

DEVELOPMENT OF BIOACTIVE GLASS COATING ON MEDICALGRADE IMPLANT SURFACE FOR BIOMEDICAL APPLICATION

Submitted in partial fulfilment of the requirements
for the award of Bachelor of Technology degree in Biotechnology

By

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(DEEMED TO BE UNIVERSITY)**

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May – 2023



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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Deeksha R V (Reg.No 39230010) who carried out her Project titled "Targeting Breast Cancer using Pimozide and its effect on Cell Proliferation, Invasion and Mitochondrial metabolism" under my supervision from January 2023 to May 2023.

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DECLARATION

I, **Deeksha R V** (Reg. No- 39230010), hereby declare that the Project Report titled "**Targeting Breast Cancer using Pimozide and its effect on Cell Proliferation, Invasion and Mitochondrial Metabolism**" done by me under the guidance of **Dr. R.P Oviya, MS (Research). , Ph. D.** is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology in **Biotechnology**.

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SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of **SATHYABAMA** for their kind encouragement in doing this project work and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V. RAMESH KUMAR**, Head of the Department, Department of Biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Internal Guide **Dr. M. BAVANILATHA**, Department of Biotechnology, and my External Guide **Dr. K. GOPI SARAVANAN**, Scientist-D in IRC (International Research Centre) for their valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-Teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT

Bioactive glasses (BG) are the most promising biomaterials for bone repair due to its superior bioactive and biocompatibility properties. However, it doesn't express antimicrobial property which restricts its wide applications. Hence, in the present study, we synthesized silver containing mesoporous bioactive glass by acid assisted sol-gel method and it was further deposited onto medical grade 316L SS using thermal spray technique. The deposited films were characterized by XRD analysis to determine crystallinity, surface morphology of the coated samples. Wettability study was performed by contact angle goniometer and the coated samples exhibited super hydrophobic nature. Further, antimicrobial efficacy, biodegradation, and cytotoxic behaviour was studied in detail. From overall study, it concluded that the silver containing mesoporous bioactive glass coating is a good candidature for biomedical applications.

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LIST OF ABBREVIATION

ACRONYM	EXPANSION
BG	Bioactive Glass
Ag-BG	Silver-doped Bioactive Glass
SS	Stainless Steel
Co-Cr	Cobalt - Chromium
SiO ₂	Silicon dioxide
B ₂ O ₃	Borate trioxide
P ₂ O ₅	Phosphorus pentoxide
MgO	Magnesium Oxide
Na ₂ O	Sodium Oxide
K ₂ O	Potassium Oxide
UV-Vis	Ultraviolet-visible spectroscopy
XRD	X-Ray Diffraction
SEM	Scanning Electron Microscope
BSA	Bovine Serum Albumin
AO	Acridine orange
MEM	Minimum Essential Medium
P.aeruginosa	Pseudomonas aeruginosa
MBG	Mesoporous Bioactive Glass
TEP	Tetra ethyl phosphate
TEOS	Tri ethyl orthosilicate
Ca (NO) ₃	Calcium nitrate
Ag (NO) ₃	Silver nitrate
Na (NO) ₃	Sodium nitrate
CHA	Carbonated hydroxyapatite
HA	Hydroxyapatite
MTT	Methyl thiazolyl tetrazolium
NBO	Non bridging oxygen
ALP	Alkaline phosphate
SBF	Stimulated Body Fluids

CHAPTER-1

INTRODUCTION

1.1 BIOMATERIALS

Biomaterials are materials found in medical implants and devices. Because they have the ability to perform predeterminant activities when engaging with biological systems. The most important need for a biomaterial is "biocompatibility," or the capacity to interact with the human body without inducing an immune response. Metals, ceramics, polymers, and composition are the four composition categories for biomaterials. Each substance has its own unique set of characteristics. Metals, for example, have high toughness and mechanical characteristics, whereas ceramics have high wear resistance and hardness, and many polymers have chemical, physical, and mechanical properties akin to those of living soft tissues. Furthermore, biomaterials may be classified depending on their interaction with the human body, with 'bioinert,' 'bioactive,' and 'bioresorbable' materials being the most common (Sola *et al.*,2011).

Metallic materials such as stainless steel, Co-Cr alloy, titanium, and their alloys are now widely used in implantology as prosthetic implant materials. Regardless of their strength, implant materials must all remain stable when exposed to living tissues or cells in a dynamic and hostile environment. Metallic prosthetic materials, according to clinical data, are prone to localised corrosion in the human body as a result of prolonged exposure. 316L SS prosthetic implants, in particular, typically fail because of the ability of crevice and pit corrosion to release metallic ions into the surrounding tissues (Oliver *et al.*,2019).

SS is one of the most often used implant materials in treatments such as hard tissue repair, cardiovascular tents and valves production, orthopaedics, prostheses, dental surgery, craniofacial surgery, and application (Bekmurzayeva *et al.*,2018).

The exceptional fatigue properties, ductility, and work hardenability of SS-316L are well recognised (Zardiackas *et al.*,2006). The lack of bio-features is a common limitation that limits the use of SS-316L for many implant applications, and research is being performed to study alternative pre-treatments in the form of surface alterations to improve its anti-fouling properties (Bekmurzayeva *et al.*,2018). Another disadvantage of using SS-316L in this biomedical application is its inability to continuously integrate with the body in locations where there is insufficient oxygen to sustain the passive film and in areas where service is unavailable. This can easily lead to the failure of popular application components, such as formed components, due to fracture (Elias *et al.*,2018).

1.2. BIOACTIVE GLASS

Bioactive glass is defined by its name, which includes "bioactive," which means one that elicits a specific biological response at the material's interface, resulting in the formation of a bond between the tissues and the material, and "glass," which is often defined as a solid with a non-crystalline (that is, amorphous) structure at the atomic scale and that exhibits a glass transition when heated towards the liquid state. Bioactive glasses are a type of surface-reactive glass-ceramic biomaterial that includes Bioglass, the original bioactive glass. These glasses have been used as implant devices in the human body to repair and replace ill or damaged bones due to their biocompatibility and bioactivity (Boccaccini *et al.*,2017). In brief, bioactive glass was created to evoke a specific biological reaction at the material's interface, which increases cell proliferation, gene response, and the establishment of a link between live tissues and the material. Its surface forms a physiologically active apatite layer (HCA) that starts bone bonding. The chemical and structural formation of apatite parallels the mineral phase of bone.

1.3 COMPOSITION OF BIOACTIVE GLASS

The basic elements are typically SiO_2 , Na_2O , CaO , and P_2O_5 , and the percentages of weight for the most typical BAGs are shown below. Bioglass content in weight percent.

SiO₂: 45wt%;

Na₂O: 24.5 wt%; CaO:

24.5 wt%; and P₂O₅:

6 wt%.

The Bioglass 45S5 has received the most research. The acronym signifies that it has a molar ratio of 5:1 for Ca/P and that SiO₂ (an oxide creator) makes up 45% of its weight. CaO and P₂O₅ glasses, which have a substantially lower molar rate, do not make connections with the bone.

Table 1.1 Composition of different bioactive glasses (Khalid et al., 2017).

	Na ₂ O	K ₂ O	MgO	CaO	SiO ₂	P ₂ O ₅	B ₂ O ₃
45S5	24.5	0	0	24.5	45.0	6.0	0
13-93	6.0	12.0	5.0	20.0	53.0	4.0	0
6P53B	10.3	2.8	10.2	18.0	52.7	6.0	0
58S	0	0	0	32.6	58.2	9.2	0
70S30C	0	0	0	28.6	71.4	0	0
13-93B1	5.8	11.7	4.9	19.5	34.4	3.8	19.9
13-93B1	5.5	11.1	4.6	18.5	0	3.7	56.6

1.4 STRUCTURE OF BIOACTIVE GLASS

The glass structure may be used to predict the majority of glass qualities. The typical description of the glass structure is that it is made up of an uneven, threedimensional network produced by network formers like SiO_2 , B_2O_3 , and P_2O_5 . The SiO_4 tetrahedron, which is linked to other tetrahedra by the siloxane Si-O-Si bond, creates the network of silicate glasses. The SiO_4 tetrahedron's connections are severed by the network modifiers. As a result, there are fewer oxygen links (also known as bridging oxygen bonds) between the tetrahedra and more nonbridging oxygen ions. Network modifiers include oxides of Na, K, Ca, Mg, etc (Jones *et al.*,2013). While the link between Si and the bridging oxygen is covalent (-): $\text{Si-O} \dots \text{M}^+$, the bond between the modifier metal ion and the nonbridging oxygen is mostly ionic (---). Some metal oxides, such as Al_2O_3 , function as intermediaries. Depending on their composition, the intermediates either operate as network formers to strengthen the glass structure or as network modifiers to depolymerize it. Network connectedness, or the average number of bonding oxygen ions per Si tetrahedron, has been used to define the degree of polymerization of the bioactive glass network (Edén *et al.*,2011; Hill and Brauer.,2011). Theoretical bioactive glasses' NC (network connection) connectivity According to Srivastava *et al.*,2012. silica in glass is a network former that may keep the glass structure together. Therefore, a reduced silica concentration might guarantee quicker dissolution and quicker bioactivity (Jones *et al.*,2013).

1.5 CATEGORIES OF BIOACTIVE GLASS

Silicate – based bioactive glasses

Silicate glasses are considered as a reliable material when considering the design of novel medical devices boasting specific properties. A large surface area, a high degree of purity index, and porosity within the bulk of the material contribute significantly toward the high level of reactivity exhibited when employing this material for research purposes and as a component of a biomaterial (Aguilar *et al.*, 2009). The open-ended structure of these BGs enables the manifestation of free space within the bulk of the material. This allows for the addition of Na^1 , K^1 , Ca^{21} , and Mg^{21} cations into the glass matrix. These cationic species are termed as network modifiers. Network modifiers are known to induce network disruption—a crucial preliminary step if realization of nonbridging oxygen (NBO) groups is to occur within the glass structure (González *et al.*, 2003; Sen and Youngman, 2003). The presence of NBOs has a strong influence on the connectivity of the material as a whole.

The commercial variant 45S5 (based on silicate glass) has been the focus of multiple studies pertaining to biomedical applications (Hench *et al.*, 2006). The inherent properties of this version of 45S5 allow for it to bond to mineralized bone tissue—a phenomenon which has been the subject of numerous investigations for the better part of four decades and counting. The silicate glass 45S5 structure can be best visualized as a three-dimensional (3D) SiO_2 network with a central silicon ion surrounded by four coordinating oxygen ions arranged in a tetrahedron configuration. The SiO_2 content in silicate BG compositions is in direct relation to their chemical stability. Moreover, glass network modifiers in variable ratios are the primary structural and compositional determinants of the bioactive nature of this material. Common glass network modifiers include Na_2O and CaO , whereas magnesium (Mg^{21}) and zinc (Zn^{21}) fall on the border of modifiers and intermediate ions (which increase the processing window to avoid crystallization). The role of these modifiers is to break some part of the SiO bonds, thereby creating NBOs and disturbing the tetrahedral silicate network (Benoit *et al.*, 2001).

