CHIDI WILSON NWEKWO
THE EFFECTS OF SILK FIBROIN BASED BIOSCAFFOLDS AND BIOFILMS FOR CARTILAGE TISSUE ENGINEERING
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A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES OF NEAR EAST UNIVERSITY

By
CHIDI WILSON NWEKWO

In Partial Fulfillment of the Requirements for The Degree of a Doctor of Philosophy in Biomedical Engineering

NICOSIA, 2021

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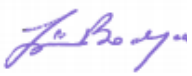
Approval of Director of the Institute of Graduate Studies

Prof. Dr. K Hüsnü Can BAŞER

We certify this thesis is satisfactory for the award of the degree of Doctor of Philosophy in Biomedical Engineering

Examining Committee in Charge:

Prof. Dr.Tulin Bodamyali		Committee Chairman, Department of Health Sciences', Faculty of Health Sciences, GAU
Prof. Dr. Terin Adali		Supervisor, Department of Biomedical of Engineering, Faculty of Engineering, NEU
Doç. Dr. Rasime Kalkan		Co-Supervisor, Department of Medical Genetics, Faculty of Medicine, NEU
Doç. Dr. Pinar Tulay		Department of Medical Genetics, Faculty of Medicine, NEU
Yrd. Doç. Dr. Ayşe Sarioğlu		Department of Medical Microbiology, Faculty of Medicine, NEU
Doç. Dr. Aylin Şendemir		Department of Bioengineering, Faculty of Engineering, EGE University

I hereby declare that all the information in this document has been obtained and presented in accordance with the academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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I should mention my one-of-a-kind housemates Coston, Emmanuel, and Ifeanyi. Then last but not the least my NECF family, from all the leaders to every one of the members helped me not be an island in an island (not alone). I really thank everyone including you who would be reading this and wonder where is my name on the list. I assure you, you are in my heart more importantly and not on a paper temporarily.

To my parents and family…

ABSTRACT

In tissue engineering and regenerative medicine, there is a need for systems delivering therapeutic agents effectively to the target area. Using injectable hydrogels as carriers is one application for this purpose, but thrombogenicity of the hydrogel can create challenges in the delivery and treatment. The polymers used in injectable hydrogel production should be biocompatible and biodegradable with structural activity, and should have the desired blood compatibility. In this study, we prepared hydrogels as combinations of silk fibroin, gelatin, polyethylene glycol and added a drug, curcumin, which has wound healing and anti-inflammatory properties. The purity of curcumin which was extracted from the rhizome of *Curcuma longa* was determined by NMR spectroscopy. Chemical, physical and thermal properties of hydrogel were determined by FTIR spectroscopy, SEM, DSC, respectively. Swelling tests, *in vitro* anticoagulation analysis and the fibrinogen concentration were determined. In our study, the presence of curcumin showed increased fibrinogen concentration and delayed blood coagulation in some formulations. The results of this study presents suitable candidates for biomedical applications with continuous blood contact and establishes the anticoagulant property of curcumin with its ability to increase fibrinogen concentration as well.

***Keywords*:** Silk fibroin; curcumin; gelatin; injectable hydrogel; anticoagulant activity

ÖZET

Doku mühendisliği ve rejeneratif tıpta, hedef alana terapötik ajanları etkili bir şekilde veren sistemlere ihtiyaç vardır. Enjekte edilebilir hidrojellerin taşıyıcı olarak kullanılması bu amaç için bir uygulamadır, ancak hidrojelin trombojenikliği, uygulama ve tedavide zorluklar yaratabilir. Enjekte edilebilir hidrojel üretiminde kullanılan polimerler biyolojik olarak uyumlu ve yapısal aktivite ile biyolojik olarak parçalanabilir ve istenen kan uyumluluğuna sahip olmalıdır. Bu çalışmada, ipek fibroin, jelatin, polietilen glikol kombinasyonları olarak hidrojeller hazırladık ve yara iyileştirici ve iltihap önleyici özelliklere sahip bir ilaç olan kurkumin ekledik. Curcuma longa rizomundan ekstrakte edilen kurkuminin saflığı NMR spektroskopisi ile belirlendi. Hidrojelin kimyasal, fiziksel ve termal özellikleri sırasıyla FTIR spektroskopisi, SEM, DSC ile belirlendi. Şişme testleri, in vitro antikoagülasyon analizi ve fibrinojen konsantrasyonu belirlendi. Çalışmamızda kurkumin varlığı bazı formülasyonlarda fibrinojen konsantrasyonunun arttığını ve kan pıhtılaşmasını geciktirdiğini göstermiştir. Bu çalışmanın sonuçları, sürekli kan teması olan biyomedikal uygulamalar için uygun adaylar sunmakta ve fibrinojen konsantrasyonunu artırma yeteneği ile kurkuminin antikoagülan özelliğini de oluşturmaktadır.

Anahtar Kelimeler: İpek fibroin; curcumin; Jelatin; enjekte edilebilir hidrojel; antikoagülan aktivite

TABLE OF CONTENTS

APPENDICES

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3D:	Three Dimensional
APTT:	Activated Partial Thromboplastin Time
CUR:	Curcumin
DSC:	Differential Scanning Calorimetry
DMSO	Dimethyl Sulfoxide
ECM:	Extracellular Matrix
FTIR:	Fourier-Transform Infrared
G:	Gelatin
INR:	International Normalised Ratio
iPSCs:	Induced Pluripotent Stem Cells
MTT assay	Methyl thiazolyl tetrazolium assay
NFC:	Nanofibrillated Cellulose
NHAC-kn:	Normal Human Articular knee chondrocyte
NMR	Nuclear Magnetic Resonance
PEG:	Poly(ethylene glycol)
PT:	Prothrombin Time
SEM:	Scanning Electron Microscopy
SF:	Silk Fibroin
TCP	Tissue Culture Plastic
TE:	Tissue Engineering

CHAPTER 1
INTRODUCTION

1.1Thesis Problem

Connective tissue composed joint cartilage of the knee is very specialized to reduce friction where two of the long bones interface one another and the avascular structure has chondrocytes. The restoration of injured joint cartilage continues to be challenging because of its poor internal repair capacity. Thus, until now no surgical procedure could produce biological composition and biomechanical properties local cartilages. The emergence of tissue engineering revolutionized the potential in the treatment of cartilage related diseases. The articular cartilage tissue engineering will exceed the current limitations of surgical treatment by offering functional regeneration in the area of defect. This technology involves ex vivo culture chondrocytes from autologous or allogenic sources ECM-based structures and later cartilage defects. Artificial ECMs and growth factors or mechanical stimuli are successful because they produce artificial cartilage suitable for tissue repair. This is the hope we have in the solution to this problem hence the aim of this thesis.

1.2 Aim of the Study

The aims of the study are;

i. To synthesize pure silk fibroin from cocoons, pure curcumin from turmeric roots and load it into a formulation with gelatin and polyethylene glycol.
ii. To evaluate the Structural, morphological and thermal properties of the prepared hydrogels using NMR, FTIR, SEM and DSC.
iii. To also evaluate blood compatibility by measuring prothrombin time (PT %), activated partial thromboplastin time (APTT %), international normalised ratio (INR), and fibrinogen (Fib, mg/dL) studies.

We aimed to develop a silk fibroin based and curcumin containing injectable hydrogel formulations with improved anticoagulant activity.

1.3 Importance of the Study

The medical science of orthopedics has the treatment of articular cartilage damage as a major task. The importance or significance of this study is to fabricate a silk fibroin based/curcumin containing bioscaffolds that can successfully be as local and sustainable drug delivery systems. Providing more formulations to the promising therapeutic strategies in the repair or regeneration of damaged or diseased cartilage tissues.

1.4 Limitations of the Study

The study had a major setback with the loss of our Normal Human Articular knee chondrocyte (NHAC-kn) due to a technical failure to the refrigerator in the laboratory at the university. In addition, another limitation of the study is the need to adjust the manner of degradation, polymeric classification and cellular interaction.

1.5 Overview of the Thesis

In this study, bioscaffolds were synthesized by blending and freeze drying method then some had been loaded with isolated curcumin and characterized as well. The results of this study presents suitable candidates for biomedical applications with continuous blood contact and establishes the anticoagulant property of curcumin with its ability to increase fibrinogen concentration as well.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Scaffold serve as a three-dimensional (3D) structure suitable for desired cellular growth, differentiation, and tissue formation. Providing a temporarily or permanently support for the process. The nature of the scaffold materials can either be natural source or synthetized in the laboratory. There are similarities in the scaffold design principles in TE for different tissue types. Hence, the mimicking of biological structure and functions of the native ECM with respects to physical properties and chemical composition (Horst et al., 2010).

Scaffolds are a 3D network of ECM fabricated to be similar to native structures, making available structural support and internal space for the incorporated cells to adhere, proliferate, and differentiate. For the best biological conditions for growth in tissue culture scaffolds should mimic the native ECM microenvironment of cartilage chondrocytes (Kim et al., 2011). Therefore, the general requirements of 3D artificial ECMs include, biocompatibility, regulated degradation, stiffness, high porosity and mechanical strength. Also, the ECM surface should exhibit suitable properties for fitting tissue formation of chondrocytes. The need for a high level of porosity is to allow the migration and proliferation of cells adhered or adhering to the ECM and also the product exchange of nutrient and waste. Controlled biodegradability is vital for the formation/development of newly regenerating tissue in the ECM (Park & Cho, 2010).

Scaffolds should also have tuneable surface properties, porosity and pore size, external geometry, biocompatibility, interface adherence, biodegradability, healing efficacy, surface properties, and mechanical competence (Chan & Leong, 2008).

In cartilage TE, biodegradable polymers of synthetic or natural origin have always been used (Lu, Zhu, Valenzuela, Currier, & Yaszemski, 2001). These polymers are usually fabricated into sponges, hydrogels, or nanofibers (Kim et al., 2011). Scaffolds used in tissue engineering include collagen (Wilke, Heuer, Neidlinger-Wilke, & Claes, 2006), atello-collagen (Sakai et al., 2006, 2003), alginate (Leone, Torricelli, Chiumiento, Facchini, &

Barbucci, 2008; Mizuno et al., 2004), gelatin (Wan, Feng, Shen, Laurencin, & Li, 2008), chitosan (Dang et al., 2006), collagen/ glycosaminoglycan (Saad & Spector, 2004), collagen/hyaluronan (Alini, Roughley, Antoniou, Stoll, & Aebi, 2002), poly-L-lactic acid (Richardson et al., 2006), silk (Chang, Kim, Kaplan, Vunjak-Novakovic, & Kandel, 2007), polycaprolactone (Nerurkar, Elliott, & Mauck, 2007), polyglycolic acıd/polylactıc acid (Mizuno et al., 2004), and bioglass (Wilda & Gough, 2006). The three scaffold materıals are natural biomaterials, synthetic polymeric materıals, and composite materials.

2.2 Cartilage Tissue Regeneration

The rigorous mechanical movement of articulating bones is facilitated by the Hyaline articular cartilage. The latter consısts of various specialized macromolecules and proteıns, capable of reducing friction at the joints. Furthermore, it should be noted that chondrocytes in an avascular structure are merely present in articulating joints such as knees, hip, and shoulders (Athanasiou, Darling, & Hu, 2009; Cao, Dou, & Dong, 2014). Similarly, cartilage TE has been widely employed in the fabrication of 3D scaffolds. Cartilage TE confers excellent mechanical properties and enhanced production of extracellular matrix upon the generated cartilage tissue by ensuring the uniform deposition of the cells (Cui, Gao, Yonezawa, & Dai, 2014). All of this is in a bid to provide a substitute to old and painful therapies for cartilage replacement caused by injuries, repeated loading or torsional loading in addition to the presence of foreign bodies in the joint space (Camarero-Espinosa, Rothen-Rutishauser, Foster, & Weder, 2016).

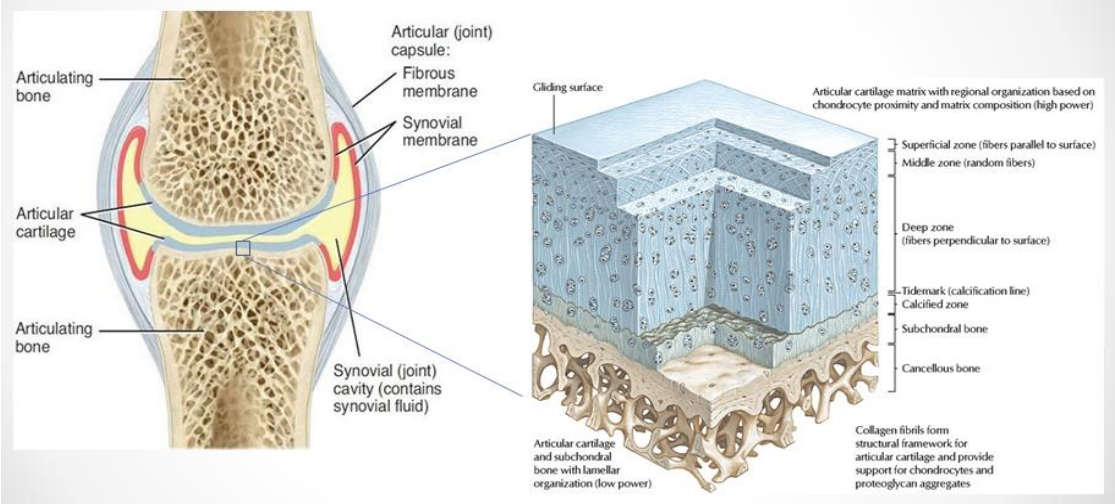

Figure 2.1: Detailed structure of a articular cartilage (Clark, 2007; Shinohara, Hasegawa, Tsunoda, Hosogi, & Sakai, 2016).

