MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES

INVESTIGATION OF SULFATE-MODIFIED LEVAN POLYSACCHARIDES AND THEIR BIOACTIVITY

AYÇA AKÇAY

MASTER THESIS
Department of Bioengineering

ADVISOR
Prof. Ebru TOKSOY ÖNER

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ÖZET

SÜLFATLANMIŞ LEVAN POLİSAKKARİTLERİNİN VE BİYOAKTİVİTELERİNİN İNCELENMESİ

İstenmeyen kan pıhtıları kan akımını sınırlayabilir ya da engelleyebilir. Bu durum vücudun organlarına zarar verebilir ve ölüme bile neden olabilir. Doğal veya modifiye yolla elde edilen sülfatlanmış polisakkaritler, antikoagülan (pıhtı önleyici) etkinlik gösteren biyoaktif maddelerdir. Heparin, hayvansal kaynaklı ve kendiliğinden sülfatlı bir polisakkarit olup en çok kullanılan antikoagülandır. Heparin elde etmenin zorlukları, heparinin yapısal heterojenitesi ve kullanımında yol açtığı ciddi sağlık sorunları nedeniyle heparin benzeri yeni antikoagülan maddelerin geliştirilmesine yönelik kapsamlı araştırmalar yapılmaktadır.

Levan, fruktoz monomerlerinden oluşan fruktan tipi bir doğrusal polimerdir. Suda çözünebilir, güçlü yapışkan ve film oluşturabilir bir ekzopolisakkarit (EPS) olması sebebiyle işlevsel bir biyopolimer olarak medikal ve kimyasal endüstrilerinde gittikçe yükselen bir popülariteye sahiptir. Heparin benzeri alternatiflere olan ihtiyacın yanında levan bazlı biyomateryallerin potansiyel biyomedikal uygulamaları da göz önüne alınarak başlatılmış olan çalışmalarda, levan türevlerinin antikoagülan madde olarak kullanım potansiyelinin aydınlatılması hedeflenmiştir.

Bu amaçla halofilik *Halomonas smyrnensis* AAD6^{T} bakterisi tarafından üretilen levan polimeri, yapısına sülfat grupları katılacak şekilde kimyasal olarak değiştirilmiştir. Daha sonra, sentezlenen levan sülfat polimerlerinin antikoagülasyon aktivitelerinin hangi mekanizma üzerinden gerçekleştiğini açıklamak üzere çeşitli koagülasyon testleri (PT, APTT, TT ve Heptest) gerçekleştirilmiştir. Sonuçlar, sülfatlanmış levanın, kan pıhtılaşma mekanizması üzerinde, hem dozaj hem de izlediği yol bakımından heparin benzeri bir antikoagülan etkiye sahip olduğunu göstermiştir.

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ABSTRACT

INVESTIGATION OF SULFATE-MODIFIED LEVAN POLYSACCHARIDES AND THEIR BIOACTIVITY

Undesired blood clots can limit or block blood supply. This situation can harm organs of the body and even cause death. Natural or modified sulfated polysaccharides are bioactive substances which exhibit anticoagulant (clot inhibitor) activity. Heparin, which is an animal sourced and naturally sulfated polysaccharide, is the most frequently used anticoagulant. Due to the difficulties in obtaining heparin, its structural heterogeneity as well as serious health problems associated with its use, considerable research has been directed towards the development of new anticoagulant agents that could mimic heparin.

Levan is a linear, fructan-type homopolymer of fructose residues. It is a water soluble, strongly adhesive and film forming exopolysaccharide (EPS), which is gaining escalating popularity as a functional biopolymer with diverse applications in medical and chemical industries. Considering the potential biomedical applications of levan-based biomaterials as well as the urgent need for heparin-mimetic alternatives, studies were initiated to elucidate the potential use of levan derivatives as anticoagulation agents in various applications. For this, levan polysaccharide produced by halophilic *Halomonas smyrnensis* AAD6^{T} bacteria were chemically modified to integrate sulfate groups to its structure. Then, the synthesized sulfated levan polymers were subjected to several blood coagulation tests (PT, APTT, TT, and Heptest) in order to understand the mechanism associated with the observed anticoagulation activity. Results have shown that sulfated levans had similar anticoagulant effect to heparin on blood clotting mechanism in terms of both dosage and inhibition pathway.

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SYMBOLS

α : Alpha

β : Beta

µg : Micrograms

µl : Microliters

g : Gram

h : Hour

k : Reaction rate

m : Mass

mg : Miligram

min : Minute

ml : Mililiter

nm : Nanometer

sec : Second

v/v : Volume per volume

w/v : Weight per volume

ABBREVIATIONS

APTT	: Activated Partial Thromboplastin Time
BRM	: Biological Response Modifier
C	: Control Group
CNT	: Carbon Nanotubes
CPS	: Capsular Polysaccharide
CSA	: Chlorosulfonic Acid
DS	: Degree of Substitution/Degree of Sulfation
DVT	: Deep Vein Thrombolism
EC	: Enzyme Commision
EPS	: Exopolysaccharide
F	: Factor
GAG	: Glycosaminoglycan
Hep	: Heparin
L	: Pure Levan
LMW	: Low Molecular Weight
MW	: Molecular Weight
NK	: Natural Killer
OD	: Optical Density
PE	: Pulmonary Embolism
PF	: Platelet Factor
PS	: Polysaccharide
PT	: Prothrombin Time
SL	: Sulfated Levan
TCA	: Trichloroacetic Acid
TF	: Tissue Factor
TT	: Thrombin Time
UF	: Unfractionated
VT	: Venous thromboembolism

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1. INTRODUCTION

Exopolysaccharides (EPSs) are high-molecular-weight polymers that are composed of sugar residues and are secreted by microorganisms to the surrounding environment. Many microorganisms including several species of gram-positive and gram-negative bacteria, archaea, fungi and some algae are known to produce EPSs. These natural, non-toxic, and biodegradable polymers not only protect microorganism against environmental extremes such as Antarctic ecosystems, saline lakes, geothermal springs or deep sea hydrothermal vents but also play important roles in various biological mechanisms such as immune response, adhesion, infection and signal transduction (Jones et al., 2014; Poli et al., 2011; Kumar et al., 2007; Sutherland, 1998) as well as in biofilm formation, biofouling and quorum sensing (Garg et al., 2014; Fazli et al., 2014).

Some EPSs produced by microorganisms such as cellulose are also produced by higher order plants. But, due to their slow production rate (3-6 months) as well as their dependence on seasonal conditions and high agricultural land, controlled microbial fermentations are preferred over plants for sustainable and economical production of EPSs at industrial scale (Toksoy Oner, 2013).

Due to their useful physicochemical and rheological properties and diverse functionality, the EPSs have been recognized as new biomaterials and have found wide range of applications. They can be used as thickeners, bioadhesives, stabilizers, probiotic, gelling agents, emulsifier, biosorbent and bioflocculant not only in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and food additives but also in health and bionanotechnology sectors (Kreyenschulte et al., 2014; Toksoy Oner, 2013; Donot et al., 2012; Freitas et al., 2011).

Levan polysaccharide is a naturally occurring β(2→6)-linked fructose polymer (fructan). As a water soluble, non-toxic, self-assembling, strongly adhesive and film-forming biopolymer with many distinguishing properties like low viscosity, high solubility in oil, compatibility with salts and surfactants, stability to heat, acid, and alkali media, high holding capacity for water and chemicals, and good biocompatibility,

levan is gaining escalating popularity as a functional biopolymer with diverse applications in medical and chemical industries (Kang et al., 2009).

Besides the diverse array of microbial levan producers, the halophilic bacterium *Halomonas smyrnensis* AAD6^T was reported to be the first extremophilic producer of levan by our research group (Poli et al., 2009). Further research on the potential use of levan by *Halomonas* sp. AAD6^T as a bioflocculating agent (Sam et al., 2011) and on its suitability for peptide and protein based drug nanocarrier systems (Sezer et al., 2011) were reported. An economical production scheme via cheap carbon resources was also proposed (Kucukasik et al., 2011). The antioxidant potential of this levan polysaccharide in high glucose condition in the pancreatic beta cells by demonstrating a correlation between reduction in oxidative stress and apoptosis with its treatment was also reported for the first time by our research group (Kazak et al., 2014). Moreover, levan and aldehyde-activated levan were successfully deposited by matrix-assisted pulsed laser evaporation (MAPLE) resulting in uniform, homogeneous, nanostructured, biocompatible, thin films (Sima et al., 2011, 2012). Interaction of these thin films with human osteoblasts was investigated and distinct areas of the binary gradient have been shown to modulate the osteoblasts' extracellular signal-regulated kinase signalling with different propensity (Axente et al., 2014). Anticancer activity of levan and aldehyde activated derivatives was also recently reported (Kazak Sarilmiser and Toksoy Oner, *in press*). Phosphonated levan-based bioactive surfaces obtained by layer-by-layer sequential assembly were found to have high biocompatibility and adhesive strength (Costa et al., 2013). Ternary blend films of chitosan and polyethylene oxide (PEO) with levan were prepared to evaluate the morphological, thermomechanical, surface and biological properties of the films (Sennaroglu Bostan et al., 2014).

1.1. Aim

Use of biodegradable polymers for biomedical applications has increased in recent decades due to their biocompatibility, biodegradability, flexibility, and minimal side effects. Applications of these materials include creation of skin, blood vessels, cartilage scaffolds, and nanosystems for drug delivery. The understanding of both cell–extracellular matrix (ECM) and cell–material interactions is crucial for the success of implantable biomaterials including tissue engineering devices. ECM is rich in sulfated

and aminated glycosaminoglycans (GAG) and proteoglycans (PG). The development of synthetic models containing those chemical groups is thus of major interest (Oliveira et al., 2013).

The glycosaminoglycan (GAG) heparin is a naturally occurring negatively charged highly sulfated water-soluble polysaccharide that is used clinically for more than 70 years as drug of choice in the prevention and treatment of thromboembolic disorders (Alban & Franz, 2001). However, the use of heparin as an anticoagulant has limitations due to serious adverse effects posed by its structural heterogeneity such as bleeding, osteporosis, skin rashes, contact dermatits, urticari or skin necrosis, among others (Maas et al., 2012; Ye et al., 2012). Hence considerable research has been directed towards the development of heparin-mimetic compounds.

Considering the potential biomedical applications of levan-based biomaterials, ongoing studies of our research group are focused on obtaining bioactive formulations using different techniques with both levan polymer as well as its chemical derivatives. As part of these activities, studies were initiated to elucidate the potential use of levan derivatives as heparin-mimetic molecules in various applications. Hence the main objective of this thesis was to obtain sulfated derivatives of levan polysaccharide with anticoagulant activity. For this, main parameters for the sulfation reaction were optimized and the synthesized sulfated levan polymers were subjected to several blood coagulation assays in order to understand the mechanism associated with the observed anticoagulation activity. The outcomes of this thesis will enlighten and strengthen the further studies on finding heparin alternative substances.

1.2. General Background

1.2.1. Bacterial Exopolysaccharides

Extracellular polymeric substances are metabolic products (Mishra & Jha, 2013). Bacterial cells are often surrounded by a polysaccharidic layer which is referred as exopolysaccharide and constitutes the interface with the environment (Cescutti, 2009). Bacterial exopolysaccharides are biopolymers that are secreted to the medium by the cells, and form a capsule that remains associated with the cell surface, or a slime that is loosely bound to the cell surface (Freitas et al., 2011). Because of their position they are

characterized as exopolysaccharides, to distinguish them from any polysaccharides that might be found within the cell (Morris & Harding, 2009).

Bacterial exopolysaccharides are mainly composed of carbohydrates like glucose, fructose, or mannose, and some non-carbohydrate substituents like pyruvic acid ketal, or phosphoric acid linked with diester linkages (Freitas et al., 2011; Cescutti, 2009). Exopolysaccharides can have a linear or branched structure, and also can be divided into two groups according to their monomer structures: homopolysaccharides and heteropolysaccharides (Cescutti, 2009; Donot et al., 2012). Homopolysaccharides are made up of a single type of monosaccharide, like levan or dextran, and are synthesised by a specific secreted enzyme. This synthesis can occur either outside the cell or within the cell wall. Heteropolysaccharides are made up of several types of monosaccharide like xanthans or gellans, have complex structures and are usually synthesised inside the cell in the form of repeating units (Donot et al., 2012). Through the diversity of sugar components, there is a wide range of possible molecular structures. Moreover, this variability is increased by the different glycosyl linkages and configurations that are also possible as seen in Table 1.1 (Freitas et al., 2011).

Table 1.1. Common exopolysaccharides and structures (Mishra & Jha, 2013).

Exopolysaccharides	**Source**	**Structure (backbone)**
Acetan	*Acetobacter xylinum*	Branched homopolysaccharide of glucose (1 → 4 *β*) linkage with side chain: *α*-(1,3)- tetrasaccharides [*β*–D–Glc–(1 → 4)–*β*–D–Glc–]$_n$ ↓[*α*(1 → 3)] Rha–*α*(1 → 6)Glc–*β*(1 → 6)Glc–*α*(1 → 4)GlcA *β*(1 → 2)Man
Alginate	*Pseudomonas aeruginosa* *Azotobacter* sp.	Polysaccharides with irregular structure: composed of variable proportions of 1,4-linked β-D-mannuronic acid (M) and its C-5 epimer *α*-L-guluronic (G) M blocks: [–D-ManUA–(1 → 4)β–]$_n$ G blocks: [–L-GulUA–(1 → 4) *α*–]$_n$ Mixed block: [DM(1 → 4)–LG–(1 → 4)–]$_n$
Alternan	*Leuconostoc mesenteroides*	Homopolysaccharide of glucose linkage alternating (1 → 3 *β*) and (1 → 6 *β*) [*β*–D–Glc–(1 → 3)–*β*–D–Glc–(1 → 6)]$_n$

Table 1.1. (Continued)

Biodispersion	*Alteromonas* sp. *Alteromonas haloplanktis* *Alteromonas macleodii*	Heteropolysaccharide containing repeating tetrasaccharide units [L-Rha → 2-acetamido → 2-deoxy-D-Glu → 3-O-[(R)-1-carboxyethyl]-D-Glu] [D-Gal → 3-O-acetyl-2-acetamido-2-deoxy-D-Glu → 2-acetamido-2-deoxy-L-guluronic acid → 3,6-dideoxy-3-(4-hydroxybutyramido)-D-Gal]
Cellulose	*Acetobacter xylinum* *Agrobacterium* sp. *Pseudomonas* sp. *Rhizobium* sp.	Linear homopolysaccharide of glucose (1 → 4 *β*) linkage [*β*–D–Glc–(1 → 4)–*β*–D–Glc–]$_n$
Curdlan	*Alcaligenes faecalis* var. *Myxogenes*	Linear homopolysaccharide of glucose (1 → 3*β*) linkage [*β*–D–Glc–(1 → 3)–*β*–D–Glc–]$_n$
Cyclosophorans	*Agrobacterium* sp. *Rhizobium* sp. *Xanthomonas* sp.	Cyclic (1 → 2)- *β*-D-glucans
Dextran	*Lactobacillus hilgardii* *Leuconostoc mesenteroides* *Leuconostoc dextranicum* *Streptococcus mutans*	Branched homopolysaccharide of glucose (1 → 6 *α*) linkage with side chains *α*-(1,3)-, *α*-(1,4)- or *α*-(1,2)- [*α*–D–Glc–(1 → 6)–*α*–D–Glc–]$_n$ ↓[*α*(1 → 3)] [D–Glc(1 → 6)–*α*–D–Glc–]$_m$ The exact structure of each type of dextran depends on the microbial strain
Emulsan	*Acinetobacter calcoaceticus*	Heteropolysaccharide containing repeating trisaccharide [GalNAc → GalNAcUA → an unidentified N-acetylamino sugar]
Gellan	*Aureomonas elodea* *Sphingomonas paucimobilis* *Sphingomonas elodea*	Linear heteropolysaccharide of glucose, glucuronic acid, and rhamnose [–D–Glc–(1 → 4)*β*–D-GlcUA–(1 → 4)*β*D–Glc(1 → 4) *β* –L–Rha(1 → 3)*α*–]$_n$
Hyaluronan (Hyaluronic acid)	*Streptococcus* sp.	Heteropolysaccharide of glucuronic acid and N-acetylglucosamine linkage alternating (1 → 4 *β*) and (1 → 3 *β*) [–DGlcUA–(1 → 4)*β*–DGalNAc–(1 → 3)*β*–]
Kefiran	*Lactobacillus hilgardii* *L. rhamnosus* *L. kefir* *L. kefiranofasciens*	Heteropolysaccharide of glucose and galactose [→6)*β*–DGlc–(1 → 2{6})*β*–DGal–(1 → 4)*α*–DGal–(1 → 3)*β*–DGal–(1 → 4) *β*–DGlc–(1]$_{21}$ ↓ ↓ → 6)*β*–DGlc–(1 → 2{6})*β*–DGal
Levan	*Alcaligenes viscosus* *Zymomonas mobilis* *Bacillus subtilis* *Halomonas smyrnensis*	Branched homopolysaccharide of fructofuranosyl (2 → 6)*β* linkage with side chains *β*(2,1) [→6)*β*–DFruf–(2 → 6)*β*–DFrucf–]$_n$ ↓[*β* (2 → 1)] [→6)*β*–DFruf–(2 → 6)*β*–DFrucf–]$_m$

Table 1.1. (Continued)

Exopolysaccharides	**Source**	**Structure (backbone)**
Succinoglucan	*Alcaligenes faecalis* var. *Myxogenes*	Heteropolysaccharide of glucose and galactose linkage alternating (1 → 4 β) and (1 → 3 β) [β–D–Glc–(1 → 4)–β–D–Gal–(1 → 3)]$_n$
Welan	*Alcaligenes* species	Heteropolysaccharide of glucose, glucuronic acid, and rhamnose with side chains of n repeat of either L-rhammose or L-mannose (1 → 4 α) or n repeat of glucose(1 → 6 α) units substituted on C3 of every 1,4 linked glucose repeating unit of backbone [D–Glc–(1 → 4)β–D-GlcUA–(1 → 4)β-D–Glc(1 → 4) α –L–Rha/Man(1 → 3) β–]$_n$
Xanthan	*Xanthomonas campestris*	Branched homopolysaccharide of glucose (1 → 4 β) linkage with side chains α-(1,3)- triasaccharides [β–D–Glc–(1 → 4)–β–D–Glc–]$_n$ ↓[α(1 → 3)] Man–β(1 → 4) GlcA β(1 → 2)Man

Exopolysaccharides do not appear to function as energy sources, and bacteria are unable to catabolise the exopolysaccharides produced. The major role of exoplysaccharides is to protect the cell in its environment. For example; surrounding itself with a layer of extracellular polysaccharides, the bacteria regulates the diffusion of certain molecules between the extracellular and intracellular environments (Donot et al., 2012). Bacterial exopolysaccharides are used for food, pharmaceutical, cosmetical, and medical applications as seen in Table 1.2 because of their great diversity in structural and functional properties (Morris & Harding, 2009).