Borate-based bioactive glasses

The trace element boron is essential for maintaining bone health. The first borosilicate glass formulation to be considered for biomedical applications was conceived in 1990. These glasses are considered as a reactive species, which accounts for their low chemical stability index. This translates to a more rapid conversion rate into HAP for this group of BG as compared to their silica based counter parts (Yao *et al.*, 2007). The substitution of SiO_2 with B_2O_3 allowed a significant escalation in the conversion rate of borate glass to HAP post immersion in aqueous phosphate solutions (Huang *et al.*, 2006). Although the mechanism by which borate-based BG undergoes conversion to a layer of apatite is similar to its silica-based equivalent, in that a borate-rich layer ensues in place of a silicate-rich layer, borate-based BGs are more amenable to precise tinkering with the degradation profile and sintering behavior, which can be achieved over a range of time lines in contrast to silicate-based BGs (Yang *et al.*, 2012). Spanning the course of a few decades, a significant amount of research has been conducted, and consequently, key data have been generated from studies pertaining to the behavior and properties of the original formulation of 45S5 BG. On the other hand, probing research questions exploring borate-based compositions are only just beginning to surface in the literature (Rahaman *et al.*, 2011; Kaur *et al.*, 2014)

1.6 BIOACTIVE GLASS AS A COATING MATERIALS

Bioactive glasses are a class of surface reactive glass-ceramic biomaterials that include the original bioactive glass, Bioglass. Because of their biocompatibility and bioactivity, these glasses have been employed as implant devices in the human body to repair and replace sick or damaged bones (Boccaccini *et al.*, 2017). Because they are both bioactive and biodegradable, bioactive glasses (BGs) have been developed as bone-filling materials for bone replacement. Bioglass[®] 45S5, the first commercially available bioactive glass (BG), was developed and successfully used by Hench *et al.* in orthopaedic bone defect repair, scaffolds, micro-bone grafting, bioactive coatings, and periodontal restoration (Rui Zhao *et al.*, 2021).

Among the BGs, 45S5 BG with SiO₂ (45%), CaO (24.5%), Na₂O (24.5%), and P₂O₅ (6%) composition meets the mechanical competency requirements and has undergone considerable study. 45S5 BG contains the same amounts of silicon dioxide (SiO₂), calcium (Ca), sodium oxide (Na₂O), hydrogen (H), and phosphorus (P) as bone. When biological fluids come into contact with the surface of the 45S5 BG implant, silica-CaO/P₂O₅ gel layers develop and are mineralized to hydroxycarbonate. Furthermore, the rich gel layers encourage the formation of the apatite-like matrix. Furthermore, dissolution studies demonstrated a rapid proliferation of osteoblast cells, which develop into new bone structures (ammudha *et al.*,2020).

In 2004, a new form of bioactive glass, mesoporous bioactive glasses (MBGs), was also introduced. MBGs have opened up new application areas for these materials, including medication delivery, due to their customised (ordered) nanometer-scale porosity, which is generated by combining the supramolecular chemistry of surfactants with sol gel chemistry. (Anahí Philippart *et al.*,2016). The most notable property of MBGs is their highly structured nanoscale pore structure (pore size range: 2-50 nm), which also contributes significantly to their capacity to produce apatite. Because of its mesoporous shape, it can load and distribute a wide range of medications, including antibiotics, as well as bioactive chemicals like growth hormones (Kargozar *et al.*,2018). In the biomedical industry, coatings have been used in a variety of ways to modify the surface of implants and, in some cases, to fully develop a new surface that gives the implant with extra attributes that are substantially different from those of the uncoated device. Bioactive coatings aid orthopaedic and dental implants because they have the potential to boost device stability by attaching to host bone and avoiding corrosion, hence reducing the release of potentially hazardous metal ions *in vivo*. In contrast, metal implants placed alone typically become encapsulated with fibrous tissue after implantation (Francisco Baineo *et al.*,2017).The application of bioglass coatings on metallic implants connects the bone to the materials and prevents metal corrosion in internal fluids. 45S5 bioglass-silica coatings on 316L stainless steel were created in this study using the plasma spray method.

For many years, 316L stainless steel has been used as a permanent surgical implant material due to its low cost, excellent production capabilities, exceptional corrosion resistance, and broad availability. Nevertheless, the mechanical integrity and biocompatibility of metallic implants could be severely damaged by corrosion when exposed to physiological fluids. Unfortunately, these materials are frequently very brittle and prone to fracture propagation, which can result in catastrophic failure. As a result of their weak mechanical properties, they cannot be used as a bulk material in load-bearing applications. As a result, bioactive coated implants are great options for overcoming the aforementioned limits because they combine the remarkable bioactivity of bioactive glasses with the advantageous mechanical properties of metals (Pourhashem *et al.*, 2014).

Cell proliferation and differentiation are improved by Ag-BG. An ideal treatment for bone fractures combines efficient tissue engineering with continuous bactericidal action to prevent bone infections, reducing the danger of antibiotic resistance while simultaneously boosting bone regeneration. Heavy metal ions (e.g., silver, copper, zinc) have been shown to be powerful antibacterial agents against drug-resistant microorganisms (Lu *et al.*, 2013). Nevertheless, the release of heavy metal ions in the body raises general toxicity concerns, preventing widespread usage. Silver ions (Ag^+) are widely recognised for their broad-spectrum biological activity against many Gram-negative and Gram-positive bacteria, including resistant species (Natalia Pajares *et al.*, 2021).

Because bioactive materials are either biostable (such as calcium phosphates) or bioresorbable (such as bioactive glasses), they are often placed as bioactive coatings to increase the chemical resistance, durability, and biocompatibility of these metallic implants. Surface coatings can be applied to implants for medical purposes, such as antibiotics and other medications, in addition to increasing bioactivity and osseointegration. (Zafer *et al.* 2019). A BAG coating on implants can emit metal ion leakage and boost corrosion resistance, resulting in a well-structured environment for osseointegration. BAG may reduce prosthesis corrosion in body fluids and improve implant osseointegration (Oshida *et al.*, 2010; Zafar *et al.*, 2019).

In addition, BAG can aid in matrix deposition, cell adhesion, and proliferation. Moreover, it stimulates osteoblast development and recruitment, and bone forms and replaces the place of fractured glass in BAG-filled regions over time (Hulsen *et al.*, 2017).

One of the major benefits is coating a bioinert material with a bioactive glass to use as a surface for biofunctionalization. The interactions that take place between the biomaterial and the physiological media at the surface of an implant are significantly influenced by a bioactive surface. Following implantation, a variety of physiological processes take place concurrently in the layer that lies between the targeted tissue and the implant. High bioactivity is the main advantage of Bioglass, while its disadvantages include mechanical weakness, low fracture resistance due to amorphous 2-dimensional glass network (Mesquita *et al.*, 2018).

1.7 PLASMA SPRAY COATINGS

Plasma spray coating is a flexible coating process that can easily produce a highperformance surface coating material. In a plasma spray process, hot ionized gas is used to melt BG powder and drives the powder particle in such a way that it can easily adhere to and agglomerate on the coating surface. Thermal spray technology is a coating process that provides functional surfaces to protect or improve the performance of a substrate or component. Thermal spraying of CaP on the implant devices can be compared with plasma spray-coating technique, having the advantage of high deposition rate and low cost. Thermal spray technique has the ability to produce HA layer with thickness from 30 to 200 μm depending on the coating condition. However, films deposited by thermal spraying suffer from poor coating–substrate adherence and nonuniform crystallinity, which reduce the lifetime of implant.

With the plasma spraying technique, the same coatings can be produced at a very rapid rate. Plasma-sprayed coated implants have shown a number of benefits for implant applications such as increased surface area leading to enhanced osseointegration both in vivo and clinically (Sun *et al.*, 2001; Paulsen *et al.*, 2007).

Plasma spraying can be carried out under a variety of conditions, for instance, in the air or under vacuum or at a very low pressure.

In addition, it can be performed using combustion to achieve high velocity at low temperature. In the present study, we synthesizing bioactive glass and silver doped bioactive glass and it was coated by using plasma spray method on medical graded 316L stainless steel used as a substrate.

1.8 VARIOUS IONS DOPED BIOACTIVE GLASSES

Several ions including Silver (Ag), Strontium (Sr) manganese (Mn), zinc (Zn), Magnesium (Mg) and copper (Cu).

1.8.1 Ag-BG

Silver is recognised as a promising metal element for inclusion with BGs to create BG implants and scaffolds for tissue engineering due to its exceptional antibacterial capabilities. Antibacterial activity of an Ag-doped BG has been demonstrated. The porous structure promotes silver ion release and germ suppression. Mesoporous BGs with silver have better antibacterial characteristics. Silver-doped BG is a promising dental material since dental implants are constantly exposed to microorganisms. Silver-doped BG/mesoporous silica composite particles have excellent antibacterial activity, and the rate of dentinal tubule occlusion is high (Xing Quin *et al.*,2018).

Because of silver's antibacterial qualities, there has been a new emphasis on the creation of silver-doped implants. Several researchers have examined the antibacterial characteristics of bioactive glasses containing silver. The fundamental advantage of integrating silver ions in a gel-glass system is that the porous glass matrix allows for regulated, sustained antibacterial agent delivery. Some researchers discovered that high concentrations of silver ions (2 wt%) cause cytotoxicity, although silver concentrations of 0.75–1 wt% had no hazardous effect. Silver's increased effectiveness has piqued the curiosity of scientists, and following substantial investigation, many theories for its antibacterial activity have been proposed: Electron transport interface Interaction, cell components and DNA binding (Luo *et al.*,2010).

1.8.2 Mg-BG

Magnesium (Mg) is a mineral that is required by the bone matrix. Magnesium can also help with wound healing, bone metabolism, fracture prevention, and bone density. Mg can operate as a network former or network modifier when doped. This suggests that increasing Mg concentration may result in more Mg^{2+} ions participating in the silica network by forming weaker Si-O-Mg bonds than stronger Si-O-Si bonds, causing the total glass network to deteriorate. Glass breakdown slows as MgO concentration increases, and the creation of an apatite layer is impeded (Ma *et al.*, 2011).

The surface reactivity of Mg-BG rises as the MgO/CaO ratio increases, which may play a key role in glass bioactivity. Another study discovered that the role of Mg^{2+} in the production of the HCA apatite layer in the $\text{SiO}_2\text{-CaO-Na}_2\text{O-P}_2\text{O}_5$ system was minimal. These contradicting data spawned a slew of ideas based on ionic potential, structural structure, or network connection. Varanasi *et al.* found that MgO had a substantial influence on osteoblast (Varanasi *et al.*, 2009). Other investigations have found that greater osteoblast proliferation and differentiation are beneficial. These results demonstrated the beneficial effect of magnesium doping on the bioactivity of bioactive glass.

