ANTI-MICROBIAL ACTIVITY OF CHITOSAN-SEA URCHIN BIOCERAMIC FOR TISSUE ENGINEERING APPLICATIONS

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By

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ABSTRACT

The main aim of this work is to synthesize and characterise antimicrobial chitosan- sea urchin bioceramic by using N, N' menthylenediacrylamide as a crosslinker via UV irradiated photo-polymerization reaction. Bioceramics were prepared by UV irradiation at $\lambda = 254$nm in the absence of the photoinitiator under heterogenous conditions and solvent casting method. Scanning Electron Microscope (SEM), X-ray Diffraction (XRD) analysis, and antimicrobial susceptibility testing (disc diffusion method) were applied to characterise the chitosan sea urchin (CU) blended bioceramics.

Five CU blend bioceramics samples were tested against six human pathogens using the disc diffusion method. CU1, CU2 and CU4 were found to be the most effective against *E.coli*. CU1 and CU2 were effective against *B.cereus*, *P.aeruginosa* and showed a larger inhibition zone when compared with E.coli. The XRD analysis of CU showed that the degree of crystallinity of the CU is higher than that of the pure chitosan. SEM analysis indicated that the bioceramic have porous structures, elongated but bonded to each other. The porous structure of the CU bioceramic suggests the likelihood of cell attachment and possibly proliferation.

These results demonstrated that the chitosan- sea urchin bioceramic have potential applications in tissue engineering.

Keywords: Chitosan; sea urchin; bioceramics; photopolymerization; antibacterial

ÖZET

Bu çalışmanın amacı, biyomedikal uygulamalarda kullanılmak üzere, N, N' metilen diakrilamid çapraz bağlayıcı kullanarak fotopolimerizasyon reaksiyonu ile kitozan-deniz kestanesi biyoseramikleri sentez ve karakterizasyonunu yapmaktır. UV-fotopolimerizasyon tekniği ileλ = 254 nm, heterojen koşullarda oluşturulan biyoseramikler Taramalı elektron Mikroskopu (TEM), X-ışın difraksiyonu (XID), antibakteriyel duyarlılık testi ve Disk yayılım Metodu kullanılarak karakterize edildiler.

Biyoseramik örneği altı insan patojeni kullanılarak disk yayılım metodu ile test edildiler. CU1, CU2 ve CU4 örnekleri *E.Coli* ye karşı en etkili byoseramik örnekleri olarak gözlemlendi. *B.cereus* ve *P.aeruginosa* karşı ise *E.coli* den olduğından daha etkili oldukları gözlemlenmiştir.Biyoseramiklerin XID analizleri sonucu, kitozan-deniz kestanesibiyoseramiklerin, saf kitozandan daha kristal bir yapıya sahip olduğunugöztermiştir. TEM ile yapılan analizlerde Kitozan-deniz kestanesibiyoseramklerin, dağınık gözenekli ve birbirine bağlı olarak yayılmış bir yapıya sahip olduğu göstermiştir. Bu yapının hücre yayılımı ve bağlanmasını destekleyici olduğu önerilmektedir.

Çalışma sonuçları, kitozan-deniz kestanesibiyoseramiklerinin doku mühendisliği çalışmalarında kullanma alanlarının çok fazla olabileceğini göstermektedir.

Anahtar Kelimeler: Kitozan; deniz kestanesi; UV-işin fotopolimerizasyonu; anti-bateriyel özellik

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LIST OF ABBREVIATIONS

CU:	Chitosan-Urchin
DD:	Degree of deacetylation
SEM:	Scanning electron microscope
FTIR:	Fourier Transform Infrared Spectroscopy
XRD:	X-ray diffraction
Mw:	Molecular weight
MHA:	Muellen Hinton Agar
MIC:	Minimum inhibitory concentration
C. albicans:	*Candida albicans*
E.coli:	*Escherichia coli*
B. cereus:	*Bacillus cereus*
S. aureus:	*Staphylococcus aureus*
P.aeruginosa:	*Pseudomonas aeruginosa*
E.facealis:	*Enterococcus faecalis*
mm:	Millimetres
PM:	Peristomal membrane
P.lividus:	Paracentrotus lividus
TD:	Test Diameter
EDTA:	Ethylene-Diamine-Tetra-Acetic acid
S.enterica:	*Salmonella enterica*
V.Vulnificus:	Vibrio Vulnificus
Spp:	Species
CLSI:	Clinical and Laboratory Standards Institute
ATCC:	American Type Culture Collection
W/v:	Weight per volume

CHAPTER 1
INTRODUCTION

1.1 Chitosan

Chitosan is an amino-polysaccharide biopolymer produced from chitin by partial deacetylation with sodium hydroxide. It is the main structural component of the exoskeleton of arthropods (crustaceans and insects) and can be found in some fungal cell walls, marine diatoms and algae (Kumar, 2000; Tharanathan and Kittur, 2003).

Chitosan has three reactive functional groups; an amino group, primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions (Furaski et al.,1996). Hence, it is composed of random beta (1-4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Tharanathan and kittur, 2003).

Singla and Chawla (2001) noted that different properties of chitosan such as degree of deacetylation (DD; 75-95%), molecular weight (Mw; 50-2000kDa), pKa values, and viscosities are largely dependent of the variation of proportions of the two monosaccharides in chitosan. Commercial chitosan have deacetylated units >85% and molecular weights ranging from 100- 1000kDa. Molecular weights (Mw) can be grouped into low, medium and high molecular weights though not specified standard but it is accepted that Low Mw < 50kDa, Medium Mw 50-150kDa and High >150kDa (Rejane et al.,2009).

Chitosan is a weak base that is soluble in dilute aqueous acidic solution as long as it is below pka (~6.3) but its insoluble in water. At its pka of ~6.3 it can change glucosamine units ($-NH_2$) into a soluble protonated form ($-NH_{+3}$). Chitosan's solubility is dependent upon its biological origin, molecular weight and degree of acetylation (Shepherd et al., 1997).

Chitosan is soluble in acidic aqueous media because of the presence of free amine group present along its chain and due to the protonation of these groups rendering the chitosan salt soluble. Nature of the salt counter-ion, degree of deacetylation, molecular weight, pH, ionic strength are important values that should be accounted for when working with chitosan solutions (Aranaz et al., 2009).

1.2 Biological Properties of Chitosan.

Chitosan and its derivative have shown its relevance commercially in the medical, industrial and pharmaceutical industry. Chitosan has several applications as shown below in table 1.1 and it is due to the possession of properties such as high biocompatibility, low toxicity, ability to biodegrade (Kong et al., 2010).

Table 1.1: Principal properties of chitosan in relation to its use in biomedical applications (Rinaudo, 2006)

Biomedical applications	Principal properties
Surgical sutures	Biocompatible
Dental Implants	Biodegradable
Artificial skin	Renewable
Bone Remodelling	Film forming
Corneal contact lenses	Hydrating agents
Controlled drug release	Non-toxic, biological tolerance
Encapsulating material	Hydrolysed by lysosyme
Wound healing properties	Efficient against bacteria, viruses, fungi

There are other properties of chitosan such as antibacterial property which has been tested invivo (Lee et al., 2009; Moon et al.,2007; Rabea et al., 2003), antimicrobial activity against viruses (Kurita, 2006), hemostatic, analgestic, antitumor, hypocholesterolemic and antioxidant (Koide, 1998; Kumar, 2000; Kumar et al., 2004).

1.2.1 Biodegradation

Biodegradation of chitosan refers to the breakdown of the polymer compound into its monomeric units (N-acetyl-glucosamine and D-glucosamine) and is catalysed by chemicals, enzymes (lysozyme, pepsin, papain, cellulose, pectinase, lipase and proteases) in-vitro and by physical methods (Pantaloen et al., 1992; Darmardji and Izumimoto, 1994; Yalpani and Pantaloen, 1994; Kumar et al., 2004).

The key players in the control of degradation rates are the degree of deacetylation (DD) and molecular weight (Mw). Enhanced cellular adhesion and elongated degradation time is

obtained by using chitosan with a DD close to 0% or100%, intermediate DD shows rapid degradation rates causing a drawback of limited cell adhesion (Frieier et al., 2005). A study reported that chitosan membranes with intermediate DD of 65% to 80% triggered inflammatory reactions that subsided overtime resulting in degradation of the films, granulation tissue formation and osteogenesis while the membranes of about 94% DD elicited minimal degradation, minimal osteogenesis and mild inflammatory reactions (Hidaka et al, 1999).

1.2.2 Biocompatibility

A natural polymer such as chitosan has the ability to interact with living cells, tissues and organs without being toxic or injurious to the body and this property is known as biocompatibility. Chitosan does not trigger immune system reactions and rejections while functioning invitro or invivo. Biocompatibility also considers cytocompatibility, genocompatibility and hemocompatibility. Modifying chitosan to an applicable form by alterations of its surface properties (physical and chemical) through varying degrees of deacetylation, crosslinking, polyethylene glycol treatment, heat treatment, wheat germ agglutinin treatment, graphene reinforcement and oxygen plasma treatment may affect chitosan's biocompatibility and biodegradation (Albanna et al., 2013). In 1995, Shigemasa and Minami documented that chitosan is well tolerated in living tissues which includes the skin, ocular membranes and nasal cavities.

