GREEN SYNTHESIS OF NANOSTRUCTURED BIOACTIVE GLASS FOR DENTAL APPLICATIONS

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ABSTRACT

GREEN SYNTHESIS OF NANOSTRUCTURED BIOACTIVE GLASS FOR DENTAL APPLICATIONS

Bioactive glass is a biomaterial commonly used in dental care products and bone tissue engineering applications due to its biocompatibility, bone-forming ability, and remineralization capability. Bioactive glasses form a hydroxyapatite-like layer on dentinal tubules by releasing calcium and phosphorus ions after interaction with saliva. Bioactive 45S5 glass traditionally synthesized by wet chemical methods which require high-temperature heating and the use of a strong acid catalyst, bringing into question of the possibility of introducing toxic acid residues into the final product. Therefore, there is a need to develop environmental-friendly bioactive glass synthesis methods or to modify existing ones in a way to uplift their environmental friendliness. To satisfy this need, we *greenized* the traditional sol-gel method by replacing the acid catalyst with an environment-friendly alternative and successfully used it for the synthesis of nano-structured 45S5 bioactive glass. First, physicochemical characterization of the synthesized bioactive glasses was performed. Then, the apatite formation capability of bioglasses were investigated in saliva. Next, the mineralization kinetics of bioglasses were tested in Ca/P buffer. *In vitro* toxicity tests were performed to assess the cytotoxic potential of the synthesized bioactive glass. All analyses were repeated for the traditional synthesis method for comparison purposes. The results confirmed that green synthesis is more advantageous in terms of bioactivity and functionality required for dental applications. Increasing the safety and functionality of bioglass at the same time during the production phase has critical importance for ensuring the sustainability of current applications as well as creating new uses in the biomedical field.

ÖZET

DİŞ HEKİMLİĞİ UYGULAMALARINDA KULLANILMAK ÜZERE YEŞİL SENTEZ İLE NANO-YAPILI BİYOCAM SENTEZİ

Biyoaktif cam biyouyumluluk, kemik rejenerasyonu ve diş sert doku remineralizasyonunu arttırması özellikleri sebebiyle diş bakım ürünlerinde ve kemik doku mühendisliği uygulamalarında yaygın olarak kullanılan bir biyomalzemedir. Biyoaktif camlar tükrük ile etkileşim haline girdiğinde kalsiyum ve fosfor iyonu salımı yaparak dentin tübülleri üzerinde kalıcı hidroksiapatit benzeri bir tabaka oluşturur. 45S5 biyoaktif cam geleneksel kimyasal yöntemlerle sentezlenebilmektedir. Ancak bu yöntemler yüksek sıcaklık ve güçlü asit katalizleri gereksinimleri dolayısıyla son üründeki toksisiteyi ve üreticinin üretim aşamasındaki sağlığını etkileme riski taşımaktadır. Bu nedenle; çevre dostu biyoaktif cam sentez yöntemlerinin geliştirilmesine veya mevcut yöntemlerin çevre dostu olma özelliklerini artıracak şekilde değiştirilmesine ihtiyaç vardır. Bu ihtiyacı karşılamak için, bu çalışmada asit katalizör çevre dostu bir alternatif ile değiştirilerek sol-jel yöntemi *yeşilleştirildi* ve nano yapılı 45S5 biyoaktif cam başarıyla sentezlendi. Sentezlenen biyoaktif cam örneklerinin fizikokimyasal karakterizasyonları yapıldı. Daha sonra, biyoaktif camın apatit oluşturma kabiliyeti yapay tükürük içinde araştırıldı ve mineralizasyon kinetikleri Ca/P solüsyonu içinde test edildi. Sentezlenen biyoaktif camın sitotoksik potansiyelini değerlendirmek için *in vitro* toksisite testleri yapıldı. Tüm analizler karşılaştırma yapılabilmesi için geleneksel sentez yöntemi için de uygulandı. Sonuçlar, yeşil yöntem ile sentezlenen biyoaktif camın diş hekimliği uygulamaları için gerekli olan biyoaktivite ve işlevsellik açısından daha avantajlı olduğunu doğruladı. Biyocamın üretim aşamasında güvenliğinin ve işlevselliğinin arttırılması mevcut uygulamaların sürdürülebilirliğinin sağlanmasında ve yeni biyomedikal kullanım alanlarının yaratılmasında kritik öneme sahiptir.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AS	Artificial Saliva
BET	Brunauer-Emmett-Teller
BJH	Barrett-Joyner-Halenda
CaPs	Calcium Phosphate Ceramics
CSBG	Chemically-synthesized Bioactive Glass
DMEM	Dulbecco's Modified Eagle's Medium
DI	De-ionized
EDX	Energy-dispersive X-ray
FAP	Fluorapatite
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GSBG	Green-synthesized Bioactive Glass
HA	Hydroxyapatite
HCA	Hydroxy Carbonate Apatite
ISO	International Organization for Standardization
MBGs	Mesoporous Bioactive Glasses
PBS	Phosphate Buffer Saline
SEM	Scanning Electron Microscope
TCP	Tri-Calcium Phosphate
XRD	X-ray Diffraction

CHAPTER 1

INTRODUCTION

Materials designed for use in contact with the living body are called *biomaterials* (Benvenuto 2022). The use in medical applications requires specific material properties such as biocompatibility, bioactivity, excellent mechanical properties, and high chemical resistance. To date, various biomaterials have been designed to interact with living tissues and biological structures for medical purposes including implantable biomedical devices, wound dressings, drug delivery, and a range of other non-load-bearing applications (Chelu and Musuc 2023). Today, the principles of basic sciences (chemistry, biology, and physics) are combined with new fabrication techniques (e.g., additive manufacturing) and recent advances in all areas of material science and engineering (e.g., tissue engineering and scaffolds) to design biomaterials that can closely mimic natural tissues and structures (Han et al. 2023).

Depending on the type of source material, biomaterials can be divided into four different groups: metallic, ceramic, polymeric, and composites. Ceramics that are used in medical and dental applications are generally called *bioceramics.* Bioceramics have been used in the treatment of injuries and diseases of the musculoskeletal systems for the last ~50 years. They possess superior characteristics compared to conventional alternatives such as (1) high biocompatibility, (2) high antibacterial activity, (3) low shrinkage, (4) ability to bond dentin, and (5) hydrophilicity.

Bioactive glasses can readily interact with and mechanically bond to hard or soft tissues in the body. Bioglass was founded in 1969 by Larry Hench, based on the hypothesis that "if a material can form hydroxyapatite *in vivo*, it will not be rejected by the body". This discovery led to significant developments in the field of medicine, as it was the first material that can not only bond to existing bones but also stimulate the formation of new bones. Their controllable chemical properties, high bioactivity, and ease of manufacturing make bioactive glasses one of the top candidates for biomedical applications. Following the discovery of bioactive glass in the 1970s, scientific research

and industrial interest on bioactive glass increased exponentially over time. Figure 1.1 shows the increase in the number of publications including bioactive glass during the last 40 years.

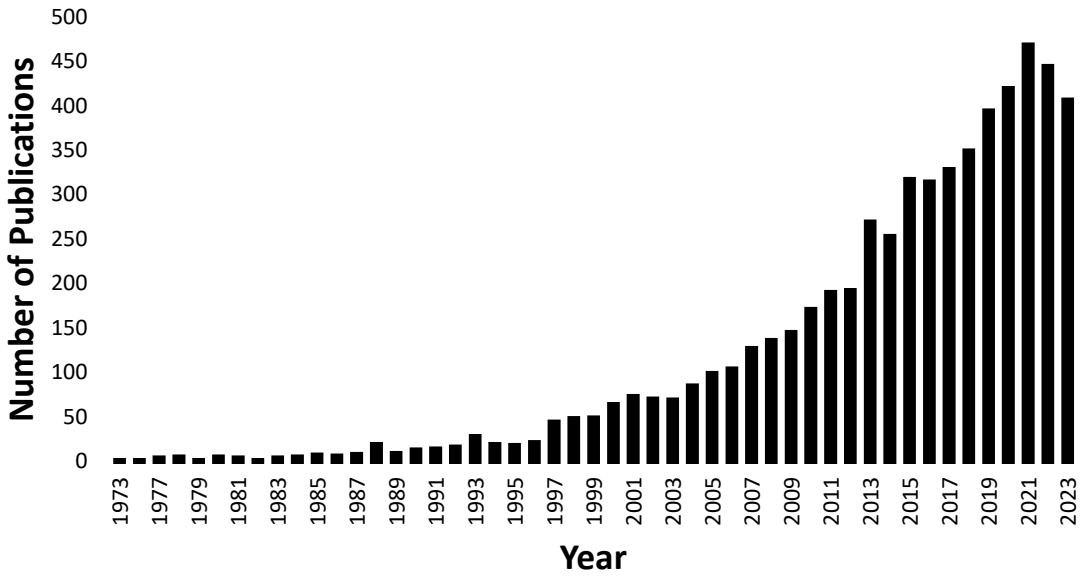


Figure 1.1. Increase in cumulative publications in the field of bioactive glass [source: Pubmed search engine].

Today, bioactive glass is one of the most widely used biomaterials in tissue engineering applications for bone grafting/regeneration (orthopedics), fillings and dental implants (dentistry), and skin rejuvenation purposes (cosmetics). In dentistry, it is commonly used as a dental restoration and mineralizing agent or multifunctional dental implant coating material. A more recent use of bioactive glass in oral healthcare is bioactive glass-containing toothpaste which is reported to help strengthen the enamel and protect against acid erosion. When bioglass interacts with saliva, it releases calcium and phosphorus ions, forming a permanent hydroxyapatite-like layer on dentinal tubules. The ability of bioglass to form a protective mineral layer on the surface of teeth made them a material of interest in protective dentistry, particularly for both healing and remineralization of the dentine surface and lesions.

Bioactive glass can be synthesized through sol-gel and melt-quenching methods. Current bioactive glass synthesis methods require high temperatures and strong acid catalysts such as nitric acid or hydrochloric acid. The use of acid-containing components at high temperatures for the synthesis of biomaterials that will be used in close contact with human tissues brings into question the risks associated with toxic acid residues in the final product. As an alternative to traditional chemical synthesis, green routes have been used by different researchers for the synthesis of bioactive glasses to improve both environmental and human health safety. For example, the acid-free hydrothermal method was used for synthesizing bioactive glass with a molar composition of 70% SiO_2 - 30% CaO (Hoa et al. 2020). They successfully synthesized spherical bioactive glass particles with a size of 20-30 nm. In a similar study, a bioactive glass of the same composition was synthesized by a modified sol-gel method in hot water without using an acid catalyst (Dang et al. 2020). The resulting bioactive glass was reported to have a particle size of 11-20 nm. While the earlier attempts to *greenize* the synthesis route of nano-structured bioactive glass are promising, there remains a need to develop environmental-friendly bioactive glass synthesis methods or to modify existing ones in a way to uplift their environmental friendliness.

To satisfy this need, we greenized the traditional sol-gel method by replacing an acid catalyst with an environment-friendly alternative and successfully used it for the synthesis of nano-structured 45S5 bioactive glass that contains 45 % SiO_2, 24.5 % CaO, 24.5 % Na_2O, and 6.0 wt% P_2O_5 by weight. The synthesized bioactive glass was characterized in terms of composition, size, surface charge, morphology, and mesoporous structure using Scanning Electron Microscope (SEM), Brunauer-Emmett-Teller (BET), Ultraviolet–Visible–Near Infrared (UV–Vis–NIR), Fourier Transform Infrared Spectroscopy (FTIR), X-ray spectroscopic methods (XRD and EDX). The apatite formation capability of the bioactive glass was investigated in artificial saliva (AS). *In vitro* toxicity tests were performed to assess the cytotoxic potential of the synthesized bioactive glass on Saos-2 human osteosarcoma cell line, while their functionality was tested by a set of mineralization experiments.

The ultimate aim of this thesis is to synthesize nano-sized bioactive glass without acid catalysts while conserving the desired bioactivity and functionality of the material. The specific aims are:

- to *greenize* the traditional sol-gel method by replacing an acid catalyst with an environment-friendly alternative,

- to synthesize nano-structured 45S5 bioactive glass using both chemical and green methods,
- to characterize the synthesized bioactive glasses in terms of size, morphology, composition, and mesoporous structure,
- to assess the mineralization potential of synthesized bioactive glasses,
- to test the apatite formation capability of the synthesized bioactive glasses in artificial saliva,
- and to measure the cytotoxic potential of the synthesized bioactive glasses on Saos-2 human osteosarcoma cell line.

This thesis consists of five chapters. In Chapter 1, brief information about the course of the thesis is given. Chapter 2 describes different aspects of biomaterials including their characteristics, synthesis methods, and dental applications. The experimental studies performed are explained in Chapter 3. The corresponding results of these experiments are presented in Chapter 4. The conclusion of the thesis with a discussion of the obtained results and suggestions for future research are discussed in Chapter 5.

CHAPTER 2

LITERATURE REVIEW

2.1. Biomaterials

Biomaterials are defined as synthetic materials that are used to replace or restore the function of damaged body tissues. They come into contact with body fluids continuously or intermittently (Agrawal 1998). Although it is a relatively new scientific field, the use of biomaterials dates back to ancient times. Artificial eyes, noses, and teeth in Egyptian mummies are the best examples of this. The usage of gold in dentistry dates back 2000 years. In ancient times, bone implants were made of iron, copper, and bronze. Despite the poisonous effects of copper ions, its use in orthopedic devices have continued until the mid-19th century, since a more suitable material could not be found. From the mid-19th century, significant improvements have been made in the use of foreign materials inside the body to replace, support, or enhance biological structures. The first metal prosthesis was produced from vitalium alloy in 1938. They were used until the 1960s but it was shown to pose serious dangers as metal corroded. In 1972, two ceramic structures named alumina and zirconia were started to be used without causing incompatibility issues. However, these inert ceramics were weakened very quickly because they could not bond to the tissue. This problem has been mostly solved with bioactive ceramics (e.g. bioglass and hydroxyapatite) developed by Larry Hench (Demirkıran 2003).

In order for any material to be used as biomaterial, it must be compatible with the body (Williams 1988). Biocompatibility is the most important feature of a biomaterial. Biomaterials can be grouped as bioinert, bioactive, biostable, and biodegradable according to the properties they show after contact with the body. First generation biomaterials were produced as bioinert as possible to minimize unwanted reactions and

interactions at the biointerface. Second generation biomaterials are biomaterials that have the ability to form bonds between the surface of an implant and tissues. In particular, the discovery of the bonding of bone to the specific composition of glasses, so-called bioactive glasses, led to the rise of second generation biomaterials (L. L. W. J. Hench 1993).

Table 2.1. Major applications of biomaterials, together with their cons and pros

MATERIALS	ADVANTAGES	DISADVANTAGES	APPLICATIONS
Metals	High strength, toughness,	High density, high corrosion	Joint prothesis, bone plates, screw, dental implants
Polymers	Elastic, easy to manufacture	Lower structural rigidity	Stitches, blood vessel, hip replacement socket
Ceramics	High biocompatibility, inert	Brittle, inelastic, hard to produce	Teeth, hip prosthesis, cranium, implant coating
Composites	High strength, good mechanical properties	Hard to produce	Joint implants, heart valves

2.1.1. Properties of Biomaterials

Biomaterials should satisfy the following properties for them to be used for a long time without causing any harm to the body: biocompatibility, bioactivity, high mechanical strength, and chemical resistance. Each of these essential characteristics of biomaterials is explained below.

2.1.1.1. Biocompatibility

Biocompatibility is the ability of a material to show an appropriate biological response without triggering any adverse reactions such as inflammation, allergic reactions, or infections (Perrotti et al., 2017; Schmalz, 2014). Biocompatibility is not a static quality because there is a change in material properties and host responses over the time. For instance, the pH of the body fluid varies between 1 to 9 depending on contacting-tissues or the load applied to our bones, joints, and tendons varies during our daily activities and routine. That is why biomaterials should be resistant to each of these changing conditions and states. Researchers have used the terms "biomaterial" and "biocompatible material" to refer to material's ability to perform the beneficial tissue responses and the required functions without causing any undesired effects. While the terms biocompatibility and biomaterials are highly correlated and often used interchangeably, *biocompatibility* expresses a property of material whereas *biomaterial* is a type of material that possesses certain properties. In other words, the ability of the material to respond appropriately within the body is called biocompatibility, and materials that are biocompatible are often called biomaterials. If the same thing is biocompatible, it is safe to be in the body. Biocompatibility testing involves measuring how reactive the body (or certain tissue/cell) is to the material and whether it is perceived as a threat by the immune cells.