1.8.3 Zn-BG

MBGs (mesoporous bioactive glasses) with zinc enrichment are antibacterial and osteogenic bioceramics. Furthermore, by regulating the expression of alkaline phosphatase (ALP), osteopontin, and osteocalcin, Zn^{2+} stimulates the differentiation of osteoprogenitor cells towards the mature osteoblast phenotype, indicating that this ion has potential osteogenic activity, making it very interesting for bone regeneration purposes. Thus, Zn^{2+} ions may stimulate osteogenesis by stimulating osteoprogenitor differentiation while limiting it by reducing HA formation on the glass surface, resulting in osteoprogenitor cell adhesion to the substrate (SánchezSalcedo *et al.*, 2014).

Balamurugan et al. used the sol-gel technique to create a bioactive glass in the CaO-P₂O₅-SiO₂-ZnO system that boosted ALP activity and osteoblast proliferation. They also investigated if the addition of zinc reduced the bioactivity of the bioactive glass. When submerged in SBF solution, the higher surface area of Zn-substituted glass can be a superior nucleation site, resulting in a more crystalline calcium phosphate phase (Balamurugan *et al.*,2007). Atkinson *et al.* recently discovered that up to 5 mol% zinc substitution in a sodium-free bioactive glass composition may cause apatite production alongside a calcite phase. Increased Zn concentration tends to diminish the calcite phase but has little effect on apatite deposition. This calcite phase can also form bonds with bone without forming an appetite layer (Atkinson *et al.*,2016).

1.8.4 Cu-BG

MBG with Cu has increased antibacterial action as well as angiogenesis potential. It has strong bioactivity and may create hydroxyapatite layers on surfaces (Xiaoju Wang *et al.*,2016).

1.8.5 Sr-BG

It increases osseointegration and antimicrobial efficacy. Strontium-containing bioactive glasses (BGs) are promising materials for addressing this issue. Because of Sr's beneficial effects on osteogenesis, it is commonly used in the therapeutic field in the form of strontium ranelate (SR), particularly in patients with osteoporosis. Strontium-based bioactive glasses have been shown to improve osteoblast metabolic activity while decreasing osteoclast activity. The reduction in osteoclasts might be induced by a decrease in tartrate-resistant acid phosphate activity and an inhibition of calcium phosphate film resorption (Gentleman *et al.*,2010). The incorporation of mesoporosity in bioactive glass was discovered to improve bone formation, disintegration, and drug transport capabilities. As a result, there is increased interest in ion-doped mesoporous bioactive glasses and their characteristics. Zhang *et al.* discovered that Sr-MBG has excellent mechanical stability in comparison to its original counterpart, which is necessary for bone healing. They also discovered that the Sr-doped MBG has a high apatite formation capacity (Zhang *et al.*,2014).

1.9 PROPERTIES OF BIOACTIVE GLASS

Bioactivity is an important feature that promotes osseointegration for bonding and implant stability. Bioglass coatings have a large surface area and are highly reactive, resulting in an effective interaction between the coating material and the underlying bone tissues. The production of a layer of carbonated hydroxyapatite (CHA) in the biological environment promotes attachment to bone tissues (Jones *et al.*, 2013).

The bioglass surface coating is subjected to ion leaching and exchange, glass dissolution, and the creation of the HA layer, which enhances tissue biological response. The increased specific surface area of bioactive glasses is believed to increase material solubility, ion availability in the surrounding area, and protein adsorption capacity. All of these elements contribute to the bioactivity of bioglass coatings (Mačković *et al.*, 2012).

Bioactive materials can be either osteoconductive or osteoinductive. The bioactive properties are determined by the structure and rate of ionic dissolution. A pH rise in bioglass 45S5 produced by excessive Ca^{2+} and Na^{+} leakage might cause cytotoxicity and delay the development of hydroxyapatite. Various studies have been conducted to investigate its effects on various cell types such as fibroblasts, cementoblasts, and osteoblasts. Bioactive glass has the ability to stimulate fibroblast proliferation and migration, increase extracellular matrix protein production, enhance osteoblast growth, and improve bone matrix mineralization. It can also interact with immunological cells like macrophages and lymphocytes, causing them to activate and produce cytokines. These effects are governed by the cell type and growth conditions, as well as the composition and structure of the glass (Muhammad saad shalik *et al.*, 2023).

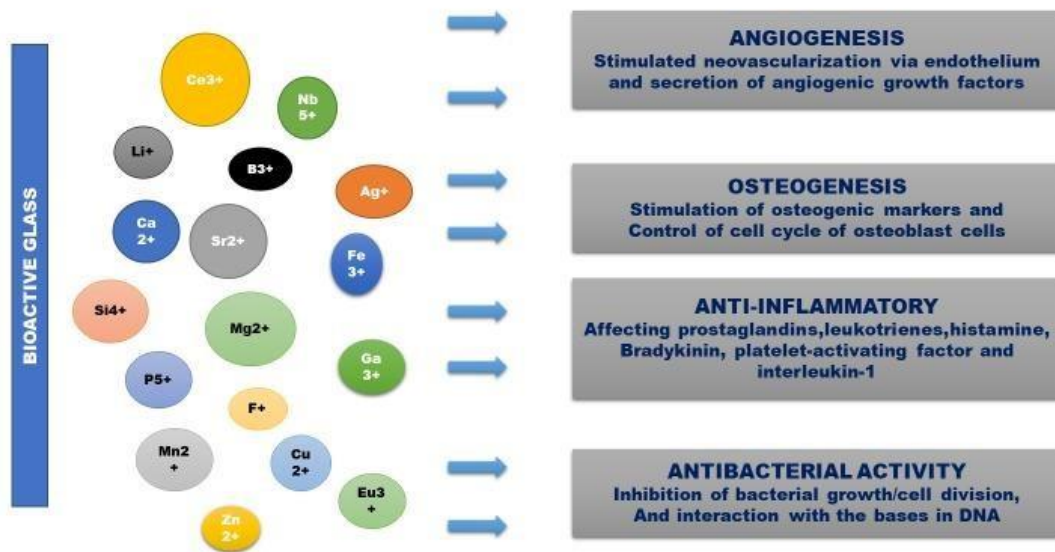


Fig 1.1 Bioactive glass with their properties

1.10 APPLICATIONS OF BIOACTIVE GLASS

- Coating materials
- Scaffolds
- Tissue Engineering
- Wound Dressing
- Immobilised Enzymes
- Drug Delivery

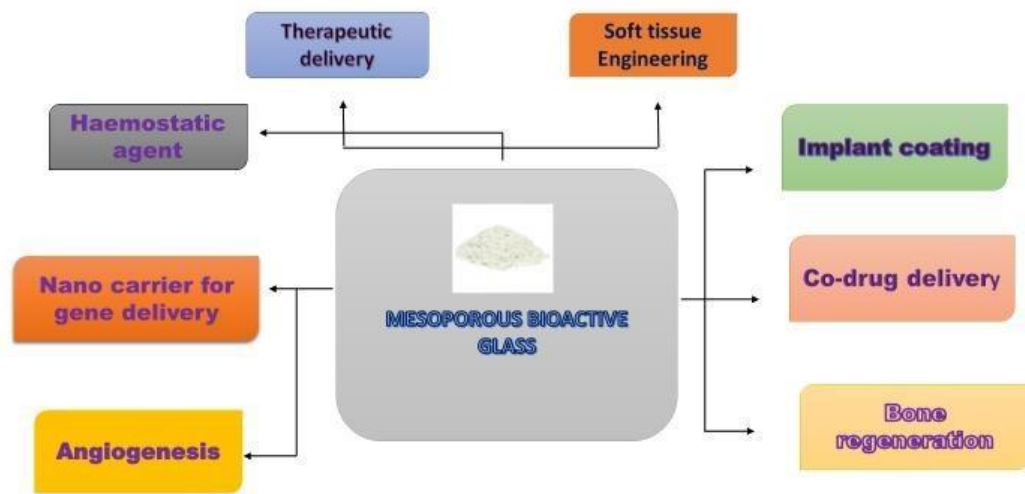


Fig 1.2 Bioactive glasses in different applications

CHAPTER-2

LITERATURE SURVEY

Nicola Gargiulo et al.,2013 synthesized the silver containing mesoporous bioactive glass by evaporation induced self-assembly method. The main aim of this work is to verify the actual bioactivity and the antibacterial properties of a silver-containing MBG. Ag-MBG showed a very good antibacterial effect against *S. aureus* strain, with strong evidence of bacterial activity already registered as 0.5mg/ml. It shows better bioactivity.

Ranga et al.,2019 concerned adding strontium along with silver into the glass matrix because of various characteristic of the metal ions in the bioglass and to enhance its bioactivity and antimicrobial properties. The introduction of silver into the prepared glass matrix shows excellent antimicrobial effects and the addition of strontium to the glass composition plays a beneficial role in orthopedic coatings and osteoporosis treatment. These materials are especially promising because of its biological effects and its bioactivity in SBF. Bioactivity was investigated by in-vitro synthesis involving immersion of the samples in SBF for different time intervals. The XRD, FTIR and FE-SEM results confirmed the formation of a hydroxylapatite layer on the surface of the finally prepared bioglass materials. An antimicrobial study revealed that the prepared bioactive glass showed a significant effect on two bacteria *E. coli* and *S. aureus*.

Wilkinson et al.,2018 tested the antimicrobial efficacy of a novel BG incorporating silver, against both planktonic and biofilm forms of the wound-relevant bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Ag-BG was stable, long lasting, and potently effective against planktonic bacteria in time-kill assays (6log reduction in bacterial viability within 2 h) and in agar diffusion assays. AgBG reduced bacterial load in a physiologically relevant *ex vivo* porcine wound biofilm model; *P. aeruginosa* (2-log reduction) and *S. aureus* (3-log reduction). AgBG also conferred strong effects against *P. aeruginosa* biofilm virulence, reducing

protease activity and virulence gene expression. Co-culture biofilms appeared more resistant to Ag-BG, where a selective reduction in *S. aureus* was observed. Finally, Ag -BG was shown to benefit the host response to biofilm infection, directly reducing host tissue cell death. Taken together, the findings provide evidence that AgBG elicits potent antimicrobial effects against planktonic and single-species biofilms, with beneficial effects on the host tissue response.