Table 1.2. Microbial exopolymers and their applications (Mishra & Jha, 2013).

Biopolymers	**Possible applications**
Acetan	Viscosifier and gelling agent
Alginate	Immobilization and microencapsulation
Cellulose	Temporary artificial skin, natural nondigestable fibers, hollow fibers or membranes, and acoustic membranes in audiovisual equipment

Table 1.2. (Continued)

Curdlan	Gelling agent
Cyclosophorans	Encapsulation of drugs and food component
Dextran	Blood plasma extender or blood flow improving agent, cholesterol lowering agent, and microcarrier in tissue/cell culture
Emulsan	Emulsification and immobilization
Gellan	Solidification/gelling agent
Hyaluronic acid	Moisturization and synovial fluid replica
Kefiran	Gelatination and viscoelasticity
Levan and Alternan	Similar as dextran
Succinoglycan	Gelling agent and immobilization
Welan	Stabilizer and viscosifier
Xanthan	Emulsification and gelatination

Bacterial exopolysaccharides are classified into two different types according to their structural properties: capsular polysaccharides (K-antigens) (CPSs) and exopolysaccharides (EPSs) (Cescutti, 2009).

1.2.1.1. Capsular polysaccharides (CPSs)

CPS is defined as an exocellular polysaccharide secreted in such a large amount that it encapsulates a bacteria, facilitating adhesion and helping screen from immune response (Morris & Harding, 2009). The capsule, which is the outmost layer of bacterial cell wall and is not present in all bacteria, is the source of CPSs (Leung et al., 2006). These biopolymers linked to the cell surface via covalent bond to phospholipid or lipid molecules (Cescutti, 2009). CPSs are amorphous in the capsular layer and highly hydrated. The water content is over 95%.

There are no exact descriptions to summerize the structure of all CPSs. They are very heterogeneous in terms of both composition and structure even in the same species. At least 80 different CPSs have been described for *E. Coli* and 90 different CPSs have been reported for *Staphylococcus pneumonia* shown in Figure 1.1 (Leung et al., 2006). CPSs are highly immunogenic, and may have evolved their unusual diversity as a way of avoiding antibody responses (Morris & Harding, 2009).

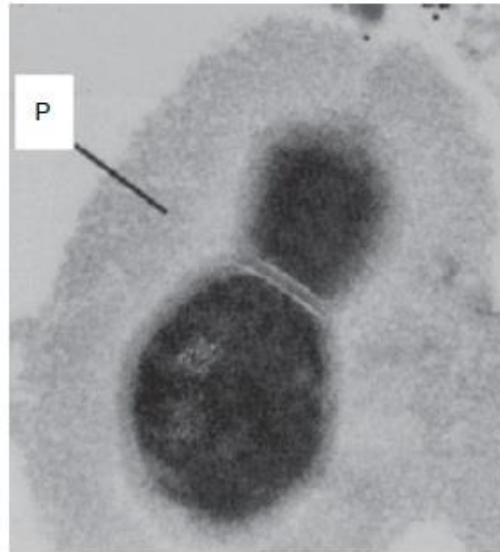

Figure 1.1. Exocellular or 'capsular' polysaccharide layer (labeled P) from *Streptococcus pneumoniae* (Morris & Harding, 2009).

1.2.1.2. Exopolysaccharides (EPSs)

Polysaccharides which appear to be released on the cell surface with no attachment to the cell and are often sloughed off to form slime are referred as exopolysaccharides (EPSs) (Cescutti, 2009). The composition and structures of EPSs are relatively simple when compare with CPSs (Leung et al., 2006). Most bacterial EPSs are synthesized intracellularly and exported to the extracellular environment as macromalocules. There are a few known exceptions like levan and dextran whose synthesis and polymerization occur outside the cells by the action of secreted enzymes that convert the substrate into the polymer in the extracellular environment (Freitas et al., 2011). Figure 1.2 represents the pathways involved in sugar fermentation and the types of exopolysaccharide synthesis.

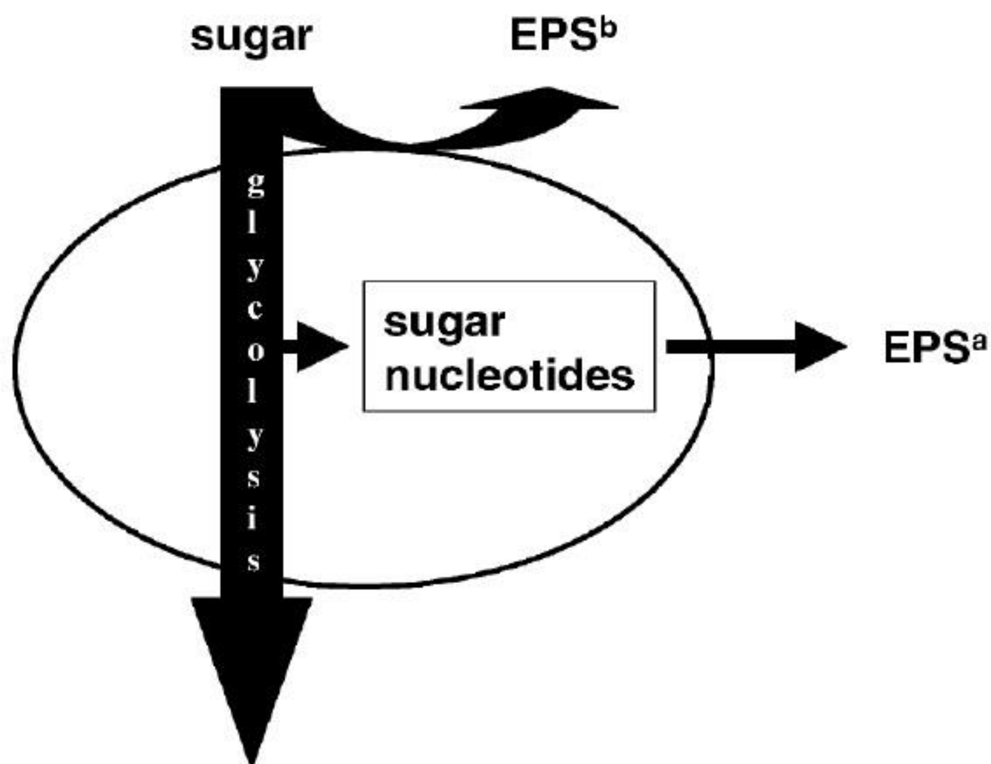

Figure 1.2. Schematic representation of pathways involved in sugar fermentation. Via glycolysis to lactate and/or other acids and biosynthesis of exopolysaccharides (EPSs) from intracellular sugar nucleotides (EPS[a]) and extracellular EPS biosynthesis (EPS[b]) (Boels et al., 2001).

The properties of bacterial EPSs are determined by their chemical composition, molecular structure, average molecular weight, and distrubition (Freitas et al., 2011). EPSs produced by bacteria have great potential, and physicochemical characteristics of EPS decide its possible commercial application ranging from pharmaceutical to food-processing, extended to detoxification, bioremediation, paints, biotechnology, and petrochemicals (Mishra & Jha, 2013). Polysaccharides are not biological inert molecules, but play active parts in cell-signalling processes by interactions with proteins and other carbohydrates (Sletmoen et al., 2003). Polysaccharides from all kind of sources, and EPSs from bacteria in particular, have great effect on immune cells triggering to show many biological activities like antiviral, anticancer, anticoagulant, and antioxidant activities as biological response modifiers (BRMs). Figure 1.3 represents shematic illustration of the activation of immune cells for the destruction of pathogens by polysaccharide BRMs.

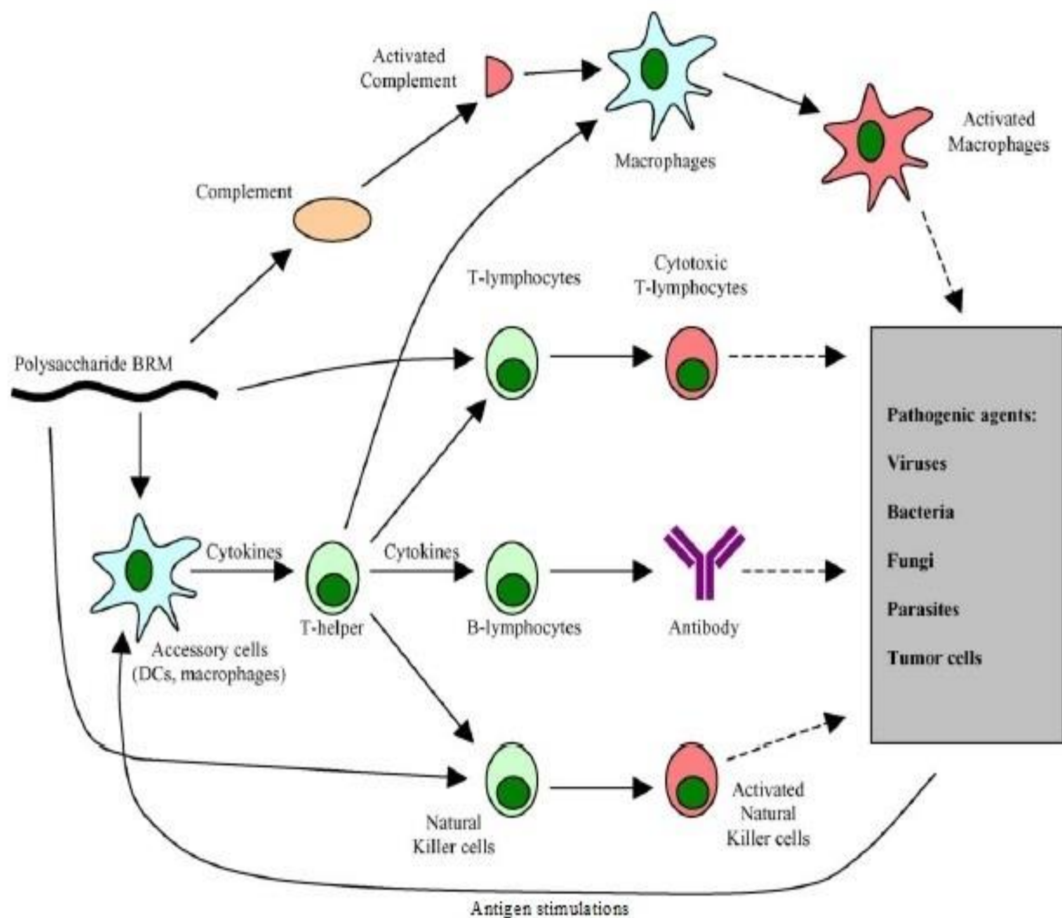

Figure 1.3. Shematic illustration of the activation of immune cells for the destruction of pathogens by polysaccharide BRMs. Polysaccharide BRMs can directly activate complement pathways, macrophages, T-lymphocytes, B-lymphocytes and naturel killer cells, or activate macrophages, T-lymphocytes, B-lymphocytes and naturel killer cells indirectly through accessory cells like DCs and macrophages. Polysaccharide stimulated accessory cells secrete cytokine like IL-1 which stimulates T helper (Th) lymphocytes to secrete cytokines. The cytokines modulate/activate macrophages, T-lymphocytes, B-lymphocytes and NK cells which become or produce effector gadgets for the clearance of pathogens. Solid arrows represent activation and dashed arrows represent suppression or destruction (Leung et al., 2006).

1.2.2. Levan by *Halomonas smyrnensis* bacteria

Levan is a fructose biopolymer mainly linked by β-(2,6)-glycosidic bonds, with β-(2,1)-linked side chains and functions as a reserve carbohydrate of monocotyledons (Yoon et al., 2004; Dahech et al., 2013). It is also produced from various microorganisms such as

Pseudomonas sp., *Xanthomonas* sp., *Bacillus* sp., and *Streptococcus* sp. (Yoo et al., 2004). Microbial levans are produced from sucrose-based substrates extracellularly and are synthesized by levansucrase enzymes (E.C. 2.4.1.10) (Poli et al., 2009; Costa et al., 2013). The chemical structure, which is shown in Figure 1.4, and physical properties of levans have been extensively characterized, in terms of molecular weight, linkage type, sugar components, and viscosity (Yoon et al., 2004).

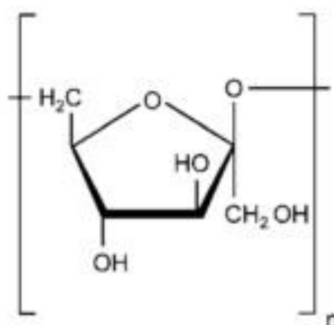

Figure 1.4. Chemical structure of levan (Costa et al., 2013).

Levan produced by different organisms differ in their weight and degree of branching (Abdel-Fattah et al., 2012). Production of microbial levans as exopolysaccharides is largely affected by the concentration of nutrients in culturing medium and the environmental conditions (Yoo et al., 2004).

Many polysaccharides have been shown to express biological activities such as immunostimulating, antitumor, and anti-inflammatory activities (Yoo et al., 2004). Since levan is biodegradable, biocompatible, non-allergic, non-toxic and water-soluble, it is shown to exert excellent cell-prolifitaring, antitumor and immunostimulating activities (Yoo et al., 2004; Yoon et al., 2004; Abdel-Fattah et al., 2012; Costa et al., 2013). Not only a bioactive substance, levan also has a variety of applications in the fields of cosmetics, medicine, printing, foods and pharmaceuticals (Yoo et al., 2004; Abdel-Fattah et al., 2012). Novel uses of levan derivatives such as levan sulfates, phosphates, and acetates polymers in medicine as anti-AIDS agents; food processing as food additive with prebiotic and hypocholesterolemic effects, and as an environmentally bening adhesive are also reported (Abdel-Fattah et al., 2012).

Halophilic *Halomonas smyrnensis* AAD6^T from Çamaltı Saltern area in Turkey (Poli et al., 2013) was described as a levan producer microorganism by our research group for the first time (Poli et al., 2009). *Halomonas smyrnensis* AAD6^T is the first

extremophilic producer of levan and produces unbranched levan. Excreting high molecular weight levan by this halophile to a highly saline environment for adhesion and protection points to the superior bioadhesive properties of levan that were retained under such unusual conditions (Costa et al., 2013).

1.2.3. Sulfated polysaccharides (SPSs)

Sulfated polysaccharide (SPS) is a kind of polysaccharide with sulfated groups in its hydroxyls and comprises a complex group of macromolecules with a range of important biological properties (Farias et al., 2000; Wang et al., 2009). These chemically anionic polymers are widespread in nature, occuring in a great variety of organisms such as plants, animals and microorganisms (Farias et al., 2000; Mestechkina & Shcherbukhin, 2010; Wijesekara et al., 2011; Raveendran et al., 2013). On the basis of occurance, SPSs can be classified as natural and chemically modified (Raveendran et al., 2013).

