THE EFFECT OF SURFACE FUNCTIONALIZATION ON CELL BEHAVIOR

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Submitted to the Institute of Biomedical Engineering
in partial fulfillment of the requirements
for the degree of
Doctor
of
Philosophy

Boğaziçi University
2024

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DATE OF APPROVAL: 15 March 2024

ACKNOWLEDGEMENTS

This thesis marks the culmination of an extraordinary learning and discovery journey, for which I would never possibly be able to give due credit to many people and organizations without their support and guidance.

I would like to extend my thanks and gratitude to my supervisor, Dr. Bora GARÍPCAN, whose expertise, understanding, and patience brought great value to my graduate experience. His unwavering support and invaluable advice were at the epicenter of my research and personal growth all along.

I would like to express my deepest thanks to the Scientific and Technological Research Council of Turkey (TÜBİTAK) and the Council of Higher Education (YÖK) for awarding me with 2211-C Domestic Priority Doctoral Fellowship Program, 2214-A International Research Scholarship and Council of Higher Educational 100/2000 Ph.D. Scholarship, which provided financial support, gave me confidence and strength at the same time to pursue my studies. These scholarships have been instrumental in enabling me to focus on my research without financial burdens.

I would also take this opportunity to express my heartfelt thanks to all the members of my thesis committee, Dr. Emine Elif GÜZEL MEYDANLI, Dr.Duygu EGE, Dr.Benay UZER YILMAZ and Dr. Mustafa Kemal RUHÍ for their invaluable comments and suggestions. Some of these guiding comments and critical judgments have added a new flavor to this research work. Truly, their expertise and dedication were great inspirations and motivation for me.

I would like to express my thanks and appreciation to Dr.Antonios MIKOS who hosted me as a research assistant in his laboratory and his laboratory members especially, Katie J. Hogan, Marissa Perez, Emily Jiang. His guidance and mentorship have been crucial in expanding my research horizon and skills. The opportunity to

work under his mentorship in such a stimulating laboratory environment has actually been one of the pinnacles of my academic career.

In my academic journey, I feel deeply compelled to express my heartfelt gratitude to my friends, especially to Hatice KAYA, Fatma Zehra ERKOÇ BÍRADLI, Bengü AKTAŞ, Sedef YUSUFOĞULLARI, Deniz KILINÇ, Berkay ERENAY, Sezin EREN DEMÍRBÜKEN and Şeyma SOYKAN whose unwavering support and companionship have been invaluable. This thesis, though reflective of my academic endeavor, is equally a testament to the friendships that have sustained me throughout this process. They lent me an ear, a word of advice, or a timely joke to help ease the mood. Their support has been a source of hope through some very daunting times. Their ability to make me smile, especially during deadlines loomed large, has been blessing.

I would like to express a special word of gratitude to my family, whose unceasing support and indefatigable encouragement have been the cornerstones of my journey. Thanks to their belief in me every bit, even when I doubt myself, they were my constant motivation. The sacrifices they have made and the love they have shown are the foundation upon which this achievement stands.

This is a culminating thesis, representative not only of my own hard work but representative of the combined effort and support of all those named above. I am eternally thankful to each one of you. Your inputs really did not only make a difference for this thesis, but also they have contributed to mold my academic career in the most affirmative way.

ACADEMIC ETHICS AND INTEGRITY STATEMENT

I, Hayriye Öztatlı, hereby certify that I am aware of the Academic Ethics and Integrity Policy issued by the Council of Higher Education (YÖK) and I fully acknowledge all the consequences due to its violation by plagiarism or any other way.

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ABSTRACT

THE EFFECT OF SURFACE FUNCTIONALIZATION ON CELL BEHAVIOR

Bone-on-a-chip platforms that replicate the native bone microenvironment are indispensable for research of complex physiological conditions. Polydimethylsiloxane (PDMS) has widespread use for these platforms and needs biochemical modification to derive suitable biocompatibility and bioactivity for bone research. In this study, PDMS surfaces were coated with Hydroxyapatite (HA) using immobilization and biomineralization techniques with the aid of small molecules, namely Aspartic Acid (AA), Glutamic Acid (Glu), and Citric Acid (CA). The effectiveness of biomineralization and immobilization techniques and the impact of small molecules on HA deposition on PDMS surfaces were investigated by chemical and morphological analysis of the surfaces, besides *in vitro* cell culture studies. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) and X-ray Photoelectron Spectroscopy (XPS) spectra confirmed that the small molecule modification on both PDMS and onto nHA particles was achieved successfully. X-ray Diffraction (XRD) patterns showed characteristic peaks of HA deposited by either biomineralization or immobilization on PDMS surfaces, which were also observed with scanning electron microscopy (SEM). The interaction of human fetal human osteoblasts cells (hFOB 1.19) with HA-deposited PDMS surfaces was evaluated with *in vitro* cell culture studies, which revealed increased cell viability across all groups, with HA deposited on AA-PDMS surfaces being the most stimulating group. Overall, it is further worthy of study on the effect of small molecules on HA deposition to the PDMS substrates, especially for those mimicking chemical and morphological features of the bone surface.

Keywords: Hydroxyapatite, Polydimethylsiloxane, L-Aspartic Acid, L-Glutamic Acid, Citric Acid, Biomineralization, Immobilization.

ÖZET

YÜZEY FONKSİYONLAMANIN HÜCRE DAVRANIŞI ÜZERİNDEKİ ETKİSİ

Kemik mikroçevresini taklit eden çip-üstü kemik modelleri, karmaşık fizyolojik koşulların araştırılması için değerli araçlardır. Bu platformlar için yaygın bir kullanıma sahip olan Polidimetilsiloksan (PDMS), biyolojik uyumluluğunu ve biyoaktivitesini artırmak için biyokimyasal modifikasyona ihtiyaç duyulmaktadır. Bu tez çalışmasında, PDMS yüzeyleri L-Aspartik Asit (AA), L-Glutamik Asit (Glu) ve Sitrik Asit (CA) küçük moleküllerin yardımıyla immobilizasyon ve biyomineralizasyon teknikleri kullanılarak HA ile kaplanmıştır. Biyomineralizasyon ve immobilizasyon tekniklerinin ve küçük moleküllerin PDMS yüzeyi üzerine HA birikimi etkinliğinin, *in vitro* hücre kültürü çalışmalarının yanı sıra yüzeylerin kimyasal ve morfolojik özellikleri karakterize edilmiştir. Azaltılmış Toplam Yansıma- Fourier Dönüşüm Kızılötesi (ATR-FTIR) ve X- ışını Fotoelektron Spektroskopisi (XPS) spektrumları hem PDMS hem de nHA partikül yüzeyleri üzerinde küçük molekül modifikasyonunun başarıyla gerçekleştirildiğini doğrulamıştır. X-ışını Kırınımı (XRD) ve SEM analizleri de, PDMS yüzeylerinin hem biyomineralizasyon hem de immobilizasyon yoluyla HA partiküllerinin başarılı bir şekilde kaplandığını göstermişlerdir. İnsan fetal insan osteoblast hücrelerinin (hFOB 1.19) üzerinde HA biriktirilmiş PDMS yüzeyleri ile etkileşimi *in vitro* hücre kültürü calışmaları ile değerlendirildiğinde, düz PDMS grubuna kıyasla diğer bütün gruplarda hücre canlılığının daha çok artmış olmasıyla birlikte, üzerinde HA biriktirilen AA-PDMS yüzeylerinin hücre canlılığını en çok artıran grup olduğu görülmüştür. Sonuç olarak, küçük moleküllerin PDMS yüzeyler üzerine HA birikimi üzerine ileri çalışmaların gerçekleştirilmesi kemik yüzeyinin kimyasal ve morfolojik özelliklerini taklit eden platformların geliştirilmesi için büyük önem taşımaktadır.

Anahtar Sözcükler: Hidroksiapatit, Polidimetilsiloksan, L-Aspartik Asit, L-Glutamik Asit, Sitrik Asit, Biyomineralizasyon, İmmobilizasyon

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LIST OF SYMBOLS

Al-Kα	K-alpha X-rays of aluminum
eV	electrovolt
mg	milligram
mL	milliliter
mM	millimolar
°C	degree Celcius
μg	microgram
μL	microliter
μm	micrometer

LIST OF ABBREVIATIONS

AA	L-Aspartic Acid
APTES	(3-aminopropyl) triethoxysilane
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
BSA	Bovine Serum Albumin
BoC	Bone-on-a-chip
CA	Citric Acid
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EDC	1-ethyl-3-(3-dimethylamino propyl) Carbodiimide Hydrochloride
FBS	Fetal Bovine Serum
Glu	L-Glutamic Acid
HA	Hydroxyapatite
HA-SM	Small Molecule Modified Hydroxyapatite
hFOB 1.19	Human Fetal Osteoblast Cell 1.19
HMDS	Hexamethyldisilazane
MTT	3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2-H-tetrazolium bromide
HMDS	Hexamethyldisilazane
nHA	Nano Hydroxyapatite
NHS	N-hydroxysuccinimide
OoC	Organ-on-a-chip
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
PDMS-AA-HA	HA deposited on AA Modified PDMS Surfaces
PDMS-APTES	APTES Modified PDMS Surfaces

PDMS-CA-HA	HA Deposited on CA Modified PDMS Surfaces
PDMS-Glu-HA	HA deposited on Glu Modified PDMS Surfaces
PDMS-nHA-AA	AA Modified nHA Particles Deposited on PDMS Surfaces
PDMS-nHA-CA	CA Modified nHA Particles Deposited on PDMS Surfaces
PDMS-nHA-Glu	Glu Modified nHA Particles Deposited on PDMS Surfaces
PDMS-nHA-SM	Small Molecule Grafted nHA Particles Coated on PDMS
PDMS-SM-HA	HA Deposited on Small Molecule-Modified PDMS Surfaces
SBF	Stimulated Body Fluid
SEM	Scanning Electron Microscopy
SM	Small Molecule
SM-nHA	Small Molecule Grafted nHA
TCP	Tissue Culture Plate
UP	Ultrapure
UV	Ultraviolet
WCA	Water Contact Angle
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction

1. INTRODUCTION

1.1 Motivation

The bone is a highly complex, rigid, and dynamic tissue that provides the structural framework of the human body while also preserving internal organs. However, these physiological functions of the bone can be hindered by a variety of bone disorders [1]. The ability of researchers to investigate the mechanisms related to (patho)physiology of the bone tissue has been restricted by conventional *in vitro* models that cannot represent the bone microenvironment. Recent developments in tissue engineering and microfluidic technologies serve as the foundation for the development of bone-on-a-chip models, emulating essential characteristics of the native bone microenvironment [2]. Bone-on-a-chip models were frequently fabricated by using polydimethylsiloxane (PDMS), which is an extremely versatile material with good biocompatibility, gas-permeability, highly tunable mechanical properties, and the ability to replicate fine surface features through soft lithography [3]. However, the biological and chemical inertness of PDMS hinders their applicability in the development of organ models [4, 5].

Hydroxyapatite (HA) is an inorganic mineral that constitutes the inorganic phase of the calcified bone structure in its carbonated form. HA is meticulously studied in bone tissue research due to its remarkable biocompatibility, bioactivity, and osteointegration in both *in vivo* and *in vitro* environments [6–8]. Due to its excellent biocompatibility, HA is a widely utilized and well-studied material in bone tissue research. Incorporating HA into other biomaterials either as a composite element or as a surface modification generally results in more favorable overall structure and mechanical properties for bone tissue research applications, such as increased tensile and compressive strength, higher porosity, and improved biocompatibility/biointegrability of the composite material [9–11].

Utilization of PDMS and HA individually in biological systems is possible; however, due to inertness of the former and the brittleness of the latter limit their use alone [12]. Composites of these two materials have already been used successfully for *in vitro* applications, ranging from preparation of a dynamic bone microenvironment through using HA as a component in a PDMS microfluidic system, synthesizing of HA/PDMS nanocomposites that are surface modifiable and mechanically tunable, mimicking a pre-existing extracellular matrix (ECM) topography, and bone matrix components through PDMS soft lithography that is enhanced by addition of HA nanoparticles to pre-polymer [13–15]. Nevertheless, the primary emphasis of these studies has been on the incorporation of HA into PDMS as an ingredient of composite, not as a coating material for the development of biointerfaces, possibly due to the limitation in interaction with PDMS and HA. To overcome this, small molecules can be employed as a surface modifier/crosslinker to prone the substrates to chemical reactions with available active functional groups.

A small molecule (SM) is a broad classification encompassing molecules with a molecular weight below 1000 Da, including various biomolecules like fatty acids, monosaccharides, amino acids [16]. Aspartic acid (AA) and glutamic acid (Glu) as amino acids act as initiation sites for calcium phosphate nucleation, which are found in regulatory proteins osteopontin, osteocalcin, and bone sialoprotein for mineralization process in bone [17, 18]. Citrate and citric acid (CA) also discovered to be an essential component in HA crystal structure and formation along with providing additional nucleation cites, apart from its crucial roles in Krebs cycle [19, 20]. These small molecules (AA, Glu, and CA) have negatively charged functional groups which modulate their adsorption to HA surfaces through electrostatic interaction [21, 22]. Studies have indicated that the extent of negativite charge present on functional groups of small molecules can impact their affinity and coverage ability to HA [21, 23–25]. Hence, the efficacy of HA coating onto PDMS substrates depends on the small molecules which are grafted onto either on a substrate or HA surface.

