

**Project Report**

**Galileo Scholarship Program**

**30<sup>th</sup> JULY 2004.**

# **Surface coating and covalent attachment of Heparin**

**Sadia Afrin**

**Raritan Valley Community College**

**Science Department**

## Abstract

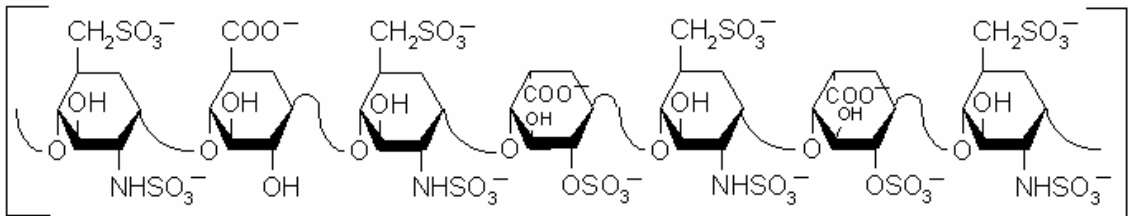
Scientists have long been trying to create non-thrombogenic surfaces. Such surfaces are very important for medical devices. Covalent attachment of heparin shows great potential in creating haemocompatible coating. This article shows the effectiveness of covalent attachment of heparin in developing non-thrombogenic coating.

## Goal

Goal of this project is to develop coating for non-thrombogenic surface by covalent attachment of heparin.

## Background

Heparin is a naturally occurring highly sulfated polysaccharide, characterized by a wide molecular weight range and powerful anticoagulant properties. Heparin was first discovered by Mc Lean in 1916. Since it's discovery it has become a widely used anticoagulant for the treatment and prevention of thrombotic diseases and for maintaining blood fluidity in extra corporeal diseases and during operative procedures.



**Figure 1:** Key structural unit of Heparin

## How Heparin works as an anticoagulant:

Heparin is a naturally produced glycosoaminoglycan. The basis for heparin's anticoagulant activity in plasma is that it inactivates the formation of fibrin clots by inhibiting two principle pro-coagulant proteases, factor Xa and thrombin. Heparin acts primarily at the following sites in the normal coagulation system:

- Heparin binds with antithrombin molecule (heparin cofactor). Binding induces a conformational change in antithrombin molecule, which accelerates the neutralization of thrombin and prevents conversion of fibrinogen to fibrin.
- Inactivates factor Xa that prevents conversion of pro-thrombin to thrombin.
- Heparin prevents the formation of a stable fibrin clot by inhibiting the activation fibrin-stabilizing factor.

### **Application**

Heparin is widely used as an injectible anticoagulant during operative procedures. But long-term use can cause life-threatening bleeding.

Efforts are on to attach heparin to polymeric materials. This means improved haemocompatibility and lubricity. The goal is to achieve non-thrombogenic surfaces to be used in blood interfacing medical devices. Implantable cardiac stents, catheters, blood filters etc are just a few of such devices.

### **Materials Required:**

- 1) **Heparin Sodium:** Eastman Kodak Co., 140 Unit/mg
- 2) **Water Based Formula (2234-172):** Hydromer Inc.
- 3) **Polyurethane film:** It is a polymer and is hydrophobic in nature.
- 4) **Cross linker:** Crosslink and make the coating dense
- 5) **Iso-propyl alcohol (IPA):** Solvent
- 6) **Phosphate Buffer (PBS):** Prepared by dissolving 16g NaCl (MW 58.4) 2.3g Na<sub>2</sub>HPO<sub>4</sub> (MW 142.0), 0.4g KCl (MW 74.5) and 0.4g KH<sub>2</sub>PO<sub>4</sub> (MW 136.1) in 2L of water.
- 7) **Toluedine Blue:** It is a basic biological dye. It binds to heparin and changes it's color from blue to purple.

### **Instruments:**

- 1) **Infra Red Spectrophotometer:** Nicolet, AVATAR-360 FT-IR.
- 2) **UV Spectrophotometer:** Beckman, DU-640.
- 3) **Coa Data 2000:** American Labor Inc.

