SYNTHESIS & CHARACTERISATION OF Ag, Cu AND Fe BIOCERAMIC

MAJOR PROJECT- REPORT SUBMITTED FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

in

POLYMER TECHNOLOGY

Submitted By

SIDDHANTH SHARMA

(2K13/PTE/13)

Under the esteemed guidance of

Dr. DEENAN SANTHIYA

ASSISTANT PROFESSOR

Department of Applied Chemistry & Polymer Technology

DELHI TECHNOLOGICAL UNIVERSITY

(FORMERLY DELHI COLLEGE OF ENGINEERING)

MAIN BAWANA ROAD, NEW DELHI - 110042

DEPARTMENT OF APPLIED CHEMISTRY & POLYMER TECHNOLOGY

DELHI TECHNOLOGICAL UNIVERSITY,

NEW DELHI -110042

CERTIFICATE

This is to certify that the major project entitled "**SYNTHESIS AND CHARACTERIZATION OF Ag, Cu AND Fe DOPED BIOCERAMICS**" research carried out by Mr. **SIDDHANTH SHARMA, M. TECH (2K13/PTE/13),** under my guidance and supervision. This is an indigenous work and no part of the same has been submitted in part or full to any other institute or university for the award of any diploma or degree.

This project was carried under my supervision in year 2014-2015 and being submitted in partial fulfilment for the award of degree of Master of Technology in Delhi Technological University.

Prof. Dr. DEENAN SANTHIYA
Assistant Professor
Delhi Technological University

Prof. Dr. D. KUMAR
Head of Department
Delhi Technological University

DECLARATION

I, **SIDDHANTH SHARMA (2K13/PTE/13)**, student of Master of Technology, POLYMER TECHNOLOGY under DEPATMENT OF APPLIED CHEMISTRY & POLYMER TECHNOLODY, DTU, DELHI hereby declare that all the information published in this thesis is based on my own intensive research and is genuine.

This report does not, to the best of my knowledge, contain any part of my work which has been submitted for the award of any other degree either in this university or any other university without the proper citation.

Place: DTU, NEW DELHI **SIDDHANTH SHARMA**

Date: 2K13/PTE/13

M.TECH (Polymer Technology)

ACKNOWLEDGEMENT

First of all I express my deepest sense of gratitude to "almighty" for bestowing me with opportunities and instincts for pursing this career. A special thanks to **Professor Dr. DEENAN SANTHIYA, DEPARTMENT OF APPLIED CHEMISTRY AND POLYMER TECHNOLOGY, DTU** for giving me an opportunity to work with her the chance to carry out my project, and for his guidance, suggestion and interaction to put in the best of my efforts in project work. Her dynamic cooperation and encouragement helped me in carrying out my work in systematic manner.

She has been a friend and guide to me. I am grateful to his supervision, guidance and direction throughout the course of the project. She has been a constant source of inspiration to me and her overflowing optimism is awe-inspiring and has helped me develop a positive attitude in life. She has encouraged me to go ahead.

I would like to express our heartfelt thanks to my parents and almighty. Their motivation, affection, inspiration and faith in me have been incredible. I will always remain indebted to them.

I am also thankful to Ms. Nidhi Gupta for her valuable suggestions and support provided during this project work. I would like to extend our thanks to all the non-teaching staff of the Applied Chemistry and Polymer Technology Department.

Siddhanth Sharma

2K13/PTE/13

M.TECH (Polymer Technology)

Department of Applied Chemistry & Polymer Technology, DTU

Bawana Road, Delhi - 110042

CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

ABSTRACT

The bio-active glass and the bioceramic samples were prepared by bio-inspired root process using CTAB as template. The synthesized materials were characterized by FTIR, SEM, EDS, XRD, UV-vis, Anti-Microbial testing and TEM. The SEM and TEM confirmed the microstructure and linkages so formed. XRD Analysis showed that the bioceramic/bioglass so prepared by bio-inspired root process are partially crystalline in nature. Further the synthesized product was subjected to *in-vitro* tests by immersing the samples into Stimulated Body Fluid(SBF). After such immersion one sample was characterized with TEM. The purpose of this thesis is to look into effect of silver, iron and copper doping on the characteristics of the prepared bioglass. The results shows that although anti-bacterial properties of the bioglass improves.

CHAPTER 2

INTRODUCTION

Nature stands as the most important source of inspiration in the development of new implant materials or any of the technological innovation. In this regard, the design of new and novel biomaterials relies on imitating the structure and function of biological systems. The bio-mimicry approach is based on the fact that after billions of years of evolution, nature knows which systems are most efficient and appropriate in terms of optimal properties. Thus, biomimetic or bio-mimicry takes advantage of these natural strategies for the solution of problems that affects humans in real life, and in all the branches of materials science and engineering.

Bioglass has superior ability to bond with the living hard (soft as well) tissues. The main structure consists of silicate, SiO_2 network etc. along with the use of some network modifiers. Bioglass is highly bioactive in nature and results into formation of apatite layer. This happens when we induce it onto the surface along with Simulating Body Fluids (SBF)'s presence. This has rendered huge application in this field especially in bone regeneration and scaffolds in Tissue Engineering.

Bioceramic is a class of biomaterials which communicates with the biological system. Bioceramics are ceramic materials that shows biocompatibility (similar to Bioglass which are also ceramic materials and shows biocompatibility). They find suitability under variety of medical applications. The primary medical application in which they find use is in the form of Implants (e.g. surgical implants).

The nanoparticle based bioglass have gained much response owing to their superior osteo-conductive properties as compared to conventional (micron sized) bioglass materials. Generally speaking the dissolution rate and microbial infection of scaffolds/implants/biomaterials in medical field is of vital importance and is still a health concern [1, 2]. Application of Nano based material allows us to develop potential product to avoid such health concerns.

CHAPTER 3

OBJECTIVE

The major objective of this project (thesis as well) is to look into (and develop too) a bioceramic (i.e. doped bioglass, rather than nanoparticle induced) that could also serve as a novel material in answering to all those health concerns and risk in a more effective way.

In the current study of Bioglass/Bioceramic, we have used bio-inspired root process for synthesis of bioglass and then doping is done (of the synthesized bioglass) with Ag, Fe and Cu (based appropriate compounds).

CHAPTER 4

REVIEW OF LITERATURE

BIOGLASS:

Bioglasses belong to the family of bio-active glasses, it is made up of Silicon Dioxide, Sodium Oxide, Calcium Oxide and Phosphorus pentaoxide in different ratios. As compared to traditional and conventional glasses, these have high concentration of sodium, calcium, Ca/P ratio but low silica content. It is due to this high ratio of Ca/P ratio, there is high degree of formation of apatite crystals.

Since we can formulate different ratios, thus different compositions of bioglass can be formed. And according to different compositions, many binds to soft tissues and bones, some to bones only while some do no bonding and gets encapsulated after implantation, and rest gets reabsorbed(in few weeks).

When some of these bind to bones only then they are called as Ceravital (or simply bioceramics). Thus we can say that both Bioceramics and Bioglasses, are subset of bioglasses.

BIOCERAMICS:

Ceramics have been used by humans since time immemorial. Humans used to obtains ceramics by heating clay to transform it into pottery (e.g. to store food etc.). However, when we implemented the use of ceramics in the treatment of diseased or in-vitro use, then those ceramics came to be known as bio-ceramics. Before 1930's only pure metals were used in implant surgeries. Post 1930's marked the beginning of better surgical equipment's and methodologies. But it was in 1969, Professor L.L Hench et al found suitability of glasses and ceramics and their bonding capabilities to human body (i.e. bones specifically)[1]. It was with his discovery of bioglass that led to forays into the field of bioceramics. After 60s, a strong wave engulfed the field of Bioceramics, for its potential use into medical applications due to its favored bio-mechanical properties. The resorbable ceramics began to be used in 1969. These types of bioceramics gets

dissolved with time and they thus are gradually replaced by natural tissues. A very thin or non-existent layer of (of bioceramic) interfacial thickness is the final result.

Bioceramics are those novel, as well as engineered, materials that find their applications in the field of medical technology and medicine as well [1]. Traditionally it was the low mechanical fracture toughness, high brittleness and the low resistance to the impact that were the factors that limited the applications of the ceramic materials. However, a strong wave engulfed this field and interest in the use of ceramics for biomedical engineering applications was developed at the latter part of 1960s. New ceramics with greatly improved properties and characteristics contributed to increase the possibilities and probabilities of using ceramics (and bioceramics) in biomedicine. Their use has extended considerably since then (i.e. 1960s) [2, 3]. The high compression strength coupled with their great chemical inertia along with their aesthetic appearance, made sure that these materials find entry into the field of dentistry, mainly in dental crowns/caps etc. Such great advantages and the desirable characterisitics ensured that their use is extended to orthopedic applications as well [4-6].
It may be noted that they were initially used as an alternative to metallic materials in order to increase the biocompatibility of implants to be used. Now the bioceramics can be classified from different points of views [7, 8], these various point of views are as described below:
(a) According to the type of answer of the living host
(b) According to the application to which they are destined [9]
(c) According to the characteristics of the material [10]

Then, accordingly, bioceramics can be divided in Bioinert Ceramic Materials, Bioactive Ceramic Materials (or Surface Reactive Ceramic Materials) and Biodegradable (or Resorbable Materials) Ceramic Materials. Now these are described as below :

BIOINERT CERAMICS

Relatively Bioinert ceramic materials undergo (or show) a very little or close to no chemical change when they get exposed to physiological environments. They maintain their mechanical as well as their physical properties while they are in the host. The response of the host to these bioceramics is -- the formation of a

very fine fibrous tissue capsule of varying dimension (thickness to be precise),generally it ranges from several micrometers or is less than that, that encapsulates the implant materials (which is the bioinert material in this case). The fixation of the implants in the body, when it enters, is done via very strong mechanical interlocking, by tissue in-growth onto the surfaces having wavy, edge appearance [11]. When high strength is needed, then the bond is made by drilling holes or puncturing or piercing in the implants using threads or cements and other such materials. But when high strengths are not required, they can be used as porous inert bioceramics, generally with sizes (of pore to be precise) ranging in between 100 -150μm, it is due to this which guarantees that the growth of the tissue towards within implants and thereby assuring its fixation [12-14]. Examples of these bioinert ceramics includes : Alumina(α-Al_2O_3); Zirconia(ZrO_2), Alumina-Zirconia and Pyrolytic Carbon.

BIOACTIVE CERAMICS

Now, when an artificial material is inculcated within the body, it is encapsulated by the un-calcified fibrous tissue that secludes or cut it off from the surrounding. This is a normal reaction which aims to defend, protect and save the body from foreign substances. It was however, in the early 70s, Hench *et al.* [15] found that a glass, called as Bioglass. Is was of the convoluted system Na2O – CaO - SiO2 - P2O5, which introduced the formation of non-fibrous tissue, but rather, it itself came into direct contact with the surrounding bone. This resulted into formation of a strong chemical bond with it.

After this discovery other types of bioglasses (i.e. variety of different composition of bioglasses) and glass - ceramics have been found so as to bind to living bone [16-18], Hench *et al.* [19- 20], Gross *et al.* [21-22], Karlon *et al.* [23-24] and Kokubo *et al.* [25-26]. These so discovered materials that are also bone-binding materials came to be known as bioactives materials.

The appearance of these type of bioceramics discovered with the need to remove and/or bar the interfacial movement that takes place with the implantation of bioinert ceramics. Consequently, L.L.Hench propounded in 1967 a research paper based upon the modification of the chemical composition of ceramics and glasses so that they have chemical reactivity with the physiological system and thus they form chemical bond between the surfaces of implant and the coterminous or the bordering tissue. Upon implantation inside the host, bioactive ceramics form a very intense bond with

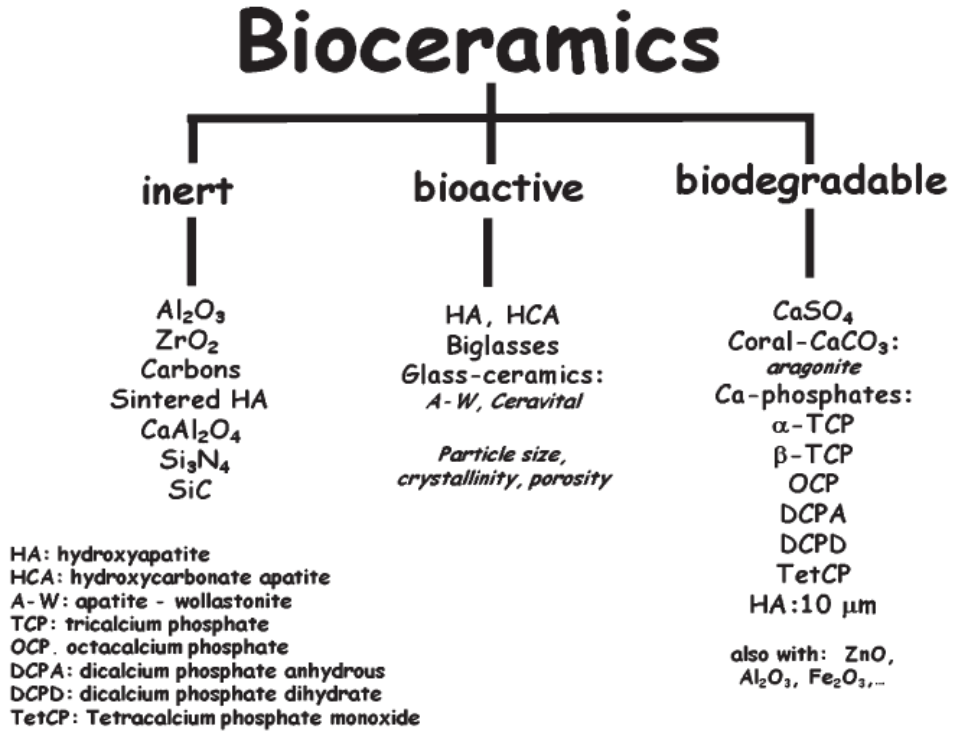
nearby or the adjacent tissues. Except hydroxyapatite, which forms a bond directly to living bone, the rest of bioactive materials bond to the bone with the help of carbohydroxyapatite layer (CHA), it is a biologically active layer which provides for a surface forming common boundary between the bioactive material and the host. Now this phase is chemical and structurally equivalent to the mineral phase of the bone, and is responsible for the above described surficial union. The surface of union between the bioactive material and the tissue is generally extremely stout. But in multiple cases, the surficial strength of adhesion is almost same to or greater than the cohesive strength of the implanted material (or the tissue that bonded to the bioactive implant). Generally speaking, the fracture or any breaking takes place, if any, happens at the implant or at the bone but never in the interface, where the bonding took place [27-28].