2.3 Current Treatment Techniques & Approaches

Cartilage TE involves the fabrication of 3D scaffolds to generate cartılage tissue that exhibits excellent mechanical properties and improved ECM production (Cui et al., 2014), thus providing a near perfect replacement for injured cartilage (Camarero-Espinosa et al., 2016). The fabricated cartilage consists of specialized proteins and macromolecules that work together to reduce joint friction at the interface of two long bones in a rigorous mechanical environment. Articulating joints such as knees, hips, and shoulders merely contain chondrocytes in an avascular structure (Athanasiou et al., 2009; Cao et al., 2014). Products used in cartilage repair therapies include the following;

- BioCart™II
- Bioseed®-C
- CaReS®
- Cartipatch®
- Chondrosphere®
- Hyalograft® C
- INSTRUCT
- NeoCart®
- NOVOCART® 3D
- MACI and
- RevaFlex™

All of these are some of repair therapy products used currently in cartilage TE (Huang, Hu, & Athanasiou, 2016).

Bioprinting is an important development in tissue engineering. Scaffold, cells, and growth factors are deposited rapidly and with accuracy under digital control and using high-throughput printheads on desired two-dimensional and three-dimensional positions. Many

successful applications have been developed using this technology. Cui et al. (2014), for example, employed a bioprinting platform consisting of a modified Hewlett-Packard Deskjet 500 thermal inkjet printer and a simultaneous photopolymerization system. Photo-crosslinkable PEG is highly soluble in water with low viscosity, making it ideal for simultaneous polymerization during 3D bioprinting. The result was a consistent distribution of cells on the 3D scaffold, with remarkable mechanical properties and improved ECM production of cartilage tissue (Cui et al., 2014).

Nguyen et al. (2017) used a composite bioink of nanofibrillated cellulose (NFC) to 3D-bioprint human-derived iPSCs into cartilage. The bioinks was composed of a formulation of alginate with NFC (NFC/A) and hyaluronic acid with NFC (NFC/HA). The pluripotency was maintained initially on the 3D-bioprinted NFC/A constructs and five weeks later, what was observed was collagen type II with hyaline-like cartilaginous tissue without tumorigenic Oct4 expression. Nguyen et al. concluded that NFC/A bioink is suitable to support cartilage production for bioprinting iPSCs in cocultures with irradiated chondrocytes (Nguyen et al., 2017).

Cheng et al. (2017) examined the effects of disrupting the prolyl hydroxylase domain–containing protein (*Phd2*) gene in chondrocytes on the superficial zone of the articular cartilage phenotype in mice. They demonstrated that *Phd2* is a negatively regulates the differentiation of chondrocyte and that hypoxia signalling is involved in the pathogenesis of osteoarthritis (Cheng, Pourteymoor, Alarcon, & Mohan, 2017; Kalkan, Nwekwo, & Adali, 2018).

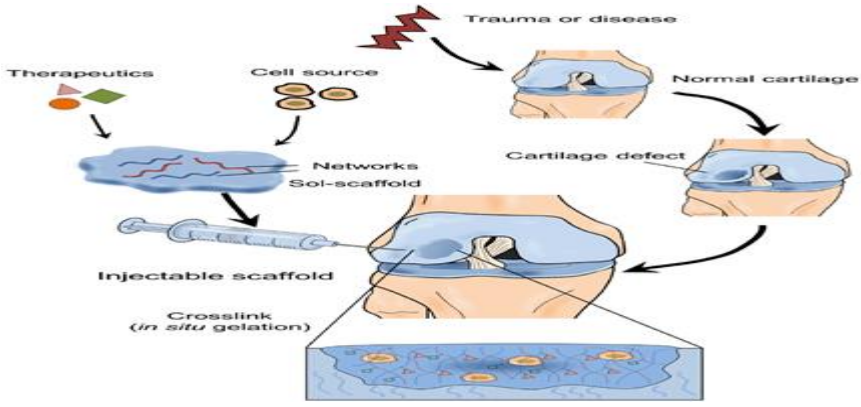

Figure 2.2: The schematic of the applications of injectable scaffolds for cartilage regeneration (Li et al., 2019).

CHAPTER 3
MATERIALS AND METHOD

3.1 Materials

SF was purified from *bombxy mori* cocoons obtained from a local market, by following protocols from our previous work (Adali, Kalkan, & Karimizarandi, 2019). Curcumin was obtained from *Curcuma longa* (Turmeric) rhizome which was obtained from a local market. Gelatin (G), Tween 80, Polyethylene glycol (PEG, molecular weight 10,000 $gmol^{-1}$) and SnakeSkin® Dialysis Tubing membranes (molecular weight cut out 3,500 $gmol^{-1}$) were purchased from Thermo Scientific (USA), anhydrous sodium carbonate (Na_2CO_3) and anhydrous calcium chloride ($CaCl_2$) were obtained from EMSURE® Merck chemicals (Darmstadt, Germany), Sodium triphosphate penta-basic was a product of Sigma-Aldrich (Germany). All experiments involved the use of deionized water and ultra-pure water. All other solvents used in thıs study were purchased from Merck and Sigma Aldrich (Germany). For the preliminary studies of the earlier purchased but later lost chrondrocytes tested on silk fibroin biofilms, Normal human articular knee chondrocytes (NHAC-kn) were obtained from Lonza (Walkersville, MD, USA) and MTT (Methyl Thiazolyl Tetrazolium) assay purchased from Thermo Fisher Scientific – USA. All cells were cultured in normal human chondrocyte cell growth medium (BulletKit®, Lonza) and maintained in 5% CO_2, 95% air and a 37°C incubator.

3.2 Preparation of Pure Silk Fibroin

SF was from *bombxy mori* cocoons with application of processes in three steps; degumming, dissolution, and dialysis. For this purpose, the cocoons were cut into little bits (about 1 mm^2 in size). 1g of the chopped pieces were put into an Erlenmeyer flask containing 100 mL of 0.1M of sodium carbonate (Na_2CO_3) solution. The flasks were placed on a hot plate at 75°C, magnetically stirred at a speed of 1.5 revolutions per minute for three hours. The obtained degummed silk were thoroughly washed severally with ultra-pure water to remove the glue-like protein called sericin from the silk fibers and finally left overnight to dry at 37°C. A strong electrolyte solution of methanol/water/calcium chloride (C_2H_5OH: H_2O: $CaCl_2$; with

2:8:1 molar ratio) was used to dissolve the silk fibers at 75°C with continuous stirring. The dissolved silk fibroin was placed in the snake-skin dialysis membrane (MWCO: 3,500 $gmol^{-1}$) and placed in deionized water for firstly an hour and changed afterward. The process of dialysis was continued for about 2 days in the deionized water that is changed regularly. After which the pure silk fibroin is extracted and stored at 4°C (Adalı & Uncu, 2016). The Pure SF solutions were firstly used to prepare the biofilms used in the preliminary studies by pouring it into a petri-dish and dried at room temperature overnight. Methanol treatments were used to remove the biofilms from the petri-dishes; the films were immersed in 90% (v/v) methanol for 1 h and then removed to dry at room temperature. The final product is seen in G of the Figure 3.1 below.

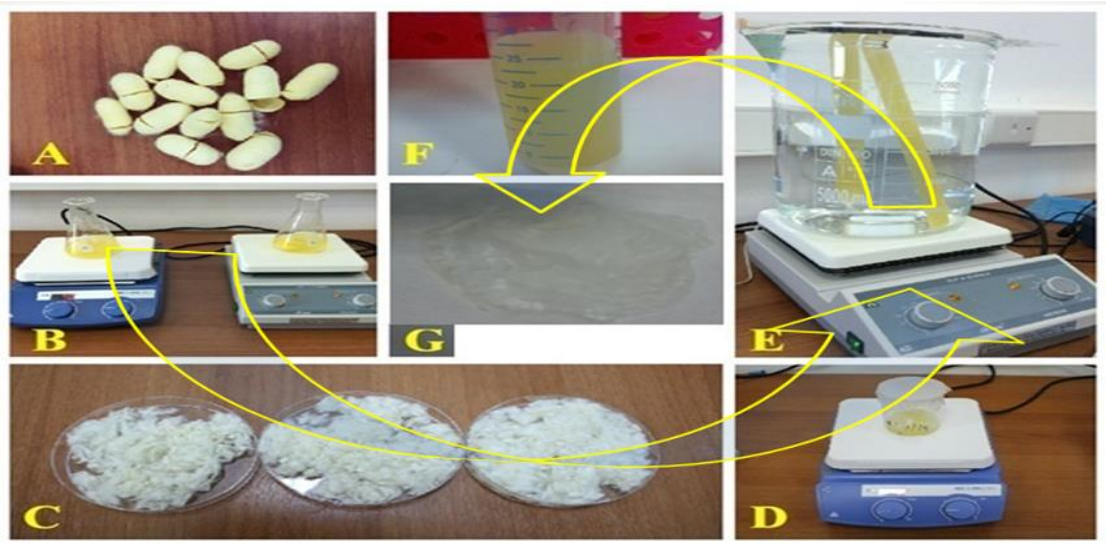

Figure 3.1: Showing the process of silk fibroin synthesis to sample preparation

3.3 Isolation of Pure Curcumin

CUR was extracted from rhizome of *Curcuma longa*. For this purpose rhizome of *Curcuma longa* (145 g) was pulverized using miller and extracted by acetone (500 mL) at 40°C. The process of extraction was repeated two more times by using 200 mL of acetone in each process. The extracted residue was finally washed with 100 mL of acetone and then the combined extracts were dried by evaporating the solvent under vacuum. Büchi R-210 and Heidolph 4001 rotary evaporators were used.

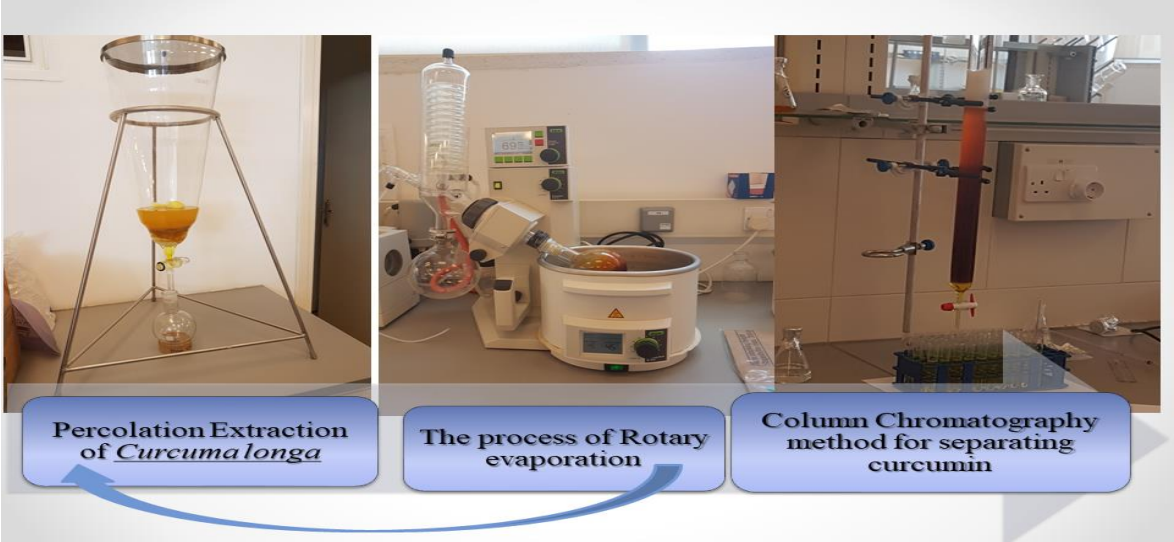

Figure 3.2: The curcumin isolation process

The crude extract (10.65 g) gotten from the initial process was dissolved in dichloromethane-methanol (1:1) mixture and silica gel (20.6 g) was added. This mixture was dried at 50°C in a vacuum and applied on a silica gel column (150 g; column dimensions: 5cm × 15 cm). Fractionation was performed with the increasing amount of methanol in dichloromethane (1 - 5% MeOH in DCM; fraction volume 100 mL). The fractions rich in curcumin were combined and evaporated until dryness (fractions 8 – 10; 3.52 g curcumin was obtained). The combined fractions rich in curcumin was applied to silica gel column chromatography (120 g; column dimensions: 3cm × 40 cm; fraction volume 10 mL) using a dichloromethane-methanol mixture (98:2, v/v) as the solvent system. Monitoring the fractions with the Thin Layer Chromatography (TLC) using dichloromethane-methanol (95:5, v/v) on silica gel plates. For this purpose, classical column chromatography (40 cm × 4 cm), Silica gel (0.063 - 200 μm, Merck) and silica gel alumina plates (Silica Gel 60 F_{254}, Merck) were used.

