Faculty of Engineering

Department of Biomedical Engineering

Citosan-graft-Silk Fibroin Hydrogels for Biomedical Applications

BME 400/402

GRADUATION PROJECT

Supervisor : Assoc. Prof. Dr. Terin ADALI

Students : Öztürk Hakan ZENCİR (20091747)

Ruhsan ONBAŞI (20101199)

LEFKOŞA – 2014

ACKNOWLEDGEMENT

This study of carried out at the Department of Biomedical Engineering,Near East University during the 2013-2014 academic year.

We express our warmest gratitude to our supervisor Assoc.Prof.Dr. Terin ADALI for introducing us to the interesting world of Silk Fibroin, Chitosan and their thrombogenic surface interaction, antimicrobial property and their behaviour in the acidic and basic environment, for guidance and endless optimism during the years.

Our special thanks go to Dr.Kaya SÜER and Meryem GÜVENİR for their valuable comments.

Many thanks to my parents, Menderes and Emine ZENCİR,and my brother Onur,for their unfailing encouragament and loving support during my whole life.(Öztürk Hakan ZENCİR)

Many thanks to my parents, Ahmet ONBAŞI and Kezban ONBAŞI and my brother Ali ,for their unfailing encouragament and loving support during my whole life.(Ruhsan ONBAŞI)

Finally,thanks to all our friends and colleagues (İlke KURT,Fatih Veysel NURÇİN,Ahmetcan YALÇIN,Hasan KUTAY,Harun ÖZTÜRK and Çağatay URFALI)for their friendship,support and for taking our mind out of the work from time to time.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CS	Chitosan
SF	Silk Fibroin
Ser	Serine
Gly	Glycine
Ala	Alanine
Tyr	Tyrosine
PBS	Phospahate Buffer Saline
ABS	Acetic acid Buffer Saline
KCl	Potassium Chloride
HCl	Hydrochloric Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
Glys	Glyserine
$CaCl_2$	Calcium Chloride
Na_2CO_3	Sodium Carbonate
CH_3OH	Methanol
$N_2HPO_4.2H_2O$	di-Sodium hydrogen phosphate dehydrate
KH_2PO	Potassium dihydrogen phophate
CAN	Cerric Ammonium Nitrate
C_2H_5OH	Ethanol
CH_3COOH	Acetic Acid
H_2O	Pure water
$C_6H_{11}O_4N$	2-amino- 2-deoxy-b-D-glucopyranose
HepG2	Hepatocellular carcinoma cells
ACF-HS	Alginate, chitin/chitosan and fucoidan hydrogel sheet

1-INTRODUCTION

The aim of this project is to synthesize and characterize Silk-Fibroin(SF) grafted Chitosan(CS) hydrogels and scaffolds modified the thrombogenic properties by adding chlopidegral as a anticoagulation agent.

- Swelling (PH:1,2 PBS PH:7,4)
- Creating SF and CS Biofilms
- Antimicrobial Activity

1. 1.CHITOSAN

Chitosan is a polysaccharide extracted from the shells of crustaceans, such as shrimp, crab and other sea crustaceans, including Pandalus borealis and cell walls of fungi. Chemical name is 2-amino- 2-deoxy-b-D-glucopyranose.molecular formula is (C6H11O4N)n. Chitosan is also known as soluble chitin. Chitin consists mainly of unbranched chains of beta-(1 → 4)-2-acetamido-2-deoxy-D-glucose (=N-acetyl-d- glucosamine). It is similar to cellulose, in which the C-2 hydroxyl groups are replaced by acetamido residue. Chitin is practically insoluble in water, dilute acids, and alcohol, with variation depending on product origin. Chitosan, the partially deacetylated polymer of N- acetyl-D-glucosamine, is water-soluble [1].

1.2.PHARMACEUTICAL APPLICATIONS OF CHITOSAN

Chitoasn has received considerable attention as a possible pharmaceutical excipient in recent decades, due to its good biocompatibility and low toxicity properties in both conventional excipient applications as well as in novel application. Some of the general applications of Chitosan in pharmaceutical fields are: Diluents in direct compression of tablets. Binder in wet granulation,Slow-release of drugs from tablets and granules,Drug carrier in micro particle systems,Films controlling drug release,Preparation of hydrogels, agent for increasing viscosity in solutions. Wetting agent, and improvement of dissolution of poorly soluble drug substances,Disintegrant,Bioadhesive polymer,Site-specific drug delivery (e.g. to the stomach or colon)Absorption enhancer (e.g. for nasal or oral drug delivery).Biodegradable polymer (implants, microparticles), Carrier in relation to vaccine delivery or gene therapy [1].

1.3.Preparation of Chitosan (CS) from Raw Materials:

CS is not a single chemical entity, but varies in composition depending on the source and method of preparation and also on physiological conditions. CS could be defined as sufficiently deacetylation of chitin to form a soluble amine salts. The degree of deacetylation must be 80 to 85% or higher or the acetyl content must be less than 4- 4.5% to form the soluble product. CS is manufactured commercially by a chemical method. Firstly the sources such as crab or shrimp shells are washed and grinded in to powdered form and then it is deproteinized by treatment with an aqueous 3-5% solution of sodium hydroxide. After that it is neutralized and demineralized at a room temperature by treating it with aqueous 3-5% of hydrochloric solution to form a white or slightly pink precipitate of chitin. Then chitin is deacetylated by treatment with an aqueous 40-45% of sodium hydroxide solution and the precipitate is then washed with water. The insoluble part is removed by dissolving in an aqueous 2% acetic acids solution. The supernatant solution is then neutralized with an aqueous sodium hydroxide solution to obtain a purified CS [7].

In the solid state, chitosan is a semicrystallinepolymer. Its morphology has been investigated, and many polymorphs are mentioned in the literature. Single crystals of chitosan were obtained using fully deacetylated chitin of low molecular weight. The unit cell contains two antiparallel chitosan chains, but no water molecules [10].

It consists of two types of monomers; chitin-monomers and chitosan-monomers.Chitosan is also reported to accelerate wound healing and enhance bone formation [9].

