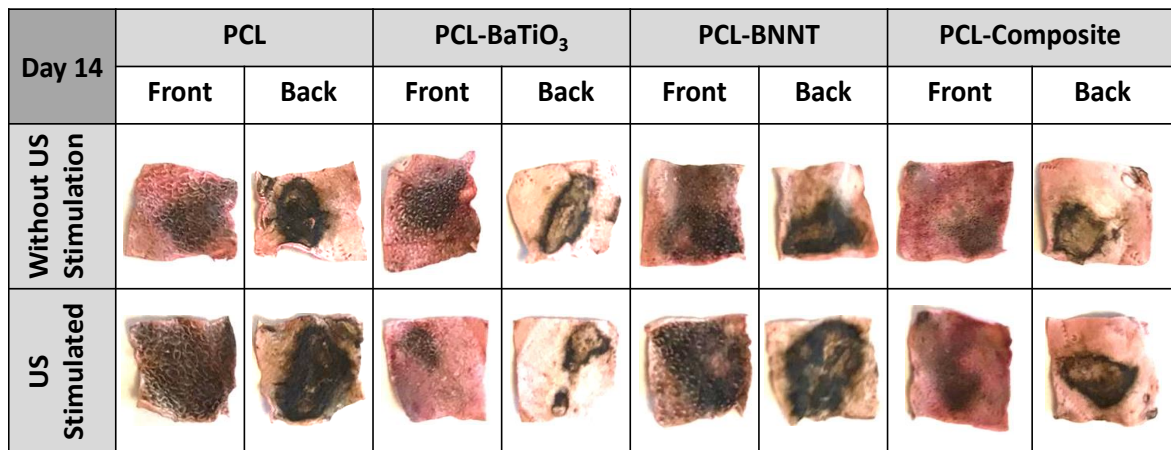


Figure 4.55. Von Kossa staining images of PCL scaffolds at day 14

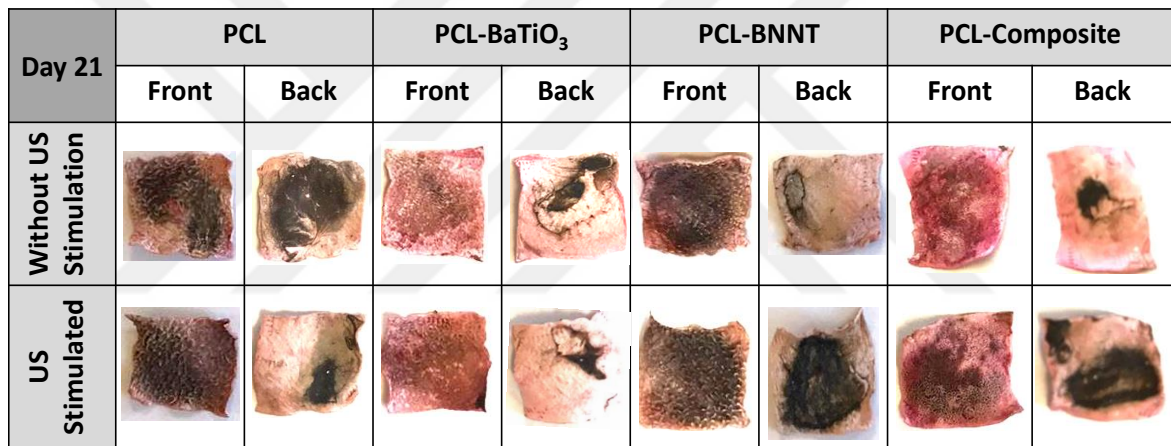


Figure 4.56. Von Kossa staining images of PCL scaffolds at day 21

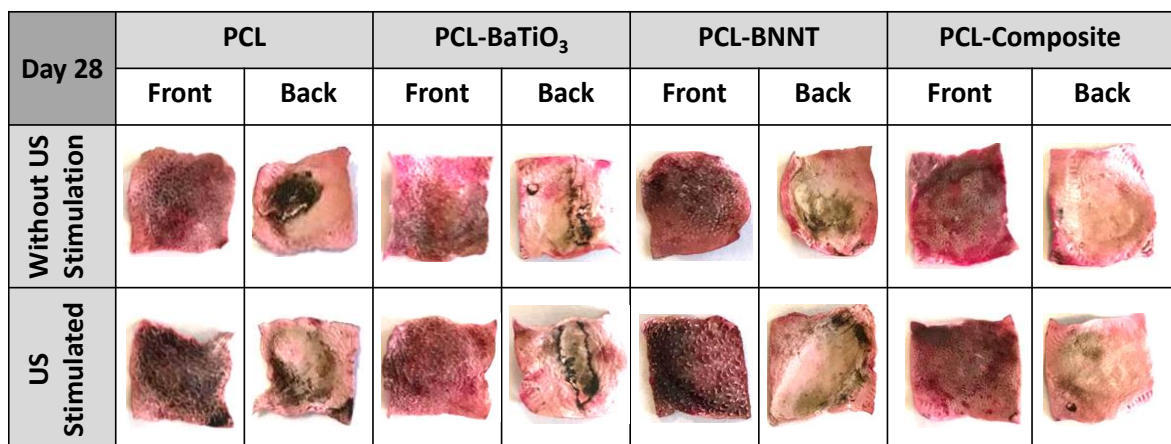


Figure 4.57. Von Kossa staining images of PCL scaffolds at day 28

4.5.5.2. Determination of Mineralization on Chitosan Scaffolds by von Kossa Staining

Front and back images of stained chitosan scaffolds with and without US stimulation at day 7, 14, 21 and 28 are presented in Figure 4.58.-4.61. BaTiO₃, BNNT, and composite embedded chitosan scaffolds with and without US stimulated were darker compared to only chitosan scaffolds at day 7 as seen front and back images in Figure 4.58. Nanomaterials supported cell attachment, proliferation and also mineralization in the early days compared to only chitosan scaffold.

Chitosan only were also good as a substrate for the cells at enhancing mineralization. US stimulated only chitosan scaffolds were darker at increasing days compared to not US stimulated scaffolds, which might be correlated the ALP activity results. HOb cells reacted to mechanical stimulation, increasing mineral deposits.

Front images of US stimulated chitosan-BaTiO₃, chitosan-BNNT and chitosan-Composite scaffolds showed more darkness compared to not US stimulated at 14th day (Figure 4.59.). Front and back images showed higher darkness on US stimulated chitosan-BaTiO₃, chitosan-BNNT, chitosan-Composite scaffolds at day 21 (Figure 4.60.). US stimulated all scaffolds were darker then the scaffolds not stimulated with US at day 28 (Figure 4.61.).

Day 7	Chitosan		Chitosan-BaTiO ₃		Chitosan-BNNT		Chitosan-Composite	
	Front	Back	Front	Back	Front	Back	Front	Back
Without US Stimulation								
US Stimulated								