3.4 Hydrogel Preparation

Silk fibroin based hydrogels were prepared by blending silk fibroin (SF, 3% $w.v^{-1}$), gelatin (G, 10% $w.v^{-1}$) and polyethylene glycol (PEG, 40% $w.v^{-1}$). Curcumin (C) was added as a bioactive agent into the solutions. Since curcumin has very low solubility in aqueous media, it was dissolved in Tween 80 (Kasoju & Bora, 2012). The solution was prepared by

dissolving 0.3 g of curcumin in 1 mL of Tween 80 which was then added into the formulations containing curcumin (Table 3.1).

Table 3.1. Hydrogel Formulations

Samples	SF (mL)	G (mL)	PEG (mL)	CUR (mL)
H4	2	4	-	-
HCP	2	4	-	0.3
SS1	5	3	2.5	-
SS2	5	3	2.5	1

SF: Silk fibroin (3% $w.v^{-1}$)
G: Gelatin (10% $w.v^{-1}$)
PEG: Polyethylene glycol (40% $w.v^{-1}$)
CUR: Curcumin (11.8 mg / 1mL tween 80)

The hydrogel coded as H4 contains SF and G, while HCP also has curcumin in it. The mixture was left to gelate and kept overnight at 4°C. The hydrogel coded as HCP was prepared in the same way and curcumin was added into these samples. For this purpose firstly, 0.0118g of pure curcumin was dissolved in 1 mL of Tween 80. 0.235g of the mixture was mixed with 1 mL of 3% silk fibroin and 5 mL of 10% gelatin. The mixture was mixed evenly on a magnetic stirrer and left to gelate. The hydrogel was later stored at 4°C.

Two formulations were prepared by addition of PEG to previous solutions to adjust their viscosity and to induce gelation (Wang et al., 2015). The sample coded as SS1 was prepared SS1 by carefully mixing 5 mL of 3% silk fibroin with 2.5 mL of 40% polyethylene glycol and 3 mL of 10% gelatin on a magnetic stirrer. After an hour of the gelation process, it was kept at 4°C, then the sample was transferred into a balloon and left overnight at -20°C. For the other hydrogel, coded as SS2, similar procedure was carried out with addition of curcumin (0.0118g curcumin dissolved in 1 mL of tween 80 was added to the solution) (Table 3.1).

3.5 Characterization Analysis

3.5.1 Nuclear magnetic resonance (NMR) spectroscopy

Bruker DRX 500 spectrometers operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C respectively was used to measure the nuclear magnetic resonance in Deuterated chloroform ($CDCl_3$). For data acquisition and processing, the software package XWIN NMR was used.

3.5.2 Swelling ratio analysis

Swelling tests were carried out at 37°C in an acidic media of pH 1.2 (acid phosphate buffer saline, ABS, 0.1M) and in a slightly basic media of pH 7.4 (phosphate buffer saline, PBS, 0.1M), by measuring the changes in weight of the samples at various time points. The formula was used to find the percent swelling values.

$$\text{Swelling \%} = \frac{\text{Ws} - \text{Wd}}{Wd} \times 100\% \qquad (3.1)$$

Where W_d denotes the initial dry weight of the hydrogel sample and W_s denotes the weight of the swollen hydrogel sample after been submerged in 10 mL of the buffer solution at different time points.

The pH of certain fluids in the body varies depending on the condition of the environment. Milošev et al., 2017 carried out research on 167 patients measuring the pH of the synovial fluid of diseased native knee and hip joints. The mean pH value of synovial fluid in the joints was 7.78 ± 0.38. In their paper they mentioned the pH range of health synovial fluids as 7.43 (range, $7.31 - 7.64$) and 7.77 (range, $7.72 - 7.81$) (Milošev, Levašič, Vidmar, Kovač, & Trebše, 2017). The result of the study indicates that the pH of diseased areas can be elevated above the normal.

3.5.3 Fourier Transforms Infrared Spectroscopy (FTIR)

Fourier Transform Infrared-Attenuated Total Reflectance (FTIR-ATR) spectroscopy analysis (Frontier, Perkin Elmer, USA) was used in the characterizing of chemical structures of hydrogels prepared.

3.5.4 Differential scanning calorimetry (DSC)

The thermal properties of the hydrogels were determined by differential scanning calorimetry (DSC, Perkin Elmer Diamond, USA), by using approximately 5-10 mg of sample placed in aluminum DSC pans. The hydrogel samples underwent heating scan with a consistent heating rate of 10°C. min^{-1} under a nitrogen atmosphere from 0°C to 350°C.

3.5.5 Scanning electron microscopy (SEM)

Morphology of the hydrogel samples were investigated using scanning electron microscopy (SEM, Quanta 400F Field Emission, FEI, Eindhoven, Holland). The hydrogel samples were sputter-coated with Au-Pd prior to examinations.

3.6 *In vitro* Anticoagulant Activity

In vitro coagulation tests were carried out following the Ethical Board decision of Near East University. Healthy volunteers donated blood plasma used for the analyses at Near East University Hospital, North Cyprus. The color-coded blue cap tube containing sodium citrate were used to collect the whole blood in the standard ratio of 1:9 (v/v). Sodium-citrated anticoagulated whole blood placed in the centrifuge at 1100 rcf for 15 minutes. Afterwards 100 µL of the plasma were taken to mix with uniform amounts of the biomaterials, incubate for 15 minutes at 37 °C, and placed in the STA Compact device to carry out all the tests and acquire the results.

Activated partial thromboplastin time (APTT), prothrombin time (PT) and fibrinogen concentration were measured by using STA – Cephascreen reagents, STA – NeoPTimal reagents, STA – Liquid Fib respectively with the STA Compact device. The quantitative determination of fibrinogen levels in plasma on interaction with biomaterial samples was carried out using the clotting method of Clauss. The tests were achieved at least 5 times and the mean were taken for further analysis.

3.7 Statistical Analysis

Data collected from the *in vitro* studies were analyzed using one-way analysis of variance (ANOVA) using OriginPro 8 software. Comparing the hemolytic activities between the samples (H4, HCP, SS1, SS2, CUR and HB as health blood). Results with $p < 0.05$ were considered statistically significant.

3.8 Normal human articular chondrocyte cell culture on SF Biofilm

The NHAC-kn (Lonza, Clonetics™ Normal Human Articular Chondrocyte Cell System) were cultured in Chondrocyte Medium (Sciencell Research Laboratories) supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 1000 U/ml streptomycin solution and specific growth factors according to the manufacturer protocol. The samples were sterilized by ultraviolet irradiation. The cell morphology and distribution were observed by a phase contrast microscope Olympus IX53 with camera Olympus DP22. Before cell seeding, the SF biofilm was conditioned inside distinctive wells of 24-well plates in culture medium for 4 hours at 37 °C. The chondrocytes were seeded on to top of the SF biofim (1 cm^2) in total volume of about 1.25 × 104 cells/ml. After 24 hours, each construct was transferred to a blank well in a different plate and replenished with fresh medium. SF biofilm was incubated for 14 days with media changes every other day. Attachment of NHAC-kn on tissue culture plastic (TCP) was used as a control. Cell attachment at different time points (30 minutes, 1, 2, and 3 hour) after seeding was evaluated with a phase contrast microscope. All images were taken at 10 and 40 magnification.

3.8.1 Method of analysis of cell proliferation

Cells loaded on each biofilm were quantitatively assessed for cellular metabolic activity with 3 (4,5 dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide (MTT). Relative cell viability was measured by methyl thiazolyl tetrazolium (MTT, Sigma) assay. The MTT is a pale yellow substrate, which was reduced by living cells to a dark blue formazan. This colorimetric process is an accurate measure of the viability of cells in culture because the formazan crystal formed has a positive correlation to the number of surviving cells and their metabolic activity that were measured by finding the absorbance or optical density. After the NHAC-kn were cultured in 24-well plates for 2 days, 7 days, 9 days and 15 days, the cell viability was evaluated using the MTT assay. MTT (100 l; 5 mg/ml) (Invitrogen) was added to each well and incubated at 37 °C for 4 hours. At the end of the assay, the blue formazan reaction product was dissolved by adding 50 μL DMSO and was transferred to a 96-well plate. The absorbance was measured at 540 nm using a Bio-Rad 500 spectrophotometric microplate reader. The absorbance of formazan indirectly reflected the level of cell metabolism and this process is the measure of the viability of cells in culture.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preliminary NHAC-kn cell culture studies

The NHAC-kn (Lonza) were cultured in Chondrocyte Medium (Sciencell Research Laboratories) supplemented with 5% fetal bovine serum. The cell morphology and distribution were observed by a phase contrast microscope. Before cell seeding, the Biofilm were conditioned inside distinctive wells of 24-well plates in culture medium 4 hours at 37°C. The chondrocytes were seeded on to top of the SF biofilms in total volume of about $1{,}25x10^4$ cells/mL. Twenty-four hours later, each construct was transferred to a blank well in a different plate and replenished the fresh medium. Biofilm were incubated for 14 days with media changes every other day. The scale of the images below are all at 200 µm.

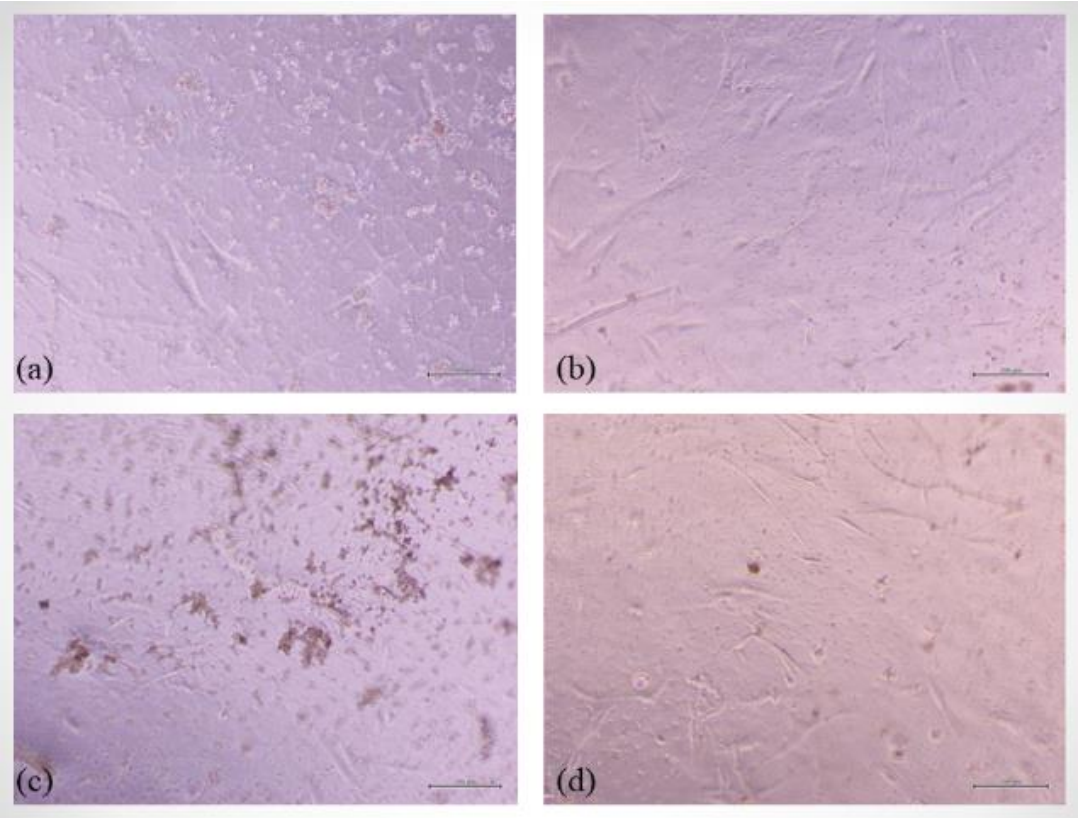

Figure 4.1: (a) 24 hours after seeding on biofilm (b) 7 days after seeding on biofilm (c) 9 days after seeding on biofilm and (d) 15 days after seeding on biofilm

We observed the progressive change of our cultured chondrocytes to a fibroblast-like morphology with passage of time. It is sometimes expected that passaged chondrocytes display features of a dedifferentiated phenotype, including a portion of cells with fibroblast-like morphology (Otero et al., 2012).