1.2.3 Low toxicity

Chitosan is safe when compared with other natural polysaccharides and this property of low toxicity makes it a biomaterial of choice. In 1968, Arai in his study deduced that chitosan showed low toxicity levels and that the LD50 (lethal dose for 50% of test population) in mice exceeded 16kg and this is similar to that of salt and glucose (Singla and Chawla, 2001).The DD has great effect on chitosan's toxicity and the molecular weight has less effect on its viability (Richardson et al., 1999). DD also affects the solubility, hydrophobicity and the electrostatic interaction with polycations by affecting the number of protonatable amine groups of chitosan. Lower DD nanoparticles showed lower toxicity in-vitro (Huang et al., 2004).

An article by Ylitalo et al. (2002) reported no side effects following chitosan's ingestion for about 12weeks in human studies. The intestinal microbial flora, however, can be affected after prolonged clinical use of chitosan (Tanaka et al., 1997).

1.3 Physical Forms of Chitosan

Chitosan with its blends can be found to exist in some various physical forms. These forms of chitosan are as a result of the process and nature of applications. Chitosan forms includes

1. Resins (beads)/microspheres
2. Hydrogels
3. Films/membranes
4. Fibers
5. Sponge

1. Resins (beads) /Microspheres

This form of chitosan is prepared by various methods which are the solvent evaporation, coacervation and emulsion methods (Peniche, 2003; Genta, 1997). The emulsion technique can be used to prepare smaller and uniform beads(Lim, 1997).The formation of the beads involves a crosslinker and dissolving chitosan in acetic acid which is then mixed for sometime like about 6-7 hrs and the amount of ciprofloxacin hydrochloric is added to the polymeric solution followed with mixing/stirring for some 2hrs (Bodemier et al.,1989; Srinatha et al., 2008).The pH of the mixed chitosan is controlled by a dilute alkali solution at a pH of 4-4.5, after which the solution is collected with a syringe, the beads are formed and are allowed for few minutes. The beads are collected by filtration, washed and dried at 50^0c for 4hr, then left at room temperature for 12hrs and they can be used in different areas of applications.

2. Hydrogels

The application of the hydrogels as a form of chitosan can be seen used in fields of pharmaceutical, biomedical and environmental industries. Chitosan hydrogels can be obtained from several procedures (Berger, 2004a; Berger, 2004b).The process involves dissolving the chitosan into an organic solvent, the emulsions or coercervates are formed then crosslinking of the polymer occurs (Koseva, 1999; Kumbar, 2002). Improvement of hydrogel stability can be achieved when modifications to the chitosan is increased.

3. Films/Membranes

Chitosan can be made into films easily by the casting method (Kanke, 1989; Bonvin, 1993) especially because chitosan is now easily used in skin care, contact lens production,

cosmetic industries, membranes, separators and in other technologies. These films of chitosan are homogeneous, clear, and flexible with good oxygen barrier mechanical properties (Hoagland, 1996; Kittur, 1998). Chitosan films have low water vapour barrier characteristics (Butler, 1996). The films are also dense and do not possess pores (Muzzarelli, 1977; Hirano, 1982).

Chitosan degrades before melting hence; it has to be dissolved in a right amount of solvent prior to casting into films. Chitosan is affected by its molecular weight due to its properties that depends on morphology (Butler, 1996; Chen, 1996), the origin, level of deacetylation, the process of film preparation, the mechanism of the free amine regeneration and the solvent used in dissolving it (Samuels, 1981; Lim, 1995).

4. Fibers

This form of Chitosan was reported firstly in 1926 (Kunike, 1926). There is some advantages of fibers such as superior mechanical properties which is better compared to the same material in heavy form. There have been studies on blends of chitosan fiber with collagen (Chen, 2006; Chen, 2008a), starch (Wang, 2007), polyethyleneoxide (Bhattarai, 2005) polyvinylalcohol (Jia, 2007; Zhou, 2006) silk fibroin (Park, 2004) and alginate (Liao, 2005).

5. Sponge

In drug carrier systems, the sponges from chitosan have great value and high interest. This is based on the biocompatibility, biodegradability, antibacterial and non-toxicity of chitosan. In this study, the chitosan solution was combined with gelatine (Poole, 1989), which led to the formation of polyionic complexes possessing slower dissolution rate that native chitosan at the same pH value (Tharachodi, 1995). These complexes could be used in drug release in a wound where the spongy form absorbs the wound fluid (Oungbho, 1997). There are also blends of chitosan/alginate sponges (Shapiro, 1997; Kofuji, 2001; Kataoka, 2001; Singla, 2001; Kumar, 2000; Coppi, 2002; Lai, 2003).

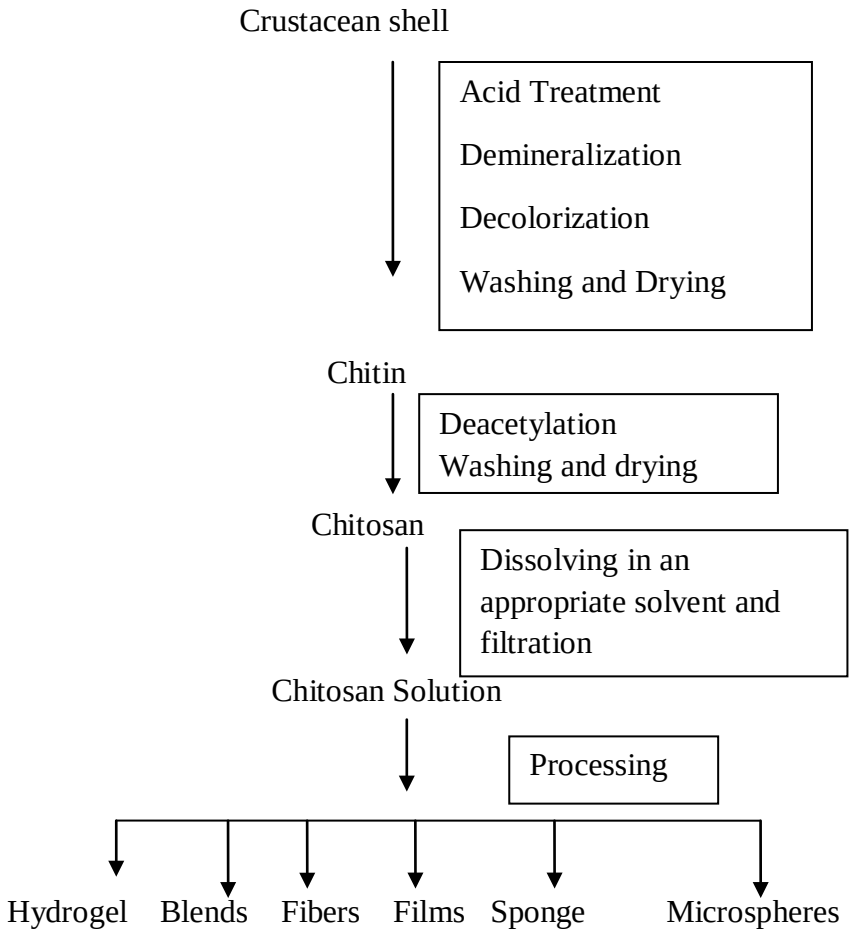


Figure 1.2: Routes for preparation of various chitosan physical forms (No and Meyers, 1995; No et al., 2000).

1.4 Mechanical properties of chitosan

The physical forms of chitosan such as hydrogels, films, fibers or sponges aforementioned are used in the biomedical domain owing to the biocompatilibity of chitosan. Chitosan; a derivative of chitin is much easier to process but the stability of chitosan forms is lower because of its hydrophilic character and pH sensitivity. Hence, various techniques are employed to control both mechanical and chemical properties (Rinauldo, 2006)

1.4.1 Enhancing mechanical properties of chitosan

A study showed that the low toxicity capability of chitosan can be maintained even after enhancing its mechanical properties. For example chitosan fibers were crosslinked with heparin and the following were observed increased diameter, lower strength, higher breaking strain values and stiffness properties but when all individual properties were taken into consideration, a higher mechanical property was observed. The mechanically

improved fibers and heparin crosslinked fibers promoted valvular interstitial cells attachment and growth in ten -day cultures (Albanna et al., 2013).

1.5 Sea Urchin

Sea urchin (*Paracentrotus lividus*) is a marine invertebrate can be found along the shallow waters (sublittoral to 20m) of the Mediterranean and north east atlantic. It forms permanent burrows on rocky substrates and along sea grass meadows. Their growth rates ranges from 40mmTD (4-5years) to 70+mm (>12years) and 75mmTD as the maximum size. They mature at about 13mmTD and reproduction occurs in spring to early summer (Boudouresque and Verlaque, 2001).

1.5.1 General overview of the sea urchin; *Paracentrotus lividus*

Paracentrotus lividus is the edible specie of the sea urchin family. Generally sea urchin is important in both basic and applied biology because of its application as a renowned experimental model (Di Benedetto et al., 2014).