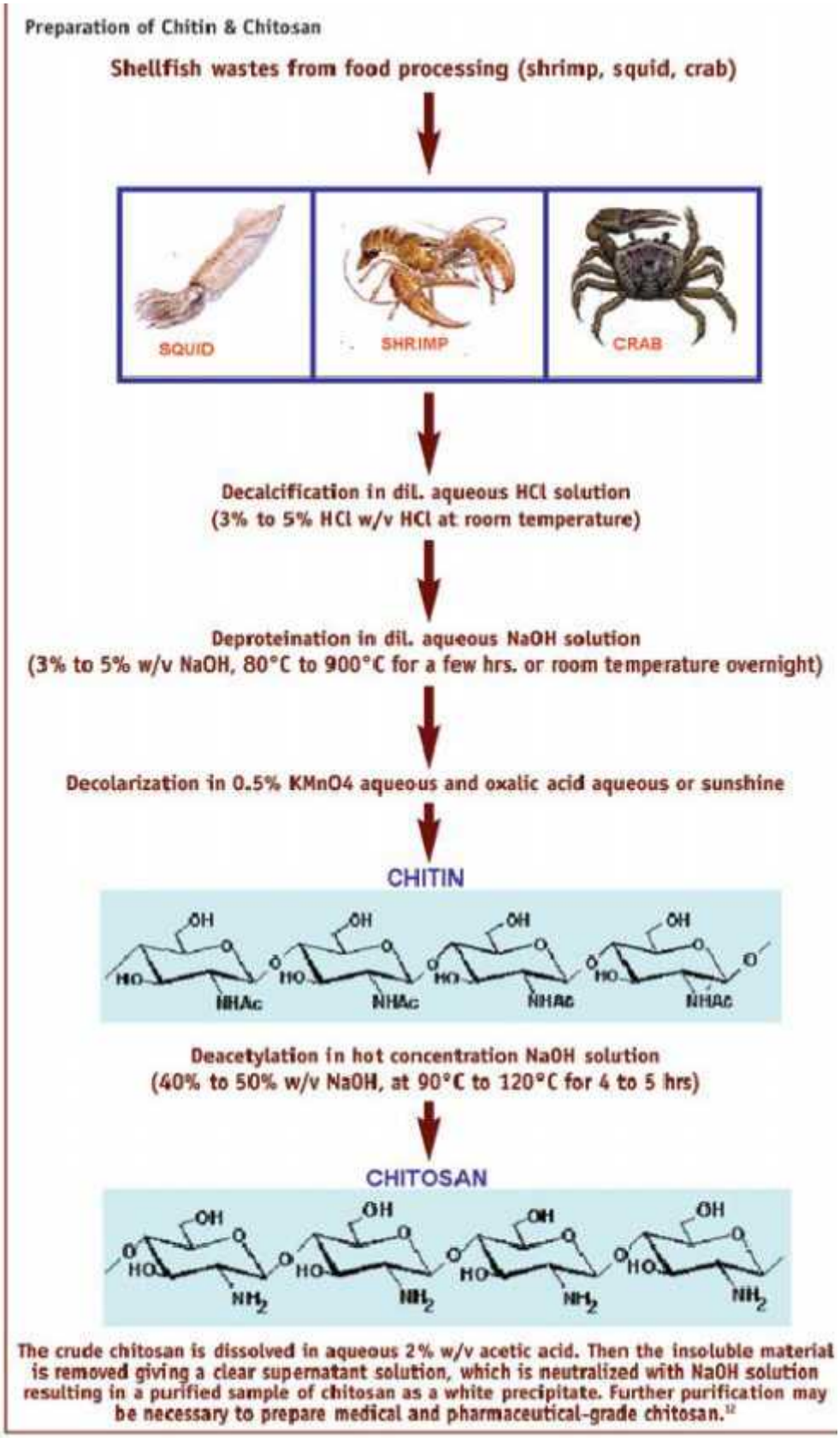

Figure-1:Properties of Chitin and Chitosan

1.4.Derivatives of Chitosan (CS): CS has a large no. Of application in pharmaceutal dosage form; its further application can be exploited by modification of basic structure to obtain polymers with a wide range of properties.

1.4.1.N-Trimethylene Chloride Chitosan:

Hamman and coworkers showed that the degree of quaternization of trimethylene chloride influences its drug absorption-enhancing properties . The charge on chitosan has a role in providing intestinal permeability. Hence, a quaternary derivatized chitosan (N-trimethylene chloride chitosan) is found to demonstrate higher intestinal permeability than chitosan alone. Polymers with higher degrees of quaternization (> 22%) are able to reduce the trans-epithelial electrical resistance and thereby epithelial transport (in vitro) in a neutral environment (pH 7.4). The maximum reduction in trans-epithelial resistance is found to be reached with trimethylene chloride with a degree of quaternization of 48%. This degree of quaternization is optimum for in-vitro transport of model drugs across a Caco-2 monolayer.

1.4.2.Chitosan Esters: Chitosan esters, such as chitosan succinate and chitosan phthalate have been used successfully as potential matrices for the colon- By convertingpolymer from an amine to succinate form, the solubility profile is changed significantly . The modified polymers are insoluble under acidic conditions and act as sustained release for the encapsulated agent under basic conditions and also for colon-targeted system[1].

1.4.3.Chitosan Conjugates: Guggi and Bernkop attached an enzyme inhibitor to chitosan. The resulting polymer retained the mucoadhesivity of chitosan preventing drug degradation by inhibiting enzymes such as trypsin and chymotrypsin. This conjugated chitosan has promising role in delivery of sensitive peptide drugs such as calcitonin[1].

1.5.Properties of Chitosan: The amino group in Chitosan has a pKa value of ~6.5, which leads to a protonation in acidic to neutral solution with a charge density dependent on pH and the %DA-value. This makes Chitosan water soluble and a bioadhesive which readily binds to negatively charged surfaces such as mucosal membranes. Chitosan enhances the transport of polar drugs across epithelial surfaces, and is biocompatible and biodegradable. Purified quantities of Chitosans are available for biomedical applications. (Fig.1)

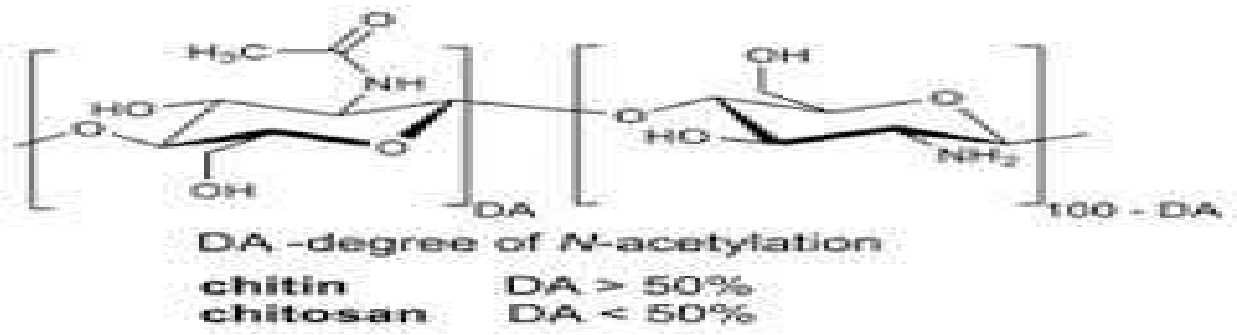

Figure. 2: Structural units of chitosan (right) and chitin (left)[5]

1.6.Characteristics of chitosan

1.6.1.Biocompatibility

Both chitin and chitosan show very good compatibility but this property depends on the characteristics of the sample (natural source, method of preparation, Mw and DD). Due to its higher versatility and biological properties the majority of the assays have been carried out on chitosan samples.Although the gastrointestinal enzymes can partially degrade both chitin and chitosan, when both polymers are orally administered they are not absorbed. For this reason, they are considered as not bioavailable. Chitosan shows a LD50 of around 16g/kg, very similar to the salt and glucose values in assays carried out on mice . Toxicity of chitosan is reported to depend on DD. Schipper et al. Reported that chitosans with DD higher than 35% showed low toxicity, while a DD under 35% caused dose dependant toxicity.