2.1.1.2. Bioactivity

Bioactive materials react with surrounding tissues at the interface of the material to form mechanically strong bonds between host tissue and material (Hench et al., 1971; Hench et al., 1996). Bioactive materials can stimulate cell differentiation and proliferation, can stimulate tissue regeneration, or release bioactive molecules for repairing the damaged functionality of the organs (Zhao 2011). In the late 1960s, Larry Hench found that certain glasses had the capability of bonding to existing bones and it led to the creation of the bioactive material concept (Hench et al., 1971). Before this

discovery, scientists and manufacturers were focused on designing materials that were passive in the human body, assuming that being passive made them safe for the body. The discovery of bioactive materials (e.g., bioactive glasses containing SiO_2, CaO, P_2O_5, Na_2O) that can readily bond to biological tissues has caused a paradigm shift in the design of materials to be used in the body, from a passive is equal to safe approach to bio-active approach focusing on mimicking and complementing body functions.

2.1.1.3 Mechanical Properties

In addition to biocompatibility, mechanical properties that describe material behaviour under force or load are important in biomaterial design. Materials undergo various forces such as primarily stress, strain, and shear. External forces acting on the area of the contact cause a reaction called *stress*, describing the applied force per unit area. Depending on the direction of the force, the applied stress is called normal stress (when the force is acting perpendicular to the area) or shear stress (when the force acts parallel to the area). The deformation caused by the shear force (acting parallel to the surface) is called shear strain. Before implantation, especially for hard tissue applications, mechanical properties such as tensile strength, yield strength, elastic modulus, corrosion, and hardness should be evaluated carefully (Kiran and Ramakrishna 2021). Expanded testing of mechanical properties includes creep tests where a fixed load is applied while monitoring the strain, fatigue (failure) tests to assess a material's ability to withstand cyclic fatigue loading conditions, viscoelasticity tests and, hardness testing.

2.1.1.4. Chemical Resistance

Another critical issue to be considered when designing biomaterials is corrosion. Corrosion is a natural process that refers to the formation of compounds on metallic surfaces following exposure to air, water, or electrolytes, converting metals into oxides or hydrated oxides. Corrosion of a biomaterial may cause its degradation in the body, resulting in the release of potentially-harmful corrosion products. The reaction of the

material within the human body can release ions from implants due to corrosion that can trigger inflammation. The product from the corrosion might pose a danger to the body or may cause implant loosening. Our body is a dynamic environment that will cause high corrosion for metallic biomaterials. Therefore, biomaterials should be designed to withstand the potential damage of an extremely-corrosive physiological environment (body fluid) to avoid the release of corrosion products which can initiate a series of adverse reactions.

2.1.2. Classification of Biomaterials

Biomaterials are typically classified as natural or synthetic according to their origin. Natural biomaterials involve protein-derived, polysaccharide-derived, glycosaminoglycan-derived, and tissue-derived biomaterials. Synthetic biomaterials are divided into four different classes depending on their compositions: metallics, polymerics, ceramics, or composites.

2.1.2.1. Metallic Biomaterials

Metals are the most widely used biomedical materials. They are preferred as biomaterials because of their strong mechanical properties. High strength, superior ductility and fracture toughness, abrasion resistance, high elastic modulus, and high electric conductivity are advantages of metallic materials (Nakano 2019). Because of these properties, they are extensively used for load-bearing applications such as orthopedic implants and restorative dentistry. They are also used in cardiovascular surgeries as stents and stent-grafts. However, they have generally low biocompatibility due to their corrosion potential in physiological environments, causing significant problems in clinical applications.

To overcome this problem, some bioactive materials such as stainless steel, titanium, cobalt, and their alloys can be used as coatings.

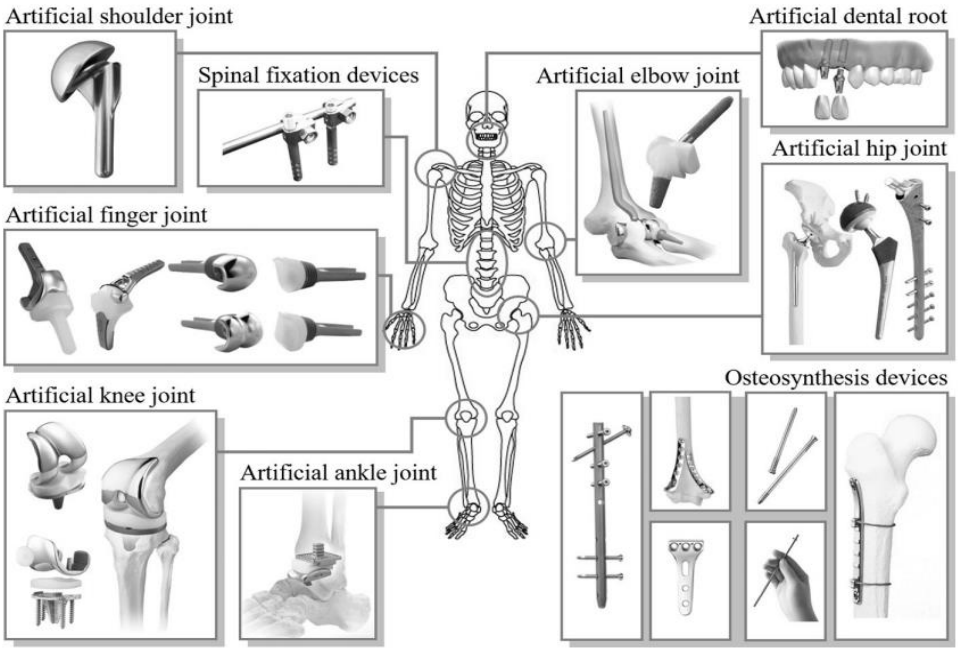


Figure 2.1. Bone-related implants made up of metallic biomaterials (Nakano 2019).

2.1.2.2. Polymeric Biomaterials

Polymers are macromolecules consisting of long chains of repeating groups of atoms. Polymeric biomaterials are widely used in dental applications, artificial hearts and heart valves, contact lenses, drug delivery systems, prosthetic materials, and tissue engineering products. Polymeric biomaterials have advantages over metallic and ceramic biomaterials in that (1) they can be produced in different shapes and compositions, (2) have low manufacturing costs, and (3) have a biodegradable nature which could be an advantage and disadvantage depending on the application (Bahadır 2008). Insufficient strength properties of polymeric biomaterials, especially in orthopedic applications, are their disadvantages compared to metallic and ceramic biomaterials.

Polymers can be grouped as synthetic and natural polymers. Natural polymers are generally biocompatible but synthetic polymers can contain impurities that may cause toxicity. Synthetic polymers have good mechanical properties compared to natural polymers. In the last three decades, researchers blended synthetic and natural polymers

to improve mechanical properties and biocompatibility compared with those of single-origin polymers to use in biomedical applications (Sionkowska 2011).

2.1.2.3. Composite Biomaterials

A group of materials formed by combining at least two materials that have different properties and that are insoluble in each other is called composite materials. Wood is one example of a natural composite material but it can also be engineered where materials with different constituents are combined to improve properties. The purpose of combining materials is to obtain, through the other material, a feature that the components do not have on their own. In other words, it is aimed to produce a new material that has superior properties than a single component. The development of composite materials with unique and tailored properties has allowed for solving engineering problems that were once considered unsolvable.

Composites are categorized based on dispersed/matrix phase or type of the matrix material which could be polymer, ceramic, or metal. Composite materials consisting of polymer, ceramic, or metallic biomaterials differ in physical, chemical, and mechanical properties. As a result, the most important purpose of producing composite materials is to combine the strengths of different materials (Ersoy 2001). The most-widely used composite materials include glass or carbon fiber reinforced polymers, metal or ceramic matrix composites, and natural polymer matrix reinforced with natural fibers. Composites used for biomedical and bioengineering applications are called biocomposites. The only difference here is the biocompatibility requirements. Biocomposites are typically used in orthopedics, tissue engineering, and restorative applications due to their high strength and low elastic modulus properties. In particular, carbon fibers- or ceramic-reinforced composite materials have found applications in a variety of orthopedic and dental applications during the last 50 years.

2.1.2.4. Ceramic Biomaterials

Bioceramics are considered as inorganic biomaterials consisting metallic and nonmetallic elements that bound together by ionic bonds. They have both crystalline and amorphous compounds. Bioceramics have an important place in bone tissue engineering applications (Figure 2.3).

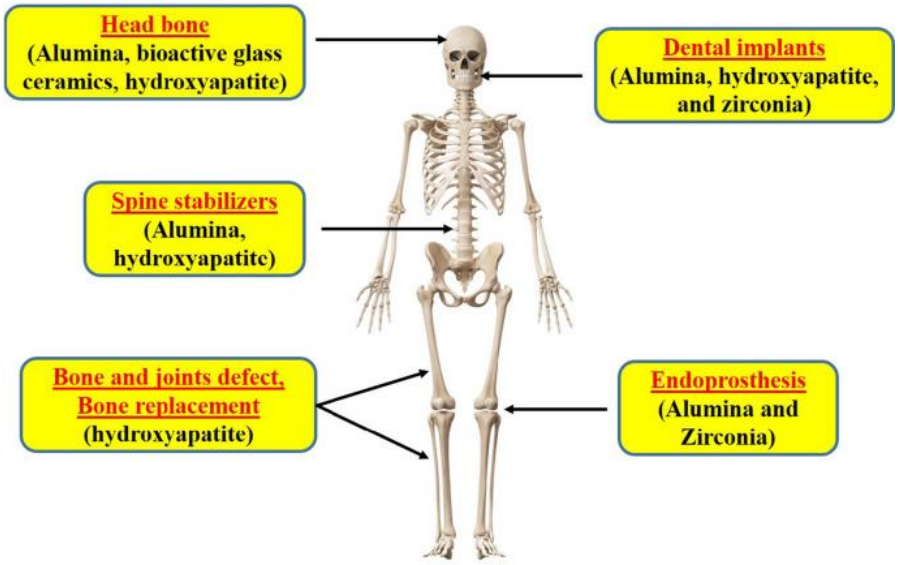


Figure 2.3. Uses of bioceramics in human body (Kumar et al. 2023).

They are used for repair and replacement of diseased or damaged parts of musculoskeletal systems. These materials can be found in crystal, glass, or partially crystal form (Vallet-Regí and Ruiz-Hernández 2011). The main characteristic features of ceramic materials are high stiffness and strength, high corrosion resistance, great hardness, and high wear (Kiran and Ramakrishna 2021). However, they have low toughness which leads to great fragility, a major problem of ceramic biomaterials. It is not possible to use ceramic biomaterials directly, especially in hard tissue applications, due to their low mechanical properties. However, it can be used as a surface coating material to improve the surface properties of metallic implants. They are widely used in orthopedics and dentistry applications. Bioceramics can be classified as bioinert,

biodegradable, or bioactive depending on molecular bioactivity when interacting with human organisms (Vaiani et al. 2023).

Table 2.2. Characteristic features and biomedical applications of bioceramics adapted from (Vaiani et al., 2023)

Material	Young's Modulus (GPa)	Compressive Strength (MPa)	Desity (g/cm^3)	Bioactivity	Applications
Alumina	380	4000	>3.9	Inert	Orthopedics, load-bearing application, dentistry
Zirconia	150-200	2000	6.0	Inert	Orthopedics, load-bearing application, dentistry
Porous hydroxyapatite	70-120	600	3.1	Bioresorbable	Dentistry, coatings, scaffolds
Tricalcium phosphate	120-160	540	3.1	Bioresorbable	Dentistry, scaffolds
Bioactive glasses	75	1000	2.5	Bioactive	Dentistry, spinal surgery

2.1.2.4.1. Bioinert Ceramics

Bioinert ceramics do not stimulate any tissue response and do not promote connection with living tissue when implanted into or interacting with biological systems. Once bioinert ceramics are implanted, varying thickness of fibrous connective tissues surrounds the material. This fibrous tissue network holds the implant and isolates it from adjacent tissues. Bioinert ceramics are commonly used for permanent implants due to their high chemical stability and corrosion-resistant nature. Alumina (Al_2O_3) and Zirconia (ZrO_2) are the two most important bioinert ceramics that are widely used in total-hip and -knee arthroplasty, dental implant, crown, and damaged bone tissue due to their excellent mechanical properties such as tensile, hardness, high wear resistance and good anticorrosion in biological systems (Anjaneyulu et al., 2019).

2.1.2.4.2. Bioresorbable Ceramics

Bioresorbable ceramics degrade and dissolve in living organisms. These ceramics are gradually absorbed and replaced by the host tissue after they are implanted in the body. TCP, CaPs, and porous HAp are examples of bioresorbable ceramics (Farid 2019). Bioresorbable and bioactive ceramics are two distinct terms used to describe highly specialized ceramics. While both are of interest for biomedical applications, they differ mainly in their chemical composition and microstructure. Resorbable ceramics provide mechanical properties for tissues in the healing process. Ceramic bioresorption is a critical aspect that must be considered to predict the in vivo fate of ceramics, particularly in applications where the mechanical integrity of biological entities should be restored. Otherwise, it may cause failure in the application of it (Bedir et al., 2023). The most frequently used ceramic for bioresorbable bone healing devices is beta-TCPs as they facilitate the bioresorption.

2.1.2.4.3. Bioactive Ceramics

Bioactive ceramics are intermediate between resorbable and bioinert ceramics. Bioactive ceramics induce tissue response by chemically bonding to surrounding tissues when interacting with the physiological environment. Many biological processes start after this interaction such as differentiation of stem cells and osteoblast adhesion (Ferraris et al. 2020). The bone-bonding properties of bioactive ceramics are referred as ‘osteoconductivity’ or ‘bioactivity’, expressing the capacity to connect with bone. Due to their osteoconductive behaviours, bioactive ceramics are utilized as coating materials to increase the mechanical and corrosion resistance of bone graft implants (Punj, Singh, and Singh 2021). HAp, bioglasses, and bioactive glass-ceramics are the most common bioactive ceramics used in biomedical applications.

2.2. Bioactive Glasses as Ceramic Biomaterials

Biomaterials were designed to be inert when immersed in body fluids before the discovery of bioactive glasses which caused a paradigm shift in the design of biomaterial from passive to active. Bioactive glasses changed the concept of designing chemically inert biomaterials, opening up possibilities to better reflect *in vivo* conditions and functions. Chemical bonding occurs between tissue and implant in bioactive glass materials and silica groups are replaced with calcium and phosphorus in the body. Biologically active ions are released to facilitate osteogenesis (Baino, Hamzehlou, and Kargozar 2018), since they have the ability to form HA and stimulate osteogenesis in physiological systems (Hench, 2006).

By introducing the bioactive glass to the physiological system, the bonding of bioactive glass particles to bone starts with the exchange of Na^+ ions in the glass with H^+ ions from the environment, resulting in an increase in the pH level. Ca^{2+} and PO_4^{3-} ions released from the glass create a calcium-phosphate rich layer deposited on the outer surface, which gradually becomes a silica-rich surface (L. L. Hench et al. 1971). The presence of a silica-rich layer facilitates the use of bioactive glass as a potential desensitizing agent for dental hypersensitivity treatment.

Bioactive glasses are composed of SiO_2, CaO, Na_2O and P_2O_5. The most-widely studied and the commercially-successful glass is known as 45S5. It contains 45% SiO_2, 24.5% CaO, 24.5% Na_2O, and 6% P_2O_5 by weight and the Ca/P ratio is 5:1. This 45S5 glass material was later trademarked as Bioglass (Larry L. Hench 2006). The low percentage of SiO_2 (below 60%), high CaO and Na_2O, and high Ca/P ratio discriminate bioactive glasses from other commercial glasses. The binding rate of bioactive glasses to living tissues such as bone tissue largely depends on their composition, and the phase diagram given in Figure 2.4. (Drahansky et al. 2016). The composition range in which bioactive glass and glass ceramics can bind to bone is shown as region A. The boundaries are kinetic boundaries, not phase equilibrium boundaries. Glasses with the highest level of bioactivity and the ability to bind to bone tissue most quickly are located in the middle of the Na_2O-CaO-SiO_2 diagram (Region E). All compositions contain 6% P_2O_5 by weight. Compounds exhibiting slower binding are located in the region containing 52-60% SiO_2 by weight. Compounds containing more than 60% SiO_2 (Region B) do not bind to bone and are bioinert. Studies have shown that only fast-reacting bioactive glasses can bind to soft tissue (Region S). When the SiO_2

ratio in the glass composition exceeds 52%, bioactive glasses cannot bind to soft tissue and may bind to hard tissue (Larry L. Hench 2006).

