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HEMOCOMPATIBILITY OF SURFACE MODIFIED SILK FIBROIN MATERIALS: A REVIEW

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Abstract. Hemocompatibility test is very common and important during development of biomaterials in direct contact with blood. Moreover, silk fibroin based biomaterials have been used in biomedical application for more than one hundred years and increasing sharply. Recent reports from our group and others have demonstrated that it has acceptable mechanical properties and patency rates in animal models. Unfortunately, pure silk fibroin shows poor hemocompatibility. Thus, the surface of a silk fibroin should be modified to improve its blood compatibility when it is used as a blood-contacting material especially for small diameter arterial prostheses. Researchers have studied hemocompatibility of silk fibroin biomaterials; however, the testing procedures vary greatly throughout different publication. Thus it is not easy to get the exact information from the literatures to performing hemocompatibility assays. Therefore, this paper reviews the studies on effects of surface modification on hemocompatibility in terms of hemolysis, anticoagulation, platelets adhesion, plasma recalcification time (PRT), activated partial thromboplastin time (APTT) and plasma prothrombin time (PT) assays of silk fibroin biomaterials. At last, other physical and biological tests which are also essential and related to the hemocompatibility testing, were also discussed in this paper.

1. INTRODUCTION

Hemocompatibility is a very important factor to decide the application of implantable biomaterials such as, artificial blood vessels and orthopedic implants. With the development of blood-contacting materials or implantable devices, it is necessary to improve the hemocompatibility by surface modification or re-design. When blood contacts with a foreign biomaterial surface, the first process that occurs is the competitive adsorption of plasma proteins [1]. Many studies have aimed to improve hemocompatibility of biomaterials by surface modification [2-6]. The improvement of hemocompatibility on a biomaterial surface aims at reducing protein adsorption with the eventual goal of decreasing platelet adhesion.

Today vascular disease is the major cause of mortality worldwide, and the most common therapy for this condition is vasotransplantation. More than 1.4 million bypass procedures are performed in the US alone, and about three times this number are performed worldwide [7] . Generally, surgical bypass is the only option in limb salvage from vascular occlusion. The ideal bypass grafts are autologous vessels harvested from patient's own saphenous vein or internal mammary artery. However, harvesting vessels with sufficient length for effective bypass without causing additional morbidity can be difficult to achieve in many patients, due to previous surgery or injury [8] . Artificial vascular grafts are therefore widely used to replace or bypass diseased arteries.

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Currently available synthetic grafts, such as expanded polytetrafluoroethylene (ePTFE, Goretex®) and polyethylene terephthalate (PET, formerly Dacron®), are successful at the macro vascular level, but fail at smaller diameters (inner diameter <6 mm) due to thrombosis and/or compliance mismatches [9-11] because this is the actual scale of interest for coronary and femoral artery bypass procedures. After 50 years development, large-diameter PET vascular grafts (inner diameter > 6 mm) can remain functioning for more than 10 years while small-diameter PET vascular grafts still fall short in meeting the biological challenges. They may cause blood clots as soon as being implanted because of the low-flow, high resistance condition [12]. Bioactive compounds have been coated or grafted onto the luminal walls of PET vascular grafts to improve their hemocompatibility [13-16].

Silk fibroin (SF) is a fascinating protein used for biomaterial application. SF in raw and regenerated versions have been used extensively in biomedical applications such as sutures, cell culture media [17], antithrombogenic material [18,19], wound healing [20], drug carrier [21-25], scaffolds in tissue engineering [26,27], bone [28-31], cartilage [32-34] engineering. SF could be an attractive promising biomaterial used for small diameter arterial prostheses [11,35]. Prostheses based on fibroin fibers provide excellent patency when implanted in the rat abdominal aorta, with overall 1 year patency rate of 85% which is much higher than that of PTFE (30% patency rate) [36]. Unfortunately, pure SF shows poor anticoagulant activity [37] when it's used as a blood contacting material. Hence, the issue of how to improve the hemocompatibility, of the silk fibroin based implant material used in direct contact with blood, is one of the most important problems to be solved.

2. TYPES OF HEMOCOMPATIBILITY ASSAYS

As for the hemocompatibility tests of medical devices, such as endovascular grafts, shunts, rings, patches, heart valves, balloon pumps, stents, pacemakers, hemopheresis filters, ISO 10993-4 (issued in 2002 and updated in 2009 by the International Organization for Standardization, the American National Standards Institute and the Association for the Advancement of Medical Instrumentation) provides a list of recommended assays (Table 1).

However, the following hemocompatibility assays are generally observed in literatures, such as: hemolytic assay, anticoagulant assay, platelet adhesion and activation assay blood coagulation time assay (PRT, APTT and PT). Standards which are generally followed for hemocompatibility tests, are given in Table 2.

3. PROCUREMENT AND PROCESSING OF BLOOD PERFUSATES

Blood collection and preparation are the vital steps to carry out hemocompatibility testing. Blood can be drawn by venipuncture from aspirin-free healthy adult human donors or from rabbits and anti-coagulated with dipotassium EDTA (1.5 mg/L) or tri sodium citrate (3.2%) or heparin, or ACD (acid citrate dextrose). Anticoagulant removes ionized calcium (Ca^{+}) through a process referred to as chelation. This process forms an insoluble calcium salt that prevents blood coagulation. The amount of blood

Table 1. List of hemocompatibility assays according to ISO 10993-4 standard.