Figure 4.58. Von Kossa staining images of chitosan scaffolds at day 7

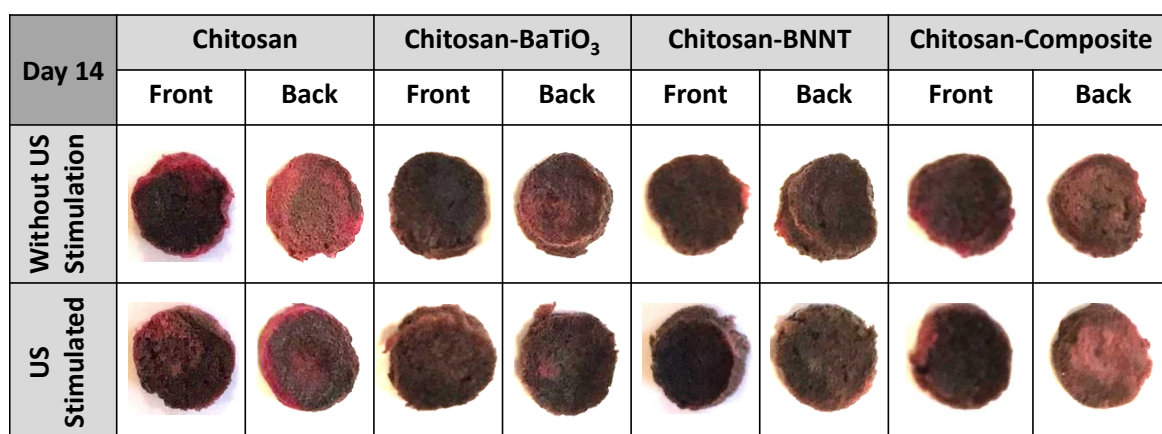


Figure 4.59. Von Kossa staining images of chitosan scaffolds at day 14

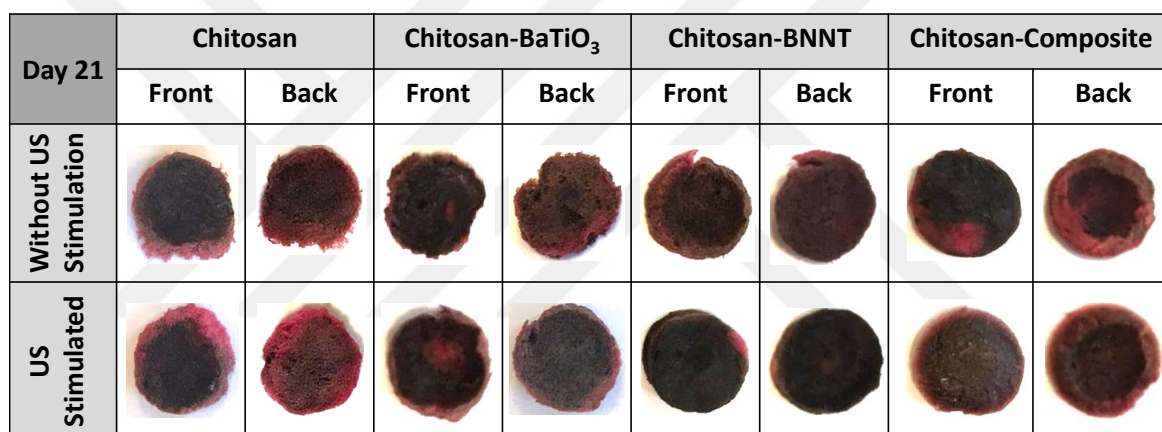


Figure 4.60. Von Kossa staining images of chitosan scaffolds at day 21

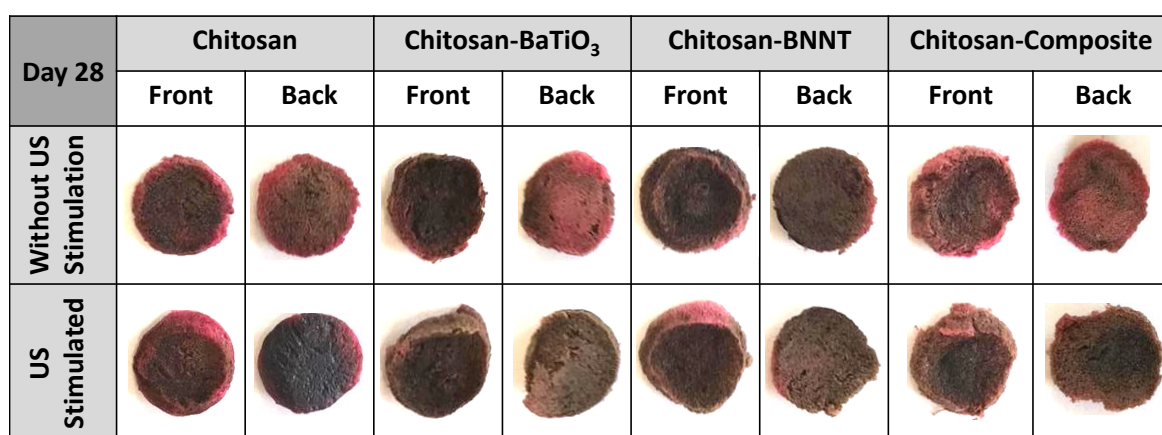


Figure 4.61. Von Kossa staining images of chitosan scaffolds at day 28

5. CONCLUSIONS AND OUTLOOKS

The aim of the completed project was the development of piezo-composite scaffolds for bone tissue defects using poly- ϵ -caprolactone (PCL) and chitosan polymers by separately embedding three piezoelectric nanomaterials including barium titanate (BaTiO_3) nanoparticles, boron nitride nanotubes (BNNTs) and the inventively synthesized “composite”, mainly composed of BaTiO_3 and BNNT, and the investigation of their interactions with human osteoblast (HOb) cells. The applicability of piezo-materials including BaTiO_3 , BNNT, and composite in bone tissue engineering was exhibited in the present study with favorable observations.