HA coating can be applied on the substrates using a variety of promising approaches. Among them, biomineralization techniques for calcium phosphate deposition

on material surfaces are reported to have better control over crystal structure, morphology, and thickness with the added benefit of ease of fabrication and being low-cost [26]. However, native-like biomineralization processes on biomaterials require an initial nucleation site for HA deposition. The introduction of small molecules on the subjected surfaces was demonstrated to initiate the nucleation and subsequent growth of the HA particles [27, 28]. Another viable alternative for introducing HA onto material surfaces is the immobilization of aforementioned small molecules grafted onto synthetic nHA particles, and functionalizing the material surfaces with small molecule modified hydroxyapatite (HA-SM) which are already reported previously to result in an increase in osteoblast bioactivity and shown to support osteoblast differentiation [18, 29, 30].

The primary objective of this study is to assess the efficiency of the small molecule assisted HA coating on PDMS substrates through biomineralization and immobilization approaches. Hence, possible chemical and physical differences of HA deposited on small molecule modified-PDMS surfaces (PDMS-CA-HA, PDMS-Glu-HA, PDMS-AA-HA) and PDMS surfaces coated with small molecule grafted nHA particles (PDMS-nHA-CA, PDMS-nHA-Glu, PDMS-nHA-AA) were evaluated through characterization with Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), X-Ray Photoelectron Spectroscopy (XPS), X-Ray Diffraction (XRD), Water Contact Angle (WCA) and Scanning Electron Microscopy (SEM) analysis. Also, the effect of adherent human fetal osteoblast cell (hFOB 1.19) interaction with these HA deposited PDMS surfaces on behavior and morphology of cells were evaluated with *in vitro* cell studies, namely, MTT assay, SEM imaging, and actin cytoskeleton staining.

1.2 Objectives

The hypothesis of this study is that small molecule-assisted hydroxyapatite particle deposited Polydimethylsiloxane (PDMS) substrates could be employed as bone mimicry platforms to provide an ideal microenvironment for osteogenic cells. The main objective of this study is to explore the efficacy of PDMS substrates coated with HA through small molecules chemistry in improving osteogenesis There are four specific

objectives of this study as follows;

1. Modification nHA particles with small molecules
2. Modification of PDMS surfaces with small molecules
3. Coating the surface of PDMS substrates with HA
4. Investigation the interaction of HA-coated PDMS substrates with Osteoblast Cells

1.3 Outline

The chapters of this thesis are arranged in the order indicated below;

- Background, which gives brief information on bone structure, materials used, and technologies employed in this research
- Materials and Methods, which describes the experimental methodologies
- Results and Discussions, in which the obtained experimental data are analyzed and compared to existing literature findings
- Conclusions, which summarize key findings and explains the contribution of the study to the biomedical applications

2. BACKGROUND

2.1 Bone Structure

Bone, a mineral-rich tissue, has important functions in the human body, notably supporting mobility, providing anchorage sites for soft tissues, storing calcium, and regulating hormones. It also functions as a specialized microenvironment encapsulating the bone marrow, which is the main supplier of mesenchymal stem cells and blood cell progenitors [31, 32].

Bone is an exceptionally dynamic tissue characterized by a continuous cycle of production and resorption process, known as "bone remodeling", in which osteoclasts demolish old or damaged mineralized matrix and successively osteoblast synthesize new bone matrix, particularly during growth or in response to injury [33, 34]. Osteocytes, another crucial type of bone cells, meticulously control and coordinate the actions and metabolic process of osteoclasts and osteoblasts during the remodeling process by detecting physical stimuli inside the bone structure [35]. Hence, this complex bone remodeling process hinges on the orchestrated actions of these three types of bone cells, and any disruption in this process can cause bone disorders [36].

Bone diseases like osteomyelitis, osteoporosis, and bone metastases impair normal bone functional processes, which drastically impacts the quality of patient life and increases healthcare expenditures for patients. Osteoporosis, which is mostly brought on by excessive bone resorption, is the most common ailment in this category [37]. It affects around one in every three women over the age of 50 [38]. According to available data, it is predicted that osteoporosis is expected to result in three million fractures and cost the economy $25.3 billion annually by 2025 [39]. The metastatic malignancy is another cause of bone tissue disorders. Bone is the third most affected tissue by metastasis and the most prevalent metastatic site for breast, prostate, and lung cancers [40]. Hence, understanding of patho(physiological) mechanisms of these disease

holds immense importance in developing novel and more efficient approaches for diagnosis, prevention, and therapeutic interventions. As a result, developing a biomimetic bone platform for *in vitro* research of healthy and diseased bone models is critical for biomedical, pharmaceutical, and toxicological applications.

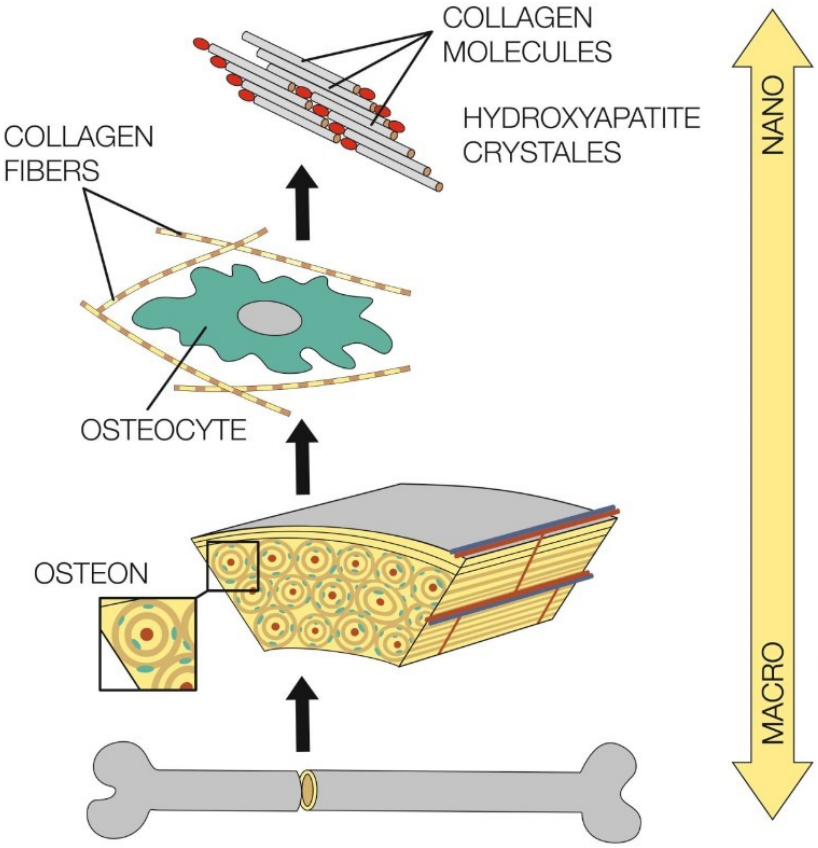

Figure 2.1 Diagram illustrating the hierarchical structure of the bone [41].

2.2 Bone-on-a-Chip Models

BoC uses advanced and highly complex technology that deliberately mimics the native complex microenvironment of bone tissue in miniaturized controllable units. The concept of the BoC platform was conceived based on the cooperation of diverse scientific disciplines related to the area of biomaterials, cell technology, and microfluidics. Such multidisciplinary work aimed to come over the pitfalls of traditional *in vitro* models and minimize the use of animals, hence offering the ability to more closely mimic bone physiology and pathology [42]. BoC platforms help obtain an understanding into

the biological processes and mechanisms mediated by the associated diseases through better emulation of the native structure of the bones. Such an advance contributes to science in many ways, especially considering the potential for BoC platforms to give more relevant insights into the effectiveness and safety profile of drugs in the field of pharmaceutical development. These platforms do have the potential to reduce reliance on animal models, which oftentimes do not precisely simulate disease status or treatment responses in humans. This is because the data presented are more compatible with human responses [43].

Besides drug development, BoC technologies have a comprehensive impact in the area of personalized medicine. Researchers can culture cells on these systems from individual patients to develop a customized microenvironment that represents, in the most accurate way, the unique biological profile of an individual. This would then enable examination of the drug response at a personal level, which would support the development of much more individualized treatment strategies. Even further, such personalized models could provide improved accuracy in the prediction of treatment outcomes, improving the modulation of treatment approaches more directly to the specific needs and biological traits of the respective patients [44, 45].

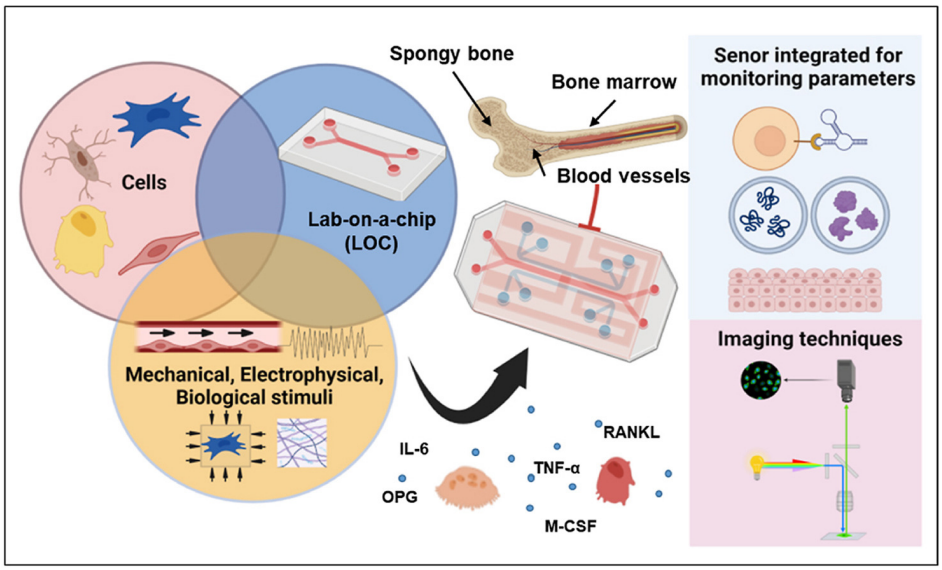

Figure 2.2 The schematic representation of the possible entities included in BoC platforms[46].

Additionally, the BoC platforms provide dynamic microenvironments that are

supposed to guide cells into the desired phenotype and functionality. This platform design is built from structural, biochemical, and cellular components that can exactly mimic the structure and function of natural bone tissue. Biomaterials, that hold the potential of mimicking the three-dimensional architecture of the extracellular matrix (ECM) of bone, provide exact mechanical properties along with the specific biochemical signals that are needed for cell growth and differentiation. All these properties make the platforms excellent for providing an environmental niche with adequate conditions for the attachment, growth, and differentiation of the cells as in native bone tissue. This provides a new way for the development of therapeutics for the repair or regeneration of damaged or lost bone tissue [46].

The success of BoC platforms in reliably mimicking native complexity in bone tissue is critically dependent upon their ability to reproduce with extremely high fidelity the various interactions that represent native bone. These platforms involve not only the emulation of the structural complexity of bone but even the high degree of complexity of the network of biochemical signaling and cellular communications which are necessary to maintain the bones and enable them to regenerate [47, 48]. One of the key studies shedding light on how BoC platforms try to reproduce such a complex environment includes the incorporation of mechanical stressors, chemical gradients, and cell–cell interactions that are part of *in vivo* conditions and bear a significant influence on bone cell behavior and the consequent morphogenesis of tissues [48]. That is, Aleman and colleagues, pointed on the importance of a comprehensive approach in the design of a BoC platform via an integrated system, indicating the dynamic and reciprocal relationships between bone cells and the extracellular matrix and biochemical signals. These studies have become exemplary with a lot of promise shown toward inspiring improved research in the field of development BoC platforms for exploration in the area of bone tissue engineering and regenerative medicine.

Research in the field of BoC platforms development revealed that the controlled physical and chemical stimuli prevailingly act as crucial factors in guidance the differentiation and maturation of bone cells. This field leverages microengineering techniques to design microenvironments that mimic the natural conditions of bone tissue, enabling

precise control over the factors that influence bone cell behavior [49]. Mechanical stress, for example, is a significant determinant of osteogenic differentiation. The application of cyclic loading, which simulates the physiological forces experienced by bones, has been demonstrated to enhance the commitment of progenitor cells to the osteoblast lineage, a process essential for bone formation and regeneration. This is supported by evidence showing that mechanical stimuli activate signaling pathways that promote the expression of osteogenic markers [50].

In addition, the biochemical environment within such platforms should be engineered in a manner that includes some guiding signals that direct the fate of osteogenic cells. In this regard, soluble factors, including small molecules, growth factors, cytokines, and hormones, have been found to play key roles in driving stem cells toward osteogenic pathways [51–53]. Their integration enabled platforms to offer a more reliable bone tissue-mimicked environment [54]. Studies demonstrated that both mechanical and biochemical stimuli are of crucial relevance in guiding differentiation toward phenotypes of the bone cell [55, 56]. This complex interplay of these environmental factors brings in the dynamism of bone cell development and sophisticated approaches that need to be taken toward the study and modulation of these factors *in vitro* [57].

Furthermore, the engineering of BoC platforms requires the use of highly selective, state-of-the-art materials and advanced fabrication techniques. That is to say, this strategic selection can allow for the development of surfaces that are supportive of adhesion and proliferation or differentiation of the cells. The design of the platform has to be such that the physical and chemical properties of the native extracellular matrix can be replicated. Hence, the integration of advanced material science with highly developed fabrication techniques becomes a critical issue, so that biomimetic surfaces can be developed for the success of BoC platforms [58–60].

Research shine on the potential of bone-on-a-chip platforms as formidable tools for scientists in the area of bone tissue engineering and regenerative medicine by lighting up complex cell differentiation and maturation. These platforms mimic the multifaceted conditions of the bone microenvironment and are capable of investigating in

detail the activities and synergies of the different stimuli in bone development. Each of them gives invaluable information on both the pathologic mechanisms of bone and the way to treat the pathologies by recruiting osteoblasts.