## Procedure:

**Cutting Polyurethane film:** Cut the polyurethane film into approximately 1inch X 1 inch size.

**Washing polyurethane Films with IPA:** Wash those films by keeping in iso-propyl alcohol for 30 minutes and then dry them in air in room temperature.

**Preparation of 5% Heparin solution:** Measure 5g water in a 10 ml screw cap glass vial and add 0.25 g of Heparin Sodium into the vial. Screw the cap of the vial and shake it for a while or until the heparin is dissolved into water.

**Preparing Five Different Solutions With Different Concentration of Heparin (5% solution):** Take five 10ml screw cap glass vials. Prepare five different solutions of the materials listed below by adding one after another serially in the glass vials. Shake each vial carefully until the solution looks homogeneous.

Sample #1: 10g Water Based Formula + 0.1 g 5% Heparin solution+ 0.05% cross linker

Sample #2: 10g Water Based Formula + 0.2 g 5% Heparin solution+ 0.05% cross linker

Sample #3: 10g Water Based Formula + 0.5 g 5% Heparin solution+ 0.05% cross linker

Sample #4: 10g Water Based Formula + 1.0 g 5% Heparin solution+ 0.05% cross linker

Sample #5 (Control): 10g Water Based Formula + 0.0 g 5% Heparin solution+ 0.05% cross linker.

**Coating polyurethane film:** coat those IPA washed films with the different Heparin concentrated solutions by dipping them into the solutions. Try to make a homogeneous coating on the surface. First dry those coated films in air at room temp for about 30 minutes. Then dry the films in an oven at 70 degree for another 30 minutes.

**Table 1: Comparison of cloudiness and homogeneity of samples**

<b>Sample No</b>	<b>Cloudiness</b>	<b>Homogeneity of coating</b>
<b>Sample #1</b>	Clear	Homogeneous
<b>Sample #2</b>	Clear	Homogeneous
<b>Sample #3</b>	Little Cloudy	Homogeneous
<b>Sample #4</b>	Cloudy	Not Homogeneous
<b>Sample #5</b>	Clear	Homogeneous

**Observation:** All the samples look clear. Sample no 4 looks more cloudy because it has the highest concentration of Heparin solution. Sample no 5 is the control, which does not have any heparin solution in it and it looks clear.

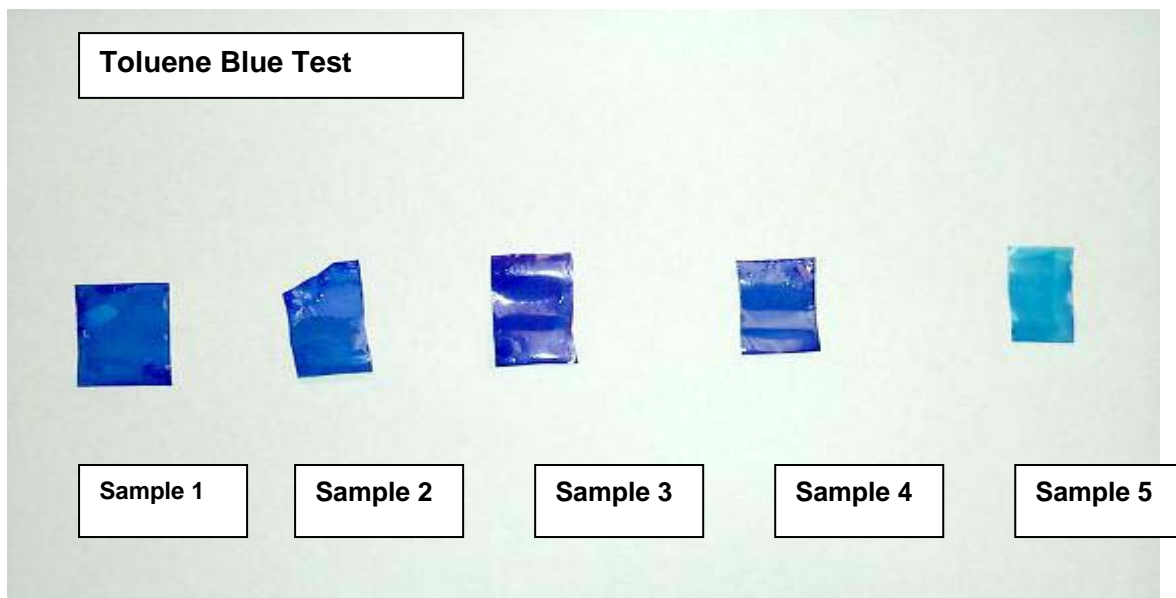
**Washing coated poly films with PBS to wash excess Heparin from the Surface:**