Hemo-incompatibility	Assays
Thrombosis	Percent occlusion, flow reduction, pressure drop across device, antibody binding to thrombus components, thrombus mass, light microscopy and SEM of adhered platelets, leukocytes, aggregates, erythrocytes, fibrin, etc.
Coagulation	Coagulation PTT (non-activated), thrombin generation, specific coagulation factor assays, FPA, D-dimer, F1+2, TAT
Platelet activation	Platelet count/adhesion, platelet aggregation, template bleeding time, platelet function analysis, PF-4, thromboxane B2, platelet activation markers, platelet microparticles, gamma imaging of radiolabelled platelets, 111 In- labelled platelet survival
Blood cell changes	Leukocyte count with or without differential, leukocyte activation, haemolysis, reticulocyte count
Complement activation	C3a, C 5a, Bb, iC3b, C4d, SC5b-9, CH50, C3 convertase, C5 convertase

Table 2. Standards generally used to perform hemocompatibility tests.

ASTM F2888-13	This test method assists in the evaluation of cardiovascular device materials for their ability to induce thrombus formation. Thrombus formation is assessed by means of a reduction in human platelets and leukocytes when consumed by thrombus after activation on the material surface. This assay may be part of the hemocompatibility evaluation for devices and materials contacting human blood, as per ANSI/AAMI/ ISO 10993-4.
ASTM F 756-00	ASTM F 756-00 is a standard test method to evaluate whether direct contact with the material or an extract of the material would cause in vitro red blood cell hemolysis.
ISO 10933	ISO 10993-1, the international guidance on the selection of biocompatibility tests for medical devices that has been developed by the International Organization for Standardization (ISO), requires an evaluation of hemocompatibility for any medical device that has contact with circulating blood, directly or indirectly, during routine use. Guidelines for such evaluations are subsequently presented in ISO 10993-4, "Selection of Tests for Interactions with Blood". This standard provides a structured test-selection system based on the intended use of the device. Although it does not provide detailed test methods or evaluation criteria, it cites various applicable references.

withdrawn must be within prescribed limits so as to maintain the proper ratio with the anticoagulant; otherwise, the blood cells may be damaged and/or anticoagulation may be unsatisfactory.

Followings are the reviews of blood preparation from some papers.

1. Whole blood was collected from healthy donors at hospital with citrated (3.2%) 1.8 ml vacuum blood-collection tubes and transported on ice. The blood samples were then, unless mentioned otherwise, centrifuged at 2000 x g for 10 min at 4 °C to obtain Platelet-poor plasma (PPP), which was then used in the plasma recalcification and factor XII activation assays [38].

2. Platelet-rich plasma (PRP), is defined as a plasma fraction containing a higher concentration of platelets than the peripheral blood [39]. To obtain PRP, the ACD blood was centrifuged at 100 x g for 20 min at 4 °C to separate the blood corpuscles. Subsequently, a portion of the PRP was further centrifuged at 2000 x g for 20 min at 4 °C to obtain PPP [40].

3. Citrate anti-coagulated human whole blood (30 ml) from a healthy volunteer supplied by Blood Center was centrifuged at 3000 rpm for 15 min to separate the blood corpuscles. The PPP obtained was used for the APTT test [41].

4.The human whole blood containing 10% ACD was centrifuged at 3000 x g for 10 min to separate the blood cells, and the remaining PPP was used for the PRT experiments [42].

5. Blood was drawn from a healthy volunteer and mixed with ACD. Fresh human blood was centrifuged at 3000 rpm (revolutions per minute) and 1500 rpm for 15 min at room temperature to obtain PPP and PRP, respectively [43].

6. Whole blood was drawn from aspirin free donors into 10 ml syringes containing 1 ml of 3.8% sodium citrate (Sigma). The whole blood was centrifuged at 1000 rpm for 15 min to obtain PRP [44].

7.Venous blood of rabbit containing 3.8% buffered sodium citrate (7:1, v/v) was centrifuged at 1500 rpm for 10 min and at 3000 rpm for 10 min at 4ºC, respectively to obtain the PPP and PRP from the supernatant [45].

8. PRP was prepared by collecting rabbit blood in plastic syringes with 3.8 w/v-% trisodium citrate solution at a ratio of 9:1, the complex of rabbit blood and trisodium citrate solution were centrifuged at 3,000 rpm for 10 min to obtain PRP [46].

4. HEMOLYSIS ASSAY

Hemolysis essentially results from the increase in osmotic pressure of red blood cells (RBCs). During hemolysis, the rupture of RBCs occurs and the hemoglobin is released from RBCs. Hemolysis percentage (HP) represents the extent of RBCs broken by the sample in contact with whole blood. The more the number of the broken red cells, the larger the value of HR is.

Fig. 1. shows the sketch map of hemolysis assay. Anti-coagulated blood is centrifuged and washed with phosphate buffer saline (PBS) five times in a centrifuge machine according to the procedure reported in literatures [47,48] to completely remove serum and obtain human red blood cells (HRBCs).