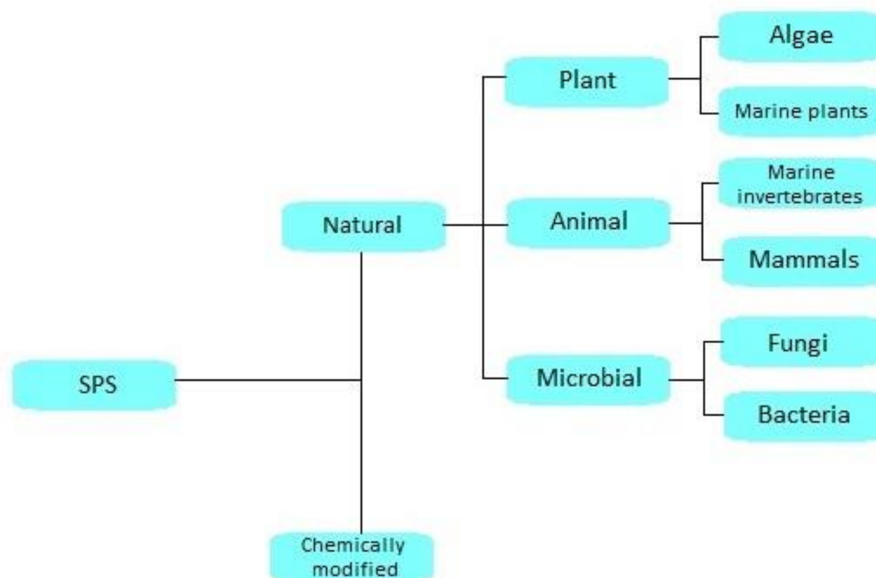

Figure 1.5. Classification of SPSs (reconstructed from Raveendran et al., 2013).

As seen in Figure 1.5, natural SPSs are extracted from plants (e.g. fucoidan), animals (e.g. heparin), and microorganisms (e.g. mauran from *Halomonas maura*) (Wijesekara et al., 2011; Wijesinghe & Jeon, 2012; Raveendran et al., 2013). The majority of plant SPSs are present in algae (Mestechkina & Shcherbukhin, 2010). Animal SPSs are endogenous in origin and are found in pericellular locations like outside the cell membrane contributing to cell to cell interaction, cellular adhesion and prevention from

external invasion of pathogens. They also act as reservoirs for many growth factors. Microbial SPSs are mainly secretory in nature and are seen as slime layer or capsule outside the bacterial cell (Raveendran et al., 2013).

There is no universal method to modify polysaccharides with sulfate. Sulfation reaction is performed in organic solvents. These organic solvents are formamide (FA), dimethylformamide (DMF), dimethylsulfoxide (DMSO), and pyridine (Py) (Mestechkina & Shcherbukhin, 2010). The sulfating reagents are mainly chlorosulfonic acid (CSA), complexes of SO_3 with pyridine ($Py{\cdot}SO_3$), concentrated sulfuric acid, and oleum (Mestechkina & Shcherbukhin, 2010; Yang et al., 2013). CSA-Py method is the most popular method to sulfate polysaccharides. The ratio of CSA to Py, reaction temperature and the reaction time are the most important factors in this method (Wang et al., 2009).

SPSs are effective for the treatment of various diseases, and also used in various industrial applications as seen in Table 1.3 (Raveendran et al., 2013). Even polysaccharides without sulfate groups exhibit biological activities after sulfation, e.g. cellulose sulfate shows anticoagulant and antiviral functions (Zhang et al., 2010). Bioactivity of sulfated polysaccharides mainly depens on several structural parameters including sugar composition, molecular weight, type of glycosidic bond of the main chain, and degree of sulfation (DS, degree of substitution, i.e., sulfate groups per sugar monomer) (Alban & Franz, 2001; Xiong et al., 2011). DS is the most important parameter for same kind of polysaccharide. Generally, the higher DS value, the stronger biological activities (Yang et al., 2013).

Table 1.3. Various types of sulfated polysaccharides with nanotechnological, biological and pharmaceutical applications (Raveendran et al., 2013).

Type of SPS	Nanotechnology application	Biological and pharmaceutical applications
Carrageenans	Gelling agent Thickening agent Stabilizing agent Capping agent for biomineralizing metal oxides Stabilizing micelles Microbeads for controlled release Drug encapsulation and delivery Hydrogels Composite gels Composite nanoparticles	Anticoagulant activity Antiviral activity Antioxidant activity Free radical scavenging activity Vaginal gel formulations Prevention of sexually transmitted diseases Microbicidal activity
Fucoidans	Stabilizing agent Fucospheres or microspheres	Anticoagulant activity Antiviral activity Anti-angiogenic activity Antitumor activity Anti-inflammatory activity Antioxidant activity Anti-proliferative activity Immunomodulating property Treating dermal burns in rabbit
Ulvan	Nanofibrous scaffolds	Resist attack of necrotic pathogens in plants Antifungal activity
Chondroitin sulfate	Nanoparticles Stabilizing agent Amphiphilic polymeric micelles Hydrogels Microspheres Tissue engineering scaffolds	Anti-inflammatory activity Fucosylated CS possess anticoagulant activity and antithrombogenic activity Tablets, ophthalmic formulations, soft capsules Treating osteoarthritis and cartilage regeneration Nutraceuticals

Table 1.3. (Continued)

Type of SPS	Nanotechnology application	Biological and pharmaceutical applications
Heparin/Heparan sulfate	Tissue and bone engineering Nanoparticles Amphiphilic nanoparticles Composite nanoparticles Blood compatible CNT's Drug delivery using modified scaffolds	Anticoagulant activity Anti-proliferative activity Antitumor activity Immunomodulating properties Antiviral activity
Dermatan sulfate	Stabilizing agent Tissue engineering scaffolds	Imaging and treatment of large human tumor metastases
Dextran sulfate	Nanoparticles Drug delivery DNA and insulin delivery Composite nanoparticles	Antiviral activity

1.2.4. Blood coagulation cascade

Hemostasis depends on a dynamic balance between coagulation and fibrinolysis. The coagulation system is poised to rapidly generate a hemostatic plug at sites of vascular injury, thereby limiting blood loss from rents or tears in blood vessels. Intricately connected to coagulation pathways, the fibrinolytic system degrades fibrin, the primary matrix protein of venous or arterial thrombi, into soluble components. Solubilization of thrombi restores blood flow and prevents ischemia in downstream organs and tissues. Perturbation of the balance between coagulation and fibrinolysis can lead to thrombosis or bleeding. Thus, excessive activation of coagulation or impaired fibrinolysis can result in thrombosis, whereas enhanced fibrinolysis can lead to bleeding (Liaw & Weitz, 2006). Since blood clotting is regarded as a chain reaction, this cycle is referred as a reaction cascade (Mestechkina & Shcherbukhin, 2010).

Physiologically, the coagulation cascade requires platelets and plasma factors to seal defects in vessel walls (Renné et al., 2006). It comprises 13 plasma factors, 10 platelet factors, and the factors contained in erythrocytes and leukocytes (Mestechkina & Shcherbukhin, 2010). Coagulation factors are primarily produced by the liver. Factors

II, VII, IX, and X are vitamin K-dependent proteins, which require gamma-glutamyl carboxylation to become biologically active. In addition to the liver, small amounts of protein may be produced in other organs, but the quantities and function of such enzymes remain elusive (Spronk et al., 2003).

The coagulation cascade is divided into two initiating pathways, the tissue factor (TF) (extrinsic) and the contact factor (intrinsic) pathway, which meet in a final common pathway (Quinn & Bellamy, 2006; Wijesekara et al., 2011).

The extrinsic pathway, which is considered to be the physiologic trigger for fibrin formation, is initiated when the plasma protease factor VIIa (FVIIa) forms a complex with the integral membrane protein tissue factor (TF), which is ubiquitously expressed on cells in subendothelial layers of the vessel, but not normally expressed on the quiescent vascular endothelium. The primary substrates of the FVIIa/TF complex are factor X and factor IX. Activity of the FVIIa/TF complex is tightly regulated by the TF pathway inhibitor, which binds to and inactivates FVIIa/TF and activated factor X on membranes (Renné et al., 2006). This pathway is termed 'extrinsic' because it depends on exposure of TF, which is not normally found within the vessel (Liaw & Weitz, 2006).

The intrinsic or 'contact' pathway is initiated when factor XII (FXII) comes into contact with negatively charged surfaces, in a reaction involving high molecular weight kininogen and plasma kallikrein. FXII can be activated by macromolecular constituents of the subendothelial matrix such as glycosaminoglycans and collagens, nucleotides, sulfatides, soluble polyanions or non-physiological materials such as glass or polymers, but the physiologically relevant mechanism for FXII activation is not certain (Renné et al., 2006). This pathway is termed 'intrinsic' because all the necessary coagulation factors are found within the blood (Liaw & Weitz, 2006).

As seen in Figure 1.6, both of extrinsic and intrinsic pathways come to a common pathway at the level of inactivated factor X, which, once activated, forms factor (Parvizi, 2010).

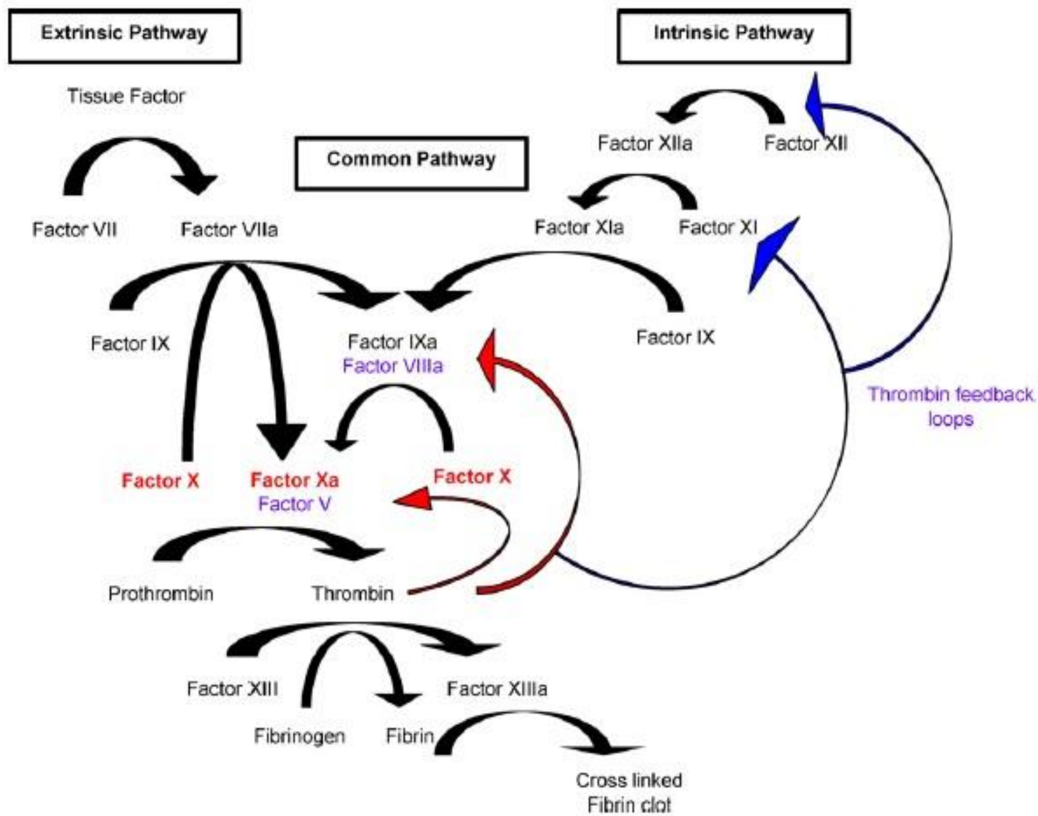

Figure 1.6. The coagulation cascade. F###: blood coagulation factor denoted in Roman numerals. Active forms are denoted by a small 'a' added to the Roman number. FXa is activated by the stepwise activation of coagulation proteinases of both the intrinsic and extrinsic pathway of the coagulation cascade following tissue injury. Activation via the extrinsic pathway is initiated when injury to the vessel wall causes blood components to come into contact with the glycoprotein receptor, tissue factor (TF). At sites of vascular endothelial damage, upregulation of TF expression on extravascular cells allows blood components such as FVII to come into contact with TF which subsequently activates and thus converts FVII to FVIIa. The resulting vitamin K-dependent TF/FVIIa complex binds FX to form the so-called extrinsic tenase complex (TF/FVIIa/FX) which catalyses the conversion of FX to FXa. Activation of the intrinsic pathway mediates FXa activation via the interaction of FXIa, FVIIIa in the presence of calcium ions and acidic phospholipid surfaces (Krupiczojc et al., 2008).

Venous thromboembolism (VT) (the expression of deep vein thrombolism (DVT) and pulmonary embolism (PE) as a single disease) is a multifactorial disease characterized by excessive formation of clots in veins, without occurance of any type of vascular

injury (Mourão & Pereira, 1999; Maas et al., 2012). It affects one in every 20,000 people per year, the risk increasing exponentially with age. Risk factors for the development of thromboembolism are divided into genetic factors and acquired, like immobility, pregnancy, and surgery (Maas et al., 2012). Cardiovascular and cerebrovascular disease has become a major cause of death behind cancer (Ye et al., 2012). Most of thromboembolism patients require anticoagulant therapy for a lifetime.

For more than 70 years, the glycosaminoglycan (GAG) heparin is the drug of choice in the prevention and treatment of thromboembolic disorders (Alban & Franz, 2001). Unfractionated (UF) heparin is a naturally occurring negatively charged highly sulfated water-soluble mucopolysaccharide organic acid that has been found in mast cells in a large number of mammalian and nonmammalian vertebrates (Günther & Ruppert, 2006; Quinn & Bellamy, 2006). It is a heterogeneous mixture of linear polysaccharide chains composed of 15–100 alternating 1→4-linked mucosaccharide units of D-glucosamine and L-iduronic acid or D-glucuronic acid (Günther & Ruppert, 2006; Volpi & Buzzega, 2012) as seen in Figure 1.7. The molecular weight of UF heparin ranges from 5 to 40 kDa with an average of ~14 kDa. Low molecular weight (LMW) heparins are much smaller (mean MW, ~5 kDa) and are produced from UF heparin by chemical or enzymatic depolymerization (Günther & Ruppert, 2006). Different LMWHs not only may have different end groups generated by the specific depolymerization procedures, but may differ from each other also in compositional terms (Casu et al., *in press*). Material for clinical use is derived from bovine lungs or pig intestinal mucosa and is prepared either as UF heparin or depolymerized LMW heparin (Günther & Ruppert, 2006). Enoxaparin is the most widely studied LMW heparin (Nguyen et al., 2010).

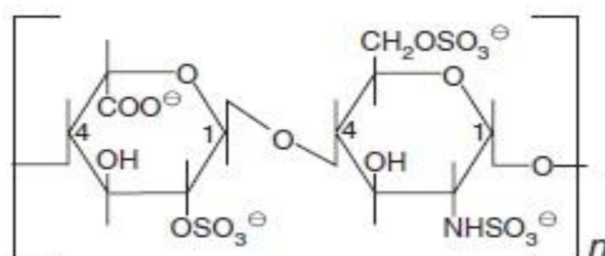

Figure 1.7. Chemical structure of heparin (Günther & Ruppert, 2006).

It is generally accepted that the anticoagulant activity of the sulfated polysaccharides partly results from the strong interaction between the negatively charged sulfate groups and some positively charged peptidic sequences (Huang et al., 2003). Heparin acts as an accelerator of antithrombin (formerly called antithrombin III, ATIII). The pentasaccharide sequence of heparin, which is shown in Figure 1.8, binds to the lysine site in antithrombin inducing a conformational change of the antithrombin molecule, which facilitates binding to specific clotting factors and accelerates the rate at which antithrombin inhibits these factors by approximately 1000 times. While its interactions with proteins are largely electrostatic, there are clearly contributions from hydrophobic effects and hydrogen bonding, as well as the promotion of secondary structure in the proteins binding to heparin, which imparts some selectivity and specificity (Sakiyama-Elbert, 2014). UF heparin inhibits factors Xa, IIa (thrombin), IXa, XIa, and XIIa, with FXa and thrombin representing the most responsive and most critical factors within the clotting cascade. Heparin, antithrombin, and thrombin form a ternary complex in which thrombin initially binds to the heparin-antithrombin complex in a non-specific manner to any site of the heparin molecule and then slides along the surface until it binds to the inhibitor. The affinity of heparin for the antithrombin-thrombin complex is much lower than that for unreacted antithrombin. Thus, heparin can dissociate from the complex and bind to additional unreacted antithrombin molecules, resulting in a continuing anticoagulant effect. The complex, however, is not effective in inhibiting fibrin-bound thrombin (Günther & Ruppert, 2006).

Low molecular weight (LMW) heparins have a relatively specific effect against factor Xa compared with other clotting factors. Both UF and LMW heparins possess the pentasaccharide sequence which binds to ATIII, but only the UF heparins have the adjacent ternary sequence which is necessary for potentiation of the interaction between ATIII and factor IIa. This sequence is not necessary for potentiation of the interaction between ATIII and factor Xa, hence the relative specificity shown by LMW heparins for Xa (Quinn & Bellamy, 2006).

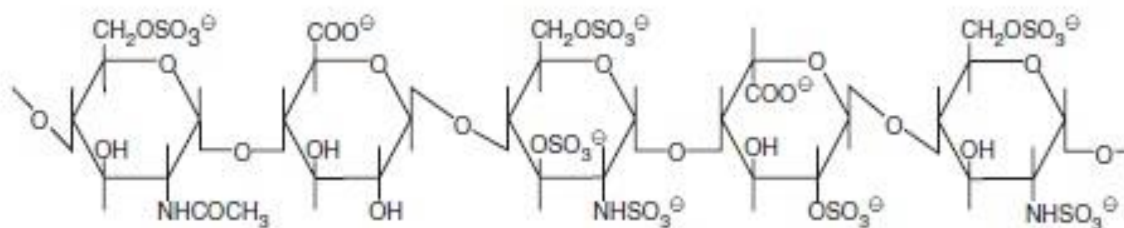

Figure 1.8. The specific pentasaccharide sequence that is responsible for high affinity binding to antithrombin (Günther & Ruppert, 2006).