BIODEGRADABLE CERAMIC

These types of bioceramics are dissolved with time and are gradually (or eventually) replaced by natural tissues. A very thin or non-existent interfacial thickness layer is the final results. They would serve as ideal implants inside the body, since they can only remain in the body till their utility and they disappear as the tissue regenerates itself. However, their main disadvantage is that their mechanical strength lowers during the reabsorption process.
The function, however, of these materials is to engage themselves in the dynamic process of reabsorption and formation which takes place inside the body (or bone tissues). So they are used like scaffolds or filling material, allowing for facilitating the tissues in their infiltration and substitution [43].
All the resorbable ceramics except plaster of paris ($CaSO_4.½H_2O$) are based on phosphates of calcium, with varying biodegradability.
The biodegradation rate is increased, as: a) specific surface increases (powders which gets biodegraded faster than that porous solids which in turn gets biodegraded faster than the dense solids). b) With decreasing crystallinity. c) When the size of grains and crystal decreases. d) In cases when there are ionic substitutions of Mg^{2+}, Sr^{2+} and $(CO_3)^{2-}$, in HA.

The factors that in turn tend to lower or reduce the rate of biodegradation include e.g.: a) F^{-} substituting in HA. b) Mg^{2+} substituting in β- TCP and c) Small values of β- TCP/HA ratios in bi-phasic compounds.
Now, the biodegradation or reabsorption of calcium phosphates is caused by three factors:
1) Physio - chemical dissolution, which depends upon solubility of the material and pH of its local environment.
2) Preferential attack on walls of boundaries of grains and the physical dissociation and splitting into various small particles. &
3) Numerous Biological factors, such as phagocytosis etc., which causes a reduction in the local pH condition, and the cellular activity.

In general, so, we can draw the above as follows:



Main bioceramics classified as a function of its reactivity with living tissues.

FIG. 1 TE APPROACH BASED ON USE OF BIOCERAMICS (ANTONIO, MARIA & PEDRO, JOUR. OF BIOMED. SCIENCE, JAN 2013)

CHAPTER 5

THEORY

Till now, the microscopic understanding of bone's mechanical behaviors is not fully understood, but the need of implants, scaffolds and bioactive fillers has showed us the way for taking up a different strategy for linking and combining the characteristics of HAP(hydroxyapatite) and other materials. More specially the biocompatibility of the HAP with the strength and/or brawness of the latter. Bioglasses and the bioceramics are more and more studied because of their surface chemical reactivity when in contact with bodily fluids[31–33]. This occurs by a convoluted mechanism of ions filtrating and partial disintegration of the glass surface, the drizzle of bone-like apatite from the solution provides an able and steady chemical bonding with the tissues. Since bioglasses and bioceramics are frail materials, they are thus used in the field of small bone faults restoration, or as coatings on dormant substrates for load-bearing prosthetic devices.

More specifically, these biomaterials have found surgical and medical applications as coating for prosthetic devices, bone-fillers as bone substitutes[34-38].

Bonding between bioglass or bioceramic and the surrounding tissues takes place through the development of a hydroxyapatite layer (HAP Layer), which is very analogous to the mineral phase of bone. When the bioglass is placed in contact with anatomical and bodily fluids, this layer is developed through a convoluted ion-exchange mechanism with the engulfing fluids, also called as bioactivity. This biological operating layer of hydroxyapatite can construct on the surface of glasses having a broad configurational range, and is considered as self by the surrounding living tissues. Also its existence is extensively recognized to be a tolerable requirement for the implants to chemically connect with the living bone. Kokubo *et al.* [30] proposed Tris - buffered SBF (Stimulated Body Fluids) for the *in-vitro* study of bioglass and bioceramics, it is because that its ion-concentration is approximately identical to that of human blood plasma. Since then, *in-vitro* tests in Stimulated body fluids have been widely employed as prior tests on new novel materials showing bioactivity. The ion filtrating eventuation involves the transfer of monovalent cations from the glass, such as Na^+/K^+ with H_3O^+ from the solution, which in turn causes an escalation in the

pH of the solution. Now, it is known that osteoblasts commit oneself a slightly alkaline medium, but it is also known that excruciating modification to the pH values can constrain the osteoblast's activity and can lead to cell necrosis or apoptosis.
Different bioglass and bioceramics have been compounded in order to get the desired mechanical, chemical characterisitics by attaining the wished and needed microstructure. Some of familiar components used are Na_2O, CaO, P_2O_5, and SiO_2 for synthesis of common bioglass. Apart from to these above components, varying adaptation of K_2O, MgO, and B_2O_3 are also used to develop different compositions. There are some other glass and bioceramics which also include ZnO, Ag and Al_2O_3.

Now in above, Hydroxyapatite layer (HA) is the dominant mineral content of bone defining as ~43% weight. HA layer is a calcium phosphate layer whose stoichiometric blueprint conform to a: $Ca_{10}(PO_4)_6(OH)_2$, with a Ca/P molar ratio = 1.67. Hydroxy Apatites fit the mineral family of Apatites whose name was derived from Greek depiction 'απαταω' which connotes deception, and it was due to this property it was puzzled with auxiliary mineral species like the beryl or the tourmaline[39]. HA demonstrates an ionic personality, and its crystalline architecture can be described like a bunched or packed hexagonal packing of oxygen atoms with the metals commandig the tetrahedral and octahedral holes of the periodic structure. The basic apatite architecture is hexagonal in nature and the rough lattice parameters are viz. a=9.4 and c=6.9 Å. Nevertheless, HA presents a low harmony and symmetry due to the detortion of the OH^- ion with respect to the quintessential model that would serve the position of sphere F^- ion in the fluorapatite. But, in most of the entirety with respect to the biomaterials it is considered that the HA layer has a fluor-apatite structure. The unit cell encompasses viz. $10Ca^{2+}$, $6(PO_4)^{3-}$ and $2OH^-$ [40]. HA also grants the exchange and trade of many other ions in their structure. These exchanges can take place in the location or area of the Ca^{2+} ions or in the $(PO_4)^3$ group or the $(OH)^-$group. Ramification of these exchanges (or substitution per se) are modification in its characteristics like lattice variables, framework, solubility etc., without compelling variation to symmetry. Now, many other ions can also infiltrate in the HA architecture affecting the attributes vis-à-vis its crystallinity, thermal stability etc. The mechanical characteristics of the HA are analogous to those of the defiant components of the bone.
Now, HA is dissimilar from biological apatites such as enamel, dentin, bone etc. in proviso of physical and mechanical properties, stoichiometry, configuration, crystallinity and other such peculiarities. For, the biological apatites are in

general calcium-lacking and are ever carbonate supplemented. Thus the more appropriate way biological apatites should be referred is carbohydroxyapatites (carbonate apatite) and not as HA[42, 43].

Now, there exist various types bioceramics-tissue interaction. It is clear that all implanted material will show a response by the host tissue. This response by the host tissue occurs (mostly) at the tissue-implant interface. So whenever the implant behaves toxic, then in such cases the tissue dies. In cases when the implant is biologically inert, then in those cases, the implants gets encapsulated by the fibrous layer by the tissue, thereby preventing any kind of interaction with the host. While, there are some implants who gets replaced by the tissue in some time. These implants gets dissolved into physiological body fluids. So, in nutshell, a good replaceable implants should have its composition very close to the body's physiological fluids.

Now the mechanism of attachment of tissue to an implant is directly related to the tissue response at tissue–implant interface. There are four types of bioceramics each with a different type of tissue attachment. The related chemical activity of different types of bioceramics depends on rate of bonding with bone. The relative level of reactivity of an implant also influences the thickness of the interfacial layer between material and tissue.
Type 1, nearly inert, implant form a non-adherent fibrous layer at the interface. However if these implant are loaded such that interfacial movement occurs, the fibrous capsule can become several micrometer thick and the implant loosens very quickly leading clinical failure.
Porous ceramic and HA coating, a type 2 bioceramics, on porous metal are developed to prevent loosening of implants. The growth of bone into surface porosity provides a large interfacial area between the implant and its host. This method of attachment is often called BIOLOGICAL FIXATION. It is capable of withstanding of more stress than type 1 implant which achieve only morphological fixation. A limitation of type 2 porous implant is the necessary for the pores to be at least 100 micrometer in diameter. Large pore size is required so that capillaries can provide a blood supply to the ingrown connective tissue. If pores < 100 micrometer then even if the micro movements occur, Capillary can be cut off leading to tissue death. When the porous implant is metal, interfacial area can provide a focus for corrosion of implant and loss of metal ion into the tissue, which may cause a variety of medical problem. Coating of these porous metals with HA, diminishes some of these limitations. The Ha coating also improve the rate of bone growth into pores. But coating

dissolves with time which limits its effectiveness.
Bioactive implant (type 3) is another approach to achieve interfacial attachments. This is intermediate concept between resorbable (type 4) and bio inert behavior. A bioactive material undergoes chemical reaction in the body, but only at surface leading to bonding of tissue at the interface. Thus a bioactive material is defined as "a material that elicits a specific biological response at the interface of material which results in the formation of a bond between the tissue and the material. The bioactive concept has been expanded to include many bioactive materials with a wide range of bonding rate and thickness of interfacial bonding layer. These include bioglass, bioglass-ceramics, dense synthetic hydroxyapatite, bioactive composites, bioactive coating. The time dependence of the bonding, strength of bond, the mechanism of bonding, the thickness of bonding zone, and the mechanical strength differ for various materials.
Type 4 is resorbable implants which are designed to degrade gradually with time and be replaced with natural tissue. A very thin or nonexistent interfacial thickness is the final result. This is the optimal solution to problem of interfacial stability. It leads to the generation of tissue instead of their replacement. The difficulty is meeting requirement of strength and short term mechanical performance of implant while regeneration of tissue is occurring. The resorption rate is must be matched to repair rate of body tissue but some material dissolve too slowly and others too fast. Since large quantities of materials being handled by cells so the constituents of resorbable implants should be metabolically acceptable.

To understand Tissue Response to an implant it is necessary to understand the nature of tissue at the interface and the significance of any alterations seen there. The significance of such changes will vary with the material and will vary with the material and will be governed both by their severity and by their persistence, a transient change or a continuing one may both appear to be identical shortly after implantation. Every organ in body is made up from a combination, in varying proportion of four tissue types: Epithelium, Muscle, nervous and connective tissue. Epithelial tissue secretes a wide variety of substance either through ducts or into blood stream. Glands are made of this tissue. Muscle tissue is found wherever movement is required. Nervous tissue is responsible to transmit signal between outside world, the brain and other parts of body. Fourth, connective tissue is named as such because it connects all other. It includes blood supply to and from organs. No organ in body is without connective tissue and it is with connective tissue that ceramic biomaterials interact. An

inflammatory response will always be there immediately after surgery while the damaged tissue, blood clot, and the bacteria introduced at the time are removed. The reddening and swelling occurs increasing the blood supply produced by the chemical released by damaged tissue. With the blood reaches cell involved in repair process. These include many cell known as phagocytes, for their ability to digest and remove foreign material. It is the presence of these phagocytes at any time other than immediately post-implantation, which can indicate problems with a material or an implant.

CHAPTER 6

RESEARCH GAP

One issue that arises during the clinical trials is because of bacterial infestation. So bacteria colonizes, which leads to failure of implants. It is thus desired that the Bioglass has good anti-microbial properties to avoid the above said failure. So for many years we've used Copper to avoid above said infestation. The use of copper has even helped humans in day-to-day operations for sanitation purpose. E.g. copper door knobs, food packaging, waste water treatment etc. It also has been proven that there are various forms of copper that is used as an anti-microbial agent vis-à-vis Cu^{2+}, CuO or simply Cu. Each of these possess different level of response. E.g. Copper nanoparticle are more toxic to bacteria that CuO nanoparticles.

Other such antimicrobial agent used is Silver (Ag). The biggest advantage of using silver over antibiotics is that the micro-organism develops less resistance when silver is used. Another advantage of using silver is its easy availability. The most common use of Ag- substituted Bioglass is its coating on medical sutures.

CHAPTER 7

LIST OF CHEMICALS/BIOCHEMICALS

1. Tris(hydroxymethryl)aminomethane base, Sisco, Research Laboratories Pvt. Ltd., Catalogue Number 2044122
2. Tris(Hydroxymethyl)aminomethane HCL, Sisco Res. Lab. Pvt. Ltd., Cat. no. 2044123
3. Cetyl trimethylammonium bromide($C_{19}H_{42}BrN$), Sigma Aldrich, Cat. No.52369
4. Tetraethylorthosilicate(TEOS), Sigma Aldrich, Cat. no. 333859
5. Triethylphosphate(TEP), Sigma Aldrich, Cat. no. 538728
6. Sodium Acetate(CH_3COONa), Sigma Aldrich, Cat. no. 8750-250G
7. Calcium Acetate($Ca(C_2H_3O_2)_2$), Sigma Aldrich, Cat. no. 402850-100G
8. Ethyl Alcohol(C_2H_5OH)
9. Silver Nitrate($AgNO_3$), Sigma Aldrich, Cat. no. S5506
10. Cupric Chloride($CuCl_3$)
11. Ferric Chloride($FeCl_2.2H_2O$)-hydrated
12. Luria Bertani Broth Media, Sigma Aldrich, Cat. No. L3522
13. E. Coli culture
14. Agar-Agar, Sigma Aldrich, Cat. no. A1296
15. Stimulated Body Fluid(SBF), Sigma Aldrich, Cat. no. H8264

CHAPTER 8

SYNTHESIS:

I. PREPARATION OF BIOGLASS:

(i) 10mM Trizma Buffer (which is Hydroxymethyl-Aminomethane) is prepared from 30mM Tris Buffer. For this preparation we mixed 0.222g of TRIS Base in 0.182g of Tris HCL. For the 30mM TRIZMA Buffer produced, we now prepare 10mM TRIZMA Buffer by taking out 33.33mL of 30mM TRIZMA Buffer and adding it to 66.67mL of Milli-Q water. This gives us the desired TRIZMA Buffer i.e. 10mM.