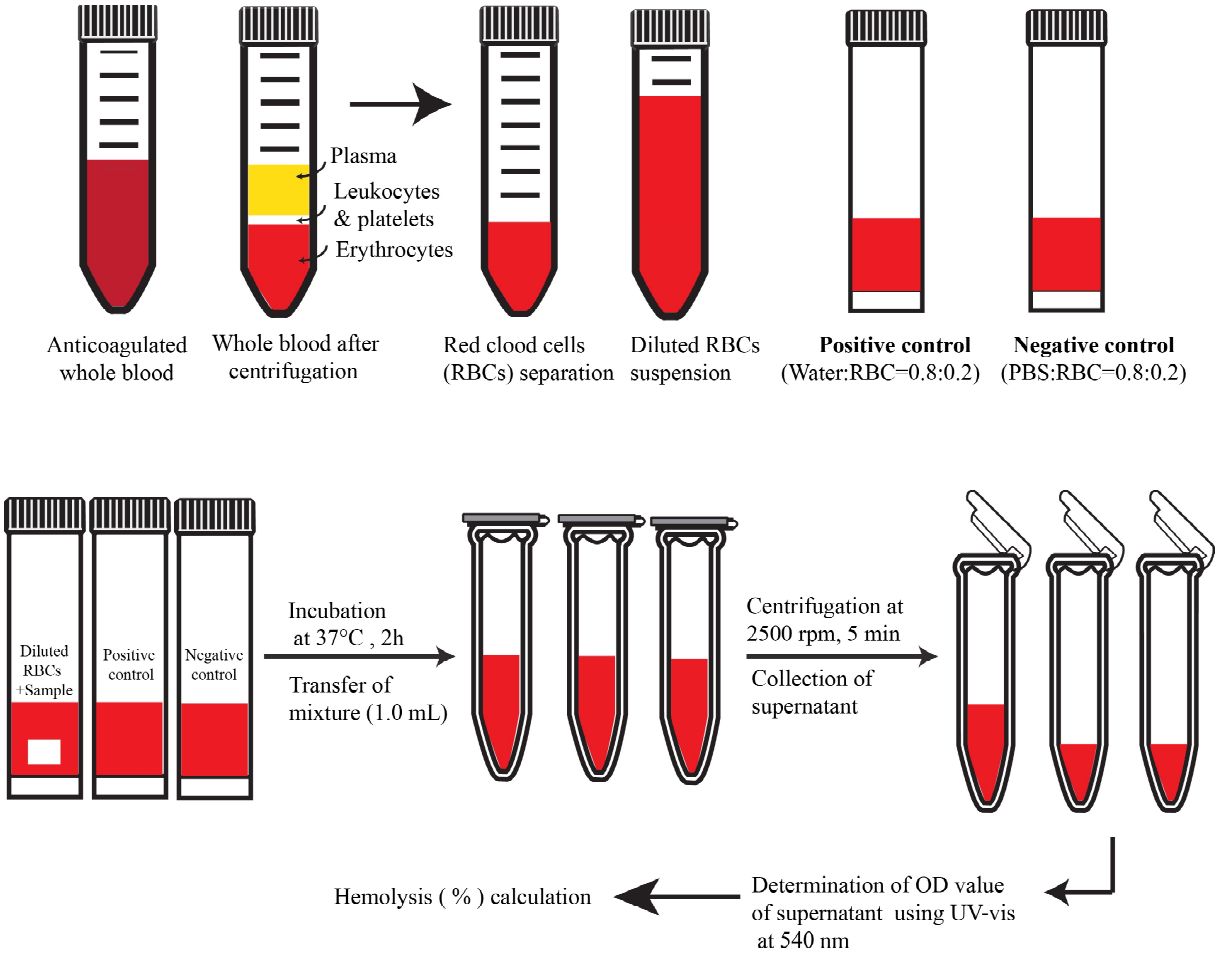


Fig. 1. Sketch map of hemolysis assay. Diluted blood prepared from RBCs is the basic requirements to perform hemolysis assay. Positive and negative solutions are also needed to compare the hemolysis property with substrates. Finally hemolysis percentage is calculated according to the equation discussed above and be compared with ASTM F756-00(2000) standard.

After that, the HRBCs are firstly diluted with PBS to make HRBCs suspension. Then, 0.2 mL of the diluted HRBCs suspension is transferred to a 5 mL tube which is filled with 0.8 mL of water (as positive control) and PBS buffer (as negative control), respectively. Samples are then exposed to HRBCs suspension containing 0.2 mL diluted HRBCs suspension and 0.8 mL PBS buffer, respectively. The above mixtures are then incubated at 37 °C for 2 h, followed by centrifugation according to different researchers (10,000 rpm; 3 min [48], or 2000 rpm ; 5 min [47] or 2500 rpm; 5 min [43]) . Then the supernatant was determined by Perkin Elmer Lambda 25 UV–vis spectrometer to record the absorbance at 540 nm. The hemolytic percentage (HP) can be calculated using the following Eq. (1) [49,50].

$$HP(\%) = \frac{(D_t - D_{nc})}{(D_{pc} - D_{nc})} \times 100, \qquad (1)$$

where, D_t is the absorbance of the test sample: D_{pc} and D_{nc} are the absorbance of positive and negative control, respectively.

According to ASTM F756-00(2000) materials can be classified in three different categories to their hemolytic index (hemolysis %). Materials with percentages of hemolysis over 5% are considered hemolytic; while the ones with hemolytic index between 5% and 2% are classified as slightly hemolytic. Finally, when the material presents a hemolysis percentage below 2% it is considered as a non-hemolytic material.