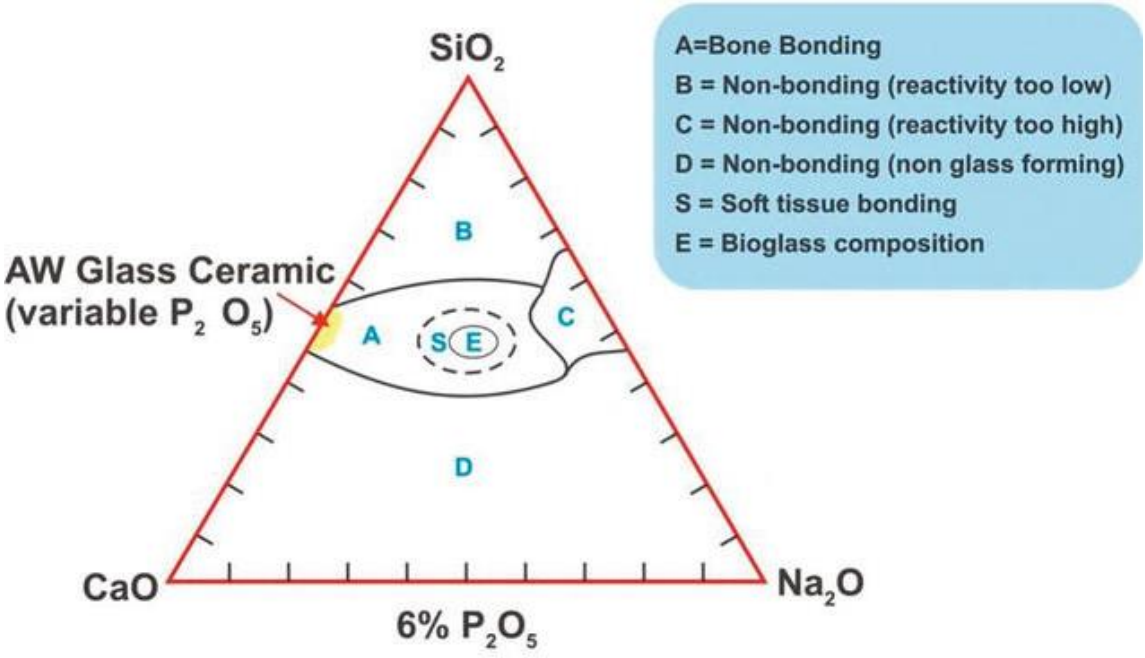


Figure 2.4. Schematic compositional diagram for bioactive glasses (Drahansky et al., 2016).

The ability of a material to form an HA-like surface layer when released into artificial body fluid is considered as an indicator of the bioactivity of that material. Since the hydroxyl carbonated apatite (HCA) layer is very similar to the mineral composition of bone, HCA formation enables the bioactive glass to form a tight bond with living bone tissue. The formation of the HCA layer in artificial body fluid *in vitro* and on the bioactive glass surface *in vivo* occurs in 5 stages. These stages are:

1. The ion exchange reaction that occurs rapidly between Na^+ and Ca^{+2} ions and H^+ (or H_3O^+) ions in the solution, leads to the hydrolysis of silica groups and the formation of silanol (Si-OH) group on the glass surface. The amount of H^+ ions decreases as a result of participation in the reaction, and this can be followed by an increase in the pH value in the simulated body fluid (Rahaman et al. 2011).

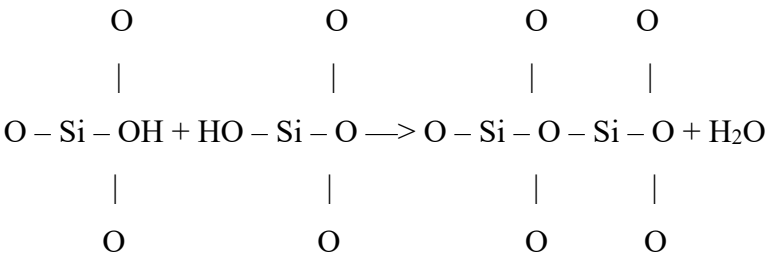
$$Si—O—Na^+ + H^+ —> Si—OH^+ + Na^+ (aq)$$

2. The increase in the pH value of the solution accelerates the dissolution of the SiO_2 glass network, allowing silica to pass into the solution in the form of $Si(OH)_4$ (silicic acid). Meanwhile, Si-OH formation continues on the glass surface according to the following reaction:

$$Si—O—Si + H_2O —> Si—OH + OH—Si$$

Although the solubility of silica is low under normal conditions, an increase in Si concentration is observed when 45S5 bioactive glass and glass ceramics are dissolved in aqueous solutions (Rohanová et al. 2011).

3. By condensation and polymerization of neighboring Si-OH groups, a 1-2 μm thick silica gel layer is formed on the surface (Larry L Hench 1991).



4. Ca^{2+} and $(PO_4)^{3-}$ ions, released as a result of dissolution in the glass and also coming from the solution, precipitate together on the layer rich in SiO_2 and form the amorphous calcium phosphate (ACP) layer.

5. As the glass continues to dissolve, the ACP layer interacts with $(OH)^-$ and $(CO_3)^{2-}$ ions in the solution and they crystallize as the HCA layer.

The nucleation and growth mechanism of HCA is accelerated by the presence of hydrated silica on the surface. Since this surface is chemically and structurally very similar to natural bone, it is possible for body tissues to attach to the surface. As the reaction continues, the HCA layer grows up to 100 μm to form a binding site. These reactions occur within the first 12-24 hours after the bioactive glass is placed in the body. After the formation of the HCA layer, osteoprogenitor cells proliferate and differentiate, allowing the bioactive glass to biologically bond to the bone.

2.2.1. Applications of Bioactive Glasses

Bioactive glasses have high biocompatibility, antimicrobial features, and bioactivity in physiological environments. These properties make bioactive glasses useful in extensive biomedical and dental applications.

2.2.1.1. Biomedical Applications

Application in Bone Tissue Engineering Scaffolds. The porous structure (coexistent macro and mesopores) of bioactive glass is suitable for tissues and blood vessel ingrowth. Together with the capability to form a HAP surface layer, these features make bioactive glass useful material in bone tissue engineering applications (Hong, Reis, and Mano 2009). Due to their bioactivity, biocompatibility, osteoconductivity, and biodegradability characteristics, bioactive glasses are widely used in tissue engineering. For example, borate-based glasses are used as scaffolds for tissue engineering as they provide support and help the growth of tissues by angiogenesis (Cannio et al. 2021).

Application in Cosmetics. The use of bioactive glasses extends to cosmetic applications. For example, a commercial bioactive glass powder named Vitryxx is used in a range of cosmetic products. It uses the 45S5 bioactive glass composition with finely ground powder whose particle size is around 3 μm (Shearer et al. 2023). To date, it has been used in various cosmetic products ranging from anti-aging creams and skin-care products to post-procedure and nail products. It has anti-odor and anti-oxidant properties such as reducing redness and wrinkles (*Schott AG*, 2023).

Application in Drug Delivery. Mesoporous bioactive glasses (MBGs) have large pore volume and high specific surface area which make them a good candidate for drug delivery since they can have high drug-loading capacity while maintaining a biocompatible nature (Vichery and Nedelec 2016). Antibiotics, anti-inflammatory agents, growth factors, other proteins, peptides, and drugs can be loaded into MBGs (Hum and

Boccaccini 2012). The mesoporous structure of bioactive glasses allows the loading of different drugs that can be released later in a controlled manner (Vallet-Regí et al. 2022). For example, one study used Fick's diffusion law to treat osteomyelitis with teicoplanin. They used borate bioactive glass as a solid carrier. Their result showed an increase in the bioactivity of hydroxyapatite formed by bioactive glass when the drug was released. This system was shown to heal the osteomyelitis in the tibial bone of rabbits *in vivo* (Zhang et al., 2010). Another bioglass-based material in drug delivery is a bioactive glass-chitosan-gelatin composite enriched with gold nanoparticles which was shown to provide higher loading for doxorubicin (81.6%) (Jayalekshmi and Sharma 2015) than magnetic-core silica nanopartices (4%) (Thomas et al. 2010).

Application in Implant Coating. Metallic implants are coated with bioactive glasses to inhibit corrosion and facilitate binding with tissues. By coating ceramic scaffolds with bioactive glass, both bioactivity and mechanical properties can be improved. For example, a 14-fold increase in compressive strength and a 3-fold increase in compressive modulus was achieved in literature by coating the biphasic calcium phosphate scaffold with nanosized bioactive glass particles in polycaprolactone (30 wt%) (Roohani-Esfahani et al., 2011).

2.2.1.2. Dental Applications

The Verified Market Research group announced that the market size of Bioglass was valued at USD 161.65 Million in 2021 and is projected to reach USD 235.90 Million by 2030, growing at a CAGR of 4.3% from 2022 to 2030. The report shows that dental application dominates the market (Report ID: 251090). A broad range of applications in dentistry are presented in Figure 2.5 while the major dental applications are explained below.

Application in Oral Care Products. Bioactive glasses have been used in oral healthcare products such as toothpastes (Tai et al. 2006). NovaMin® is one of the bioactive glasses used in toothpaste as an active agent to reduce tooth sensitivity and increase

remineralization (Gjorgievska and Nicholson 2011). NovaMin contains amorphous calcium sodium phosphosilicate and, when introduced into the oral environment, it releases calcium and phosphate ions which leads to an increase in the pH level of the surrounding environment and results in the formation of crystalline hydroxycarbonate apatite (HCA) layer (Burwell, Litkowski, and Greenspan 2009). BiominF is another commercial product of bioactive glass used in toothpaste technology. It contains fluoride and phosphate ions and forms fluorapatite (FAP) in/around exposed dentine tubules (Brauer et al. 2010).

Application in Periodontics and Implant Dentistry. Periodontitis inflammatory disease of the periodontium can cause loss of attachment, gingival bleeding, and alveolar bone loss which may lead to loss of the tooth structure if not treated properly (Profeta and Prucher 2015). PerioGlass is one of the bioactive glasses that is commonly used in bone grafting operations. It repairs periodontal defects when used as a grafting material. It is also used in periodontal surgical applications to activate bone regeneration (Lovelace et al. 1998). Most dental implants are made of titanium, and bioactive glass has been used to coat titanium implants (Koller et al. 2007). Titanium implants are biocompatible and osteoconductive materials but they are bioinert. To overcome this problem, bioactive glasses are used as a surface-modifying material to increase the bonding ability of titanium implants to the bone (Talreja, Gayathri, and Mehta 2013). Covering dental implants with bioactive glass also minimizes the infection and inflammation around the implants due to their antimicrobial properties (López-Píriz et al. 2015).

Application in Orthodontics. In orthodontics, dental adhesives facilitate the bonding of a compound like dental composites or orthodontic brackets to natural tooth tissue (Skallevold et al. 2019). The dental adhesive adheres the hydrophobic dental resins to the hydrophilic tooth surface by acting as an interface between two materials. With the use of orthodontic brackets, adhesion of the bracket to the tooth surface initiates favorable conditions for bacteria which may lead to demineralization of the tooth and the formation of white spot lesions (Gange 2015). Bioactive glasses have the ability to remineralize these white spot lesions (Milly et al. 2014).

Application in Oral and Maxillofacial Surgery. Bioactive glass used as synthetic bone adjunctive material increases bone formation in maxillofacial surgeries at a higher rate

than other calcium phosphate-based compounds (Peltola et al. 2003). In 2005, the use of bioglass as a bone stimulant was approved by US Food and Drug Administration (FDA) (Larry L. Hench and Jones 2015). Novabone and Perioglass are commercial names of bioglass used as a synthetic bone graft in orthopedics and maxillofacial surgeries, respectively (Fetner et al., 1994). The effect of bioactive glass on bone stimulation and bone regeneration has been shown in the literature (Larry L. Hench 2013). BonAlive, StronBone, Bioglass 45S5, and Biogran are some of the commercial products of bioactive glass commonly used in oral and maxillofacial surgeries.

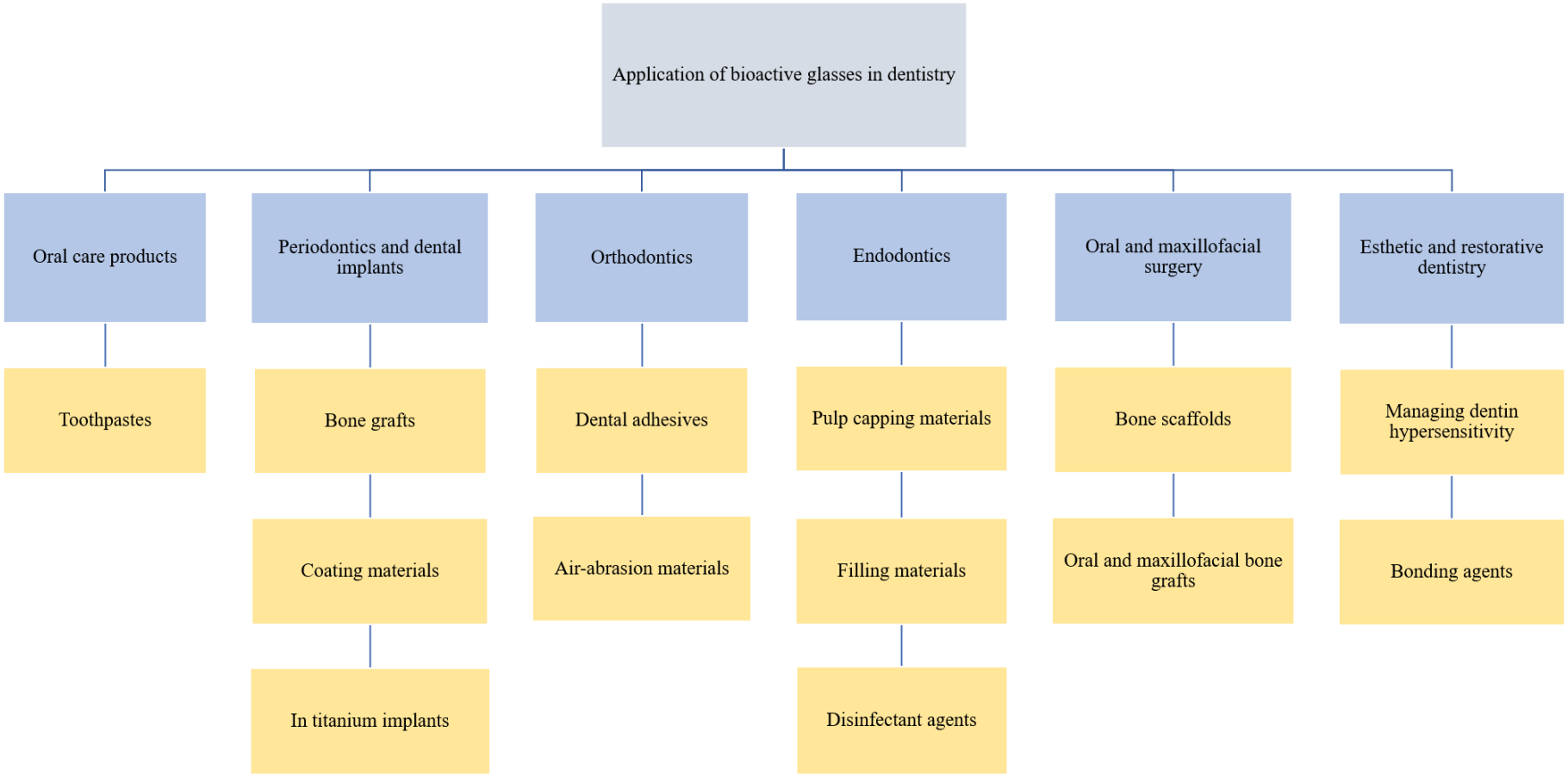


Figure 2.5. Application of bioglasses in denistry (Jafari et al. 2022)

2.2.2. Synthesis Methods of Bioactive Glasses

Bioactive glasses are commonly produced by two different methods: the melting method and the sol-gel method. While the melting method is based on melting glass at high temperatures, the sol-gel method involves synthesis at lower temperatures compared to melt-quenching (typically around 1500 °C).

2.2.2.1. Melt-Quenching Method

The melt-quenching method is a traditional bioactive glass production technique. The procedure includes the following steps (Gao, Seles, and Rajan 2023):

- Ingredients are ground into powder form by a ball mill,
- Mixing of ingredients and heating to > 1300 °C in a platinum crucible,
- The melt is poured into the mold to get the desired shape.
- The molten glass is quenched in cold water to obtain a glass frit.
- The quenched glass is annealed at 500 °C to remove internal stress from the glass (Kaur et al. 2014).