UF and LMW heparins are the current anticoagulant polysaccharide drugs (Ye et al., 2012). However, the use of heparin as an anticoagulant has limitations due to serious adverse effects that it may entail, such as heparin-induced thrombocytopenia-II (HIT), bleeding, osteporosis, skin rashes, contact dermatits, urticaria and skin necrosis, eosinophilia, among others (Maas et al., 2012; Ye et al., 2012). HIT is caused following the formation of heparin/platelet factor 4 (PF4) complexes (Nguyen et al., 2010). If the patient was occurred by HIT, 51% of patients will be lead to thromboembolic complications and 37% of patients will die (Ye et al., 2012). Some patients are at particular risk for heparin-induced bleeding (e.g. patients who have vitamin K deficiency (antibiotic therapy, liver disease) or who have had recent surgery) (Quinn & Bellamy, 2006). Osteoporosis is a complication of long-term treatment with therapeutic doses of heparin and appears to be the result of heparin binding to osteoblasts with subsequent osteoclast activation (Nguyen et al., 2010). In addition to these limitations, due to its animal origin, biological contamination by animal pathogens is a major concern in heparin therapy (Maas et al., 2012). Still, since LMW heparins have better defined chemical compositions, they provide more predictable anticoagulant dose, longer half-lives and reduced side effects (Liang & Kiick, 2014).

The pharmacochemistry of blood has a large number of well-developed tests for determining anticoagulant activity of anticoagulants via prolongation in clotting time (Wijesekara et al., 2011). The main test are prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and determination of fibrinogen-clotting time of factor Xa (Heptest) (Mestechkina & Shcherbukhin, 2010). Figure 1.9 represents the simplified classic scheme of the coagulation cascade with the anticoagulation tests recording different stages within this process.

- PT measures the integrity of the extrinsic pathway and is performed by adding blood to tissue thromboplastin derived from brain tissue and calcium (Minors, 2007). The time for clotting of normal plasma is approximately 12 to 15 seconds (Liaw & Weitz, 2006).
- APTT is a measure of the integrity of the intrinsic pathway, and is performed by mixing blood with kaolin (which provides an electronegative surface), a phospholipid (as a substitute for PF3) and calcium. The normal APTT is 30–40 seconds (Minors, 2007).
- TT measures the time to clot formation after the addition of thrombin and calcium to citrated plasma (Liaw & Weitz, 2006). The normal TT is 14–16 seconds (Minors, 2007).
- The Heptest, especially developed for heparin, measures the inhibition of factor Xa (Alban & Franz, 2001).

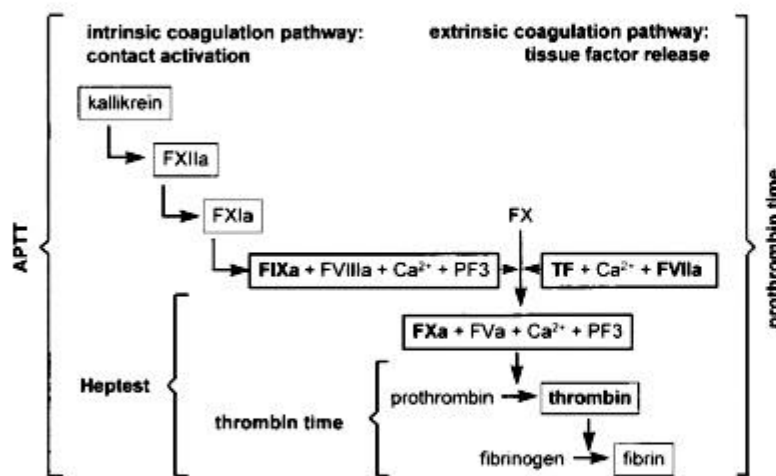

Figure 1.9. Simplified classic scheme of the coagulation cascade with the anticoagulation tests recording different stages within this process (to avoid complexity, the positive feedback loops, and the interactions between the classically differentiated intrinsic and extrinsic pathway are not shown) (Alban & Franz, 2001).

2. MATERIAL AND METHOD

2.1. Materials

2.1.1. Polymer

Pure levan polymer used in this study was obtained from *Halomonas smyrnensis* AAD6^T strain (GenBank accession number DQ131909) which had been isolated from Çamaltı Saltern Area in Turkey (Poli et al., 2009; Poli et al., 2013).

2.1.2. Chemicals

All chemicals and solutions used in this study were supplied by MERCK (Germany), SIGMA (USA), and TOKRA (Turkey). LMW heparin was purchased from Sanofi Aventis under the brand of Clexane that contained enoxaparin as active ingredient. All experiments were carried out in the laboratories of Bioengineering Department and Chemical Engineering Department at Marmara University.

2.1.3. Standard solutions

Solutions Used in DS Analysis

Reagent Solution

Gelatin 2 g

dH_2O 400 ml

After the gelatin was dissolved in 400 ml distilled water (60-70 ^oC), the solution was allowed to stand at +4 ^oC for overnight. Afterwards 2 g $BaCl_2$ was added to the solution.

Cl_3CCOOH solution, 4% (w/v)

Cl_3CCOOH 4 g

dH_2O 100 ml

K_2SO_4 solution, 3 mg/ml

K_2SO_4 0.3 g

dH_2O 100 ml

Solutions Used in Levan Sulfation Process

Na_2CO_3 solution, saturated

Na_2CO_3 40 g
dH_2O 100 ml

2.2. Laboratory Equipment

Analytic Balances (0.1 mg, 1 mg, 10 mg)	Ab204-S, Pg403-S, Pg40002-S (Mettler Toledo, Sweden)
Autoclaves	Mod3870 Elv (Systec, Germany) OT 032 (Nüve, Turkey)
Automatic Pipettor Sets	4500080 (Finipipette, Finland) 4500110 (Finipipette, Finland)
Coagulometer	MT 4C (Tokra, Turkey)
Deepfreezes	-20 °C (Arçelik, Turkey) -20 °C (Heto Holten, Denmark)
Freeze-Dryer	Lyovac GT 2 (Steris, USA)
Heating Magnetic Stirrer	Mr3003s (Heidolph,Germany)
Laminar Flow Cabinet	Class II Safety Cabinet, Safe 2010 0.9 (Heto Holten, Denmark)
Refrigerators	+ 4 °C (Arçelik, Turkey)

Spectrophotometer	UV/Vis, Lambda 35 (Perkin Elmer, USA)
Vortex	Reax Top (Heidolph, Germany)
Water Purification Systems	Primary Grade Water Purification System, Purelab Prima 30 Ultrapure Water System, P.Maxima Ls, (Usf Elga, UK)

2.3. Experimental Methods

2.3.1. Sulfation of levan polymer

To obtain sulfated levan polymer, powdered pure levan was allowed to suspend in pyridine (C_5H_5N) by continuously stirring for two days. Chlorosulfonic acid ($ClSO_3H$) (CSA) was added to the mixture in an ice bath, dropwise. The mixture was allowed to react by continuously stirring at room temperature and the reaction was ended by adding saturated sodium carbonate (Na_2CO_3) solution (1 ml sodium carbonate solution per 1 ml pyridine) to the mixture. The phases were allowed to separate (Figure 2.1) and after one day, the upper phase, which was pyridine, was removed. The remaining polymer phase was obtained as sodium salts. This polymer phase was dialysed against distilled water for 4-5 days to remove sodium salts completely. Then, dry sulfated levan polymer was obtained after freeze-drying.

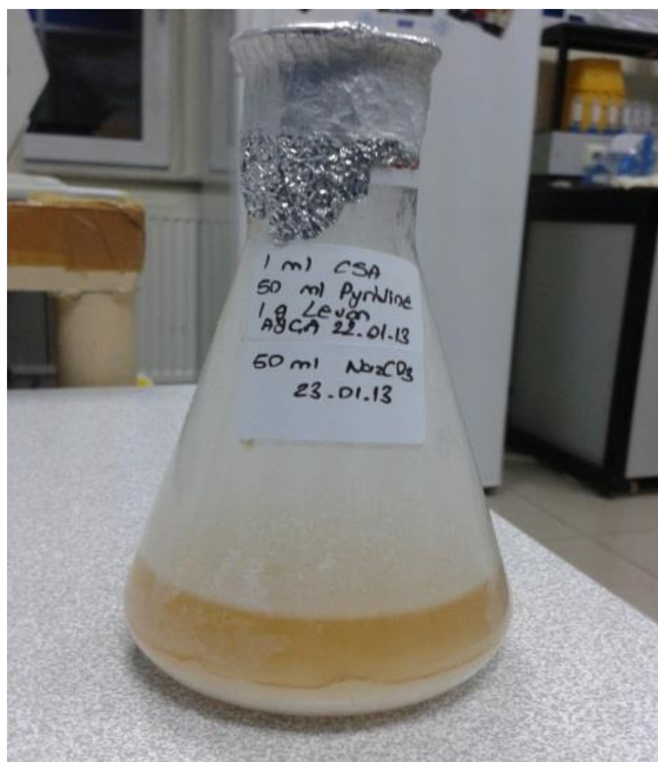

Figure 2.1. Dissociation step of levan sulfation reaction. The upper yellow phase is pyridine phase, and the lower white phase contains the sulfated polymer as sodium salts.

2.3.2. DS analysis

Degree of substitution (DS) is the most important factor for the sulfation process. To assay for the DS, a calibration chart was prepared with potassium sulfate (K_2SO_4) solution according to the Dodgson's (1961) method. For this, two grams of gelatin was dissolved in 400 ml distilled water (60-70 ^oC) and the prepared solution was allowed to stand at +4 ^oC overnight. Then, two grams of barium chloride ($BaCl_2$) was added to the solution, and after three hours, the reagent was ready to be used. 4% trichloroacetic acid (Cl_3CCOOH) (TCA) (w/v) solution was prepared. K_2SO_4 solutions at different concentrations were prepared. 1 ml of reagent, 3.8 ml of TCA solution and 0.2 ml of K_2SO_4 solution dilutions per test tube were mixed and allowed to stand for 15 minutes. A blank solution was prepared with distilled water instead of K_2SO_4 solutions. After 15 minutes, 1 ml of mixture from each tube was taken and the spectrophotometric optical density (OD) measurements were carried out in 1.5 ml plastic cuvettes at 500 nm wavelength against the blank solution.

To assess the DS of levan sulfate samples, levan polymer solutions at different concentrations were prepared. The above-mentioned procedure was followed by using the sample solutions instead of K_2SO_4. A blank solution was prepared with distilled water instead of sulfated levan polymer solution dilutions. Spectrophotometric OD measurements were carried out at 500 nm wavelength against the blank solution.

2.3.3. Blood coagulation tests

Healthy human plasma was used as control plasma in all of the coagulation tests. Sulfated levan anticoagulant with different concentrations within control plasma was referred as sample plasma. Forsaken the mistakes, it was assured that plasma/anticoagulant ratio was 9/1 (v/v) in PT, APTT, and TT tests. LMW heparin (enoxaparin) was regarded as a reference.

2.3.3.1. PT tests

The appropriate application for PT testing was entered to the coagulometer. The mixture of PT reagent and $CaCl_2$ solution in the reagent slot on the instrument were pre-heated for 10 minutes in order to reach 37 ^{o}C prior to the start of the study. Plastic cuvettes were placed into the incubation wells with a metal ball in each. 50 μl of the sample plasma/control plasma to be tested was pipetted into the ball-containing cuvettes in the incubation wells. The plasma-containing cuvette was placed into the measuring well. The incubation timer on the instrument was started and sample/control plasma was incubated for 2 minutes (120 seconds) to reach 37 ^{o}C. Following the audible warning of the instrument upon the completion of the incubation, 100 μl of the PT reagent at 37 ^{o}C was withdrawn using the instrument's pipette. Once the channel where the test was to take place had been activated, the reagent was pipetted into the cuvette. The timer was started to count simultaneously with the pipetting procedure. The timer stopped upon the formation of the clot and thus the test ended. The value that the timer read was recorded on the basis of seconds.

2.3.3.2. APTT tests

The appropriate application for APTT testing was entered to the coagulometer. The $CaCl_2$ solution in the reagent slot on the instrument was pre-heated for 10 minutes in

order to reach 37 ^oC prior to the start of the study. The APTT reagent was kept at room temperature for 10 minutes before start. Plastic cuvettes were placed into the incubation wells with a metal ball in each. 50 µl of the control plasma to be tested was pipetted into the ball-containing cuvettes in the incubation wells. The plasma-containing cuvette was placed into the measuring well and 50 µl of APTT reagent mixture was added into the plasma-containing cuvette. The incubation timer on the instrument was started and sample/control plasma was incubated for 3 minutes (180 seconds) to reach 37 ^oC. Following the audible warning of the instrument upon the completion of the incubation, 50 µl of the $CaCl_2$ solution at 37 ^oC was withdrawn using the instrument's pipette. Once the channel where the test was to take place had been activated, the $CaCl_2$ solution was pipetted into the cuvette. The timer was started to count simultaneously with the pipetting procedure. The timer stopped upon the formation of the clot and thus the test ended. The value that the timer read was recorded on the basis of seconds.

2.3.3.3. TT tests

The appropriate application for TT testing was entered to the coagulometer. The TT reagent was kept at room temperature for 10 minutes before start. Plastic cuvettes were placed into the incubation wells with a metal ball in each. 100 µl of the sample plasma/control plasma to be tested was pipetted into the ball-containing cuvettes in the incubation wells. The incubation timer on the instrument was started and sample/control plasma was incubated for 3 minutes (180 seconds) to reach 37 ^oC. Following the audible warning of the instrument upon the completion of the incubation, 50 µl of the TT reagent at 37 ^oC was withdrawn using the instrument's pipette. Once the channel where the test was to take place had been activated, the reagent was pipetted into the cuvette. The timer was started to count simultaneously with the pipetting procedure. The timer stopped upon the formation of the clot and thus the test ended. The value that the timer read was recorded on the basis of seconds.

2.3.3.4. Heptest

A blank solution was prepared for Heptest by mixing 200 µl acetic acid, 200 µl standard human plasma, 200 µl factor Xa reagent, 200 µl factor Xa substrate, and 200 µl distilled water in a 1.5 ml plastic cuvette.

According to the manifacturer's instructions, a kinetic method was used and a calibration chart was prepared for Heptest. 40 µl of standard human plasma was dissolved in 960 µl distilled water. This solution was assumed as having 100% factor Xa activity. 500 µl of this reference solution was dissolved in 500 µl distilled water to give the second solution with 50% factor Xa activity. By further dilutions, solutions with 25% and 5% factor Xa activities were prepared. For each of these solutions, 200 µl solution was added in a plastic cuvette, and the cuvette was incubated at 37 ^{o}C for one minute in the incubation wells of the coagulometer. 200 µl factor Xa reagent was pipetted to the cuvette, and the cuvette was incubated at 37 ^{o}C for another one minute. After adding 400 µl distilled water and 200 µl factor Xa substrate to the cuvette, the spectrophotometer was started to take measurements at 405 nm wavelength immediately. The change of absorbance was recorded for 5 minutes against the blank solution.

100 µl standard human plasma and 100 µl sulfated levan anticoagulant solution at different concentrations were added in a plastic cuvette, and the cuvette was incubated at 37 ^{o}C for one minute in the incubation wells of the coagulometer. 200 µl factor Xa reagent was pipetted to the cuvette, and the cuvette was incubated at 37 ^{o}C for another one minute. After adding 400 µl distilled water and 200 µl factor Xa substrate to the cuvette, the spectrophotometer was started to take measurements at 405 nm wavelength immediately. The change of absorbance was recorded for 5 minutes against the blank solution.

3. RESULTS AND DISCUSSION

Sulfated polysaccharides have many biological activities such as anticoagulant, antiviral, antifungal activities. Sulfated polysaccharides can exist naturally, or can be obtained by modifying with sulfating agents. Strength of biological activities of a given sulfated polysaccharide takes form according to its DS value. Thus, the knowledge of DS value is essential for sulfated polysaccharides.

3.1. DS Analysis Results

To determine DS, a calibration chart was prepared with K_2SO_4 solutions at different concentrations. $BaSO_4$ was the resulting compound in the solutions. OD results at 500 nm wavelength are shown in Table 3.1.

Table 3.1. OD values for given K_2SO_4 concentrations at 500 nm wavelength.