5. ANTICOAGULANT ASSAY

The anticoagulant properties of a sample can be determined by a kinetic clotting time method described in the previous studies. [51-53] In brief, specimens are cut into small pieces in a dimension of 20x20 mm^2 in triplicate and are put into individual well of 12-well tissue culture plate. Glass cover

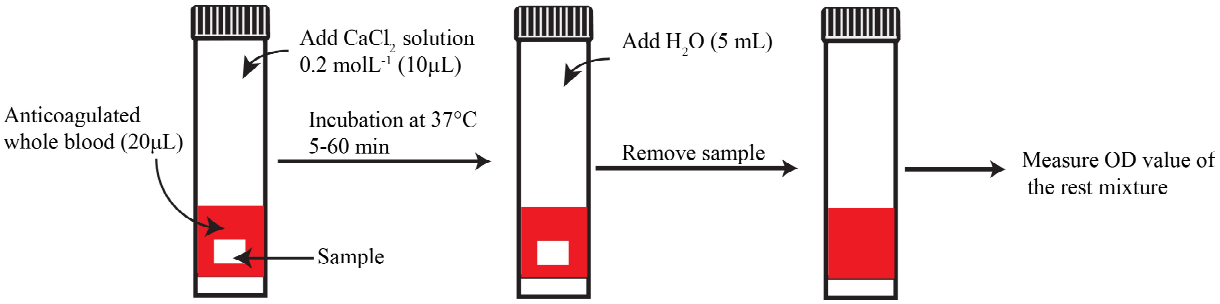


Fig. 2. Process of anticoagulant assay. Incubation time of anticoagulated blood with substrate maybe extended from 5-60 min as observed from various papers.

slips without sample are used as positive control. Then anti-coagulated human blood (20 μL) is dropped onto the surface of the specimens and the cover slips, respectively. As a trigger for blood coagulation, 10 μL of $CaCl_2$ solution (0.2 mol L^{-1}) is then added to each blood solution and incubated at 37 °C for a predetermined period of time (5, 10, 20, 40, and 60 min). After that, 5 mL water is added into each well carefully and incubated at 37 °C for 5 min. The concentration of hemoglobin in water is measured by monitoring the absorbance at 540 nm using a UV–vis spectrophotometer. The process of anticoagulant assay is depicted in Fig. 2.

6. PLATELET DEPOSITION AND ACTIVATION ASSAY

Upon contact with blood, an ideal artificial vascular graft surface should not adsorb platelets. An artificial surface should neither activate nor change the morphology of the platelets. Once platelets adhered, they become activated and aggregate to form platelet thrombus, and then gradually diminish blood flow, consequently lead to vascular occlusion [54]. Activation of platelets can be judged by change in their morphology: as activation proceeds, the platelets lose their round shape, form pseudopodia (tangled) and spread on the biomaterial. The assay of platelet adhesion can be used to examine the activation of platelets, fibrin clots, etc. Anticoagulated blood is centrifuged at 1500 rpm for 15 min at room temperature to obtain platelet-rich-plasma (PRP). Volume of plasma can be adjusted in BSGC buffer, pH 7.3 [218 mg of K_3PO_4, 1.2 gm of NaH_2PO_4, 7.0 gm of NaCl, 4.0 gm of sodium citrate, and 2.0 gm of glucose in one litre of deionized water] to obtain physiologic stock platelet count of $1.5x10^5$ μL [55].

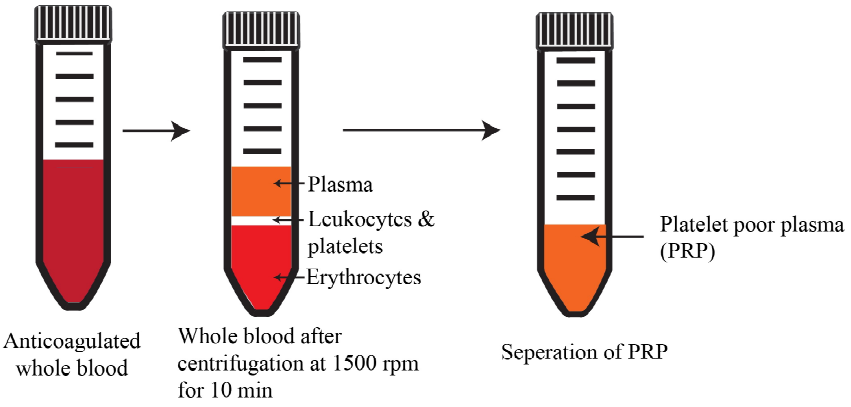
The process of platelet adhesion assay is illustrated in Fig. 3. Samples are cut into squares of 8 mm x 8 mm and are allowed to contact in 0.5 mL of stock PRP suspension for 1-3 h at 37 °C under the static conditions [41,43] . Test samples are washed gently with PBS several times to remove non-adhere platelets. The PBS-platelet suspension is recovered and counted in a coulter counter (n = 6). The stock suspension of platelets is also counted. Platelet deposition on the substrate is estimated by quantifying depletion of platelets from the stock suspension as per Eq. (2).

$$\text{Platent adhesion \%} = \left[1-\left(\frac{C_{stock}-C_{substrate}}{C_{stock}}\right)\right]\times 100, \quad (2)$$

were C_{stock} is the platelet count in the stock suspension (platelet #/μL), $C_{substrate}$ is the platelet count measured in the stock suspension following incubation with the test or control substrate.