when the films are dry keep those films separately (according to their sample No) in 50 ml Phosphate Buffer (PBS) solution for about 45 minutes at room temperature. After 45 minutes take those films out of the PBS solution and rinse with DI water for a while and dry them in air at room temp.

**Preparation of 0.005% Toluedine Blue Solution:** Take a 200 ml screw cap plastic jar and measure 100 g of PBS into it. Carefully Measure about 0.005 g of Toluedine Blue powder and add it into the 100 g PBS. Screw the cap of the plastic jar and shake it very well until all the Toluedine Blue is dissolved and the solution looks homogeneous.

## Detection of Heparin Presence On Film Surface:

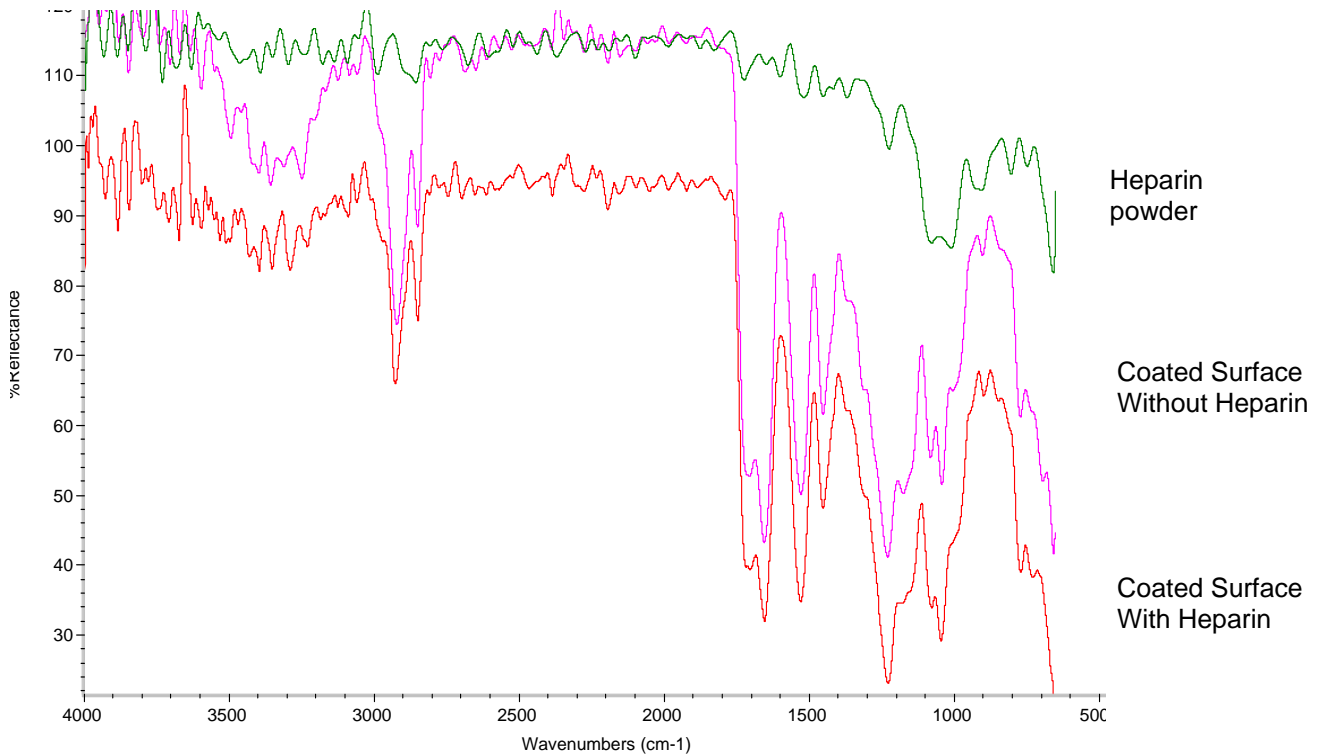
**Toluedine Blue Test to Observe the Presence of Heparin on the film:** Take five 200 ml plastic containers and measure about 25g of 0.005% Toluedine Blue solution. Take (film size”) coated films of each sample and put them in The Toluedine Blue solution separately. After 30 seconds take those films out of the solution and put them on a white paper serially by sample number to observe and compare color change.