2.2.2.2. Sol-Gel Method

The sol-gel synthesis is an alternative low-temperature synthesis method for bioactive glasses. ‘Sol’ is comes from the solution and ‘gel’ comes from the gelation. Sol is the suspension of colloidal particles formed in a liquid. These particles undergo many reactions such as hydrolysis and polycondensation to form a gel-like network structure. The chemical structure of the starting material determines the solvent to be used in the reaction. For example, alcohol is used as a solvent for metal oxides, while water is used for some oxides and ceramics. Strong acids like nitric acid and hydrochloric acid at high concentrations are used as catalysts in sol-gel reactions (Vafa, Bazargan-Lari, and

Bahrololoom 2021). In the sol-gel synthesis of bioactive glasses (BGs), tetraethyl orthosilicate (TEOS) is the widely used silicate precursor. Sol-gel synthesis mainly involves acid hydrolysis and polycondensation reaction to form the Si-O-Si bond. Then, it is cast in a teflon container and sealed at room temperature (Gao, Seles, and Rajan 2023). Synthesis of glass materials at relatively low temperatures compared to melt-quenching allows doping of various inorganic, organic, and biomolecules for different applications during the formation of a glassy matrix. Sol-gel treatment takes place in seven steps: mixing of the precursors, casting, gelation, aging, controlled drying, stabilization, and densification (Larry L. Hench and West 1990).

When compared to bioglass synthesized using the melting method, the sol-gel method provides several advantages in bioglass synthesis such as low-temperature processing, improved control over composition, size and shape, high homogeneity and purity, and higher bioactivity (J. Faure et al. 2015). The silica content should be less than 60% mol percent to allow glass to bond with bone when it is obtained by the melt-quenching method. Whereas, if the glass is obtained by sol-gel method for HA layer formation and bone bonding silica content can have up to %90 by mol (Kaur et al. 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

SiO_2-Na_2O-CaO-P_2O_5 containing 45S5 system is chosen for bioactive glass synthesis. Materials used for synthesizing nano-structured bioactive glass, their formula and, the companies that they were purchased from, are given in Table 3.1.

Table 3.1. List of materials used in this thesis

Preparation of Bioactive Glass Nanoparticles		
Material	**Formula**	**Company**
Tetraethyl Orthosilicate (TEOS)	$(C_2H_5O)_4Si$	Sigma- Aldrich
Triethyl Phosphate (TEP)	$(C_2H_5O)_3PO$	Sigma- Aldrich
Calcium Nitrate Tetrahydrate (CNT)	$Ca(NO_3)_2$	Sigma- Aldrich
Sodium Nitrate	$NaNO_3$	Carlo Erba
Nitric Acid	HNO_3	Sigma- Aldrich
Preparation of Artificial Saliva		
Material	**Formula**	**Company**
Calcium Chloride	$CaCl_2$	AFG Bioscience
Potassium Chloride	KCl	Sigma- Aldrich
Sodium Chloride	NaCl	Merck
Potassium Dihydrogen Phosphate	KH_2PO_4	Sigma Aldrich
HEPES 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid	$C_8H_{18}N_2O_4S$	Sigma Aldrich

3.2. Methods

3.2.1. Bioactive Glass Synthesis

For the green and chemical synthesis of bioactive glass, existing sol-gel methods which were used fort he synthesis of another type of bioglasses were modified and used in this study (Dang et al. 2020) (Pirayesh and Nychka 2013).

3.2.1.1. Chemical Synthesis of Bioactive Glass

The bioactive glass 45S5 is composed of SiO_2: Na_2O: CaO: P_2O_5 with the respective molar percentages of 46.1: 24.4: 26.9: 2.6. Synthesis steps are summarized below:

- 33.5 ml TEOS was added dropwise into the reaction vessel which contained 2.25 ml (1M) pure HNO_3 with 48.6 ml of hot water (at 60℃).
- The mixture was continuously stirred for 1 h.
- The following reagents were added sequentially at 45-minute intervals: 0.017 mol (2.9ml) of TEP, 0.085 mol (20.13g) CNT, 0.16 mol (13.52 g) of $NaNO_3$.
- The mixture was stirred after observing clear sol.
- The sol was placed in an oven to transform into a gel for 1 day at 70 ℃.
- The wet gel was aged for 1 day at 100 ℃ and dried for 6 h at 150 ℃.
- The dried glass was sintered for 3 h at 700 ℃ to extract residual nitrates.
- The obtained powder was ground by a porcelain mortar pestle for further characterization.

3.2.1.2. Green Synthesis of Bioactive Glass

The procedure for the green synthesis of bioactive glass was the same as the chemical synthesis method, with the exception that HNO_3 was replaced with the same amount of water.

3.2.2 Preparation of Bioactive Glass Discs

After bioactive glass powders are obtained, pellet pressing is used to press bioactive glass discs to minimize surface-area-related differences in bioactivity experiments. Firstly, 0.3 g bioactive glass powders were measured. By using a 13 mm pellet pressing die, we applied 4.5 metric tons with Carver manual hydraulic press for 5 minutes under vacuum condition.

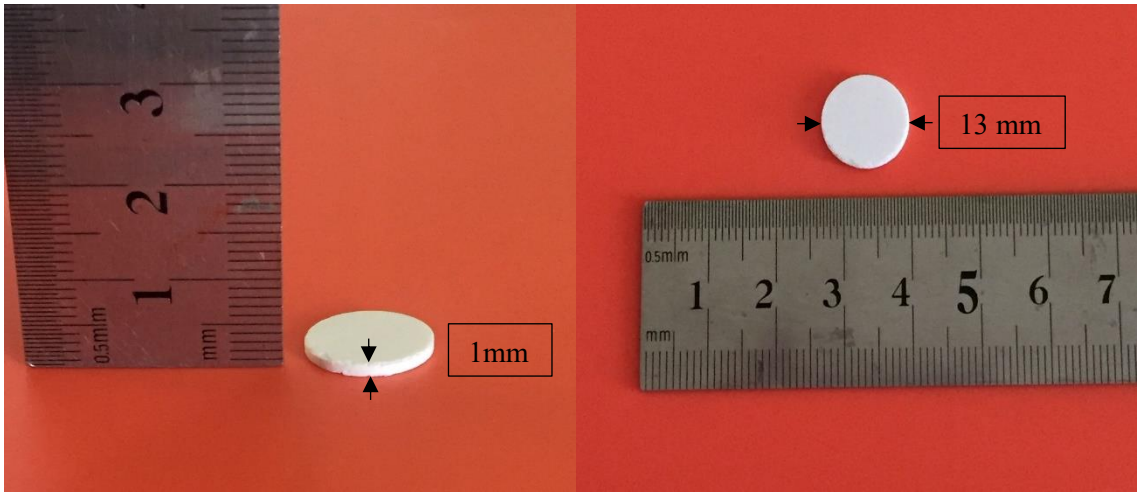


Figure 3.1. Bioactive glass discs with height and diameter.

3.3. Physicochemical Characterizations

The synthesized bioactive glasses were characterized in terms of composition, size, morphology, mesoporous structure, and surface area using Scanning Electron Microscope (SEM), Energy Dispersive X-ray Spectroscopy Analysis, Fourier Transform Infrared Spectroscopy (FTIR), Brunauer-Emmett-Teller (BET), and X-ray Diffraction Analysis. All analyses were done in Iztech Integrated Research Center (IRC).

3.3.1. Scanning Electron Microscope with Energy Dispersive X-ray Spectroscopy Analysis

SEM analysis was done in Material Research Center in IZTECH IRC. SEM analysis was carried out using the scanning electron microscope (FEI, Quanta™ 250 FEG) equipped with backscattered electron and secondary electron detectors to analyze the shape and size of the bioactive glass nanoparticles. The working voltage was set to 10 kV and samples were examined at different magnifications. A double-sided adhesive carbon tab was placed on the SEM stub and a clean aluminum plate was stuck on the carbon tab. After synthesizing the bioactive glass, different concentration of 5 µl of the dispersed samples was dropped on an aluminum plate. The sample was dried in an open atmosphere overnight before analysis. By using energy dispersive x-ray spectroscopy (via Oxford Instruments, Aztec software), the main elemental components of the samples were obtained. Elements less than 1% atomic weight were excluded from the results.

3.3.2. Fourier Transform Infrared Spectroscopy Analysis

FTIR analysis was done in Biotechnology and Bioengineering Application and Research Center in IZTECH IRC. Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer, Spectrum Two FT-IR Spectrometer) was performed to identify the

chemical bonds in the molecules of the sample. The transmission spectra were obtained between 400-4000 cm^{-1}. The frequencies formed by the vibration of the bonds between the atoms that make up the material correspond to absorption peaks in infrared spectroscopy which enables the characterization of the functional groups (Lin and Wang 2012).

3.3.3. X-ray Diffraction Analysis

XRD analysis was done in Material Research Center in IZTECH IRC. The X-ray diffraction method was performed to analyze the crystallographic structure of bioactive glasses. The XRD analysis is done with an X-ray diffractometer (Philips, X'Pert Pro) with an X-ray source of Cu Kα radiation (k = 1.54178 Å). Data is collected by scanning 2θ range from 10 to 80° at a step size of 0.02°.

3.3.4. Brunauer-Emmett-Teller (BET) Analysis with Barrett-Joyner-Halenda (BJH) Method

BET analysis was done in Material Research Center in IZTECH IRC. The Brunauer Emmet and Teller (BET) analyses were conducted to determine the surface area, pore volume, and pore size of the bioactive glasses by gas adsorption. Nitrogen gas is used as an adsorbent. The pore size distributions and pore volumes were obtained with the BJH desorption method by using the desorption isotherms. Samples were degassed at 250 K for 3 hours under vacuum conditions before using the surface area analyzer device (Micromeritics, Gemini V).

3.4. *In-Vitro* Bioactivity Tests

Bioactivities of the synthesized bioactive glasses were tested with 2 different methods. The mineralization feature of the synthesized glass powders was tested with (1) mineralization kinetics experiments and (2) by quantifying the mineral occurrence on the surface of the bioglass discs in artificial saliva.

3.4.1. Mineralization Kinetics of Bioactive Glass

A 2-hour absorbance-reading (OD-value) was taken in the presence of HEPES, KH_2PO_4 and $CaCl_2$ to examine the effect of bioactive glass on mineral formation. The following steps were followed:

- 1mg/ml concentration of bioactive glass was prepared in DI water and incubated for 1 h at 37 °C.
- 75 µl Calcium (19.2 mM), 75 µl Phosphate (11.52 mM), 30 µl HEPES (50 mM), and 20 µl prepared bioactive glass was placed in a 96-well plate for green and chemical synthesis experiment sets.
- 75 µl Calcium (19.2 mM), 75 µl Phosphate (11.52 mM), 50 µl HEPES (50 mM) was prepared for control experiments.
- Experiment was carried out for 2 h while shaking every 10 seconds and the absorbance was recorded (at 820 nm wavelength) with a spectrophotometer (Thermo Scientific, Multiskan Sky).
- After 1 hour and 2 hours, a 10 µl sample was taken from control, green synthesis, and chemical synthesis experiment sets and SEM characterization was performed to compare mineral formation. All experiments were repeated 5 times (3 repeats in each independent experiment).

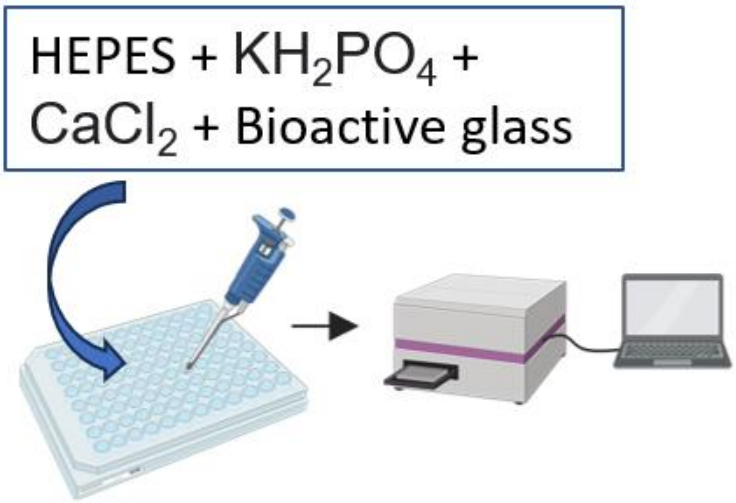


Figure 3.2. Illustration of the mineralization kinetics measurements.

3.4.2. Bioactivity Test in Artificial Saliva

The prepared and compressed bioactive glass discs were immersed in artificial saliva and incubated for 1, 3, 7, and 14 days at 120 rpm and 37 °C. Each disc containing bioactive glass synthesized with and without acids was incubated separately in 30 ml of artificial saliva. The required amount of artificial saliva volume for each disc was calculated according to the formula below:

$$V_s = 100 \times S_a$$

($V_s$volume of the solution, S_a: surface area of the discs)

All experiments were repeated for at least 3 times.

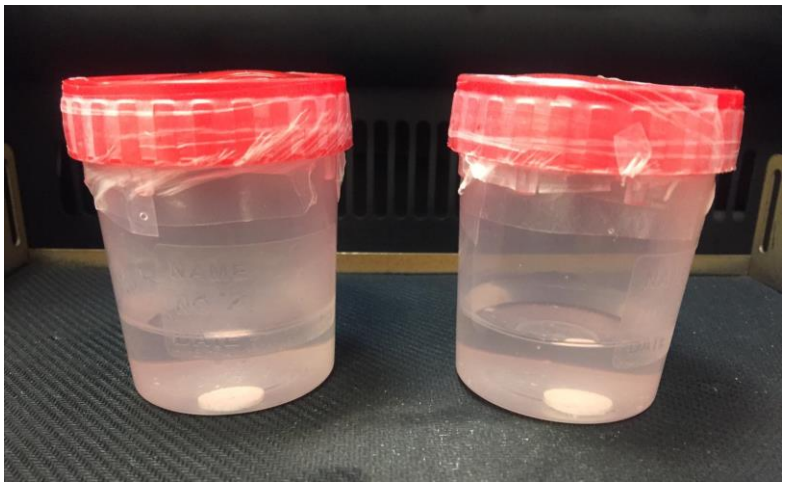


Figure 3.3. Bioactive glass discs in artificial saliva.

3.4.2.1. Artificial Saliva Preparation

Artificial saliva was prepared by using the component listed in Table 3.2 to create a medium similar to a natural oral environment. It was prepared according to the protocol reported in the literature (Yucesoy et al. 2023). Briefly, all ingredients were weighed and dissolved in ultrapure water. After mixing all ingredients, the pH was set to 7 with the addition of 1 M NaOH. The prepared artificial saliva solution was stored in a glass container.

Table 3.2: The Composition of Artificial Saliva Solution

COMPOSTION	CONCENTRATION
$CaCl_2$	1.5 mM
KCl	130 mM
NaCl	1 mM
KH_2PO_4	0.9 mM
HEPES	20 mM

3.4.2.2. pH Change Analysis

After incubation for 1-14 days, bioactive glass discs were removed from artificial saliva, rinsed with DI water, and dried at room temperature. The pH change was measured with a pH meter (Orion Star A211, Thermo Scientific) and used as an indicator of bioactivity level. Data were collected from triplicates of each incubation period and the results were presented as a pH curve.

3.4.2.3. Weight Loss Analysis

The weight of the bioactive glass discs was measured before being immersed in artificial saliva. After immersion in artificial saliva, discs were rinsed with DI water, and dried at room temperature. Dried discs were weighted to calculate the weight loss percentage of the discs according to the formula given below:

$$W(\%) = \frac{W_0 - W_f}{W_0} \times 100$$

(W_0: weight before incubation, W_f: weight after incubation)

Discs were stored for further characterizations to observe mineral formation on the surface of the discs. Since the conversion reaction is associated with loss in the mass of bioactive glass, weight loss measurements are helpful in understanding the kinetics of the conversion process of bioactive glass to hydroxyapatite in artificial saliva. Results were compared and discussed for green and chemical synthesis.

3.5. *In-Vitro* Cytotoxicity Studies

Cytotoxic and cell proliferation potential of bioglass synthesized through chemical or acid-free routes was measured with the MTT assay.

3.5.1. Saos-2 Cell Line Culture

Saos-2 (human bone osteosarcoma cells, ATCC HTB-85) cell line was used in the cell viability assay. The cell were routinely cultured in Dulbecco's modified Eagle's (DMEM High Glucose, Sigma-Aldrich, D6429) medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco™, 26140079) and 1% (v/v) Penicillin/Streptomycin (Gibco™, 15140122) and incubated in a cell incubator at 37 °C and 5% CO_2 humidified atmosphere. Briefly, all materials used in cell culture were heated in a water bath at 37 °C. Saos-2 cell stocks thawed fast in a water bath at 37°C. The 1 mL of the thawed cell was taken into a 15 mL falcon tube, and 4 mL of the DMEM high glucose complete medium was added on top of the falcon. The falcon tube was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded to remove the DMSO (Sigma-Aldrich, D8418). The cell pellet that remained in the bottom of the tube was dissolved with DMEM high glucose complete medium and seeded into a 25 cm^2 cell culture flask. Cells were controlled daily and transferred to a 75 cm^2 cell culture flask when they reached 80% confluency. Medium in the cell culture flask was discarded and the flask was washed with 3 mL of sterile PBS solution (Pan Biotech, P04- 36500). Then, cells were trypsinized with 2 mL trypsin (Gibco™, 25200-056) for detachment and incubated for 5 minutes at 37°C and 5% CO_2. Detachment of the cells was observed under a microscope and 4 mL of DMEM high glucose complete medium was put into a culture flask. All media was transferred into a 15 mL falcon tube which was centrifuged at 1000 rpm for 5 minutes and supernatant was discarded to remove the trypsin. Then, the cell pellet was dissolved with an appropriate amount of DMEM high glucose complete medium and passaged 1:3 ratios for 75 cm^2 cell culture flasks. The volume of the flask was completed to 10 mL to incubate at 37 °C and 5% CO_2 for cells to grow. To freeze the cell pellets for storage, they were put into a freezing medium containing 95% FBS and 5% DMSO. The medium was

refreshed every third day. Prior to analysis, cells were harvested after they reached 80% confluency, and viable cells were counted with a hemocytometer. $1x10^4$ cells/well were seeded into a 96-well plate for MTT cell viability assay.