On the other hand, Mw of chitosan did not influence toxicity.Chitosan presents higher cytocompatibility in vitro than chitin. The cytocompatibility of chitosan has been proved in vitro with myocardial, endothelial and epithellial cells, fibroblast,hepatocytes, condrocytes and keratinocytes.This property seems to be related to the DD of the samples.When the positive charge of the polymer increases, the interactions between chitosan and the cells increase too, due to the presence of free amino groups. The adhesion and proliferationof keratinocytes and fibroblasts on several chitosan films with different DDs depend on both, DD and cell type. In both cells, the percentage of cell adhesion was strongly dependent of the DD, increasing with this parameter. The type of cell was a factor that also affected the adhesion, being more favourable for fibroblasts which exhibit a morenegative charge surface than for keratinocytes. On the other hand, the proliferation decreased considerably by increasingthe DD.

Residual proteins in chitin and chitosan could cause allergic reactions such as hypersensitivity. The protein content in a sample depends on the source of the sample and, especially,on thet method of preparation[1].

1.6.2.Anti Cancerous Agent

Santosh Kumar and coworkers30 concluded that novel chitosan–thymine conjugate has been successfully synthesized by the acylation reaction between chitosan and thymine-1-yl-acetic acid and its dual antimicrobial and anticancer effect had been tested. The morphological study of the chitosan–thymine conjugate has shown macro porous structure for biomedical properties. The microbiological screening has demonstrated the positive antimicrobial activity against pathogenic bacteria and fungi. The assays for cell proliferation and viability showed that the chitosan–thymine conjugate was non-cytotoxic but significantly reduced the rate of proliferation in cancerous HepG2 cells. Thus, the chitosan–thymine conjugate might be a very promising candidate for practical applications in the field of biomedical and medicine vis-à-vis genetic information (transfer and function)[1].

1.6.3. Antibacterial Activity

Chitosan may also have an effect on the type of bacteria living in the intestines or on the action of these bacteria. A small human study found that taking 3-6 grams per day of chitosan for two weeks reduced indicators of putrefaction in the intestines, change that might help prevent diseases such as colon cancer. Antibacterial activity of the water-soluble N- alkylated disaccharide chitosan derivatives against Escherichia coli and Staphylococcus.

1.6.4. Wound Healing

Kaoru Murakami and co workers prepared a composite hydrogel sheet produced from blended alginate, chitin/chitosan and fucoidan powders (ACF-HS). It possesses many advantages as a wound dressing for repair of healing-impaired wounds. The application of ACF-HS significantly stimulated repair of mitomycin C-treated healing- impaired wounds in rats. Thus, ACF-HS is a promising wound dressing for healing-impaired wound repair. R. Jayakumar,M. Prabaharan and coworkers reviewed the recent progress of chitin and chitosan- based fibrous materials, hydrogels, membranes, scaffolds and sponges in wound dressing. The fibrous materials based on chitin and its derivatives have the properties of high durability, good biocompatibility, low toxicity, liquid absorption, and antibacterial activity. These properties would lead to accelerate wound healing. Chitosan/collagen membrane could be used to hasten wound healing and induce cell migration and proliferation and antibacterial activity

1.7.SILK FIBRION

Silks are generally defined as protein polymers that are spun into fibers by some lepidoptera larvae such as silkworms, spiders, scorpions, mites and flies [1–3]. Silk proteins are usually produced within specialized glands after biosynthesis in epithelial cells, followed by secre- tion into the lumen of these glands where the proteins are stored prior to spinning into fibers. Silks differ widely in composition, structure and properties depend- ing on the specific source. The most extensively characterized silks are from the domesticated silkworm, Bombyx mori, and from spiders (Nephila clavipes and Araneus diadematus). Many of the more evolutionarily advanced spiders synthesize different types of silks. Each of these different silks has a different amino acid composition and exhibits mechanical properties tailored to their specific functions: reproduction as cocoon capsular structures, lines for prey capture, lifeline support (dragline), web construction and adhesion. Fibrous proteins, such as silks and collagens, are characterized by a highly repetitive primary sequence that leads to significant homogeneity in secondary structure, i.e., triple helices in the case of collagens and b-sheets in the case of many of the silks.

1.7.1. STRUCTURE OF SILK FIBROIN

Sılk fibroin,like creatine and collogen,belongs to fibrillar proteins.The elements of the supramolecular structure of silk fibers are macrofibrils with a width of up $6.5x10^5$ nm,which,in turn,consist of helically packed nanofibers 90-170 nm in diameter.Nanpfibrils may play an important role in imparting enchanged strength to silks.The molecular weıght of natural silk fibroin reaches 370 000 Da;fibroin macrochain length,150 nm;and macrochain diameter, 0.45 nm.

Silk fibers produced by cultivated Bomby mori mulberry silkworm mainly consist of two proteins,sericin and fibroin ; they also contain minor amounts of residues of other amino acids and various impurities:falts,waxes dyes,and mineral salts.Depending on the cocoon strain,the fibroin content is 66.5-73.5,abd the sericin content ,26.5-33.5 wt%.

As for the chemical composition,Bombyx mori fibers consist of residues of no less than 16 amino acids whose ratio varies between different areas of the supramolecular structure of fibroin.”Heavy” areas of the polymer,with a mean molecular weightt of up to 350 000-370 000,mainly consist of highly ordered hydrophobic macromolecules,and in loser “light”areas

with a mean molecular weight of about 25 000 the major components are polar amino acid residues.

The mole fraction of glycine,alanine,serine,and tyrosine residues combined is 90 %; their sequnce is represent by the general formula.