Figure 1.3: *Diagram of paracentrotus lividus* (Legg and Jones, 1988)

They belong to the Echinoidea (Phylum Echinodermata). Echinoids have an internal skeleton constituted of calcitic plates within their skin.

Echinoderms can undergo hydrothermal processing which changes the mechanical and chemical properties of their structure making it similar to the human bone (Aizenberg and

handler, 2004). In order to facilitate mass transfer and tissue development, sea urchin skeletons are formed from a three- dimensional crystalline meshwork termed stereom which can be seen under high magnification in (Fig1.4). There are regular series of pores in the sea urchin's skeletal plate and are in direct contact with each other. Three quarters of the pores are exits on the tube feet while the remaining is connected to the reproductive and alimentary canal. The size of the pores and the thickness of the rods forming the stereom differs according to the type of soft tissue that attaches to that part of the skeleton. A boundary exists between two stereoms, one associated with muscle attachment (top right) and the other with normal epithelium (skin - bottom left) can be observed in figure 1.4 below. Stroma is a connective tissue that fills the pore spaces when the urchin is living. The prevention of crack propagation through the plate was assumed possible based on the design of the echinoid's skeleton (Smith, 1981).

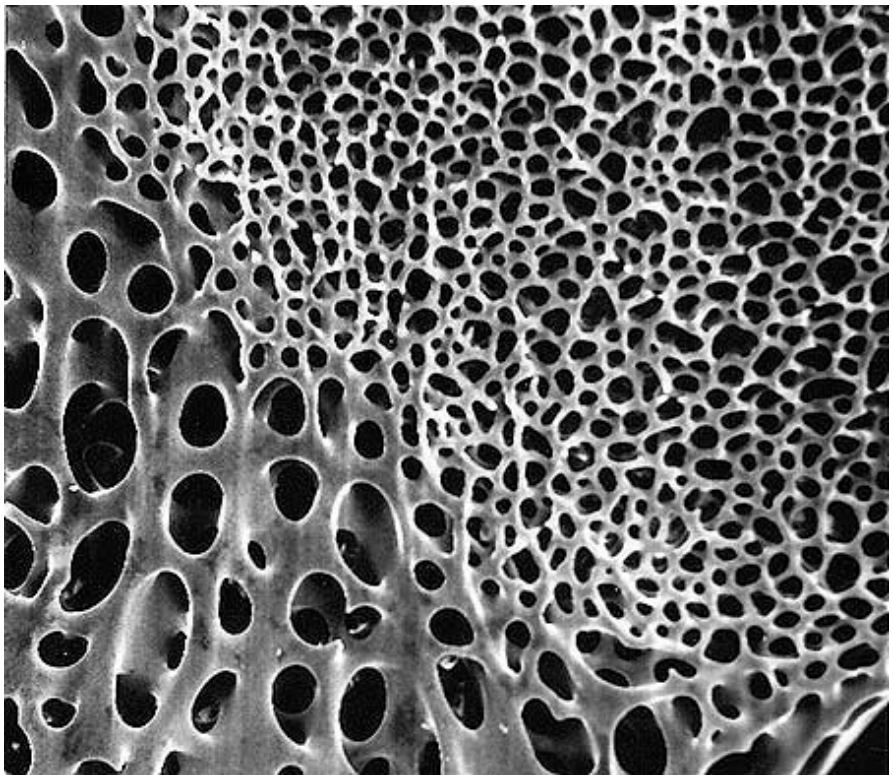


Figure 1.4: Diagram showing the 3D skeleton of a sea urchin (Smith, 1981).

The replamine form technique has been used in several studies to replicate the structural elements of echinoderms thereby generating promising hard tissue replacements to bone. The sea urchin uses the available materials in the sea water to produce its spicule, hence it is made up of organic and inorganic materials. The spicule has a starlike shape devoid of

facets. This unique morphology is attained by depositing a disordered amorphous mineral phase first, allowing it to slowly transform to a crystal with a ordered lattice structure. This unsusual transformation from amorphous to an ordered crystalline structure at room temperature needs to be understood for directional growth in next generation biomaterials (Aizenberg and Hendler, 2004; Ben- Nissan and Green, 2014).

1.5.2 Tissue engineering relevance of biomaterials from sea urchin

Adhesive protein, calcite, calcium carbonate and collagen are biomaterials that can be obtained by several processes from various parts of the sea urchin (Aritra et al., 2014; Di Benedetto et al., 2014).

1.5.2.1 Adhesive protein

Bindin is an insoluble, 30000mw protein component that is secreted from the acrosomal vesicle of the sea urchins sperm during fertilization. Bindin encourages the adhesion of the sperm to the vitelline layer (glycocalyx) covering the egg (Vacquier and Moy, 1977; Rossignol et al., 1981).

Glabe et al. (1982) suggested that the complementary egg surface ligands for the bindin protein are high molecular weight, sulphated fucose containing glycoconjugates. Sulphated fucans are constituents of the cell surfaces of marine animals, vitelline layer in species of sea urchins and eggs of algae. DeAngelis and Glabe (1986) reported that the interaction of the proteins is dependent on polysaccharide structural features such as its sulphate esters and high molecular weight. A major component of this adhesive is 3,4 dihydroxyphenylalanine (DOPA) which is a by-product of post-translational modification of tyrosine (Deming,1999). The absence of DOPA causes the loss of its adhesive ability along with characteristics such as biocompatibility and biodegradability that makes it suitable as industrial and medical adhesives (Grande and Pitman, 1987; Yu et al., 1999).

1.5.2.2 Calcite and Calcium carbonate

The sea urchin's spicule is the source of calcite and calcium carbonate in the sea urchin. The spicule is formed within a clump of specialised cells as the sea urchin lays down a single crystal of calcite from which the remaining spicules are formed. There is a directional growth that starts from the three radii at the crystalline centre of the intended location of the spicule. These three radii are amorphous calcium carbonate but slowly transform to calcite. This mechanism is not perfectly understood but theories such as

ordered precipitation and growth mechanisms at crystallographic regions are assumptions yet to be fully verified (Ben- Nissan and Green, 2014; Milev et al.,2004).

Calcium carbonate is the most abundant form of calcium in the marine environment. It exists in a crystalline form as calcite in sea urchins (Stupp and Paul, 1997; Wilt, 2002). Calcium carbonate of marine origin has a lot of features that makes it a good candidate for orthopaedic and dentistry applications such as porosity, pore interconnectivity and architecture (Laine et al., 2008).

1.5.2.3 Collagen

Collagen is the main structural constituent of animal tissues which functions mainly by conferring elasticity and strength to the tissues thereby enhancing its mechanical properties (Barnes et al., 2007; Satmov and Pombe, 2012). Due to its high biocompatibility, collagen type 1 is considered as the gold standard for tissue engineering applications such as the basic for a cell culture system. Injectable matrices and scaffolds for bone regeneration are obtained for fibril forming collagen which includes type I, II, III, V, XI. Scaffolds derived from marine sponges displayed good cell viability and osteo-inductive potential (Oliveira et al., 2009; Paranteau-Bareil et al., 2010).

The peristomial membrane (PM) of the sea urchin is a soft tissue which surrounds its mouth. It is composed of mammalian-like collagen. A recent study showed that sea urchin P.lividus can be a valuable low cost source of collagen for mechanically resistant biomedical devices (Di Benedetto et al., 2014). Collagen fibrils were synthesized from sea urchin P.lividus without any damage, in substantial amount and purity for fibrillar scaffold production (Barbaglio et al., 2012).

1.6 Antimicrobial activity of sea urchin

Biomaterial associated infections can be attributed largely to staphylococcal biofilms. Polymer related infections could be rooted more to staphylococcus epidermis while metal related infections is attributed to staphylococcus aureus (Gotz, 2004).

Conventional antibiotics to a large extent has been unable to treat these infections because of the high resistance of the microbial biofilms (Gilbert et al., 1997). Hence, an important goal was established with the discovery of anti- infective agents which are active against both plankton microoorganisms and biofilms (Projan and Youngman, 2002).

The immune system of marine organisms was found to be an under utilised source of antimicrobial agents. A study focussed on the coelomocytes effector cells of the sea urchin

P.lividus, a wide range of immunological functions was observed as well as cellular recognition, phagocytotosis and cytotoxicity (Arizza et al., 2007).

P.lividus survival shows that its innate immune system is active, potent and effective. Antibacterial activity was observed from a 60kDa protein which was isolated from lysates of coelomocytes from P.lividus (Stabili et al., 1996).

Another study centered on the antimicrobial peptides in P.lividus being the first of its kind. The antimicrobial activity of a 5-kDa peptide fraction from the coelomytes cytosol (5- CC) against human pathogens was evaluated. Furthermore the invitro inhibitory capacity of the 5-CC against *S.epidermis* 1457 biofilm and reference staphylococcal biofilm was also evalauted using static chamber system. Minimal inhibitory concentrations (MICs) ranging from 253.7 to 15.8 mg ml was determined. They observed an inhibitory activity and anti-biofilm properties of 5-CC against staphylococcal biofilms of reference strains *Staphylococcus epidermidis* DSM 3269 and *Staphylococcus aureus* ATCC 29213. The biological activity of 5-CC could be attributed to three peptides belonging to the sequence segment 9–41 of a beta-thymosin of P. lividus. It was concluded that the effector cells of P. lividus profers a source of marine invertebrates-derived antimicrobial agents in the development of new strategies to treat staphylococcal biofilms (Schillaci et al., 2010).