**Figure 2:** Color change of coated films after toluene blue test

**Observation:** We cannot see the actual color change in this picture for some technical reasons. But actually sample No 1 and sample No 2 looked slightly purple. Sample No 3 appeared more purple. And sample No 4 was very brightly purple since it has the highest concentration of heparin. Sample No 5 appeared blue since it does not have any heparin in it's coating.

**Infra Red Spectrophotometer:** Use Infra Red Spectrophotometer to get the IR spectrum of powder Heparin, coated polyurethane film without any heparin (control) and heparin coated polyurethane film to compare the difference of heparin peaks.



**Observation:** In this IR spectrum we don't see the individual heparin peak in the coated surface with heparin and without heparin. Individual peaks of heparin might be overlapped by the peaks of polyurethane film. And that's why the heparin coated polyurethane film surface has the same peaks as the without any heparin coated film surface.

**Result:** IR spectrum does not help to detect the presence of heparin on polyurethane film surface.

## **Detection of Heparin Release:**

**Washing Coated Films for different Time period:** Take five 200 ml screw cap plastic container and measure about 50 g of PBS in each container. Take four coated films (1 inch X 1 inch) from each sample and put them separately into the PBS solution in five different containers and label them with sample number. Keep those containers at room temperature.

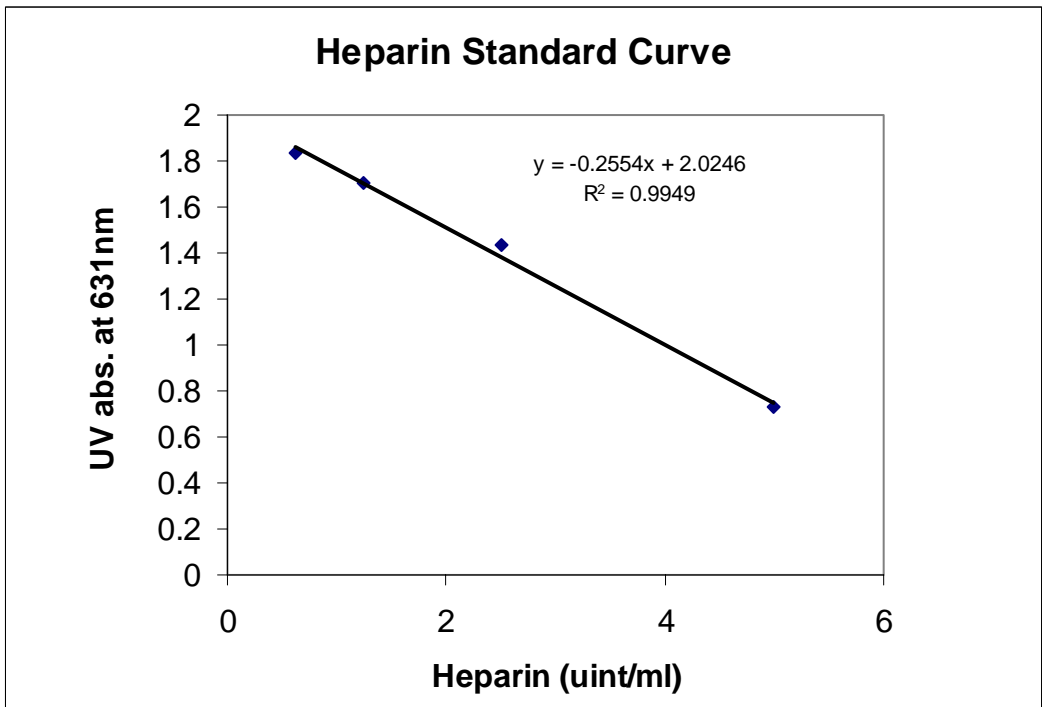
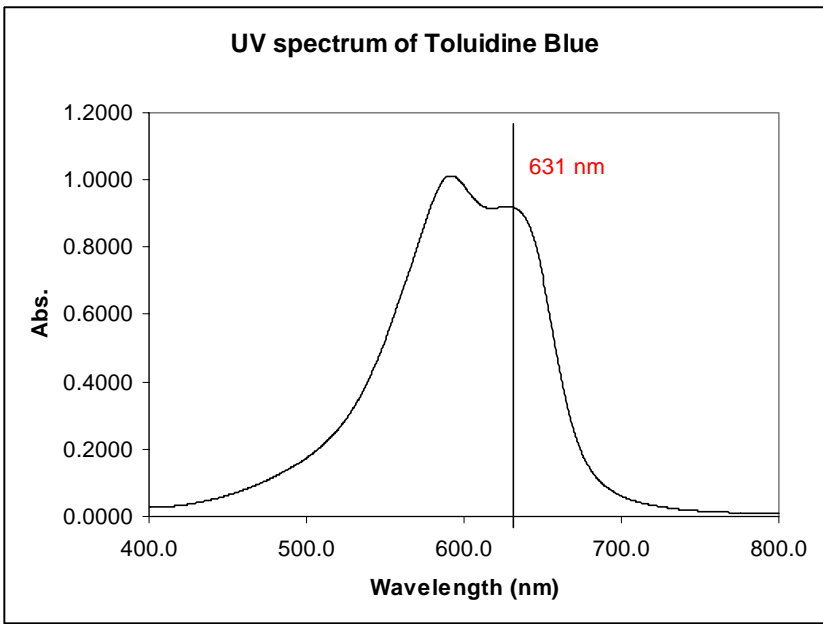
After 1 week collect one film from each sample, rinse them with DI water and dry in air. Change the Buffer solution. Follow the same procedure for collecting samples after 2 week, 3 week and 4 week. Keep them separately.