2.3 PDMS for Bone-on-a-Chip Platforms

The advancement of BoC platforms has brought a new dawn to both tissue engineering and regenerative medicine. These new platforms are truly designed to replicate the complex *in vivo* microenvironment of bone tissue, which is very useful for the study of various valuable aspects of bone physiology, disease progression, and related treatment. One of the design issues of the BoC platform includes the selection of the most biocompatible materials that may closely emulate specific characteristics of the targeted tissue microenvironments. PDMS is the most commonly used material for fabricating BoC platforms due to its excellent properties [61, 62].

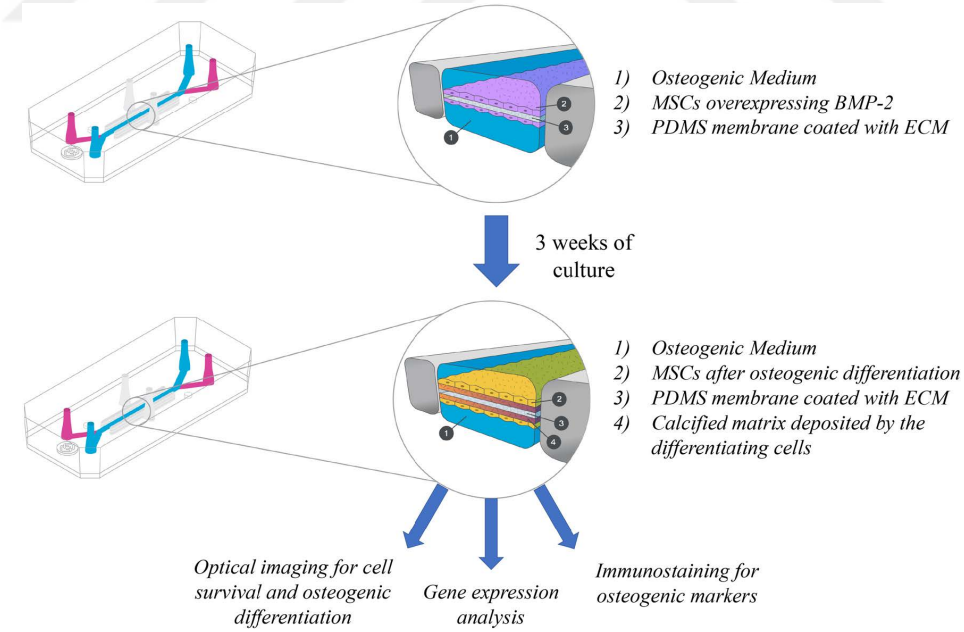

Figure 2.3 The schematic representation of the Bone-on-a-chip platform [63].

PDMS is an organosilicon-based polymer. Therefore, it has a great advantage in its use due to its excellent biocompatibility, which characterizes it as one of the indispensable materials in the field of biomedical application. The widespread use of

PDMS in the forefront of organ-on-a-chip technology research has played a major role in the solidification of its importance for further development toward the realization of BoC platforms. The high prominence of PDMS can be linked to many important advantages, congruent with the requirement of tissue engineering [64, 65].

Advantages of PDMS in BoC Platforms;

Biocompatibility:

The material is of high biocompatibility, with a very low risk of cytotoxicity - one of the most important factors to be considered while deciding about the usage of a material as a biomaterial [64, 65].

Gas Permeability:

The permeability of PDMS to gases such as oxygen and carbon dioxide is particularly vital for BoC platforms. This property enables mimicking the *in vivo* environment where, due to *in vivo*, the tissue is always in contact with a gas in which it supplies oxygen and carries away the given carbon dioxide. Hence, the cells cultured on the platform should reach the necessary gases for respiration as closely to *in vivo* environment [66].

Optical Transparency:

Another entity of PDMS material that makes it highly crucial for BoC applications is its optical transparency. This presents the possibility of tracking cell behavior and tissue development in real-time, in a non-invasive way, thus the possibility of enabling detailed studies in the dynamics and nature of cellular interaction in the microenvironment [67].

Flexibility and Mechanical Properties:

One main property of PDMS is that it enables to preparation of flexible substrates and microfluidic channels, which could allow the stiffness of native tissues to be exactly reproduced. This approach provides a fine-tuning of the PDMS mechanical properties and allows the reaching of the stiffness and elasticity of the bone tissue, both being factors among others very critical for cell behavior and function. This tunability allowed the designing of dynamic conditions able to simulate mechanical stresses and strains similar to those present in the native bone tissue to which cells are subjected. Hence, resulting in platforms of higher physiological relevance [68, 69].

PDMS finds many applications with many advantages in tissue engineering and organ-on-a-chip models, but it has its own disadvantages, especially when used in *in vitro* cell culture studies. One of the major problems with PDMS surfaces is that they are intrinsically hydrophobic and not have functional groups that are essential for the adhesion of cells. However, this property of PDMS may be one of the reasons for which PDMS is rendered it less than ideal in attachment support to the cells, which is the most basic requirement in the successful culture of cells on the surfaces [38, 70, 71].

The hydrophobic nature of the PDMS surface restricts cell attachment which is essential for the proliferation of cells and accurate simulation of the native bone tissue environments. The extracellular matrix (ECM) acts as an adhesion surface that regulates cell signaling and control of cellular behavior through specific binding sites to cells in the human body [72]. As a result, since PDMS does not naturally provide for these binding sites, cells would have difficulties in attachment and proper orientation on an untreated PDMS surface. In a result, this drawback is rectified through surface modification techniques that enhance the hydrophilicity of PDMS for cell culture application [73, 74].

Surface modification is crucial for PDMS substrates since modifications could significantly enhance cell attachment to the substrate through the incorporation of functional groups or coatings mimicking the binding domains of cells in the natural ECM. The surface modification of PDMS is mostly carried out by plasma treatment, UV-ozone treatment, and grafting of hydrophilic polymers, and biomolecules on its

surface. Such tailoring increases the wettability of PDMS and enables simple means to adhere the molecules on its surface [75, 76].

Further, the regulation of cell behaviors on PDMS platforms is directly related to their ability to adhere to substrates properly. The more the surface hydrophilicity of PDMS, the easier it becomes for researchers to control cell-substrate contacts during cell growth and differentiation and the overall cellular microenvironment [77–79]. This indeed strengthens cell-cell and cell-material interactions and gives a major contribution to the development of a more physiologically relevant organ-on-a-chip model, expediting the development in this most promising area of tissue engineering research [80].

As this technology in this area of science develops further in the future, the research is directed toward optimizing the properties of PDMS or look for some other possible biocompatible material that can complement or even improve its properties on a BoC platform. In the future, these advances will surely allow a more open door toward the possible applications of BoC technology and, accordingly, develop models both more complex and realistic of human bone tissue and other organ systems.

2.4 Surface Modification Strategies for BoC Platforms

Surfaces of engineered bone platforms represent intimate sites for numerous biochemical processes. Hence, the surface properties of these BoC platforms have a decisive role in directing biomolecular activities, including diffusion, adsorption, and reactions that are essential for physiochemical process in native bone. In that respect, surface engineering approaches can provide research with tools to control biomolecular processes and cellular activity by designing the surface properties of the platforms.

The engineering surface with particular characteristics entails the physical and chemical surface modifications to replicate the physiological and chemical environment of native bone tissue. A variety of materials and strategies have been employed for

surface modification of BoC models, depending on the design objective of the models and the intended degree of biomimicry [81].

A novel approach has demonstrated the potential of bone surface-mimicked polydimethyl siloxane (BSM PDMS) membranes, which could enhance the proliferation of osteoblasts and calcification of bone matrix. Topographical cues of the natural bone surface were faithfully patterned onto PDMS features using soft lithography, which as a result allowed for more activation of osteoblast-specific differentiation and maturation markers. This technique not only offers a native-like platform for cell growth but also offers new insight into intracellular pathways related to osteoblast growth and differentiation [82].

Surface chemical modification has become an eminent strategy in the realm of organ-on-a-chip field, used more specifically in improving cell-relevant behaviors of adhesion, proliferation, and differentiation. Surface modifications are engineered with chemical functional groups simulating the native extracellular matrix (ECM) to engineer the environment that becomes favorable for development of *in vivo*-like environments. It may be further surface-functionalized to introduce the bioactive cues that can help cell-material interactions to the maximum possible extent.

Recent studies in PDMS surface modifications have included metal layer coating, layer-by-layer deposition, dynamic surfactant treatment, and amphipathic protein adsorption. Besides this, surface modification by gas-phase processing methods such as plasma processing, sol-gel coatings, and chemical vapor deposition has also been tried to achieve stable modifications [83]. This means these methods aim to make the change in the naturally hydrophobic nature of PDMS to a more cell-friendly, hydrophilic surface that could have a high impact on the performance of the microfluidics-based devices used in bone-on-a-chip platform.

One approach to the surface modification is the covalent bonding of functional groups or biomolecules to the PDMS surface. For example, silane coupling agents such as (3-Aminopropyl)triethoxysilane (APTES) have been reported to significantly en-

hance further conjugation of the PDMS surface with proteins like collagen for adhesion and proliferation of mesenchymal stem cells (MSCs) by means of glutaraldehyde (GA) cross-linking. Not only does this increase the biocompatibility of the PDMS, it also introduces specific biochemical cues that can guide stem cell differentiation towards the osteogenic lineage [84, 85]. This is very important in osteogenic differentiation and a point emphasized in the biological and biomedical applied environment of bone tissue engineering. One of another methods employed for PDMS surface functionalization is oxygen plasma treatment, while its purpose is to replace the hydrophobic methyl (-CH3) groups of the PDMS with the hydrophilic silanol groups (SiOH) to enhance wettability. This modification can be further augmented by the bio-active molecule deposition of collagen-I. The molecule can covalently bind to the PDMS surface through its polar groups, which also allows it to self-assemble to support cell attachment [85].

In addition, HA modification on PDMS for BoC platforms represent valuable step in the development of more biologically relevant microenvironments for cell culture and drug screening. The use of HA, whose presence in microfluidic chips increases the mimicry of the structure and function of the bone, is more conducive to the proliferation and osteogenic differentiation than by the use of PDMS alone. The study exerts that HA modification on PDMS surface aligns with the need for the development of a high-throughput platform for bone-related disease research [13].

These results clearly show the high flexibility and great potential of surface modification strategies of PDMS for bone-on-a-chip applications and represent the most promising way toward more physiologically relevant models that may help the study of bone physiology, disease, and tissue regeneration.

2.5 Hydroxyapatite

Bone exhibits highly organized structures consisting of mineralized collagen fibrils, which feature a precise arrangement of calcium crystals, predominantly HA within organic matrix. This matrix primarily comprises about 85-90% Type-I Collagen and

various non-collagenous proteins. The HA crsytals exhibit alignment with c-axis parallel to the longitudinal axis of collagen fibril, form a microenvironment where different types of bone cells, involving osteoblasts, osteoclasts and osteocytes are able to grow [86].

HA crystals have a significant function in the stimulation and regulation of diverse cellular activities, namely, adhesion, proliferation, differentiation, and vascularization of cells. Accordingly, modification of the bone model surfaces with these crystals may be the ideal strategy to provide the signals necessary for the modulation of cellular behaviors in a way that enables the construction of the physiological environment of bone *in vitro* [87, 88].

The functionalization of HA onto PDMS provides a novel approach to improve the mimicry capabilities of BoC platforms. This modification approach is intended to use the biocompatibility and osteoconductivity of HA, a mineral with properties akin to the natural bone composition, and improve the microenvironment for bone cells on the chip. In study conducted by Tang *et al.*, a HA-PDMS microfluidic chip was fabricated for bone-related cell culture and drug screening. The study aimed to construct an environment that is biomimetic with the potential to mimic the structure and function of bone, hence providing a strong platform for any research in a bone-related biomedical field. These studies have shown that, with HA integration into PDMS-based chips, the cellular microenvironment can be further tailored to appropriate biochemical signaling and mechanical properties needed for bone cell growth and differentiation [13].

To summarize, the surface modification of HA on PDMS for bone-on-a-chip applications presents an innovative design with sophisticated fabrication techniques and strategies for surface modification. Besides, these advancements enhance the biomimetic functionality of the platforms and address the issues related to the inherent material properties of PDMS, thereby paving the way for more precise and effective studies in bone tissue engineering.

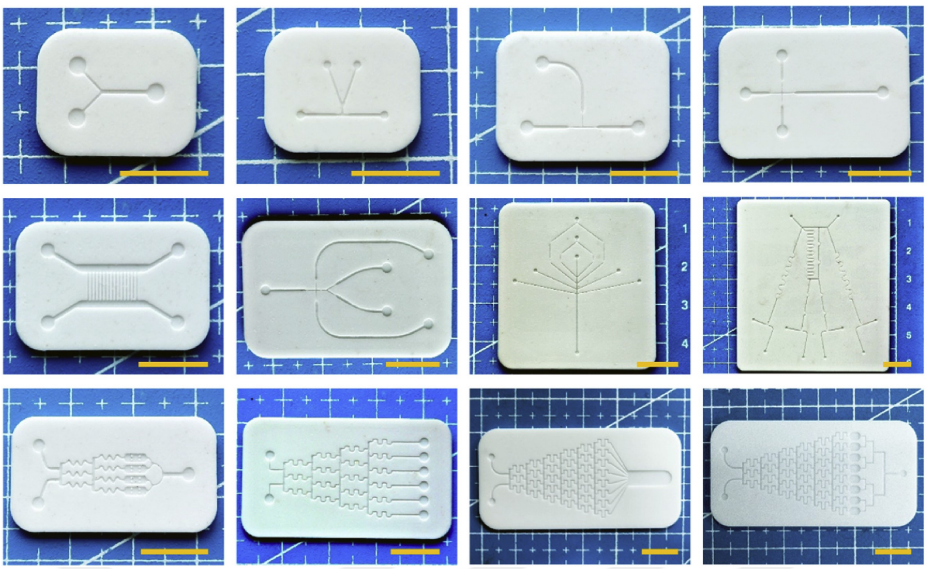

Figure 2.4 The micrographs of HA incorporated PDMS Microfluidic Channels [13].