1.7 Antimicrobial activity of chitosan

1.7.1 Factors affecting the antimicrobial activity

Several studies exploring the antimicrobial activity of chitosan from different sources under diverse experimental conditions have been performed. Numerous irregularities were observed in the results from these studies. It was reported that 0.1% of chitosan had greater inhibitory effect on Gram-positive than Gram-negative bacteria (No et al., 2002). Chhabra et al. in 2006 also stated that chitosan had stronger antimicrobial activity against S. aureus than S. enterica and V. Vulnificus thereby suggesting that chitosan has greater inhibitory potential against Gram-positive than Gram-negative bacteria. A discrepancy to the earlier studies was observed by Helander et al. (2001), the study demonstrated that chitosan presented a higher antimicrobial activity against Gram-negative than Gram-positive bacteria. These discrepancies observed is not surprising because chitosan's in-vitro antimicrobial activity is based on various intrinsic and extrinsic factors which is related to chitosan itself (type, Mw, DD, viscosity, solvent and concentration) and environmental conditions (pH, test strains, temperature, physiological state and culture medium, EDTA,

metal ions e.t.c) (Raafat and Sahl, 2009). Various methodologies applied in studies are other factors contributing to different results of antimicrobial activity of chitosan.

1.7.1.1 Intrinsic factors

Despite the knowledge from many studies about the intrinsic and extrinsic factors affecting the antimicrobial activity of chitosan, determining the influence of Mw or the DD value on the antimicrobial activity of chitosan is still a challenge. For example, it was noted that oligosaccharides and D-glucosamine possessed little or no antibacterial activity, hence, chitosan is said to be largely affected by its Mw (Rhodes and Roller,2008; No et al.,2002). Jeon et al., (2001) also stated that for proper microorganism inhibition a minimum of 10kDa chitosan is required. Although Raafat et al. (2008) did not observe any appreciable antimicrobial activity at a molecular size above 10kDa, having tested a large number of chitosan preparations. There is a minimum degree of polymerization required for antimicrobial activity. Higher DD depicted a higher degree of bacterial growth inhibition than those with a lower DD maybe due to the higher percentage of protonated amine groups (Shigemasa et al., 1994; Liu et al., 2001). No et al. (2002) suggested that the minimal inhibitory concentrations (MICs) of chitosans ranged from 0.05% to above 0.1% for different bacteria tested and Mw of chitosan used. Studies showed that Campylobacter spp. was the most sensitive microorganisms to chitosan, and the MIC of chitosan for Campylobacter ranged from 0.005 to 0.05% (Raybaudi-Massilia et al., 2009).

1.7.1.2 Extrinsic factors

The antimicrobial activity of chitosan is strongly dependent on pH. Yoshihiko et al.(2003) reported that antimicrobial activities might be the resulting effect of dissolving chitosan in an acidic media such as acetic acid, hence, stronger antimicrobial activity was observed at lower pH. The inability of chitosan to remain bactericidal at pH 7 (neutral) is probably due to the presence of a majority of positively uncharged amino groups and poor solubility of chitosan (Aiedeh and Taha, 2001, Papineau et al., 1991 and Sudarshan et al., 1992).

While its antimicrobial activity is inversely affected by pH, it is directly affected by temperature and in the presence of EDTA (Tsai and Su, 1999; Jumaa et al., 2002; No et al., 2002).

Contradictory evidences as regards ionic strength of chitosan exists, where one study reported that the presence of divalent cations reduces the antimicrobial activity of shrimp chitosan against *E.coli* (Tsai and Su, 1999), probably because the increase of metal ions,

especially divalent ions, could decrease the chelating capacity of chitosan (Kong et al., 2010). In contrast, Chung et al. (2003) suggested that the higher ionic strength could enhance the solubility of chitosan and therefore increase its antimicrobial activity. It is probably due to existing cations in medium may interact with the negative-charged components mainly on the cell wall of bacteria besides polycationic chitosan, consequently weakening the antimicrobial activity.

1.7.2 Antimicrobial Mode of action

Although a lot of factors influence the antimicrobial activity of chitosan, the exact mode of action is yet to be determined and fully understood.

The polycationic structure is essential to its antimicrobial action. It forms unnecessarily in acidic conditions because grafted groups of chitosan's derivatives may change the pKa(6.3) of chitosan and cause protonation at higher pH values (Yang et al., 2005). In the native chitosan, when pH is below pKa, NH_3^+ groups of glucosamine interact electrostatically with the anionic components of the microorganism's surface. This causes leakage of proteins and intracellular microbial cell components thereby impairing the vital bacteria activities. Hence, it is said that chitosan possesses growth inhibitory capacity as a result of the physiological adaptation of the bacteria cell to chitosan stress (Muzzarelli et al., 1990; Helander et al., 2001; Je and Kim, 2006; Raafat et al., 2008; Kong et al., 2010).

Electrostatic interaction is dependent on the number of amino groups linked to the C2 on chitosan backbone. This is indicative that antimicrobial activity can be enhanced by large amounts of amino groups. Therefore higher DD chitosan shows stronger inhibition than chitosan with lower DD (Kong et al., 2010).

Distinctive modes of antimicrobial action can be observed from different molecular weights (Mws) of chitosan and its physical states. For HMw water soluble and solid chitosan, interaction occurs only at the surface, no cell wall penetration but formation of an impermeable layer around the cell, hence blockage of vital solutes into the cell (Kong et al., 2010). For LMw water soluble chitosan, penetration of the cell wall occurs and interaction with DNA to prevent mRNA synthesis and transcription of DNA (Sudharshan et al., 1992).

1.7.3 Method to Detect Antimicrobial Activity of Chitosan

1.7.3.1 Disk diffusion method

In the disk diffusion method a large number of antimicrobials can be tested at the same time in an easy and flexible manner. The microorganism inoculum is fine tuned to certain concentration, inoculated to the whole surface of a (150mm in diameter) Mueller-Hinton agar (MHA) plate with a sterile cotton-tipped swab to form an even lawn. The paper disks are infused with diluted antibiotic solution and placed on the surface of each MHA plate (6mm in diameter) using a sterile pair of forceps. Plates were incubated aerobically for 16-24 hrs at 35-37^{o}C and the diameter of zone inhibition was measured by a ruler or vernier calliper. The results are then allotted to three categories, susceptible, intermediate, or resistant based on the diameter of inhibition zone and the CLSI interpretative data (CLSI, 2012). The larger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial. The major advantages of this method are that it does not require any special equipment; selection of disc is flexible and can be easily interpreted. The results are qualitative hence unable to generate the MIC value (i.e., not quantitative) and difficult to examine the susceptibility of majority of fastidious and slow-growing bacteria (Wilkins and Thiel, 1973; Dickert et al., 1981; Jorgensen and Ferraro, 2009).Other drawbacks is that disk diffusion is labor intensive and time-consuming (Klancnik et al., 2010). Disk diffusion was used to determine the antimicrobial activities of chitosan in many studies (Kulkarni et al., 2005; Pranoto et al., 2005; Coma et al., 2006; Kim and Kim, 2007; Cao et al., 2009; Mayachiew et al., 2010); however, chitosan was reported to be effective against bacteria, yeast, and fungi without mentioning MIC values.

1.8 Bioceramics Synthesis

Samur et al. in 2013 conducted a study where apatite structures, such as hydroxyapatite (HA) and fluorapatite (FA), from precursor calcium phosphates of biological origin (sea urchin) was synthesized by mechano-chemical stirring and hot-plating conversion method. The resulting materials were heat treated at 800°C for 4 hours. The experimental results suggests that sea urchin skeleton may be an alternative source for the production of mono or biphasic calcium phosphates with simple and economic mechano-chemical (ultrasonic) conversion method.

1.9. Aim/objective of thesis

1 The development of a stepwise solvent casting method in order to obtain composite materials containing chitosan and sea urchin for tissue engineering applications.

2 Applying the disc diffusion method on the chitosan-urchin based bioceramic to determine its potential as anti-bacterial filler for tissue engineering applications.

3 Physicochemical characterizations of composites with respect to their composition, structure, and properties will be achieved. Analytical methods and techniques such as Antimicrobial susceptibility testing, Scanning electron microscopy (SEM), X-ray Diffraction (XRD) will be involved. A range of analytical methods must be employed to investigate the composite samples since each of these methods has its own limitations and therefore it is necessary to combine all results to obtain a comprehensive view.

1.9.1 Problem Statement

Several research is in progress as regards the synthesis of calcium phosphates from natural materials such as eggshells, shells of some marine molluscs e.g. sea urchins, mussels, sea and land snails and cuttlefish bones (Coelho et al., 2006; Goller and Oktar, 2002; Agaogullarý et al., 2012; Vecchio et al., 2007; Kel et al., 2012). It is safe to say calcium phosphate can be obtained from sea urchin proving relevant to dental and orthopaedics applications.