## **Toluedine Blue Test to detect the amount of heparin after washing coated Films for different time Period:**

**Preparation of Standard Solutions:** Take five 200 ml screw cap plastic container to prepare five different standard solutions by serial dilution.

To prepare a 10 unit standard solution measure about 5.0 mg of sodium heparin and add 70.0g phosphate buffer or PBS in one of the containers. Shake the container well for a while. To prepare a 5 unit standard solution measure 25g of 10-unit heparin in another container and add 25 g of PBS into it and shake it well. For 2.5 unit solution take 25g of 5-unit solution and add 25g of PBS in it. For 1.25 unit and 0.625 unit standard solutions follow the same procedure of serial dilution.

For the Toluedine blue test take five 20 ml screw cap glass vials and add 1.5 ml of 0.005% toluedine blue solution in each vial. Then add 1.5 ml of five different standard solutions into five vials and Vortex vigorously for 30 seconds. After that add 3.0 ml hexane in each vials and again vortex vigorously for another 30 seconds. The aqueous layer (lower layer) in those vials are the samples. Using a spectrophotometer take the reading of absorbance of Toluedine Blue at 631 nm wavelengths. Use PBS as blank.



**Observation:** the standard curve is linear. This standard curve shows that 0.625 unit has the lowest concentration of heparin representing the highest value of absorbance. And 5unit has the highest concentration of heparin which, represents the lowest value of absorbance,

**Result:** Higher absorbance value at 631nm wavelength represents the lower amount of heparin unit/ml.

**Toluedine blue Test for unwashed, 1 week washed, 2 week washed, 3 week washed and 4 week washed films:** Cut 1.5cm x 2.5cm size film of all five samples. Take five 20 ml screw cap glass vials and put those five samples separately in those vials. Label them by sample number. Add 1.5 ml of Toluedine blue and 1.5 ml of PBS into each vial. Vortex each vial vigorously for 30 seconds. After that add 3 ml of Hexane in each of those vials and vortex vigorously for another 30 seconds. The aqueous layer (lower layer) in those vials are the samples. Using a spectrophotometer take the reading of absorbance of Toluedine Blue at 631 nm wavelengths. Use PBS as blank. Follow the same procedure for all unwashed, 1 week washed, 2 week washed, 3 week washed and 4 week washed films to get the absorbance value at 631nm.