Activation of adhered platelets could be assessed through material-induced changes to their morphology using SEM. In preparation for SEM, the adhered platelets are fixed with 2.5% buffered glutaraldehyde solution overnight in a refrigerator at 4 °C [43], dehydrated at increasing ethanol solution in sequence (55%, 70%, 80%, 90%, 95%, and 100%) and finally dried in a desiccator. The samples are then coated with gold over 60 s in a sputter-coater. The morphology of adhered platelets can be classified on a five-point scale of increasing degree of activation as rounded, dendritic (i.e., with emerging pseudopods), spread-dendritic, spread, and fully spread (i.e., flattened) as described previously [14].

Platelet activation can be additionally confirmed by visualizing the expression of the platelet surface activation marker p-selectin (CD62P) using immunofluorescence. The number of platelets adhered to the samples are determined by measuring the lactate dehydrogenase (LDH) activity of the cells lysed with Triton X-100 [56]. The LDH activity can be determined by measuring the initial rate of nicotinamide adenine dinucleotide hydride (NADH) oxidation in the presence of pyruvate. A linear relationship is thereby obtained between the LDH activity



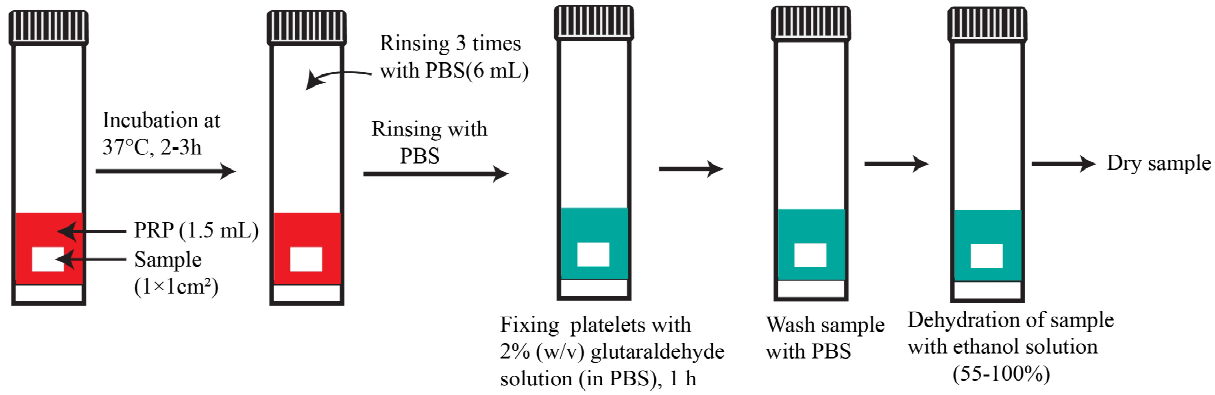
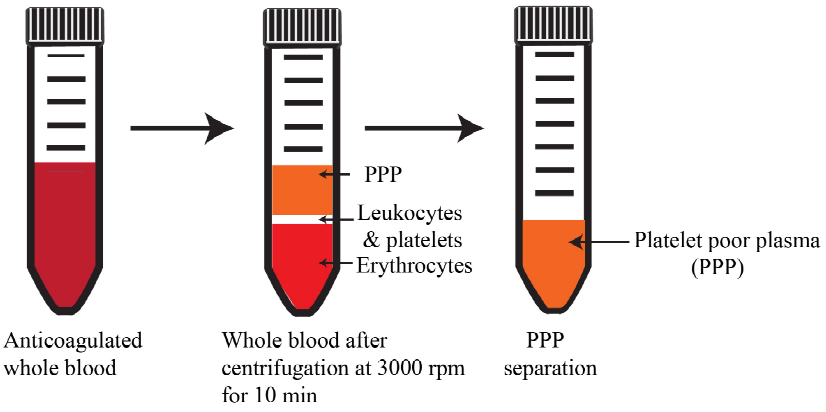


Fig. 3. Schematic platelet adhesion assay. Platelet-rich plasma is produced first using appropriate centrifugation speed and time, after that platelet adhesion assay is carried out. The sequence of operations may be varied from various authors.



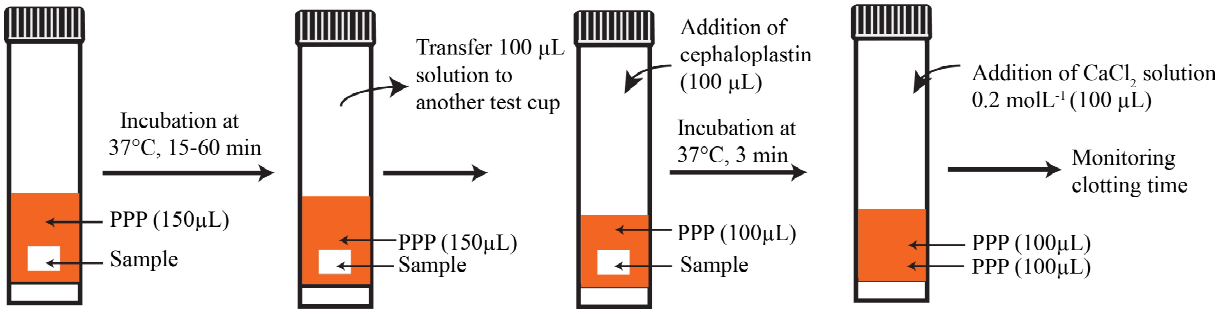


Fig. 4. Schematic activated partial thromboplastin time (APTT) assay. Platelet-rich plasma is prepared first followed by incubation of substrate with PPP solution.

of the aliquots of the cell suspension and the number of platelets which can be counted with a hemocytometer. The NADH oxidation is followed by the decrease in absorbance at 340 nm. The experiment is carried out in triplicate and a mean value is calculated.