Composite materials are composed of a polymer matrix and inorganic fillers such as bone, dentine (collagen and apatite), shell of crustaceans (chitin, proteins and calcium carbonate), shell of eggs (glycoproteins and calcium carbonate) having either role of protection, mechanical support or strength are just some examples from nature. Being inspired by nature we try to imitate such composite biomaterials whereby chitosan is the polymer matrix and the sea urchin as the inorganic phase. The motion to use chitosan is based on several factors such as: biocompatibility, biodegradability, low toxicity, antibacterial etc.

Chitosan has been established as an antimicrobial agent though lacking adequate mechanical properties. Its use has been limited as a result of poor mechanical properties, weak barrier properties of the film and high moisture sensitivity (Domard, 2011). Doping chitosan with sea urchin to confer mechanical stability to the composite and anti-bacterial ability to the composite thereby developing infective resistant materials.

CHAPTER 2
MATERIALS AND METHODS

2.1 Bioceramic Synthesis

2.1.1 Materials Used

Sea urchin (Echinoidea) was obtained from the marine area of the saltwater bed of the Girne sea located in the Turkish Republic of North Cyprus. Medium molecular weight chitosan with a viscosity of 200-800cp, 1wt% in 1% acetic acid with a deacetylation degree of 75%-85% (Batch 448877-50G, Sigma Aldrich, U.S.A), Glacial acid and N, N'-methylene diacrylamide used as a crosslinker from Merck chemicals, Germany.

2.1.2 Preparation of the CU Blended Bioceramic

Crosslinker (N, N' methylene diacrylamide)- 1M was prepared by dissolving 1.54g in 100ml of distilled water.

Sea Urchin was air dried and blended to a powdery form.

0.2g of chitosan was acetylated with 0.1M acetic acid (1%) at 25^{o}C on a magnetic stirrer and refrigerated for 24hours.

Figure 2.1: Diagram showing air dried blended sea urchin.

Varying concentrations of the chitosan and urchin powder were sonicated in the presence of a cross-linking agent (N, N-methylene diacrylamide) for 20 minutes.

Table 2.1: Weight/volume ratio of CU blended bioceramics.

Samples	Urchin(g)	Chitosan(ml)	CH_2=CHCONH)$_2CH_2$(ml)
CU1	0.200	2	0.25
CU2	0.100	2	0.25
CU3	0.025	2	0.25
CU4	0.050	2	0.25
CU5	0.010	2	0.25
control 1	____	2	_____
control 2(urchin)	0.200	____	_____

Figure 2.2: Mixing on a magnetic stirrer.

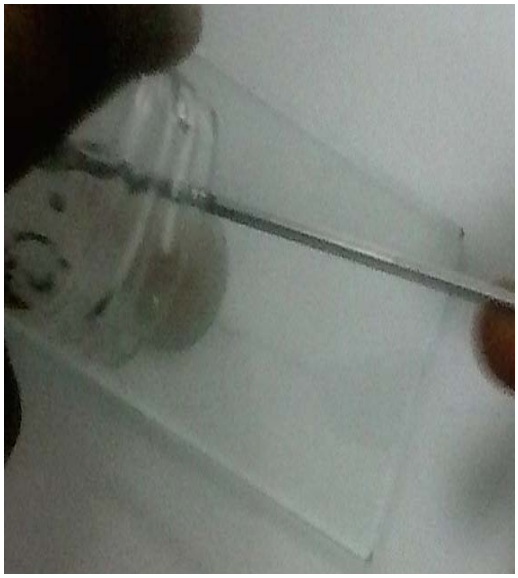


Figure 2.3: Casting on a glass slide

The heterogeneous suspension was immediately cast on a clean glass plate and was irradiated in a vacuum ultraviolet device using a short wavelength (λ= 254nm) for about 3hours. The composite was air dried overnight and lifted off the glass plate with a thin spatula by immersion in distilled water. Cs-Urchin composite was conditioned at room temperature for further analysis.

Fig 2.4(a) Cs-Urchin composite in distilled H_20 2.4(b) Pure chitosan swelling in distilled H_2O

2.2 Sterilisation

The samples were sterilised with hydrogen peroxide prior to further analysis at the sterilisation unit of Near East hospital, Lefkosa. The hydrogen peroxide solution was introduced into a vaccum chamber thereby creating a plasma cloud. Hydrogen peroxide inactivates the microorganism by oxidising their cellular components.

An energy source is required for the process to occur and when it was turned off, water vapour and oxygen were formed, hence absence of toxic residues or harmful emissions. The samples were wrapped before sterilisation; the sterilisator was maintained at a temperature of 40^oC-50^oC and takes between 45-55minutes to complete s cycle (Reichert and Young, 1997; Rutala 1998)

2.3 Antimicrobial Activity

2.3.1 Microorganism Strains and Culture Conditions

The six strains of micro-organisms used in this study included gram-positive bacteria *Enterococcus faecalis* ATCC 25922, *Bacillus cereus* ATCC 17666, *Staphylococcus aureus* ATCC 25923 and gram-negative bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and fungi *Candida albicans* ATCC 29212. Cultures were incubated at 35^oC for 24hrs.

2.3.2 Anti-microbial Susceptibility Testing

The microorganism suspension were prepared by suspending 3-5well isolated colonies from agar plates into 3ml Liquid broth culture medium (adjusted to a pH of 5.9)(No et al, 2002) and the turbidity was adjusted to the 0.5McFarland standard.

100µl of the 0.5McFarland suspension was further diluted in 10ml of the liquid broth culture medium, which was used as the final inoculums.

2.3.3 Disk Diffusion Method

The microorganism suspension prepared above was inoculated onto the entire surface of a Mueller-Hinton agar plate with a sterile cotton-tipped swab to form an even lawn. Disk-like sizes of the chitosan-urchin composite were placed on the surface of the plates using a sterile forceps. The treated plates were placed at room temperature for 10 minutes and then incubated at 35±0.1 °C for 24-48 hours. At the end of the incubation period, inhibition zones of the bioceramic formed on the medium were measured with a transparent ruler in millimetre.

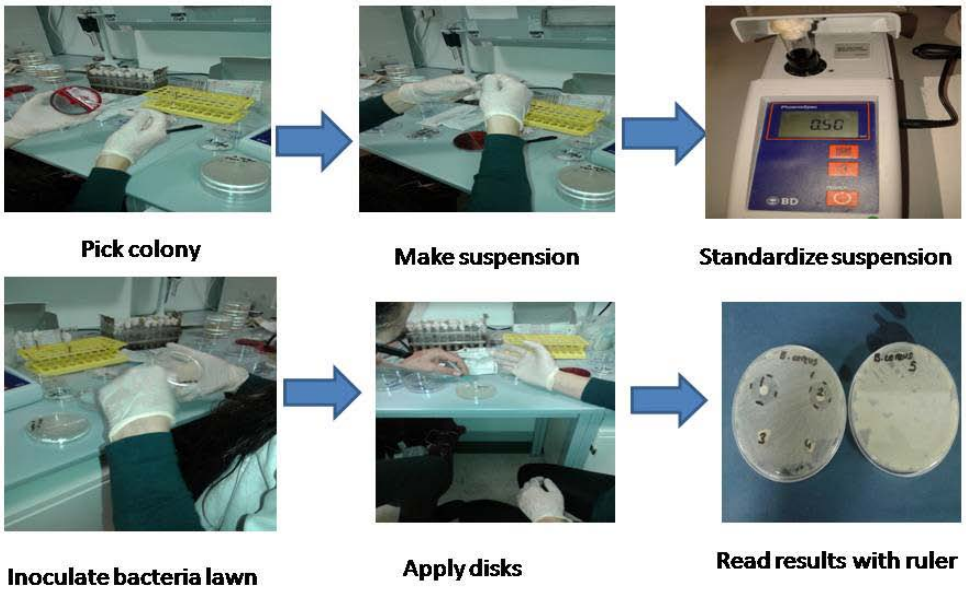


Fig 2.5- Disk diffusion method procedure

2.4 Material Characterizations

2.4.1 X-ray Diffraction Analysis (XRD)

Powder X-ray diffraction analysis was carried out at Tubitak Marmara Research Institute, Turkey by using a shimadzu XRD-600 model diffractometer with a Cu- X-ray tube (L=1.5405A^{o}). The diffractometer scans at the rate of 2^{o}/min within the region of 2θ and

resulting diffraction intensity curves obtained was produced. The crystallinity index was calculated by using the principle of Jaworska et al., (2003).