2.6 Small Molecule Chemistry

In bone structure, the HA crystals are aligned with the major protein structure, an insoluble collagen framework to which noncollagenous proteins and small molecules have also adhered. [89, 90]. Recent research has demonstrated that both proteins and small molecules contribute to the synthesis of HA and the modulation of cellular functions in biological systems. These studies have motivated the researched to delve into this field to gain thorough understanding of the molecular level interaction between small molecules and HA. The results of these studies revealed that acidic molecules residing in bone ECM contribute in the modulation of growth rate and orientation of crystals [20, 89–91].

Negatively charged aspartic acid (AA), glutamic acid (Glu) and Phosphoserine are exemplary to acidic amino acids that are particulary abundant in the structure of noncollagenous proteins such as osteopontin, bone sialoprotein, dentin matrix protein 1 and dentin phosphoryn [92]. Recent studies also demonstrated the presence of aminoacids in the hole sites of collagen which is a intrafibrillarly nucleation region of HA. These aminoacids revealed to be essential in regulation of numerous processes in bone matrix [92, 93]. Glu have been shown to be engaged in intercellular commu-

nication and regulation of homestasis within the body as well as bone cell signalling processes. Also, Glu may be secreted via eocytosis upon stimulation of the glutamate receptors on osteoblasts to initiate localized osteogenic reaction [94, 95]. Furthermore, AA has been discovered to enhance the mineral deposition in bone matrix and trigger the osteoblast development. Additionally, *in vitro* studies demonstrated that can trigger the accumulation of apatite crystals by serving as a catalyst for their nucleation [96, 97]. Other than acidic amino acids, citric acid (CA) has also been reported to have substantial function in bone formation by interacting with calcium ions during bone formation [98].

Recent research endeavors have been directed towards synthesizing calcium phosphates in presence of acidic small molecules within the environment. These studies investigated the inflence of small molecules on HAp in different respects, involving the interaction small molecules with HA particles, the effect of adsorption of small molecules on HAp characteristic features such as purity, particle size distribution, structural and dielectric properties, the impact of small molecule adsoprtion on HAp on biological functionality *in vivo*. These studies revealed that acidic small molecules could be highly promising in tailoring the features of HA based materials to fulfill specific qualifications during the development of bone-related structures and devices [92, 93, 99, 100].

The incorporation of AA and Glu has been highlighted as a promising approach to improve the bioactivity and functionality of HA for bone regeneration applications. These amino acids take part in the growth of apatite crystals and are involved, not only in nucleating them but also in their crystal formation, which is extremely critical to form bone mineralization. Hence, the modification of HA with these amino acids mimics the natural composition of the bone. Studies conducted by Boanini *et al.*, indicated that the HA nanocomposites doped with AA and Glu increase the interaction with osteoblast-like cells, thus suggesting enhanced bioactivity and, therefore, supporting the important factors for efficient bone regeneration, such as proliferation and differentiation of bone cells [18].

In addition, the modification of HA with AA and Glu improved the mechanical properties of HA based composites. This enhancement has paramount importance in the development of biocompatible bone grafts and implants that, enable to withstand physiological loads. These HA materials, which have been modified with amino acids, may also serve as a novel platform for developing a novel, targeted drug delivery system, particularly for bone-related diseases. In this way, modified derivatives, which increase HA affinity towards certain cellular receptors of bone tissue, allow localization of drug targeting with increased effectiveness of treatment and minimum systemic side effects [101]. These studies suggested that the integration of amino-acid-modified HA in BoC platforms provides opportunities for targeted drug delivery studies within a highly controlled microenvironment. This HA can be used to study the interactions of the bone tissue with different therapeutics toward developing new treatments for bone-related diseases, all due to enhanced bioactivity by modification with AA and Glu.

The studies on the modification of HA using CA bear immense advancement in the field of biomaterial engineering, most importantly related to bone regeneration, dental applications and orthopedic implants. These studies have suggested that CA is of prime importance in the improvement of physicochemical properties and biological functionalities of HA. For example, nHA nanoparticles coated with CA have been found to have higher solubility. Hence, CA-modified HA can be the material of preference for its use in drug-delivery applications and bone-tissue engineering [22, 102]. Besides, the development of HA composites with CA for orthopedic implant use suggests that these materials have the advantage of better biocompatibility and osteoconductivity than other such materials [91, 103].

Moreover, the research establishes the varied applications of CA-modified HA, ranging from the promotion of osteoblastic differentiation to improving degradation behavior and biological properties for *in vitro* applications of nHA particles [102, 104]. The clinical evaluations also emphasized the effectiveness of nHA composites with CA towards periodontal regeneration, representing a new material that could hold great promise for regenerating intra-bony defects [105]. The studies provide an incisive perspective on the possibility of CA as a modifying agent for HA and hence laid

the foundation for possibilities in the development of next-generation biomaterials for biomedical applications.

In the context of this thesis study, the impact of acidic small molecules namely AA, Glu, and CA on HA immobilization onto PDMS surfaces has been investigated in this thesis study in an effort to develop potential functional BoC platforms for future research.

3. MATERIALS AND METHODS

3.1 Functionalization of nHA Particles with Small Molecules

L-aspartic acid (AA), L-glutamic acid (Glu) and citric acid (CA) were used as small molecules for functionalization of nano-hydroxyapatite (nHA) particles. These small molecules were chosen based on charges of functional groups present in their structure, as listed in Table 3.1, nHA particles were functionalized with small molecules through a well-known EDC/NHS chemistry. To that end, carboxyl groups of small molecules were initially activated by dissolving 250 mg of each molecule in 100 mL of ultrapure water (UP water) containing 250 mg of 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC) and 250 mg of N-hydroxysuccinimide (NHS) and stirred for 2 hours. Then, 250 mg of nHA particles were added into the activated small molecule solutions and stirred for 24 hours at room temperature to allow the reaction between carboxyl groups of small molecules and OH groups of nHA particles. Subsequently, small molecule functionalized nHA particles were washed three times by centrifugation at 5000 rpm for 15 minutes and resuspension in UP water each time. The resulting small molecule functionalized nHA particles were freeze-dried, grounded into fine particles, and stored at 4°C until use [29, 106].

Table 3.1
Dissociation constants of functional groups on the structure of small molecules.

	pKa1	pKa2	pKa3	Reference
AA	2.1	3.7	9.8	[107]
Glu	2.2	4.2	9.7	[108]
CA	3.1	4.8	6.4	[109]

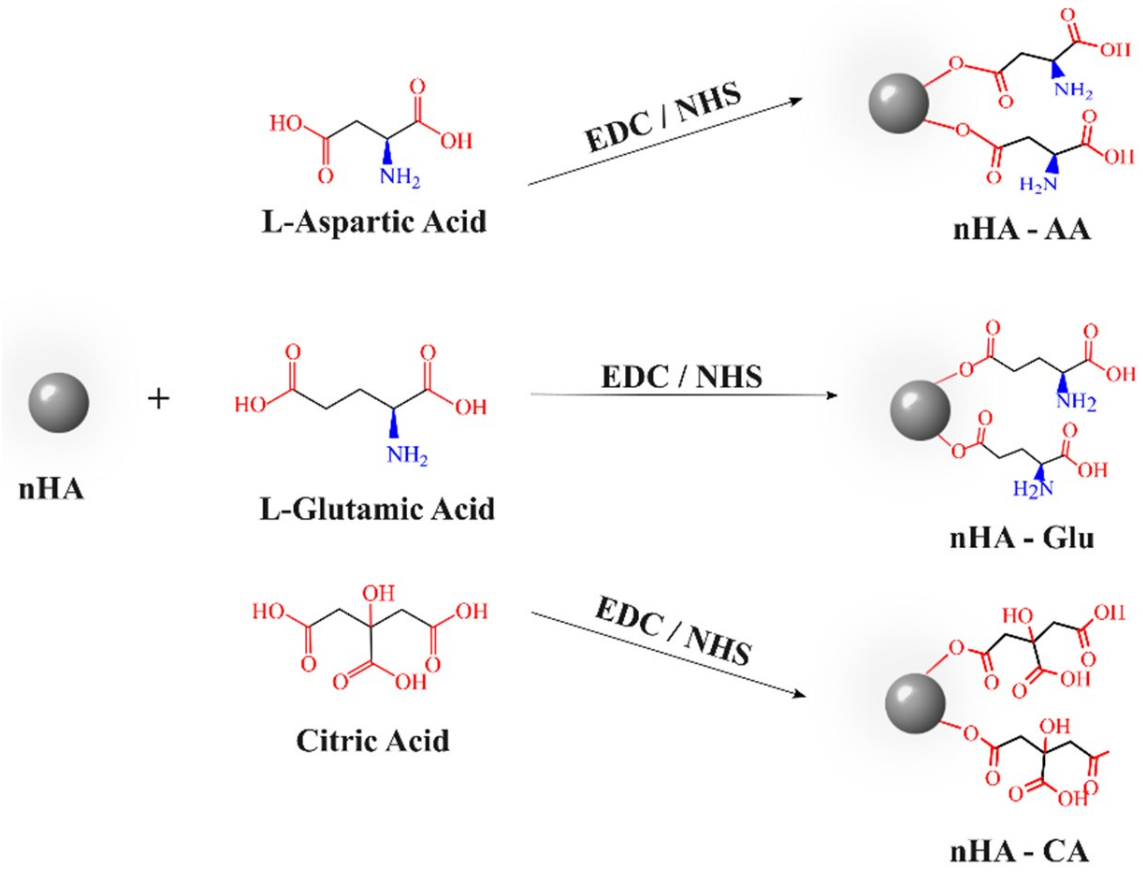

Figure 3.1 Schematic representation of the surface grafting reaction for nHA particles with L-Aspartic Acid (AA), L-Glutamic Acid (Glu), and Citric Acid (CA).

3.2 Immobilization of Small Molecule Grafted nHA Particles on PDMS

Poly(dimethylsiloxane) (PDMS) substrates were prepared by using 10:1 mixture of silicone elastomer base and curing agent (Sylgard 184, Dow Corning, USA), degassing under vacuum, and followed by curing at 75°C for 4 hours as described by Erenay *et al.* [82].

After that, PDMS substrates were modified with the small molecule functionalized nHA particles using silanization and carbodiimide reactions. Hence, the sur-

faces of PDMS substrates were silanized through treatment with oxygen plasma for 60s under 50 sccm oxygen flow at 200 mTorr pressure and followed by incubation in 10% (3-aminopropyl) triethoxysilane (APTES) solution in absolute ethanol at 50°C for 2 hours [110]. Meanwhile, the free carboxyl groups of small molecules introduced onto nHA particles were activated using the carbodiimide reaction. Briefly, 100 mg of small molecule modified nHA particles were added into 20 mL of aqueous solution containing 100 mg of EDC and 100 mg of NHS and stirred for 2 hours at room temperature to obtain the activated nHA suspension solution. After washing silanized PDMS substrates with UP water three times, the samples were incubated in activated nHA suspension solution for 24 hours at room temperature under gentle stirring. Hereafter, PDMS substrates modified with nHA particles underwent with washing process with UP water, repeated three times to remove excessive and physically adsorbed nHA particles and then dried under vacuum conditions at room temperature for subsequent use [29, 111]. AA grafted nHA particles immobilized on PDMS, Glu grafted nHA particles immobilized on PDMS and CA grafted nHA particles immobilized on PDMS substrates were abbreviated as PDMS-nHA-CA, PDMS-nHA-Glu and PDMS-nHA-CA. From this point on, these abbreviations will be used throughout the manuscript.

3.3 Surface functionalization of PDMS substrates with small molecules

As a second approach, the functionalization of PDMS surfaces with small molecules was conducted through the reaction between amine groups of silanized PDMS surfaces with carboxyl groups of small molecules as shown in Figure 4.3. Accordingly, the protocol described in the previous section was followed for the silanization of PDMS surfaces. Meanwhile, the carboxyl groups of small molecules were activated by mixing 100 mg of small molecules with 100 mg of EDC and 100 mg of NHS in 20 mL of UP water under continuous stirring at room temperature for 2 hours. Subsequently, silanized PDMS substrates were separately incubated in each small molecule solution at room temperature under gentle shaking [28, 111]. After 24 hours, the samples were

taken out of the solution and rinsed with UP water. Finally, samples were dried under a vacuum and stored in the desiccator for a maximum of 24 hours before subsequent use.