7. APTT, PRT AND PT

The blood coagulation cascade pathway includes intrinsic, extrinsic, and common pathway. The intrinsic and extrinsic pathways lead to the formation of fibrin clots [57]. The APTT for a biomaterial reflects the duration in which the intrinsic pathway, especially the Factor XII, is activated over the contact with blood. The longer the time, the more difficult the onset of the intrinsic pathway is. So, a prolonged APTT suggests an improved anticoagulant activity of a biomaterial [58].Therefore, the blood plasma APTT test is commonly used to evaluate the *in vitro* anticoagulation properties of different biomaterials. Fig. 4. illustrates the APTT process. Substrate of 1 cm x 1 cm is placed carefully in tubes and incubated in 0.1-0.5 ml of PPP at 37 °C for 1-30 min [41,40]. Afterwards, 0.1 mL PPP solution is transferred and mixed well with 0.1 mL of actin activated cephaloplastin reagent at 37 °C for 3 min in another tube. Simultaneously with addition of 0.1 mL $CaCl_2$ solution (0.025 M), a stopwatch is started and the solution is mixed well. Cephaloplastin activates the coagulation factors of the intrinsic pathway of the coagulation mechanism in the presence of calcium ions. The clotting time of the plasma solution is recorded with a chronometer [40] or with a clot time analyzer [41] at the sign of fibrin formation. The tests are repeated three times for each substrate.

Plasma recalcification time (PRT) is measured to compare sample-induced delay in clotting of PPP following activation of prothrombin (Factor II) in the presence of Ca^{2+}. PRT is an indicator for the activation of the intrinsic coagulation cascades in the blood and thus a useful marker in testing the hemocompatibility of a biomaterial [44]. The plasma recalcification time (PRT) reflects the delay in the intrinsic coagulation process. The PRT is generally delayed in the absence of coagulation factors, such as fibrinogen, thrombinogen and so on, or in the presence of anti-coagulant substance. The longer the PRT for a biomaterial, the better is the anticoagulant activity of the biomaterial [58].

The schematic PRT assay can be seen in Fig. 5. Substrate of (1 cm x 1 cm) is placed carefully in tubes and incubated in 0.1-0.5 mL of PPP at 37 °C for 1-30 min [40,43]. Afterwards, 0.1 mL of PPP solution is taken out and mixed with 0.1 mL of $CaCl_2$ solution (0.025 M) in another tube. The clotting time of the mixture is monitored automatically [41,43] with a clotting time analyzer or by manually [58] dipping a stainless-steel hook into the mixture to detect fibrin threads. Clotting time is recorded at the first signs of any fibrin formation on the hook. The experiment is conducted in quadruplicate and a mean value is calculated.

Prothrombin time (PT) is an indicator of extrinsic and common coagulation activation [59]. PT represents the duration in which the extrinsic pathway is started, and a prolonged PT also signifies an enhanced anticoagulant activity of a biomaterial [58]. Graphical representation of PT is portrayed in Fig. 6. PPP (0.1-0.5 mL) is layered atop the substrate(1 cm x 1 cm) and incubated at 37 °C for 1-60 min [58,43] . Afterwards, 0.1 mL of PPP is taken out to the test cup, simultaneously with the addition of 0.1 mL thromboplastin reagent at 37 °C. The time taken for the onset of fibrin (clot) formation is detected using a steel hook (n = 3) [58].

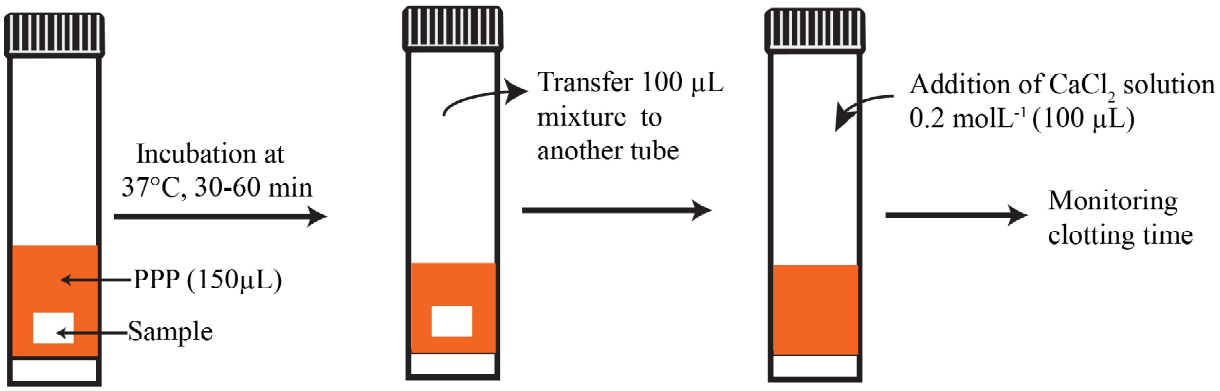


Fig. 5. Figure illustrating plasma recalcification time (PRT) assay using platelet-poor plasma (PPP) solution.