-Gly-Ala-Gly Ala-Gly-Ser-Gly-Ala-Ala-Gly-[-Ser-Gly-$(Ala-Gly)_n$-]$_8$-Tyr-

1.7.2. PROPERITES OF SILK-FIBROIN

The enhanced environmental stability of silk fibers in comparison to globular proteins is due to the extensive hydrogen bonding, the hydrophobic nature of much of the protein, and the significant crystallinity. Silks are insoluble in most solvents, including water, dilute acid and alkali. Detailed structural analysis of spider dragline silk proteins has yielded information on the organiza- tion and orientation of the numerous but very small b-sheet crystals in the fibers, and a high level of organization of the protein even in the less crystalline domains. Liquid crystalline phases and conforma- tional polymorphism have been implicated in the biological processing of these proteins to contribute to the architectural features within the fibers . These nanoscale features, factoring in the small, orientated and numerous b-sheet crystals, a fuzzy interphase between these crystals and the less crystalline domains, and the shear alignment of the chains, provides a basis for the origin of the novel mechanical properties exhibited by silk fibers. A comparison of mechanical properties suggests that they provide a remarkable combination of strength and toughness. The distinguish- ing features of the spider silks are the very high strength in combination with excellent elasticity in comparison with these other biomaterials. In addition, these fibers display resistance to failure in compression that distinguishes them from other high performance fibers, such as Kevlar.

1.7.3. BIOMEDICAL APPLICATIONS OF SILK-FIBROIN

1.7.3.1. Tissue Engineering

Silk based tissue engineering system which search new methods and materials to create synthetic tissue mimics that can be implanted in vivo to spur regeneration of diseased tissue or injured.

1.7.3.2. Drug delivery

Drug delivery is the method or process of administering a pharmeceutical compound to achieve a therapeutic effect im humans or animals. Drug delivery release profile,absorption,distribution and elimination for the benefit of improving product effiency and safety as well as patient convenience and compliance.

1.7.3.3. Blood-Contacting Material

Several approaches have been used to improve blood compatibility of SF. Silk fibroin have been modeled after the structure of the highly sulfated polysaccharide heparin, which is anticoagulation.

SF derivatives produced through the reaction with sulfuric acid, and sulfonated silk blends were shown to be effective anticoagulation, suggesting that type of chemical modification of SF would be useful for applications where these materials will be in contact with blood.

2-EXPERIMENTAL

2.1. Materials and Methods

Ultrapure water were supplied by Near East University medicine faculty. CAN(ceric ammonium nitrate) was purchased from Sigma-Aldrich(St.Louis,MO,USA), CH3COOH (acetic acid)were purchase from E.Mecrk D-6100 Darmstadt. Ultrapure water was used to prepared silk fibroin. Acetic acid and ultrapure water were used to prepared Chitosan.

2.2. Preparation of Chitosansolution

Firstly measured 0,2g chitosan, 0,1M 100ml acetic acid. After we put the magnetic strirrer no temprature and spined at 1 rpm.

Figure-3: Solution of CS

2.3. Preperation of Silk Fibroin

2.3.1 Silk Degumming;

1 gram of Bombyx Bori was measured and boild for 3 hour in a 0.1 M Sodium carbonate solution at 70 ^{o}C on a magnetic strirrer with speed of 1 rpm.Then rinsed throughly with warm ultrapure water to extract the glue like sericin proteins.This procedure is repeated three times and then dried at room temperature.

2.3.2 Preperation of Electrolyte

Electrolyte formation is dissolving the silk fibres to have liquid form of silk fibroin by breaking down the H-bonding in β-sheet to get aqeous solution.
In this step,$CaCl_2$,C_2H_5OH,H_2O(1:2:8 mole ratio)and degummed silk fibres were mixed at 75 ^{o}C with stirring.

2.3.3. Silk Fibroin Dialysis

Dialysis is removal of the ions within the solution obtained from the dissolution step.Electrolyte solution was dialyzed continuously for 72 h against running ultrapure water to remove ions using a cellulse semi-permeable membrane (made of Carboxymethyl, diameter: 2.7 cm).The liquid silk fibroin was stored to be used in nanoparticle preparation.

Figure-4:Degumming process

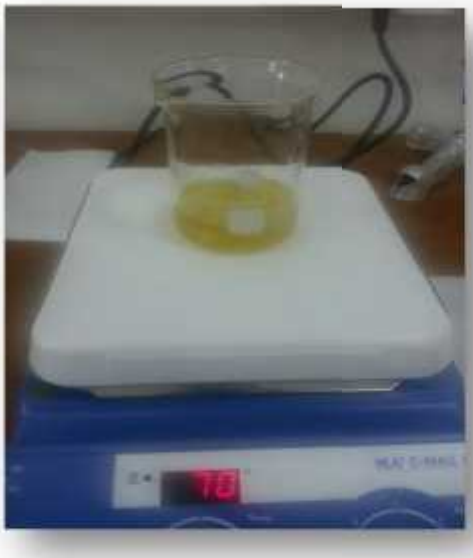

Figure-5:Electrolyte procedure

Figure-6: Dialysis system

2.4. Preperation of Phosphate Buffer Saline

There are many different ways to prepare Phosphate Buffer Saline.Some formulations do not contains potassium,while others contain calcium or magnesium.Generally,PBS contains the following constituents:

Salt	Concentration	Concentration
(-)	**(mmol/L)**	(g/L)
NaCl	137	8.01
KCL	2.7	0.20
$Na_2HPO_4.H_2O$	10	1.78
KH_2PO_4	2.0	0.27
Ph	7.4	7.4

Table-1:Phosphate Buffer Saline Contents

After preparing the PBS solution the PH is adjusted to 7.4 by adding either Hydrochloric acid HCl or Sodium hydroxide NaOH depending on the PH value whether it is below or above 7.4

2.5.Acetic Acid Solution Preperation

Glacial acetic acid is diluted by water to get 0.5M acetic acid after that the ph is adjusted to 1.2 by HCl if the PH value goes beyond that point it can be reversed back by NaOH.

2.6. Preparation of Chitosan Graft and Silk Fibroin Hydrogels

First Graft used 18ml dissolved chitosan ,5ml of liquid silk fibroin and 100 ml 0,1M CH3COOH(acetic acid)and 0,05g CAN(ceric ammonium nitrate) were obtained solution. In the process used 5 bar nitrogen gas 2 hour at 60C to protected to crystalline structure. After form of grafting we are used pure Aseton to precipitated. We repeated four times grafting operations.We took best result at CAN 0.0756g.

3.RESULTS AND DISCUSSIONS

3.1.Creating SF and CS Graftings

CS(Chitosan)	SF(Silk Fibroin)	CAN(Ceric Ammonium)	Time	Temperature	rpm
18 ml	5 ml	0.0527 g	2h	60 ^{o}C	1 rpm
18 ml	5 ml	0.0756 g	2h	60 ^{o}C	1 rpm
18 ml	5 ml	0.1 g	2h	60 ^{o}C	1 rpm
18 ml	6 ml	0.0756 g	2h	60 ^{o}C	1 rpm

Table-2:Proportions and Properties of CS and SF Grafts

Figure-7: Preparation of CS graft and SF hydrogels

Figure-8: Before filtration operation

3.1.2 Filtration of Graft

Consisted chitosan solution filtration with filter paper and we seperated particles. After chitosan filtration we obtained;

Figure-9:Filtration of CS graft and SF hydrogels **Figure-10:Waste products of CS graft andSF**

Figure-11 : Result of CS graft and SF hydrogels

3.1.3. Swelling Test for SF and CS Grafts

After the grafts were prepared,they were tested for their swelling properties in the PBS and ABS solutions.