4.1.1 MTT assay

The attachment and cell growth of chondrocytes by 3-(4,5-dimethythiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) assay at 2 days, 7 days, 9 days and 15 days. A significant increase in cell proliferation was observed confirming that the biofilm is a suitable biomaterial. As seen in the graph below showing progress over a span of days.

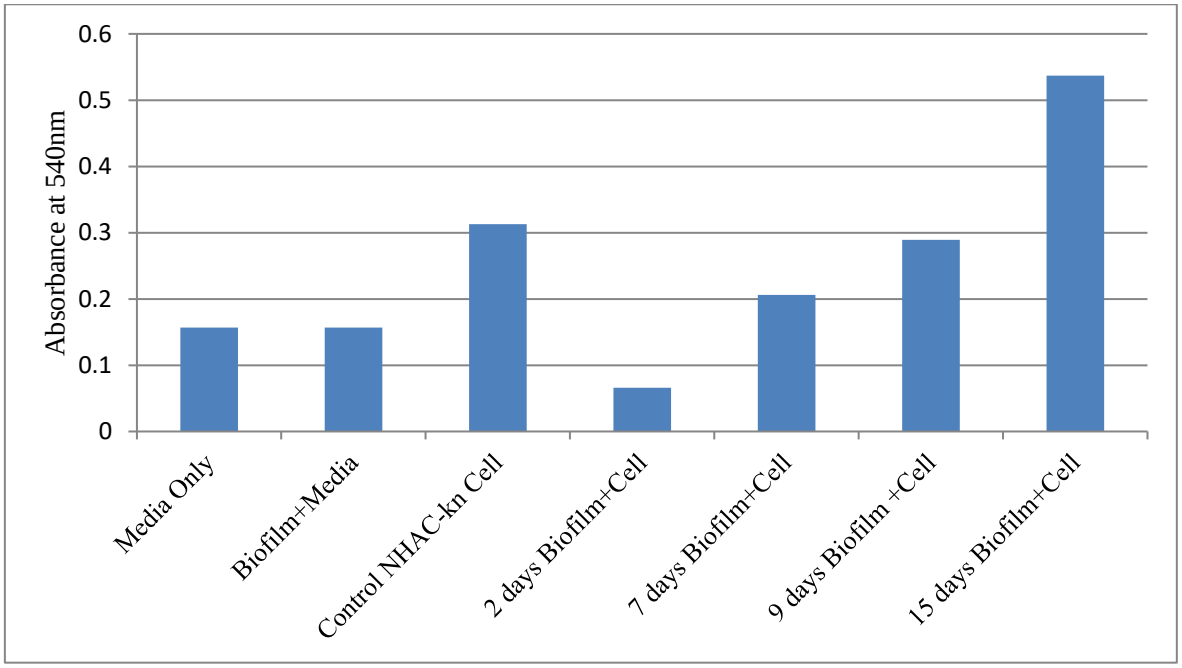

Figure 4.2: MTT cell proliferation assay

4.2 Curcumin analyses

4.2.1 Thin layer chromatography (TLC) analysis

Curcumin, after isolation and purification steps, was monitored by TLC using dichloromethane-methanol on silica gel plates. The amount of curcumin obtained was 262 mg. The result of several rounds of TLC was necessary for all the curcumin derived from the first run. Furthermore, the fractions were collected according to thin-layer chromatography and pure curcumin as the orange crystals is the yield, after all, containing curcumin were combined and worked on following the literature (Berger & Sicker, 2009).

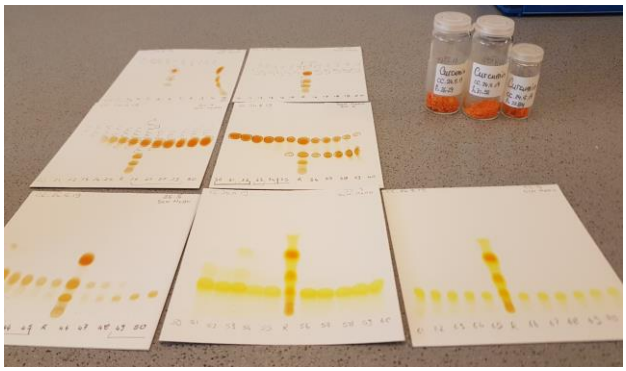

Figure 4.3. TLC of isolated curcumin

4.2.2 Nuclear magnetic resonance (NMR) spectroscopy

The amount of pure curcumin gotten from the isolation process was 262 mg. Portions from it were used to verify the purity of the curcumin and afterward used to prepare our hydrogels. The purity and structure elucidation of curcumin was established by ^{1}H and ^{13}C~NMR spectroscopy. The ^{1}H and ^{13}C~NMR data for the isolated compound correlated with those reported for curcumin (Berger & Sicker, 2009). The data seen in Table 4.1, indicates the presence of enolic form of curcumin. The singlet at 5.92 ppm integrating for one proton was assigned to H-4 which confirms the enolic form of curcumin. Figure 4.3 shows the ^{1}H~NMR, ^{13}C~NMR and DEPT-135 Spectra results of curcumin. The NMR data of curcumin was in accordance with those reported by Berger and Sicker (Berger & Sicker, 2009).

Table 4.1: ^{1}H and ^{13}C~NMR data of Curcumin (^{1}H: 500 MHz; ^{13}C: 125 MHz; $CDCl_3$)

H/C Atom	DEPT-135	δ_C, ppm	δ_H, ppm, *J* (Hz)
1/1	CH	140.8	7.62 d (15.8)
2/2	CH	122.0	6.51
3/3	C	183.5	-
4	CH	101.2	5.92 brs
1′/1′	C	127.9	-
2′/2′	CH	109.8	7.07 d
3′/3′	C	147.0	-
4′/4′	C	148.0	-
5′/5′	CH	115.0	6.96 d (8.2)
6′/6′	CH	122.9	7.14 dd (8.2/1.6)
OMe	CH_3	56.2	3.97 s

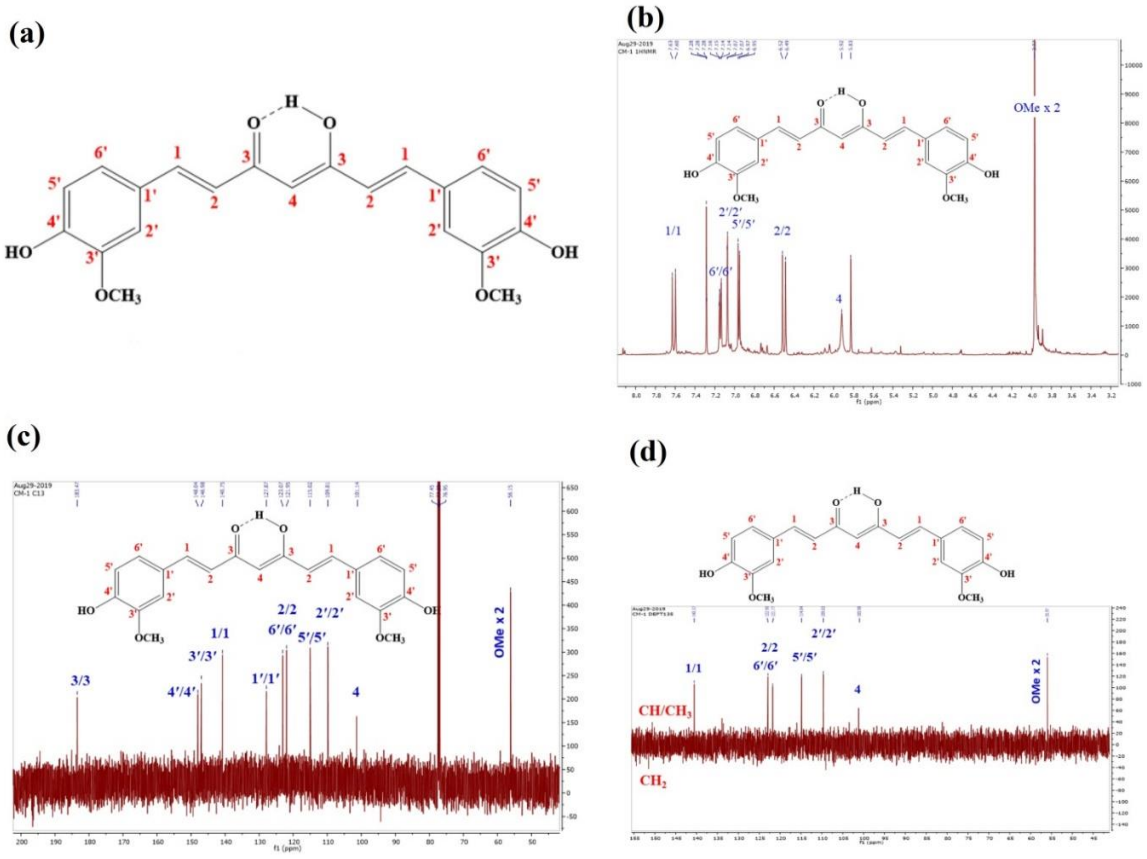

Figure 4.4: (a) Structure of curcumin (b) ^{1}H NMR Spectrum of Curcumin (500 MHz; $CDCl_3$), (c) ^{13}C NMR Spectrum of Curcumin (125 MHz; $CDCl_3$), (d) DEPT 135 Spectrum of Curcumin (500 MHz; $CDCl_3$)

4.3 FTIR Spectra Analysis

The absorption band is shown in Figure 4.5 of SS1 was observed at 1651 cm^{-I}, 1466 cm^{-I}, and 1279 cm^{-I} indicates the presence of amide I, II, and III respectively which is present in SF with random coil conformation (Adalı & Uncu, 2016). The SS2 loaded with curcumin has absorptıon bands at 1648 cm^{-1} (amide I), 1466 cm^{-1} (amide II) and 1279 cm^{-1} (amide III) which exactly corresponds to that of SS1 and similar to Figure 4.5 (Lian, Zhan, Zhang, & Mo, 2014). H4 and HCP had almost entirely similar 1642 cm^{-I}, 1466 cm^{-I}, 1238 cm^{-I} and 1626 cm^{-I}, 1466 cm^{-I}, 1239 cm^{-I} respectively for the amides. The spectra indicative of PEG showed wagging of CH_2 at 1341 cm^{-1} and C–C stretching at 1097 cm^{-1} present in only SS1 and SS2 that contains PEG (Vrandečić, Erceg, Jakić, & Klarić, 2010). In the FTIR spectra of H4, HCP, SS1 and SS2, which all have the gelatin band at 3280 cm^{-1} is credited to –NH stretching together with hydrogen bonding (Takei et al., 2020). Gelatin showed bands at 1651 cm^{-1} in SS1, 1648 cm^{-1} in SS2, 1642 cm^{-1} in H4, 1628 cm^{-1} in HCP and 1538 cm^{-1} in SS1, 1544.0 cm^{-1} in SS2, 1546 cm^{-1} in H4 and HCP similar to SF which were due to the CO and CN stretching vibrations of amide I and amide II linkages, respectively (Khade et al., 2014). The spectra indicative of gelatin and curcumin as well, showed at 3288 cm^{-1} and C-H stretching vibration at 2922 cm^{-1} present evidently in SS1, SS2 and HCP (Eren, Baysal, & Doğan, 2020; Muyonga, Cole, & Duodu, 2004).