From all the absorbance data and using the standard curve equation calculate the actual amount of Heparin IU/cm<sup>2</sup> on the surface of the films.

**Table: Calculated amount of Heparin IU/cm<sup>2</sup> on the surface of the films**

Sample NO	Unwashed	1 week washed	2 week washed	3 week washed	4 week washed
Sample #1	0.35	0.27	0.44	0.24	0.25
Sample #2	0.51	0.31	0.61	0.42	0.40
Sample #3	0.38	0.41	0.60	0.61	0.37
Sample #4	0.43	0.45	0.57	0.62	0.48
Sample #5	0.00	0.00	0.00	0.00	0.00

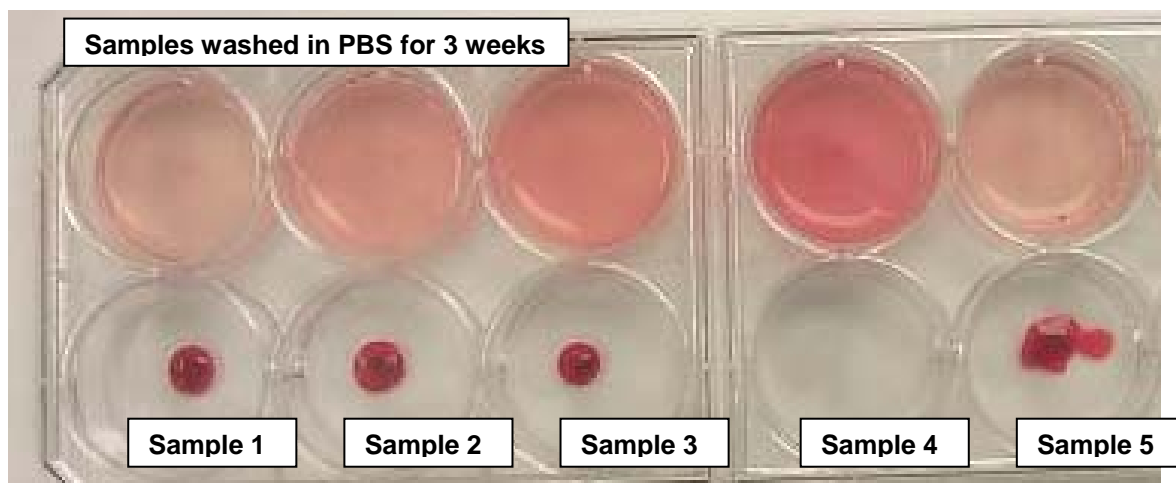
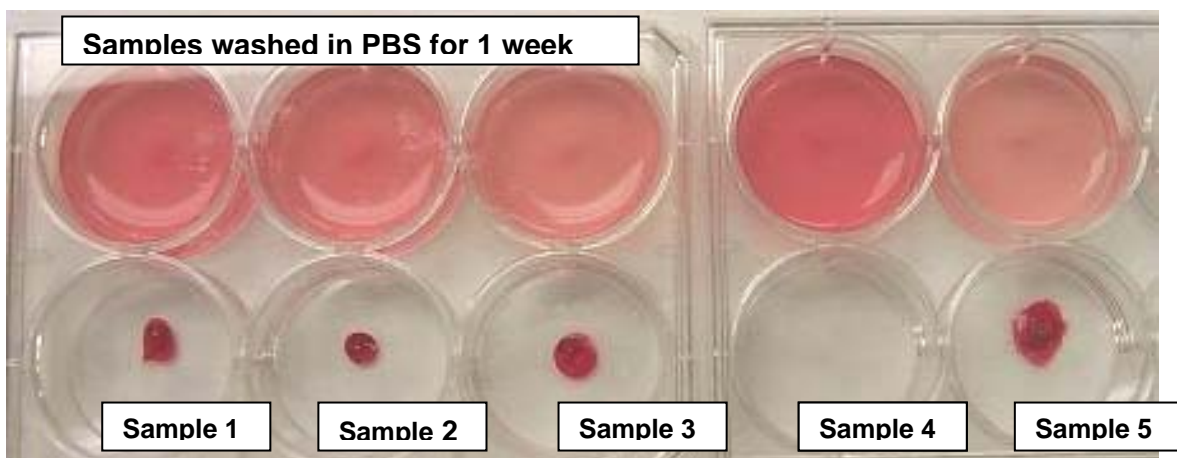
**Observation:** Sample no 5 has no heparin detected by using Toluedine Blue Test, while other samples have haperin from 0.3 to 0.6 unit/cm<sup>2</sup>. The samples still have haprin on the surface even after 4 weeks washing in PBS. The amount of heparin is