Ching Wang et al.,2020 synthesized a silver- containing MBG and it was coated by spin coating on the surfaces of titanium to improve their surface bioactivity and antibacterial activity. The surface morphology and their effects on the bioactivity and antibacterial activity of the coating were investigated. The invitro bioactivity test showed MBG-Ag coated Ti to have good mineralization ability and biocompatibility. It exhibited significant antibacterial activity against both aggregatibacter actinomycetemcomitans and streptococcus mutans. The wettability and adhesion strength of the films were evaluated using contact angle measurements and adhesion strength tests, respectively. The phase composition, chemical bonding, morphology, and oxidation states of the films were analyzed via X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS), and X-ray photoelectron spectroscopy (XPS). In vitro bioactivity analysis of the films was performed by immersion in a simulated body fluid (SBF) for 24hrs. A silvercontaining mesoporous bioactive glass film with excellent biocompatibility and antibacterial properties was successfully prepared.

Naghib et al.,2012 investigated the 45S5 bioactive glass was produced and coated onto a 316L stainless steel substrate. It increases the bioactivity and provided high mechanical strength. The surface morphology of coated specimens were investigated using SEM. Then the coated specime were immersed in stimulated body fluid (SBF) for 14 days at 37^o C. All the coated specimens were analysed by FTIR and XRD for formation of hydroxyapatite layer.

Natalic pajaras et al.,2021 developed a sol-gel (solution-gelation) derived bioactive glass doped with silver ions (Ag-BG), tailored to provide non-cytotoxic antibacterial activity while significantly enhancing osteoblast-lineage cell growth in vitro and bone regeneration in vivo. We observed that Ag-BG inhibits bacterial growth and potentiates the efficacy of conventional antibiotic treatment. Ag-BG microparticles enhance cell proliferation and osteogenic differentiation in human bone marrow stromal cells (hBMSC) in vitro. Moreover, in vivo tests using a calvarial defect model in mice demonstrated that Ag-BG microparticles induce bone regeneration. This novel system with dual biological and advanced antibacterial properties is a promising therapeutic for combating resistant bacteria while triggering new bone formation.

Wren et al., 2012 developed the silver containing bioactive glass coating to determine the antimicrobial properties compared to the coated and uncoated particles. Low Ag concentration can encourage cell proliferation of human cell lines. Surface area analysis determined that Ag coating of the glass particles resulted in increased the surface area from 2.90 to 9.12 m²/g (90 µm) and 1.09-7.71 m²/g (425-850 µm). Scanning electron microscopy determined that the Ag coating remained at the surface and there was little diffusion through the bulk. Antibacterial (*Escherichia coli*--13 mm and *Staphylococcus epidermidis*--12 mm) and antifungal testing (*Candida albicans*--7.7 mm) determined that small Ag-coated glass particles exhibited the largest inhibition zones compared to uncoated particles. pH analysis determined an overall higher pH consider in the smaller particles, where after 24 h the large uncoated and Ag coated particles were 8.27 and 8.74 respectively, while the smaller uncoated and Ag coated particles attained pH values of 9.63 and 9.35 respectively.

Mehdipour et al.,2012 demonstrated that bioactive glass (BG) particles were synthesized by a sol–gel process and were then coated on a 316L stainless steel substrate using an electrophoretic deposition (EPD) technique. Thickness, structure and electrochemical behavior of the coating were characterized.

SEM studies showed that increasing EPD voltage leads to a coating with more agglomerated particles, augmented porosity and micro cracks. The results of Fourier transformed infrared (FTIR) spectroscopy revealed the adsorption of TEA via methyl and amid groups on bioactive glass particles. Presence of bioactive glass coating reduced corrosion current density (i_{corr}) and shifted corrosion potential (E_{corr}) toward more noble values in artificial saliva at room temperature. Percent porosity of the coating measured by potential dynamic polarization technique increased as EPD voltage was raised. The results of impedance spectroscopic studies demonstrated that the coating acts as a barrier layer in artificial saliva.

Singh et al.,2022 synthesized a new family of bioactive glass and coated on a 316L stainless steel. It showed better corrosion resistance in SBF. *In vitro* test by soaking the BAG in simulated body fluid (SBF) showed good bioactivity due to ion exchange between alkali ions from the BAG and the ions present in the SBF. The 316L SS substrates were textured using laser before application of BAG using sol-gel dip coating. Pull off test showed that the adhesion_strength in case of textured and nontextured surfaces were 4.2 MPa and 2.46 MPa, respectively. This implies that texturing of the surface is helpful in increasing the adhesion strength of the coating. The analysis of scanning electron microscopy (SEM) images of the coating after immersion in SBF shows the formation and growth of the apatite layer that will further inhibit the corrosion of the substrate. These results demonstrate the potential of the synthesized BAG as a coating material for 316L SS implants.

Xuan Vuong Bui et al.,2017 synthesized the bioglass 45S doped with Ag (45S-Ag). The influence of doping Ag on the glass matrix was highlighted by DTA and XRD methods. The presence of Ag element was controlled by EDX analysis. “In vitro” of synthesized glass was effectuated by soaking of glass powder in SBF solution. EDX result indicated that silver was released when immersing derivative bioglass in SBF solution and silver is an antibacterial agent. XRD and SEM confirmed the bioactivity of glass 45S-Ag by the apatite formation on its surface.

Edathazhe et al.,2018 investigated the phosphate bioactive glass was coated on biomedical implant materials such as stainless steel 316 L, duplex stainless steel (DSS) 2205 and Ti6Al4V alloy by thermal enamelling method. The structural properties and composition of glass coated substrates were studied by XRD, SEM and EDS analysis. The coatings were partially crystalline in nature with porous structure and pore size in nanometre. The corrosion resistance of 316 L substrate increased after coating, whereas it decreased in case of DSS 2205 and Ti6Al4V. The XRD and SEM/EDS studies indicated the bioactive hydroxyapatite (HAp) layer formation on all the coated surfaces after electrochemical corrosion test, which improved the corrosion resistance. The observed electrochemical corrosion behavior can be explained based on protective HAp layer formation, composition and diffusion of ions on glass coated surfaces. The *in-vitro* bioactivity test was carried out at 37 °C in HBS solution for 14 days under static conditions for uncoated and coated substrates. pH and ion release rate measurements from the coated samples were conducted to substantiate the electrochemical corrosion test. The lower ion release rates of Na⁺ and Ca²⁺ from coated 316 L supported its higher electrochemical corrosion resistance among coated samples. Among the uncoated substrates, DSS showed higher electrochemical corrosion resistance. Amorphous calcium-phosphate (ACP) layer formation on all the coated substrates after *invitro* bioactivity test was confirmed by XRD, SEM/EDS and ion release measurements.

Thet Swe et al.,2022 investigated the silver doped bioactive glass were synthesized by the sol-gel method. The structural properties of synthesized bioglass were characterized using X-ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR). XRD results showed that after Ag was doped into the bioglass formulation, the phase transformation from amorphous phase to crystalline phase was detected. However, there is not much difference in FTIR spectra for all synthesized samples (with and without doped Ag₂O). Therefore, in this study, it can be concluded that Ag₂O doping does not affect the functional groups of bioglass.

Halin Zhu et al.,2014 developed mesoporous 58S bioglass (SM58S) was prepared through surface modification of the mesoporous 58S bioglass (M58S) with

γ -aminopropyl triethoxysilane (KH550). The results of Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA) showed that the amino groups were grafted to the surface of M58S after modification with KH550. The silver-containing SM58S (Ag-SM58S) and M58S (Ag-M58S) were prepared by the dipping method. The Ag⁺ loading capacity, release rate and antibacterial properties of Ag-SM58S and Ag-M58S were investigated. It is indicated that surface modification of M58S with KH550 can improve the Ag⁺ loading capacity. The result of antibacterial property showed that Ag-SM58S exhibited significant anti-bacterial effects against Escherichia coli and Staphylococcus aureus. The sustained release of Ag⁺ from Ag-SM58S for 768 h ensured excellent antibacterial property of AgSM58S. In vitro osteoblast proliferation and differentiation tests showed that AgSM58S was a good matrix for the growth of osteoblasts. Consequently, the results of the study suggested that Ag-SM58S might be a promising bone repair material.

Rabiee et al.,2012 investigated the effects of zirconia on microstructure of bioactive glass coating. Coating without zirconia was obtained different grain size. In case of with zirconia, the microstructure of bioactive glass coating. Coating were performed by sol-gel method. Coating without zirconia was obtained different micro structure are composed of nanophase. It seems that an activation barrier on the growing of nanoplate excites.

Balamurugan et al.,2006 investigated the Bioactive glass was successfully synthesized by sol-gel method. FT-IR analysis confirms the absorbance of SiO group. The biocompatibility analysis confirms the growth of HAP on the surface of the bioglass particles.

Ehsan Vafa et al.,2021 investigated two different type of 45S5 bioactive glassceramic were compared. Both of them were prepared using the sol-gel method, with two different catalysts: 2 M nitric acid (NACBG) and 10 mM vinegar. Homemade vinegar (HMOV) extracted from apples which contained up to 5% acetic acid, as an affordable and low concentration catalyst, was used for the synthesis of 45S5 bioactive glass

ceramic called Homemade Vinegar Catalyzed Bioactive Glass (HMVCBG). The effect of these two catalysts on the structure, morphology, and properties of the bioactive glass ceramics were investigated. SEM with EDXS and BET were used to assess surface characteristics and specific surface area, which showed more specific surface area in HMVCBG. Crystallinity and composition were analyzed by XRD, XRF and FTIR which showed some crystalline phases in BG. In-vitro bioactivity was studied in Phosphate Buffer Saline and SEM results showed that HMVCBG has a more uniform hydroxyapatite layer and formation occurs more quickly. HMV can form more numerous porosities and a more specific surface area because of higher pH of acetic acid. Hence, PBS solution penetrates to the depth of HMVCBG and hydroxyapatite nucleation form in more spots in the depth of HMVCBG. Thus, HMVCBG has a higher bioactivity level than NACBG.

Newman *et al.*,2014 developed the strontium-substituted bioactive glass (SrBG) implant coating. The coating can be successfully applied to Ti6Al4V and after implantation into the distal femur and proximal tibia of twenty-seven New Zealand White rabbits for 6, 12, or 24 weeks, it produced no adverse tissue reaction. The glass dissolved over a 6week period, stimulating enhanced peri-implant bone formation compared with matched HA coated implants in the contralateral limb. Furthermore, superior mechanical fixation was evident in the SrBG group after 24 weeks of implantation.

Pouraghaei *et al.*,2016 synthesized Silver doped-bioactive glass and was coated on titanium substrate using dip coating method. Scanning electron microscopy (SEM) depicted a crack-free and homogeneous coating. The energy dispersive Xray spectroscopy corresponding to the SEM image affirmed the presence of Si, P, Ca and Ag as main elements of Ag-BG coating. Contact angle measurement confirmed positive effect of silver on the wettability and adhesion of Ag-BG on the Ti surface. X-ray Diffraction analysis confirmed the amorphous nature of Ag-BG. Fourier transform infrared spectroscopy confirmed successful inclusion of silver in glass structure. X-ray Fluorescence Spectroscopy demonstrated that composition of Ag-BG was very

close to the designed composition. Results of acellular in vitro bioactivity test showed formation of ball-like particles including a layer of entangled rough apatite crystals on the surface.