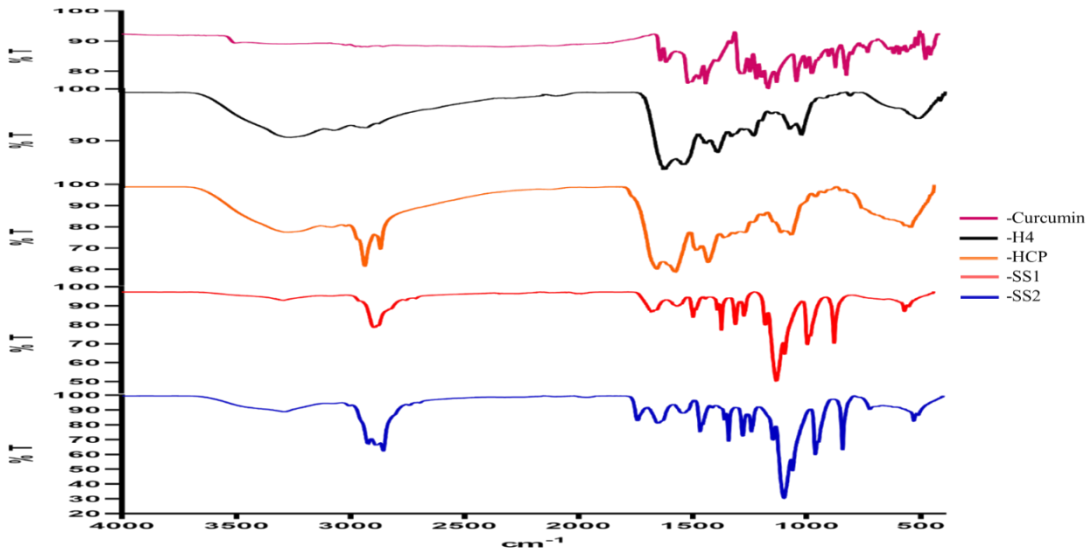

Figure 4.5: FTIR spectra of Curcumin, H4, HCP, SS1, and SS2

4.4 DSC Analysis

The thermograms showing the various peaks in Figure 4.6 of the hydrogels H4, HCP, SS1 and SS2 are indicative of the melting of samples (endotherms). The peaks as observed for H4 on the first round of heating before cooling was at 150.45°C (an endothermic peak) to 257.87°C (an exothermic peak), on cooling had an extrapolated value of 129.47°C and on the second heating had also an extrapolated value of 146.37°C. The DSC peaks of H4 on the first heating indicates an initial absorption of heat and then release in crystallization. The sample HCP had two peaks on the first heating 168.22°C to 243.25°C, on the second heating 89.41°C to 225.62°C was extrapolated. The DSC peaks of HCP is indicative of water loss from the matrices and followed by decomposition. While that of SS1 was 60.72°C, 41.18°C and 60.37°C for first heating, cooling and second heating respectively. Then SS2 was 55.43°C, 30.48°C and 55.28°C for first heating, cooling and second heating respectively. The DSC peaks of SS1 and SS2 are quiet similar in that changes are observed in the underlying heat capacity through the denaturation endotherm as seen in Figure 4.6 of SS1 and SS2 respectively in the onset of dehydration and structural reconfiguration (Holland et al., 2019).

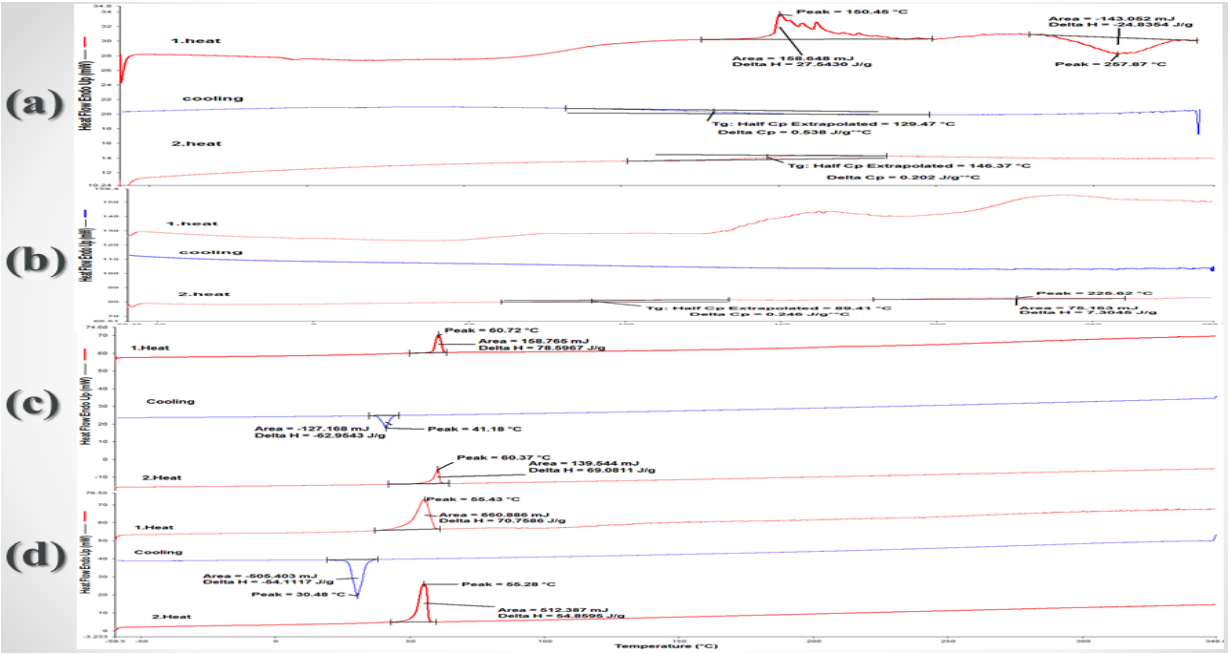

Figure 4.6: DSC Thermograms of (a) H4, (b) HCP, (c) SS1 and (d) SS2 Hydrogels

4.5 Swelling ratio Analysis

The samples were tested for their swelling behaviour in PBS and ABS by gravimetric analysis. The swelling properties are seen to be dependent upon several factors, including the pore size of the network, interactions between polymer chains and cross-linkers, and the solvent used (ABS and PBS). Table 4.2 shows the percentage swelling ratio of HCP, H4 and G (Gelatin only) in ABS and PBS.

From the graph in Figure 4.7 (a) gelatin showing its behavior in PBS and ABS, we see a better swelling ratio than the rest of the other samples. We notice by comparison the difference between H4 and HCP over the same period in Figure 4.7 (b), seeing that HCP is different from H4 in constituent by the addition of curcumin and 1mL extra of gelatin. SS1 and SS2 had degraded into particles in both acidic and basic buffers because of the presence of PEG and the absence of a crosslinking agent to effectively maintain the structural integrity in the buffers (McBath & Shipp, 2010; Shah, Saha, & Saha, 2015). The samples form particles that can be used to transport cells which can be secreted into matrices in cartilage treatment therapies (Dhote et al., 2013).

Table 4.2: Showing the %swelling ratio samples in ABS and PBS

Time (mins)	G-ABS	G-PBS	H4-ABS	HCP-ABS
0	0	0	0	0
5	75.94	4.03	3.52	10.03
10	142.39	7.47	8.52	15.82
20	238.95	10.72	12.97	19.47
30	340.43	16.08	21.42	23.72
60	473.81	32.15	26.68	27.21

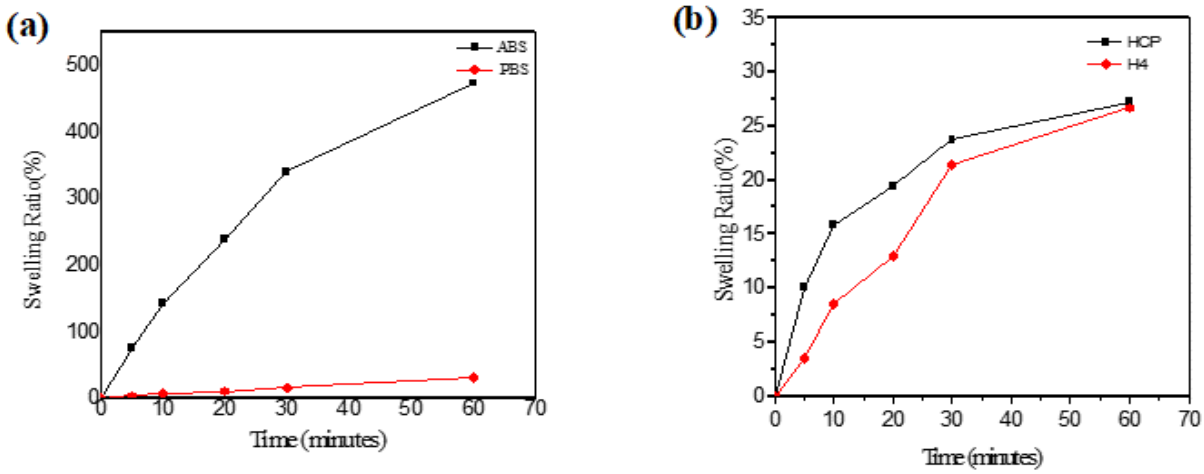

Figure 4.7: (a) The swelling ratio of Gelatin in both ABS and PBS. (b) The swelling ratio of both HCP and H4 in ABS

4.6 Scanning electron microscopy (SEM) Analysis

The morphology of SS1 as shown in Figure 4.8, the combination of SF, PEG and Gelatin without Curcumin and SS2 is the same formulation as SS1 and including Curcumin. We observed the changes in morphology with the presence of pure curcumin. Visible pores are seen in SS1, which are also present in SS2 that includes curcumin in a slightly rough conformation. This will favor protein adhesion. The porous nature of SS1 and SS2 is due to PEG in both samples.(Jarman-Smith, Brady, Kurtz, Cordaro, & Walsh, 2012; Zhang, Sun, Yuan, & Cao, 2017)

There is a noticeable distinction in the composition of the pairs of SS1/SS2 and H4/HCP, noting again that the first pair of SS1 and SS2 is with PEG while H4 and HCP are without PEG. The pair of SS1 and SS2 is more porous unlike the pair of H4 and HCP that contains many visible microspheres (Figure 4.8). The microspheres will favor transport and controlled release. HCP hydrogel has some rough microspheres and is porous as well, with the difference in formulation with H4 is the presence of curcumin. This is confirmed in SS1 and SS2 as well with the presence of curcumin in SS2. It is hypothesized in this study that the presence of curcumin in formulation resulted in a rough morphology as seen in HCP and SS2 (Figure 4.8).

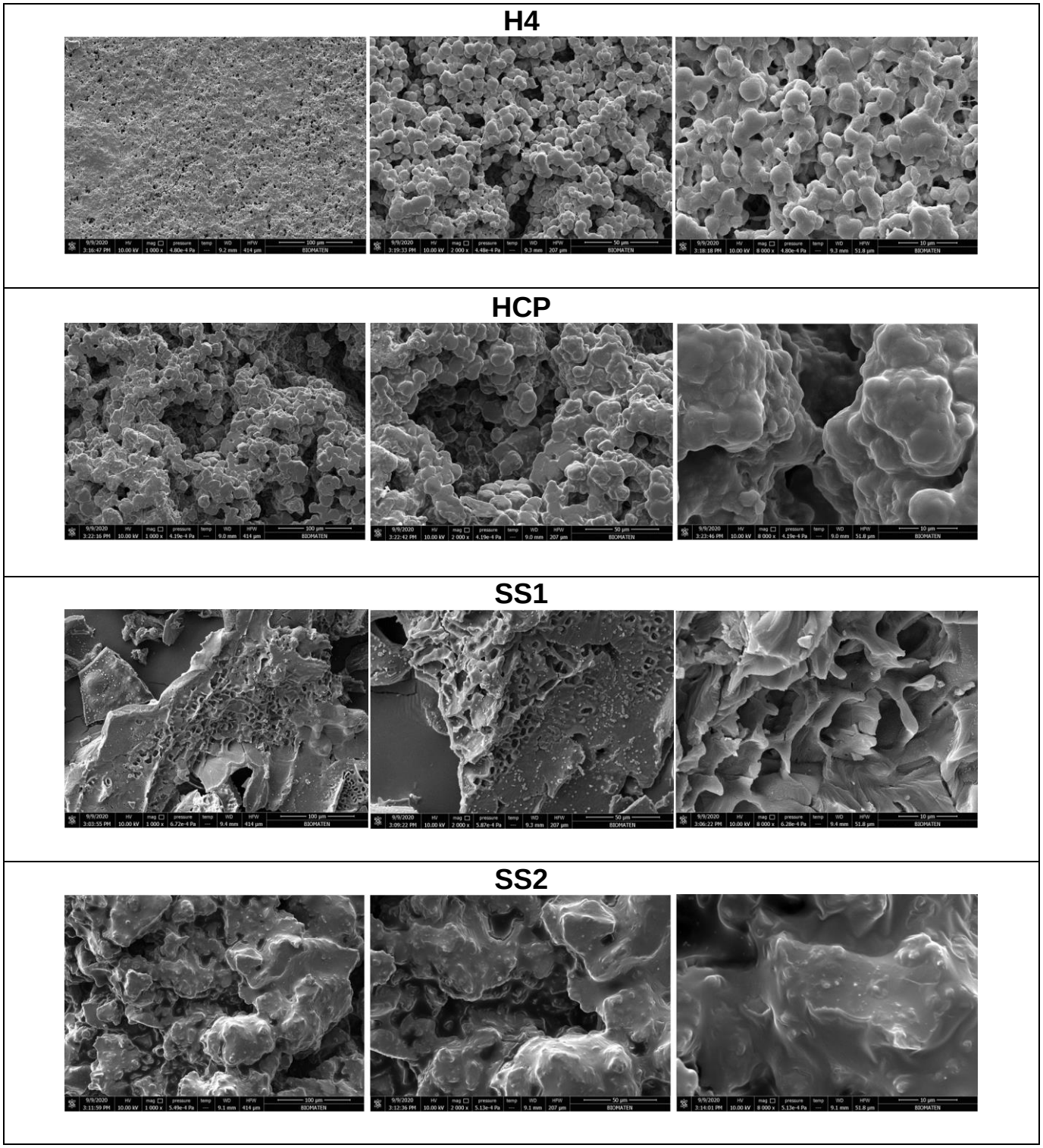

Figure 4.8: SEM images of hydrogel samples at different magnifications (Scale bars: 100 µm; 50 µm; 10 µm)

4.7 *In vitro* anticoagulation analysis

In this study, to the best of our knowledge this is the first time this blend is used to analysed the effect of curcumin on silk fibroin based injectable hydrogel prepared by the method of blending to determine plasma coagulation. In Tables 4.3, Figures 4.9 and Figure 4.10, we find the mean values and error bar graph of PT, APTT, PT%, INR and fibrinogen concentration for samples pure curcumin (CUR), SF/G hydrogel (H4), SF/G/C hydrogel (HCP), SF/G/PEG hydrogel (SS1), SF/G/PEG/C hydrogel (SS2) and healthy blood (HB). An hour extension was noticed for analysis done at a temperature of 37 ± 1°C. A prolonged PT and APTT was observed and recorded which was statistically significant ($p < 0.05$). Note that CUR is just the curcumin dissolved in Tween 80 that was used in the coagulation test to make a comparison with the other samples effectively.