(ii) This obtained solution was divided into two parts. Let's name them as A and B for simplicity reasons.

(iii) In A & B which contains 50mL of 10mM TRIZMA Buffer, we add 1.4mM CTAB (which is Cetyl trimethylammonium bromide). The amount of CTAB that we added after calculations came out to be 0.0255g. CTAB was used because it is an effective antiseptic agent against bacteria and fungi. The solution was then left to be allowed to mix with the help of a magnetic stirrer for half an hour.

(iv) After half hour of continuous stirring, we add 4.64ml of TEOS in A & B. TEOS is Tetraethyl ortho silicate. This is then allowed to mix for another half hour.

(v) This is followed by addition of TEP (Triethyl phosphate). Amount we added of Triethyl phosphate is 0.5g. This was followed by rigorous mixing session for half an hour.

(vi) Now after half hour, we added Sodium acetate amounting to 3.18g to bot A & B. This was again allowed to mix for half under on the magnetic stirrer.

(vii) Again after half hour has elapsed we added Calcium acetate in the quantity i.e. 2.10g to both A & B, which were allowed to mix for half an hour at 37 degree centigrade.

(viii) We repeated the above process for adding a third doping agent, let this mark as C, so as to achieve high accuracy and simplicity. For this instead of preparing 100mL of TRIZMA Buffer, we instead prepared 50mL of TRIZMA Buffer of 10mM. For that we added 16.66mL of 30mM TRIZMA Buffer to 33.34mL of Milli-Q water. This was followed by addition of 1.4mM of CTAB, i.e. 0.0255g, followed by mixing on magnetic stirrer for half hour. This was followed by addition of TEOS, TEP, Sodium Acetate, Calcium Acetate of the above same quantity.

This marks the completion of preparation of bioglass. But we continued the process and added doping agents before the bioglass was completely formed so as to achieve better linkages and bonding with the doped agents, thereby forming a better bioceramic.

II. PREPARATION OF BIOCERAMIC

(i) To A we added Silver Nitrate ($AgNO_3$) amounting to 0.0016mM. This was done half hour past we added Calcium acetate. This was again maintained at temperature 37 degrees. The amount of $AgNO_3$ we added was 0.0011g.

(ii) To B we added Cupric chloride ($CuCl_3$) half an hour after we added Calcium Acetate to B. This was left to mix properly for half an hour at a temperature of 37 degrees. Amount of $CuCl_3$ came out to be, after calculation, 0.0136g. It may be noted that molarity we used is same for each doping agent. Thus above weight that we added came after we kept the molarity of $CuCl_3$ to be 0.0016mM.

(iii) To C we added Iron chloride di hydrate ($FeCl_2.2H_2O$) in same molarity i.e. 0.0016mM, this amounted to addition of 0.000130g of $FeCl_2.2H_2O$. This was left to mix for half an hour under magnetic stirrer at a temperature of 37 degree centigrade.

This marks the preparation of Bioceramic. But it is still highly diluted. So we concentrate it.

Washing Steps:

(i) For concentrating our developed samples, we performed centrifugation for all the developed samples i.e. A, B and C. Centrifugation was done at the rotational speed of 4000rpm per 15 minute frequency.

(ii) After every 15 minutes of continuous rotation, we performed water washing. And the sample was allowed to rotate in the centrifugation machine again at 4000rpm.

(iii) The above two steps were continued till we were able to obtain dense and concentrated (though in amorphous) state of the sample. After such sample was obtained we performed ethanol washing and the sample was centrifuged

again at 4000rpm.

(iv) The concentrated sample was then placed inside the incubator while we prepared the media for culture preparation and culture testing.

III. Bacterial Culture Media Synthesis

(i) For this task we first added 0.625g of Luria Broth Media into 2X25mL of Milli-Q water.

(ii) This was followed by autoclaving of the prepared solution. Autoclave was necessary so as to free the media from any other unwanted impurity.

(iii) In other instance we also prepared a solution composed of Luria Broth (1.25g) and added to it 1.875g of Agar, which helps in faster solidification of the media.

(iv) Now we started the UV Light of Laminar Air flow for 5-10 minutes so as to free the laminar environment of contaminants.

(v) In one instance we put 10uL of culture into the media.

(vi) This we then put into incubator at 37 degrees centigrade so as to know about characterisitcs of prepared material by observing its optical density (via UV-vis).

(vii) After completing the Optical Density measurement we divided the total prepared media + culture into four equal parts (25mL in each petridish)

(viii) One was allowed to contain only the Medium and the culture in it while in the other three we added Silver doped Bioglass (A), Iron-Doped Bioglass (B), Copper-doped bioglass (C) respectively. Thus we prepared four samples in such a way.

It may be noted that we entered these A, B and C in diluted form. For preparation of them into diluted form we took equal amount of each of the bioceramic and added equal amount of water into it. This after addition was allowed to be placed inside a sonicator for sonification and uniform mixing of the bioceramic in the water we added. We then placed Simulating Body Fluids (SBF), we added around 20uL of SBF, onto the diluted bioceramic.

(ix) So we were left with 4 samples now.

(x) We observed the four samples in large petridish for few days and the results are shown in the result section.

IV. SECONDARY BIOCERAMIC REACTION

(i) We prepared the same up till step vii. Of part IV.
(ii) We then diluted the same samples in the exact amount which we used in large petridishes.
(iii) But this time we spreaded (using the spreader) the media and the culture in small petridishes.
(iv) Instead of placing the bioceramic in diluted form we placed the bioceramic in pellet form.
(v) So for this we prepared the powedered bioceramic into the pellets (each for Ag doped bioceramic, Cu doped bioglass and Fe doped bioglass).
(vi) Also before inserting the pellets directly into the media, we submerged these pellets into the Simulating Body Fluids (SBF).
(vii) This was then inserted into the culture media inside the small petridishes.
(viii) Our assumption proved quite right, for using small petridish for bioceramics prepared with better results. This is as shown in the results section.

1. RESULTS AND OBSERVATION :

I. SEM

Following figure shoes SEM results of different doped bioglass samples. The synthesized bioglass particles are spherical shaped and size of the particle is approximately below 1µm. Interestingly the morphology of the bioglass sample was retained even after doping with the Ag, Fe and Cu. EDS spectra of the bioglass sample indicated the presence of elements such as Si, Ca, P, Na and O by showing the respective peaks in the spectra. For the doped sample EDS spectra also showed the respective presence of the dopants (Fe, Ag and Cu) in the spectra.

BG (Pure)

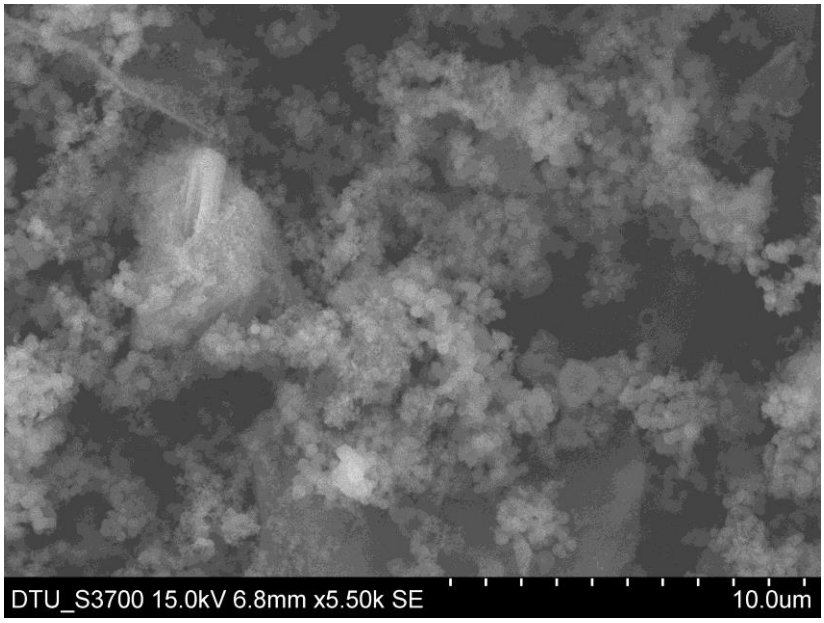


Fig.2. SEM micrograph of bioglass (pure) at 10µm scale and 5.5kX magnification

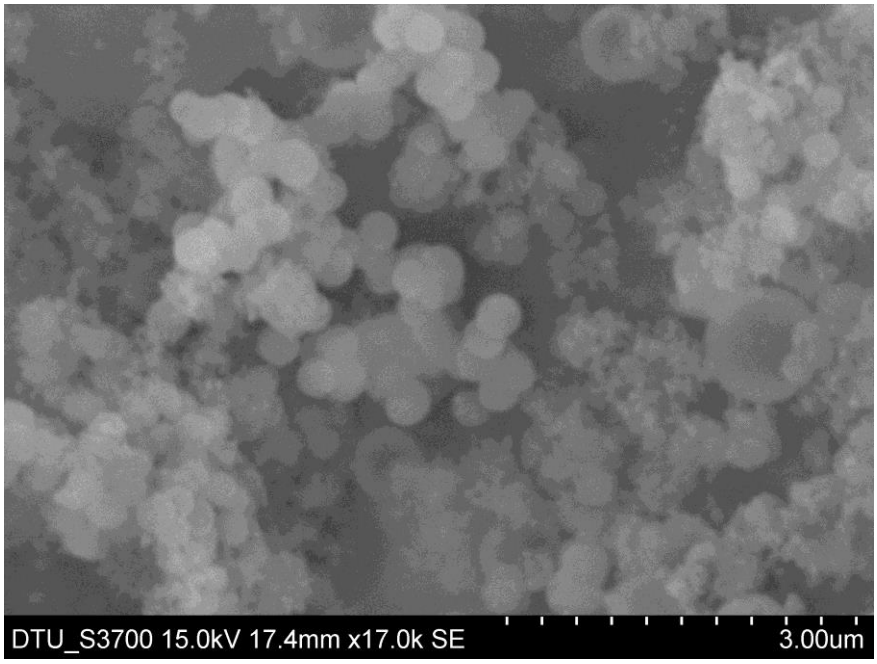


Fig.3. SEM micrograph of bioglass (pure) at 3 µm scale and 17kX magnification

i. **BG-Fe**

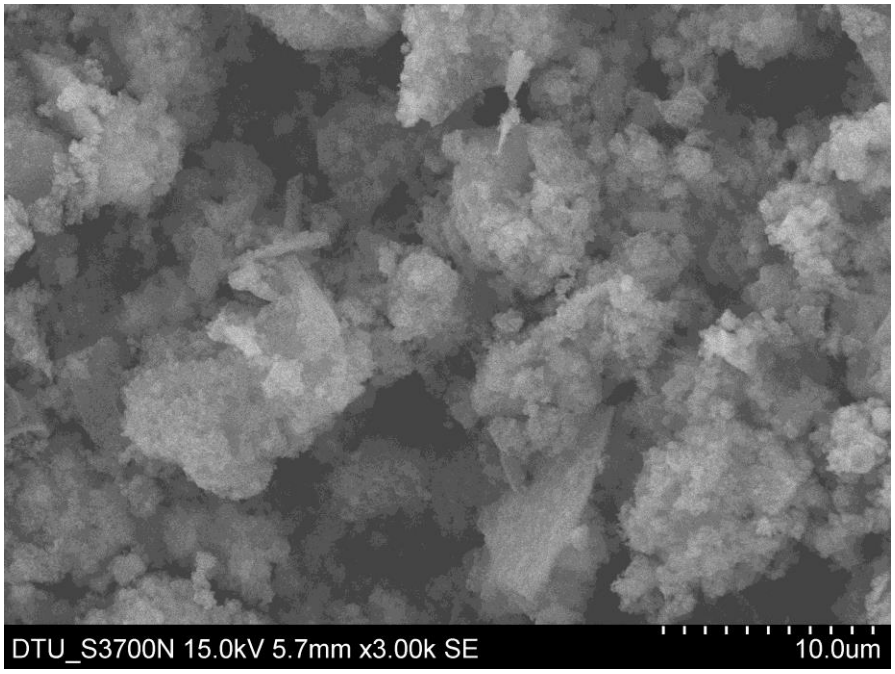


Fig.4. SEM micrograph of bioglass doped in Fe at 10 µm scale and 3kX magnification

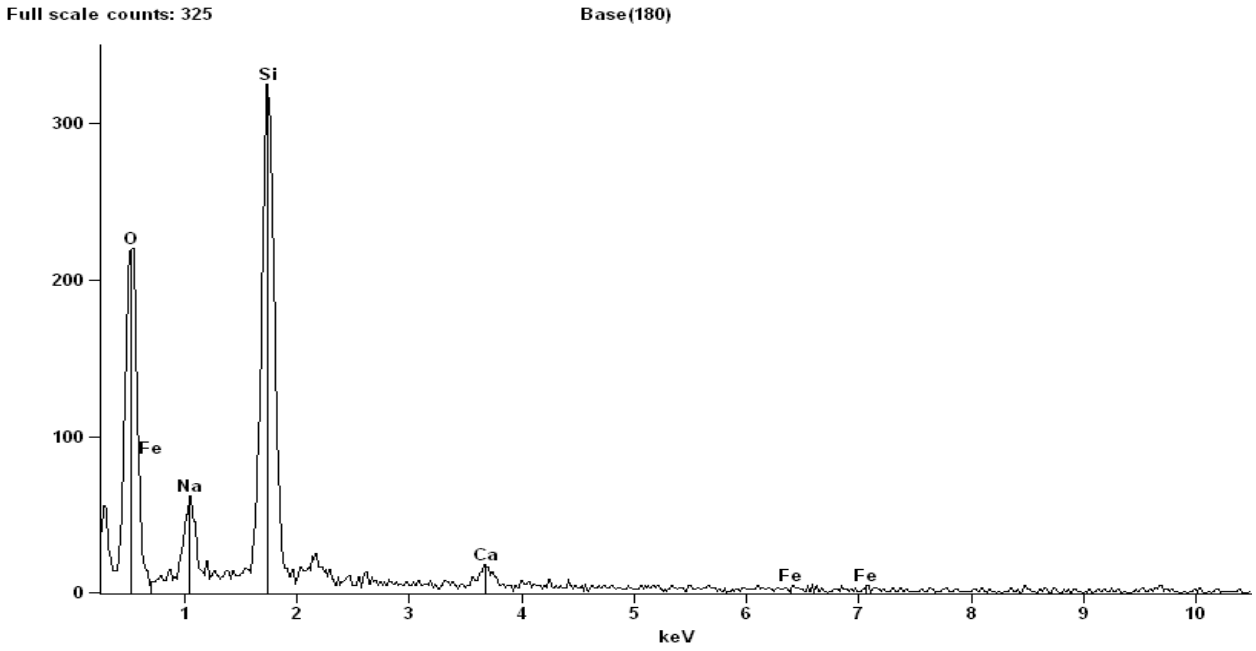


FIG.5. EDS spectra showing Si, O, Fe, Na and Ca peaks

Element Line	Net Counts	Int. Cps/nA	Weight % Error	Weight %	Atom %	Atom % Error	Formula	Standard Name
O K	2234	---	51.00	+/- 1.12	64.74	+/- 1.42	O	
Na K	497	---	6.24	+/- 0.39	5.51	+/- 0.34	Na	
Si K	3659	---	37.61	+/- 0.75	27.20	+/- 0.54	Si	
Si L	0	---	---	---	---	---		
Ca K	235	---	4.71	+/- 0.64	2.39	+/- 0.32	Ca	
Ca L	0	---	---	---	---	---		
Fe K	7	---	0.44	+/- 0.81	0.16	+/- 0.30	Fe	
Fe L	0	---	---	---	---	---		
Total			100.00		100.00			