First, purchased BaTiO_3 was characterized using SEM and AFM and it was in spherical shape and the size of BaTiO_3 was determined as nearly 50 nm. Raman spectrum of BaTiO_3 showed that BaTiO_3 was in tetragonal phase, which is attributed to its piezoelectric future, due to the sharpness of the peak at 305 cm^{-1} [238].

The use of a mechanically activated catalyst mixing ($\text{Fe}_2\text{O}_3/\text{ZnO}$) approach contributed to the production of high-yield and large-scale BNNT growth. BNNT were synthesized using chemical vapor deposition (CVD) method using a mixture of catalyst-hybrid powder, which increased the yield of BNNT up to 80.6 percent [235]. Long-term ball-milling process also increased the yield and decreased the diameter of BNNT to 50-100 nm [230].

One of the main aims of this project was to develop a nanomaterial for biomedical applications, which has higher piezoelectric property than its components, BNNT and BaTiO_3 . This idea was developed based on the ability to synthesize these two nanomaterials under similar conditions such as ball milling process before synthesis and annealing at high temperatures [146, 225]. The composite, a mixture of BNNT and BaTiO_3 , were successfully synthesized on same synthesis plate using CVD technique. Formation of both BNNT and BaTiO_3 in composite was confirmed using SEM, AFM and TEM techniques. Based on the XRD results of the composite, in addition to the formation of BNNT and BaTiO_3 , other products such as barium titanium bis borate ($\text{B}_2\text{Ba}_6\text{TiO}_6$) and titanium nitrogen boron ($\text{Ti}_4\text{N}_3\text{B}_2$) were also formed in the composite structure as foreseen. However, the formation of the desired structures, BaTiO_3 and BNNT, rich in piezoelectric property in composite structure was the main goal for the continuation of the experiments

in this study. The results were evaluated considering the formation of by-products of the composite.

A novel piezoelectric material from non-piezoelectric precursors and two different piezoelectric materials (BaTiO_3 and BNNT) with different formation mechanisms were successfully produced in the same synthesis batch. The synthesized composite has higher piezoelectricity than BNNT as hypothesized. This hybrid system synthesis showed that the piezoelectric properties of BNNTs could be supported. In addition to BaTiO_3 or BNNTs, the composite can be a good candidate for piezoelectric stimulation in medical applications.

The biocompatibility of nanomaterials should be clarified so that they can be used in biological applications. BaTiO_3 and composite did not show any toxic effect on Human osteoblast (HOb) at increasing concentrations while BNNTs reduced the cell viability at increasing concentrations. BNNTs reduced cell viability up to 73 percent at maximum concentration, which could be associated with increased cellular uptake with increasing dose. Considering the effects of nanomaterials on cell viability and the WST-8 results, maximum dose to be used in scaffold construction was determined as 500 $\mu\text{g/ml}$. This dose was also chosen for BNNT, although BNNTs reduced cell viability by 73 percent at 500 $\mu\text{g/ml}$. Because this dose is the dose embedded in the scaffold, not the dose given directly to the cell. The uptake of BaTiO_3 , BNNT, and composite by HOb cells significantly increased at elevating concentrations which could be associated with their hydrophobic character. Hydrophilic materials demonstrate higher efficiency with respect to hydrophobic materials [242]. Hydrophobic BaTiO_3 , BNNT, and composite tended to interact with medium proteins in cell culture media, which enhances their cellular uptake by increasing their hydrophilicity.