Table 4.3: *In vitro* blood coagulation test results

Samples	PT (sec)	PT(%)	INR	APTT(sec)	FIB.(mg/dL)
H4	59.3	15	4.88	46.9	173
HCP	30.5	33.3	2.42	39.9	184
SS1	16.3	73	1.24	37.7	99
SS2	16.5	72	1.26	36.2	196
CUR	15.2	81	1.15	33.8	185
HB*	16	75.3	1.22	32.2	174

*HB is healthy blood sample.

Several researchers have expressed difficulty in evaluating the thrombogenicity and haemocompatibility of biomaterials due to the lack of standards (Braune, Lendlein, & Jung, 2018). Hence, we further emphasize the need for developing standards and test protocols for evaluating the haemocompatibility of biomaterials.

Mijovski (2019) in his work, helps us with reference range and therapeutic range for unfractionated heparin using several reagents. STA-Cephascreen, one of the several reagents used in his study which is the same reagent used in our research as well. He had an APTT reference range of 24-35 seconds and an APTT therapeutic range of 62-90 seconds for the unfractionated heparin (Božič Mijovski, 2019). This gives us some form of reference in further evaluation of our results. From Table 4, samples like H4 and HCP are above the

reference range with 46.9 secs and 39.9 secs, which establishes their ability to more strongly inhibit the formation of thrombin, making them a potent anticoagulant composite biomaterial formulation.

Increased or decreased concentration levels of fibrinogen is relevant to the analysis of our biomaterials. Cell adhesion to biomaterials can be promoted by fibrinogen adhesion on the elevation of fibrinogen concentration (Horbett, 2018). The fibrinogen concentration of the samples shows that the presence of curcumin increased the fibrinogen concentration as seen in the curcumin (CUR) itself and sample SS2 in comparison to the health blood (HB). It is seen to have influence HCP which as the same constituents with H4 and differs with the presence of the curcumin (CUR). The drop in fibrinogen concentration in SS1 is because of the presence of PEG, which is highly efficient in resisting protein (Jin, Jiang, Yin, Ji, & Stagnaro, 2013). This goes further to explain the great effect of CUR on the fibrinogen concentration in SS2 to counteract that effect greatly.

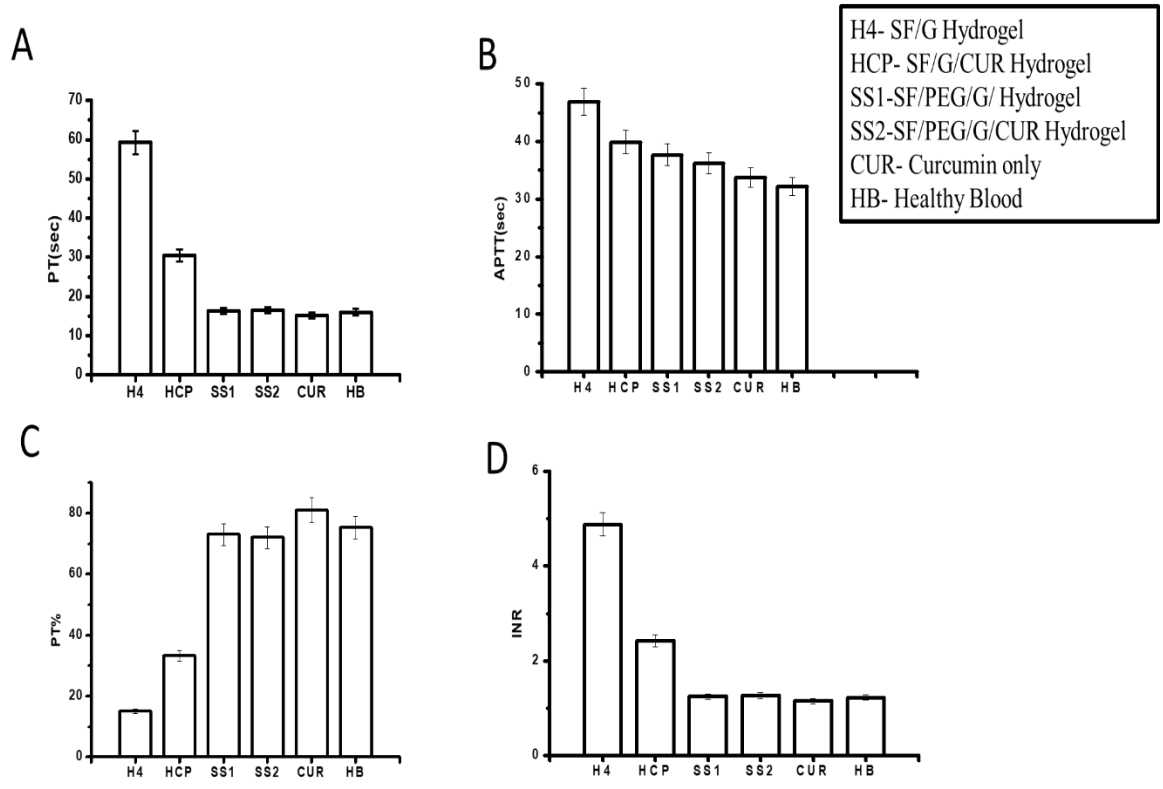

Figure 4.9: Blood analyses results (a) PT (b) APTT (c) PT% and (d) INR.

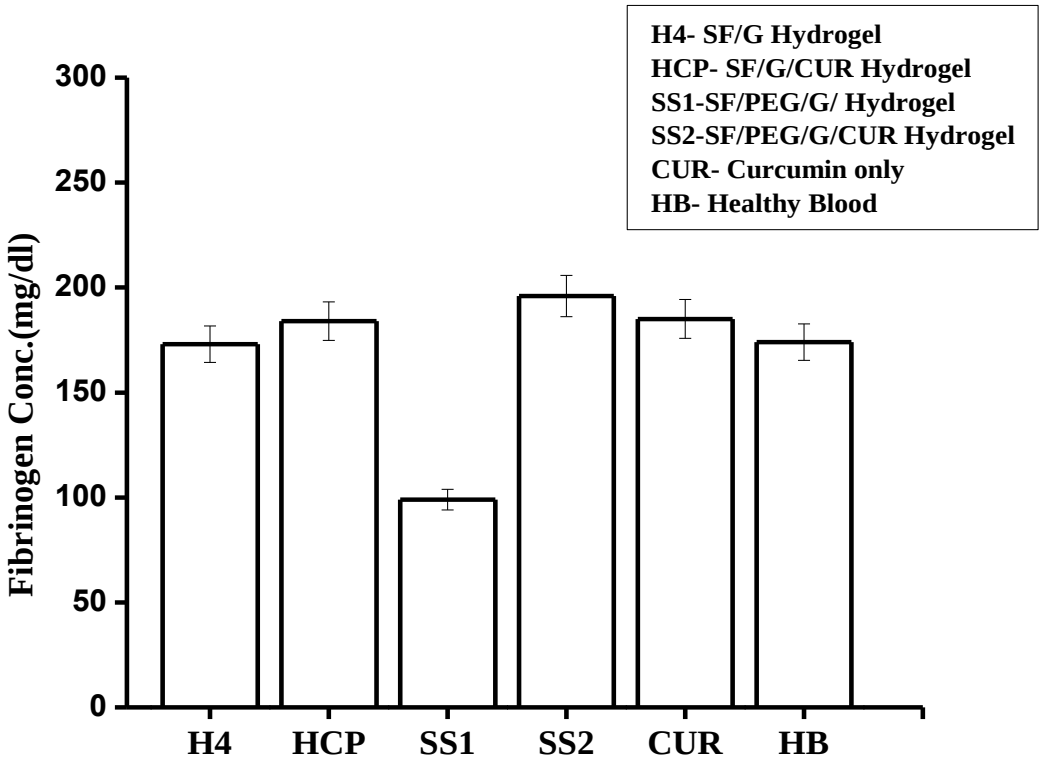

Figure 4.10: Fibrinogen concentrations of samples.

Overall, there is no significant difference in the PT, APTT, PT%, and INR values for SS1 and SS2 but in the fibrinogen concentration there is in comparison with the health blood (HB). We observed no significant difference in fibrinogen concentration for H4 and HCP but a great difference was observed in comparison to health blood and other samples, which is because of Gelatin and the ratio to silk fibroin is 2:1.

CHAPTER 5
CONCLUSION

5.1 Conclusions

In this work, the prepared composite hydrogels were characterized by SEM, FTIR, DSC, swelling tests, and *in vitro* anticoagulation analysis. The results show that all four of the hydrogel samples in this study namely H4, HCP, SS1 and SS2 are suitable for numerous biomedical applications. H4 and HCP hydrogel will be ideal for applications involving continuous contact with blood given their performance in our *in vitro* anticoagulation analysis. The plasma coagulation system activation test demonstrated that SF/C based hydrogel activated the intrinsic and extrinsic systems on prolonged interaction with fresh healthy blood using STA-Cephascreen, STA – NeoPTimal and STA – Liquid Fib reagents. One of the unique observation in this study is the increased fibrinogen concentration in the presence of curcumin and the effects of PEG and Gelatin on fibrinogen concentration and PT/APTT respectively was further established. The study also affirms the anticoagulant activity of curcumin and the numerous possibilities and potential applications of curcumin in biomaterial science. Notwithstanding, all the products proposed in this work are ideal candidates for diverse biomedical applications.

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APPENDICES

APPENDIX 1

FTIR RAW DATA FOR SS1

cm^{-1}	%T
4000	97.23
3999	97.23
3998	97.23
3997	97.23
3996	97.23
3995	97.23
3994	97.23
3993	97.23
3992	97.23
3991	97.23
3990	97.23
3989	97.23
3988	97.23
3987	97.23
3986	97.23
3985	97.23
3984	97.23
3983	97.23
3982	97.23
3981	97.23
3980	97.23
3979	97.23
3978	97.23
3977	97.23
3976	97.23
3975	97.23
3974	97.23
3973	97.23
3972	97.23
3971	97.23
3970	97.23
3969	97.23
3968	97.23
3967	97.23
3966	97.23
3965	97.23
3964	97.23
3963	97.23

3962	97.23
3961	97.23
3960	97.23
3959	97.23
3958	97.23
3957	97.23
3956	97.23
3955	97.23
3954	97.23
3953	97.23
3952	97.22
3951	97.22
3950	97.22
3949	97.22
3948	97.22
3947	97.22
3946	97.22
3945	97.22
3944	97.23
3943	97.23
3942	97.24
3941	97.24
3940	97.24
3939	97.23
3938	97.23
3937	97.23
3936	97.23
3935	97.23
3934	97.23
3933	97.23
3932	97.23
3931	97.23
3930	97.23
3929	97.23
3928	97.22
3927	97.22
3926	97.21
3925	97.21
3924	97.21
3923	97.21
3922	97.22
3921	97.22

3920	97.22
3919	97.23
3918	97.23
3917	97.23
3916	97.24
3915	97.24
3914	97.24
3913	97.25
3912	97.24
3911	97.24
3910	97.24
3909	97.23
3908	97.23
3907	97.22
3906	97.21
3905	97.2
3904	97.2
3903	97.19
3902	97.2
3901	97.2
3900	97.21
3899	97.21
3898	97.22
3897	97.22
3896	97.23
3895	97.23
3894	97.23
3893	97.23
3892	97.23
3891	97.23
3890	97.23
3889	97.23
3888	97.23
3887	97.22
3886	97.22
3885	97.22
3884	97.21
3883	97.21
3882	97.21
3881	97.21
3880	97.22
3879	97.22

3878	97.22
3877	97.22
3876	97.21
3875	97.21
3874	97.21
3873	97.21
3872	97.21
3871	97.21
3870	97.21
3869	97.21
3868	97.21
3867	97.2
3866	97.2
3865	97.2
3864	97.2
3863	97.2
3862	97.2
3861	97.2
3860	97.2
3859	97.2
3858	97.2
3857	97.2
3856	97.2
3855	97.2
3854	97.2
3853	97.2
3852	97.19
3851	97.19
3850	97.19
3849	97.2
3848	97.2
3847	97.2
3846	97.2
3845	97.2
3844	97.19
3843	97.19
3842	97.19
3841	97.18
3840	97.18
3839	97.18
3838	97.18
3837	97.19