2.4.2 Scanning Electron Microscopy (SEM).

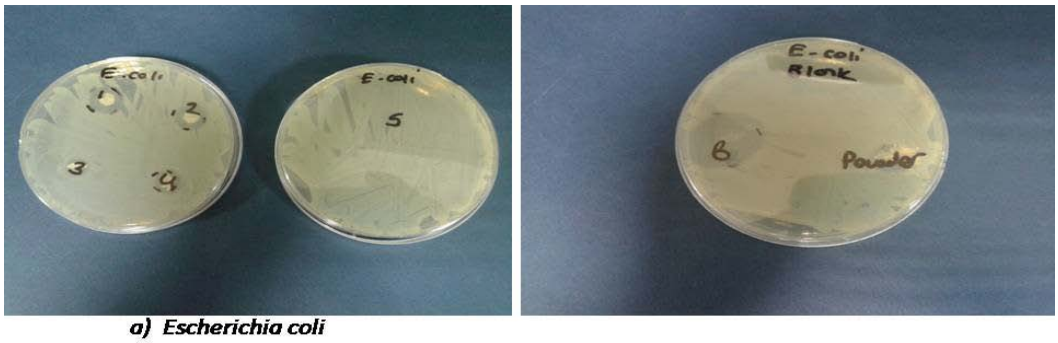
The morphology (particle size, roughness or smoothness) of CU blend bioceramic was observed by scanning electron microscopy (SEM). SEM analysis was carried out at Tubitak Marmara Research Institute, Turkey using a SEM- Jsm- 6510 model at an acceleration voltage 10kV.The device produces images of the samples by focussing a beam of electrons on it and samples were sputter-coated with gold to prevent charging.

CHAPTER 3

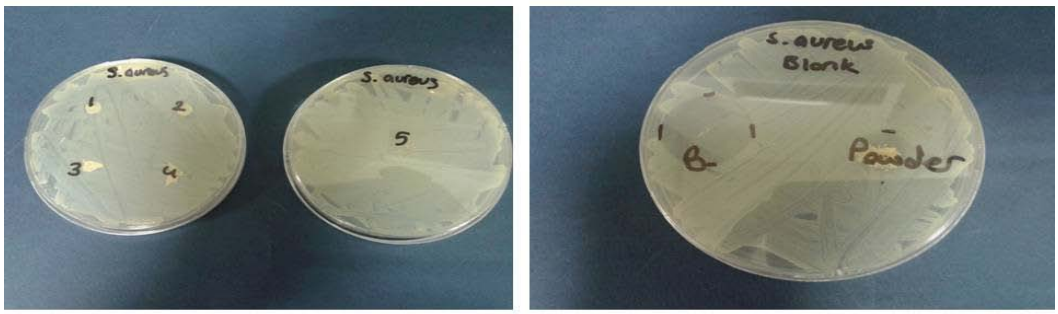
RESULTS AND DISCUSSION

Table 3.1: Inhibition zones obtained from disc diffusion method

Microorganisms	Bioceramics	Inhibition zone diameter(mm)
E.faecalis	controls,1,2,3,4,5	-
C.albicans	controls,1,2,3,4,5	-
B. cereus	control 1	10
	control 2(urchin)	-
	CU 1	17
	CU 2	20
	CU 3-5	-
E.coli	control 1	23
	control 2(urchin)	10
	CU 1	13
	CU 2	14
	CU 3	-
	CU 4	10
	CU 5	-
P.aeruginosa	control 1	24
	control 2(urchin)	-
	CU 1	18
	CU 2	20
	CU 3-5	-
S.aureus	control 1	24
	control 2(urchin)	16
	CU 1-5	-

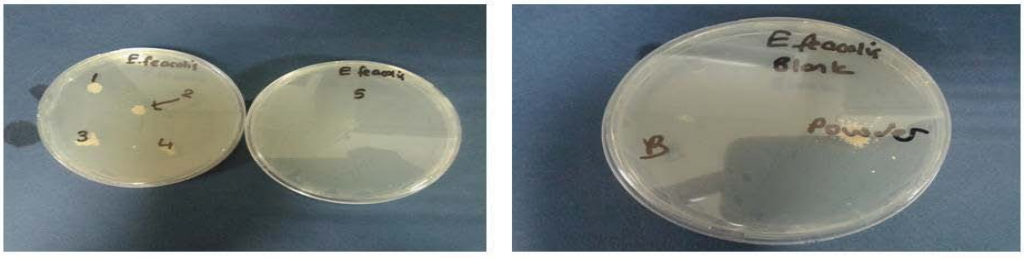


a) *Escherichia coli*

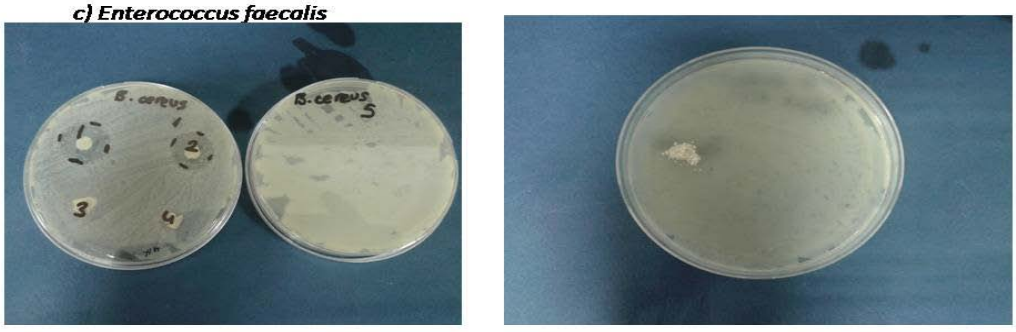


b) *Staphylococcus aureus*

Figure 3.1: Inhibition Zones of Cs-Urchin composite against a)Escherichia Coli. b) Staphylococcus aureus



c) *Enterococcus faecalis*



d) *Bacillus cereus*

Figure 3.2: Inhibition Zones of Cs-Urchin composite against c) Enterococcus faecalis. d) Bacillus cereus

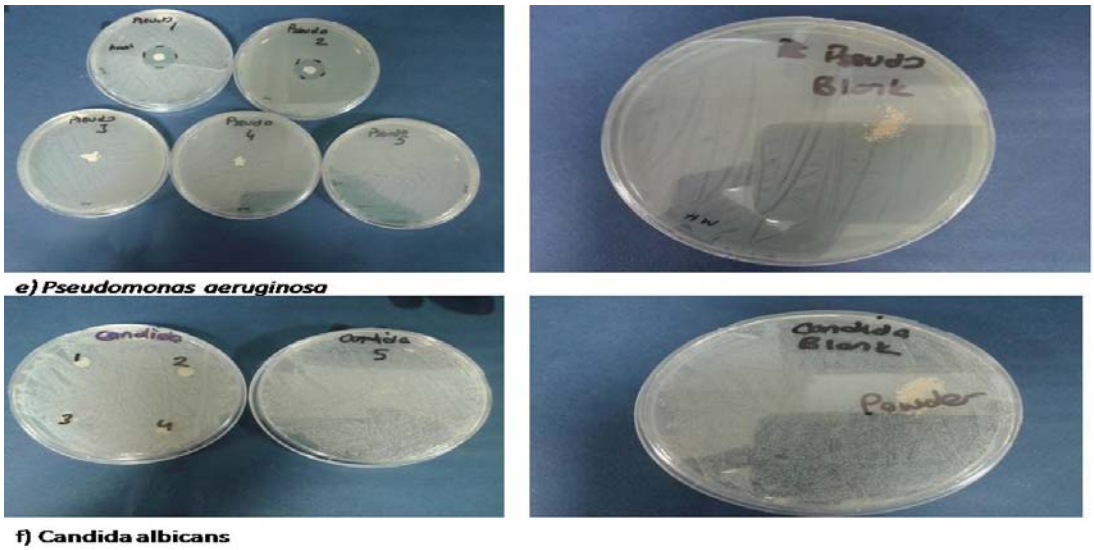


Figure 3.3: Inhibition Zones of Cs-Urchin composite against
e) Pseudomonas aeruginosa f) Candida albicans