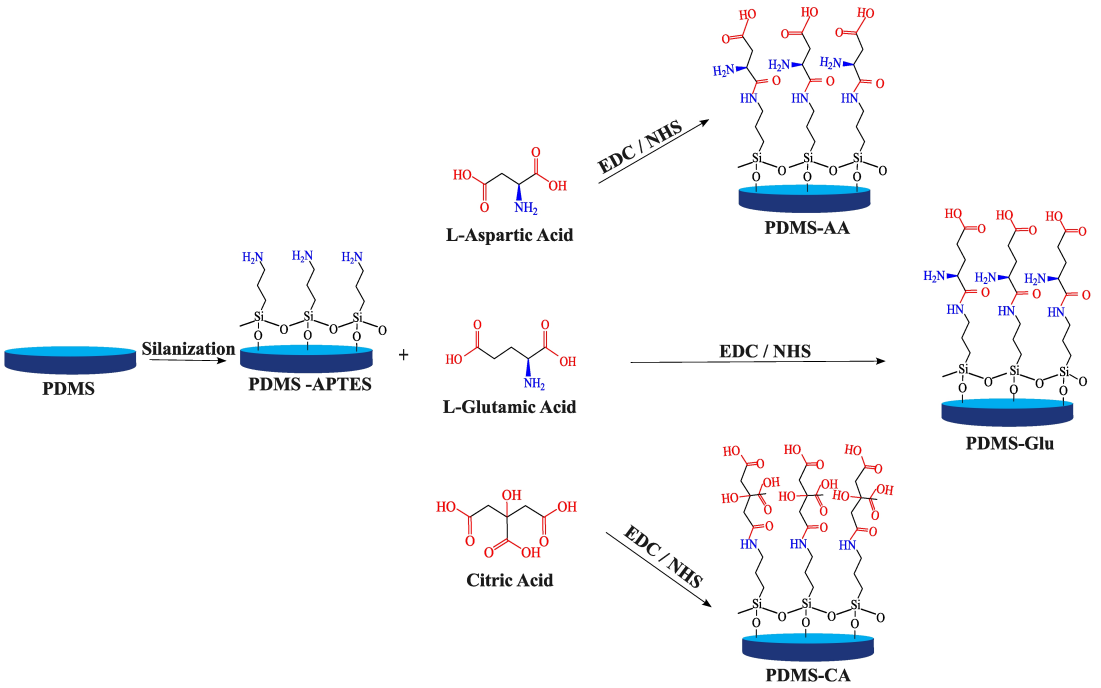

Figure 3.2 Schematic illustration of the surface modification process for PDMS with Glu, AA, and CA small molecules.

3.4 Precipitation of HA on PDMS functionalized with small molecules

The HA precipitation on PDMS substrates was investigated by incubation of small molecule modified PDMS substrates in Simulated Body Fluid (SBF) prepared by the protocol provided by Kokubo *et al.* [112]. Hence, the nucleation sites were initially formed on the small molecule modified PDMS substrates through incubation in 0.2 M $CaCl_2$ for 20s and rinsing with UP water and followed by incubation in 0.2 M K_2HPO_4 for 20s and rinsing with UP water. This pre-treatment with ionic solutions was repeated three times [113]. Thereafter, each small molecule immobilized

PDMS substrate was immersed in 40 mL of SBF inside centrifuge tubes and incubated at 37°C by gentle shaking at 100 rpm for 7, 14 and 21 days to deposit HA on the substrates. These substrates were then washed with UP water, dried under vacuum, and stored in the desiccator for further use [114]. HA precipitated on AA functionalized on PDMS, HA precipitated on Glu functionalized on PDMS and HA precipitated on CA functionalized on PDMS were abbreviated as PDMS-CA-HA, PDMS-Glu-HA and PDMS-CA-HA. From this point on, these abbreviations will be used throughout the report.

3.5 Characterization of nHA Particles Functionalized with Small Molecules

3.5.1 FTIR

The grafting nHA particles with AA, Glu and CA, the functionalization of PDMS surfaces with AA, Glu and CA and HA deposition on PDMS surfaces were confirmed with Fourier transform infrared-attenuated total reflectance (FTIR-ATR) (Bruker, Vertex 70V, USA) by recording the spectra between 400 cm^{-1} and 4000 cm^{-1} with the resolution of 1 cm^{-1}.

3.5.2 Zeta Potential

Surface potential of nHA particles and small molecule grafted nHA particles were determined with Zetasizer (Malvern Instruments, NanoS, UK). Measurements were undertaken on nHA particle colloidal dispersion in deionized water at a concentration of 1.25 mg/mL [115].

3.6 Surface characterization of PDMS substrates

3.6.1 XPS

The surface functionalization of PDMS substrates with small molecules were characterized using X-ray photoelectron spectroscopy (SPECS, FlexMod, Germany) using monochromatic Al Kα X-ray source by performing both wide scans and high-resolution scans for Si2p, C1s, N1s, and O1s region. Deconvolution of the high-resolution peaks was performed using the Gaussian-Lorentzian function.

3.6.2 XRD

The crystalline structures of HA deposited on PDMS substrates by biomineralization, and immobilization methods were examined with X-Ray diffraction (XRD) (Empyrean, Malvern Panalytical, UK) using Cu Kα radiation source (λ = 1.54, 40 kV and 20 mA) over the range between 4° and 50 of 2θ value.

3.6.3 SEM

Scanning electron microscopy (SEM) was used to evaluate the changes in the surface topography of the PDMS substrates following functionalization with HA by small molecule chemistry. The substrate surfaces were sputter-coated with 5 nm platinum and then imaged with SEM (Philips XL30 ESEM-FEG/ EDAX) using 10 kV voltage.

3.6.4 Water contact angle measurements

The wettability of PDMS substrates deposited with HA was assessed using the sessile drop technique by dropping 5 μL of UP water on the surface of substrates and

analyzing the contact angle of water drop after 10 s using the contact angle goniometer (KSV, CAM-100, Finland). The contact angle was measured at three different points for each sample, and triplicate samples were used for each group.

3.7 *In vitro* Biological Evaluation of HA coated PDMS Substrates

The interaction of HA-coated PDMS substrates with human fetal osteoblast cells (hFOB 1.19 ATCC CRL-11372 Homo sapiens bone, ATCC) was evaluated through *in vitro* cell culture studies. Briefly, the substrates were sterilized by immersing them in 70% ethanol two times for 15 minutes, exposing them to ultraviolet (UV) light for 30 minutes, and washing them with sterile phosphate buffer saline (PBS) solution. Subsequently, hFOB 1.19 cells were seeded on the substrates at a density of 8000 cells/ cm2 and cultured in DMEM F12 medium containing 2.5 mM L-glutamine supplemented with 10% (v/v) fetal bovine serum (FBS) (Biosera) and 1% penicillin/streptomycin (P/S) (Biosera). Adherent cells on the substrates were incubated at 35°C and 5% CO_2 for 14 days [116].

3.7.1 Metabolic Activity of hFOB 1.19 Cells

The viability of hFOB 1.19 cells cultured on HA deposited PDMS substrates was assessed using 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay on days 1, 4, 7, and 14. On the day of analysis, culture media from each well were aspirated and replaced with 10% (v/v) MTT in cell medium. Cells were incubated at 35°C for 4 hours. MTT solutions were aspirated and formazan crystals were dissolved in 300 μL of dimethylsulfoxide (DMSO). Resulting formazan solutions in each well were transferred into separate wells in 96-well plates. Absorbance of the wells was read at 570 nm with reference to 750 nm using a microplate reader (Bio-rad, iMark, USA). The viability of cells on PDMS substrates was calculated by normalizing the values to those from cells cultured on a tissue culture plate (TCP). Each assay was

repeated three times, and three samples were used for each experimental group [117].

3.7.2 Immunofluorescence Staining

Cytoskeletal structures of hFOB 1.19 cells adherent on HA deposited PDMS substrates were examined by double staining with phalloidin and Hoechst 33342 fluorescent dye solutions. To that end, hFOB 1.19 cells cultured on the substrates for 7 days were fixed with 4 wt% paraformaldehyde solution in PBS for 30 min. Subsequently they were permeabilized using 0.1% Triton X-100 in PBS containing 0.05% Tween 20 for 5 min and then blocked with 1 wt% bovine serum albumin (BSA) solution in PBS for 1 hour at room temperature. After each step, the cells were washed with PBS three times. Later, the cytoskeletal structure and nucleus of cells were stained by immersion in phalloidin solution in 1 wt% BSA (Alexa Fluor 488, Thermo Fischer) diluted at 1:40 ratio for 20 min and Hoechst 33342 dye solution (ab228551, Abcam) diluted at 1:1000 ratio for 30 min respectively and followed by gently washing with PBS for three times after each staining. Cells were imaged under a fluorescent microscope (Leica, DM IL, Germany) [118].

3.7.3 Morphological Evaluation of hFOB 1.19 Cells

The morphology of hFOB 1.19 cells cultured on HA-deposited PDMS substrates was imaged with SEM. After culturing cells on PDMS substrates for 7 days, they were fixed with 3% glutaraldehyde solution for 30 min. Subsequently, the cells were dehydrated by immersion in 50, 70, 90, and 100% ethanol grades for 10 min in each ethanol solution. Cells were later treated with hexamethyldisilazane (HMDS) and air-dried in the laminar hood overnight prior to imaging. Samples were sputter coated with 5 nm platinum and then imaged under SEM using 10 kV [119].

3.8 Statistical Analysis

All quantitative results were expresses as mean $\pm$ standard deviation and subjected to statistical analysis using ANOVA followed by Tukey HSD post hoc test. Statistically significant differences between the experimental groups were determined based on p-values with denotation of ns, for $p \geq 0.05$; $*$ for $p < 0.05$; $**$ for $p \leq 0.01$; $***$ for $p \leq 0.001$).

4. RESULTS AND DISCUSSION

4.1 Grafting Small Molecules to Hydroxyapatite Particles

To decipher the interaction between small molecules with commercially available nHA particles, FT-IR spectra of small molecule grafted nHA (SM-nHA) particles were compared with pristine nHA particles, as shown in Figure 4.1. All SM-nHA particles showed a slight shift in the peak of the phosphate stretching mode from 1027 to 1058 cm^{-1}. This peak shift is ascribed to the interaction between the inorganic phosphate group with functional groups present in organic compounds [22, 120]. Additionally, peaks specific to small molecules were observed at the spectra of SM-nHA particles. The C=O stretching of carboxyl groups in small molecules was assigned around 1650 cm^{-1}, which confirms the grafting of nHA particles with small molecules [29]. Furthermore, the two peaks around 2850- 2930 cm^{-1} are affiliated with C-H stretching of methylene groups, whereas the peak at 3433 cm^{-1} is attributed to O-H groups of carboxyl moieties on the structure of small molecules [106]. All these observations present enough evidence to conclude that the small molecules were successfully immobilized onto nHA particles.

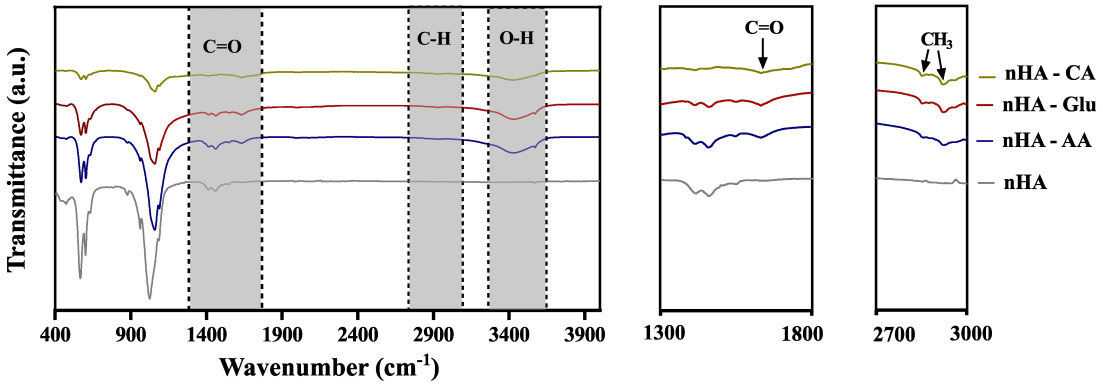

Figure 4.1 Characterization of the interaction between nHA particles with small molecules through comparing ATR-FTIR spectra of pristine and Glu, AA, and CA grafted nHA particles.

The small molecule functionalization of the nHA particles surfaces was also investigated through assessment of changes in surface charge characteristics as it can be seen in Figure 4.2. This has been majorly contributed by the existence of amino and carboxyl (NH_2-H^+ and COO^-) groups from the small molecules, coupled with differences in the availability of grafting sites on the surfaces of nHA particles [121]. The influence of these modifications on the surface charge of particles was rigorously analyzed using Zetasizer measurement.

While the zeta potential of unmodified HA particles was recorded to be -6.67 mV, it was observed to be a far more negative potential (approximately -13 mV) when functionalized with CA (CA-nHA particles) in line with previous study [122]. The shift in zeta potential not only substantiates the efficient surface modification achieved through functionalization with CA but also reflects higher stability and the possibility of interactivity of the Glu-HA particle surface. On the other hand, the zeta potential values moved to be more positive with both nHA-AA and nHA-Glu particles, as –0.41 mV and –3.09 mV, respectively.

This is indicative of the fact that CA imparts different characteristics in surface charge as compared to Glu and AA, which could give an information about the possible difference in their interaction dynamics with the nHA particles. The functionalization of nHA particles with small molecules was observed to increase the zeta potential (making it less negative) compared to unfunctionalized nHA particles, indicating an increase in surface charge neutrality. Dual roles of carboxyl and amine groups as binding agents in AA and Glu most probably contribute to the zeta potential observed, balancing the interaction with the surface of nHA particles. Implying the possibility of these factors being controllable in a strategy to tune the surface charge, the fact that the zeta potential was significantly affected independently by such a small molecule for purposes of functionalization would mean that the specific kind of small molecule can be controllable for specific applications [121].

These results strongly support the fact that complex interplay for the physico-chemical properties of the nHA particles and their functionalization with small molecules

is necessary to allow tailorability of their surfaces to support the site-specific interaction of surface design for various bone tissue engineering applications.