supposed to increase from sample 1 to sample 5. The data obtained roughly follows this. On the other hand the amount should constant or decrease as the time period is increased. However, the experimental data doesn't exactly follow this. This could have been caused by the experiment error or difference between each sample.

**Thrombin Test:** Take 1x1 inch coated films from each sample and tap them on Petri dishes separately. With a pipette put 100  $\mu\text{L}$  of Blood on the surface of each film. Then carefully add 50  $\mu\text{L}$  Calcium Chloride to the blood and mix them well with the help of a pipette. Put all the Petri dishes carefully in an incubator so all the blood stays on the surface of the films for 25 minutes. After 25 minutes take those dishes out and add 200  $\mu\text{L}$  of PBS into each dish.

After 60 seconds, collect liquid and solid part of the blood separately. Take the solid part of each sample (if there is any) in an aluminum plate and dry them in an oven for about 20 minutes and calculate the weight of each dry sample separately.

follow this same experiment with 1 week washed films and 3 week washed films.



**Table:** Comparison of thrombin weight formed on Heparin Coated Polyurethane Film to coated film without heparin:

Washing Time	Sample # 1 (0.05% Heparin in Coating Solution)	Sample #2 (0.1% Heparin in Coating Solution)	Sample # 3 (0.25% Heparin in Coating Solution)	Sample # 4 (0.5% Heparin in Coating Solution)	Sample # 5 (No Heparin in Coating Solution)
1 Week	0.0043 g (51.80%)	0.0051 g (61.45%)	0.0045 g (54.22%)	0 g (0%)	0.0083 g (100%)
3 Week	0.0113 g (100%)	0.0064 g (57.15%)	0.0049 g (43.75%)	0 g (0%)	0.0112 g (100%)

**Observation:** From this two pictures and this table we can see how the amount of blood clot has decreased with the increasing concentration of heparin in the coating. It also presents, comparing to 1 week washed samples, 3-week washed samples has higher percentage of blood clot, since 3 week washed samples suppose to have less heparin on their surfaces then 1 week washed. Sample no 5, which does not have any heparin shows the highest amount of blood clot.

**Result:** The amount of heparin on the surface of the films decreases as time period of washing increases but it remains active.

## Conclusion

The heparin coating used in this research remained active after repeated washing. The amount of heparin on the surface of the films decreases as time period of washing increases but it still remains active. All the results points to success in creating a non-thrombogenic coating by covalent attachment of heparin.

**APTT Test using Coa Data 2000:** Activated partial thromboplastin time or APTT is a conventional screening test that measures the prolonged clotting time of recalcified citrate-anticoagulated plasma in the presence of heparin, by using a phospholipids reagent and a surface activator. This is widely used to verify the effectiveness of heparin.

The method involves pipeting 200  $\mu\text{L}$  of plasma into a tube containing 4x4 mm of test material which is incubated for 1 min at 37 degree centigrade. 200  $\mu\text{L}$  of APTT reagent is then added & incubated for another 3 min at same temperature. After this 200  $\mu\text{L}$  of 20mM of calcium chloride is rapidly added & the clotting time is monitored.

This will help us compare the clotting time among different heparinized polyurethanes, control sample.

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