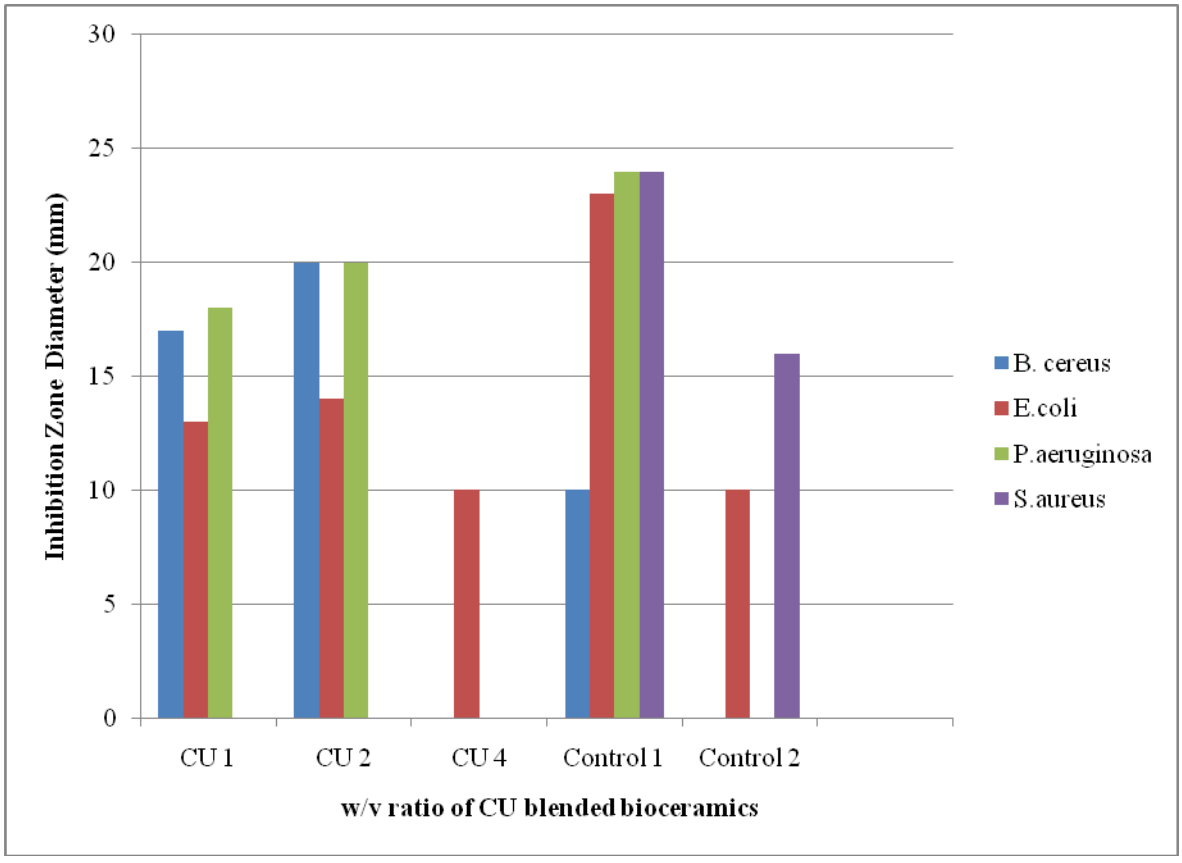


Figure 3.4: Graphical representation of w/v of CU blended bioceramic and inhibition zones against *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa* and *Escherichia coli*.

3.1 Disc Diffusion Analysis.

Antimicrobial activity of chitosan-sea urchin composite was evaluated based on the diameters of clear inhibition zone surrounding the disks. If there is no inhibition zone, it is assumed that there is no antimicrobial activity, hence, E.faecalis and C.albicans showed no inhibition zone thereby assuming little or no antimicrobial activity. Fig 3.1, Fig 3.2 and Fig 3.3 shows representative disk diffusion plates with different bacteria after 24 h incubation. The diameter of inhibition zone of Bacillus cereus and Pseudomonas aeruginosa is larger than that of Escherichia coli, indicating Bacillus cereus and Pseudomonas aeruginosa is more susceptible to chitosan-sea urchin composite than E. coli. However, three of the five proportions (0.05, 0.10, 0.20) are showed similar inhibition zones against E.Coli. This antimicrobial result is a bit surprising owing to the fact that chitosan is an established antimicrobial agents against all the microorganisms used above. The absence of antimicrobial activity against E.faecalis and C.albicans could be attributed to the concentration of the chitosan used and the form in which it was used (Shaik et al., 2014). The biological properties such as antimicrobial activity of chitosan can vary based on the form in which it exists after preparation (Kumirska, 2011). Pure chitosan and pure urchin were effective against S.aureus but in the blend were found ineffective probably as a result of the cross-linking agent used (Cai et al., 2010; Schillaci et al., 2010; Xie, et al., 2002; Yang et al., 2002)

3.2 X-ray diffraction Analysis (XRD)

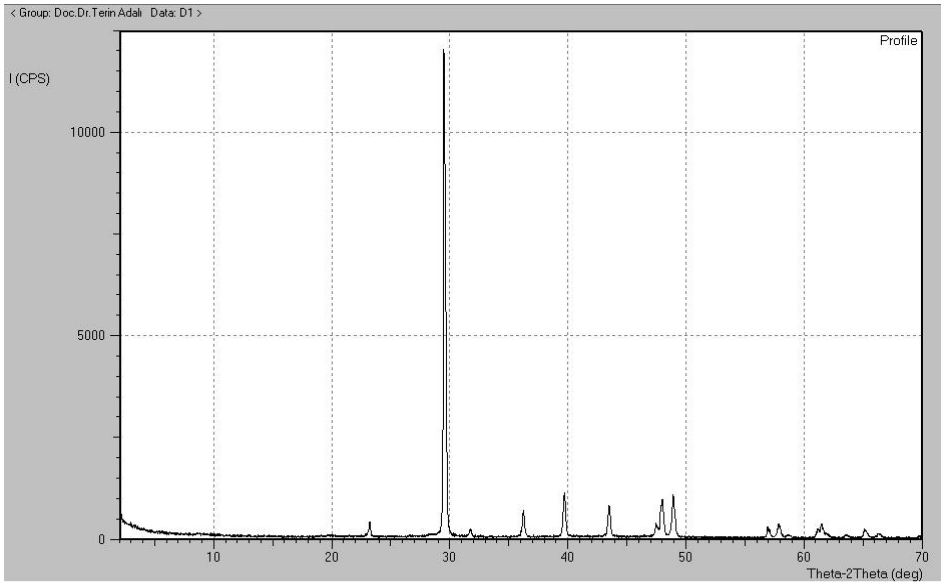


Figure 3.5: XRD diffractogram of 0.20g CU blended bioceramic.

X-ray powder diffraction analysis is one of the most powerful tools for identifying the crystal structure and mineral phases in the CU blended bioceramic (Rahman et al., 2013).

The X-ray diffraction analysis was carried out to verify the mineral phase in the CU blended bioceramic. The XRD pattern is shown in Figure 3.5 above and the strongest peak was observed at $2\theta = 29.5^{o}$, smaller peaks at 23^{o}, 36^{o}, 40^{o}, 43.5^{o}, 48^{o}, 58^{o} and 61.5^{o} were also noted from the xrd raw data. Previous studies shows an almost exact xrd pattern of CU blended bioceramic to calcium carbonate and calcite, furthermore the crystallinity of pure chitosan was found between 20^{o} and 25^{o} hence the degree of crystallinity of the Cu blended ceramic is higher than pure chitosan (Tsipursky and Buseck, 1993; Rahman et al., 2013).