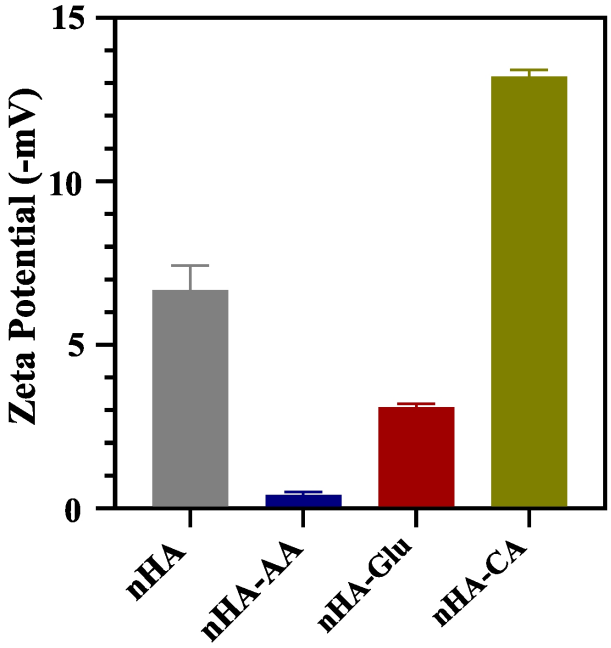

Figure 4.2 Comparisons between zeta potentials of pristine, Glu, AA, and CA modified nHA particles.

4.2 Functionalization of PDMS surfaces with small molecules

Small molecules were frequently used to enhance the deposition of HA particles on various substrates developed for biomedical applications [30, 98, 114]. In this study, PDMS surfaces were functionalized with Glu, AA, and CA small molecules through silanization and carbodiimide reactions. The reactions between PDMS, APTES, and small molecules were elucidated through high-resolution XPS, as illustrated in Figure 4.3. Wide scan survey spectra of PDMS have shown Si2p (binding energy of 99.4), Si2s (binding energy of 150.1), C1s (binding energy of 282.7 eV), and O1s (binding energy of 531.3 eV), as expected. N1s photoelectron signal was observed at 397.7 eV binding energy in the spectra of functionalized PDMS substrates, which confirms the successful immobilization of APTES and small molecules on PDMS substrates [123].

The chemical compositions of plain, APTES, and small molecule-modified PDMS substrates are presented in Figure 4.3.(a). Plain PDMS had no nitrogen content, but the introduction of APTES on PDMS substrates led to a decrease in the atomic ratio of silicon from 15.59 to 11.90 as well as an increase in the atomic ratio of nitrogen to 4.04 and oxygen from 51.58 to 66.07 compared to that of plain PDMS. Changes in the atomic ratios demonstrate effective anchoring of APTES on PDMS substrates. Upon immobilization of small molecules on PDMS substrates, the atomic ratio of nitrogen was decreased slightly; whereas, the atomic ratio of oxygen was further increased. This finding indicates the interaction of amine groups of APTES with carboxyl groups of small molecules.

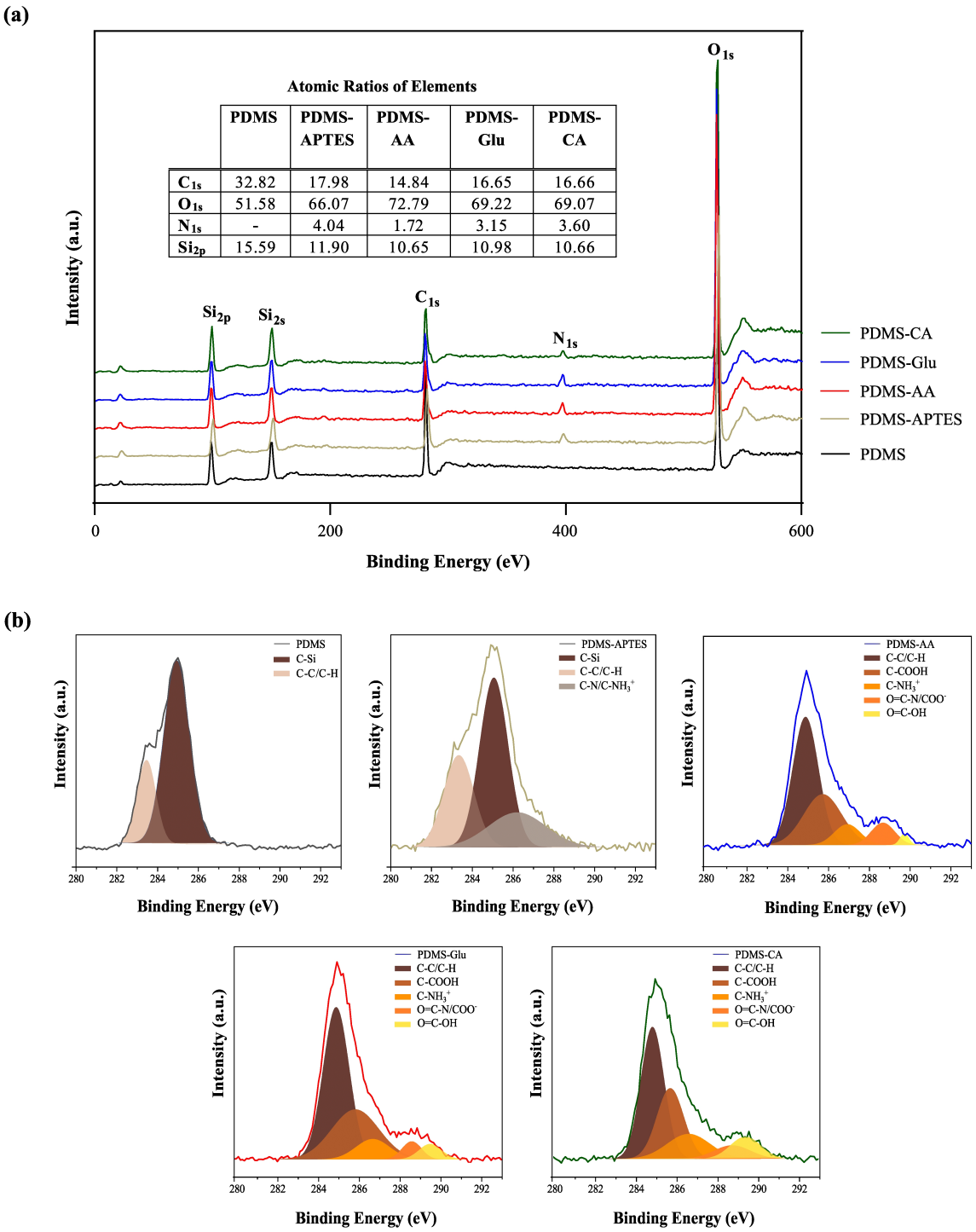

Figure 4.3 Evaluation of variation in chemical states of elements for plain, silanized, and Glu, AA, and CA-modified PDMS surfaces using XPS (a) XPS wide scan and (b) C1s core level elemental XPS spectra.

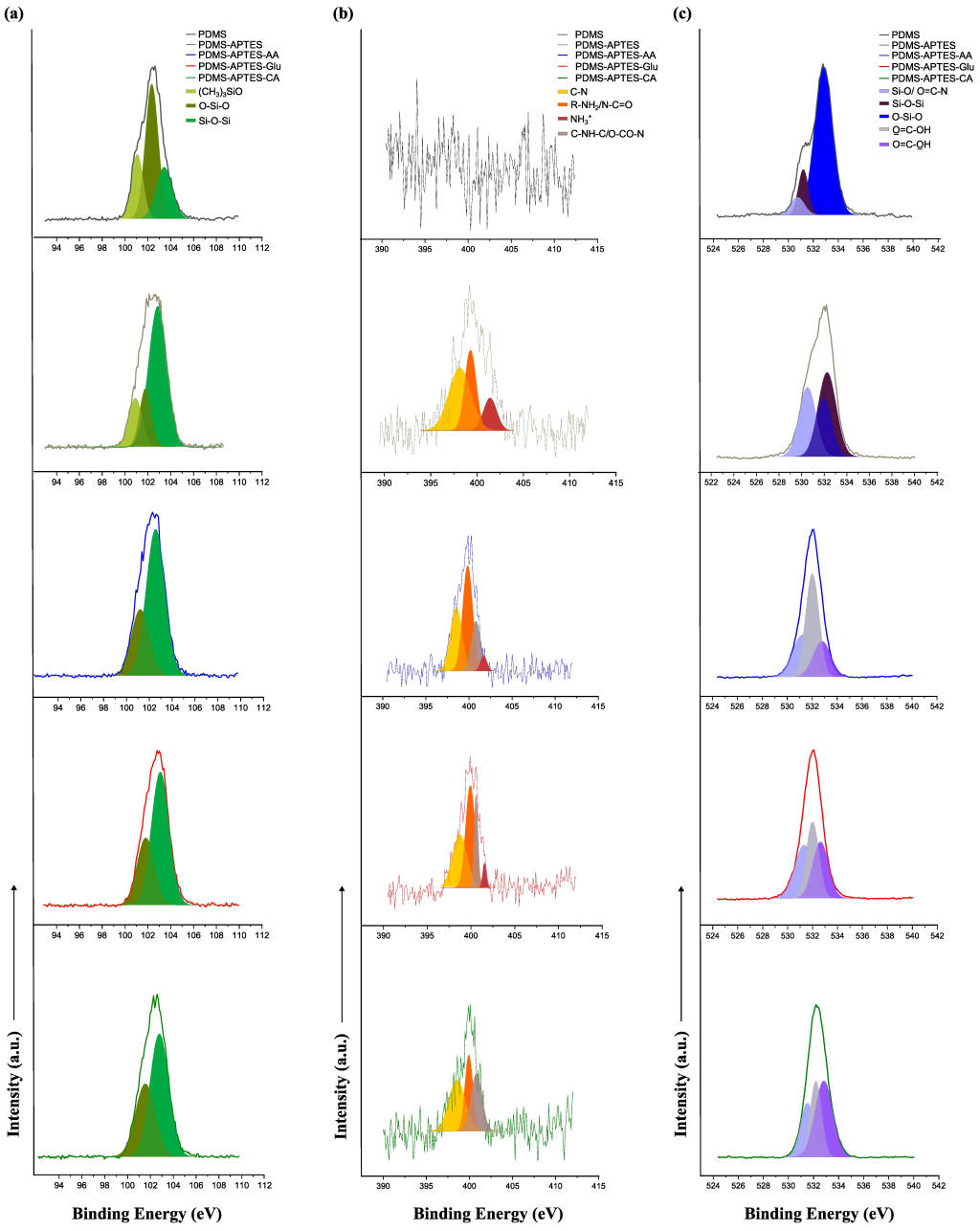

Figure 4.4 Evolution in the chemical states of the elements following small molecule modification using XPS. Deconvoluted high-resolution XPS spectra of (a.) Si2p, (b.) N1s and (c.) O1s core levels for PDMS, PDMS-APTES, PDMS-AA, PDMS-Glu, and PDMS-CA.

High-resolution XPS spectra of C1s, O1s, N1s, and Si2p peaks of PDMS substrates functionalized with small molecules (SM-PDMS) were deconvoluted into curves to identify bonds formed after the chemical modification process. Figure 4.3.(b) depicts the comparison of C1s spectra of plain and small molecule functionalized PDMS substrates to identify the changes in the carbon-based bonds after modification with small molecules. C1s spectrum of plain PDMS was deconvoluted into a double peak, attributed to C-Si bonds at 283.45 eV and C-C/ C-H bonds at 284.93 eV [123–125]. The C1s spectrum from PDMS surfaces silanized with APTES was divided into three curves at 283.33 eV, 285.06 eV, and 286.19 eV, which are assigned to the peaks of C-Si, alkyl carbon (C-C) / hydrocarbon (C-H) and carbon-nitrogen (C-N) / ($C\text{-}N^+$)/ protonated amine ($C\text{-}NH_3^+$) bonds, respectively [123]. Presence of a carbon-nitrogen peak after silanization confirms the successful immobilization of APTES onto the PDMS surface. After the functionalization of the silanized PDMS surfaces with small molecules, C1s peaks were fitted by five curves. Curves previously ascribed to C-C/C-H and C-N/ $C\text{-}N^+$/ $C\text{-}NH_3^+$ bonds were observed again after small molecule functionalization approximately at 285 eV and 286 eV. Newly formed curves approximately at 287 and 289.5 eV were assigned to the peaks of C-COOH and carboxylic acid (O=C-OH) bonds, respectively [28, 126]. These curves might indicate the presence of reacted or unreacted small molecules in the environment. Also, the curve observed around approximately 287.9 eV, was attributed to the amide (O=C-N)/ carboxylate (O=C-O-) bond, which denotes the reaction between the amine group of APTES and carboxyl groups of small molecules [127]. Moreover, N1s, O1s, and Si2p spectra shown in Figure 4.4 provided further evidence that these small molecules were grafted on the PDMS surfaces.

4.3 The Deposition of HA particles on PDMS surfaces

Change in the surface chemistry after the deposition of HA particles with two different approaches on PDMS substrates was analyzed with ATR-FTIR spectroscopy. Figure 4.5.(a) depicts the spectrum with peaks ascribed to PDMS and HA. Specifically, the deposition of HA on PDMS substrate surfaces gave rise to the appearance of the new characteristic phosphate peaks of apatite in both coating methods. These peaks at 569

and 603 cm^{-1} correlated with bending vibrations of PO_4^{3-} and stretching vibrations of PO_4^{3-}, respectively [128, 129]. However, these peaks were more pronounced in the spectra of small molecule grafted nHA particles coated on PDMS (PDMS-nHA-SM) substrates than those of HA precipitated on small molecule functionalized PDMS (PDMS-SM-HA) substrates.

The microstructure of plain PDMS and HA particles deposited on PDMS samples through small molecule chemistry were analyzed with thin film XRD spectroscopy. As shown in Figure 4.5.(b), the diffractogram of plain PDMS demonstrates two characteristic peaks at around 12.4°, proving the amorphous structure of the PDMS as indicated in the previous study [130]. Also, plain PDMS and PDMS substrates incubated in SBF for 21 days had similar diffraction patterns, confirming the inertness of non-activated PDMS surfaces in this study.