K_2SO_4 (µg/ml)	SO_4^{-2} (µg)	OD (500 nm)
30	16.5327	0.2591
60	33.0654	0.2532
90	49.5981	0.3202
120	66.1308	0.3119
150	82.6636	0.3400
180	99.1963	0.4152
210	115.7290	0.4611
240	132.2617	0.4887
270	148.7944	0.5051
330	181.8599	0.6043
360	198.3926	0.6258
390	214.9253	0.6533

SO_4^{-2} amounts in 1 ml solution were calculated according to the following equation:

$$m_{SO_4^{-2}}(\mu g) = \frac{MW_{SO_4^{-2}}(g/mol) * m_{K_2SO_4}(\mu g)}{MW_{K_2SO_4}\,(g/mol)} \quad (3.1)$$

$MW_{K_2SO_4}$: Molecular weight of K_2SO_4 (174.2 g/mol)

$MW_{SO_4^{-2}}$: Molecular weight of SO_4^{-2} (96 g/mol)

$m_{K_2SO_4}$: K_2SO_4 mass in 1 ml solution (equal to concentration)

$m_{SO_4^{-2}}$: SO_4^{-2} mass in 1 ml solution

$$m_{SO_4^{-2}} = \frac{96 * m_{K_2SO_4}}{174.2}$$

(3.2)

OD-SO_4^{-2} calibration graph (see **Appendix A**) gave an equation that would be used in the next step:

$$OD = 0.0022(SO_4^{-2}) + 0.1958$$

(3.3)

DS values were calculated according to the following equations:

$$S\% = \frac{BaSO_4(\mu g) * 0.1374 * 100}{Sample(\mu g)}$$

(3.4)

$$DS = \frac{162 * S\%}{3200 - (102 * S\%)}$$

(Mendes et al., 2009) (3.5)

S%: Sulfur percent

Sample: Sulfated polysaccharide amount in the sample solution

$BaSO_4$: Resulting $BaSO_4$ complex amount in the sample solution

$BaSO_4$ amounts in the sample solutions were calculated according to the following equation:

$$BaSO_4(\mu g) = \frac{MW_{BaSO_4}(g) * {SO_4}^{-2}(\mu g)}{MW_{{SO_4}^{-2}}(g)}$$

(3.6)

MW_{BaSO_4}: Molecular weight of $BaSO_4$ (233 g/mol)

$$BaSO_4 = \frac{233 * {SO_4}^{-2}}{96}$$

(3.7)

3.2. Sulfation of Levan Polymer Results

Different levan amounts, different CSA volumes and different reaction times were tested to get sulfated levan polymer with different DS values. As a result, 11 different sulfated levan derivatives were obtained.

200 mg levan was subjected to 100 µl CSA for 30 minutes. After all the synthesis steps, 42 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.2, average DS was found **0.19**.

Table 3.2. DS results of sulfated levan derivative (1st experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
3.25	0.3381	0.19
2.4375	0.3015	0.19
1.625	0.2701	0.20

200 mg levan was subjected to 200 µl CSA for 30 minutes. After all the synthesis steps, 52 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.3, average DS was found as **0.19**.

Table 3.3. DS results of sulfated levan derivative (2nd experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
5	0.396	0.19
3.75	0.3585	0.19
2.5	0.3045	0.19

1 g levan was subjected to 1 ml CSA for 2 hours. After all the synthesis steps, 217 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. Further calculations were done according to DS determination results. According to Table 3.4, average DS was found to be **0.28**.

Table 3.4. DS results of sulfated levan derivative (3rd experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
5	0.4948	0.29
3.75	0.4263	0.28
2.5	0.3515	0.28

1 g levan was subjected to 1.5 ml CSA for 24 hours. After all the synthesis steps, 341 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.5, average DS was found to be **0.69**.

Table 3.5. DS results of sulfated levan derivative (4th experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
5	0.8043	0.67
3.75	0.6752	0.71
2.5	0.5114	0.70

5 g levan was subjected to 5 ml CSA for 24 hours. After all the synthesis steps, 965 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.6, average DS was found to be **0.48**.

Table 3.6. DS results of sulfated levan derivative (5th experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
5	0.6689	0.47
3.75	0.57	0.50
2.5	0.4374	0.48

5 g levan was subjected to 7.5 ml CSA for 24 hours. After all the synthesis steps, 843 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.7, average DS was found to be **0.44**.

Table 3.7. DS results of sulfated levan derivative (6th experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
1.25	0.3029	0.44
0.9375	0.2775	0.42
0.625	0.2527	0.45

5 g levan was subjected to 5 ml CSA for 24 hours. After all the synthesis steps, 916 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.8, average DS was found **0.50**. This sulfated derivative named as **SL1**.

Table 3.8. DS results of sulfated levan derivative (SL1) (7th experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
4	0.5926	0.50
3	0.4954	0.50
2	0.3953	0.50

5 g levan was subjected to 7.5 ml CSA for 24 hours. After all the synthesis steps, 1.12 g polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.9, average DS was found **0.89**. This sulfated derivative named as **SL2**.

Table 3.9. DS results of sulfated levan derivative (SL2) (8th experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
2.25	0.5297	0.89
1.875	0.471	0.87
1.5	0.4211	0.90

5 g levan was subjected to 7.5 ml CSA for 24 hours. After all the synthesis steps, 1.45 g polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.10, average DS was found **2.37**. This sulfated derivative named as **SL3**.

Table 3.10. DS results of sulfated levan derivative (SL3) (9th experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
1.65	0.6043	2.36
1.5	0.5651	2.33
1.35	0.5332	2.42

The sulfated derivative of levan with 0.89 DS was resulfated via CSA. 500 mg sulfated levan was subjected to 750 µl CSA for 24 hours. After all the synthesis steps, 123 mg polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.11, average DS was found **2.72**.

Table 3.11. DS results of sulfated levan derivative (10th experiment).

Sulfated Levan (mg/ml)	OD (500 nm)	DS
2.25	0.7832	2.74
1.95	0.7047	2.71
1.8	0.6619	2.71

5 g levan was subjected to 7.5 ml CSA for 24 hours. After all the synthesis steps, 1.69 g polymer was recovered. OD was measured for different polymer concentrations at 500 nm. Further calculations were done according to DS determination results. According to Table 3.12, average DS was found **2.43**.

Table 3.12. DS results of sulfated levan derivative (11th experiment).

Sulfated Levan (mg/ml)	OD (500nm)	DS
2.25	0.7602	2.44
1.875	0.6632	2.40
1.5	0.5725	2.45

The low polymer yields can be attributed to the difficulties associated with the recovery of the aqeuous polymer phase from the organic pyridine solvent phase. Despite prolonged incubations at room temperature, no clear separation could be obtained.

Table 3.13. Summary of levan sulfation results.

Levan (g)	CSA (ml)	Reaction Time	DS
0.2	0.1	30 min	0.19
0.2	0.2	30 min	0.19
1	1	2 h	0.28
1	1.5	24 h	0.69
5	5	24 h	0.48
5	7.5	24 h	0.44
5	5	24 h	0.50[a]
5	7.5	24 h	0.89[b]
5	7.5	24 h	2.37[c]
0.5[d]	0.75	24 h	2.72
5	7.5	24 h	2.43

a: SL1, b: SL2, c: SL3, d: Sulfated levan with 0.89 DS.

Reaction time is a major parameter for sulfation process. Table 3.13 shows that when the DS was only 0.19 for 30 minutes of reaction time, it increased up to 0.28 when the reaction time was extended to 2 hours, and it increased up to 0.50 when the reaction time was extended to 24 hours for the same levan:CSA ratio. The DS value of a chemically sulfated polysaccharide depends on the CSA volume which is used in its synthesis process. Higher CSA volume gives higher DS. At the same time, it could be said that higher amounts of polymer and higher volumes of CSA may result in higher DS.

3.3. Results of Blood Coagulation Tests

Heparin is the most used polysaccharide anticoagulant because of its high inhibition efficiency on blood coagulation factors. However, it is proven that many sulfated polysaccharides (natural or synthesized) have anticoagulant activity just because they have sulfate groups in their structures (Table 1.3). Sulfated levan could be one of these anticoagulant polysaccharides, so *in vitro* blood coagulation tests were carried out. Three sulfated levan derivatives (SL1, SL2, and SL3) were used in blood coagulation tests.

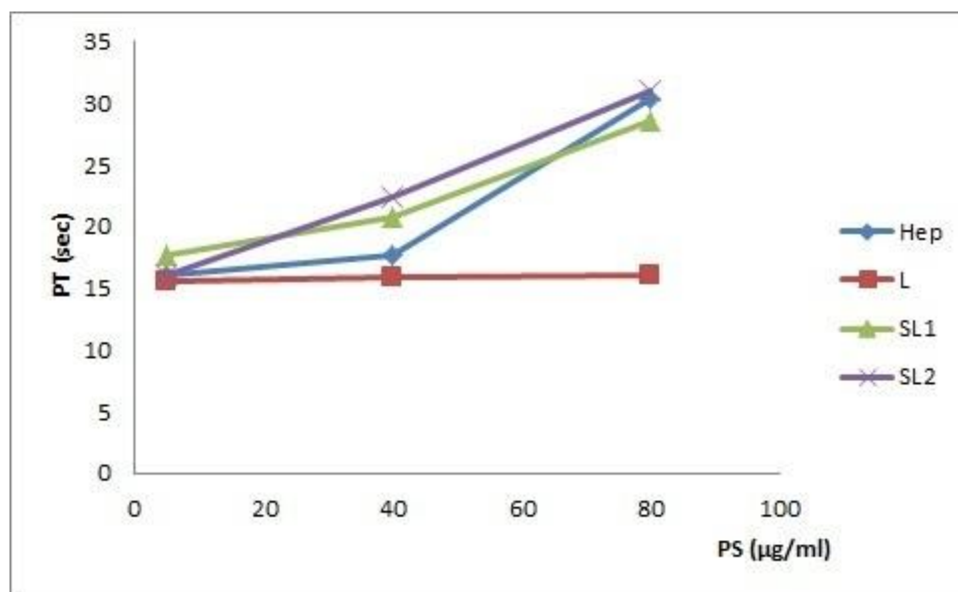

Figure 3.1. PT results for Heparin (Hep), L (pure levan), SL1, and SL2.

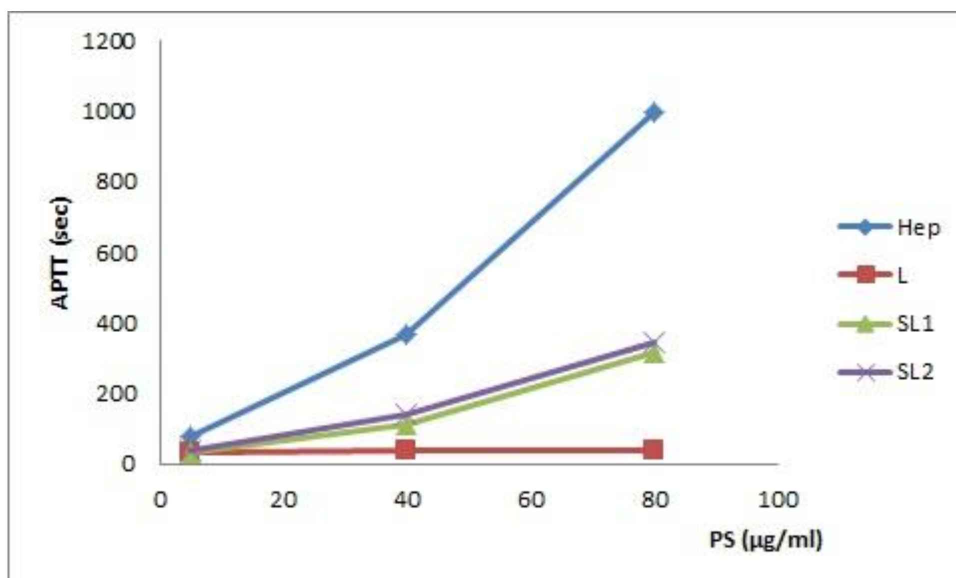

Figure 3.2. APTT results for Heparin (Hep), L (pure levan), SL1, and SL2.

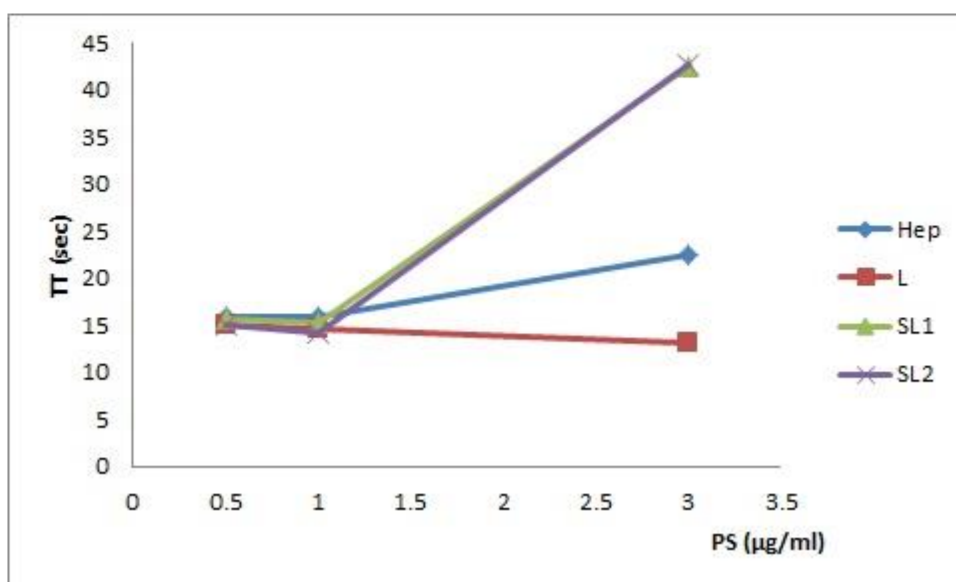

Figure 3.3. TT results for Heparin (Hep), L (pure levan), SL1, and SL2.

As shown in the Figure 3.1, Figure 3.2, and Figure 3.3, pure levan has no anticoagulant activity while heparin, SL1, and SL2 have. Also, the results suggest that sulfated levan with higher DS has higher anticoagulant activity. Moreover, the results suggest that sulfated levan polymer has its inhibition effect on the **intrinsic** blood coagulation

pathway in consequence of high APTT prolongation while PT is almost stable. However, heparin uses the same inhibition pathway according to these results.

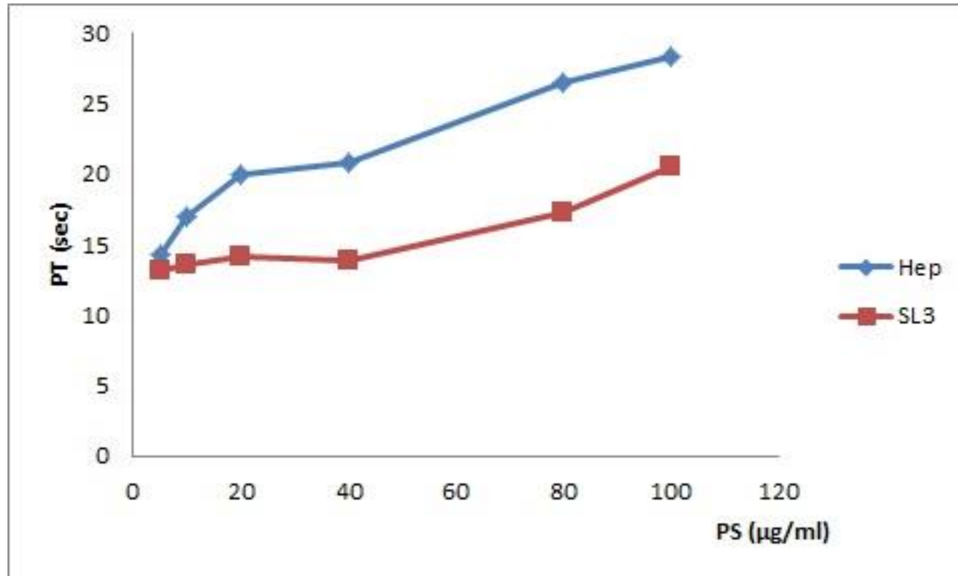

Figure 3.4. PT results for Heparin (Hep), and SL3.

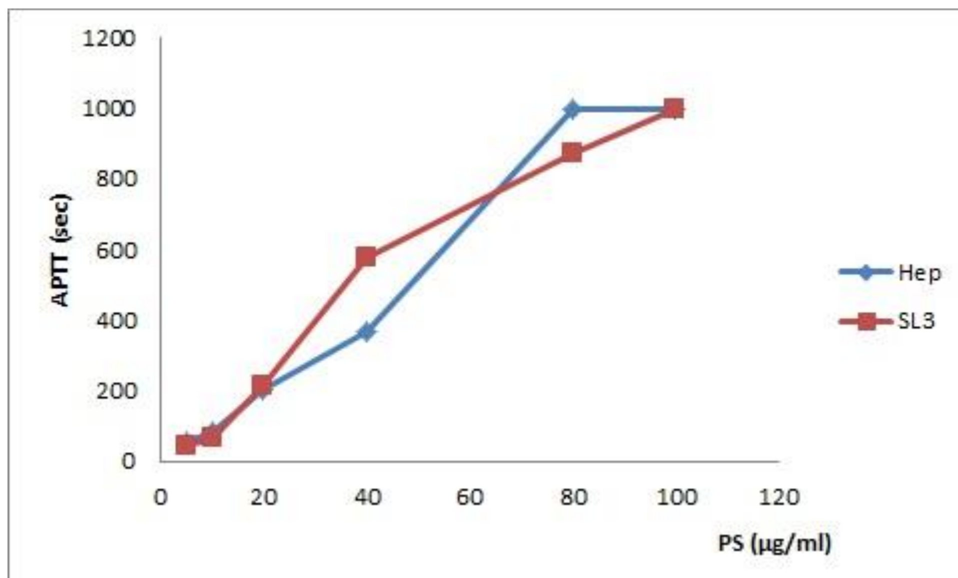

Figure 3.5. APTT results for Heparin (Hep), and SL3.