The swelling ratios were calculated by using:

$$\text{Swelling \%} = \frac{\text{weight t} - \text{weight(dry)}}{\text{weight(dry)}} * 100\%$$

Where weight(t) is the graft's weight measured at any given time and the weight (dry)is the weight of the grafts in its dry state.

3.1.3.1. Swelling Test in PBS at pH:7.4

Grafts	Ingredients	Propotions	Weight in dry state
G1	CS+SF+CAN	18 ml + 5 ml+ 0.0527 g	0.0275g
G2	CS+SF+CAN	18 ml+ 5 ml + 0.0756 g	0.0141 g
G3	CS+SF+CAN	18 ml+ 5 ml+ 0.1 g	0.0353 g

Table-3: Propeties of SF+CS Grafts which were used in PBS swelling test

Time (minutes)	G1 Weight (g)	G2 Weight (g)	G3 Weight (g)
5	0.0312	0.0153	0.0424
10	0.0323	0.0173	0.0465
15	0.0335	0.0186	0.0487
20	0.0349	0.0192	0.0555
25	0.0363	0.0218	0.0592
30	0.0389	0.0238	0.0625
45	0.0402	0.0273	0.0703
60	0.0413	0.0272	0.0747
75	0.0438	0.0264	0.0773
90	0.0456	0.0286	0.0778
120	0.0465	0.0297	0.0790
150	0.0488	0.0281	0.0810
1391	0.0501	0.0294	0.0833
1421	0.0505	0.0292	0.0838
1451	0.0517	0.0303	0.0786
1511	0.0504	0.0292	0.0848

1571	0.0510	0.0293	0.0808
2828	0.0532	0.0287	0.0812
2888	0.0542	0.0295	0.0844
4316	0.0563	0.0297	0.0802
4436	0.0536	0.0297	0.0787
9991	0.0539	0.0288	0.0937
10051	0.0551	0.0303	0.0944
10111	0.0563	0.0305	0.0927
11243	0.0575		0.0942
11423	0.0572		0.0938

Table-4: The weight results of SF+CS grafts while swelling in Phospate Buffer Solutin at pH 7.4

Swelling Ratios (%)

Time (minutes)	G1	G2	G3
5	13.45	8.51	20.11
10	17.45	22.69	31.72
15	21.18	31.91	37.96
20	26.9	36.17	57.22
25	32	54.60	67.70
30	41.5	68.79	77.05
45	46.18	93.61	99.15
60	50.18	92.90	111.61
75	59.3	87.23	118.98
90	65.81	102.83	120.39
120	69.1	110.63	123.79
150	77.5	99.29	129.46
1391	82.18	108.51	135.97
1421	83.63	107.09	137.39
1451	88	114.89	122.66
1511	83.3	107.09	140.22
1571	85.5	107.80	128.89
2828	93.5	103.54	130.02
2888	104.7	109.21	139.09
4316	94.9	110.63	127.19
4436	96	110.63	122.94
9991	100.3	104.25	165.43
10051	104.7	114.89	167.42
10111	109.1	116.31	162.60
11243	108	116.10	166.85
11423	107.6	115.86	165.72

Table-5: Swelling ratios of SF+CS grafts while swelling in Phosphate Buffer Solution at pH 7.4

Figure-12:Swelling Test for Phosphate Buffer Saline of Graft 1

Figure-13:Swelling Test for Phosphate Buffer Saline of Graft 2

Figure-14:Swelling Test for Phosphate Buffer Saline of Graft 3

We prepare 3 different samples that includes constant amount of SF and CS but different amount of CAN.

In the first sample we can see that after 75 minutes the swelling ratio reaches to equilibrium state.In the second graph it can be said after 90 minutes the swelling ratio reaches to equilibrium level.For the third graph we can say at 1391 minutes the swelling ratio reaches to equilibrium state.However it can also see that after 4436 minutes the graft 3 swells and then being stable again.

From these three graphics it can be seen PBS swelling ratio is increasing at the certain level then it reaches to the equilibrium state.However, we can say that as increasing the amount of CAN,time to reach equilibrium is increasing.

3.1.3.2.CS and SF Grafts'Swelling Test in ABS at pH:1.2

Grafts	Ingredients	Propotions	Weight in dry state
G1	CS+SF+CAN	18 ml + 5 ml+ 0.0527 g	0.0143g
G2	CS+SF+CAN	18 ml+ 5 ml + 0.0756 g	0.0167g
G3	CS+SF+CAN	18 ml+ 5 ml+ 0.1 g	0.0198g

Table-6: Propeties of SF+CS Grafts which were used in ABS swelling test

Time (minutes)	G1 Weight (g)	G2 Weight (g)	G3 Weight (g)
5	0.0169	0.0205	0.0235
10	0.0180	0.0225	0.0268
15	0.0200	0.0241	0.0287
20	0.0207	0.0261	0.0324
25	0.0211	0.0283	0.0358
30	0.0247	0.0305	0.0374
45	0.0248	0.0344	0.0423
60	0.0253	0.0352	0.0469
75	0.0272	0.0384	0.0457
90	0.0289	0.0349	0.0484
120	0.0312	0.0384	0.0476
150	0.0292	0.0349	0.0527
1391	0.0323	0.0374	0.0478
1421	0.0318	0.0355	0.0483
1451	0.0321	0.0349	0.0463
1511	0.0320	0.0351	0.0453
1571	0.0317	0.0342	0.0420
2828	0.0317	0.0347	0.0451
2888	0.0306	0.0375	0.0427
4316	0.0307	0.0339	0.0401
4436	0.0306	0.0357	0.0430
9991	0.0313	0.0343	0.0413
10051	0.0306	0.0336	0.0406
10111	0,0308	0.0321	0.0373
11243	0.0313	0.0309	0.0361
11423	0.0318	0.0306	0.0332

Table-7: The weight results of SF+CS grafts while swelling in Acetıc Acıd Buffer Solutıon at pH 1.2

Swelling Ratios (%)