XRD spectra of PDMS-nHA-SM substrates and PDMS-SM-HA exhibited similar diffraction patterns in addition to the characteristic patterns of PDMS. These diffraction peaks centered at 26.1°, 31.7°, and 45.5° correspond closely to the previously described pattern for HA particles. These findings demonstrate that HA was effectively deposited onto PDMS substrates; however, an apparent difference was observed in the intensity of the peaks between HA deposition methods [28, 131]. PDMS-SM-HA substrates have led to higher intensities in characteristic peaks of HA compared to that of PDMS-nHA-SM substrates. These results are consistent with the findings obtained with ATR-FTIR analysis (Figure 4.5.(a)). Differences in the intensities of HA-specific peaks could be observed due to grafting of the nHA particle surface with small molecules, which decreases the crystallinity of the nHA, as shown an earlier study [132]. On the other side, the immobilization of small molecules on the PDMS surfaces facilitated the precipitation of a dense Ca-P layer on the samples, which may have caused increases in the intensities of observed peaks [114].

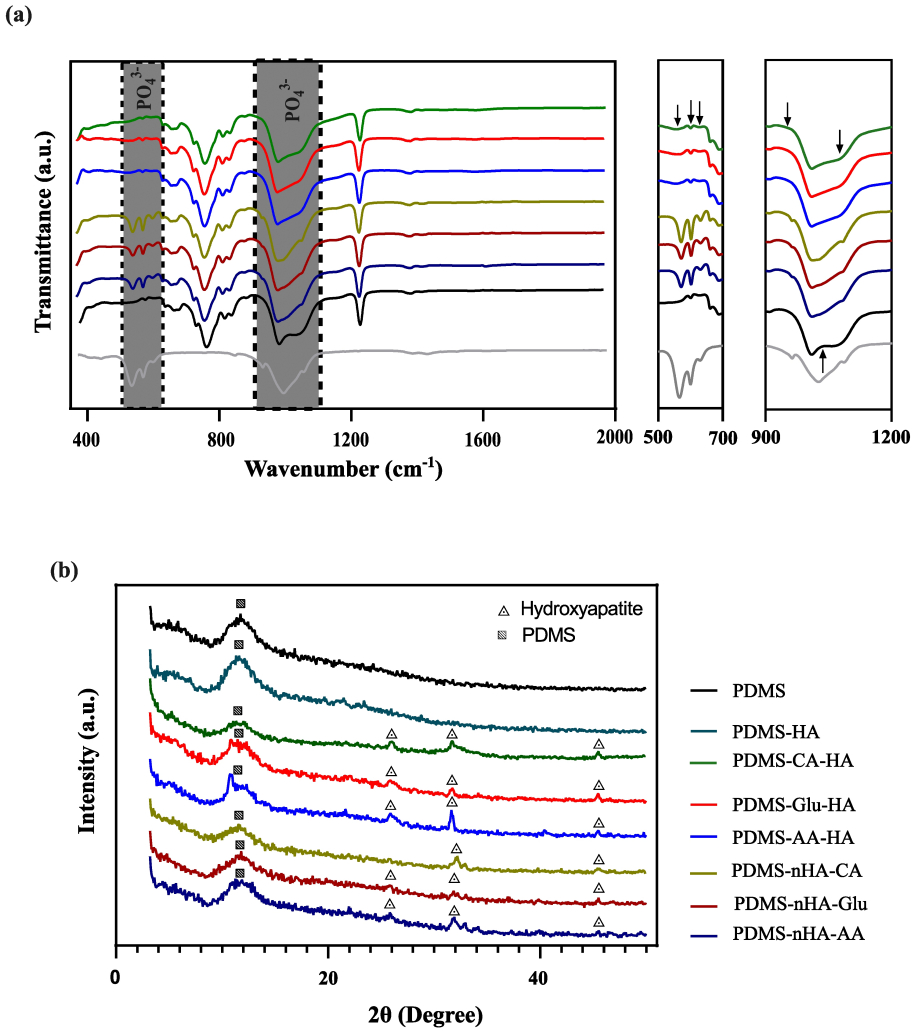

Figure 4.5 Comparisons of change in chemical composition between pristine PDMS, silanized PDMS, PDMS-nHA-AA, PDMS-nHA-Glu, and PDMS-nHA-CA substrates and PDMS-AA-HA, PDMS-Glu-HA, PDMS-CA-HA substrates using (a) ATR-FTIR and (b) XRD spectra.

4.4 Wettability of PDMS surfaces coated with HA particles

Water contact angle (WCA) values, which serve as a gauge for surface hydrophilicity, were determined by a sessile drop of deionized water on the surface of PDMS-nHA-SM and PDMS-SM-HA substrates. Figure 4.6 demonstrates the shape of water drops on HA-deposited PDMS surfaces and their contact angle with surfaces. Plain PDMS surfaces had the highest WCA values as 108° ± 3.6 °, denoting its highly hydrophobic nature, as is well known from the literature [133, 134]. Deposition of HA on PDMS substrates decreases WCA values of the surfaces below 75°,

which shows the wettability of the surfaces has been increased significantly, compared to plain PDMS surfaces due to the presence of polar functional groups on HA structure, nHA-SM particles, and PDMS-SM, as confirmed by the ATR-FTIR and XPS analyses presented above. PDMS-SM-HA had relatively lower WCA values as compared to PDMS-nHA-SM. This behavior can be ascribed to the deposition mechanism of HA particles since PDMS-SM-HA favored the deposition of particles uniformly over the surface in contrast to that PDMS-nHA-SM which tended to form partial agglomeration. As a result, the agglomeration may have caused a decrease in the hydrophilic surface contact area. Hence, an increase in the WCA value on PDMS-nHA-SM substrates, as suggested in a previous research study [135]. Even if the coating method had an influence on the hydrophilicity of substrates, no discernible difference was observed in WCA value between PDMS-AA-HA, PDMS-Glu-HA and PDMS-CA-HA substrates as well as PDMS-nHA-AA, PDMS-nHA-Glu and PDMS-nHA-CA substrates when the effect of small molecules was compared in each coating method.

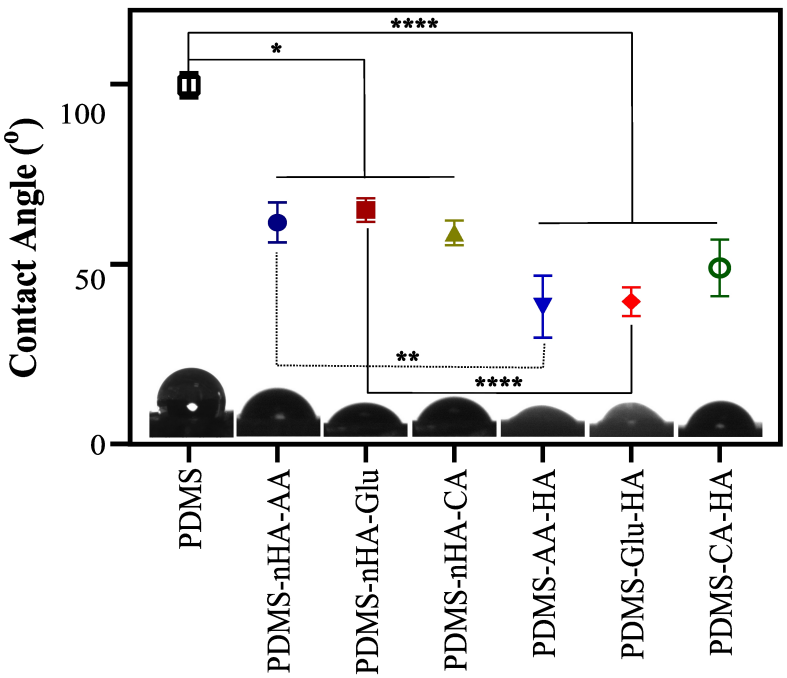

Figure 4.6 Images of water droplets on plain and HA deposited PDMS substrate surfaces and water contact angle measured on PDMS substrate surfaces.

4.5 Morphology of HA particles deposited on PDMS substrates

Morphological properties of HA deposited on PDMS substrates were analyzed from SEM micrographs as shown in Figure 4.7. HA particles were observed to be distributed uniformly over the surface of the substrates. However, HA particles formed a denser layer of coating on PDMS-SM-HA substrates than on PDMS-nHA-SM substrates. SM-nHA particles had a propensity to form aggregate, likely due to approximately neutral surface charge after functionalization, which was confirmed with zeta potential measurements depicted in Figure 4.2. This result was consistent with the results reported by Gonzalez *et al.*, where they have shown that functionalized nHA particles with relatively low surface charge were unstable in the dispersion, leading to aggregation formation [136]. Also, it is a well-known phenomenon that particles with a potential between -30 mV to 30 mV are deemed to be unstable and susceptible to agglomeration and flocculation [137].

Small molecules immobilized on PDMS substrates have essential roles in the nucleation of HA crystals. Previous studies have revealed that functional molecules on the substrates act as nucleation sites for Ca^{2+} and PO_4^{3-} ions, thus triggering the precipitation and the growth of HA particles [25, 138]. SEM images demonstrated that small molecule immobilization on PDMS substrates induced the deposition of HA (Figure 4.7), akin to the result of earlier reports [17, 97]. However, AA immobilization had a stronger nucleating ability than Glu and CA immobilization. This is attributed to the different affinity of small molecules to Ca^{2+} based on the charged state of the functional groups. A number of research demonstrated that increasing the negativity of surface functional groups improved the affinity for HA [25, 138, 139].

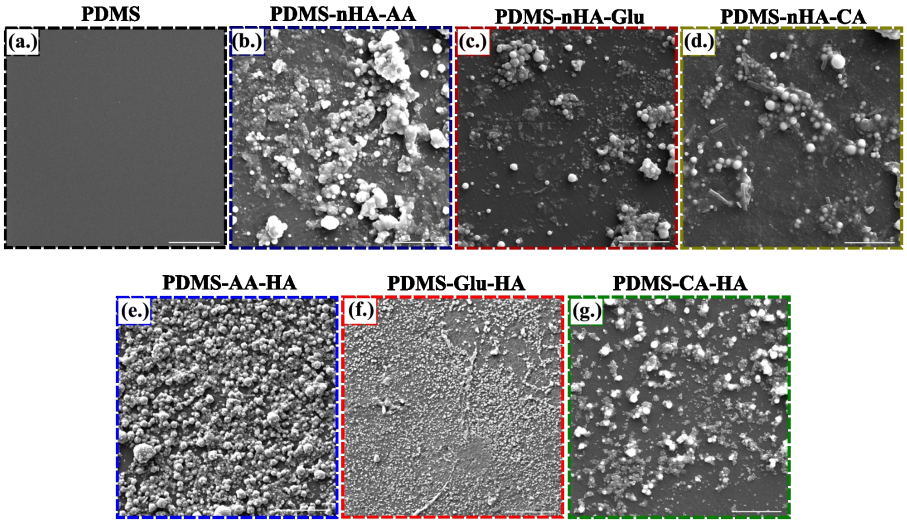

Figure 4.7 SEM micrographs showing the topography of PDMS surfaces functionalized with HA through small-molecule chemistry. (a) PDMS, (b) PDMS-nHA-AA, (c) PDMS-nHA-Glu, (d) PDMS-nHA-CA, (e) PDMS-AA-HA, (f) PDMS-Glu-HA, (g) PDMS-CA-HA (Scale bar: 5 μm).

Negatively charged carboxyl (COOH) groups of small molecules were usually responsible for interacting with the HA particles [140, 141]. Comparing the pKa values of carboxyl groups on the structure of the small molecules as tabulated in Table 1, AA was explored to be more negative than Glu and CA. Hence, it can be suggested that the negativity of small molecules and the affinity of HA to negatively charged functional groups led to observed differences in the rate of deposition of HA on PDMS substrates, which are consistent with the findings of previous reports [25, 138, 139].

4.6 hFOB 1.19 cell viability on PDMS substrates

The cytocompatibility of HA-deposited PDMS substrates was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Figure 4.8.(a) shows the cellular response as a percentage of cell viability. On the first day, the viability of hFOB 1.19 cells demonstrated only slight variations amongst HA-deposited PDMS samples through small molecule chemistry, suggesting that the efficacy of initial cell attachment on the samples was comparable to each other. Cell viability has signif-

icantly increased on all samples by the fourth day of culturing, though varying degrees, with the highest values attained on PDMS-SM-HA. On the 14th day of cell culture, the viability of cells cultured on the PDMS-SM-HA substrates was much higher than that of the cells cultured on PDMS-nHA-SM substrates. This result was anticipated due to a more uniform and denser layer of HA coating on PDMS-SM-HA than on other substrates (Figure 4.7). Homogenous HA coating on the PDMS substrates has contributed to introducing small surface features, increasing surface roughness, which is known to support cellular attachment and proliferation [131, 142].

Small molecule modifications provide potential benefits by increasing the efficiency of HA deposition by improving the physicochemical surface properties to favor the enhancement of substrate biocompatibility [28, 106, 111]. Accordingly, hFOB 1.19 cells viability on HA-deposited PDMS substrates through small molecule chemistry was significantly higher than that of cells on plain PDMS surfaces (Figure 4.8). Cell viability results have also revealed the distinct potency of each of small molecule modifications in the improvement of surface biocompatibility. When the experimental groups in the two different HA coating approaches were compared, the cell viability on PDMS-nHA-AA and PDMS-AA-HA were higher than that of other substrates. This result may have been acquired due to enhanced cytocompatibility of the surfaces with an increased HA coating density, induced by affinity of Ca^{2+} to highly negatively charged functional groups of AA, as observed in ATR-FTIR spectra and SEM images (Figure 4.5.(a) and Figure 4.7) [138, 143]. These results have demonstrated the decisive role of negatively charged functional groups on small molecule structures in directing cellular functions.

