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Original Article

Fibrin Gel Properties and Gelation Structures for Tissue Engineering Scaffold and Biomedical Engineering Applications

G N Vadival [1], Irza Sukmana [2], A Y E Risano [2], Agus Sugiri [2], and Fadil Hamdani [3,*]

[1] Faculty of Biosciences and Medical Engineering, Block V01, Universiti Teknologi Malaysia, UTM Johor Bahru, 81310 Malaysia
[2] Mechanical Engineering Department, Faculty of Engineering, Universitas Lampung, Jl. Prof. Dr. Soemantri Brojonegoro 1, Bandar Lampung, 35143 Indonesia
[3] Department of Electrical Engineering, Faculty of Engineering, Universitas Lampung, Jl. Prof. Dr. Soemantri Brojonegoro 1, Bandar Lampung, 35143 Indonesia

* Correspondence: fadil.hamdani@eng.unila.ac.id

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Fibrin gel is utilized in a wide range of medical applications, such as hemostatic agents, wound healing, drug delivery, cell delivery, cell differentiation, and tissue engineering. Notably, fibrin gel exhibits exceptional extensibility compared to other filamentous biopolymers, capable of stretching over five times its original length without breaking. Remarkably, it can fully recover from elongations exceeding 100% once the applied stress is removed. This paper presents an optimized formulation of fibrinogen and thrombin tailored for culturing human umbilical vein endothelial cells (HUVEC). We explore the mechanical and physical properties of the fibrin gel, aiming to identify ways to enhance its medical applications. The gel is synthesized in vitro through the combination of fibrinogen and thrombin, allowing us to assess how varying the proportions of these components affects the gel structures and properties

Keywords: Fibrin gel, gelation structures, thrombin, scaffolds, turbidity.

1. Introduction

Fibrin gel is a biomaterial which is widely used in many various biomedical areas including bone tissue, cardiac tissue, nervous tissue and vascular graft and other tissues. This biodegradable material functions as matrices for delivery and proliferation of living cells. Along with the development of tissue engineering and regenerative medicine, fibrin glue has been studied to find new applications [1]. Several studies have been conducted for polymers, including fibrin, gelatin, chitosan, poly (ethylene glycol) and poly (propylene fumarate) extensively to investigate the gelation and cell delivery properties. Among these polymers, fibrin appears to be one of the most commonly used hydrogel, due to the advantages that can be obtained to avoid risks of foreign body reaction and virus infection [2].

Fibrin is essential for blood clotting, fibrinolysis, cellular and matrix interactions, inflammation and wound healing. In the human body, the initial phase of wound healing is the release of fibrinogen to mediate platelet aggregation. This fibrinogen is converted into fibrin by a proteolytic enzyme called thrombin. Platelets then bind to the fibrin strands, macrophages and neutrophils attach to the fibrin strand for the disposal of injured tissues and infection pathogens. The process of wound

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healing is terminated when fibroblast anchors to fibrin when they enter the wound site to repair or replace the damaged tissues [3].

Fibrin gel can be produced in vitro by mixing fibrinogen and thrombin solutions. Studies have proved that different concentrations of fibrinogen and thrombin affects the gelation time of fibrin gel, therefore affecting its mechanical and physical properties. Due to the variation of mechanical and physical properties, fibrin gel can be adapted for various medical uses. Some production of fibrin gel lacks some mechanical properties compared to native extracellular matrix (ECM), therefore strategies have been investigated for the improvement of its properties [4]. It is well known that the mechanical and physical properties of fibrin gel produced in vitro mainly depend on the concentration of fibrinogen and thrombin solutions, This study aims to focus on the effect of fibrinogen and thrombin concentration on properties of fibrin gel and to identify and find the best composition of fibrinogen and thrombin to prepare a fibrin gel for three dimensional environment cell and tissue culture.

Some production of fibrin gel lacks some of the mechanical properties compared to native blood vessels. These properties mainly depend on thrombin and fibrinogen concentrations. Clotting time is influenced by thrombin concentration and the temperature but less influenced by fibrinogen concentration [5]. At high thrombin concentrations, tight networks are formed with more fiber bundles with finer and thinner fibers. As the thrombin concentration is decreased, the average fiber bundle size increases and the gel becomes more porous [6]. Thrombin was an important modulator in the process, thus, increasing concentrations lead to more transparent gels as clotting time decreased. Therefore, fibrin gel has potential use in various medical and bioengineering applications. Fibrin gel is biocompatible and biodegradable which has various applications in medical use. Formations of fibrin in vitro mainly depend on the concentration of fibrinogen and thrombin which affects the gelation or clotting time.