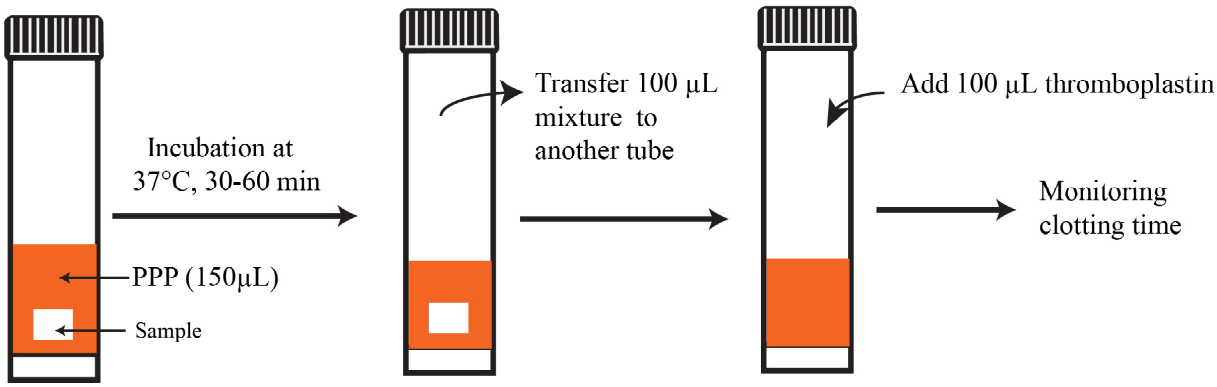


Fig. 6. Sequence of operations to carryout prothrombin time (PT) assay using platelet-poor plasma (PPP).

8. MODIFICATION OF SILK FIBROIN MATERIAL TO IMPROVE HEMOCOMPATIBILITY

In an effort to study the effect of hemocompatibility property of modified silk fibroin by an isocyanate head group. Jiang et al synthesized and constructed Zwitterionic phosphobetaine bearing a hydroxyl and a zwitterionic group, 8-hydroxy-2-octyl phosphorylcholine (HOPC), to the surface of silk fibroin (SF) film. The result revealed that zwitterionic phosphobetaine led mainly to good nonthrombogenicity in platelet adhesion test [60].

Vepari et al. [61] reported increased antiadhesion and antithrombotic properties of Poly(ethylene glycol) modified silk fibroin films. In this study, silk fibroin film surfaces were PEGylated by reaction with cyanuric chloride-activated poly (ethylene glycol) (PEG). They suggested that Surface PEGylated silk fibroin films could be useful antiadhesion and antithrombotic materials for biomedical applications when considered along with the unique mechanical and tailorable degradation profiles of silk fibroin.

Wang et al.[62] investigated the effect of ferulic acid modified silk fibroin fabric and observed that the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) and the whole blood coagulant time (WBCT) of modified silk fibroin prolonged largely than that of pure silk fibroin .

Some researchers studied the effect of heparin grafting after plasma treatment on the biocompatibility of electrospun silk fibroin nanofibers [63]. They found that *In vitro* coagulation time tests namely, the APTT, PT, and TT of the heparin-modified scaffolds were much higher than those of the pure silk fibroin scaffolds.

In another experiment, Gu et al. [64] modified *Bombyx mori* silk fibroin (SF) film by SO_2 gas plasma treatment, or by a two-step process including NH_3 gas plasma treatment and reaction with 1,3-propane sultone to improve the blood compatibility. They determined *In vitro* antithrombogenicity by the method of the APTT, PT, and TT tests. Moreover, the antithrombogencity of treated films was increased remarkably due to surface sulfonation. The results implied a potential use of sulfonated SF for blood-contacting biomaterials.

Tamada et al. [65] investigated the sulfation of Silk fibroin (*Bombyx mori*) using chlorosulfonic acid in pyridine. They observed that blood coagulation was prevented by 0.5 mg of sulfated fibroin in 1 mL of blood, while original fibroin did not show any effect. They also claimed that Anticoagulant activity of sulfated fibroin strongly depends on the amount of sulfate groups introduced.

As reported by Furuzono et al. [19], 2-Methacryloyloxyethyl phosphorylcholine (MPC) was grafted onto silk fabric in a two-step heterogeneous system through the vinyl bonds of 2-methacryloyloxyethyl isocyanate (MOI) modified on the fabric. First, habutae silk fabric was modified with the MOI monomer in anhydrous dimethyl sulfoxide using di-n-butyltin (IV) dilaurate and hydroquinone at 35 °C. Second, graft polymerization with MPC onto the MOI modified silk was conducted using 2,2-azo bis (2-(2-imidazolin-2-yl) propane dihydrochloride) (VA-044) as an azo polymerization initiator. Platelet adhesion was preliminarily tested measuring lactate dehydrogenase. They reported that the number of platelets adhering to polyMPC-grafted silk fabric decreased by about one tenth compared to original and MOI-modified silk after 60 min of contact with human platelet-rich plasma (1.0×10^6 platelets cm^{-2}).