Durgalakshmi Dhinasekaran *et al.*,2021 investigated the bioactive glass and hydroxyapatite coatings on titanium by pulsed laser deposition method. Raman and IR spectroscopic techniques based on silica and phosphate functional groups mapping have confirmed homogeneity in coatings by pulse laser deposition method. Comparative studies on nanostructured bioactive glass and hydroxyapatite on titanium surface elaborated the significance of bioactivity, hemocompatibility, and cytocompatibility of the coated surface. Notably, both hydroxyapatite and bioactive glass show good hemocompatibility in powder form. Hemocompatibility and cytocompatibility results validate the enhanced sustenance for hydroxyapatite.

2.1DIFFERENT COATING TECHNIQUES

COATING MATERIAL	SUBSTRATE	TECHNIQUE	COATING CHARACTERISTICS	REF
45S5	Pure Titanium	APS	The bonding strength of BG + bond coat averages 27.18 2.24 MPa, and the bonding strength of BG averages 8.56 0.57 MPa.	Goller G., (2004)

45S5	AISI 304	Solution precursor plasma spraying (SPPS)	Average thickness of uniform coating is 35 m.	Cañas <i>et al.</i> , (2018)
BG-Ca/HA	316 L Stainless Steel	SPS	Coatings that are compact and have a consistent thickness with little pore presence	Cattini <i>et al.</i> , (2013)
BG_Ca glass	Ti6Al4V	SPS	Coatings have a continuous and homogenous thickness of 31–40 m, a hardness of 34–98 HV, an elastic modulus of 16–23 GPa, and a critical load of 18–21 N.	Cattini <i>et al.</i> , (2013)
BG-Ca/Mix	Grade2 Titanium	HVSFS and suspension plasma spraying (SPS)	The HVSFS coating is extremely dense and thin. The hardness is 396–516 HV, and the	Bolelli G <i>et al.</i> , (2015)

			elastic modulus is 61–95 GPa. The thickness ranges from 20 to 50 mm. SPS coatings with a thickness of 50 m	
Ag-BG	Titanium	Sol–gel method	homogeneous and free of large- and small-scale fractures	Pouraghaei <i>et al.</i> , (2016)
Bioglass®	NiTi Alloy	EPD	homogeneous microstructure with uniform topography and no fractures or holes 5–15 m in thickness	Boccaccini <i>et al.</i> , (2006)
Biovetro®	Ti6Al4V	Atmospheric plasma spraying (APS)	wide superficial region of spherical grains on a surface containing microcavities	Gabbi, Cacchioli (1995)

Table 2.1 DIFFERENT COATING TECHNIQUES

2.2 PROPERTIES

2.2.1 MECHANICAL PROPERTIES

Ideally, bioactive implants for clinical applications should match the mechanical properties of the host tissue and exhibit strong interfacial bonds with hard and/or soft tissues (Minardi *et al.*,2015). Mechanical properties of bioactive glasses, which possess physical characteristics relatively close to 'hard' bone tissue, great attention has been focused on using these biomaterials in contact with bone and teeth (Gurbinder Kaur *et al.*,2019).

Glass-ceramics possess crystalline phases embedded in a residual amorphous matrix. The crystalline phases enhance the strength of glass-ceramics and give them higher fracture toughness when compared to the parent glass. In this regard, crack bridging and crack deflection were determined to be the most potent toughening mechanisms (Apel *et al.*,2008). The glass-ceramic Cerabone® AW (34SiO₂–16.2P₂O₅–44.7CaO–0.5CaF₂–4.6MgO wt%) (Hench *et al.*,2015) has higher mechanical properties than 45S5 Bioglass and hydroxyapatite HA. Therefore, apatite-wollastonite (AW) glass-ceramic is used for vertebral replacements where significant compressive strength is required (Gurbinder Kaur *et al.*,2019).

The major concern when using partially crystallized glasses is the corresponding decrease in bioactivity, as ion-exchange phenomena at the interface between implant surface and biological fluids are mainly related to the residual glassy phase. Hench and co-workers found that 40% crystallinity did not affect the bioactivity of partially crystallized 45S5 Bioglass®. However, the formation rate for an apatite layer at a surface is slowed down if crystallinity reaches 100%, although it is not totally suppressed (Gurbinder Kaur *et al.*,2019). Kokubo and co-workers reported that AW glass-ceramics did not form a HA layer when immersed in Tris-buffer solution. However, the formation of a polycrystalline HA layer occurred on their surface upon soaking in the simulated body fluid (SBF) (Gurbinder Kaur *et al.*,2019).

NiO doped bioactive glass-ceramics were prepared by melting and quenching. NiO concentration was varied between 0.4 and 1.65 mol%. Increasing the NiO content flexural strength (three-point bending) increased from 106 MPa to 120 MPa. Similarly, compressive strength increased from 112 MPa to 133 MPa and Knoop microhardness values increased from 7.65 to 8.15 (GPa). The observed increase of mechanical properties is due to formation of Ni–O–Si bonds in the silicate network (glass phase structure) and crystallization of sodium calcium silicate phases (ceramic phase structure). NiO doped bioactive glass-ceramics were confirmed to be bioactive and non-cytotoxic for rabbit osteoblast bone cells (Vyas *et al.*, 2016).

2.2.2 ANTICORROSION PROPERTY

The corrosion resistance of metal alloys plays a significant role in orthopedic applications. Generally, the implant corrosion activity is an important aspect for assessing the functionality and material biocompatibility in *in vitro* and *in vivo*. It shows the polarization curves for uncoated and coated samples and their I_{corr} and E_{corr} values. The corrosion parameters values provide a more reliable measure of biomaterial capability against corrosion. A lower I_{corr} value implies more resistance to corrosion, and a nobler E_{corr} value reveals higher corrosion protection (Poorraeisi *et al.*, 2018).

Singh *et al.*, showed that there were noticeable variations between the corrosion behaviors of samples. The Ti–13Nb–13Zr alloy attained the least E_{corr} value. The sequences of E_{corr} value for different coatings were $Fe_3O_4 < BG < BG-F5 < BG-F3 < BG-F1$. The E_{corr} value for BG and Fe_3O_4 coating depicts an improvement in resistance to corrosion and for BG-F1 coating; the E_{corr} value demonstrates that this coating has the highest corrosion protection than all the coatings. The Fe_3O_4 coating showed a less I_{corr} value than Ti–13Nb–13Zr alloy but more than the BG coating. Reportedly, the BG–CS coating enhanced the corrosion ability of the bare Ti–13Nb–13Zr alloy (Singh *et al.*, 2020).

2.3 BIOACTIVITY PROPERTY

Bioactivity is the property of interacting with a tissue to drive its repair (Margaux *et al.*, 2021).

It is commonly evaluated by an index, I_B , linked to the parameter, $t_{1/2}$, which is half the time necessary for the glass surface to be covered with cells. $I_B = 100t_{1/2}$. In 38% of MBG whose composition was indicated in the articles, the proportion of SiO_2 was greater than or equal to 80% mol. This is explained by the increase in the surface area, pore volume, and pore size when the SiO_2 content increases (Wu *et al.*, 2012). The high concentration of SiO_2 plays a key role in bone metabolism and collagen synthesis. The most frequent composition for MBG comprised $\text{SiO}_2:\text{CaO}:\text{P}_2\text{O}_5$. The porosity increased when calcium oxide increased, compared with sodium oxide (Fernando *et al.*, 2018). The compositions with high sodium interfered with the textural features by reducing the porosity because of the fusion of pores. Porosity has a significant role at the interface with cellular membranes and could enhance bioactivity as a result of enhanced surface area (Baino *et al.*, 2016). Moreover, glasses with CaO , P_2O_5 , and SiO_2 in their composition exhibit preferential layer formation on their surface involving the Si-OH group formation including a heterogeneous nucleation of apatite (Kaur *et al.*, 2016).

2.4 BIOCOMPATIBILITY ASSESSMENT

Biocompatibility in vitro ranks as one of the most important properties to investigate with regards to the behavior of biomaterials, such as MBG. The articles investigated in this review clearly show the importance of validating the biocompatibility of these materials (Margaux *et al.*, 2021).

2.4.1 HEMOCOMPATABILITY ASSAY

The hemocompatibility of the samples was evaluated with human blood to examine their hemolysis behavior. According to ISO 10993-5 standards, the range of hemolytic, less than 5%, is considered as a safe value for implant materials (Zhang *et al.*, 2009). The % hemolysis for different samples. Singh *et al.*, investigated it can be detected that the hemolysis rate of uncoated Ti-13Nb-13Zr alloy was significantly higher as compared to the coated samples. The Fe_3O_4 -coated sample showed a less hemolysis rate than the uncoated Ti-13Nb-13Zr alloy but more than the BG-coated sample. The hemolytic rate of BG- and Fe_3O_4 -coated samples indicates minor contamination of erythrocyte membranes due to scrambling and

shrinkage which leads to the discharge of intracellular hemoglobin. The composite coatings revealed the finest hemocompatibility with the lowest percentage of hemolytic values (3.3–3.9). The BG-F3 and BG-F5 samples depicted slightly hemolytic than the BG-F1 sample due to the penetration of Fe_3O_4 particles into the RBC cell membrane. It has been reported that various factors such as surface energy, electrical charge, and topography of the material play a substantial role in generating adverse influences on erythrocytes which produce hemolysis during the in vitro examination (Henkelman *et al.*, 2009). The RBCs morphology after exposure to the samples was shown. Several hemolytic RBCs were detected on the Ti–13Nb–13Zr alloy surface. The number of hemolytic RBCs was reduced for BG and Fe_3O_4 coatings, whereas all the erythrocytes almost interacted with composite coatings possess their original (approximately round) morphology. The results of blood compatibility assay suggest that the fabricated coatings can successfully prevent the RBCs hemolysis and act as a suitable material in biomedical applications (Singh *et al.*, 2020).

2.4.2 CYTOCOMPATIBILITY ASSESSMENT

For the MBG cytocompatibility assessment, most of the investigated studies used different immortalized cell lines (about 75% of the studies) such as MG-63 osteoblasts like cells (Wu *et al.*, 2019). The osteoblastic phenotype of MG-63 cells is particularly interesting for the study of bone regeneration (Liu *et al.*, 2019). Some studies have revealed that there are differences between established immortalized cell lines and primary cells in cellular response to the biomaterials in vitro (Alamouch *et al.*, 2020). Although established cell lines have the advantage of being immortal, available worldwide, and easy to grow, they remain distant from target cells and therefore do not allow the study of necessary markers (such as markers of bone remineralization). Primary cell cultures, on the other hand, are cells with a limited lifespan (because they differentiate after a few multiplications), but they are closest to the target tissues. This considerable advantage from primary cell lines makes it possible to study markers which cannot be studied with established cell lines, and to study bone remineralization in a more in-depth and relevant manner (Margaux *et al.*, 2021).