In this study, the different composition of thrombin and fibrinogen which affects the mechanical and physical properties of fibrin gel was investigated. Furthermore, the manipulation of these properties for medical use is investigated. Some aspects is considered to achieve these objectives, including the factors which influence clotting time of fibrin gel, the effect of thrombin and fibrinogen concentration on the clotting time of fibrin gel, and the effects of different clotting time to the mechanical and physical properties of fibrin gel.

2. Materials and Methods

Biomaterials are any matter, surface, or construct that interacts with biological systems. Biomaterials are essential in the development of tissue engineering as the delivery vehicles for bioactive substances or cells. Biomaterials must be designed to easily manipulate and reproducible, which must be biocompatible, bioabsorbable and biodegradable. Biomaterials can be produced in nature or synthesized in the lab using various chemical approaches [6, 7].

Fibrin gel has been recognized as a biopolymer that is formed during the wound healing process in the body. Fibrinogen will be converted to fibrin by an enzyme called thrombin. Fibrin gel can be produced in vitro by mixing fibrinogen and thrombin solutions [8]. Current clinical and bioengineering applications of fibrin gel including Haemostatic glue and wound repair, Drug Delivery, Cell Delivery, Patterning, Cell differentiation and tissue engineering [9]. Fibrin glue can be used instead of sutures or staples to enhance healing, minimizes scarring and eases application. Fibrin is an appealing drug delivery vehicle since it can be injected where it gels in situ, it is degraded naturally and it stimulates the body's own wound healing response. Keratinocytes suspended in fibrin were effective in reconstituting full thickness wounds. Human mesenchymal stem cells have been differentiated into osteoblasts and mouse embryonic stem cells have been coaxed down neural and astroglial lineages, by modulating the mechanical and chemical properties of a fibrin-based matrix.

Human fibrinogen, produced in the liver, contains three pairs of different polypeptide subunits (α, β, γ) [10]. Fibrinogen has an elongated structure with three connected nodules. Nodule at the center is called E, containing the N-terminal residues of all six chains. The chains exit this nodule in two sets of three-chained a-helical coiled coils that lead to the two distal nodules, called D. Each D nodule contains the separately folded C-terminal regions of the Bb and g chains, called bC and gC, respectively. Lastly, the C-termini of the Aa chains, called aC, exit the D-nodule, for a short distance as a fourth chain to the coiled coil, and then extend along the surface of the protein towards the central nodule where they may interact with one another and the E nodule [11,12].

The conversion of fibrinogen to fibrin is catalyzed by thrombin, which cleaves in sequence the N-termini of the Aa chain, releasing fibrinopeptide A (FpA), and the Bβ chain, releasing fibrinopeptide B (FpB). Polymerization sites on the N-termini of the α and β chains, called 'A' and 'B' knobs, respectively are exposed due to the removal of the fibrinopeptides. The newly exposed N-terminal sites interact with polymerization sites, called 'a' and 'b' holes, in γC and βC, respectively, forming 'A:a' and 'B:b' interactions. Polymerization proceeds in two steps: first the formation of half-staggered, double-stranded protofibrils, and then the lateral aggregation of protofibrils into fibers. 'A:a' interactions mediate protofibril formation and 'B:b' interactions may influence lateral aggregation [13].

Thrombin also catalyzes the activation of FXIII, which is bound to fibrinogen and fibrin. FXIIIa is a transglutaminase that catalyzes the formation of isopeptide bonds between g chains of two fibrin molecules, forming g–g dimers, and between chains of several fibrin molecules, forming a polymers. Cross-linked fibrin clots are stronger and more resistant to fibrinolysis than their uncrosslinked counterparts [14].

The study of the mechanical properties of fibrin gel is important because as an example, in hemostasis, the clot structure must be strong enough to form a plug to stop bleeding and to withstand the pressure of arterial blood flow. In thrombus formation, viscoelastic properties will determine whether the flowing blood will cause it to deform reversibly or irreversibly, rupture, or embolize. Many such applications of fibrin gel manipulate its mechanical properties for a better use. [15].