PCL and chitosan polymers were selected considering their differences in mechanical strength, hydrophobic character and degradation times. Afterwards, BaTiO_3 , BNNT, and composite were separately embedded in to PCL and chitosan scaffolds. In this study, PCL scaffolds were constructed in fibrous structure using electrospinning technique and chitosan scaffolds were constructed in porous structure using freeze-dryer technique. Physical, chemical and biological properties of composite scaffolds were investigated with many techniques. The mean diameters of electrospun fibrous PCL, PCL- BaTiO_3 , PCL-BNNT, and PCL-Composite scaffolds were under 1.6 μm , which provides huge surface

areas for better cell growth. Chitosan based scaffolds had very porous structure. When BaTiO₃, BNNT, and composite were embedded in chitosan, pore sizes increased compared to the only chitosan scaffolds. Additionally, inter pores were observed in all types of chitosan scaffolds which is crucial for cell attachment and proliferation.

Determination of BaTiO₃, BNNT, and composite distribution in PCL and chitosan scaffolds was achieved by generating 20x20x20 μm 3D Raman mapping at their characteristic peaks of each nanomaterial. For the distribution of the nanomaterials in PCL, it could be said that electrospinning technique and also hydrophobic nature of the both nanomaterials and polymer caused some aggregations. In addition, the nanomaterials could be observed in almost every region in mapping area; however, their intensity levels are variable due to aggregations owing to hydrophobic nature of the nanomaterials.

BaTiO₃, BNNT, and composite enhanced the mechanical properties of both PCL and chitosan scaffolds and they could be used as reinforcement agents in tissue engineering scaffolds. The use of BaTiO₃, BNNT, and composite improved the physical properties of PCL and chitosan scaffolds. For example, they increased wettability of PCL scaffolds and increased the swelling capacity of chitosan scaffolds, which are crucial for better cell adhesion and growth. The biodegradation rate of scaffold materials should be matching the speed of new bone tissue formation. BaTiO₃, BNNT, and composite increased the biodegradation rate of PCL scaffolds and decreased the degradation rate of chitosan scaffold. US stimulation did not make a remarkable difference in degradation rate of PCL and chitosan scaffolds compared to not US stimulated samples. In addition, the biocompatibility test performed for PCL scaffolds showed that all types of PCL scaffolds did not significantly affect the cell viability. US stimulation increased viable cell number when PCL-BNNT and PCL-Composite scaffolds used in the upcoming days. In addition, Live/Dead assay was also performed for determination of biocompatibility of all types of scaffolds. All designed PCL scaffolds were nontoxic and very good candidate for cell attachment and proliferation as understood from the excess amount of living cells. The use of BaTiO₃, BNNT, and composite resulted in better cell attachment and growth. Moreover, cell number on US stimulated BaTiO₃, BNNT, and composite embedded chitosan scaffolds increased compared to scaffolds not stimulated with US at the time points of 21st day as illustrated in fluorescence images and also mean fluorescence intensity graph.

SEM analysis demonstrated that HOb cells on PCL scaffolds were grown rapidly and produced extracellular matrices at increasing incubation times and HOb cells on chitosan scaffolds significantly increased the matrix mineralization when US stimulation was applied. ALP analyses and von Kossa staining experiments showed that the use of piezoelectric BaTiO₃, BNNT, and composite materials indicated positive results for improving and increasing bone growth and osteogenesis. BNNT and the composite enhanced the bone mineralization under US stimulation.

In brief, it has been demonstrated that piezoelectric nanomaterials could be used to fabricate piezo-composite scaffolds and these composite scaffolds could be promising alternatives to contribute to wireless tissue stimulation studies using piezoelectric phenomena. However, further experiments should be carried out to maximize the effectiveness of the future scaffolds of bone tissue engineering.

The amount of nanomaterials used in polymers can be increased and their distribution can be evenly provided by managing surface functionalization strategies without damaging their piezoelectric properties. Different scaffold fabrication techniques can be used for providing better nanoparticle distribution. Piezoelectric properties of the prepared scaffolds should also be evaluated.

Alkaline phosphatase activities should be evaluated by real time PCR analysis. Long-term in vivo experiments on laboratory rats can be carried out to examine the effects of these structures on the implantation site.

These promising piezoelectric nanomaterials can be used in other electric-responsive tissue engineering applications such as, neural and cardiac tissue engineering.

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