Yagi et al. [66] prepared small-diameter vascular grafts 1.5 mm in diameter and 10 mm in length by coating a double-raschel knitted silk fiber graft with silk fibroin aqueous solution containing poly(ethylene glycol diglycidyl ether) as a cross-

Table 3. List of studies of surface medication of silk fibroin materials by several researchers.

Type of silk fibroin	Modified by	Outcomes	Ref.
Silk fibroin film	Zwitterionic phosphobetaine	Improved the hemocompatibility properties of modified silk fibroin.	[60]
Silk fibroin film	Poly(ethylene glycol)	Exhibited good antiadhesion and antithrombotic properties.	[61]
Silk fibroin fabric	Ferulic Acid	The test results showed that the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) and the whole blood coagulant time (WBCT) of modified silk fibroin are much longer than that of pure silk fibroin.	[62]
Silk fibroin nanofibers	Heparin	The antithrombogenicity of the silk fibroin nanofibrous scaffolds was improved significantly after heparin modification according to the analysis of APTT, PT and TT tests.	[63]
Silk fibroin film	The film was modified by SO_2 gas plasma treatment, or by atwo-step process including NH_3 gas plasma treatment and reaction with 1,3-propane sultone	Anticoagulant properties were improved significantly. Obtained good platelet adhesion and cell compatibility.	[64]
Silk fibroin powder	Chlorosulfonic acid	The antithrombogencity of treated films was increased remarkably due to surface sulfonation.	[65]
Silk fibroin fabric	Graft-polymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC)	The number of platelets adhering to polyMPC-grafted silk fabric decreased by about one tenth.	[19]
Double-raschel knitted silk vascular grafts	Coating fibroin aqueous solution containing poly (ethylene glycol diglycidyl ether) as a cross-linking agent	In vitro showed no early formation of thrombosis.	[66]

linking agent. Eight weeks after implantation of the grafts in rat abdominal aorta, they observed that there was no early formation of thrombosis. Table 3 shows a list of summary of surface modification studies carried out by some researchers to enhance hemocompatibility of silk fibroin materials.

In addition to the blood compatibility tests, some other experiments are also needed for characterizing the surface of the blood compatible materials. A list of the probable tests along with their importance is given in Table 4.

9. CONCLUSION

A great number of data describing the hemocompatibility tests has been accumulated. Therefore, where possible, tests should use an appropriate model or system, which simulates the geometry and conditions of contact of the device with blood during clinical application, including duration of contact, temperature, sterile condition. When possible, the tests should be repeated a sufficient 5-6 number of times. Tests have to be performed within a minimal delay of usually 2 h since some properties of blood change rapidly following collection. Anti-coagulated blood is exposed to the material under the before-mentioned standardized conditions including aspects such as time, temperature and flow. It's hoped that this review might provide some insights into systematic evaluation of hemocompatibility of biomaterials.

ACKNOWLEGDEMENTS

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Table 4. Tests to characterize hemocompatibility of the polymeric materials.

Test name	Importance
Qualitative and quantitative analysis of immobilized bioactive molecules used for surface modification	The amount of heparin immobilized on the modified surface can be determined using the toluidine blue method [67, 5]. Heparin density on the polymer surface can be determined by comparison with standard results and expressed by mass per unit surface area ($\mu g/cm^2$) [68]. Alcian blue staining can be used to determine the presence of heparin on heparin immobilized surfaces qualitatively [69].
Wettability test by static /and or contact angle tester	In general, material hydrophobicity plays an important role in blood compatibility. A hydrophilic surface absorbed fewer platelets, thus leading to longer blood coagulation time [70].
Protein adsorption assay	It was demonstrated that higher protein absorption and protein conformational change always occurred on hydrophobic surface because proteins were difficult to de-adsorb from hydrophobic surface under the effects of hydrophobic bonds [71,72].
Adsorption of fibrinogen and ATIII	Concentration of fibrinogen should be lower and high level of ATIII adsorption is expected for a blood compatible material. Heparin modified surfaces generally shows lower concentration of fibrinogen (5-6 ng/cm^2) and high level of ATIII adsorption (660 ng/cm^2) on its surfaces [73].
Thrombogenicity of the surface	A chromogenic method could be used to test the thrombogenicity of the surface and to examine whether the covalently attached heparin is able to prevent thrombus formation [73].
Scanning electron microscopy (SEM)	The morphology of the modified polymeric surface can be studied by scanning electron microscopy [43].
Atomic force microscopy (AFM)	Surface roughness is an important parameter in biomedical materials, as it may affect cell adhesion [74]. In the case of vascular prostheses, increased surface roughness can compromise hemocompatibility as turbulent blood flow may initiate hemolysis [75]. A commonly accepted fact is that increase the surface roughness can lead to more platelet adhesion, because extra surface roughness usually means larger area exposed to the platelets [76].
X-ray photoelectron spectroscopy (XPS)	An XPS spectrum detects the changes in the chemical characteristics of material modified by certain treatments. XPS exhibits any new atomic signal on the polymers/substrate after the modification. For example, since the elemental ratio of heparin in approximately C:O:N:S=12:14:2:1, an increase in C1s,O1s,the C:O ratio and, as well the appearance of an would be expected on the heparinized surface [73].
Fourier transform infrared (FTIR) spectroscopy	Used to determine the chemical functionalities present in a sample. It may also be used to monitor migration of functional groups to the polymer bulk [77].

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