Table.1. Compositional results of bioglass doped in Fe.

ii. BG-Ag

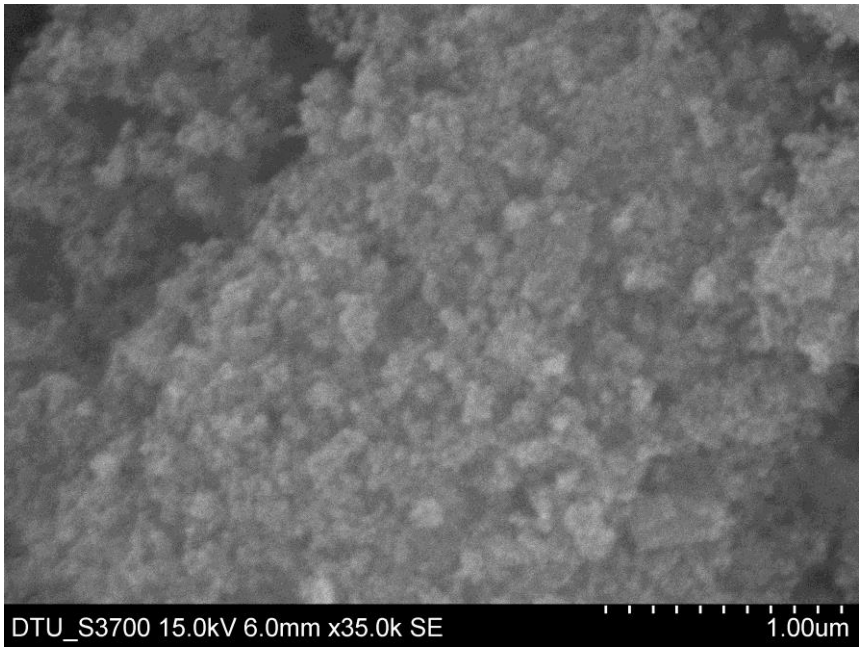


Fig.6. SEM micrograph of bioglass doped in Ag at 1 µm scale and 35kX magnification

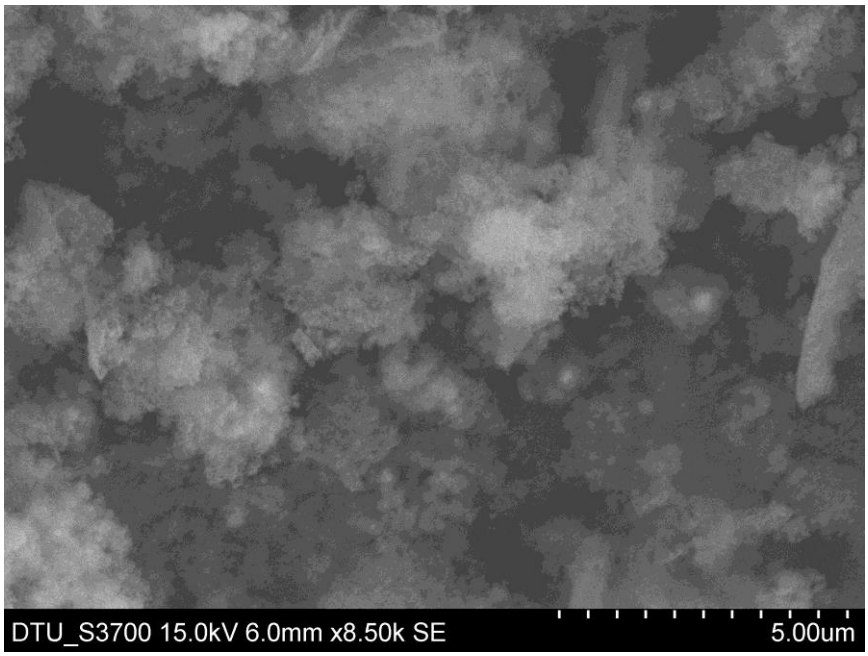


Fig.7. SEM micrograph of bioglass doped in Ag at 5 µm scale and 8.5kX magnification

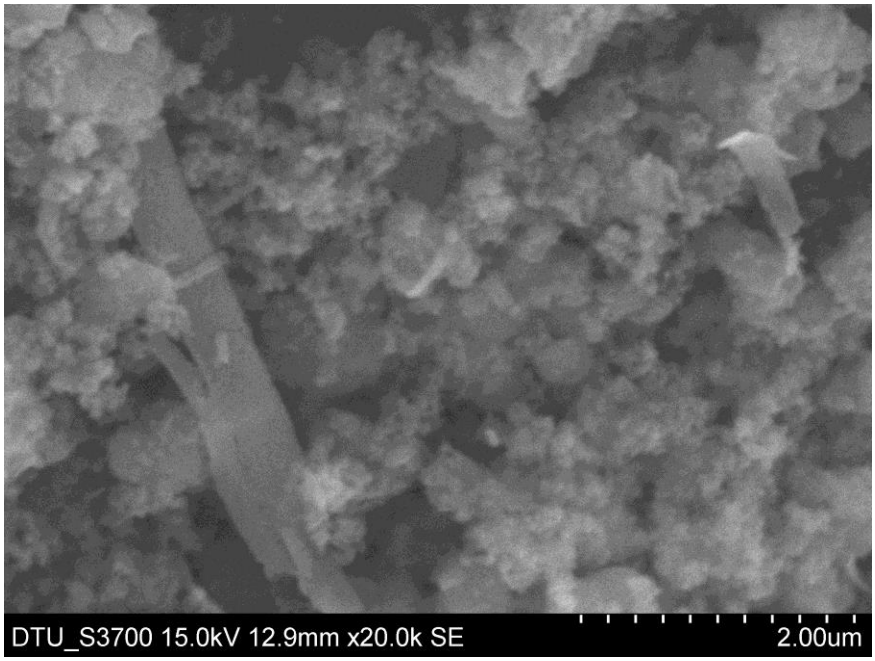


Fig.8. SEM micrograph of bioglass doped with Ag at 20kX magnification

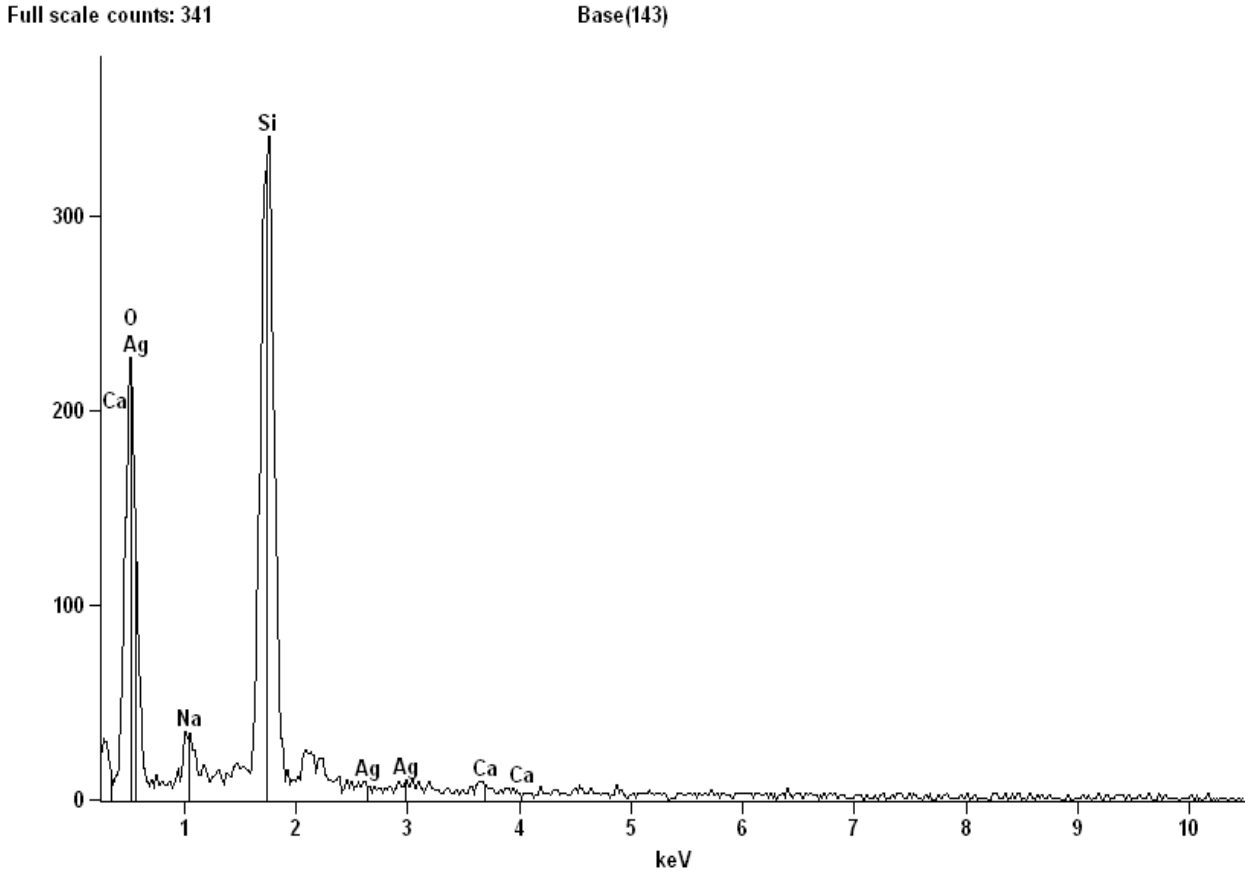


Fig. 9. EDS spectra of bioglass after doping with Ag showing peaks of Si, Ag, O, Na and Ca

Element Line	*Net Counts*	*Int. Cps/nA*	*Weight %*	*Weight % Error*	*Atom %*	*Atom % Error*	*Formula*
O K	2141	---	52.84	+/- 1.18	66.72	+/- 1.50	O
Na K	226	---	3.03	+/- 0.35	2.66	+/- 0.31	Na
Si K	4194	---	41.50	+/- 0.78	29.85	+/- 0.56	Si
Si L	0	---	---	---	---	---	
Ca K	46	---	0.87	+/- 0.27	0.44	+/- 0.13	Ca
Ca L	0	---	---	---	---	---	
Ag L	66	---	1.75	+/- 0.66	0.33	+/- 0.12	Ag
Ag M	0	---	---	---	---	---	
Total			100.00		100.00		

Table 2. Compositional spectra of bioglass doped in Ag.