Time (minutes)	G1	G2	G3
5	18.8	22.75	18.68
10	25.9	34.73	35.35
15	39.9	44.31	44.94
20	44.6	59.88	63.63
25	47.6	69.46	80.80
30	72.4	82.63	88.88
45	73.4	105.98	113.63
60	77	110.77	136.86
75	90.2	129.94	130.80
90	102.1	108.98	144.44
120	118.2	123.95	140.40
150	118.2	112.57	166.16
1391	104.2	108.98	141.41
1421	126.9	110.17	143.93
1451	122.4	104.79	133.83
1511	124.5	107.78	128.78
1571	123.8	124.55	112.12
2828	121.7	102.99	127.77
2888	114	113.77	115.65
4316	114.7	105.38	102.52
4436	114	101.19	117.17
9991	122.4	92.21	108.58
10051	115.4	85.02	105.05
10111	125.2	83.23	88.38
11243	116.8	82.60	82.32
11423	114.2	79.92	67.67

Table-8: Swelling ratios of SF+CS grafts while swelling in Acetic Acid Buffer Solution at pH 1.2

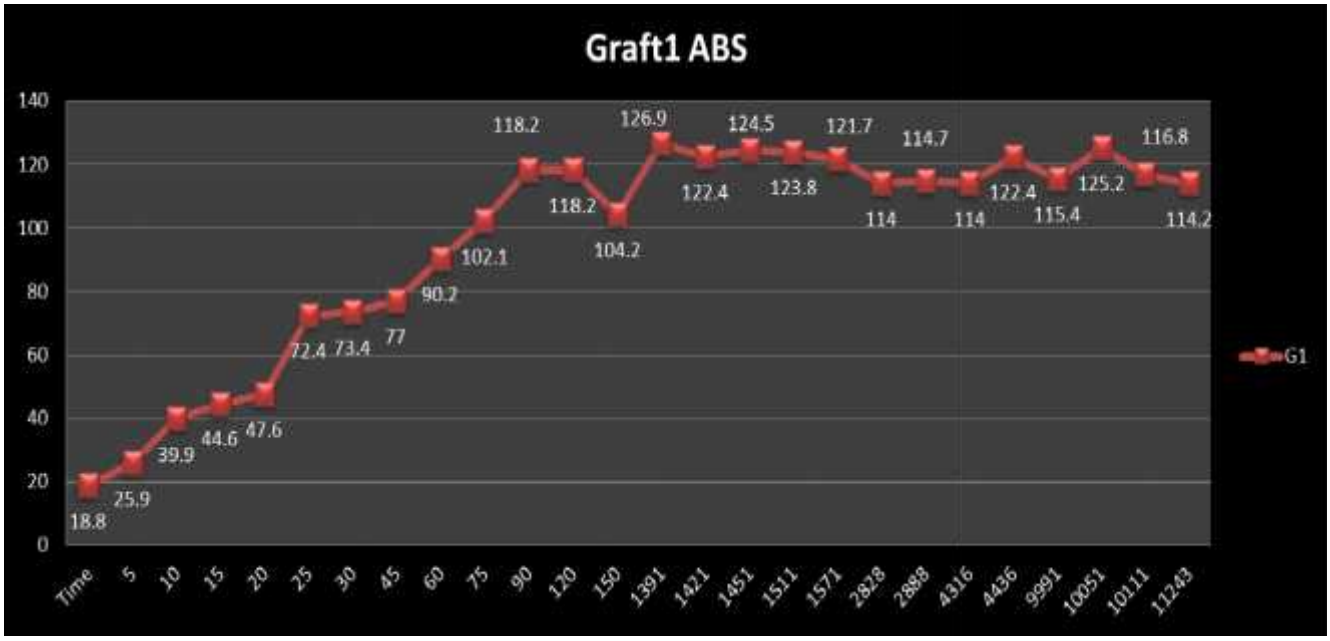

Figure-15:Swelling Test for Acedic Buffer Saline of Graft 1

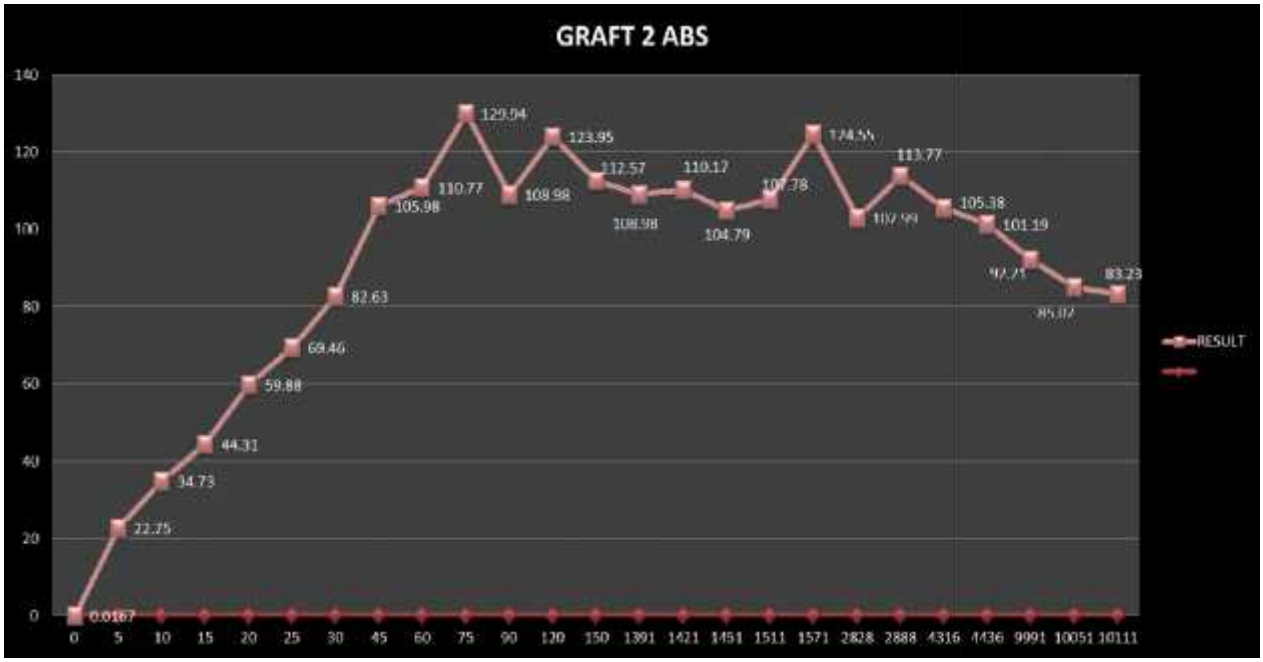

Figure-16: Swelling Test for Acedic Buffer Saline of Graft 2

Figure-17:Swelling Test for Acedic Buffer Saline of Graft 3

The graphs show that, the SF and CS graft crystalline structure swells up to the optimum swelling value.Then begins decreasing.These swelling ratio decreasing causes by the decomposition of our crystalline structure.The result prove that at basic pH,crystalline structures keep their surface stability at their saturation level.But when they faced with acidity they start to dissolve.