2.5 ANTIMICROBIAL PROPERTY

Bioactive glass is active against a variety of therapeutically significant bacterial strains, including gram-positive and gram-negative strains, as well as aerobic and anaerobic strains (Narender *et al.*, 2019). Narender *et al.*, who demonstrated that samples calcined at 600°C for 3 h were used for the antimicrobial study. The antibacterial properties of the samples were examined with respect to gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria. The agar disc diffusion method was used to check the antibacterial activity of the prepared bioactive glass. The discs were cleaned with double distilled water two to three times and soaked in a bioactive glass medium overnight for better absorption before being placed on the culture plates. The same experiment was performed in triplet to assume the accuracy of the results. The culture plates were incubated at 37°C for 24 h.

After 24 h of incubation, the zone of inhibition was measured for different samples. It was observed from the antimicrobial results that the silver-free samples had no zone of inhibition with respect to either of the bacteria but that as the silver content increased in the glass matrix, a significant zone of inhibition was seen with respect to both bacteria. The details of the zone of inhibition are given. Consequently, the experiment revealed that the antibacterial effect of the glass matrix can be tuned with very small amounts of silver. In future, therefore, there will be scope to use this glass matrix for various biomedical applications because of its biocompatibility, costeffectiveness and antibacterial effect (Narender *et al.*,2019).

Like Hu *et al.*, who investigated the potential of Ag-doped SiO₂-CaO-P₂O₅-Ag₂O silicate bioactive glass with nanoporosity (pore size of 6 nm) and high surface area (467 m² g⁻¹) prepared via the sol-gel method for wound healing applications, the bacterial growth was reduced to 0.01% of that of the control culture solution above this concentration. While the antibacterial rate reached 75% in one hour and 99% in twelve hours, the produced silicate bioactive glass with 0.02 wt% Ag displayed good antibacterial characteristics against Escherichia coli without harmful effect. The high surface area of these silicate glasses resulted in an extraordinary hemostatic performance, and they also successfully stimulated blood coagulation and achieved bleeding control in the animal model. (Hu *et al.*,2012)

CHAPTER-3

AIM

Our Aim of the project is to develop a silver containing bioactive glass coated onto 316L stainless steel substrate and analysis its mechanical strength and biological study.

OBJECTIVE

Its characteristics are surface morphology, Crystallinity were analysed using XRD. Wettability analysed using goniometer. Its properties are antimicrobial, anticorrosion, biocompatibility, bioactivity, hemocompatibility, cytotoxicity.

CHAPTER-4

MATERIALS AND METHODS

4.1 MATERIALS REQUIRED

Tetraethyl orthosilicate, triethyl phosphate, calcium nitrate, sodium nitrate, silver nitrate, nitric acid, distilled water.

4.2 SYNTHESIS METHIOD - SOL-GEL METHOD

4.2.1 *Synthesis of BG*

Dropwise additions of tetraethyl orthosilicate $\text{Si}(\text{OC}_2\text{H}_5)_4$ (TEOS) and triethyl phosphate $\text{PO}(\text{C}_2\text{H}_5)_3$ (TEP) to the solution (13.7 and 1.37 ml, respectively) were made and the solution was agitated for 30 minutes to get a clear solution. Water has a molar ratio of 12:1 to TEOS [Lin *et al.*,2009]. Next, in the agitated solution, sodium nitrate NaNO_3 powder (4.85 g) was gently added. Lastly, 7.75 g of calcium nitrate tetrahydrate $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ powder was added to the solution and agitated until it became translucent. As a result, during polycondensation processes, the solution began to change into a gel. The gel was then stored at 75 degrees Celsius for 12 hours before being dried at 200 and 700 degrees Celsius for 5 and 2 hours, respectively. According to Faure and colleagues [Faure *et al.*,2015], chemical bioactive glass-ceramic was created. After drying, the resultant powder was manually crushed to form a fine powder.

DRIED BIOACTIVE GLASS



Fig 4.1 Synthesized Bioactive Glass

4.2.2 Synthesis of Ag- BG

Dropwise additions of tetraethyl orthosilicate $\text{Si}(\text{OC}_2\text{H}_5)_4$ (TEOS) and triethyl phosphate $\text{PO}(\text{C}_2\text{H}_5)_3$ (TEP) to the solution (13.7 and 1.37 ml, respectively) were made and the solution was agitated for 30 minutes to get a clear solution. Water has a molar ratio of 12:1 to TEOS [Lin *et al.*,2009]. Next, in the agitated solution, sodium nitrate NaNO_3 powder (4.85 g) was gently added. Lastly, 7.75 g of calcium nitrate tetrahydrate $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ powder was added to the solution as well as add silver nitrate (AgNO_3) 0.169 g into the solution and agitated until it became translucent. As a result, during polycondensation processes, the solution began to change into a gel. The gel was then stored at 75 degrees Celsius for 12 hours before being dried at 200 and 700 degrees Celsius for 5 and 2 hours, respectively. According to Faure and colleagues [Faure *et al.*,2015], chemical bioactive glass-ceramic was created. After drying, the resultant powder was manually crushed to form a fine powder.

Ag-BG POWDER



Fig 4.2 Synthesized Ag-BG powder

4.3 COATING METHODS

4.3.1 Substrate preparation

Medical grade stainless steel substrates were used with 20×10×1mm² size as a substrate material. Before deposition, all substrates were polished using different grades of silicon carbide (SiC) sheets (600, 800, 1000, 2000 and 5000 grits) to obtain uniform roughness (Ra – 80 nm). After polishing, they were ultrasonically cleaned with acetone followed by distilled water for 15 min to remove surface residues. Finally, the substrates were sterilized under steam pressure (15 lbs for 15 min at 121 °C) and dried.

4.3.2 Coating preparation

The coating samples were prepared by Metco 7 M atmospheric plasma spray facility with 9 MB spray pistol and Metco 4MP-dual type feedstock system on stainless steel.

4.4 PLASMA SPRAY COATING METHOD

In the plasma spraying technique, bioactive materials such as BG powder is fed into the plasma that forces it to impinge onto the substrate in a molten state; thus, a layer of BG coating is achieved (Fig. 4.3). By using appropriate gases to create plasma and control the speed of plasma, the desired crystallinity of BG coatings is obtained. Plasma spraying can be carried out under a variety of conditions, for instance, in the air or under vacuum or at a very low pressure. In addition, it can be performed using combustion to achieve high velocity at low temperature.

The coating thickness ranges from 0.04 to 0.05mm, containing interconnected round pores of diameter (150–400µm) that approximate the pore size of bone (Lee *et al.*, 2012), hence improving the bone-implant interface

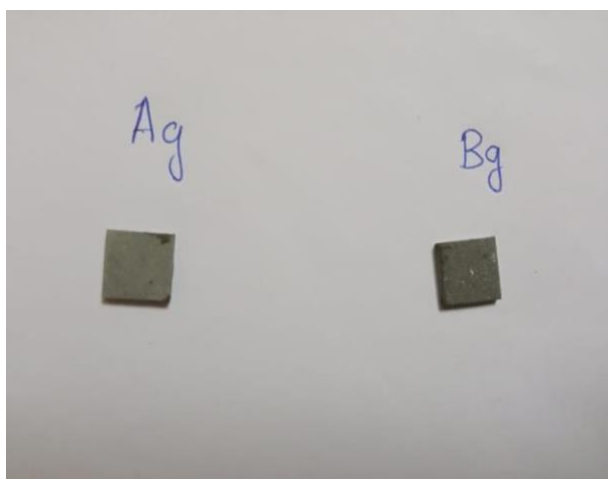


Fig 4.3 BG and Ag-BG powders coated on 316L SS

4.5 CHARACTERISTIC STUDY

4.5.1 XRD ANALYSIS

Bioactive glass and Ag-bioactive glass coated stainless steel substrates were characterized by X-ray diffraction (Rigaku Mini Flex 660 (9 kW) with a X-ray source of Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$)).

4.5.2 WETTABILITY ANALYSIS

An in-house built contact angle measuring instrument was used to measure the static contact angle of a droplet (deionized water of volume 20L) brought in contact with the thin film's surface.

A digital camera was used to capture a picture of a liquid droplet, and the enlarged image was processed using image J software to determine the acute precision of contact angle measurement.

4.6 IN-VITRO BIOLOGICAL STUDY

4.6.1 HEMOLYSIS TEST

The hemolysis test was carried out in accordance with ISO 10,993-4:2002. Initially, test samples were put in a 25 ml beaker according to the ISO 10,993-1 standard technique.

Then, 10 ml of saline was placed into a beaker holding test samples, followed by a 30-minute incubation at 37°C. The supernatant (saline solution) was then collected and centrifuged at 1500 rpm for 10 minutes. 8 ml of anticoagulant-containing whole blood was diluted with 10 ml of normal saline solution, from which 0.2 ml was removed and placed into materials extracting saline solution. As a negative and positive control, normal saline and distilled water were employed.

A UV-spectrophotometer was used to measure the solution at 545 nm. The following equation was used to calculate the rate of hemolysis (HR).

$$HR (\%) = \frac{AS - AN}{AP - AN} \times 100$$

AP - AN

Where AS denotes the absorbance of the test samples and AN and AP signify the negative and positive controls, respectively.

4.6.2 ANTIBACTERIAL ANALYSIS

4.6.2.1 BACTERIAL ADHESION STUDY

P. aeruginosa (MTCC-424) strain was obtained from Microbial Type Culture Collection and gene bank (MTCC), India. The lyophilized bacterial powder was cultured in nutrient broth at 37 °C for overnight incubation. After incubation, 0.1 ml of bacterial culture were added into a 200 ml conical flask containing nutrient broth. Finally, UV-sterilized test samples were immersed into the nutrient broth for 1, 2, 3 and 4 days at 37 °C in a shaking incubator. The incubated samples were gently washed with distilled water to remove the unbound cells on the samples. Then, acridine orange (AO) stain was added onto the samples and incubated for 10 min. After incubation, the samples were again gently washed and observed by epifluorescence microscopy (Nikon Eclipse E600, excitation filter BP 490; barrier filter 0515) to determine the proportion of viable bacteria.

4.6.3 IN-VITRO IMMERSION STUDY

The in vitro immersion studies were studied using Hanks's simulated body fluid: SBF (Hanks Balanced salt solution (HBSS, Sigma-Aldrich)) at 37 °C. Before immersion, the samples were kept under UV-C light irradiation for 15 min for sterilization. To understand the biocompatibility and biomineralization of the coated substrates, Plasma sprayed coated substrates of dimension 10x10mm were immersed in a 15 mL Eppendorf tube (Tarson, India) in a volume of 10 mL of Hank's SBF solution for 7 days. The surface of the coating is kept with the face to higher exposure to solution interaction. The procedure for immersion was followed as prescribed by Kokubo *et al.*, After 7 days, the immersed samples were dried overnight at 80 °C. Then the OD values are noted for each sample.