iii. Bg-Cu

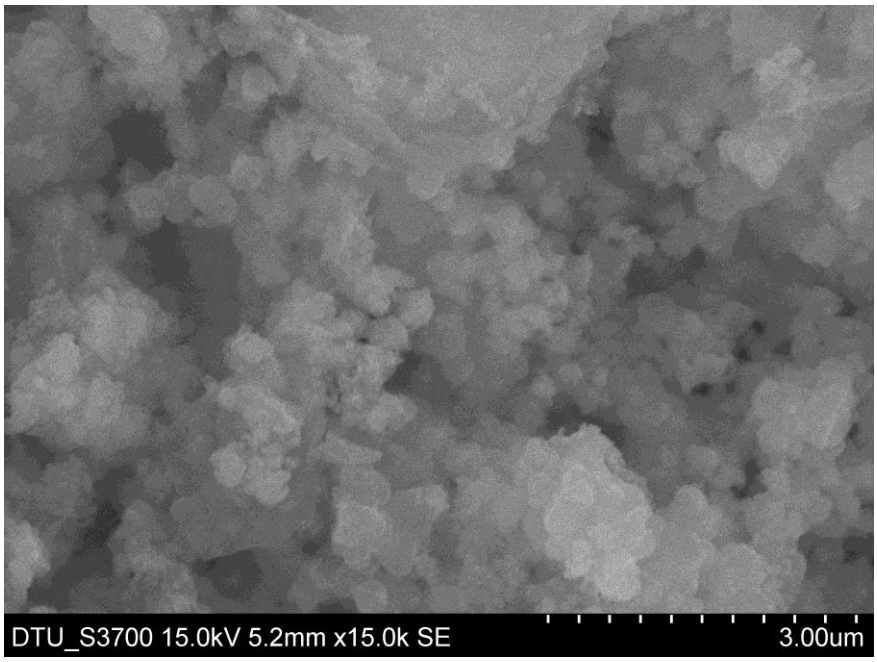


Fig.10. SEM micrograph of bioglass doped with Cu at 15kX magnification

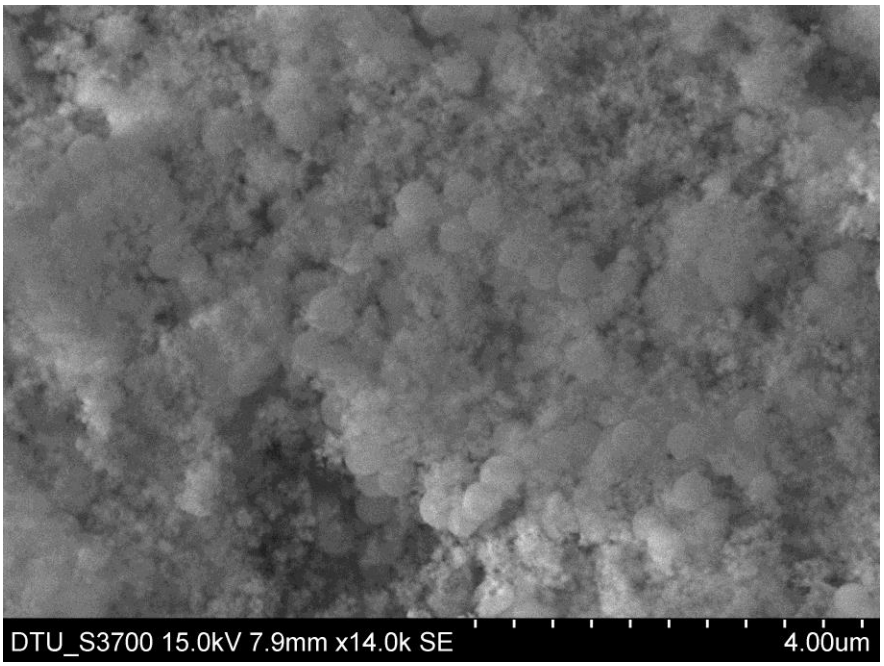


Fig.11. SEM micrograph of bioglass doped with Cu at 14kX magnification

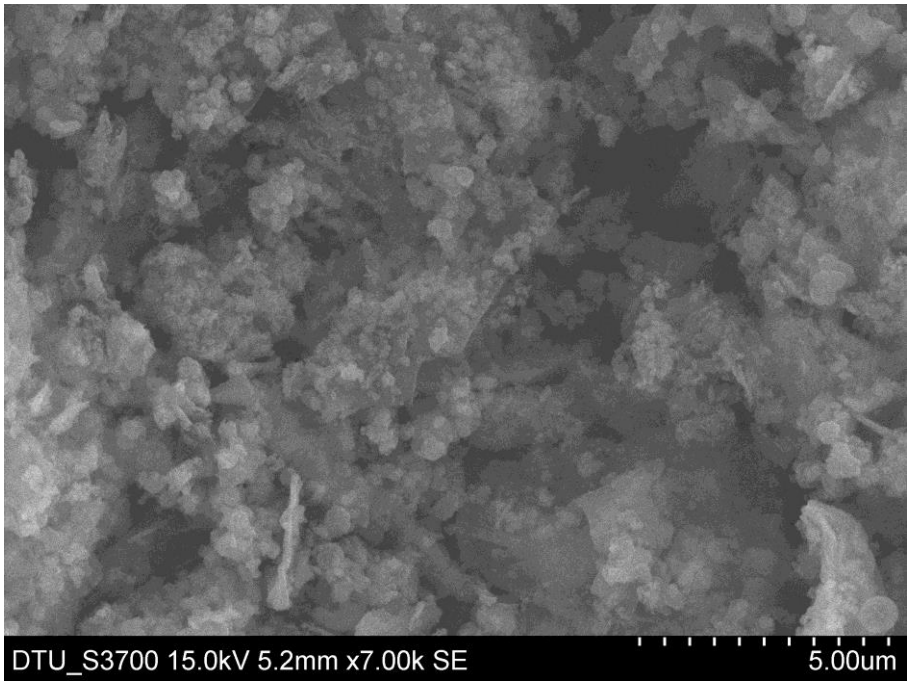


Fig.12. SEM micrograph of bioglass doped with Cu at 7kX magnification

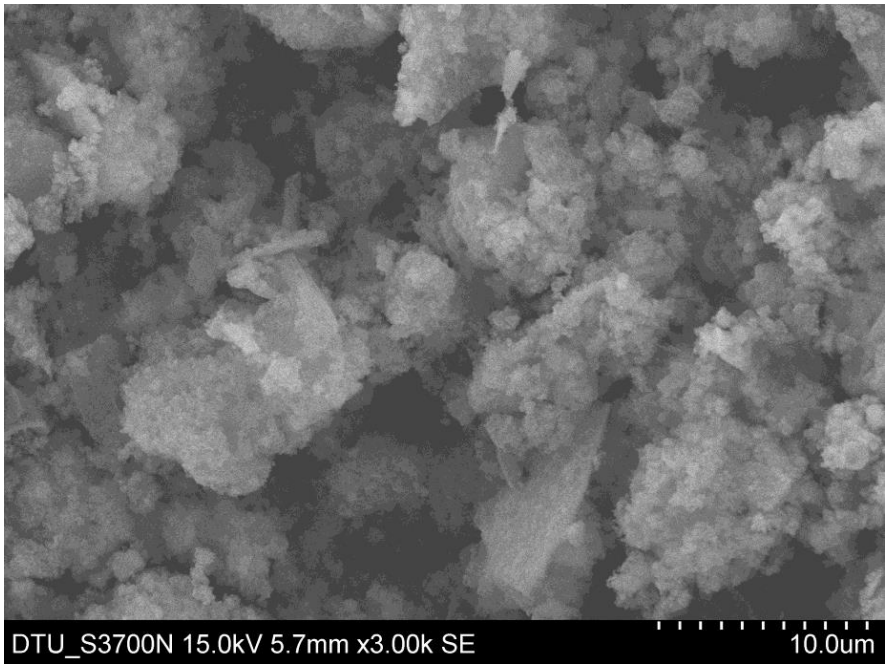


Fig.13. SEM micrograph of bioglass doped with Cu at 3kX magnification

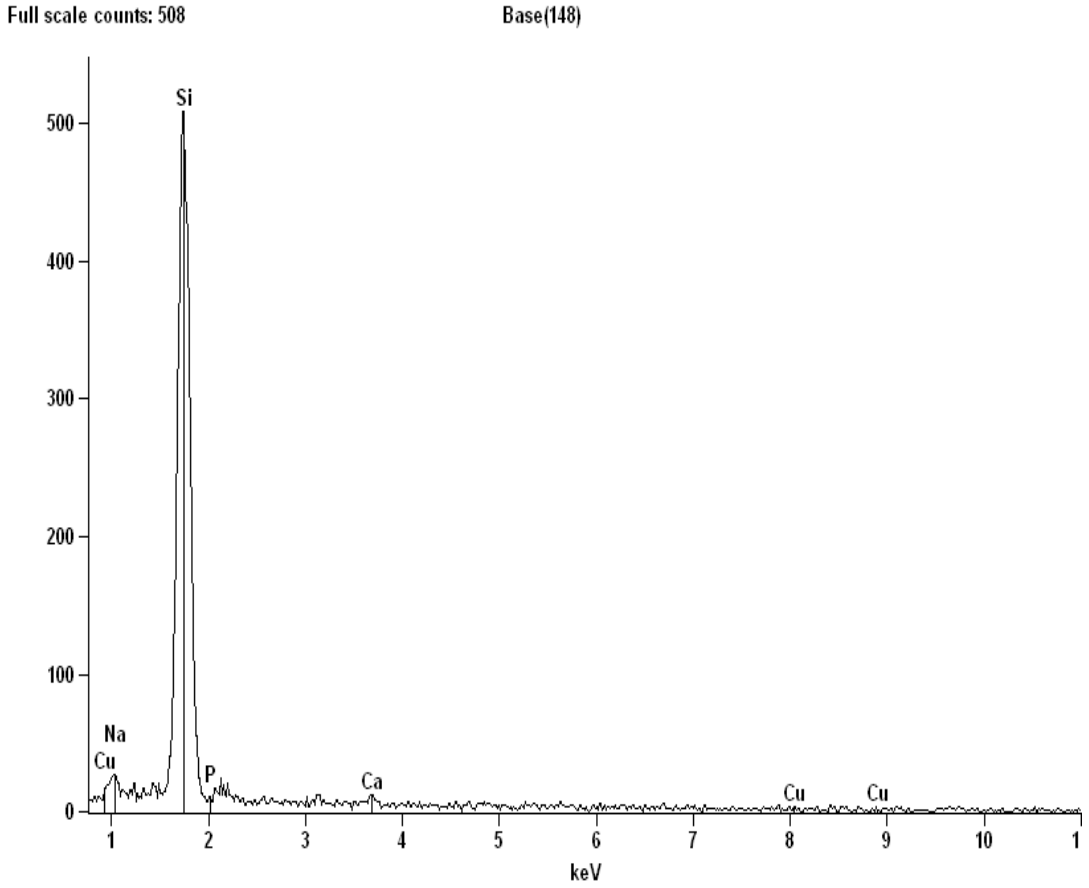


Fig.14. EDS spectra showing peaks of Cu, Si, Na, P and Ca

Element Line	*Net Counts*	*Int. Cps/nA*	*Weight %*	*Weight % Error*	*Atom %*	*Atom % Error*	*Formula*
Na K	132	---	2.04	+/- 0.29	2.50	+/- 0.36	Na
Si K	6212	---	95.49	+/- 1.49	95.76	+/- 1.50	Si
Si L	0	---	---	---	---	---	
P K	0	---	0.00	---	0.00	+/- 0.00	P
P L	0	---	---	---	---	---	
Ca K	71	---	2.47	+/- 0.52	1.74	+/- 0.37	Ca
Ca L	0	---	---	---	---	---	
Cu K	0	---	0.00	---	0.00	+/- 0.00	Cu
Cu L	64	---	---	---	---	---	
Total			100.00		100.00		

Table 3. Compositional spectra of bioglass doped in Cu

II. XRD

Following Fig.15, 16 and 17 shows wide angle XRD pattern of Ag, Fe, Cu-doped bioglass sample respectively. The appearance of a broadband from 2θ 20-30 degree shows the amorphous nature of the bioglass sample. While in the figure 15, the presence of sharp band from 2 theta 30-32 and 45-46 degree shows the presence of crystallinity in the Ag doped sample.