3.2. CS and SF Biofilms

3.2.1 Preperation of biofilms

Sample	Glyserin	Silk Fibroin	Chitosan
1	0.0563 gr	2 ml	2 ml
2	0.0563 gr	2 ml	3 ml
3	0.0563 gr	3 ml	3 ml
4	0.0563 gr	3 ml	2 ml

Table-9:Proportions of SF and CS Biofilms

Silk fibroin and chitosan biofilms were preperad by mixing different amounts of chitosan and silk fibroin showed in Table-9 and 0.0563 g of glycerine.Then the solution was placed on to

smooth glass slides at room temperature.After one day they washed with pure water in order to remove the films from glass slides.Then the films were allowed to dry.

Figure-18:CS and SF Biofilm 1

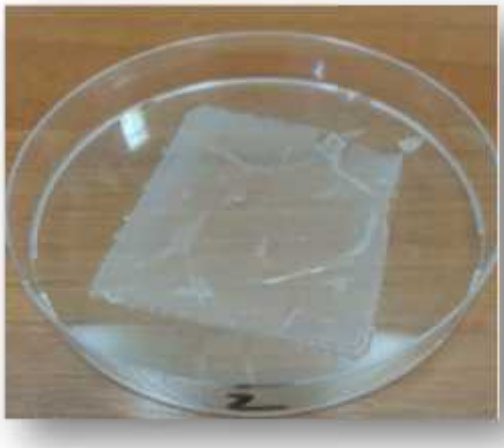

Figure-19: CS and SF Biofilm 2

Figure-20:CS and SF Biofilm 3

Figure-21:CS and SF Biofilm 4

During the experiment instead of washing with distilled water methanol treatment was applied to the films but structural damage were occured since the chitosan is a polymer.

3.3. Antimicrobial Activity

Antibiotic sensitivity is the susceptibility a bacterium to an antibiotic. Antibiotic susceptibility testing is used to determine which antibiotic will be most successful in treating a bacterial infection.

Small discs are placed onto a petri dish. Then the material is applied onto the dics to examine the bacterial growth. This method based on the diffusion of material through the disc. If materials have antibiotic behaviour they kill bacteria or fungus and create a clear ring called zone of inhibition around the discs.

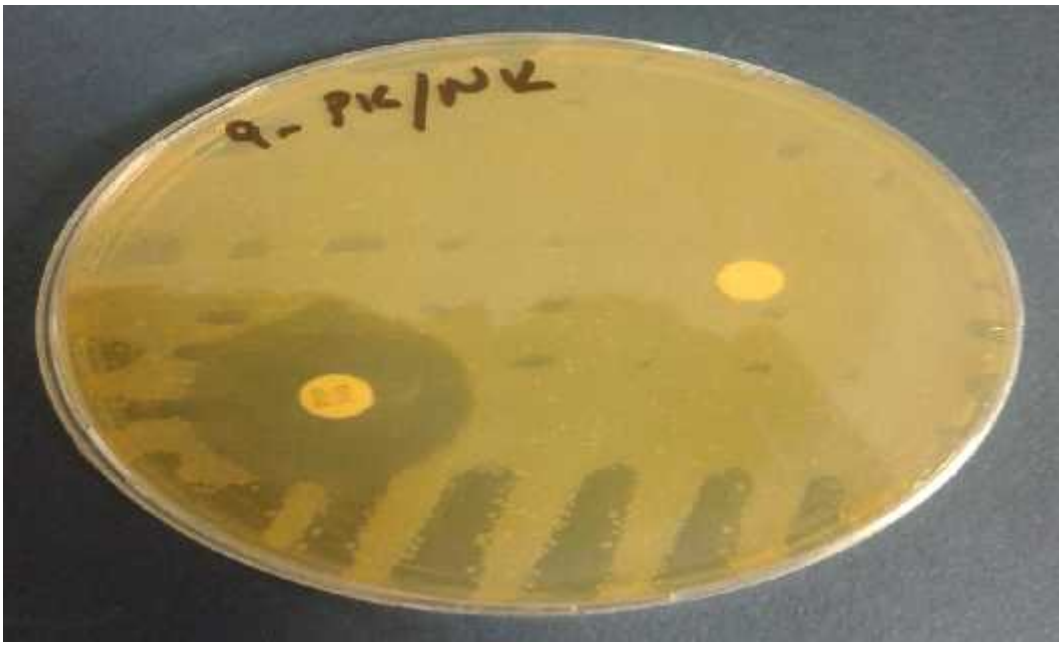

Figure-22:Positive-Negative Control of Bacterial Test

In the antibicrobial activity experiments,firstly agar was poured into the petri dishes to supply the microbiological growth medium at the pH level 7.2-7.4.Then 100µl E.coli bacteria were added to medium.The disc to be diffused.Liquid form of SF and mixture of SF and CS was applied to disc,however the SF biofilms were put in the medium without disc.Then samples incubated overnight at 37 ^{o}C.