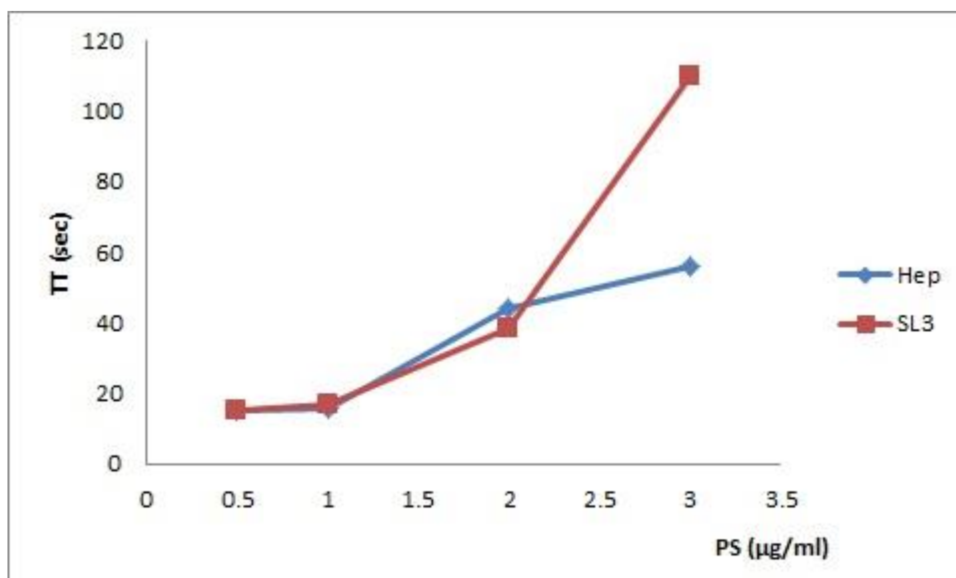

Figure 3.6. TT results for Heparin (Hep), and SL3.

SL3 has the highest DS among SL1, SL2, and SL3, so it has the highest blood clot inhibition effect. As shown in the Figure 3.4, Figure 3.5, and Figure 3.6, SL3 has nearly the same activity effect as heparin.

Lasiodiplodan (an extracellular β-glucan) from fungus *Lasiodiplodia theobromae* was investigated by Vasconcelos et al. (2013). In the study, lasiodiplodan was sulfated chemically with CSA and DS was found to be 0.95. APTT assay results for sulfated lasiodiplodan showed that 3 µg/ml of heparin was equal to 20 µg/ml sulfated lasiodiplodan.

A highly sulfated chitosan, which was sulfated Py·SO_3 complex and with a DS above 2.0, was investigated by Yang et al. (2013). APTT assay results showed that 80 µg/ml of highly sulfated chitosan was equal to 15 µg/ml of heparin.

A polysaccharide obtained from green algea *Enteromorpha linza* and named as LEP was studied by Wang et al. (2013). The polysaccharide was sulfated with CSA and its DS was 0.90. APTT assay results revealed that 100 µg/ml of sulfated LEP polysaccharide was equal to 5 µg/ml of heparin.

A naturally sulfated polysaccharide obtained from green seaweed *Codium vermilara* (Bryopsilades) and named as Ab1 was investigated by Fernández et al. (2013). APTT

assay results showed that 5.0 μg/ml of Ab1 and 0.65 μg/ml of heparin were the equivalent concentrations that prolonged APTT for the same time period.

A novel glysosaminoglycan-like polysaccharide (named as AAP) from abalone *Haliotis discus hannai* Ino was studied by Li, G. et al. (2011). This research showed that 64 μg/ml of AAP could not reach the APTT prolongation that heparin had at 16 μg/ml.

A naturally sulfated polysaccharide, which was named as PML, from green algea *Monostroma latissimum* was researched by Li, H. et al. (2011). In conclusion of APTT assays, it revealed that PML at 16 μg/ml had a bit longer APTT than heparin had at 16 μg/ml.

An extracellular polysaccharide from edible mushroom *Pleurotus sajor-caju* was studied by Telles et al. (2011). The polysaccharide was sulfated with sulfuric acid and its DS was found to be 0.17. Blood clotting assays revealed that this chemically sulfated polysaccharide did not show any anticoagulant activity.

Arivuselvan et al. (2011) investigated a naturally sulfated polysaccharide from brown seaweed *Turbinaria ornata*. 1000 μg/ml of the polysaccharide could not reach the APTT prolongation that heparin reached at 100 μg/ml.

Botryosphaeran exopolysaccharide from the fungus *Botryosphaeria rhodina* MAMB-05 was studied by Mendes et al. (2009). The polysaccharide sulfated two times with CSA to get higher DS value and its final DS was 0.80. APTT assay results revealed that 5 μg/ml of resulfated botryosphaeran was equal to 1 μg/ml of heparin.

Mao et al. (2009) investigated a naturally sulfated polysaccharide from green algea *Monostroma latissimum* (Chlorophyta). In conclusion of APTT assays, it appeared that double dose of sulfated polysaccharide was needed to reach the same APTT prolongation that heparin reached at one dose.

Taken together, these examples suggest that the anticoagulant activity of almost every sulfated polysaccharide (naturally or chemically sulfated) is weaker than that of SL3, and higher concentrations are required to achieve the same effect as that of SL3 in the APTT assays.

According to given examples, heparin:sulfated polysaccharide ratios for the same APTT prolongation vary from 1:1 to 1:20. The ones with 1:1 ratio are replaceable with heparin, and for SL3 polysaccharide, this ratio is about 1:1.

To determine % factor Xa activity, a calibration chart was prepared with the reference plasma amounts (see **Appendix B**). Determined clotting rates for given factor Xa activities at 405 nm wavelength are shown in Table 3.14 and Figure 3.7.

Table 3.14. Clotting rates for given factor Xa activities.

Factor Xa Activity (%)	Clotting Rate (min^{-1})
5	0.0041
25	0.0831
50	0.2024
100	0.5085

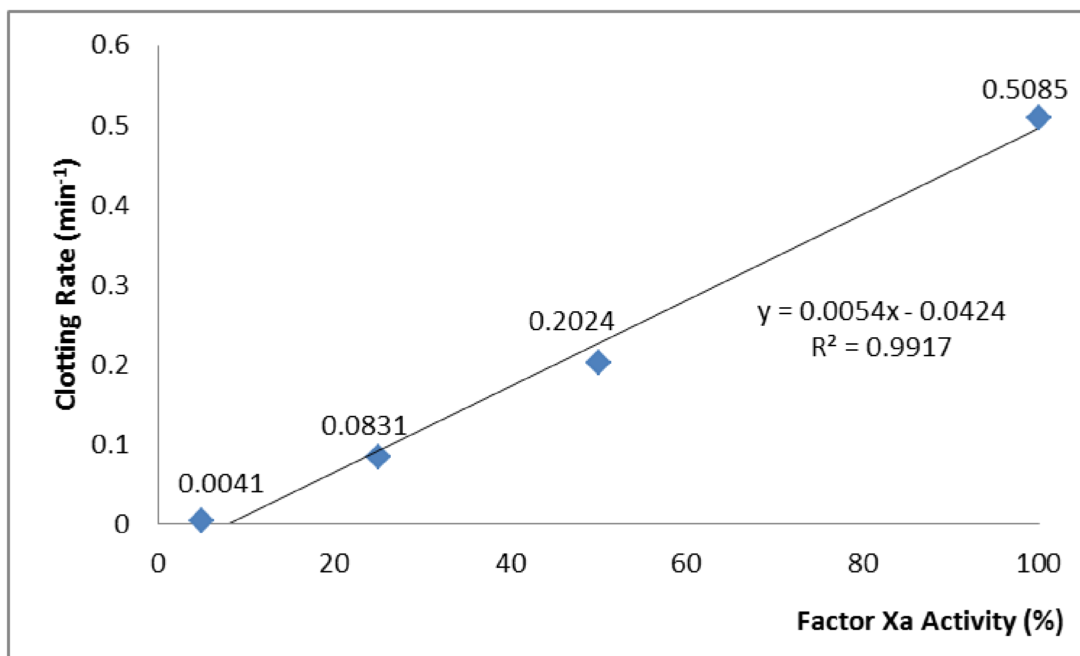

Figure 3.7. Factor Xa Activity - Clotting Rate graph.

According to Figure 3.7:

$$y = 0.0054x - 0.0424 \tag{3.8}$$

means;

$$\text{Clotting Rate (min}^{-1}) = [0.0054*\text{Factor Xa Activity (\%)}] - 0.0424 \tag{3.9}$$

Factor Xa activities for given SL3 concentrations are calculated according to this formula:

$$\text{Factor Xa Activity (\%)} = \left[\frac{\text{Clotting Rate}_{(SL3)}(\text{min}^{-1}) + 0.0424}{0.0054}\right] * 2 \tag{3.10}$$

Table 3.15. Clotting rates and factor Xa activities for given SL3 concentrations (see **Appendix C**).

SL3 (µg/ml)	Clotting Rate (min^{-1})	Factor Xa Activity (%)
0.01	0.1392	67.2593
1	0.1464	69.9259
100	0.1261	62.4074
250	0.0423	31.3704
375	0.0459	32.7037
500	0.0260	25.3333
1000	0.0263	25.4444

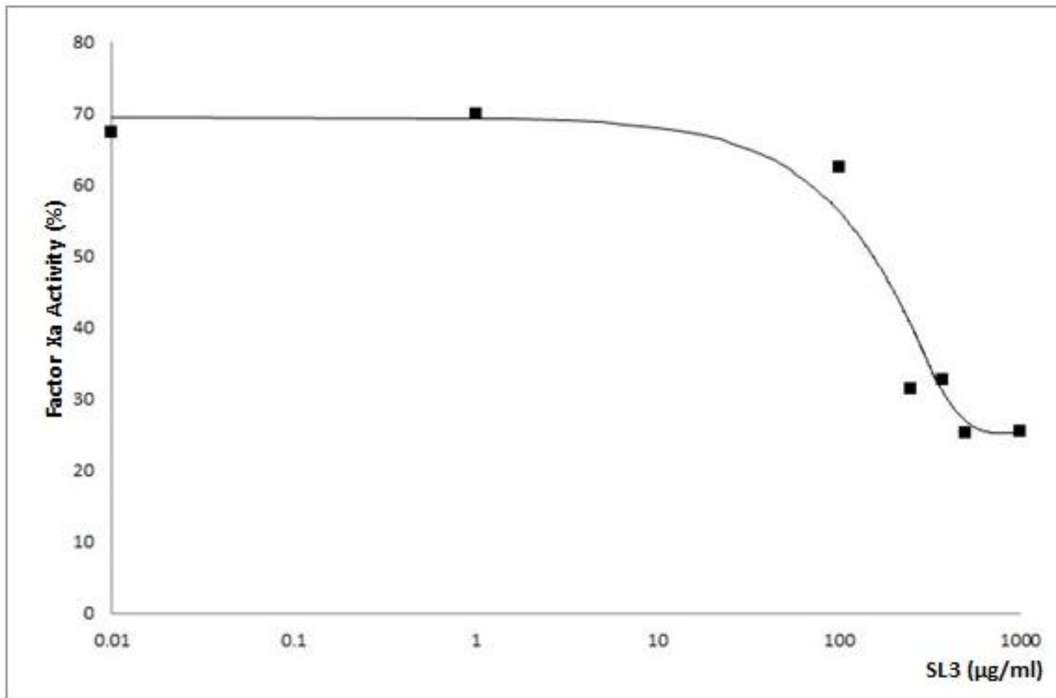

Figure 3.8. SL3 - Factor Xa Activity graph.

In consequence of Heptest, it reveals that sulfated levan inhibits factor Xa **directly** like heparin does and this is a major feature for an anticoagulant. As seen in Table 3.15, clotting rate and factor Xa activity are dose-dependent and higher dose of SL3 results less clotting rate and less factor Xa activity. Figure 3.8 shows that SL3 can reduce factor Xa activity down to 25%.

4. CONCLUSIONS AND RECOMMENDATIONS

Within the scope of this study, the levan polysaccharide produced by the *Halomonas smyrnensis* cultures was chemically modified to integrate sulfate moieties into its structure. Although the presence of sulfate groups was analytically verified by the Dodgson's (1961) method, the final structure of the polymers should be elucidated by using more precise methods like FT-IR or NMR.

In the light of this study, several recommendations can be given for the further works on sulfated levan polysaccharide. Since no sulfated levan studies have been reported on the anticoagulant activity, this thesis constitutes an important step toward understanding the sulfated levan activity on the blood coagulation cascade and its possible applications.

Future researches should concentrate on the investigation of effect of sulfated levan polymer on blood coagulation mechanism *in vivo*. Since it is possible that sulfated levan acts *in vivo* different from *in vitro*, more information on anticoagulant activity of sulfated levan would help us to establish a greater degree of accuracy on this matter.

In this thesis, *in vitro* blood coagulation assays resulted that sulfated levan inhibits blood clots as strong as heparin. But, heparin can lead to some bleeding problems for some patients. Further investigations on the optimum dose of sulfated levan polymer to be taken by patients is required in order to eliminate the particular risk for sulfated levan-induced bleeding.

Sulfated polysaccharides have many biological activities other than anticoagulant activity. In addition to all these recommendations, other biological activities of sulfated levan should be studied like anticancer, antioxidant, or antiviral activities.

This study may be used as a reference material by biomaterial and pharmaceutical industries that produce drugs, or biomedical implements for human welfare. The potential of this sulfated polysaccharide in various pharmaceutical or biomaterial applications need to be investigated and clarified.