4.6.4 CYTOTOXICITY STUDY

4.6.4.1 CELL VIABILITY TEST AND MTT TEST

The indirect technique was used to evaluate the cell's cytotoxicity. The S2S fibroblast- cells were cultured in a 1% antibiotic mixture (300 mg/mL streptomycin and 5 mg/mL amphotericin B), 10% bovine serum Albumin (Gibco), and minimal essential medium (MEM, Gibco) in the tissue culture laboratory of the HKU Faculty of Dentistry. The incubation took place at 37 °C in 5% CO₂ that was humidified. In accordance with ISO standard 10993-5, three discs from each group were put into a 6-well plate with 5 mL of MEM. The samples were gathered. A sterile 0.22-μm syringe filter (Acrodisc, Pall) was used to filter two concentrations, 100% and 50% dilutions. In 96-well plates, S2S cells (4x10⁴ cells/mL) were planted and incubated for 24 hours.

The media was then taken away, 100 μL of each substance extract was added, and the cells were plated before being incubated for 24 hours.

The MTT test (Sigma-Aldrich) was used to determine the cytotoxicity of the cells. The 10 mg of MTT was mixed with 2 mL of PBS to create the MTT stock solution. 100 μL of MEM and 10 μL of MTT stock were added to each well after the extracts had been removed. The backdrop was three wells without cell seeding. The cells were treated for 4 hours at 37°C. Following that, 85 μL of the medium was taken out, 50 μL of dimethyl sulfoxide was added to each well, and incubation at 37 °C for 10 minutes was then performed. The experiments were run in duplicate, and the absorbance at 540 nm was recorded using a spectrophotometer (SpectraMax M2, Molecular Devices, San Jose, CA, USA).

CHAPTER-5

RESULT AND DISCUSSION

5.1 XRD ANALYSIS

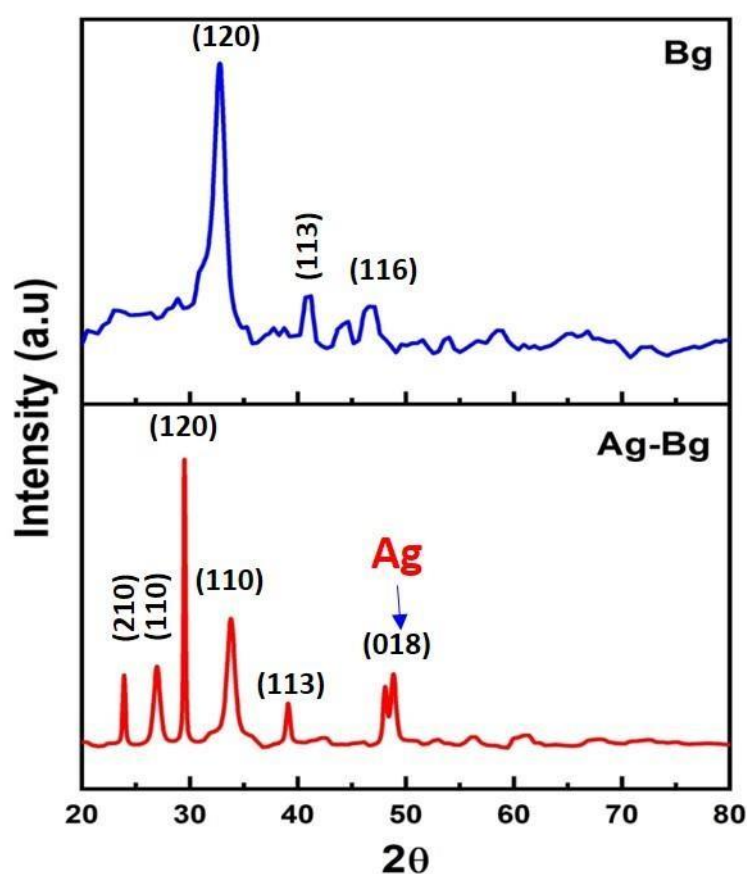


Fig 5.1 XRD analysis of Synthesized BG and Ag-BG.

The presence of crystallographic phases in the sintered samples of bioactive glass and Ag-BG was estimated from X-ray diffraction (XRD) patterns (Fig.5.1). The XRD pattern of sintered Ag-BG was well matched with the JCPDS # 09-04-32 standard of $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ crystal structure. The average crystallite size is calculated by the

Scherrer equation, $d = 0.9\lambda/(\beta\cos\theta)$, where d is the crystallite size in the direction perpendicular to the lattice planes, λ is the wavelength of the X-rays, β is the fullwidth at half-maximum of the X-ray diffraction peak in radians and θ is the Bragg angle. The crystallite size of bioactive glass and Ag-BG is calculated with their first major peaks and found to be range between ~20nm to ~18 nm. For Bioactive glass, all the major peaks were well matched with sodium calcium silicate crystal structure ($\text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9$) with the JCPDS# 75-1687. The presence of sodium-calcium silicate structure is the most expected phase in the bioactive glass 45S5 system as it contains a higher percentage of sodium and calcium next to silica. And we have chosen a sintering temperature of 700 °C with respect to our earlier work, where at this temperature there will be the formation of a prominent single phase of $\text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9$ crystal. The formed $\text{Na}_2\text{Ca}_2\text{Si}_3\text{O}_9$ crystal structure termed as combeite structure possesses good biocompatibility properties when present in the silica glass network.

5.2 WETTABILITY ANALYSIS

Wettability investigations revealed that BG and Ag-BG coatings had contact angles of 137 and 139, respectively, and are super hydrophobic. The super hydrophobic character of coated samples results from a decrease in the presence of metal ions on the coating's surface causes oxygen vacancies, resulting in a more hydrophobic character than on uncoated 316L SS substrates.



(a). BG



(b). Ag-BG

FIG 5.2 Wettability analysis of coated sample.

(a) BG shows super hydrophobic nature of contact angle 137. (b) Ag-BG shows super hydrophobic nature of contact angle 139.

5.3 IN-VITRO BIOLOGICAL STUDY

5.3.1 HEMOLYSIS TEST

(a)	(+)ve Control	(-)ve Control	Test	(+)ve Control	(-)ve Control	Test
Sterile Saline	1.25 mL	1.25 mL	1.25	1.25 mL	1.25 mL	1.25
Diluted Blood	20 μ L	-	20 μ L	20 μ L	-	20 μ L
Distilled Water	-	20 μ L	-	-	20 μ L	-
Sample	-	Ag-BG	Ag-BG	-	BG	BG
OD Value	0.898	0.027	0.040	0.898	0.028	0.039
Hemolytic Ratio (%)	-	-	1.49	-	-	1.26

Table 5.1 Percentage of hemolysis ratio of coated samples.

Hemolysis is the breakdown of red blood cells that results in the release of haemoglobin into the circulation, which causes inflammation. The hemolytic ratio for the non-hemolytic state should be less than 2.0%, according to the ASTM guideline (ASTM F 756-00). The hemolysis behaviour of BG, Ag-BG coated samples is shown in Table5.1.

Higher platelet circularity indicated that platelets on the sample surface were inactivated. The hydrophilic surface promotes the production of -OH groups, which may boost platelet activation by lowering protein adhesion, which inhibits platelet adhesion and activation. Furthermore, the non-hemolytic character of 316L SS surfaces was attributable to albumin protein adhesion, which substantially inhibits platelet activation.

5.4 ANTI-BACTERIAL STUDY

5.4.1 ANALYSIS OF EPIFLUORESCENCE MICROSCOPE

Figure5.3 shows epifluorescence pictures of 316L SS, BG and Ag-BG nanocomposite coatings stained with AO after short-term exposure (5, 15, and 30 minutes) using the direct contact approach. AO interacts with both live and dead cells, turning live cells orange and dead cells green. Figures5.3 a, b and c depict the 316L SS, BG and Ag-BG coatings after 30 minutes of exposure. The presence of bacteria is shown by the orange glow. BG coatings failed to manifest their microbicidal action but reduced bacterial adhesion as compared to 316L SS samples, as seen in Fig. 5.3 b. Approximately half of the bacteria perished after 15 minutes of exposure to Ag-BG coating. During the first 30 minutes of exposure, the survival percentage dropped dramatically.

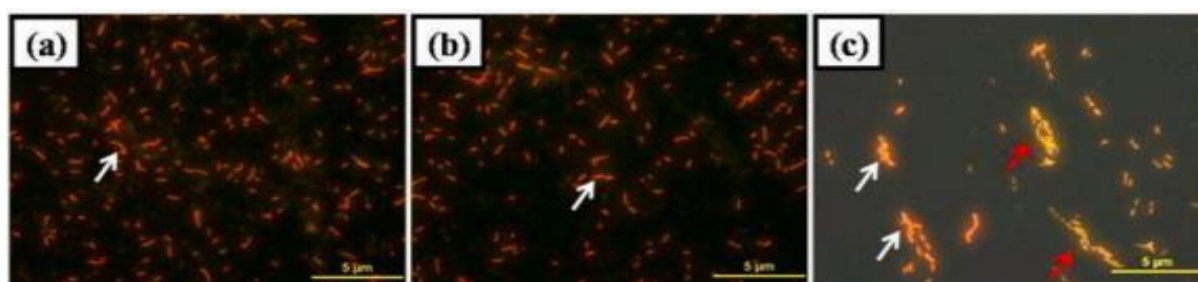


Fig 5.3 Antibacterial activity of coated samples.

(a) 316L SS, (b) BG, and (c) Ag-BG coatings against *P. aeruginosa* after 5, 15, and 30 minutes of exposure. (Note: white arrow mark denotes living cells and red arrow mark indicates dead cells)

5.5 IN-VITRO IMMERSION STUDY

The apatite formation on the surface of the bioactive material during in vitro immersion studies in simulate body fluid is proof for its bioactivity and is often used to confirm the bone-bonding ability during in vivo. The surface morphology transformation and increase in Ca and P deposition on the surface of bioactive glass (Fig.5.4) after immersion is also evidenced by the apatite formation of the bioactive material.

The in vitro SBF immersion results of the coated samples show the surface morphological transformation from spherical morphology to agglomerated apatite deposits after 7 days of immersion. Notably, the bioactive glass shows higher calcium deposition, with the absence of Si and Na presence on the surface, which signifies the bioactivity of the material. Other than structural transformation, the peak intensity of Ca variation is not much higher compared to before immersion samples in Ag- BG coatings.