3.3 Scanning Electron Microscopy (SEM)

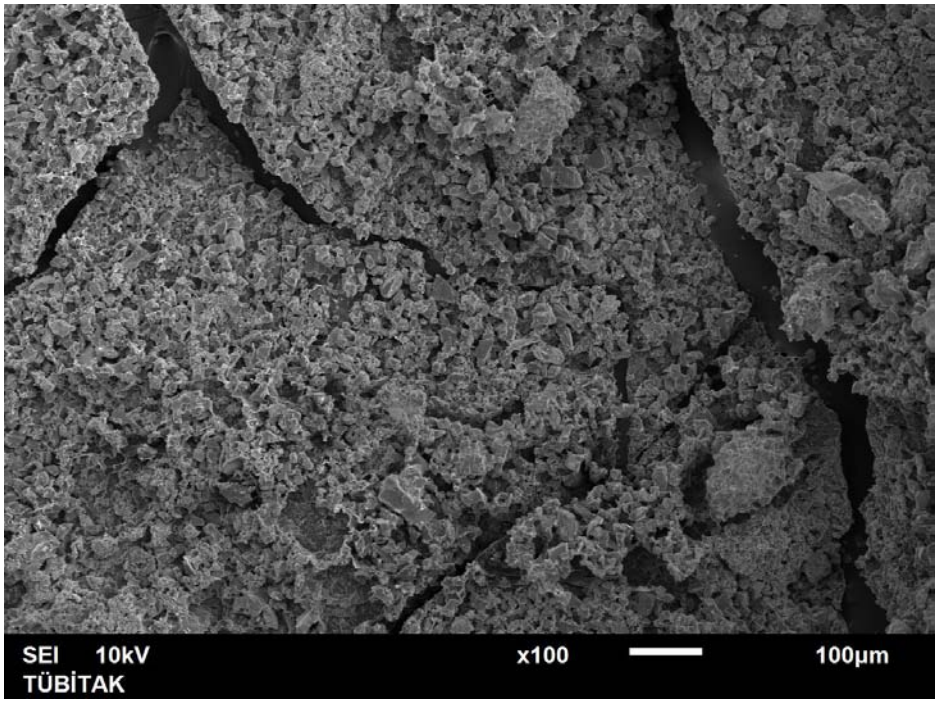


Fig 3.6- SEM micrograph 0.20g CU blended bioceramic at x100

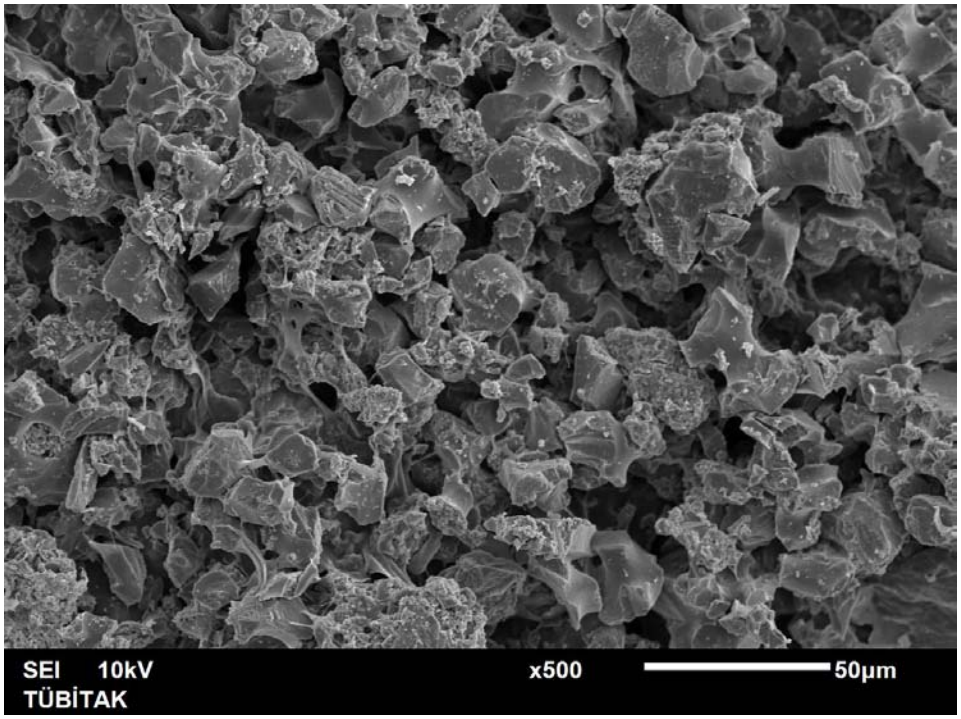


Fig 3.7- SEM micrograph 0.20g CU blended bioceramic at x500

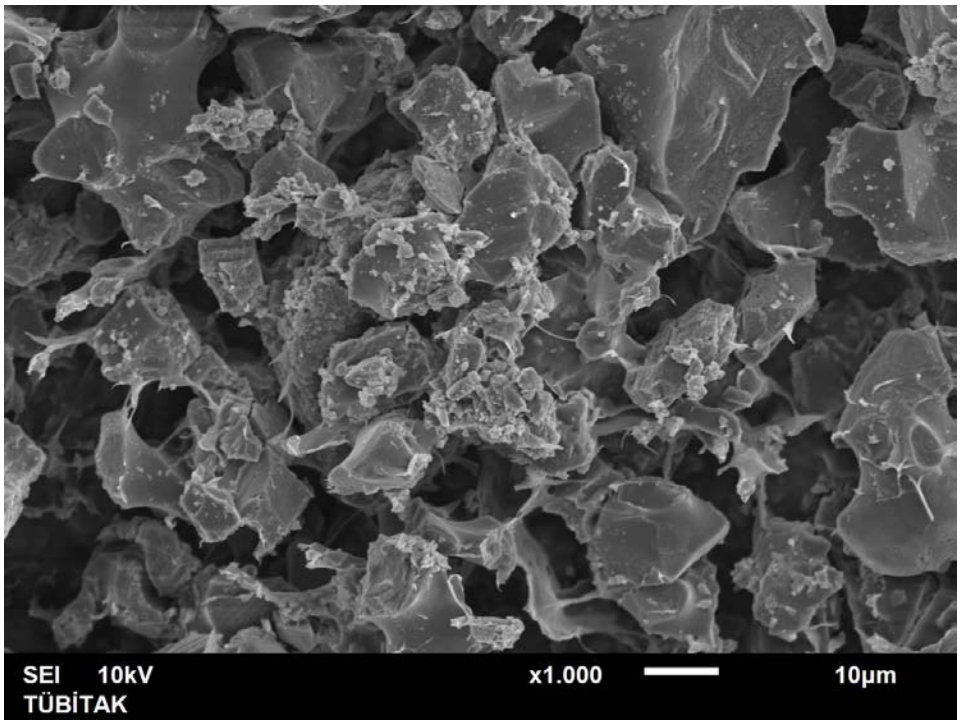


Figure 3.8: SEM micrograph 0.20g CU blended bioceramic at x1.000

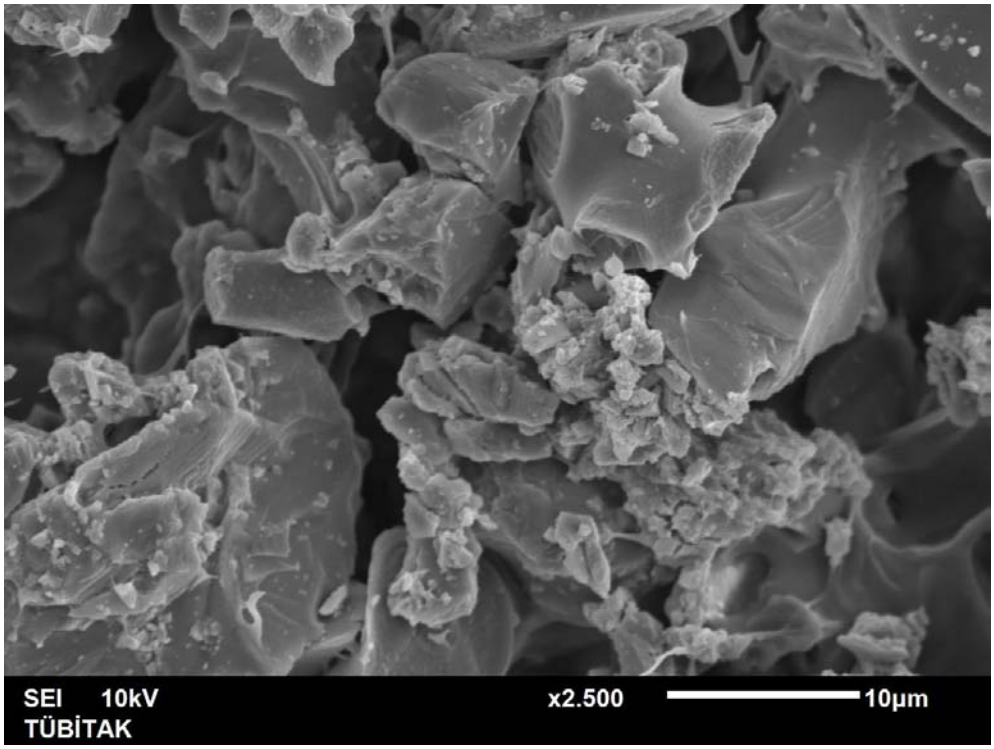


Figure 3.9: SEM micrograph 0.20g CU blended bioceramic at x 2.500

The porosity, particle size, crystal size and surface roughness of the sample were analysed to determine the composite morphology. The sample was studied under the scanning electron microscope at 10kv at different magnifications as shown in figure 3.3, figure 3.4, figure 3.5 and figure 3.6. The results showed aggregations and long interconnections of particles, this shows the function of the crosslinker (N,N'-menthylenediacrylamide) used. The presence of fiber particles was observed and the SEM result showed that the composite were rough, elongated but connected. Di Benedetto et al. (2014) also observed the presence of open porous structures that are interconnected and this corresponds with our result.

CHAPTER 4
CONCLUSION

Chitosan swells in neutral solution (water), dissolves in acidic medium and precipitates in basic medium. In this study we fixed the volume of chitosan and N,N'methylene diacrylamide to observe the effect of the sea urchin. We observed that increasing the concentration of the sea urchin reducing the swelling of chitosan thereby proferring stability to the bioceramic.

Five chitosan-urchin blend bioceramic samples made from medium molecular weight (50-150kDa) chitosan were tested against six human pathogens using the disc diffusion method. CU1, CU2 and CU4 were found to be the most effective against E. coli. CU1 and CU2 were effective against B.cereus and P.aeruginosa also showing a larger inhibition zone when compared with E.coli. CU1 – CU5 were ineffective when compared with E.faecalis and C.albicans and surprisingly pure chitosan and pure urchin were effective against S.aureus but in the blend were found ineffective probably as a result of the crosslinking agent used. Therefore, the application of CU blend bioceramic in inhibiting bacteria related infections for biomedical applications is needed for future work.

The crystallinity of the CU blend bioceramic was determined by XRD and the bioceramic had its strongest peak at 29.5^0 with small area under the peak, we can conclude its a crystalline material and that its crystallinity is higher than pure chitosan which is atabout 20^0.

The presence of fiber particles was observed and the SEM result showed that the composite were rough, elongated but connected. The roughness of the CU blend bioceramic suggests the likelihood of cell attachment and possibly proliferation.

In conclusion, this study appears to be the first of its kind where chitosan is blended with sea urchin and where disk diffusion is used to determine the antimicrobial activity of CU blend bioceramic. The resistance to some of the microorganisms suggests the need to apply other methods when conducting in vitro antimicrobial susceptibility testing of CU blend bioceramic since the antimicrobial activity results from numerous studies need to be comparable.

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