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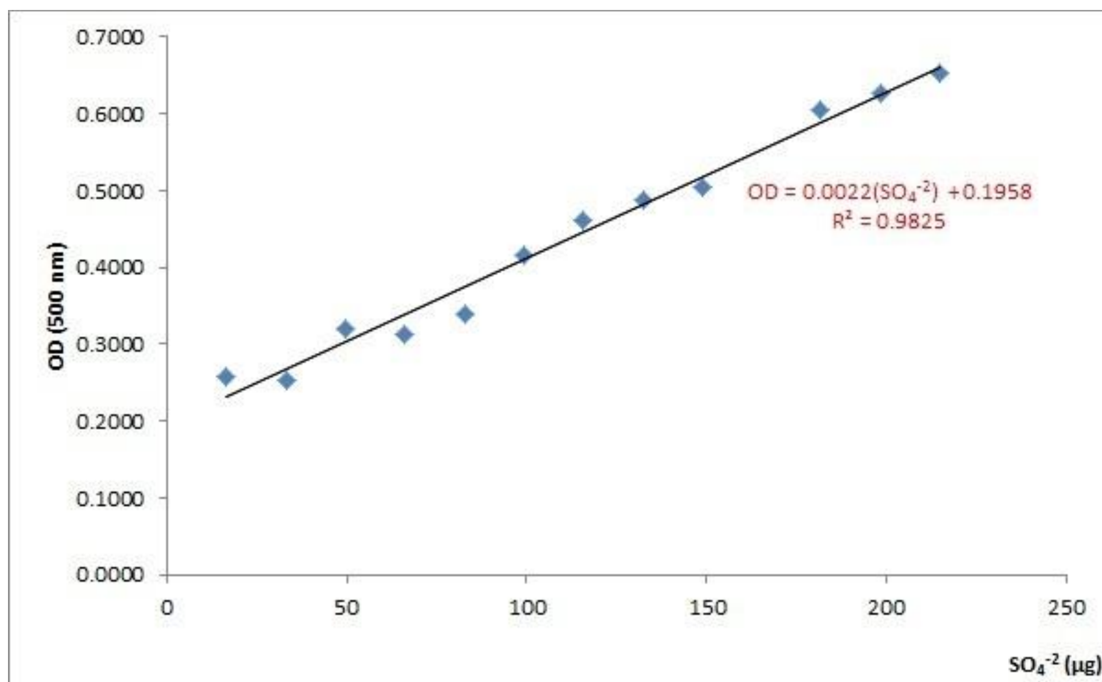
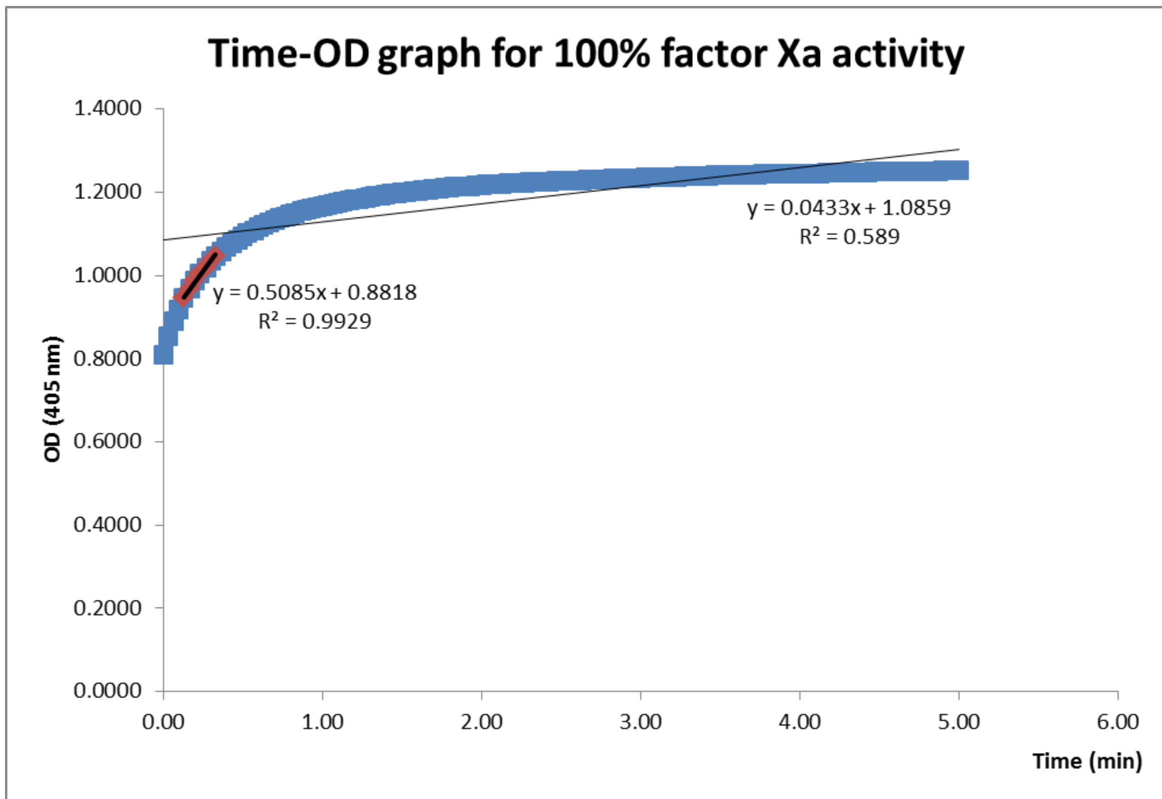
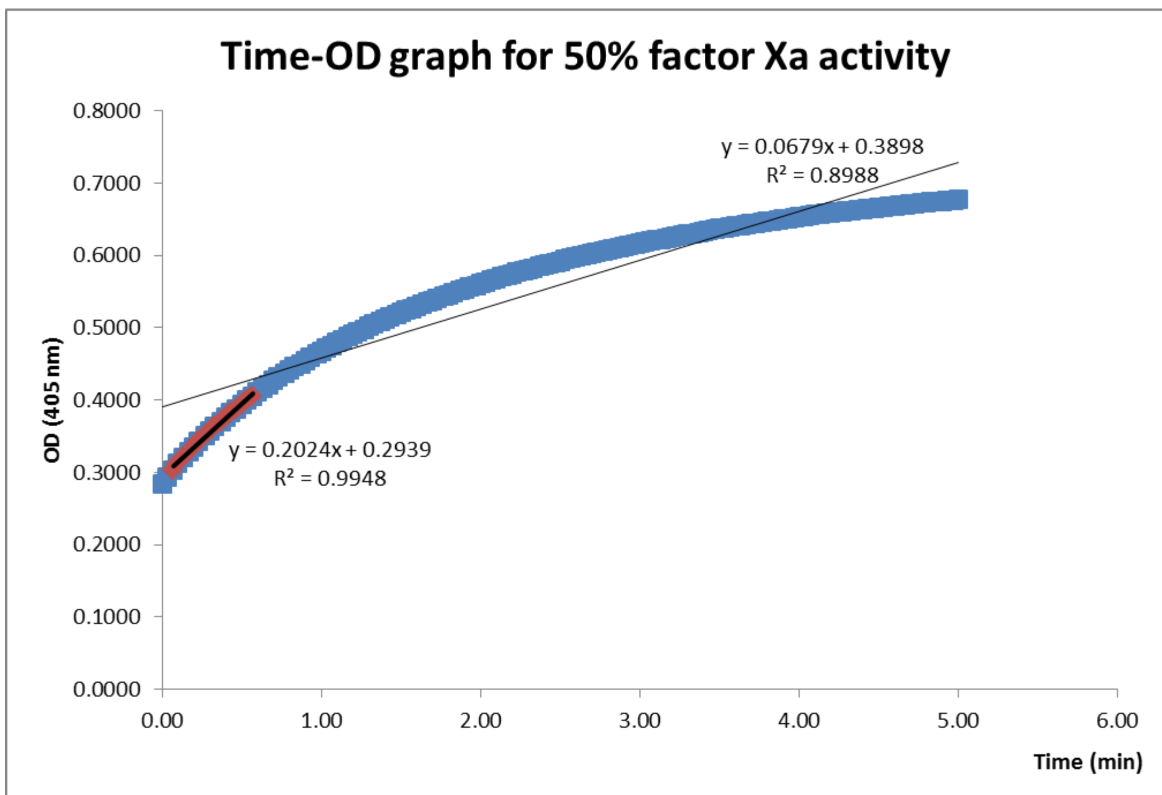
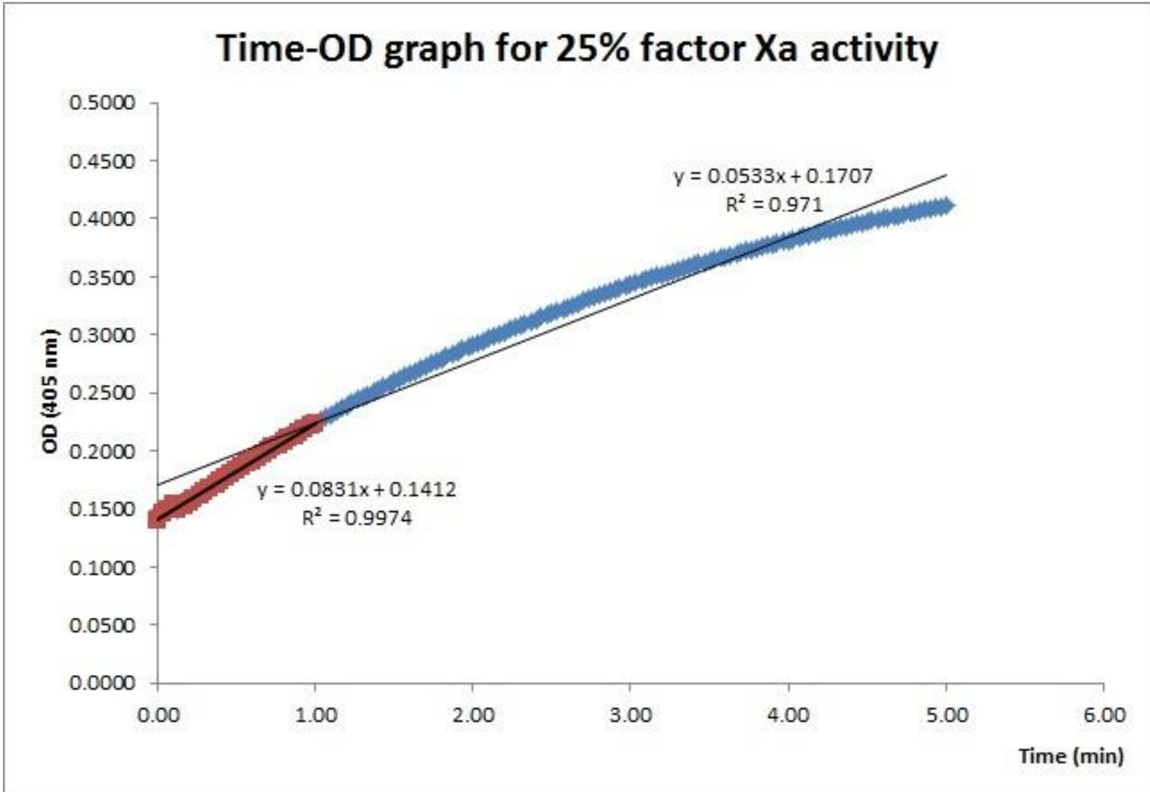
APPENDIX A