3.5.2. Preparation for Cytotoxicity Assay

Green and chemical synthesized bioactive glasses were sterilized by dry heating sterilization at 200 ^oC for 2 hours in a benchtop drying oven (FN-500, Nüve). Bioactive glass-containing extracts were prepared by incubating 50 mg of each bioactive glass sample in a 1 ml serum-free DMEM culture medium at 37 °C. After 24h incubation, extracts were passed through a 0.22 μm filter. The filtrated extract was completed with 10 % FBS and 1 % Penicillin /Streptomycin. The extract solution was diluted to different concentrations (1.25, 2.5, 5, 7.5, 10 mg/ml) using DMEM high glucose complete medium. MTT reagent was prepared in a sterile PBS solution at a concentration of 5 mg/ml in the dark.

3.5.2.1. Cell Viability Assay by MTT

Cell viability assays were used to measure the proportion of viable cells in the bioglass-exposed Saos-2 population. Cell viability analysis was carried out with a Thiazolyl blue tetrazolium bromide (MTT, M2128, Sigma-Aldrich) assay to measure the cell viability of Saos-2 cells following exposure to differently-synthesized bioactive glass samples. MTT assay measures the reduction of a tetrazolium salt into an insoluble formazan product by mitochondrial enzyme activity of living cells. MTT, or 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, is yellow in solution and it can be converted into purple formazan in living cells which makes it colorimetric assay. The following steps are followed:

- 1x 10^3 cells/well were seeded into a 96-well plate for MTT cell viability assay, and incubated for 24 hours under 37 °C and 5% CO_2 atmosphere.

- After 24 hours, cell culture media from each well was discarded and bioactive glass-containing cell culture media was added (100 µL to each well) at increasing concentrations.
- Cells were incubated for 24 hours under 37 °C and 5% CO_2 atmosphere.
- After 24 hours of incubation, 10 µL of MTT working solution (5 mg/ml in PBS) was added and cells were incubated for 3 hours at 37 °C and 5% CO_2 atmosphere in the dark.
- Later, all media inside the wells were discarded and 100 µL DMSO was added to solubilize the formazan crystals that occurred inside the cells.
- After 10 minutes of slow shaking, the optical density was measured as triplicates at 570 nm for MTT and 690 nm for background using a spectrophotometer (Thermo Scientific, Multiskan Sky).
- Background substruction was performed and cell viability % versus bioactive glass concentration graphs were plotted. Saos-2 cells cultured with cell culture medium only were used as control.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Physicochemical Characterization of Bioactive Glasses

Characterization of bioactive glass synthesized through the chemical route (CSBG) and green route (GSBG) was performed using a range of techniques including SEM, EDX, FTIR, XRD, and BET. SEM analysis enabled imaging of synthesized bioactive glasses and provided information about their morphology and size (Figures 4.1 and 4.2).

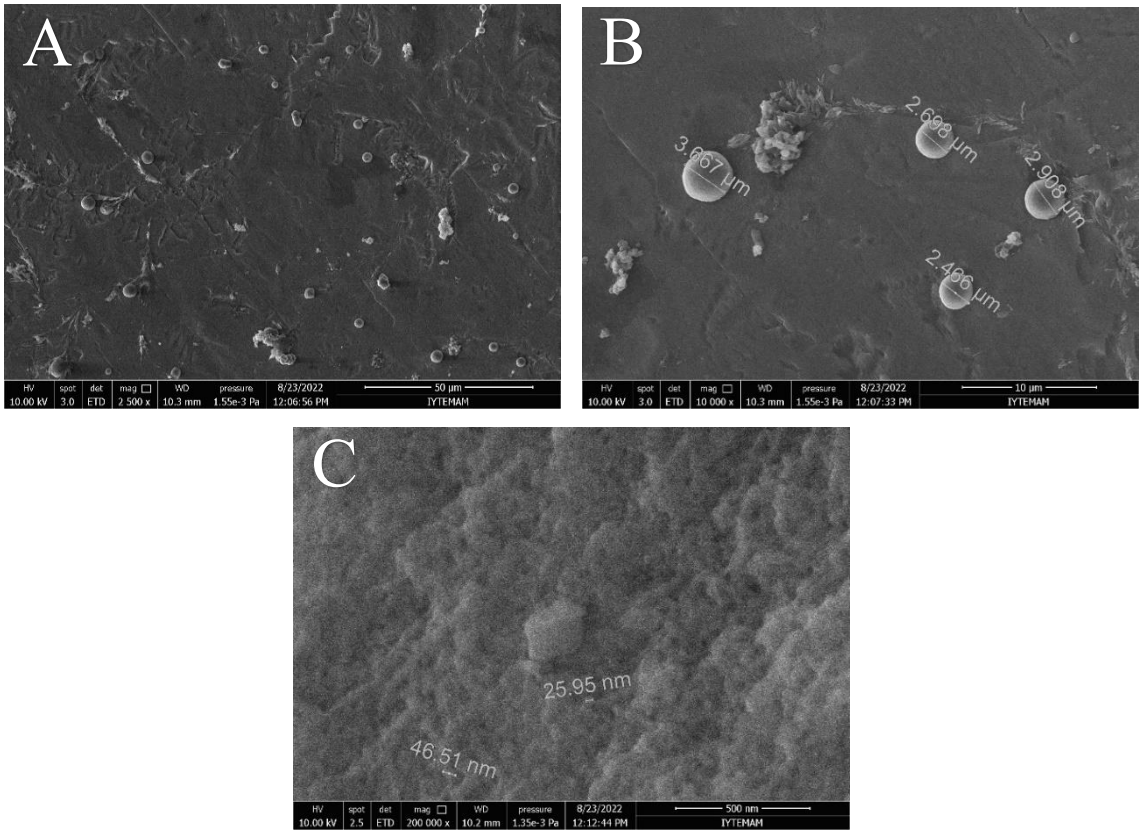


Figure 4.1. SEM Images 4 of CSBG; A) 50µm B) 10µm and C) 500 scale bar.

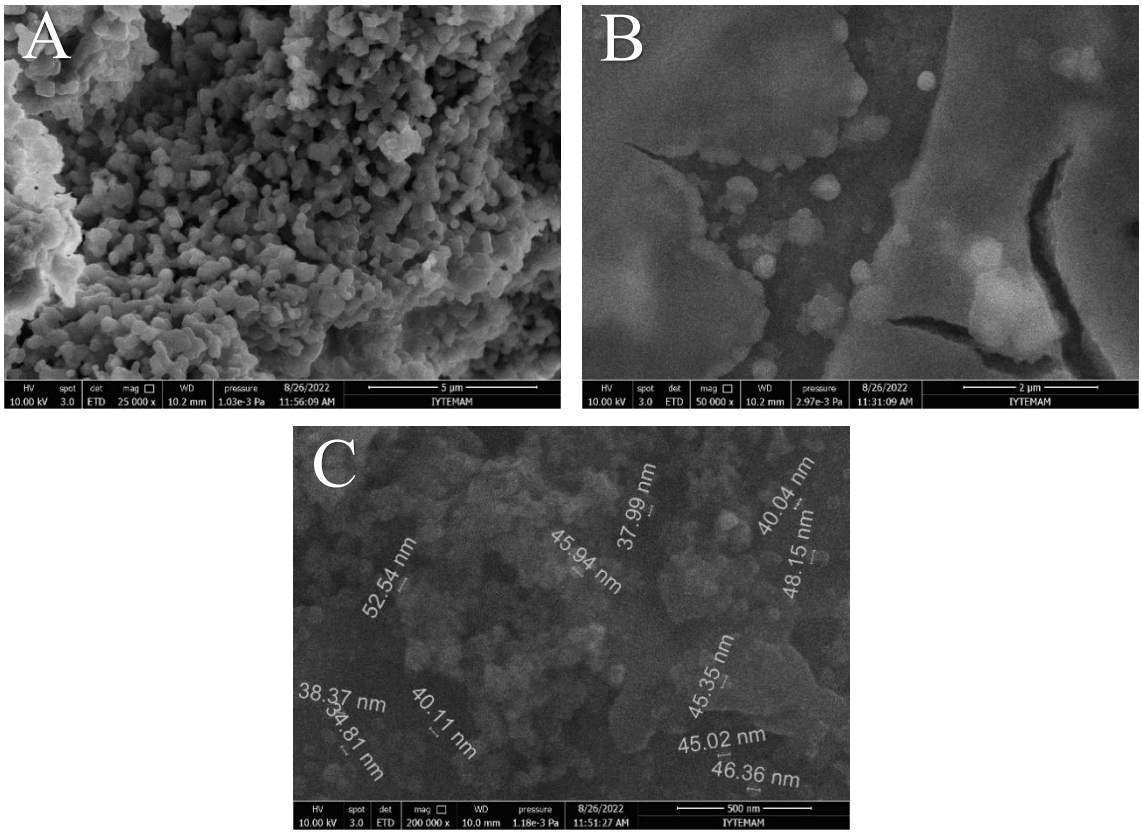


Figure 4.2. SEM Images 4 of GSBG; A) 50µm B) 10µm and C) 500 scale bar.

SEM results show that both CSBG and GSBG have a spherical shape and mean particle size of ~ 41-43 nm. The blurry nature of the SEM images suggests that particles are not well dispersed, partly due to the difficulties in the sample preparation process. Considering the correlation between the particle size and the reactive surface area, the small sizes obtained, even in the absence of an acid catalyst, suggest that no compromise has been made in terms of bioactivity level when discarding acid use. This is later confirmed with the BET specific surface area analysis.

Surface properties such as specific surface area, pore size and volume measured via BET analyses can be seen in Table 4.1. The measured BET surface areas of CSBG and GSBG are 1.48 and 2.72 m^2/g, respectively. BET surface area value of CSBG is 5.1-fold and GSBG is 10.3-fold higher than melt-derived 45S5 bioactive glass (0.24 m^2/g) (Sepulveda, Jones, and Hench 2002). The mean pore diameter of CSBG and GSBG are 4.9 nm and 5.3 nm, respectively. Therefore, according to the IUPAC, both bioactive glasses are in the mesoporous range (the pore diameter between 2 to 50 nm)(Qiao and

Huo 2017). According to the pore volume and pore size calculated by the BJH formula, GSBG has almost 2 times higher surface area and pore size than CSBG. Here, it can be concluded that not using an acid catalyst increased the specific surface area of the bioactive glass. Having a mesoporous structure with a high specific surface area would increase the interaction of glass material with a physiological solution and, therefore, may enhance the bioactivity of the glass.

Table 4.1.Surface properties of chemical and green synthesized BG obtained via BET.

	CSBG	**GSBG**
Single Point Surface Area (m^2/g)	1.4515	2.6537
BET Surface Area (m^2/g)	1.4815	2.7276
Langmuir Surface Area (m^2/g)	2.3506	4.3284
Pore Volume (cm^3/g)	0.0018	0.0036
Pore Size (Å)	49.3738	53.0505

FTIR spectroscopy was used to identify the chemical bonds of the CSBG and GSBG and to determine the functional groups present in both samples. The obtained FTIR peaks (Figure 4.3) were compared with the literature.

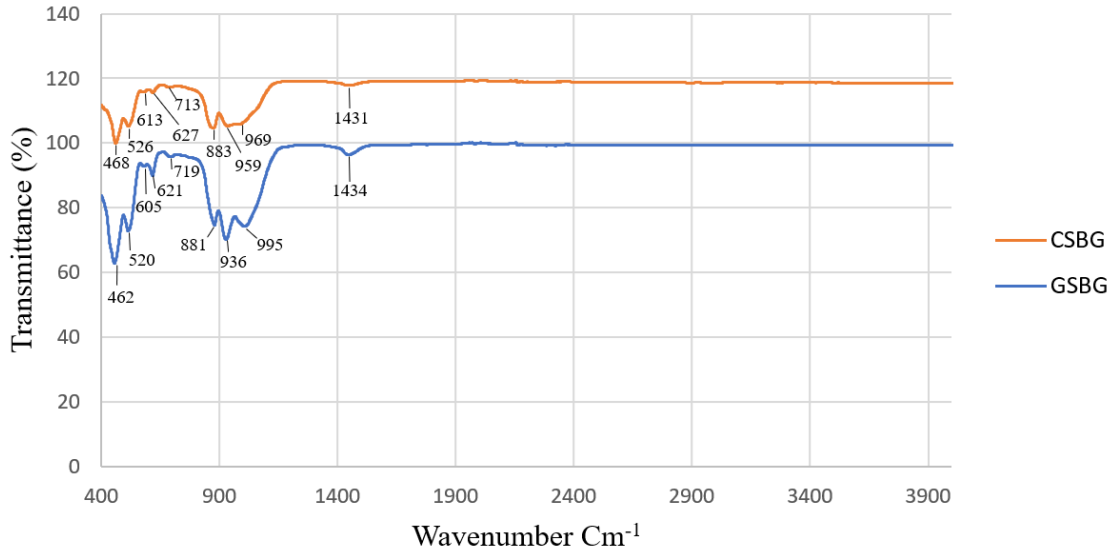


Figure 4.3. FTIR spectra of CSBG and GSBG.

For CSBG, bands occured at 468 cm^{-1}, 526 cm^{-1}, 613 cm^{-1}, 627 cm^{-1}, 713 cm^{-1}, 883 cm^{-1}, 959 cm^{-1}, 969 cm^{-1}, and 1431 cm^{-1}. For GSBG, bands occured at 462 cm^{-1}, 520 cm^{-1}, 605 cm^{-1}, 621 cm^{-1}, 719 cm^{-1}, 881 cm^{-1}, 936 cm^{-1}, 995 cm^{-1}, and 1434 cm^{-1}.

The characteristic peaks of bioactive glass at ~881 cm^{-1} and ~883 cm^{-1} correspond to the vibration of Si-O-Si stretching of non bridging oxygens. Other peaks seen at ~520 cm^{-1} and ~526 cm^{-1} indicate the silicate bonds of the Si-O-Si bending mode. The band located at ~605 cm^{-1} and ~613 cm^{-1} is attributed to the P-O bending of the PO_4^{3-} group. Also, peaks at ~936 cm^{-1}, ~959 cm^{-1}, ~969 cm^{-1}, and ~995 cm^{-1} are referring to P-O stretching mode (ElBatal et al. 2003). A weak peak at ~1431 cm^{-1} and ~1434 cm^{-1} is related to the residual carbonate group of precursors (Vafa, Bazargan-Lari, and Bahrololoom 2021). Another weak peak at ~713 cm^{-1} and ~719 cm^{-1} can be due to the presence of adsorbed CO_2 at the glass surface (Lucas-Girot et al. 2011).

Next, XRD was carried out to identify the presence of crystalline phases and to reflect crystal size and lattice strain (Figure 4.4).