3836	97.19
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3822	97.19
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3818	97.19
3817	97.2
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3815	97.21
3814	97.21
3813	97.21
3812	97.21
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3810	97.21
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3807	97.21
3806	97.21
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3803	97.21
3802	97.21
3801	97.2
3800	97.2
3799	97.2
3798	97.21
3797	97.21
3796	97.22
3795	97.22

3794	97.22
3793	97.23
3792	97.23
3791	97.23
3790	97.23
3789	97.23
3788	97.23
3787	97.22
3786	97.22
3785	97.22
3784	97.22
3783	97.22
3782	97.22
3781	97.22
3780	97.22
3779	97.21
3778	97.21
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3776	97.21
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3765	97.21
3764	97.2
3763	97.2
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3760	97.19
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3753	97.18

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3750	97.16
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3745	97.16
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3743	97.16
3742	97.16
3741	97.16
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3735	97.16
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3723	97.19
3722	97.19
3721	97.2
3720	97.2
3719	97.2
3718	97.2
3717	97.2
3716	97.2
3715	97.19
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3713	97.18
3712	97.18
3711	97.18

3710	97.19
3709	97.2
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3704	97.24
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3701	97.24
3700	97.24
3699	97.24
3698	97.24
3697	97.24
3696	97.24
3695	97.23
3694	97.22
3693	97.22
3692	97.21
3691	97.2
3690	97.2
3689	97.2
3688	97.2
3687	97.2
3686	97.21
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3684	97.21
3683	97.21
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3668	97.15
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3658	97.16
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3656	97.12
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3654	97.1
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3638	97.06
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3608	96.8
3607	96.79
3606	96.78
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3603	96.77
3602	96.77
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3598	96.71
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3596	96.68
3595	96.68
3594	96.67
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3592	96.64
3591	96.63
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3589	96.6
3588	96.59
3587	96.58
3586	96.57
3585	96.56

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3581	96.54
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3330	93.92
3329	93.9
3328	93.88
3327	93.87
3326	93.86
3325	93.84
3324	93.82
3323	93.8
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3321	93.74
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3294	93.07
3293	93.05

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1224	94.35
1223	94.62
1222	94.86
1221	95.08
1220	95.27
1219	95.44
1218	95.61
1217	95.76
1216	95.9
1215	96.03
1214	96.15
1213	96.26
1212	96.36
1211	96.46
1210	96.54
1209	96.63
1208	96.72
1207	96.79
1206	96.86
1205	96.91
1204	96.96
1203	97
1202	97.04
1201	97.08
1200	97.13

1199	97.18
1198	97.21
1197	97.23
1196	97.25
1195	97.26
1194	97.26
1193	97.26
1192	97.26
1191	97.24
1190	97.22
1189	97.2
1188	97.18
1187	97.16
1186	97.13
1185	97.1
1184	97.07
1183	97.02
1182	96.96
1181	96.89
1180	96.82
1179	96.74
1178	96.65
1177	96.56
1176	96.45
1175	96.34
1174	96.21
1173	96.09
1172	95.97
1171	95.84
1170	95.71
1169	95.57
1168	95.41
1167	95.22
1166	95
1165	94.77
1164	94.52
1163	94.24
1162	93.91
1161	93.53
1160	93.08
1159	92.56
1158	91.93

1157	91.17
1156	90.24
1155	89.07
1154	87.65
1153	86.04
1152	84.36
1151	82.81
1150	81.57
1149	80.71
1148	80.21
1147	79.97
1146	79.9
1145	79.96
1144	80.18
1143	80.64
1142	81.35
1141	82.27
1140	83.26
1139	84.2
1138	85
1137	85.62
1136	86.05
1135	86.29
1134	86.38
1133	86.33
1132	86.14
1131	85.79
1130	85.24
1129	84.47
1128	83.44
1127	82.16
1126	80.69
1125	79.12
1124	77.53
1123	76.04
1122	74.66
1121	73.4
1120	72.17
1119	70.91
1118	69.58
1117	68.17
1116	66.72

1115	65.3
1114	63.98
1113	62.76
1112	61.62
1111	60.53
1110	59.48
1109	58.44
1108	57.41
1107	56.44
1106	55.55
1105	54.71
1104	53.88
1103	53.07
1102	52.32
1101	51.65
1100	51.07
1099	50.64
1098	50.4
1097	50.35
1096	50.46
1095	50.73
1094	51.17
1093	51.7
1092	52.28
1091	52.92
1090	53.62
1089	54.36
1088	55.09
1087	55.84
1086	56.63
1085	57.44
1084	58.27
1083	59.13
1082	60.03
1081	60.97
1080	61.9
1079	62.84
1078	63.78
1077	64.69
1076	65.55
1075	66.36
1074	67.14

1073	67.87
1072	68.53
1071	69.11
1070	69.63
1069	70.05
1068	70.34
1067	70.45
1066	70.28
1065	69.73
1064	68.77
1063	67.5
1062	66.17
1061	65.09
1060	64.52
1059	64.56
1058	65.16
1057	66.15
1056	67.35
1055	68.64
1054	69.91
1053	71.09
1052	72.15
1051	73.1
1050	73.95
1049	74.7
1048	75.38
1047	75.99
1046	76.54
1045	77.03
1044	77.47
1043	77.88
1042	78.28
1041	78.67
1040	79.03
1039	79.37
1038	79.69
1037	80.01
1036	80.33
1035	80.66
1034	80.98
1033	81.3
1032	81.61

1031	81.93
1030	82.27
1029	82.63
1028	83
1027	83.36
1026	83.73
1025	84.09
1024	84.44
1023	84.77
1022	85.09
1021	85.39
1020	85.68
1019	85.96
1018	86.22
1017	86.5
1016	86.77
1015	87.06
1014	87.35
1013	87.64
1012	87.93
1011	88.21
1010	88.46
1009	88.69
1008	88.91
1007	89.11
1006	89.29
1005	89.47
1004	89.63
1003	89.8
1002	89.96
1001	90.12
1000	90.27
999	90.42
998	90.57
997	90.72
996	90.87
995	91.02
994	91.17
993	91.32
992	91.47
991	91.62

990	91.76
989	91.9
988	92.03
987	92.14
986	92.24
985	92.33
984	92.41
983	92.46
982	92.49
981	92.5
980	92.48
979	92.44
978	92.35
977	92.21
976	92.01
975	91.72
974	91.33
973	90.81
972	90.11
971	89.19
970	87.98
969	86.4
968	84.4
967	81.97
966	79.24
965	76.42
964	73.84
963	71.78
962	70.42
961	69.77
960	69.69
959	70.02
958	70.6
957	71.31
956	72.07
955	72.83
954	73.53
953	74.11
952	74.5
951	74.69
950	74.67
949	74.52

948	74.31
947	74.16
946	74.16
945	74.37
944	74.79
943	75.4
942	76.15
941	76.98
940	77.85
939	78.7
938	79.5
937	80.18
936	80.73
935	81.18
934	81.57
933	81.98
932	82.44
931	82.94
930	83.46
929	83.93
928	84.36
927	84.77
926	85.22
925	85.73
924	86.26
923	86.79
922	87.29
921	87.76
920	88.19
919	88.59
918	88.96
917	89.31
916	89.64
915	89.95
914	90.23
913	90.48
912	90.73
911	90.98
910	91.22
909	91.46
908	91.69
907	91.92

906	92.13
905	92.32
904	92.51
903	92.7
902	92.89
901	93.07
900	93.22
899	93.35
898	93.48
897	93.61
896	93.75
895	93.88
894	93.98
893	94.05
892	94.1
891	94.14
890	94.18
889	94.23
888	94.28
887	94.34
886	94.39
885	94.42
884	94.44
883	94.46
882	94.48
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878	94.61
877	94.62
876	94.61
875	94.57
874	94.52
873	94.45
872	94.38
871	94.32
870	94.27
869	94.21
868	94.15
867	94.06
866	93.94
865	93.81

864	93.64
863	93.42
862	93.16
861	92.86
860	92.51
859	92.13
858	91.73
857	91.32
856	90.95
855	90.6
854	90.23
853	89.77
852	89.1
851	88.15
850	86.85
849	85.21
848	83.26
847	81.08
846	78.76
845	76.38
844	74.1
843	72.12
842	70.76
841	70.31
840	70.88
839	72.33
838	74.34
837	76.51
836	78.54
835	80.28
834	81.7
833	82.84
832	83.77
831	84.55
830	85.24
829	85.83
828	86.35
827	86.85
826	87.4
825	88
824	88.62
823	89.21

822	89.74
821	90.19
820	90.58
819	90.9
818	91.19
817	91.44
816	91.66
815	91.85
814	92.03
813	92.19
812	92.33
811	92.46
810	92.59
809	92.74
808	92.91
807	93.09
806	93.25
805	93.41
804	93.55
803	93.69
802	93.83
801	93.97
800	94.1
799	94.24
798	94.36
797	94.48
796	94.57
795	94.66
794	94.74
793	94.8
792	94.86
791	94.91
790	94.97
789	95.04
788	95.11
787	95.17
786	95.21
785	95.24
784	95.27
783	95.31
782	95.36
781	95.4

780	95.41
779	95.41
778	95.41
777	95.43
776	95.46
775	95.5
774	95.53
773	95.56
772	95.57
771	95.58
770	95.59
769	95.58
768	95.57
767	95.57
766	95.59
765	95.62
764	95.63
763	95.64
762	95.63
761	95.62
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757	95.63
756	95.63
755	95.62
754	95.61
753	95.6
752	95.59
751	95.58
750	95.56
749	95.53
748	95.51
747	95.49
746	95.48
745	95.46
744	95.45
743	95.43
742	95.4
741	95.37
740	95.34
739	95.3

738	95.28
737	95.26
736	95.24
735	95.21
734	95.17
733	95.12
732	95.08
731	95.04
730	95.02
729	95
728	94.98
727	94.95
726	94.94
725	94.92
724	94.9
723	94.86
722	94.83
721	94.79
720	94.76
719	94.74
718	94.72
717	94.71
716	94.69
715	94.65
714	94.61
713	94.58
712	94.56
711	94.53
710	94.5
709	94.47
708	94.43
707	94.4
706	94.36
705	94.34
704	94.32
703	94.3
702	94.27
701	94.26
700	94.25
699	94.24
698	94.22
697	94.21

696	94.19
695	94.18
694	94.16
693	94.15
692	94.14
691	94.13
690	94.12
689	94.11
688	94.1
687	94.09
686	94.07
685	94.06
684	94.04
683	94.03
682	94.02
681	94.01
680	94
679	93.99
678	93.97
677	93.96
676	93.95
675	93.94
674	93.93
673	93.92
672	93.91
671	93.9
670	93.89
669	93.87
668	93.86
667	93.85
666	93.84
665	93.83
664	93.82
663	93.81
662	93.8
661	93.8
660	93.79
659	93.78
658	93.77
657	93.76
656	93.75
655	93.74

654	93.73
653	93.72
652	93.71
651	93.7
650	93.69
649	93.68
648	93.67
647	93.66
646	93.64
645	93.62
644	93.59
643	93.57
642	93.55
641	93.53
640	93.51
639	93.51
638	93.52
637	93.54
636	93.55
635	93.56
634	93.55
633	93.54
632	93.51
631	93.49
630	93.48
629	93.48
628	93.46
627	93.43
626	93.4
625	93.38
624	93.36
623	93.34
622	93.33
621	93.34
620	93.37
619	93.4
618	93.42
617	93.44
616	93.45
615	93.45
614	93.46
613	93.47

612	93.49
611	93.52
610	93.54
609	93.56
608	93.57
607	93.57
606	93.58
605	93.59
604	93.59
603	93.58
602	93.59
601	93.61
600	93.64
599	93.64
598	93.63
597	93.61
596	93.59
595	93.58
594	93.55
593	93.51
592	93.47
591	93.46
590	93.48
589	93.49
588	93.47
587	93.42
586	93.36
585	93.34
584	93.33
583	93.34
582	93.32
581	93.29
580	93.23
579	93.17
578	93.11
577	93.06
576	93.03
575	93.02
574	93.01
573	93
572	92.98
571	92.95

570	92.92
569	92.89
568	92.86
567	92.82
566	92.76
565	92.69
564	92.62
563	92.54
562	92.47
561	92.43
560	92.42
559	92.43
558	92.41
557	92.33
556	92.22
555	92.11
554	92.05
553	92.02
552	92
551	91.96
550	91.92
549	91.87
548	91.83
547	91.79
546	91.76
545	91.73
544	91.7
543	91.65
542	91.57
541	91.44
540	91.27
539	91.07
538	90.87
537	90.63
536	90.32
535	89.88
534	89.34
533	88.74
532	88.13
531	87.6
530	87.24
529	87.12