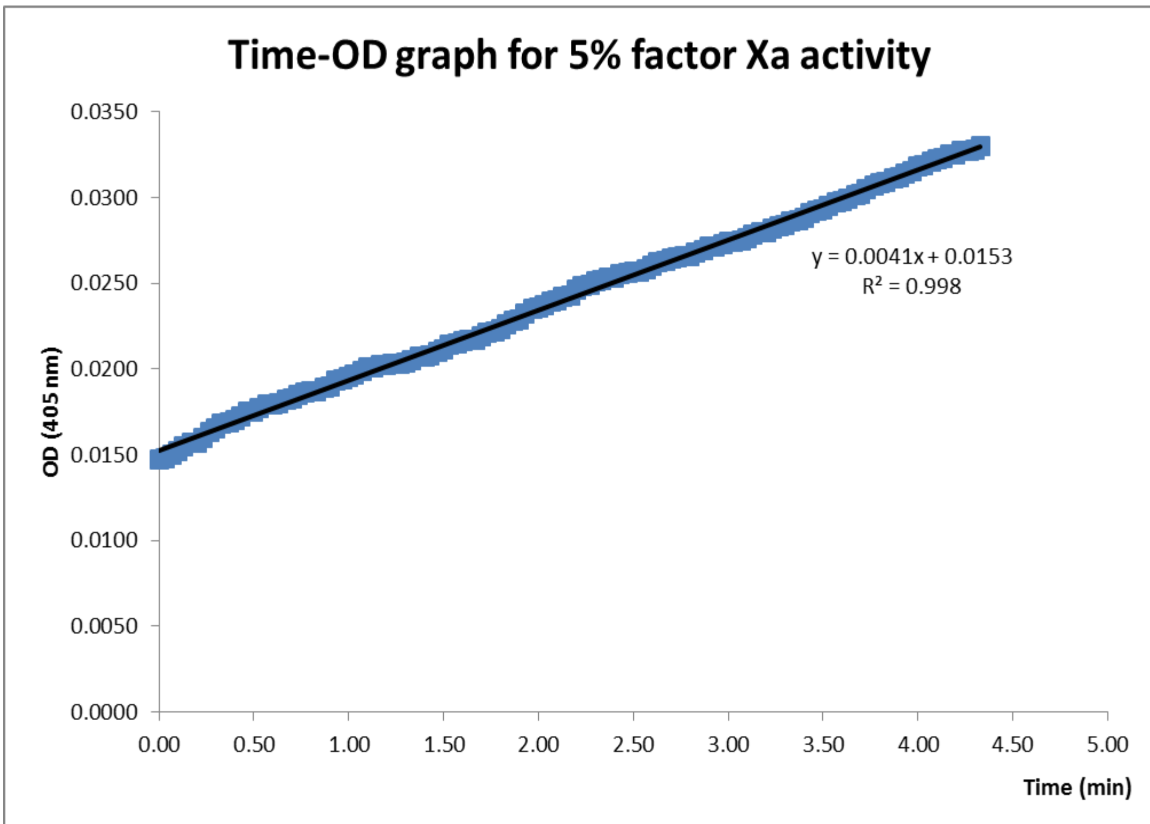


APPENDIX B

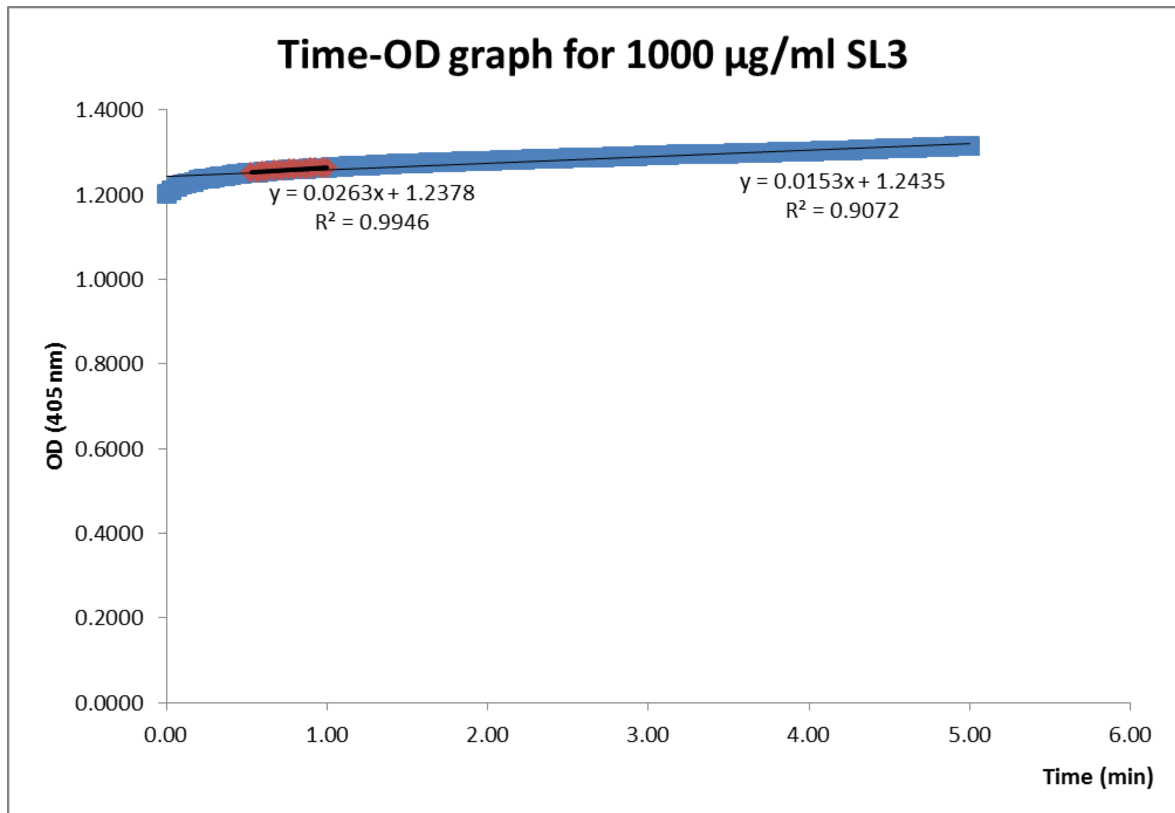


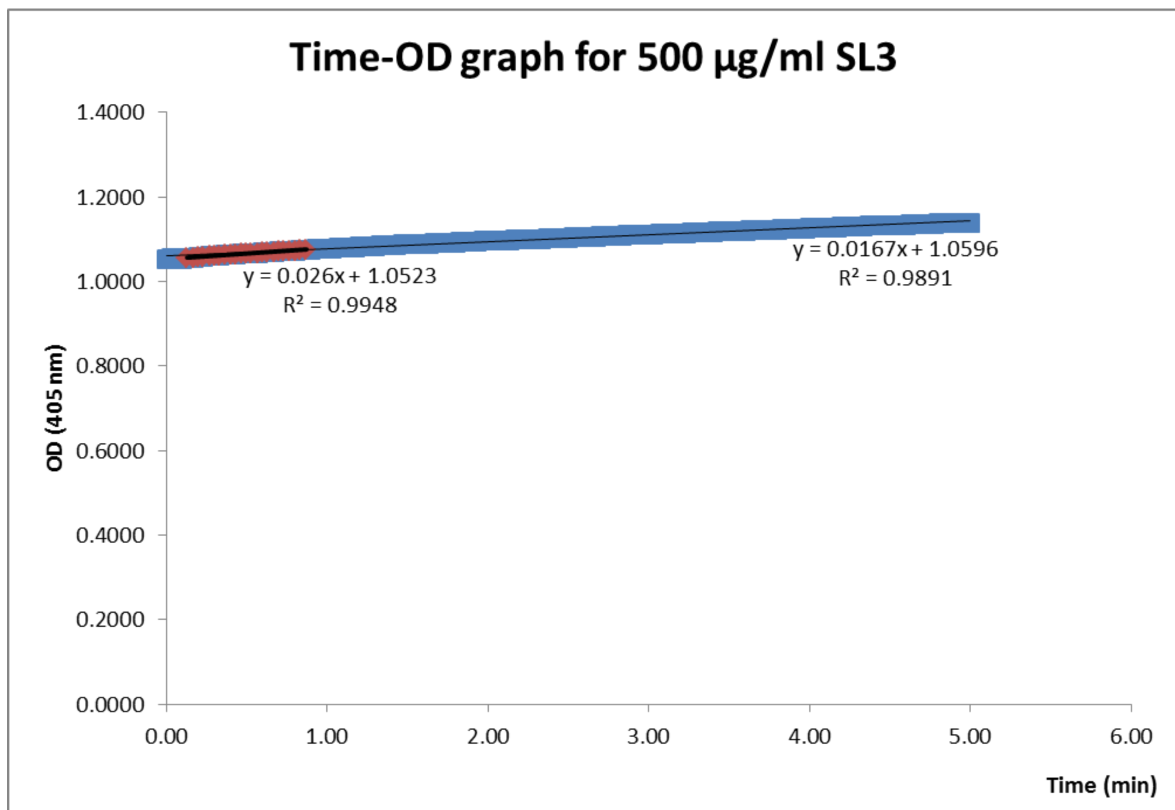


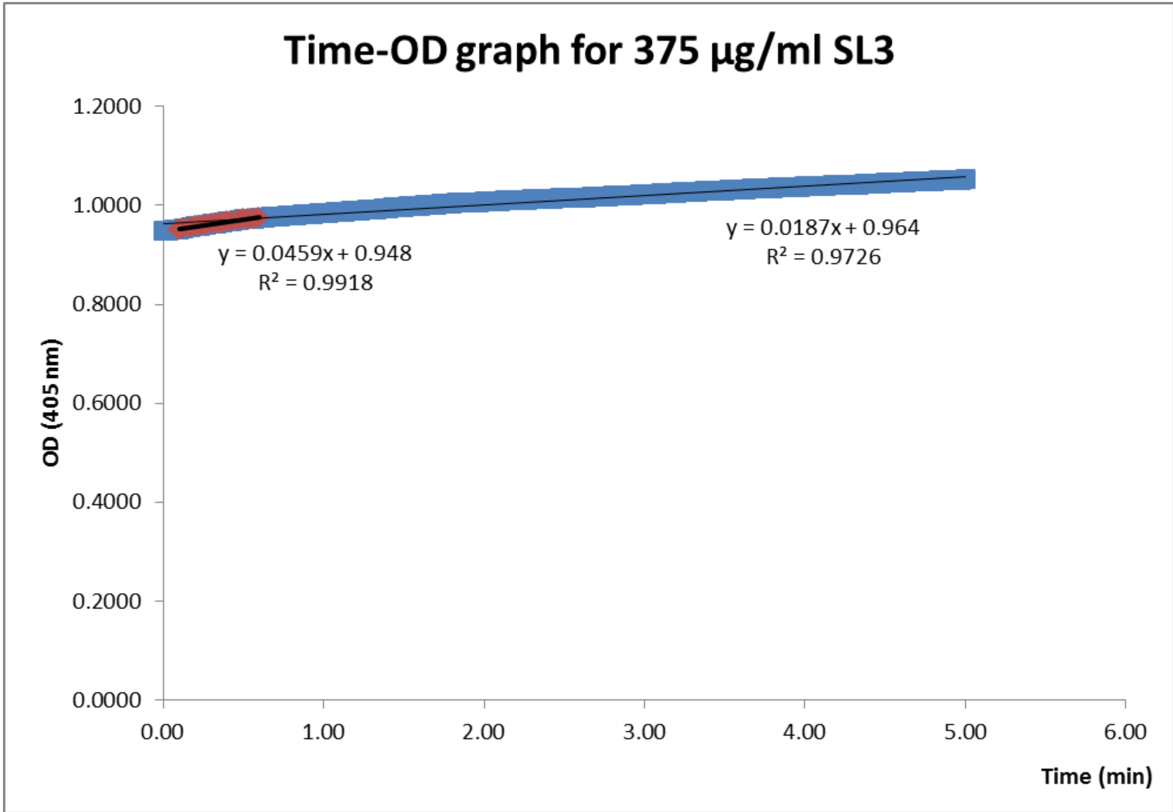

Time-OD graph for 25% factor Xa activity
OD (405 nm)
0.5000
0.4500
0.4000
0.3500
0.3000
0.2500
0.2000
0.1500
0.1000
0.0500
0.0000
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Time (min)
y = 0.0533x + 0.1707
R² = 0.971
y = 0.0831x + 0.1412
R² = 0.9974

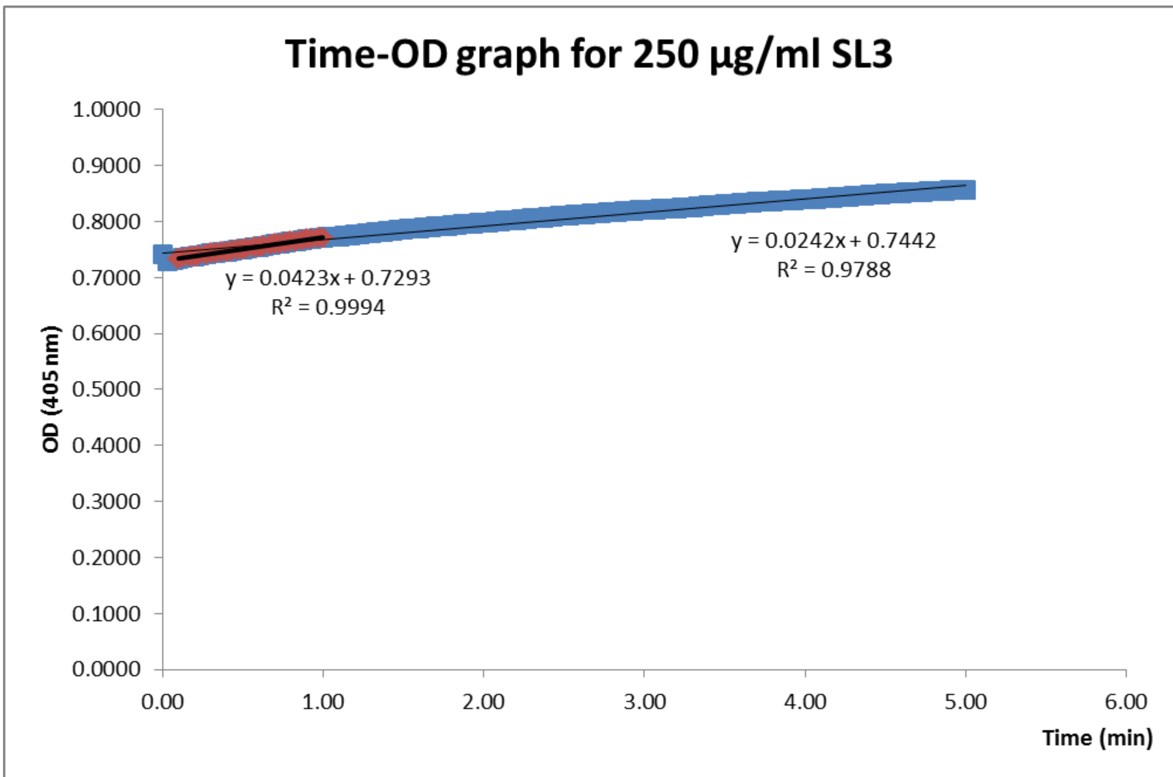

Time-OD graph for 5% factor Xa activity
OD (405 nm)
0.0350
0.0300
0.0250
0.0200
0.0150
0.0100
0.0050
0.0000
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
Time (min)
y = 0.0041x + 0.0153
R² = 0.998

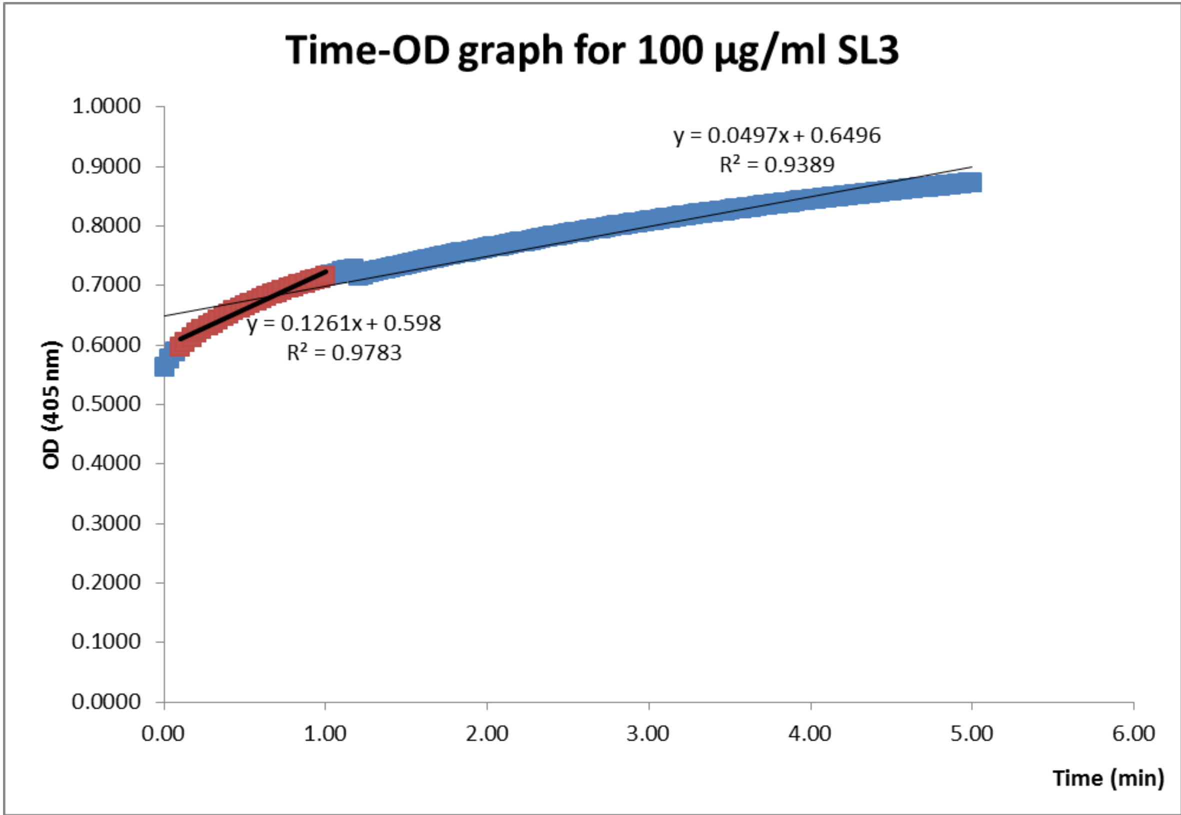
APPENDIX C

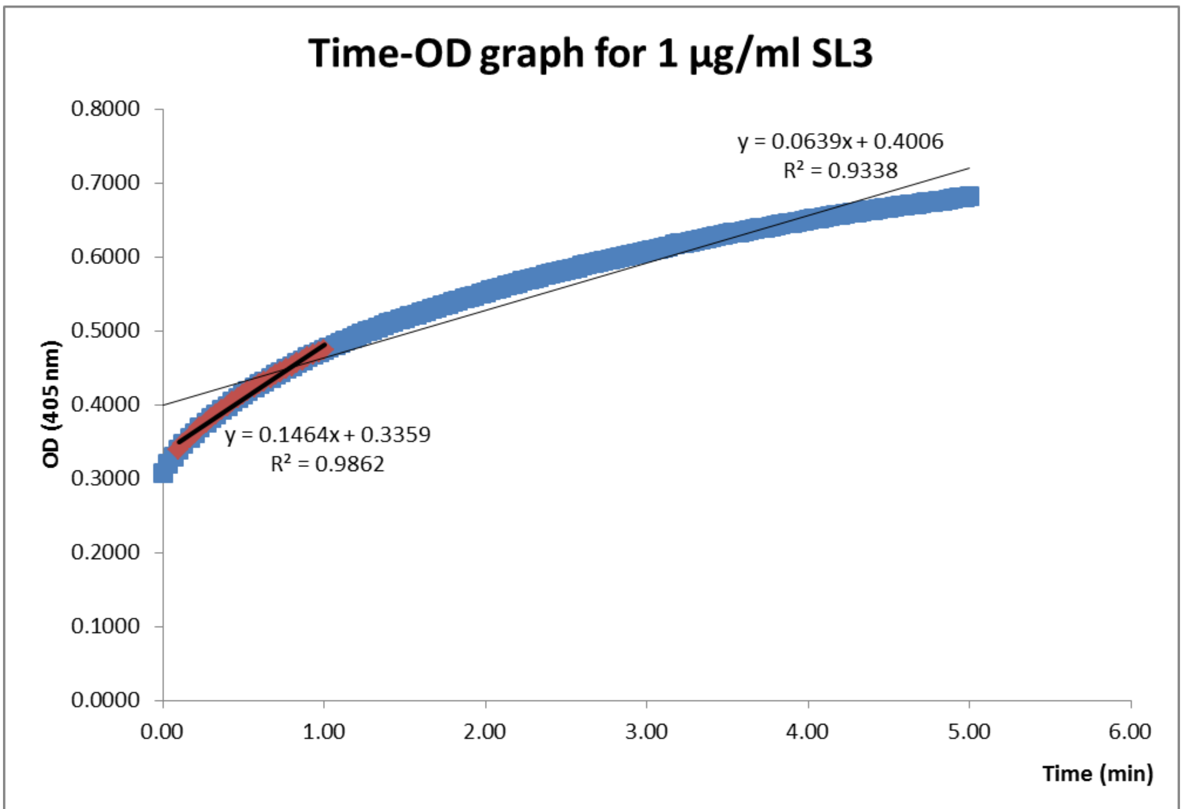


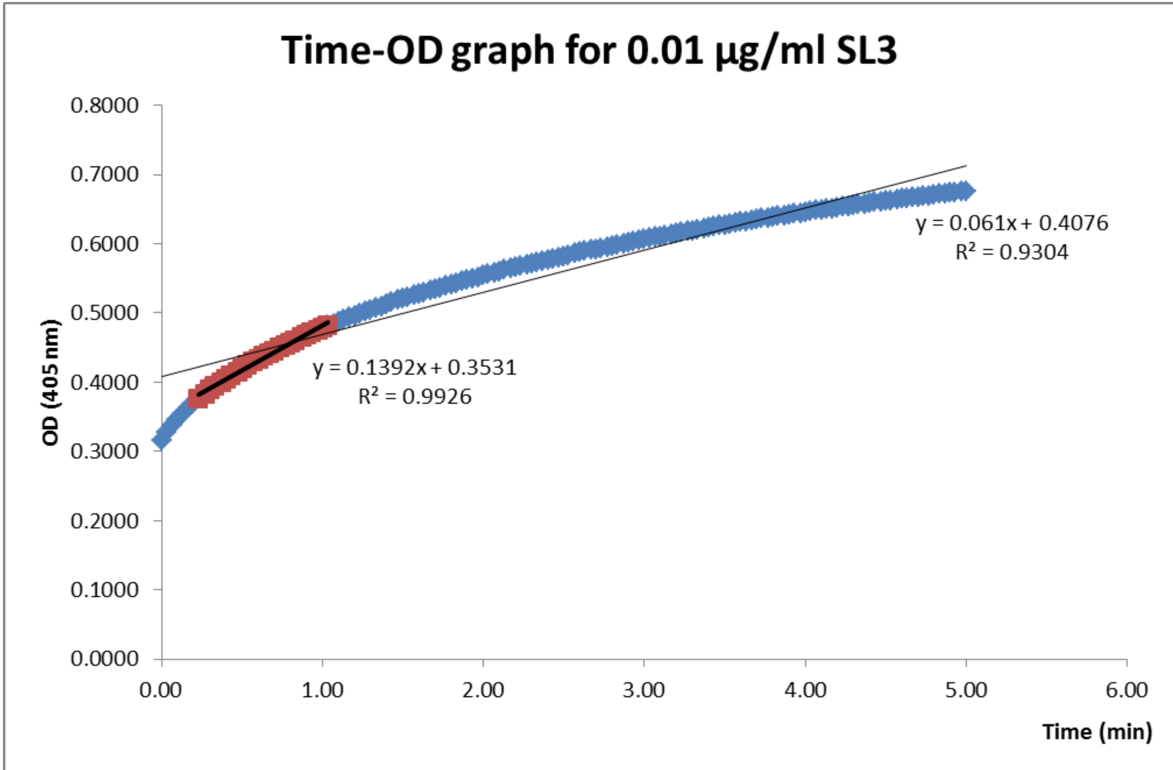



Time-OD graph for 375 µg/ml SL3
1.2000
1.0000
0.8000
0.6000
0.4000
0.2000
0.0000
OD (405 nm)
y = 0.0459x + 0.948
R² = 0.9918
y = 0.0187x + 0.964
R² = 0.9726
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Time (min)


Time-OD graph for 250 µg/ml SL3
1.0000
0.9000
0.8000
0.7000
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
OD (405 nm)
y = 0.0423x + 0.7293
R² = 0.9994
y = 0.0242x + 0.7442
R² = 0.9788
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Time (min)


Time-OD graph for 100 µg/ml SL3
y = 0.0497x + 0.6496
R² = 0.9389
y = 0.1261x + 0.598
R² = 0.9783
OD (405 nm)
1.0000
0.9000
0.8000
0.7000
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Time (min)


Time-OD graph for 1 µg/ml SL3
y = 0.0639x + 0.4006
R² = 0.9338
y = 0.1464x + 0.3359
R² = 0.9862
OD (405 nm)
0.8000
0.7000
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Time (min)


Time-OD graph for 0.01 µg/ml SL3
0.8000
0.7000
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
OD (405 nm)
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Time (min)
y = 0.061x + 0.4076
R² = 0.9304
y = 0.1392x + 0.3531
R² = 0.9926

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Year	Company	Position
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Presentations:

- Akçay, A. and Toksoy Öner, E. "Levan-based Heparin-mimetic Polysaccharides" VI. Bioengineering Congress "Human Welfare" (BEC 2013), November 12-15, 2013, Aydın, Turkey (Poster Presentation).

Projects:

- 2012-2013 TUBITAK research Project no: MAG-111M232 and title "Biological Activities of Levan-based Polymers: *In Vitro* and *In Silico* Investigations".

Professional Memberships:

- Chamber of Chemical Engineers