i. Bg-Ag

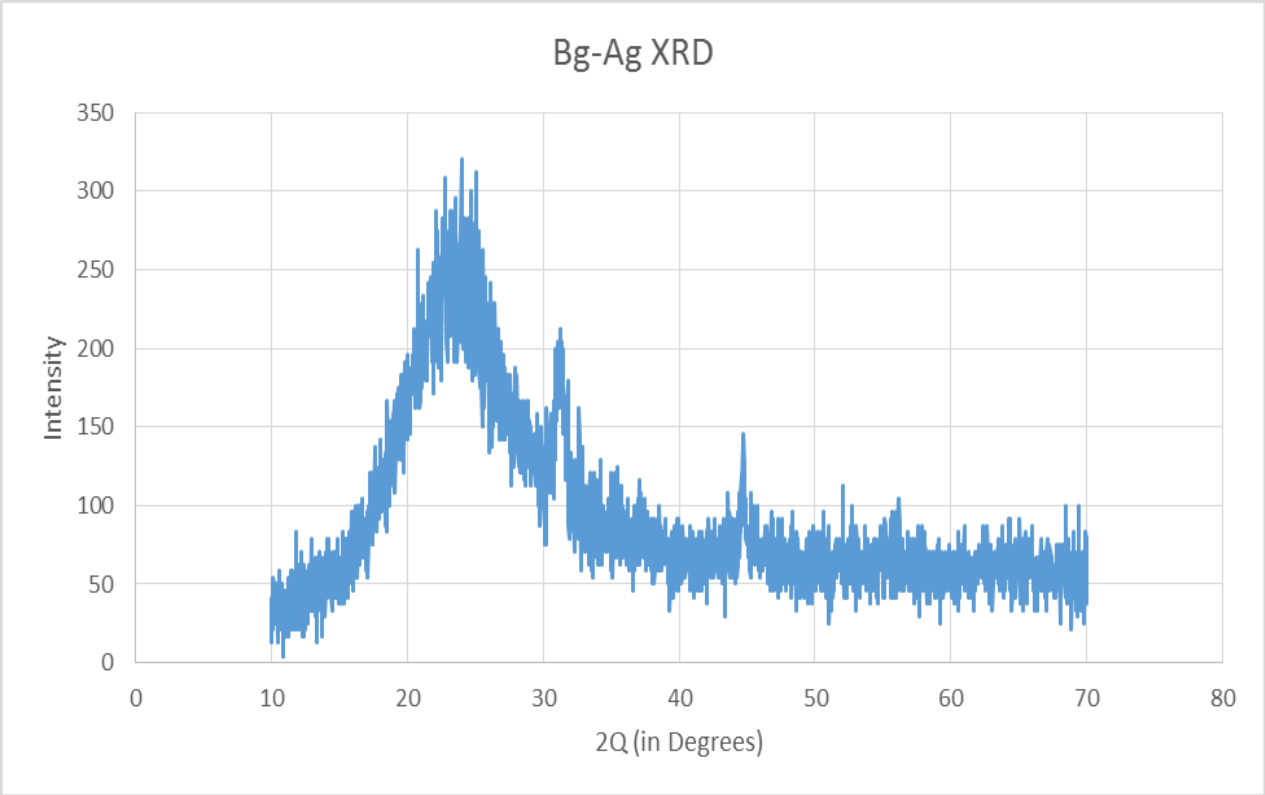


Fig.15. Wide angle XRD pattern of prepared doped bioglass in Ag

ii. Bg-Fe

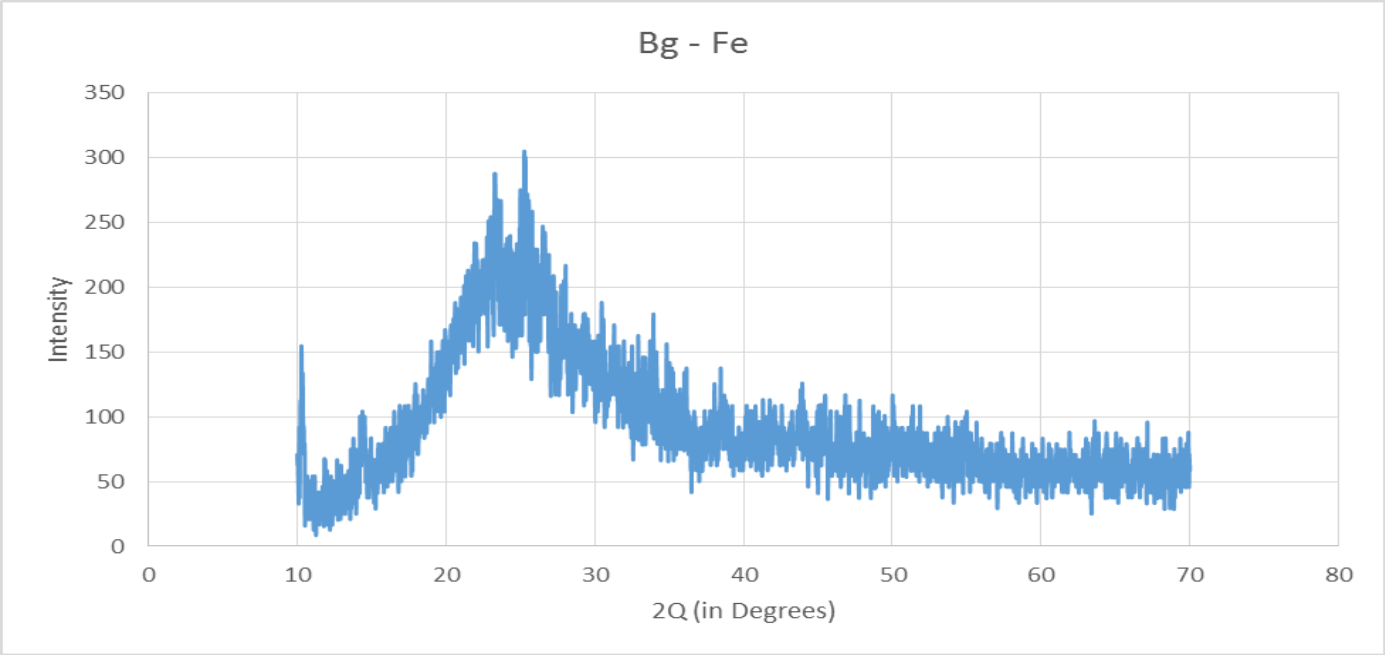


Fig.16. Wide angle XRD pattern of prepared doped bioglass in Fe

iii. Bg- Cu

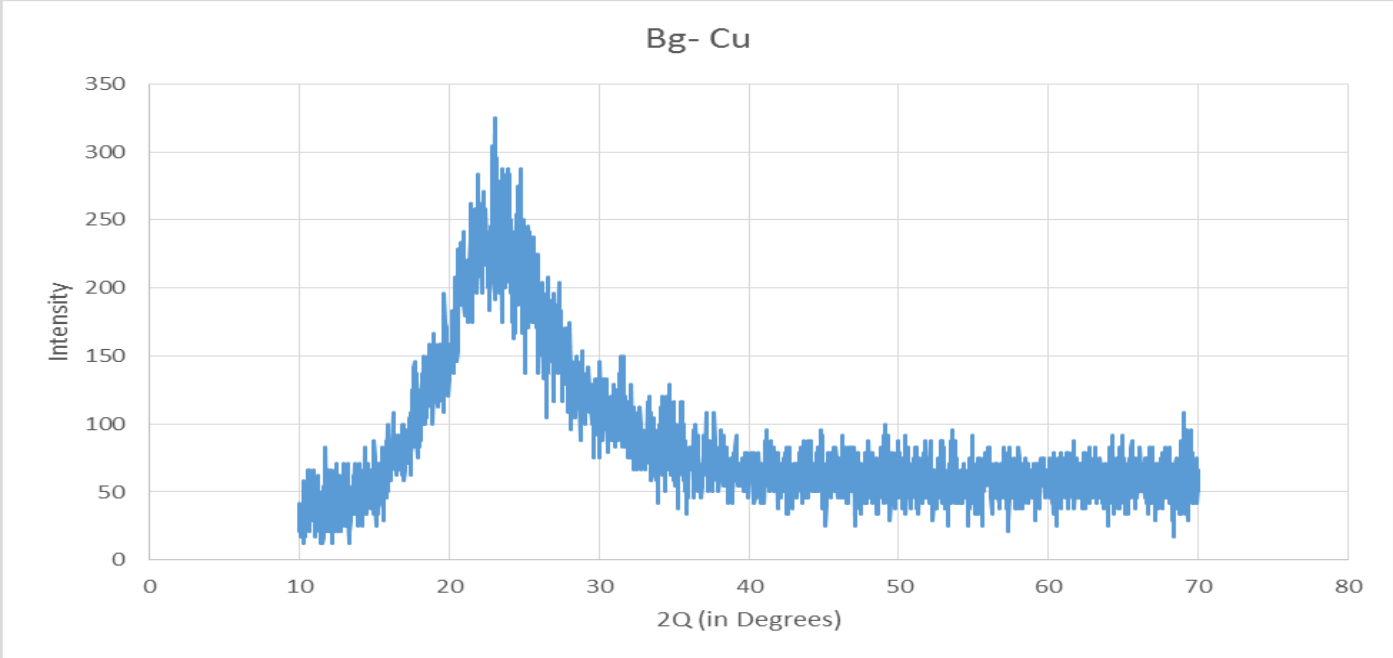


Fig.17. Wide angle XRD pattern of prepared doped bioglass in Cu

III. FTIR

Figure 18-21 shows FTIR spectra of Bioglass before and after doping with Ag, Fe and Cu. The different vibrational bonds existing in the samples are listed in the Table no. 4.

Sample Name	Wave Number	Vibrational Bonds
Bioglass	**500**	**$(Po_4)^-$**
	900	**Si – O - Si**
	1000	**$(Po_4)^-$**
	1600	**$(CO_3)^{2-}$**
	1800	**OH**
Bioceramic (Ag doped)	**499**	**$(Po_4)^-$**
	750	**Si – O – Si**
	1100	**$(Po_4)^-$**
	2300	**Si - Ag**
Bioceramic (Cu doped)	**500**	**$(Po_4)^-$**
	750-800	
	1100	**$(Po_4)^-$**
	1501	
	1700	**$(CO_3)^{2-}$**
	2400	
	3600	
	3700	
	3750	
Bioceramic (Fe doped)	**480**	
	700	
	1100 – 1200	**$(Po_4)^-$**
	1550	**$(CO_3)^{2-}$**
	2350 – 2400	
	3400	

Table 4 Vibrational bonds and corresponding wave number

i. Bioglass (pure i.e. CTAB based)