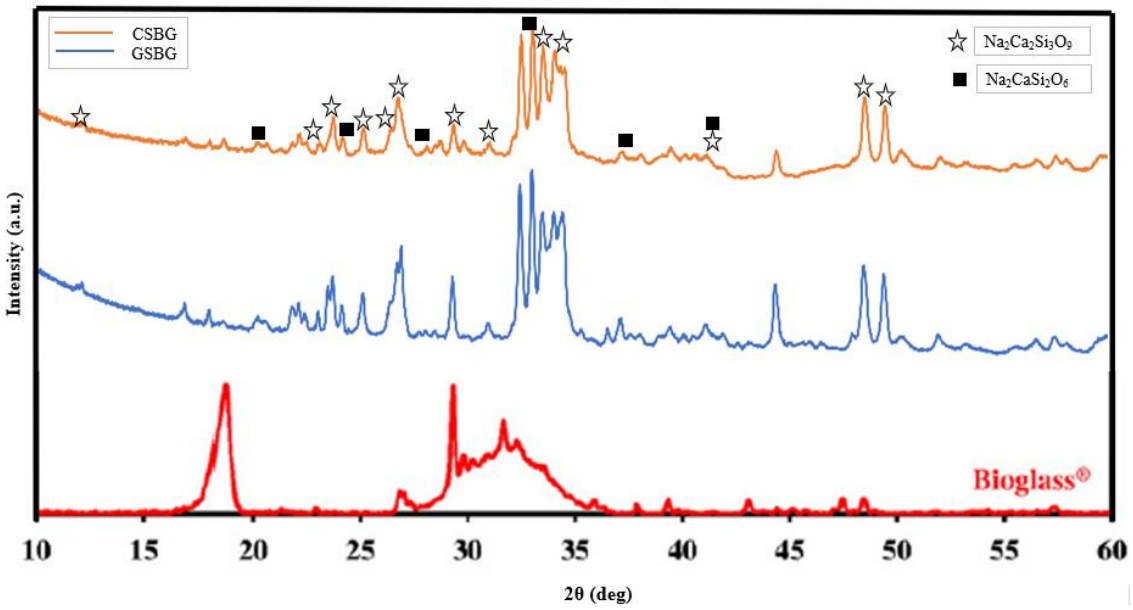


Figure 4.4. XRD spectra of CSBG and GSBG, in comparison to commercial Bioglass®.

Crystal structure analyses of CSBG and GSBG have been completed with XRD analyses. The Bioglass® powder is essentially amorphous due to the melt-qenching method process compared to the sol-gel method. Diffractogram of both synthesized bioactive glasses are very similar with more indication of crystalline phase formation than commercial Bioglass®. The crystalline phases data were also compared with the International Center for Diffraction Data (ICDD) database (Combeite, $Na_2Ca_2Si_3O_9$, ICDD PDF #22.1455) and found very consistent.

Bioactive glass (45S5) crystallizes at the $Na_2CaSi_2O_6$ phase and it can be explained partly by the fact that it is isostructural to the high temperature form of $Na_2Ca_2Si_3O_9$ (Lefebvre et al. 2007). The heat treatment at 700 °C used in sol-gel synthesis leads to the form of combeite phase, and combeite is known to influence bioactivity (J. Faure et al. 2015). The obtained XRD data is very consistent with the literature (Joel Faure et al., 2013 & J. Faure et al., 2015).

EDX analyses were performed to determine the elemental content of the CSBG and GSBG. The Ca/P ratio of CSBG was found to be 6.9 whereas the ratio for GSBG is 5.85 according to EDX results. The stoichiometric Ca/P ratios of 45S5 bioglass is 5. The reason for this change in ratio may be due to the fact that the synthesized samples contain a mixed phase, as confirmed by XRD data. Intensities of elemental constituents of CSBG

and GSBG can be seen in Figure 4.5 and Figure 4.6, respectively, while Si, Ca, Na, P, and O percentages are shown in Table 4.2.

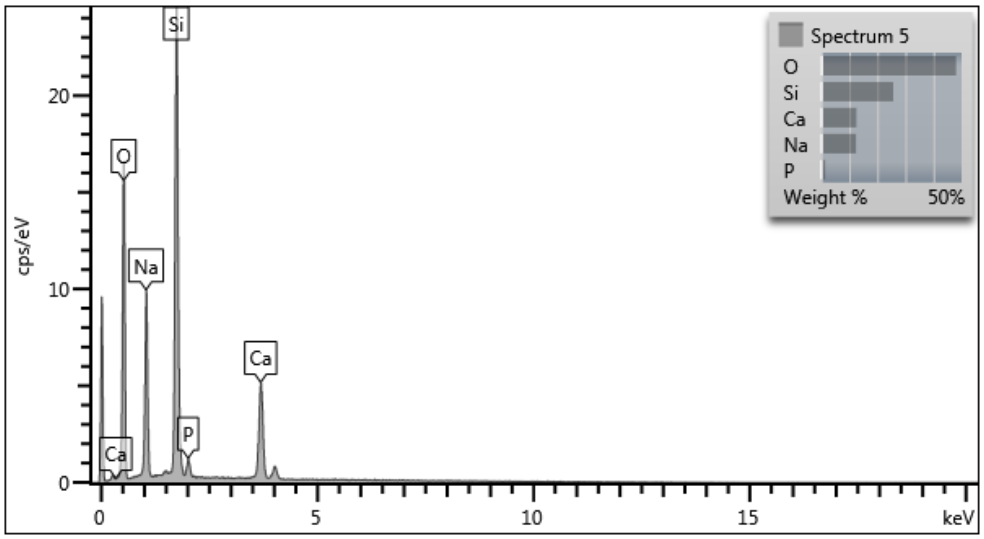


Figure 4.5. Intensities of elements in CSBG measured via EDX.

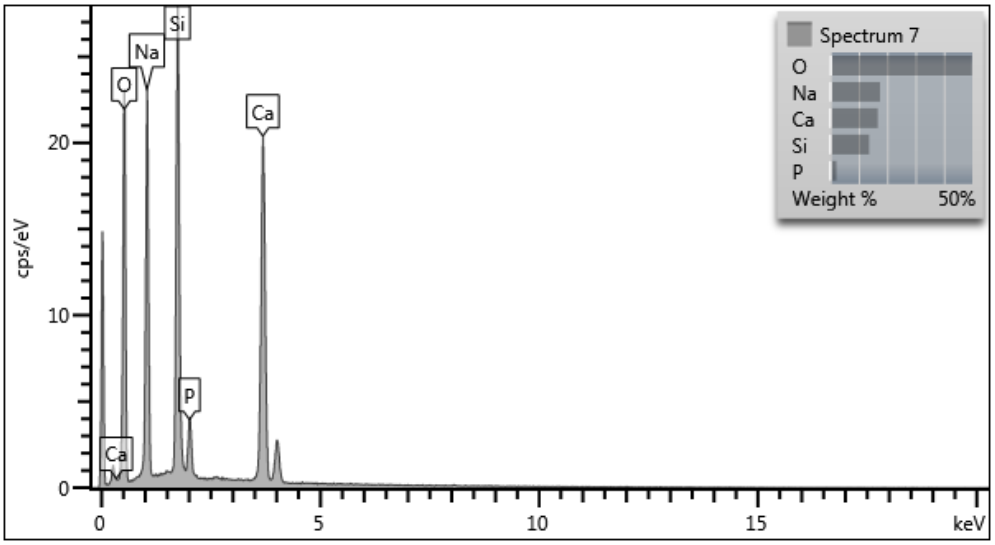


Figure 4.6. Intensities of elements in GSBG measured via EDX.

Table 4.2. Elemental composition of CSBG and GSBG.

	Atomic % in CSBG	Atomic % in GSBG
O	62.40	64.16
Na	11.18	15.74
Si	18.99	10.02
P	0.93	1.47
Ca	6.50	8.61

4.2. *In-Vitro* Bioactivity Tests of Bioactive Glasses

In-vitro tests are conducted to assess the bioactivity of the synthesized bioactive glasses. Mineralization kinetics of the CSBG and GSBG are compared to the control group explained in 3.4.1. The mineralization kinetics experiment was repeated 5 times (3 repeats in each). Every repeat included 2 h spectrophotometer incubation with 721 readings. All these absorbance data were normalized with ANOVA (Figure 4.7).

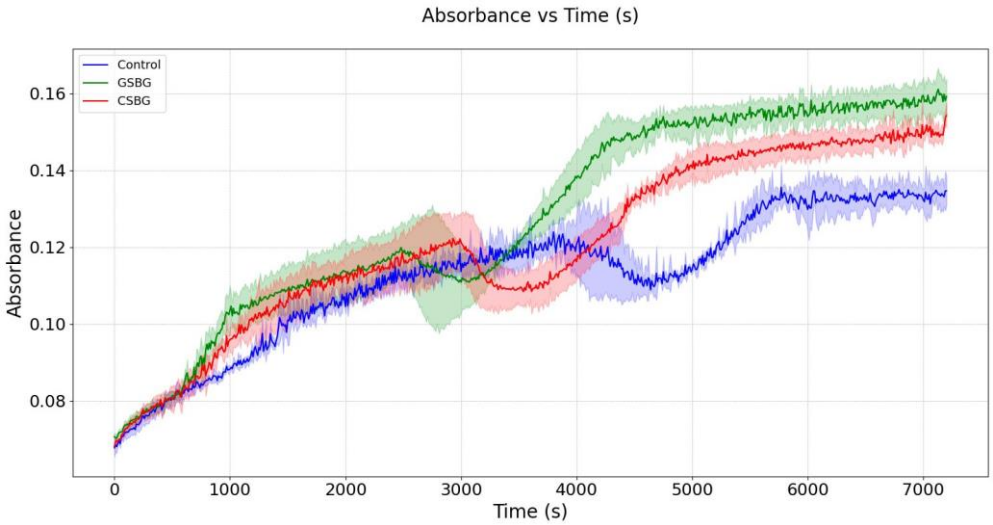


Figure 4.7. Mineralization kinetics of GSBG and CSBG with control.

The absorbance – time spectrophotometer result given in Figure 4.7 shows that in the presence of bioactive glasses, the GSBG forms more minerals compared to CSBG. Both synthesizing methods yielded higher mineralization when compared to control group. It can be speculated that after a certain point, the calcium released from the bioactive glasses begins to react. Compared to the control group, GSBG and CSBG increased mineralization by 20% and 12%, respectively.

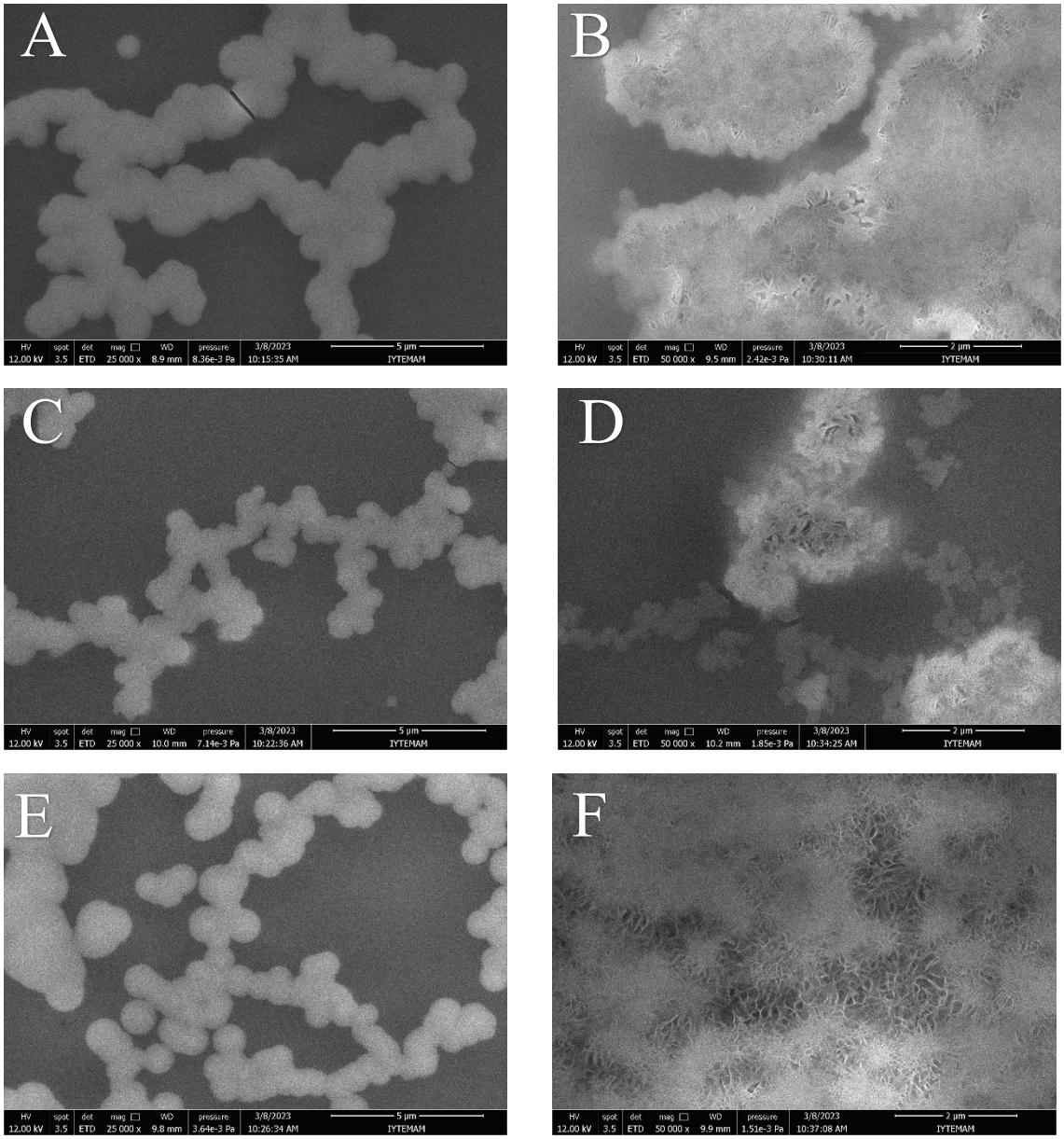


Figure 4.8. SEM results of mineralization kinetics. A) Control after 1 h, B) Control after 2 h, C) CSBG after 1 h, D) CSBG after 2 h, E) GSBG after 1 h, F) GSBG after 2 h

To confirm mineral formation, SEM analysis was performed after 1 and 2 h of the spectrophotometer readings (Figure 4.8). HA was observed in 2h SEM results, but not in 1h results. GSBG increased the mineralization rate at the highest rate

The obtained bioactive glass powders were pressed in pellet form for bioactivity tests to be performed in artificial saliva (AS). Pellets were incubated for varying time periods to test their bioactivity. After each time point, pellets were taken from the saliva solution and rinsed with DI water for 10-15 seconds and left to dry at room temperature. The pH of the AS was measured at the end of each incubation time (1 day, 3 days, 7 days, and 14 days). All experiments were repeated at least 3 times.

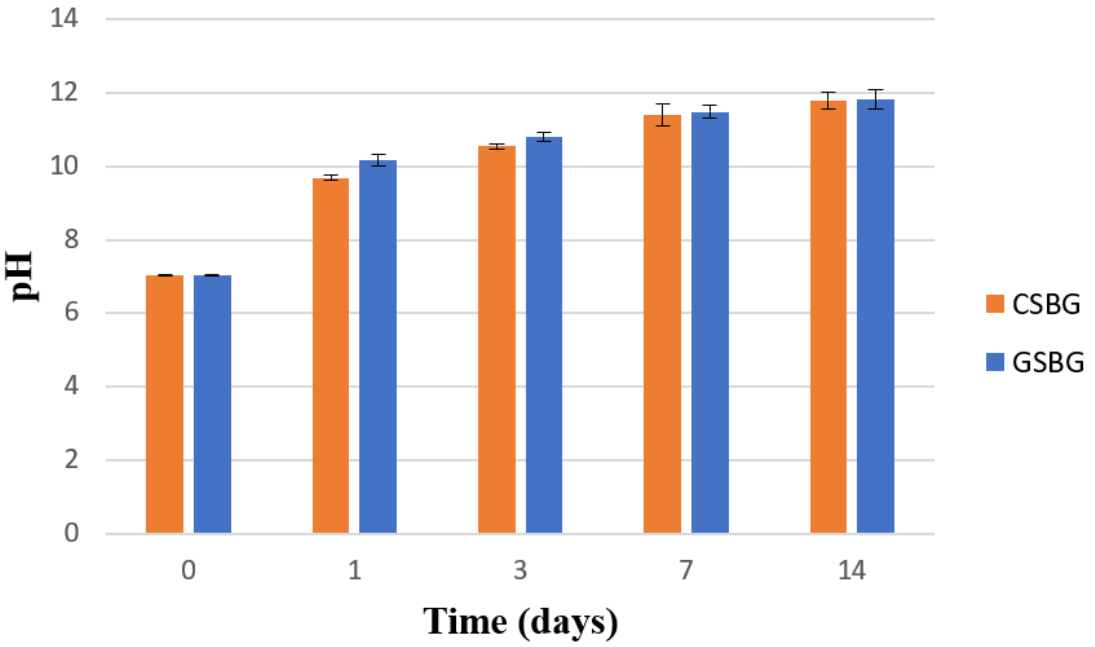


Figure 4.9. pH change graph of the bioactive glasses prior to and following AS immersion.

The pH change of the bioactive glasses was monitored to understand the apatite layer formation over time (Figure 4.9). The pH changes of the samples following AS immersion for 1-14 days were observed. The pH values increased with increasing immersion time. The rise in the pH measurement was from 7.01 ± 0.01 to 11.79 ± 0.23 for CSBG; and to 11.81 ± 0.26 for GSBG, after 14 days of immersion in AS. The pH

values show the same trend for both samples in all tested time intervals.