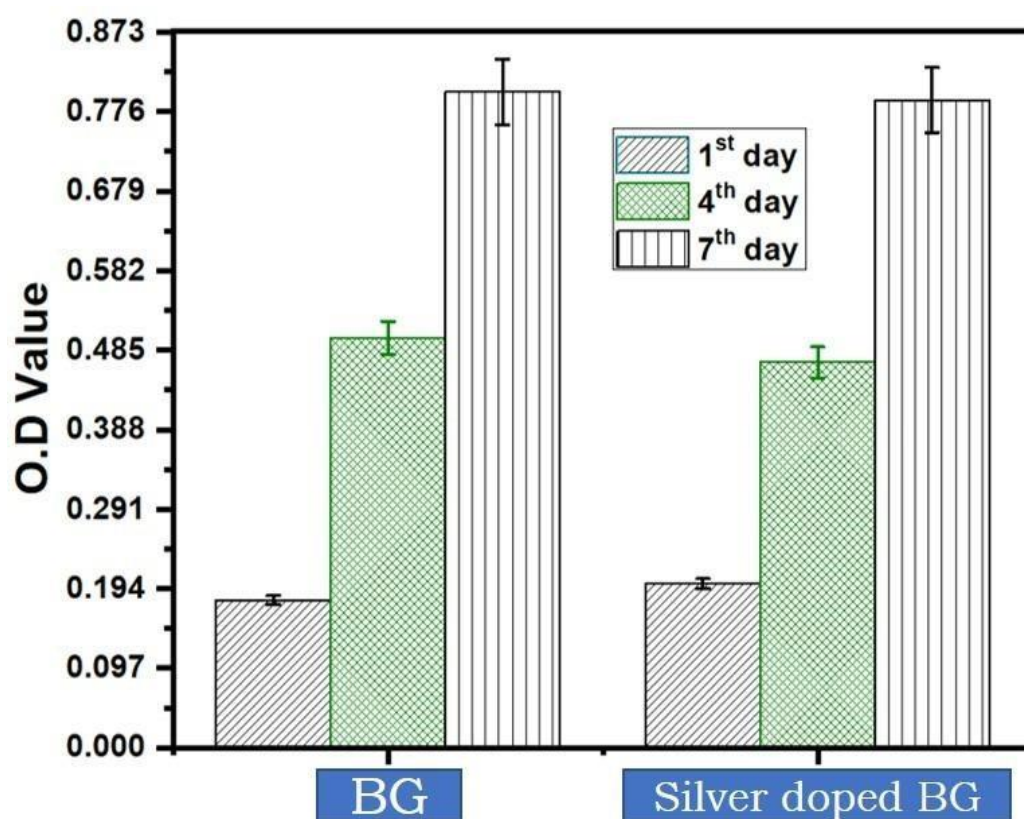


Fig 5.4 Immersion of coated samples on SBF.

5.6 CYTOTOXICITY STUDY

5.6.1 CELL VIABILITY AND MTT TEST

The proportion of S2S fibroblast cells that were still alive after growing on the bioactive glass-coated 316L SS surface. For the Ag-BG coating, the lowest cell growth was seen after 24 hours of culture at 50% and 100% extract concentrations. At 24 hours of culture, the BG coating for a 50% extract concentration and the BG coating for a 100% extract concentration showed the maximum cell growth. All groups had high cell viability (about 70%). At 24 hours at a 50% extract concentration, the cell growth on the BG coating was not statistically different from that on Ag-BG. At 24 hours at the 50% extract concentration, the cell growth on the BG coating was substantially high than Ag-BG

than Ag-BG ($p < 0.05$). At 24 hours with a 100% extract concentration, the cell proliferation on the BG coating was not different from that on Ag-BG ($p < 0.05$). statistically

The cell cytotoxicity study also suggest that the higher concentration of our glass samples proven to be toxic to the fibroblast cells. The results also demonstrate that, Ag incorporation to the glasses has vaguely reduced the viability and proliferation and increased cytotoxicity which could be due to their probable antibacterial and antiangiogenic nature. From the cell culture and their cytocompatibility study it is evident that the glasses are cytotoxic, fatal and lytic to cells at higher concentration but at lower concentration (<0.5 mg/ml) they are found to be cytocompatible.

FIBROBLAST CELLS

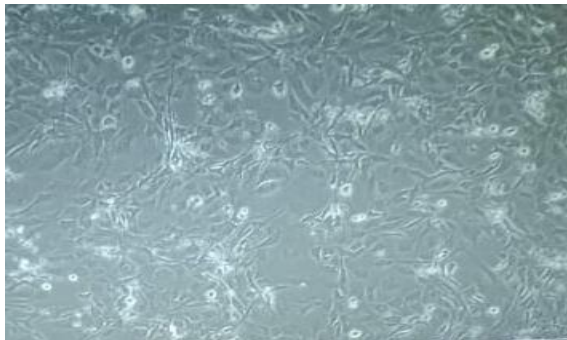


Fig 5.5 Cultured fibroblast cells

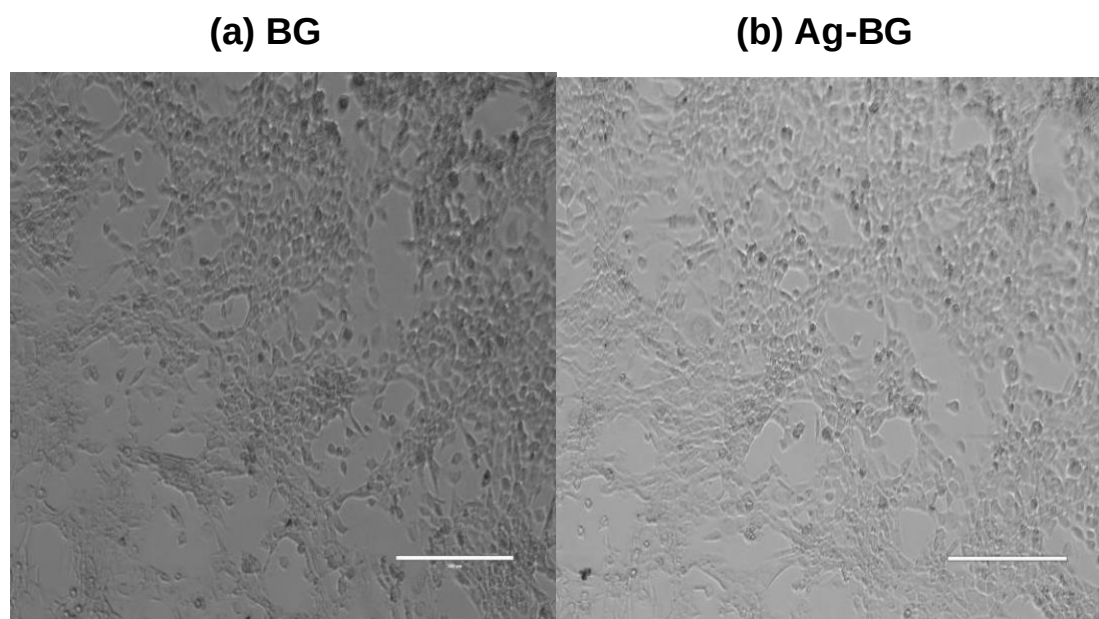


Fig 5.6 Cell attachment on coated samples.

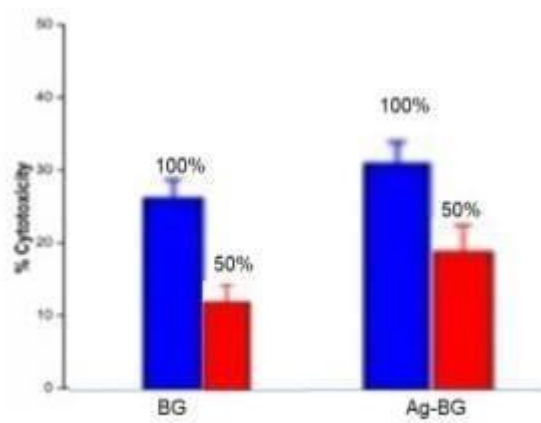


Fig 5.7 MTT assay of coated samples.

CHAPTER-6

SUMMARY AND CONCLUSION

Bioactive surface coatings are beneficial in terms of improving the surface properties of dental implants and achieving superior osseointegration. Although a majority of bioactive coatings reported no toxicity, inflammation, or foreign body response, there are issues such as the binding interface of coating and implant materials and solubility that need further research. Bioactive coatings have been reported to improve the clinical outcome in patients with systematic diseases affecting bone tissues. The bioactive coated implants can be explored further for clinical applications in medically compromised patient considering their better osseointegration.

From the present study, we synthesized bioactive glass and silver doped bioactive glass powders. Then, the synthesized glass powders are given for characteristic study for X-Ray Diffraction (XRD). It was well matched with the JCPDS CARD for both BG and Ag-BG samples. The synthesized glass powders are coated by using plasma spray method. It shows the coating thickness ranges from 0.04 to 0.05mm, containing interconnected round pores of diameter (2-50nm). The wettability behaviour of coated samples shows super-hydrophobic nature.

From the in-vitro biological study, hemolysis ratio shows less than 5% for the both samples. So, it has a better hemocompatibility property. From the antibacterial study, it reveals that the dead cells are seen in silver doped bioactive glass. So, Ag – BG has a better antibacterial property than BG. The in-vitro immersion of bioactive glass samples for 7 days. It reveals that there is a hydroxyapatite layer formation on the samples. The fibroblast cells were cultured and their cell attachment for both bioactive glass and Ag-BG are seen. For the cytotoxicity study, less concentration like 0.5mg/ml shows good cytocompatibility. For higher concentration, it was more toxic.

From this study we concluded that synthesized BG and Ag-BG by sol-gel method. BG and Ag-BG coatings were successfully developed by plasma spray method.

It exhibited better hemocompatibility, biocompatibility properties. Ag-BG coating showed better anti-bacterial property rather BG coating. Hence, the BG and Ag-BG coating is good candidature for bioimplant applications.

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PUBLICATION AND CERTIFICATE:



Presented Poster in SRM ICONN 2023 International Conference

Abstract is published in ICONN 2023 Abstract Book:

Structural and *in vitro* biological behaviour of silver containing bioactive glass coated 316L SS for biomedical applications

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ABSTRACT

Bioactive glasses (BG) are the most promising biomaterials for bone repair due its superior bioactive and biocompatibility properties. However, it doesn't express antimicrobial property which restricts its wide applications. Hence, in the present study, we synthesized silver containing mesoporous bioactive glass by acid assisted sol-gel method and it was further deposited onto medical grade 316L SS using thermal spray technique. The deposited films were characterized by XRD, SEM/EDS and XPS analysis to determine crystallinity, surface morphology and oxidation states of the coated samples. Wettability study was performed by contact angle goniometer and the coated samples exhibited hydrophilic nature. Further, antimicrobial efficacy, biodegradation, biomineralization and cytotoxic behaviour was studies in detail. In addition, corrosion resistant nature of coated samples was determined by electrochemical impedance spectroscopy (EIS) method. From overall study, it concluded that the silver containing mesoporous bioactive glass coating as good candidature for biomedical applications.

Keywords: Mesoporous bioactive glass, Silver, *in vitro* degradation, Biomineralization, Thermal spray