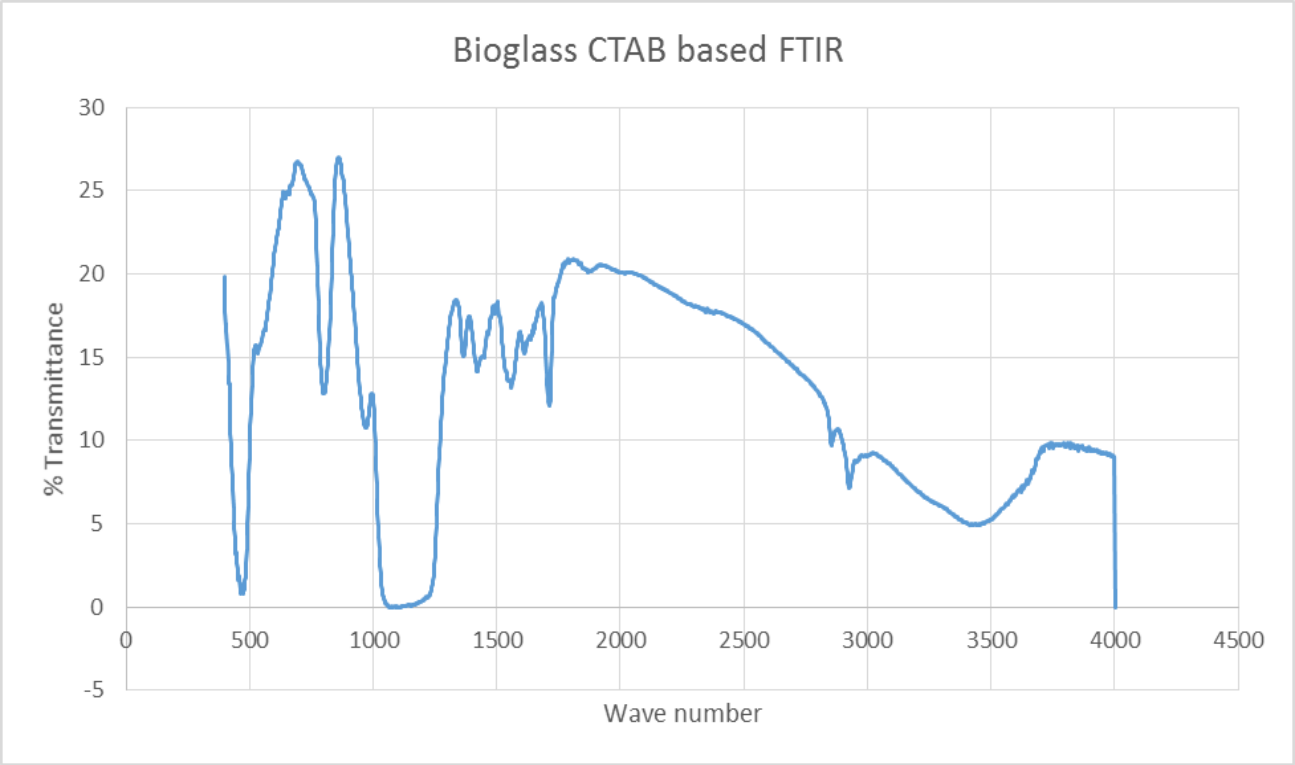


Fig.18. FTIR graph of CTAB based Bioglass

ii. Bg-Ag

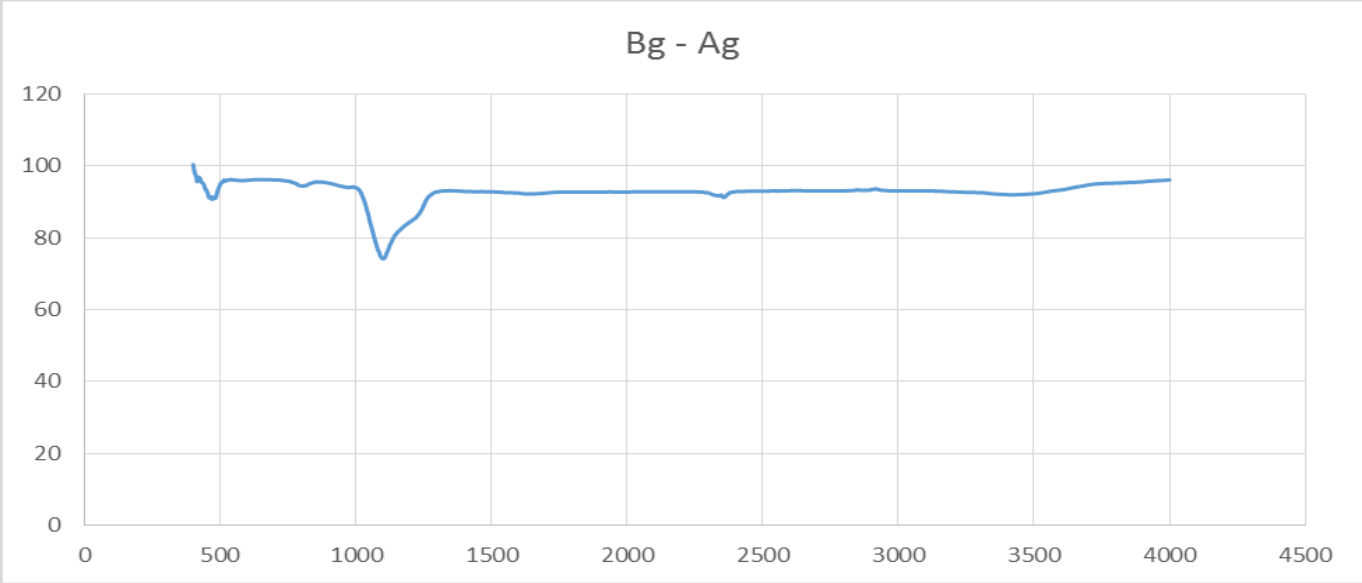


Fig.19. FTIR graph of Ag doped Bioglass

iii. Bg-Cu

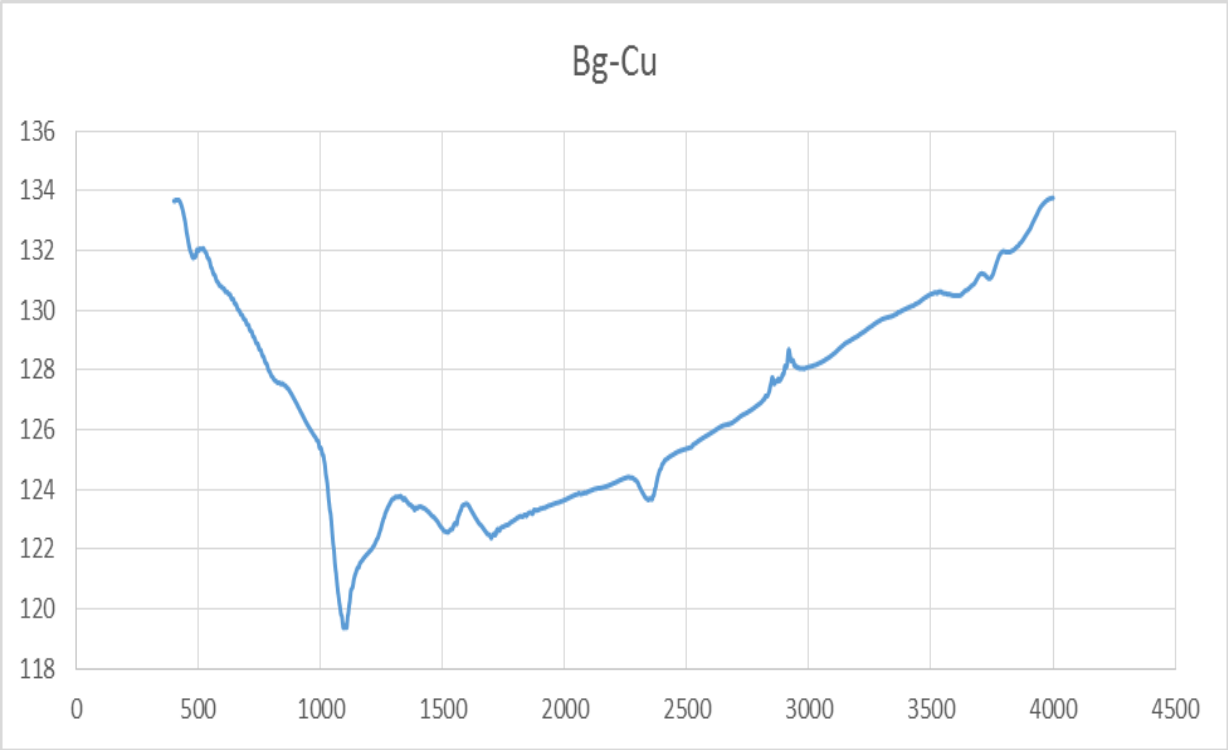


Fig.20. FTIR graph of Cu doped Bioglass

iv. Bg-Fe

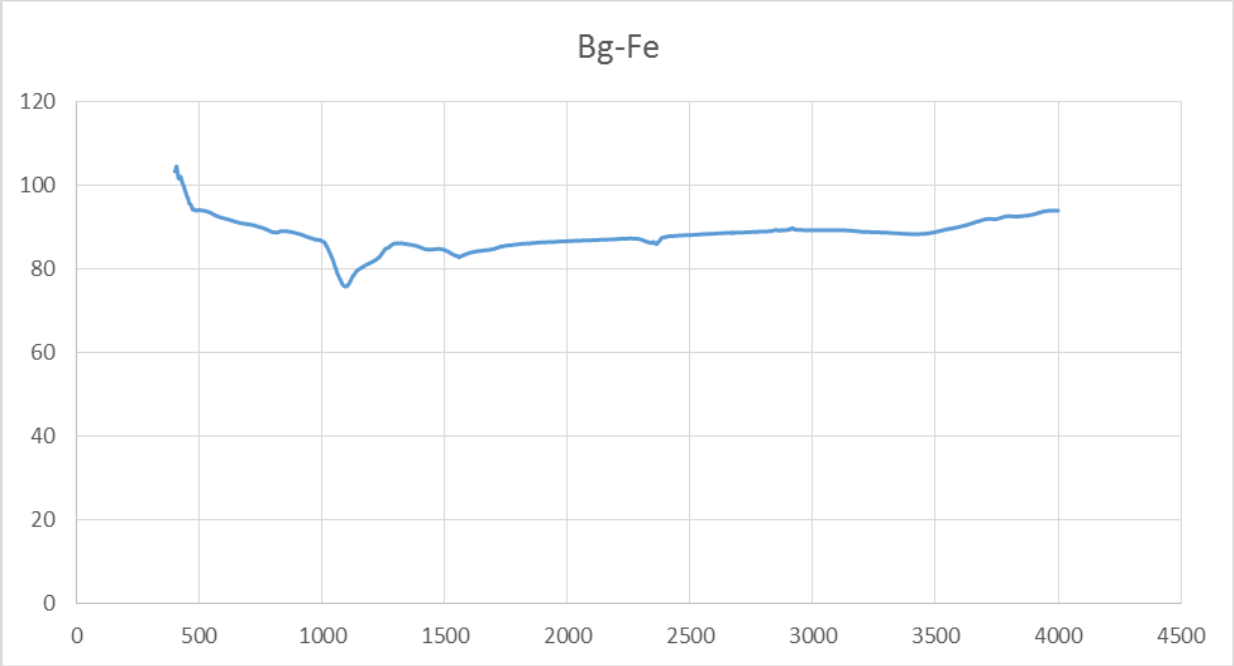


Fig.21. FTIR graph of Fe doped bioglass

IV. Uv-Vis of E-Coli

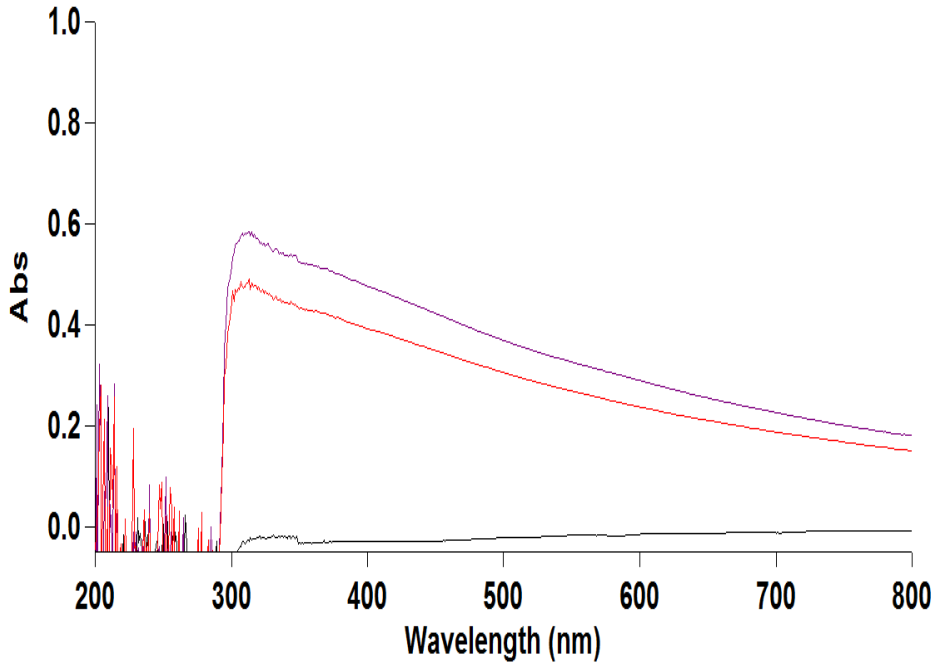


Fig.22.Optical Density of 0.6 was recorded at the wavelength of 310nm

V. Anti- Microbial Testing

A. General Preparation

In the figure 23, proper growth of the E.coli culture could be observed in our controlled petridish (without any dopant). In comparison to this, after incubating the culture with Ag, Fe and Cu dopants, a formation of inhibition zone was observed. Herein, the different doped samples were studied in two forms 1. Pellet form and 2. Diluted form.

The pellet form of sample (Fig. 24 -26) was first soaked in SBF and then incubated with the culture. After 10 hours of incubation, inhibition zone of various diameters could be observed according to the antibacterial activity of the samples.

After 10 hours of incubation, we observed that the doped bioceramic pellets also displayed the anti-bacterial propertyas displayed in the figure below. Bioglass which was doped with copper and iron showed more anti-bacterial property than the one which was doped with silver. This result was applicable to pellet form dopants.

The diluted form (Fig. 27 -29) of the doped sample was prepared according to the concentration of 1mg/mL in SBF. Of this 20uL was poured into the wells of each petridish.

After, again 10 hours of incubation, we observed anti-bacterial wall or the inhibition zone being developed due to the presence of diluted form of the dopants as shown in figure.

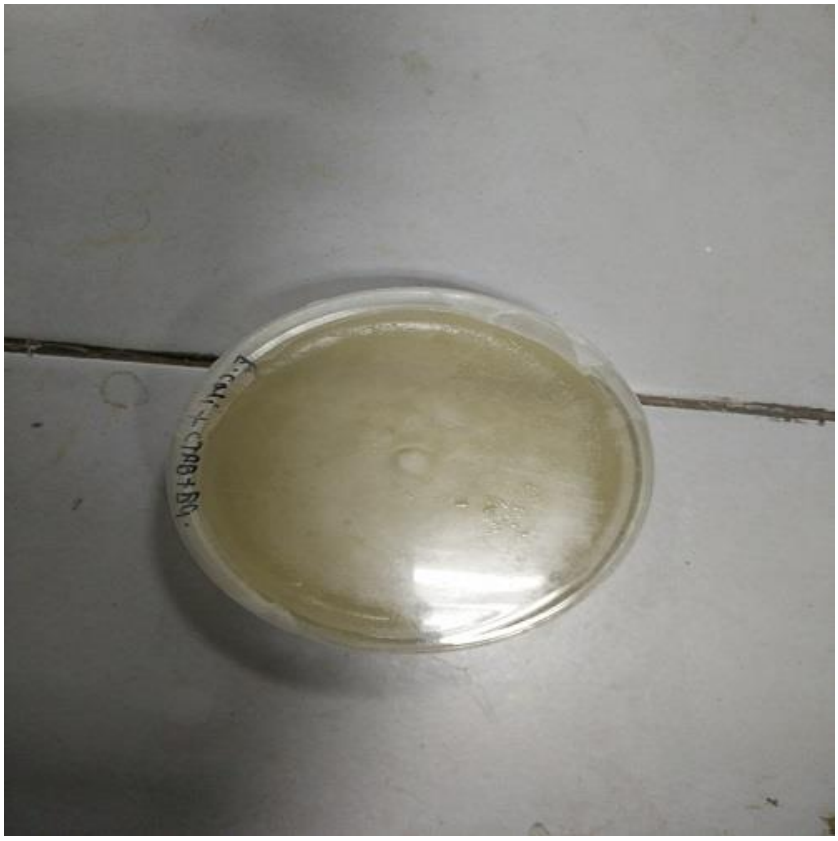


Fig.23.Petridish contains only the E. Coli, and CTAB based BG.