The initial increase in pH after immersion can be explained by the exchange of Na^+/Ca^{2+} ions from bioactive glass with H^+ ions from AS solution, leading to the formation of a high level of silanol (Si-OH). Ca^{2+} ions precipitate with OH^- and PO_4^{3-} anions from AS, forming the Ca-P layer precipitated on the Si-rich layer, known as the protective apatite layer (Loh et al. 2023). After the first day of immersion, pH increased at a decreasing rate due to the decrease in Na^+ ion content and the increase in glass solubility (Tripathi et al. 2019). Change in pH values and exchange of ions between the bioactive glass and AS confirms the formation of a hydroxy apatite-like layer.

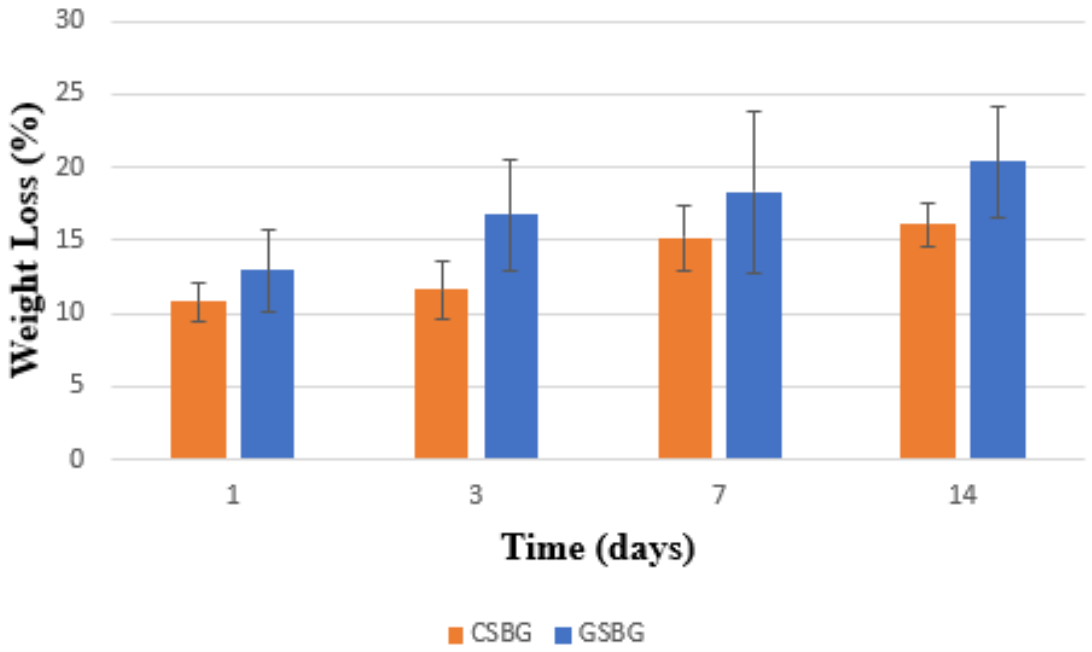


Figure 4.10. Weight loss of the bioactive glasses immersed in AS for 1, 3, 7 or 14 days.

The weight loss of the samples as a function of reaction time is shown in Figure 4.10. The weight loss measurement provides insights into the dissolution kinetics of the bioactive glass in a physiological environment. The graph shows that the weight loss of both samples is increased with increasing immersion time in AS up to 7 days. After day 7, there is no significant increase for CSBG, unlike GSBG. It seems that the degradation and deposition of CSBG has reached an equilibrium after day 7, though further data (>14)

is needed to confirm this hypothesis. In the dissolution period, bioactive glass pellets start leaching and the ion exchange between glass and AS increases. Simultaneous with the formation of a silica-rich layer, an amorphous CA-P layer forms and crystallizes which inhibits further degradation (Babu et al. 2021). The formation of the HAP layer on the surface of the sample increases over time (Zia et al. 2016). GSBG weight loss continues until day 14.

Overall, GSBG experiences a higher amount of weight loss when compared to CSBG, suggesting that the biodegradation rate of GSBG is also higher. Here, weight loss can be treated as an indicator of bioactivity. It can be concluded that both glasses are bioactive but GSBG is more bioactive than CSBG, which is consistent with the results of mineralization kinetics experiments (Figure 4.7). Both samples started to form apatite crystals on their surfaces and this finding is further supported by XRD and FTIR results.

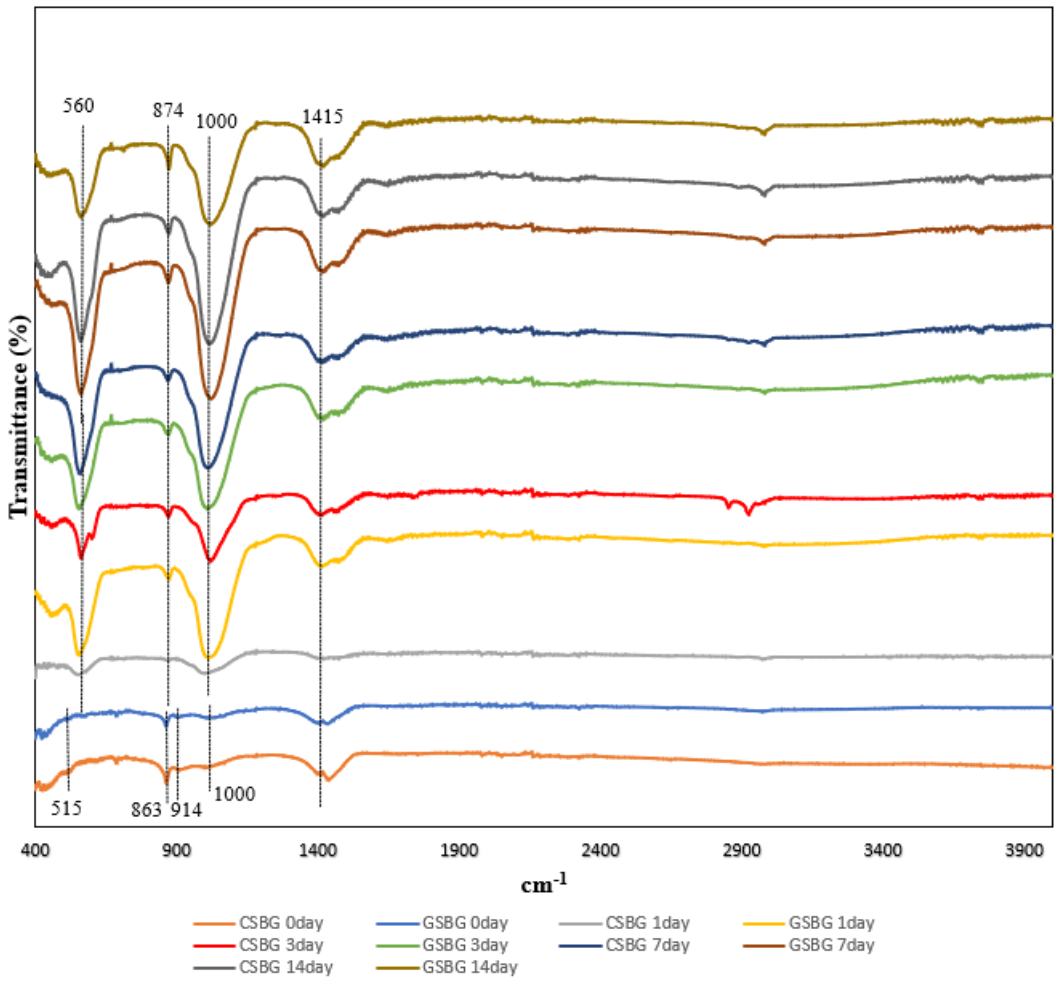


Figure 4.11. FTIR spectra of CSBG and GSBG before and after incubation in AS.

HA/HCA formation on the surface of bioglass discs was analyzed with FTIR before and after immersion in AS (Figure 4.11). Data were compared with the relevant literature (ElBatal et al. 2003).

Before incubating in AS, the FTIR spectra of both samples showed the most characteristic bands of the silica network. The bands around 515 cm^{-1}, 863 cm^{-1}, and 914 cm^{-1} are attributed to the Si-O-Si bending and stretching mode of Si–O–Si non-bridging oxygen atoms, respectively. The band around 1000 cm^{-1} corresponds to asymmetric stretching of Si-O-Si bridging of oxygen atoms.

After incubation in AS for 1, 3, 7, and 14 days, the spectra bands of the samples were modified since chemical interactions occurred between the glass pellets and the AS. The spectra bands at 515 cm^{-1} and 914 cm^{-1} are disappeared. It is associated with the glassy-network dissolution and re-polymerization of orthosilicic acid to make a SiO_2-rich layer on the surface (Tuan et al. 2021). Another possible explanation is the development of the HA or HCA layer that could cause the weakening and disappearance of Si-O-Si vibration bands (Rezaei et al. 2014).

The P-O characteristic peak at 560 cm^{-1} is attributed to the vibration of the PO_3^{4-} group in the HA, indicating the HA formation (Bui and Dang 2019). Simultaneously, the strong peak at 1000 cm^{-1} corresponds to P-O stretching. When the layer grows, P-O peaks usually become sharper (Crovace et al. 2016). C-O stretching vibration peaks are observed at 874 cm^{-1}, indicating the formation of carbonated calcium phosphate during the initial periods. The peaks around 1415 cm^{-1} refer to C-O in the CO_3^{2-} group and indicate the presence of crystalline ionic carbonate species on the glass surface (Deliormanlı 2017). The peaks around 2980 cm^{-1} refer to P-OH stretching in the HP_2^{-4} group (Babu et al. 2021).

Next, HA/HCA formation on the surface of bioglass discs was analyzed with XRD before and after immersion in AS (Figure 4.12).

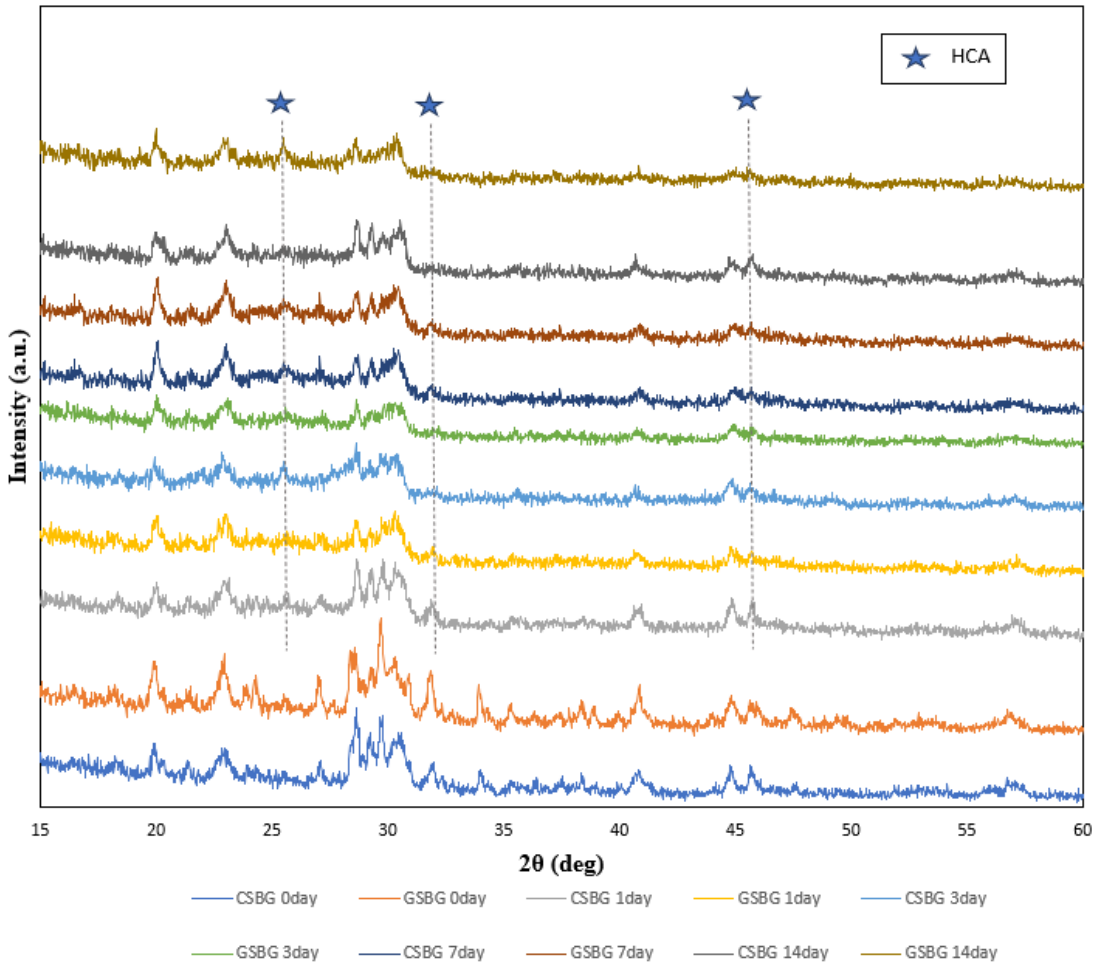


Figure 4.12. XRD spectra of CSBG and GSBG before and after incubation in AS.

After soaking the bioactive glass discs in AS for 1-14 days, the crystal peaks at 26°, 32°, and 46° matched with apatite peaks according to JCPDS cards (09-0432). Over time, glass transformed into an amorphous phase. The sharpening of the HA peaks indicates the formation of crystalline HCA. This phase transformation from crystalline to amorphous is very important to understand that glass is biodegradable in biological environments (Adams and Essien 2015). Bioactive glass discs showed bioactivity *in vitro* and their mineralization product was apatite but this result should be supported with further analyses like Confocal Raman Microscopy.

Later, SEM analyses of the bioactive glass discs prior to and following 1-14 days of immersion in AS were performed to obtain apatite crystals shown in Figure 4.13-4.15.

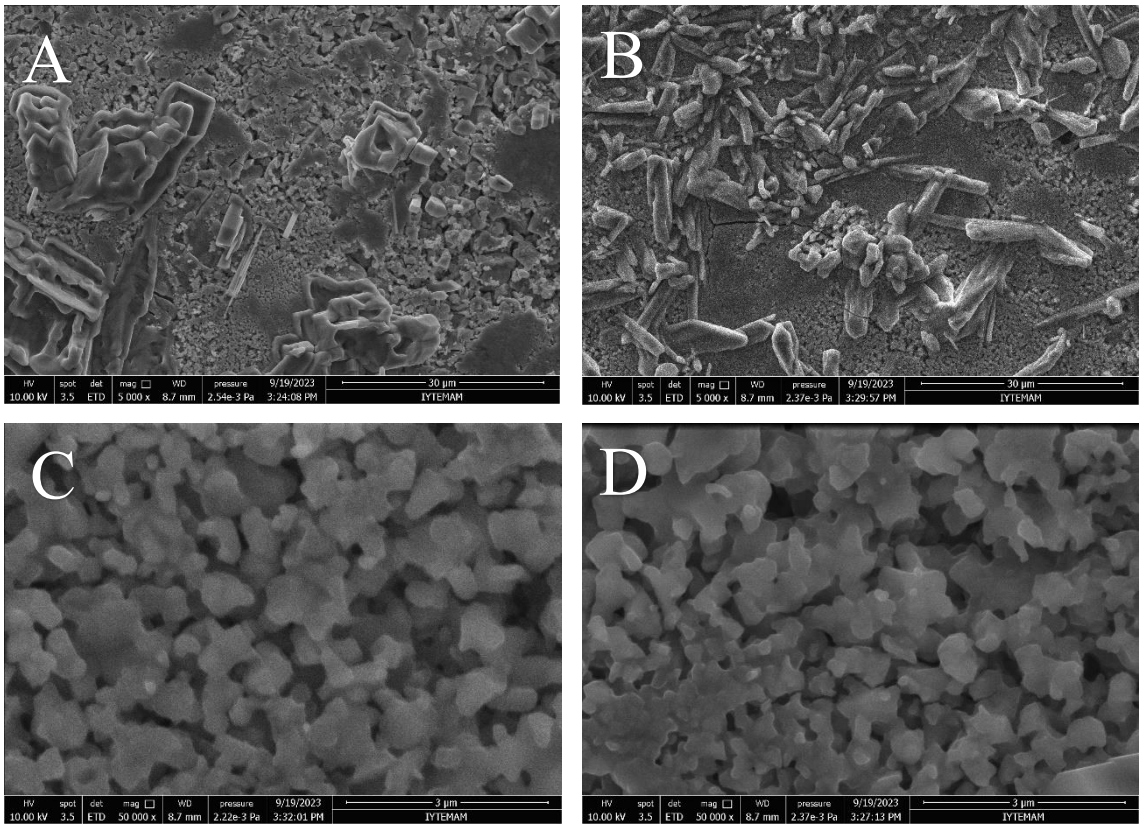


Figure 4.13. Pellets before immersion in AS. A) CSBG, B) CSBG, C) GSBG, D) GSBG. Scale bar: 30 µm (A-C), 3 µm (B-D).