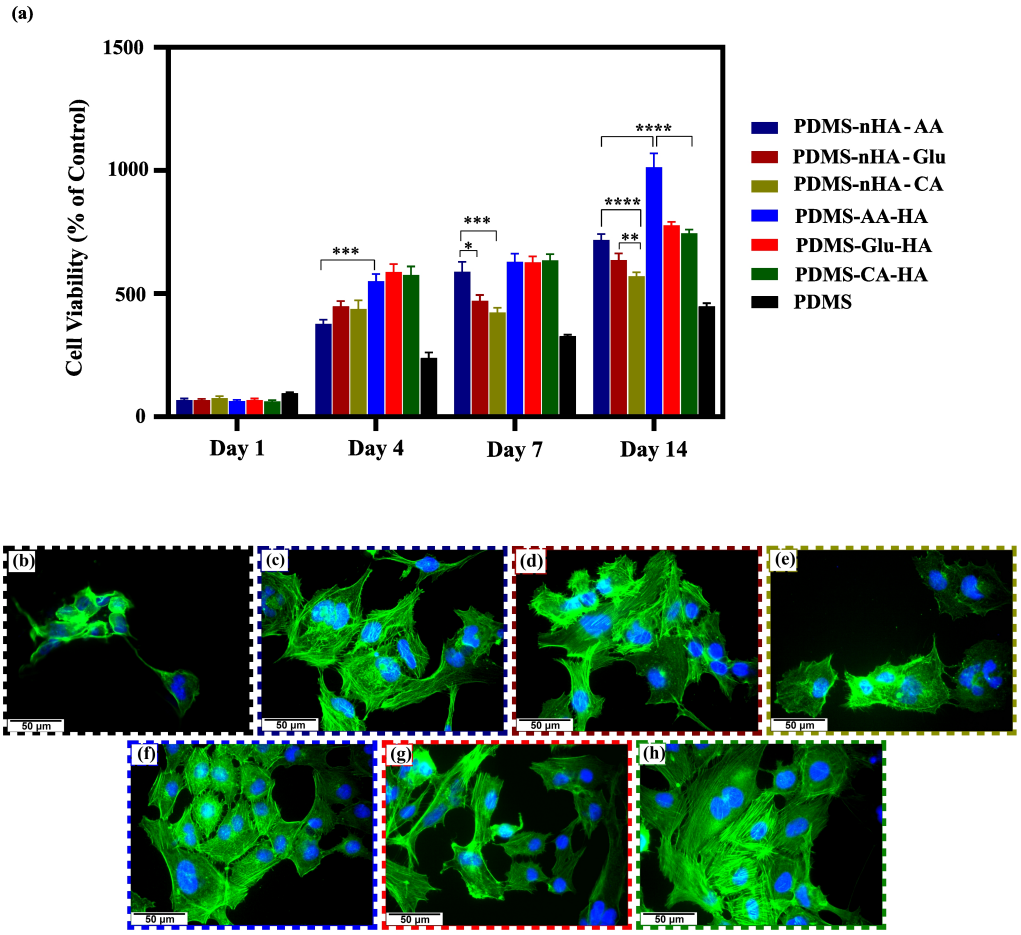

Figure 4.8 Characterization for *in vitro* cell culture of hFOB 1.19 cells on different hydroxyapatite deposited PDMS surfaces for 14 days. (a) MTT Assay results, representing viabilities of hFOB 1.19 cells cultured on PDMS surfaces deposited with HA with different methods. Fluorescence images showing immunofluorescence-stained actin filaments and nuclei of hFOB1.19 cells adherent on different PDMS surfaces on the 7th day of culture and (b) PDMS (c) PDMS-nHA-AA, (d) PDMS-nHA-Glu (e) PDMS-nHA-CA (f) PDMS-AA-HA (g) PDMS-Glu-HA and (h) PDMS-CA-HA surfaces (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; Scale bars: 50 μm).

4.7 Actin filament organization of hFOB 1.19 cells on PDMS substrates

Adhesion of hFOB 1.19 cells to HA-deposited PDMS substrates was followed by spreading, possibly mediated by profound changes in the organization of the actin cytoskeleton and the formation of focal adhesions as responses to HA on PDMS substrates. Accordingly, we examined possible interdependence between HA deposition on PDMS substrates through small molecule chemistry with cellular adhesion and spreading behaviors. For this purpose, actin filaments of hFOB 1.19 cells cultured on the HA-deposited PDMS substrates were stained with conjugated phalloidin and then counterstained with Hoechst to visualize the nuclei of cells on the 7th day of culture. hFOB 1.19 cells have shown an elongated morphology and no actin stress fibers other than at the apical border when grown on plain PDMS surfaces as can be seen in Figure 4.8.(b). On the other hand, hFOB 1.19 cells on HA-coated PDMS substrates had polygonal morphology with visible actin stress fibers across the whole cell body. Actin cytoskeleton organization of the cells on the substrates was driven by cell-material interactions. Utilizing plain PDMS surfaces in cell culture studies is known to be a challenging task due to its hydrophobic nature. HA deposition on PDMS surfaces has been shown to change its physical and chemical characteristics and has proven to be an effective approach for improving its biocompatibility [144, 145]. Accordingly, the cytoskeletal organization of osteoblast cells on HA-deposited PDMS substrates confirmed the beneficial effect of HA deposition on cellular activity.

4.8 Morphology of hFOB 1.19 cells on PDMS substrates

To acquire fundamental comprehension of the interaction between HA-deposited PDMS substrates and cells, the morphology of osteoblast cells was analyzed using FE-SEM, as shown in Figure 4.9. Previous studies highlighted the profound effect of HA coating on the induction focal adhesion and expansion of osteoblast by rendering the surface stimulating for cellular interaction [146, 147]. Accordingly, it was observed

that irrespective of coating approach all HA deposited PDMS substrates, the cells were tightly adhered to the substrates and exhibited well-developed lamellipodia with rich filopodia structures forming a network with surrounding cells and the surface. In contrast, cells cultured on plain PDMS surface showed limited expansion with the propensity to interacting with neighbor cells rather than the surface. This can be explained by the profound affinity of cells to coated surfaces triggered by enhanced hydrophilicity and chemistry provided by the inorganic crystals and organic compounds, as in this study, Glu, AA, and CA [146].

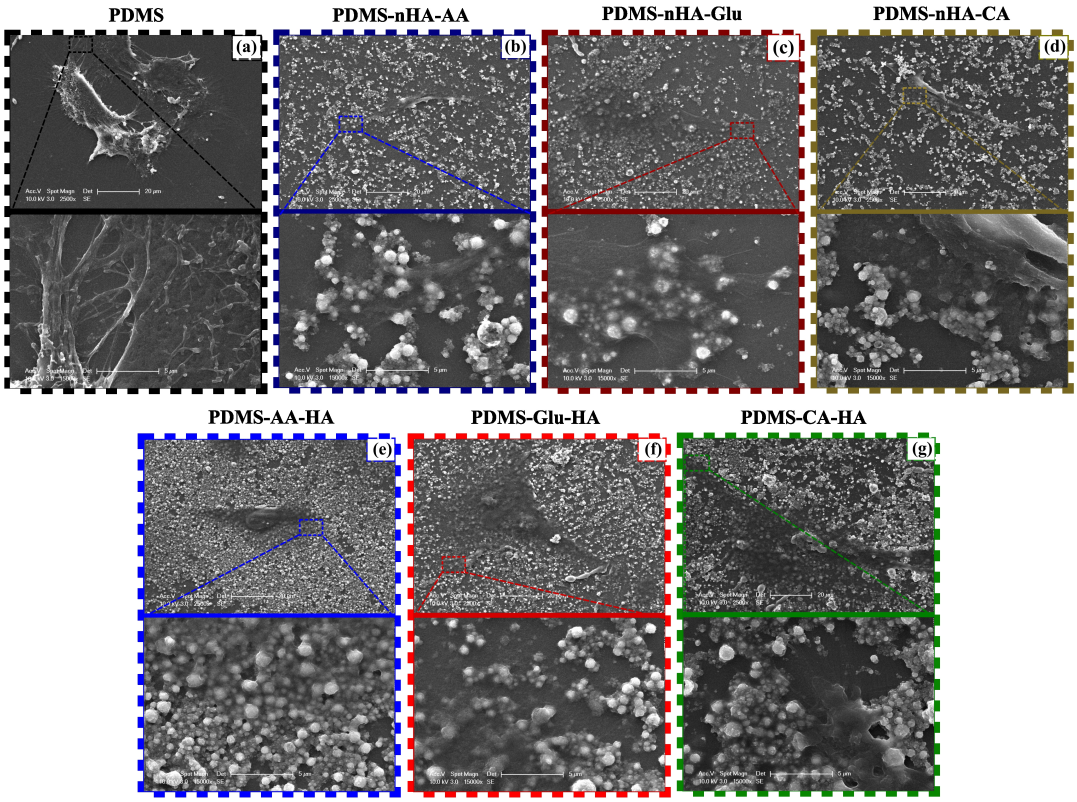

Figure 4.9 SEM micrographs visualizing the morphology of hFOB cells grown on (a) PDMS; (b) PDMS-nHA-AA, (c) PDMS-nHA-Glu, and (d) PDMS-nHA-CA surfaces (e) PDMS-AA-HA (f) PDMS-Glu-HA and (g) PDMS-CA-HA surfaces on the 7th day of culture (SEM image scale bar: 20 Î¼m and magnified SEM image scale bar: 5 μm).

To further elucidate the interaction of cells with HA particles coated on PDMS substrates through two different approaches, the morphology of cells was examined with higher magnification SEM imaging. The cells on PDMS-SM-HA tended to expand

much more than those on PDMS-nHA-SM. This variation in the spreading behavior of osteoblast cell were ascribed to the interaction between cells and deposited HA particles, especially the size of the contact area between HA particles and cells as well as the relationship between the surface roughness and the expansion of cells. This attribution was derived based on the observation in SEM figures that a denser layer of HA particles on PDMS-SM-HA substrates compared to that on PDMS-nHA-SM substrates. Accordingly, this dense layer of HA particles may have increased the surface roughness, so surface area along with contact sites available for interaction with cells, facilitating the expansion and elongation of cells on the substrates via the contact guidance phenomenon [148, 149]. Overall, it can be inferred that the surface chemistry and roughness provided by HA coating by incubation SBF resulted in a more profound effect on stimulation of the lamellipodia extension and filopodia protrusion of osteoblast cells.

5. CONCLUSION

This thesis study examined the overall characteristics of HA deposited on PDMS surfaces through a biomineralization and immobilization approach using small molecules for their potential to stimulate the bioactivity of hFOB cells. In this respect, initially, the complex mechanisms of small molecule grafting onto nHA particles, functionalization of PDMS surfaces with these molecules and HA particle deposition on PDMS surfaces were evaluated in detail. Subsequently, the effect of HA modification on cell viability, and morphology was investigated through *in vitro* cell culture studies. In this respect, this study found the groundwork for understanding deposition of HA on PDMS surfaces, where small molecules such as Glu, AA, and CA play vital roles in increasing the affinity of the PDMS surfaces for HA modification, a critical step for the development of advanced bone-on-a-chip platforms.

The study provided a detailed evaluation of the interactions between small molecules and nHA particles. The changes in FT-IR spectral peaks indicated successful grafting of these molecules on HA particles. Subsequently, the efficacy of immobilization of small molecules on PDMS surfaces was investigated through XPS analysis, revealing the changes in elemental composition, and confirming the successful immobilization of small molecules. Further chemical analysis offered a thorough understanding of HA particle deposition onto PDMS surfaces, where ATR-FTIR and XRD analyses provided a clear demonstration of the enhanced surface chemistry with HA deposition. The significant reduction in water contact angle on HA-deposited PDMS surfaces compared to plain PDMS highlights the enhanced hydrophilicity, which is a key factor in the interaction of cells with PDMS surfaces. The study also investigated the morphological changes caused by HA precipitation, using SEM imaging, revealing the uniform HA particle distribution that is critical for the intended functionality of the PDMS surfaces. The chemical and morphological analysis results proved that HA was successfully deposited on PDMS surfaces with both approaches, with some variation in the homogeneity of HA distribution across surfaces. The results demonstrated

that the biomineralization approach favored HA deposition better than immobilization techniques.

In vitro cell culture studies were conducted to examine the viability of cells on HA-deposited PDMS surfaces, demonstrating propitious conditions led by HA deposition for cell attachment. Especially, AA-functionalized PDMS surfaces were demonstrated to be the most suitable platform for osteoblast viability compared to HA deposited on Glu and CA functionalized surfaces since PDMS-nHA-SM surfaces resulted in a similar trend, where nHA-AA particles gave rise to higher osteoblast viability as compared to nHA-Glu and nHA-CA particles. Also, with *in vitro* cell culture study, the organization and morphology of actin filaments of hFOB 1.19 cells on engineered surfaces were investigated. The results showed not only enhanced cell adhesion and spreading on HA-coated PDMS, but also highlighted the impact of surface chemistry and roughness on cellular behavior. This understanding of the interaction between engineered surfaces and cells formed a background for future studies in tissue engineering and regenerative medicine applications.

In conclusion, this study investigated the complex mechanism of small molecule grafting either onto nHA particles or on PDMS surfaces, HA deposition on PDMS surfaces, and the subsequent effect of HA deposition on the viability and morphology of hFOB cells. The study revealed that the versatility of PDMS, with its ability to mimic surface features with soft lithography, can be further enhanced with HA modification by the methodology we presented in this study to fabricate bone models for exploring osteogenesis in a native-like environment. Hence, this work provides a variety of multifaceted approaches to engineering PDMS surfaces for biomedical applications. Through a detailed investigation of chemical modifications, HA precipitation, and cell-surface interactions, it elucidates the complex interaction between surface chemistry, morphology, and biocompatibility.

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