A. Pellet form Test:

i. Fe Pellet

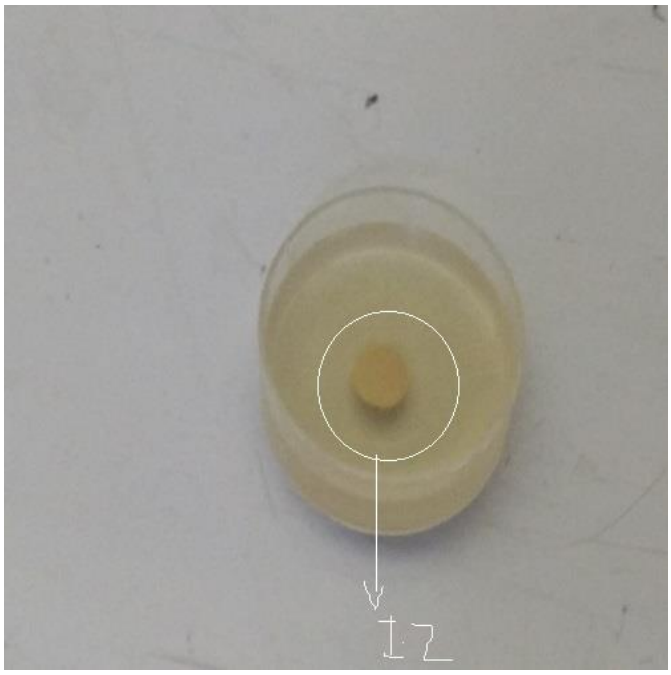


Fig.24. Pellet Fe soaked in SBF and immersed in medium containing E.Coli

ii. Cu Pellet

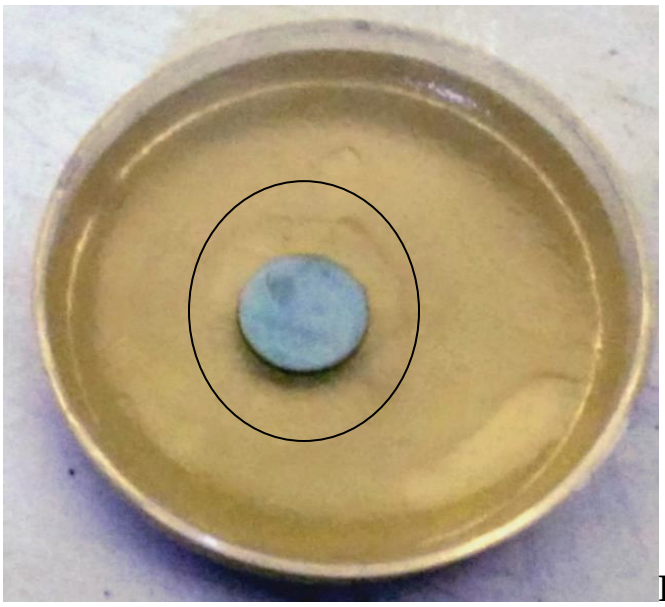


Fig.25. Pellet Cu soaked in SBF and immersed in medium containing E.Coli

iii. Ag Pellet

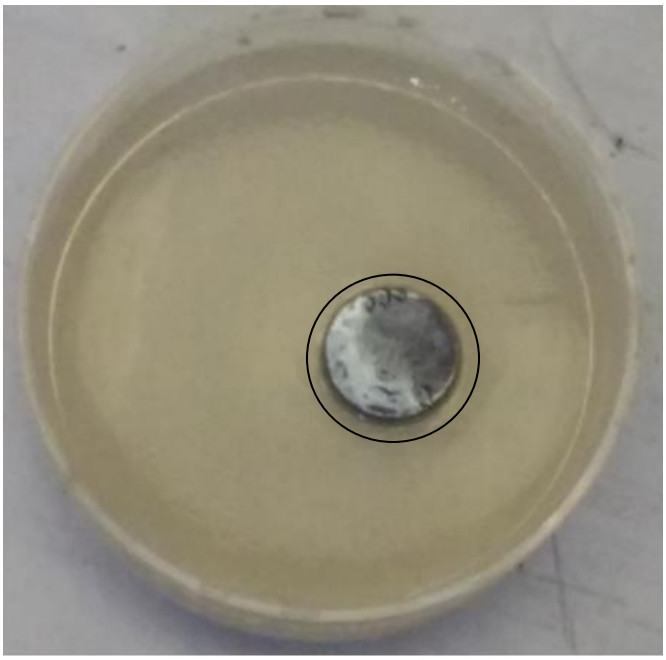


Fig.26. Pellet Ag soaked in SBF and immersed in medium containing E.Coli

B. Diluted form Test:

i. E. Coli and Fe doped Bg

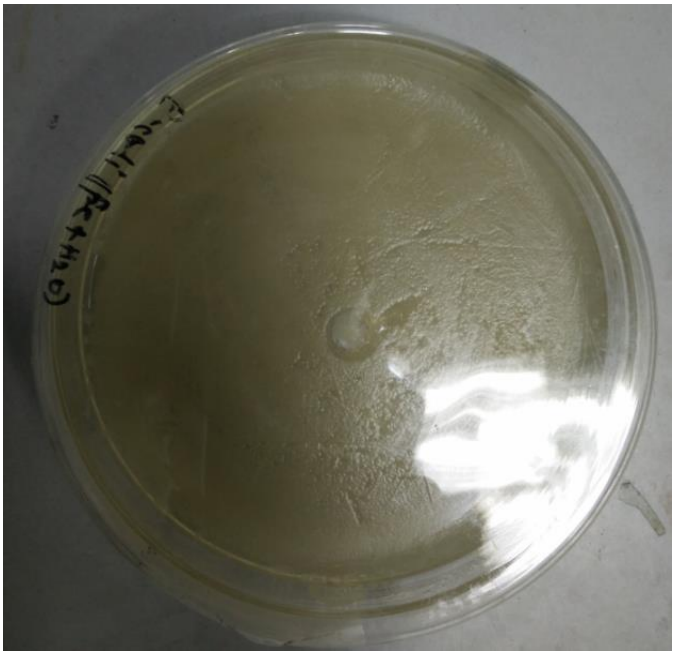


Fig.27. Diluted Fe Anti-Microbial Testing

ii. E. Coli and Cu doped Bg

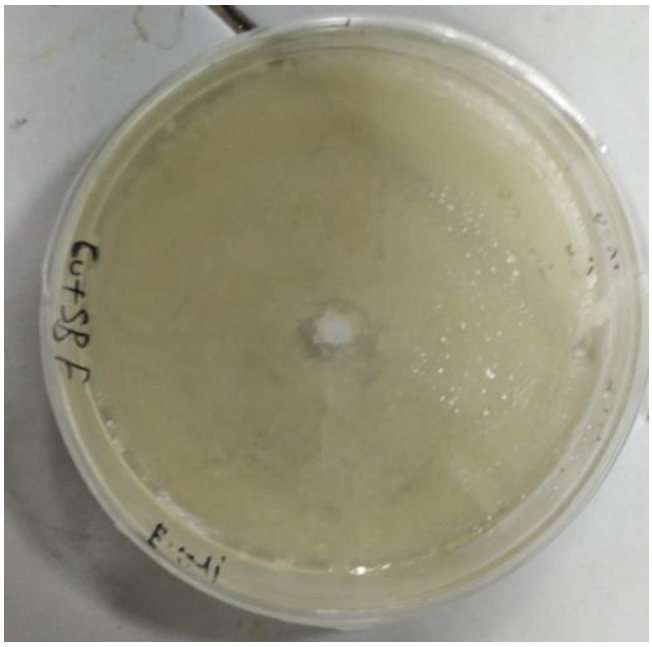


Fig. 28 Diluted Cu Anti-Microbial Test

iii. E. Coli and Ag doped Bg

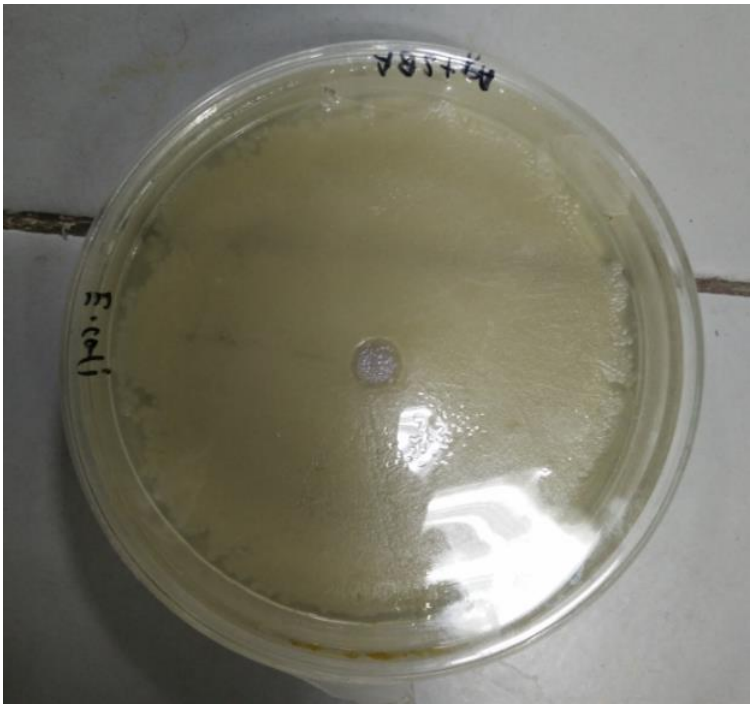


Fig. 29 Diluted Ag Anti-Microbial Test

CHAPTER 10

Application:

Bone-regeneration and repair:
Healing of small bone defects and regeneration of bones after damage or fracture is an effective process. But, the challenge arises when there are defects due to deficient healing through various causes vis-à-vis osteoporosis, fractures involving bone sites of poor vascularity etc. So because of this, the magnitude or number of fracture related to osteoporosis has increased multi-fold in the last decade.
As we know that Osteoporosis occurs frequently in women after menopause phase because of loss of estrogen, but bone loss is known to happen in both men and women with ageing. A number of other situations related to osteoporosis are e.g. diabetes mellitus etc, are known to affect skeletal healing (optimally) after trauma etc.
This is a major challenge to our healthcare systems and thus bone regeneration is an important clinical issue in regenerative medicine. Thus, therapeutic strategies to improve bone healing in these circumstances are the need of the hour and is of utmost importance.

Bone is produces through a series of Bio-mineralization processes, which is a series of physio-chemical reactions that give rise to formation of organic – inorganic nano-composites with superior and excellent mechanical properties that would be impossible to attain with pure materials. The tissue is made up of a cellular components and an extracellular matrix consisting of an organic phase, mainly type 1 collagen, and an inorganic ceramic phase of calcium - deficient carbonated hydroxyapatite (CHA) nanocrystals. Now, the main characteristics of biological apatites are: Variable composition, nano crystal size, calcium deficient, presence of carbonate groups and structural disorderness. Another characteristic of apatite surface is its ability to fit in ions in its sub – lattice surface for e.g. calcium, phosphate and hydroxyl etc.
These apatite nanocrystals grow at ordered mineralization sites of collagen molecules in bone. Greater than 200 bone pieces with varying lengths and different shapes constitute the human skeleton. All of these provide a hierarchical structure ranging from lacunae, lamella, to macroscopic materials. This hierarchical porosity thus must be reproduced in the design of new biomaterials for bone regeneration and repairing of the hard tissues.

CHAPTER 11

Future Scope:

Interestingly the anti-bacterial activity of the sample has been evaluated in the project still many aspects need to be studied to further validate the anti-bacterial activity of the sample. Such as Growth curve study of the doped sample need to be evaluated, TEM morphology of pellet samples need to be observed to view the effect of dopants doped bioglass with the E. Coli. Many other *in-vitro* experiments such as bioactivity evaluation of the doped sample in SBF need to be carried out.

In the present project anti-bacterial activity has been evaluated against E. Coli (Gram Positive bacteria) only. Further the anti-bacterial activity of above needs to be evaluated against Gram Negative bacteria. Such study is necessary to prove the anti-bacterial action of the samples against broad-spectrum of bacteria.

CHAPTER 12

References:

1. D. F. Williams, Definitions of Biomaterials, Elsevier, Amsterdam(1987)

2. J. F. Shackelford, Bioceramics: Current status and future trends, Material Science Forum 293, 99-106.

3. V.A. Dubok, Bioceramics - Yesterday, today, tomorrow, Powder metal Met. C+ 39 (7-8), 381-394.

4. M. Vallet-Regi, Ceramics for medical applications, J. Chem. Soc. Dalton(2) 97-108.

5. D.F. Williams, The Biocompatibility and Clinical Uses of Calcium Phosphate Ceramics, in Biocompatibility of Tissue Analogs (II 43-66).

6. S.F. Hulbert, J.C. Bokros, L.L.Hench, J.Wilson, & G.Heimke, Ceramics in Clinical Applications: Past, Present and Future, pp. 189-213 in High Tech Ceramics.

7. L. L.Hench, Bioceramics: From Concept to Clinic.

8. S. F. Hulbert, L.L.Hench, D. Forces & L. Bowman, History of bioceramics, pp. 3-29 in Ceramics in Surgery.

9. L.L.Hench, Prosthetic implant materials, Ann. Rev. Material Science 5, 279-300

10. K. De Groot, Medical Applications of Calcium Phosphate Bioceramics.

11. K.Hayashi, N.Matsuguchi, K.Uenoyama, *et al.*, Re-evaluation of the biocompatibility of bioinert ceramics in vivo, Biomaterials 13(4), 195-200.

12. S. F. Hulbert, & J. T. Klawitter, Potential of ceramic materials as permanently implantable skeletal prosthetic devices, J. Biomed. Mater. Res. 4, 433-456.

13. K. De Groot, Effect of Porosity and Physicochemical Properties on the Stability Resorption and Strength of Calcium Phosphate Bioceramics, Ann. N.Y. Acad. Science, 523, 227.

14. H. S. Cheung, & M. H. Haak, Growth of osteoblasts on porous calcium phosphate ceramic: an in vitro model for biocom. Study, Biomaterials 10 (1), 63-67.

15. L.L.Hench, R.J. Splinter, W. C. Allen & T. K.Greenlee, Bonding mechanisms at the interface of ceramic prosthetic materials, 117-41 (1971).

16. B.A. Blenke, E.H. Pfeilbromer & H.H.Kas. Langen bechs Arch. Chir. Suppl. Forum 107.

17. T. Kokubo, Novel Ceramics for Biomedical Applications, pp.1-16, in Third Euro Ceramics. Vol. 3.

18. G.Berger, R.Sauer, G.Steinborn, F.Wihsmann, V.Thieme, St.Kholer & H.Dressel, Clinical application of surface reactive apatite / wollast. containing glass ceramics, pp. 120-26.

19. L.L.Hench, Fundamental Aspects of Biocompatibility, D.F.Williams.

20. L.L.Hench & D.E.Clark, Physical Chemistry of glass surface, Journal Non-Crystalline Solids 28(1), 83.

21. U.M.Gross, C.Müller Mai & C Voigt.., Ceravital Bioglass Ceramic, Pp.105-124, in An introduction to bio ceramics. Hench, & Wilson, Editors.

22. U.M.Gross & V.Strunz, Clinical Application of Biomaterials, Pp. 237.

23. K.H.Karlsson, K.Fröberg & T Ringbom, A structural approach to bone adhering of bioglasses, Journal Non - Cryst. Solids, 112, 69-72(1989).

24. Ö.H.Anderson, K.H.Karlsson & K.Kanganiemi, Calcium Phosphate formation at the surface of Bioactive in vivo, Jour. Non-Cryst. Solids, 119, 290-296(1990).

25. T.Kokubo, A - W Glass – Ceramic : Processing and Properties, Pp.75.88, An
Introduction to Bioceramcs, L. L. Hench & J.Wilson Editors Sing.

26. T.Kokubo, S.Ito, S.Sakkay & Y.Yamamuro, Formation of a High Strength Bioglass - Ceramic in the System MgO – CaO - SiO2 - P2O5, Jour. Material Science, 21, 536-540(1986)

27. L.L.Hench, & J.Wilson, Surface - active Biomaterials, Science, 226, 630(1984).

28. L.L.Hench, Bioactive Ceramics, pp. 54 , Bioceramics : Mat. Char. Versus In Vivo Behaviour, Vol 523, Ducheyne, P. Lemons, J.E. editors.

29. U.Gross, R.Kinne, H.J.Schmitz & V.Strunz, The Response of Bone to surface active glass/glass – ceramics, CRC. Crit. Rev. Biocomp.(1988).

30. T.KOKUBO, H.KUSHITAI, C.OHTSUKI, S.SAKKA and T. YAMAMURO, Jour. Mat. Science Mater. Medicine, 3(1992) 79. and T.KOKUBO, H.KUSHITANI and S.SAKKA, Journal Biomedical Mat. Res. 24(1990).

31. M.Bosetti and M.Cannas, The effect of bioglasses on bone marrow stromal cells differentiation, Biomat., no. 18, pp. 3873-3879(2005).

32. L.L.Hench, Genetic design of Bioglass, J. of the Euro. Cer. Soc., vol. 29, no. 7, pp. 1257-1265(2009).

33. L.L.HENCH, Jour. Ameri. Cerami. Society, 74(7),(1991).

34. L.L.HENCH, R.J.SPLINTER, W.C.ALLEN and T.K.GREENLEE, Jour. Biom. Materiald Res. 2(1971),1176 and H.OONISHI, L.L.HENCH, J.WILSON, F.SUGIHARA, E.TSUJI, M.MATSUURA, S.KIN, T.YAMAMURO and

S.MIZOKAWA, Jour. Biomedical Material Res, 51(2000).

35. P.DUCHEYNE and J.M.CUCKLER, Rev.Clin.Ortho.Rel.Res, 277(1992).

36. M.VALLET - REGI, Jour. Chemistry Society Dalton Trans. 5(2001).

37. E.VERNE, M.BOSETTI, C.VITALE - BROVARONE, C.MOISESCU, F.LUPO, S.SPRIANO and M.CANNAS, Biomat. 23(2002)-3395.

38. E.VERNE, R.DEFILIPPI, G.CARL, C.VITALE - BROVARONE and P. APPENDINO, J.European Ceramic Society 23(2003)-675.

39. W.A.Deer, R.A.Howie & J.Zussman, Non-silicate : Sulphate, Carbonates, Phosphate & Halides, An introduction to Rock – f orming minerals. Volume 5-B.

40. R.A.Young, Some aspects of crystal structural modeling of biological apatites, Col. In. CNRS 230, 21-40(1973).

41. B.Kerebel, G.Daculsi and A.Verbaere, High res. elec. microscopy & crystallo. study of some boil. Apatites, J.Ultrastruc. Res.57, 266-275(1976).

42. S.Jackson, A.G.Cartwright and D.Lewis, The Morph. of bone min. crystals, Calcifer. tissue Res.25, 217-222(1978)

43. M.Neo, S.Kotani, Y.Fujit *et al.*, Differences in ceramic bone interface between surface-active ceramics and resorbable ceramics - A study by scanning and transmission electron-microscopy, Jour. Biomedical Material Res. 26(2), 255-267(1992).