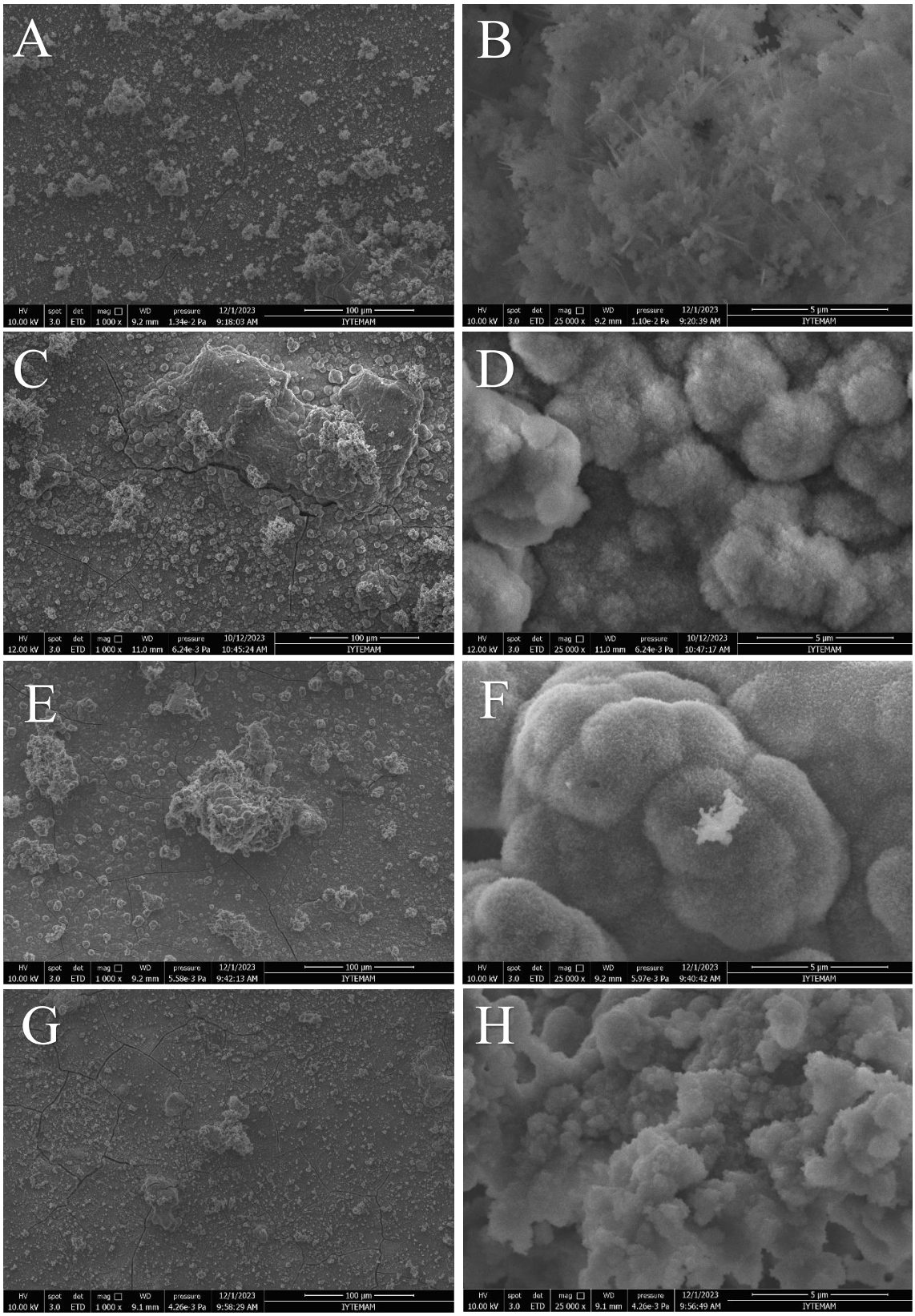


Figure 4.14. CSBG pellets after immersion in AS. A) 1 day scale bar: 100µm, B) 1 day scale bar: 5µm, C) 3 day scale bar: 100µm, D) 3 day scale bar: 5µm, E) 7 day scale bar: 100µm, F) 7 day scale bar: 5µm, G) 14 day scale bar: 100µm, H) 14 day scale bar: 5µm.

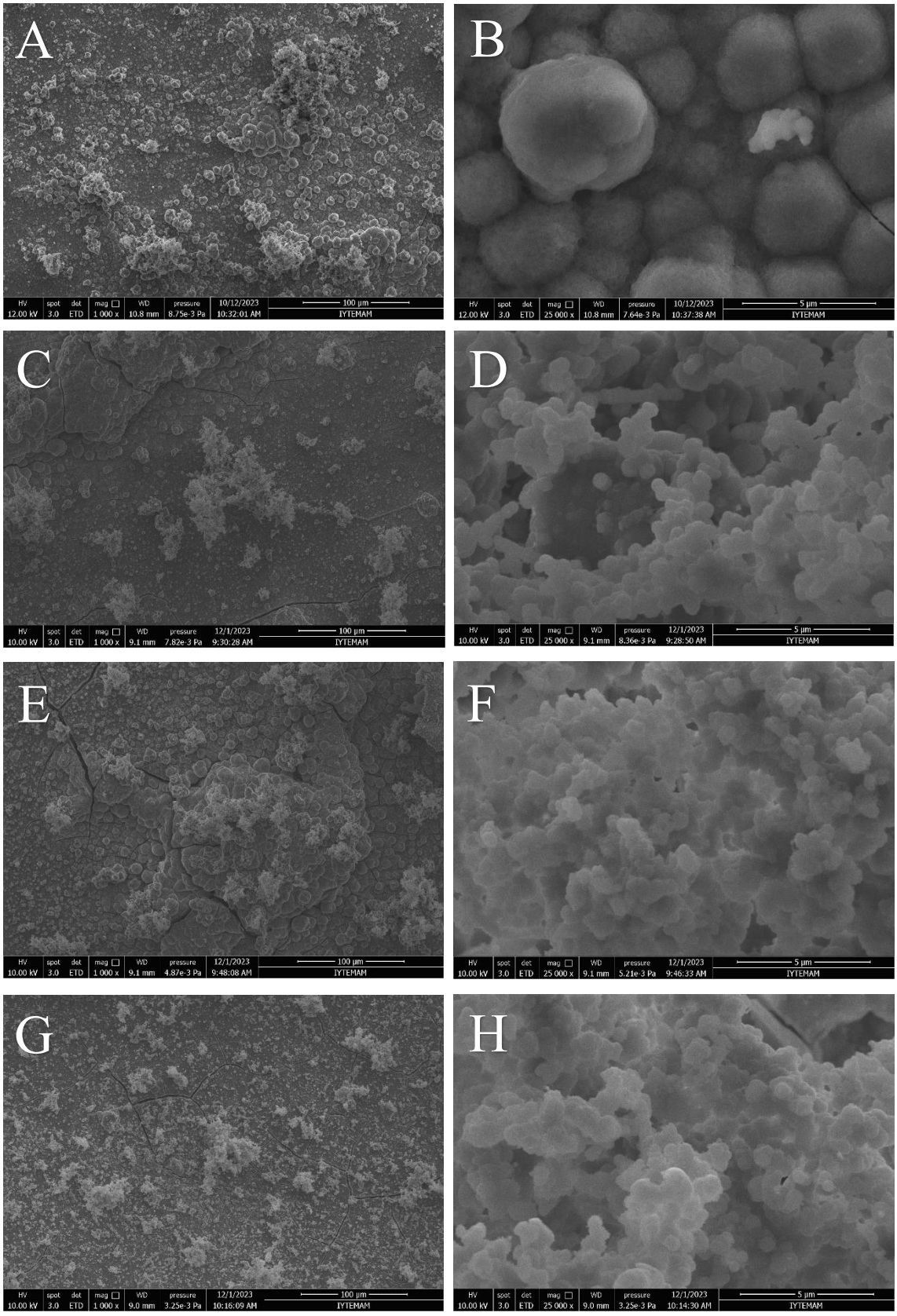


Figure 4.15. GSBG pellets after immersion in AS. A) 1 day scale bar: 100µm, B) 1 day scale bar: 5µm, C) 3 day scale bar: 100µm, D) 3 day scale bar: 5µm, E) 7 day scale bar: 100µm, F) 7 day scale bar: 5µm, G) 14 day scale bar: 100µm, H) 14 day scale bar: 5µm.

The SEM images of samples before and after immersion in AS confirmed the apatite formation on the surface of the pellets. As consistent with the literature, cauliflower-type apatite formation was observed in both synthesized bioactive glass pellets which indicate the bioactivity (Nawaz et al. 2020). The ion exchange of Ca^{2+} from glass with H^{+} from AS solution leads H_2O to break Si-O bonds and forms a porous silica gel layer on the surface of the pellets. This layer contains many Si-OH groups and OH groups and have a strong attraction to Ca/P groups in AS, ultimately leading to HCA formation (Chen et al. 2018).

Before immersion, pellets were smooth and had no cracks. As seen in the SEM images, the entire surface is covered by HCA layer. The release of ions caused cracks to form on the surface. Crack intersections provide a suitable HCA nucleation site. Cracks formed on the surface of bioactive glasses within 14 days indicate their biodegradability and the bioactivity of the HCA layer formed on their surface. HA formation within an *in vitro* physiological environment is directly related to tight attachment to bone *in vivo*.

4.3. Cell Viability Assessments of Saos-2 Cells

Cytotoxicity tests are widely used to pre-screen the toxicity of the material. The changes in the viability of Saos-2 cells after bioactive glass treatment were assessed via 3 independent (3 replicas) MTT tests. Non-treated cells were used as control. Both CSBG and CSBG showed the same behavior with the increasing concentration as shown in Figure 4.16. It has been determined that the concentration of 7.5 mg/ml is a critical value, above which BG samples became toxic for Saos-2 cells. According to ISO 10993-5 standard, the material is toxic when its cellular viability is lower than 70% (ISO 10993-5, 2009). Therefore, bioactive glasses in this study show biocompatibility after culture with Saos-2 cells for 24 h. The bioactive glass synthesized without using acid catalyst (GSBG) in this study can be safely used in potential biomedical and dentistry applications.

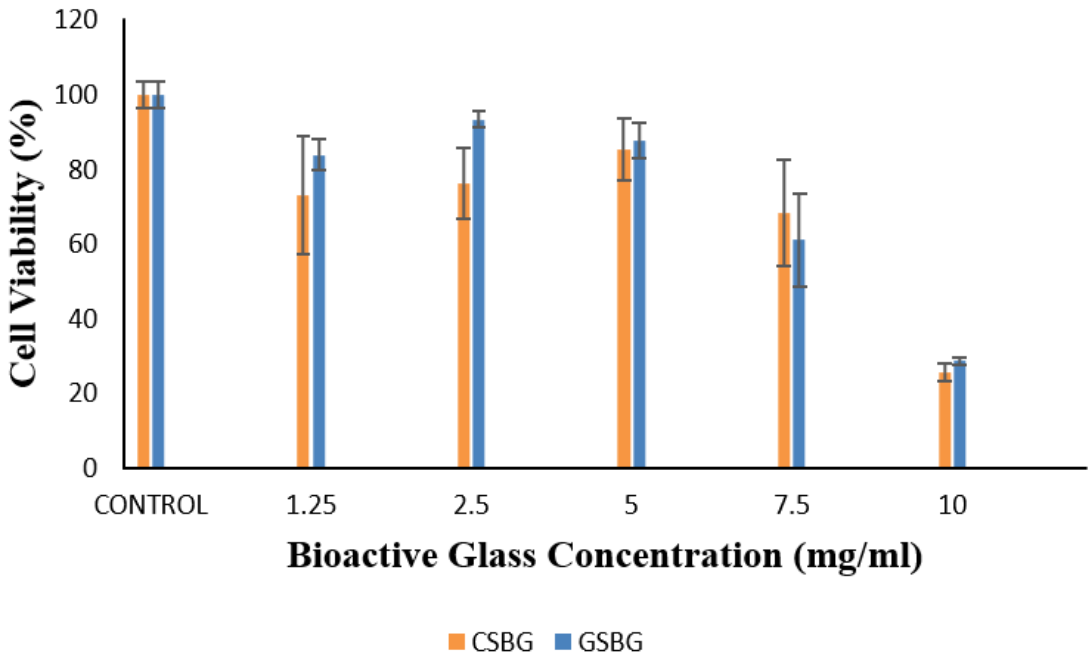


Figure 4.16. Viability result of Saos-2 cells treated with increasing concentrations of CSBG and GSBG.

CHAPTER 5

CONCLUSION

Bioactive glass is a type of bioceramic material that can readily dissolve in body fluids and releases calcium and phosphate to form hydroxy carbonated apatite. It is originally designed for use as a bone substitute in tissue engineering applications due to its proven bone-bonding properties. Later, it has been proven successful in protecting the tooth against various common problems such as early decay, acid erosion, and sensitivity. The special bioactive glass dissolves in the saliva, and as it dissolves, it releases calcium and phosphorus ions (crucial components of tooth enamel), forming a protective layer on the teeth. Today, there are several dental products that are made with different forms of bioglass particles with the aim of repairing tooth decay. Despite the advances in the design of smart bioglass-based biomaterials, their synthesis still relies (mostly) on traditional methods such as sol-gel and melting that require the use of acids and organic solutions that may be toxic. Therefore, at the same time as bioactive glass technology is evolving, there remains a need to uplift the safety and environmental friendliness of their synthesis routes. Here, we achieved this goal by replacing acid catalysts in the sol-gel method with DI-water. In other words, bioactive glass was synthesized with an acid catalyst: *chemical* (CSBG), and without an acid catalyst: *green* (GSBG). Physicochemical characterization data confirmed that our synthesis was successful. We then assessed both the functionality and toxic potential of bioactive glasses synthesized with and without acid catalysts, and GSBG showed superior performance in almost all tests. More specific conclusions drawn from physicochemical characterization, *in vitro* functionality and bioactivity tests are provided below:

- ✓ The mean particle size obtained for CSBG and GSBG was 43 nm and 41 nm, respectively, confirming that smaller sizes could be achieved without the need for acid solutions.

- ✓ Surface properties analyzed with BET suggested that both bioglasses had mesoporous structures. GSBG had a higher surface area than CSBG, an important indicator of increased functionality and bioactivity.
- ✓ Bond formation and functional groups obtained with FTIR showed similar bands for both bioglass samples. The obtained functional groups were consistent with the literature.
- ✓ Crystal structure of CSBG and GSBG was investigated with XRD and similar patterns were observed. Combeite ($Na_2Ca_2Si_3O_9$) was the dominant phase in synthesized glasses. It is expected given that it crystallizes from 45S5 Bioglass during heat treatments.
- ✓ Elemental analyses were done with EDX. Both CSBG and GSBG had a high ratio of Ca/P, which is considered essential for bone health.
- ✓ Performance of the synthesized glass is assessed with the mineralization kinetics experiment. GSBG displayed a higher mineralization rate compared to the control group and CSBG. Both bioactive glasses increased the mineralization rate.
- ✓ SEM results of the mineralization kinetics experiment confirmed that after 2 h apatite formation occurred.
- ✓ To better understand the bioactivity, pellets formed from the synthesized bioglasses were immersed in artificial saliva and the subsequent changes in the pH were measured. An increase in pH after immersion in both samples was observed, indicating HCA formation. The weight loss (%) of the GSBG was higher than that of CSBG. It was confirmed that GSBG had a higher biodegradation rate and higher bioactivity.
- ✓ Apatite formation on the pellet was confirmed by XRD and FTIR peaks. Both glasses increased mineralization.
- ✓ Apatite formation was further confirmed with SEM after different durations of immersion in AS. HCA/HA covered the surface of the pellets after incubation.
- ✓ Cytotoxic potential of bioglasses was tested with MTT assay. There was a significant decrease in the viability of Saos-2 cells treated with > 7.5 mg/ml of synthesized bioglasses regardless of the synthesis route. No sign of toxicity was observed below this dose.

We performed the mineralization tests on the pellets, rather than the powders, to understand whether GSBG is more bioactive due to its surface area or synthesis method. The results proved that GSBG is more bioactive even with the same surface area. It increases the mineralization and is not toxic when realistic exposure doses are used. The *greenized* sol-gel method is a good alternative for synthesizing nano-structured bioactive glass with increased functional properties compared to the acid catalyst sol-gel method. Future work will include the integration of remineralizing agents (such as fluorine) and the development of bioactive glass composites with added functionality in dental applications.

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