528	87.22
527	87.53
526	87.97
525	88.43
524	88.84
523	89.22
522	89.56
521	89.85
520	90.04
519	90.15
518	90.24
517	90.35
516	90.47
515	90.53
514	90.46
513	90.24
512	89.94
511	89.67
510	89.53
509	89.53
508	89.62
507	89.79
506	90.06
505	90.38
504	90.71
503	91
502	91.23
501	91.37
500	91.46
499	91.59
498	91.8
497	92.03
496	92.23
495	92.34
494	92.41
493	92.48
492	92.58
491	92.71
490	92.85
489	92.96
488	93.03
487	93.07

486	93.07
485	93.03
484	92.96
483	92.93
482	92.99
481	93.12
480	93.26
479	93.38
478	93.47
477	93.57
476	93.66
475	93.76
474	93.83
473	93.88
472	93.91
471	93.93
470	93.98
469	94.04
468	94.1
467	94.16
466	94.22
465	94.28
464	94.33
463	94.39
462	94.45
461	94.5
460	94.55
459	94.59
458	94.63
457	94.67
456	94.72
455	94.75
454	94.79
453	94.82
452	94.86
451	94.89
450	94.93
449	94.96
448	95
447	95.04
446	95.07
445	95.1

444	95.12
443	95.13
442	95.15
441	95.19
440	95.25
439	95.3
438	95.34
437	95.39
436	95.44
435	95.47
434	95.49
433	95.49
432	95.49
431	95.53
430	95.59
429	95.68
428	95.76
427	95.8
426	95.82
425	95.82
424	95.81
423	95.82
422	95.87
421	95.95
420	96.07
419	96.18
418	96.25
417	96.27
416	96.27
415	96.28
414	96.31
413	96.35
412	96.39
411	96.44
410	96.52
409	96.61
408	96.69
407	96.72
406	96.7
405	96.64
404	96.6
403	96.64

402	96.82
401	97.13
400	97.53

APPENDIX 2

FTIR RAW DATA FOR SS2

cm^{-1}	%T
4000	99.39
3999	99.39
3998	99.39
3997	99.39
3996	99.39
3995	99.39
3994	99.39
3993	99.39
3992	99.39
3991	99.39
3990	99.39
3989	99.39
3988	99.39
3987	99.39
3986	99.39
3985	99.39
3984	99.38
3983	99.38
3982	99.39
3981	99.39
3980	99.39
3979	99.39
3978	99.39
3977	99.39
3976	99.39
3975	99.38
3974	99.38
3973	99.38
3972	99.38
3971	99.38
3970	99.38
3969	99.38
3968	99.38
3967	99.38
3966	99.38
3965	99.38
3964	99.38
3963	99.38

3962	99.38
3961	99.38
3960	99.38
3959	99.38
3958	99.38
3957	99.38
3956	99.38
3955	99.38
3954	99.38
3953	99.38
3952	99.38
3951	99.38
3950	99.39
3949	99.39
3948	99.39
3947	99.39
3946	99.39
3945	99.39
3944	99.39
3943	99.39
3942	99.39
3941	99.39
3940	99.39
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3927	99.4
3926	99.39
3925	99.39
3924	99.39
3923	99.4
3922	99.4
3921	99.4

3920	99.4
3919	99.4
3918	99.4
3917	99.4
3916	99.4
3915	99.4
3914	99.4
3913	99.4
3912	99.39
3911	99.4
3910	99.4
3909	99.4
3908	99.4
3907	99.4
3906	99.4
3905	99.4
3904	99.4
3903	99.4
3902	99.4
3901	99.4
3900	99.4
3899	99.4
3898	99.4
3897	99.4
3896	99.4
3895	99.4
3894	99.4
3893	99.4
3892	99.4
3891	99.4
3890	99.4
3889	99.4
3888	99.4
3887	99.4
3886	99.4
3885	99.4
3884	99.4
3883	99.4
3882	99.4
3881	99.4
3880	99.4
3879	99.4

3878	99.4
3877	99.4
3876	99.4
3875	99.4
3874	99.4
3873	99.4
3872	99.4
3871	99.4
3870	99.4
3869	99.4
3868	99.4
3867	99.4
3866	99.4
3865	99.4
3864	99.4
3863	99.4
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3861	99.4
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3857	99.39
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3801	99.39
3800	99.39
3799	99.39
3798	99.39
3797	99.39
3796	99.39
3795	99.39

3794	99.39
3793	99.39
3792	99.39
3791	99.39
3790	99.39
3789	99.39
3788	99.39
3787	99.39
3786	99.39
3785	99.39
3784	99.39
3783	99.4
3782	99.4
3781	99.4
3780	99.4
3779	99.4
3778	99.4
3777	99.4
3776	99.4
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3773	99.4
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3771	99.4
3770	99.4
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3761	99.4
3760	99.4
3759	99.4
3758	99.4
3757	99.4
3756	99.4
3755	99.4
3754	99.39
3753	99.39

3752	99.39
3751	99.39
3750	99.39
3749	99.39
3748	99.39
3747	99.39
3746	99.39
3745	99.39
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APPENDIX 3

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APPENDIX 4

FTIR RAW DATA FOR HCP

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485	83,42
484	83,45
483	83,6
482	83,94
481	84,29
480	84,46
479	84,53
478	84,64
477	84,8

476	84,96
475	85,13
474	85,32
473	85,46
472	85,51
471	85,57
470	85,71
469	85,84
468	85,92
467	86,04
466	86,2
465	86,35
464	86,5
463	86,68
462	86,91
461	87,12
460	87,27
459	87,44
458	87,64
457	87,84
456	88,01
455	88,16
454	88,33
453	88,52
452	88,68
451	88,83
450	88,99
449	89,17
448	89,37
447	89,62
446	89,89
445	90,06
444	90,09
443	90,06
442	90,11
441	90,23
440	90,36

APPENDIX 5
ONE-WAY ANOVA RAW DATA

Table 5.1: Descriptive Statistics

Sample(s)	Mean	Standard Deviation	SE of Mean
H4	59.816	67.08976	30.00345
HCP	58.024	71.86634	32.1396
SS1	45.448	40.2831	18.01515
SS2	64.392	78.18668	34.96615
CUR	63.23	74.44904	33.29462
HB	59.744	69.63062	31.13976

Table 5.2: Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	5	1154.64642	230.92928	0.04985	0.99828
Error	24	111171.10072	4632.1292		
Total	29	112325.74714			

Null Hypothesis: The means of the levels are equal.

Alternative Hypothesis: The means of one or more levels are different.

At the 0.05 level, the population means are not significantly different.

APPENDIX 6

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name	: Nwekwo Chidi Wilson
Nationality	: Nigerian
Date and Place of Birth	: 7 June 1989, Kaduna
Marital Status	: Single

EDUCATION

Degree	Institution	Year of Graduation
M.Sc.	Biomedical Engineering, Near East University, TRNC	2015
B.Sc.	Biochemistry, Madonna University, Nigeria	2010

WORK EXPERIENCE

Year	Place	Responsibilities
2016-Present	Near East University, TRNC	Lecturer
2011– 2012	Newton College, Osun State, Nigeria	Teacher
2009	National Agency for Food and Drug Administration and Control	Lab. Intern

LANGUAGES SPOKEN

English, Hausa, Igbo, Yoruba, and Turkish

CERTIFICATES

- International Biomedical Engineering Congress 2015 (North Cyprus)
- Leadership Training Certificate of Completion (Rain Ministries) (North Cyprus)
- Nigerian Institute of Management Membership Certificate (Nigeria)
- National Drug Law Enforcement Agency, NYSC Drug Free Club (Nigeria)
- Nigeria Christian Corpers' Fellowship (Nigeria)
- National Youth Service Corps (Nigeria)

PUBLICATIONS IN PEER REVIEW JOURNALS IN COVERAGE OF ESCI AND SCIE

- Kalkan, R., **Nwekwo, C. W**., and Adali, T. (2020). The effects of silk fibroin based biofilms for cartilage tissue engineering. Cyprus Journal of Medical Sciences. **(Accepted on the 12th of November, 2020)**
- Kalkan, R., **Nwekwo, C. W**., and Adali, T. (2018). The use of scaffolds in cartilage regeneration. *Critical Reviews™ in Eukaryotic Gene Expression*, *28*(4). DOI: 10.1615/CritRevEukaryotGeneExpr.2018024574
- Ozsahin, D. U., Uzun, B., Musa, M. S., Helwan, A., **Wilson, C. N**., Nurcin, F. V., ... and Ozsahin, I. (2017). Evaluating cancer treatment alternatives using Fuzzy PROMETHEE method. International journal of advanced computer science and applications, 8(10), 177-82. DOI: 10.14569/IJACSA.2017.081024

CONFERENCE ORAL AND POSTER PRESENTATION

- International Biomedical Science and Technology Symposium 2019, Izmir Turkey. "Antıcoagulant Actıvıtıes Of Sılk Fıbroın / Curcumın Based Injectable Hydrogels" https://www.kongreuzmani.com/24-biomedical-science-and-technology-symposium-biomed-2019.html
- International Biomedical Engineering Congress 2018, Near East University (North Cyprus) "The Effects Of Silk Fibroin Based Biofilms For Cartilage Tissue Engineering" PP-11 http://ibmec2018.con.neu.edu.tr/
- 3rd Mediterranean Symposium on Medicinal and Aromatic Plants Girne TRNC, April 2017. "Synthesis and Characterization of Silk Fiber Microparticles Containing Linseed Oil" OP-25

www.mesmap.com/.../File/mesmap3abstractbook_baski_05052017_eisbnlastweb.pdf

- International Biomedical Engineering Congress 2015, Near East University (North Cyprus) "Antimicrobial activity of Silk Fibroin microparticles" PP-28 http://con.neu.edu.tr/ibmec

THESISES

Master

- Antimicrobial activity of silk fibroin microparticles. Unpublished Master thesis, Near East University, Department of Biomedical Engineering, Faculty of Engineering, Nicosia, Cyprus.

Undergraduate

- Effects of methanol extract of *Crotalaria retusa, Alchornea cordifolia, Azaderica indica* and *Carica papaya* on oxidative stress in wistar albino rats. Madonna University, Department of Biochemistry, Faculty of Natural and Applied Sciences, Nigeria.

COURSES GIVEN *(from 2016 to 2020)*

Undergraduate:

Courses given:

- General Chemistry for Biological Sciences and Engineering
- Analytical Chemistry
- Biochemistry
- Chemistry for Life Science
- Biomaterials
- Polymer Technology
- System Design on Bioengineering
- Nanotechnology
- Bioenergy Sources
- Biophysics

HOBBIES

Reading, Research, Learning new things, Playing musical instruments (Guitar, Keyboard and Ocarina), Singing, Physical Exercises, Playing Basketball, Speaking and Meeting people.

EK: 1037-202

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 23.01.2020
Toplantı No : 2020/76
Proje No :955

Yakın Doğu Üniversitesi Mühendislik Fakültesi öğretim üyelerinden Doç. Dr. Terin Adalı'nın sorumlu araştırmacısı olduğu, YDU/2020/76-955 proje numaralı ve **"Hidrojel Ve Polielektrolit Yapılarda Kan Uyumluluğu Çalışmaları"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

1. Prof. Dr. Rüştü Onur (BAŞKAN)
2. Prof. Dr. Nerin Bahçeciler Önder (ÜYE) KATILMADI
3. Prof. Dr. Tamer Yılmaz (ÜYE) KATILMADI
4. Prof. Dr. Şaban Saygı (ÜYE)
5. Prof. Dr. Şanda Çalı (ÜYE)
6. Prof. Dr. Nedim Çakır (ÜYE)
7. Prof. Dr. Nurhan Bayraktar (ÜYE)
8. Doç. Dr. Nilüfer Galip Çelik (ÜYE) KATILMADI
9. Doç. Dr. Emil Mammadov (ÜYE)
10. Doç. Dr. Mehtap Tınazlı (ÜYE) KATILMADI

APPENDIX 8
SIMILARITY REPORT

AUTHOR	TITLE	SIMILARITY	GRADE	RESPONSE	FILE	PAPER ID	DATE
Chidi Nwekwo	Abstract	0%	--	--		1504514713	08-Feb-2021
Chidi Nwekwo	Conclusion	0%	--	--		1504526081	08-Feb-2021
Chidi Nwekwo	Results and Discussion	0%	--	--		1504522218	08-Feb-2021
Chidi Nwekwo	Entire Thesis	7%	--	--		1504531563	08-Feb-2021
Chidi Nwekwo	Literature Review	7%	--	--		1504517011	08-Feb-2021
Chidi Nwekwo	Introduction	12%	--	--		1504516268	08-Feb-2021
Chidi Nwekwo	Materials and Methods	13%	--	--		1504517960	08-Feb-2021