Figure-23:SF and CS 40µl

Figure-24:SF and CS 80µl

Figure-25:SF and CS 40µl

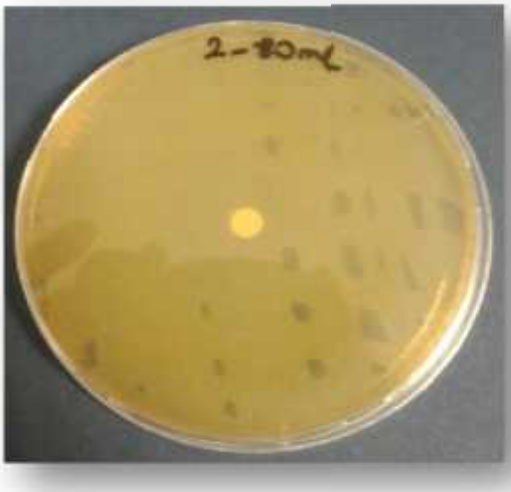

Figure-26:SF and CS 80µl

Figure-27:SF and CS 40µl

Figure-28:SF and CS 80µl

Figure-29:SF Film Tablet

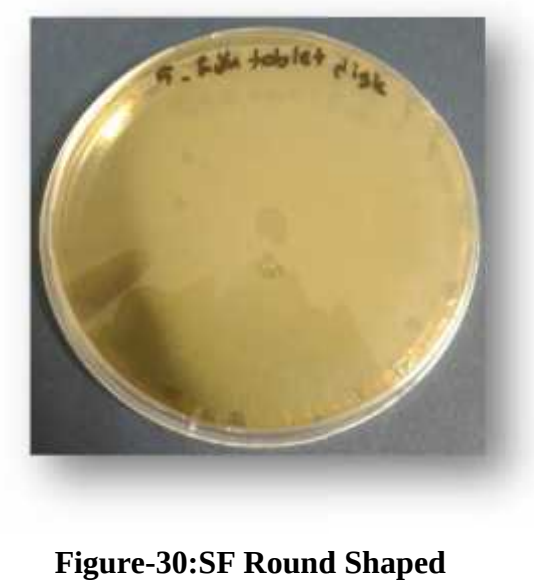

Figure-30:SF Round Shaped

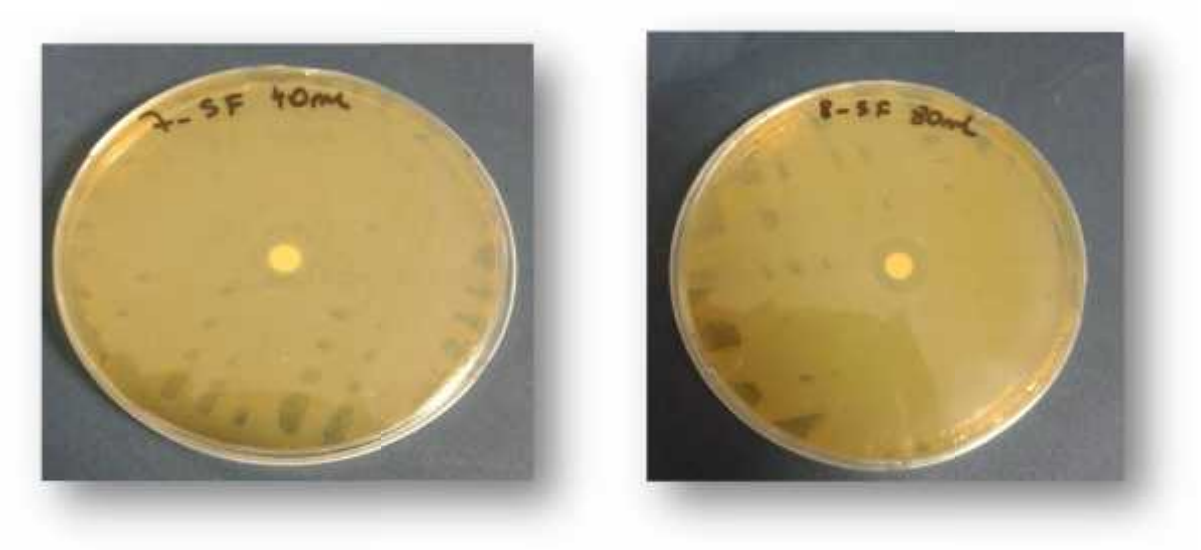

Figure-31:SF 40µl Disc Zone **Figure-32:SF 80µl Disc Zone**

The results showed that silk fibroin has bactericidal property which is mean have bacteria killing behavior.The effect of bacteria killing is increased as the amount of SF is increased.
The silk fibroin biofilms and SF and CS mixture have no bacteriocidal property but have bacteriostatic property that prevents the bacterial growth.

4.CONCLUSION

1. In this study, we achieved a crystalline structure by grafting the liquid form of silk fibroin and chitosan via using inhibitor called cerric ammonium nitrate in an inert atmosphere, supplied by nitrogen gas, instead of creating the grafts with electrospinning method like the other studies.
2. The acidic and basic behaviour of the silk fibroin and chitosan graftswere examined.From the PBS test graphics it can be seen swelling ratio is increasing at the certain level then it reaches to the equilibrium state. However, we can say that as increasing the amount of CAN, the time to reach equilibrium is increasing also.
3. The ABS graphs show that the S and CS graft crystalline structures swells up to the optimum swelling value.Then begins decreasing.These swelling ratio decreasing causes by the decomposition of our crystalline structure. From the graphics we also observed that as increasing the amount of CAN the stability of the swelling ratio is deteriorated.The result prove that at basic pH,crystalline structures keep their surface stability at their saturation level.But when they faced with acidity they start to dissolve.
4. During prepearing the chitosan silk fibroin biofilms we observed that if the amount of siik fibroin is increaed, the geleation formation is occured. For the silk fibroin based biofilms methanol is used to remove the films from the glass slides. When we applied methanol to the chitosan and silk fibroin biofilms we have no success becaouse methanol distubs the chitosan structure. Distilled water was applied for removing the films from glass slides.
5. The antimicrobial activity test results showed that silk fibroin has bactericidal property which is mean have bacteria killing behavior.The effect of bacteria killing is increased as the amount of SF is increased.The silk fibroin biofilms and the liquid form SF and CS mixture have no bacteriocidal property but have bacteriostatic property that prevents the bacterial growth.

5.REFERENCES

[1]Gavhane Yogeshkumar N.1, Gurav Atul S1 and Yadav Adhikrao V.21Department of Biopharmaceutics, Government College of Pharmacy, Karad, Tal-Karad,
Dist-Satara, Pin-415124, Maharashtra, India.
2Gaurishankar Educational and Charitable Trust's, Gaurishankar Institute of Pharmaceutical Education and Research, Limb, Satara, Pin-415015, Maharashtra, India.

[2]Gregory H. Altman, Frank Diaz, Caroline Jakuba, Tara Calabro, Rebecca L. Horan, Jingsong Chen, Helen Lu, John Richmond, David L. Kaplan*

[3] V.Kearnrs, A.C. MacIntosh, A. Crawford and P.V. Hatton-Silk-based Biomaterials for Tissue Engineering,2008.

[4] Kumar, R.M.N. V.-Eds. Handbook of Particulate Drug Delivery,2008.

[5]Jayakumar R, Prabaharan M, Reis RL, Mano JF. Graft copolymerizedchitosan—Present status and applications. CarbohydrPolym 2005;62:142–158.

[6] Inmaculada Aranaz, Marian Mengíbar, Ruth Harris, Inés Paños, Beatriz Miralles, Niuris Acosta, Gemma Galed and Ángeles Heras; Functional Characterization of Chitin and Chitosan Current Chemical Biology, 2009, 3, 203-230

[7]Nitar Nwe , Tetsuya Furuike and Hiroshi Tamura ;The Mechanical and Biological Properties of Chitosan Scaffolds for Tissue Regeneration Templates Are Significantly Enhanced by Chitosan from Gongronella butleri. Materials 2009, 2, 374-398; doi:10.3390/ma2020374

[8]Nuttapon Vachiraroja, Juthamas Ratanavaraporna, Siriporn Damrongsakkula,Rath Pichyangkurab, Tanom Banaprasertc, Sorada Kanokpanont"A comparison of Thai silk fibroin-based and chitosan-based materials on in vitro biocompatibility for bone substitutes".

[9] Mariana Agostini de Moraes, Grinia Michelle Nogueira, Raquel Farias Weska and Marisa Masumi Beppu "Preparation and Characterization of Insoluble Silk Fibroin/Chitosan Blend Films"

[10] Marguerite Rinaudo "Chitin and chitosan: Properties and applications"