Material used in the experiment includes fibrinogen from bovine plasma, aprotinin from bovine lung, thrombin from bovine plasma, Phosphate Buffer Solution (PBS) and 4% formaldehyde to fix the samples of fibrin gel before Field Emission Scanning Electron Microscope (FESEM). A series of experiments are conducted from the preparation of fibrin gel to the mechanical and physical testing on the fibrin gel. The instruments required includes UV Vis Spectrophotometer (Shimadzu UV 1201), Field Emission Scanning Electron Microscope/FESEM (Zeiss Supra 35 Vp Gemini), Freeze-drying machine (Labconco Benchtop), Water bath, electronic weighing scales, and Cell culture lab equipments (Pipette, Pipette tips, Gloves, Well plates, Spatula).

Fibrin gel was prepared by mixing fibrinogen solution and thrombin solution. Our objective is to prepare 500 μL of fibrin gel by preparing 250 μL of fibrinogen solution and 250 μL of thrombin solution. PBS solution were prepared earlier and stored in a flask. The amount of fibrinogen needed was first weighted using an electronic weighing scales and then it is mixed with PBS solution. In a different well plate, the amount of thrombin stock needed is mixed with thrombin solution. Both fibrinogen and thrombin solution is mixed together in a clean well plate to form 500 μL fibrin gel and left at room temperature for 30 minutes. This is the polymerization process of fibrinogen to fibrin. The volume of thrombin and fibrinogen were manipulated to form fibrin gel with different concentration of fibrinogen and thrombin. As an example, to prepare fibrin gel with concentration ratio of 2.0/1.0 which means 2.0mg /mL fibrinogen and 1.0U/mL were prepared in equal composition to form final concentration of 500 μL of fibrin gel. Four different concentration prepared in this work shown in Table 1. The fibrin gel that was prepared varies in concentration of fibrinogen and thrombin. Therefore some analysis of the fibrin gel required to verify its properties.

Table 1. Fibrin gel compositions

Concentration	Weight (mg)	Volume (μL)		
	Fibrinogen	*PBS*	*Thrombin*	*PBS*
2.0/1.0	4.0	250.0	50.0	200.0
2.0/4.0	4.0	250.0	200.0	50.0
0.5/4.0	1.0	250.0	200.0	50.0
4.0/0.5	8.0	250.0	25.0	225.0

Properties of fibrin gel mainly depend on the clotting time. Some properties of fibrin gel such as clotting time, water uptake property and turbidity were studied. In measurement of clotting time, after vortex mixing the solutions of fibrinogen and thrombin, the gel was left at room temperature. The clotting time was observed manually and when the tip of pipette cannot penetrate the gel that will be the clotting time of that particular fibrin gel. The clotting time of the gel was recorded. In water uptake property, the fibrin gel was soaked in PBS at room temperature for different times. Furthermore, 1000 μL of PBS is transferred into the gel using pipette and soaked in the fibrin gel. After 30 minutes, PBS which does not diffuse to the gel is collected using pipette and the volume is measured. The same procedure is repeated for intervals of one hour, two hours and four hours.

For Measurement of turbidity, another set of fibrin gel was prepared in cuvettes. A change of turbidity is recorded for different time intervals using the UV-VIS Spectrophotometer (Shimadzu UV 1201) using 550 nm wavelength. In microstructure Observation, another set of fibrin gel was prepared in a well plate. The fibrin gel is fixed with 4% formaldehyde. 500 μL of formaldehyde solution were pipette into the respective well plates and left at room temperature for 20 minutes. Then, the formaldehyde was collected using pipette and disposed of. 500 μL of PBS were then pipette to the gel and left for five minutes and then collected and disposed. These procedures were repeated thrice. After this, the samples need to be freeze dried. The well plate without the cover is covered with aluminum foil tied with a rubber band. Needle is used to prick small holes on wells that contain samples. Then the fibrin gel is freeze dried using the freeze drying machine for three days. After that, the samples are coated and observed under FESEM (Zeiss Supra 35 Vp Gemini).

3. Results and Discussions

Fibrin gel can be formed by mixing solutions of fibrinogen and thrombin. After clotting at room temperature, the solution will lose its fluidity and transform into hydrogel. Clotting time highly affects the microstructure and handling properties of fibrin gel which is mostly controlled by the concentration of fibrinogen and thrombin. The higher the thrombin composition, the faster the fibrin glue polymerizes. It can be concluded from the graph on Figure 1, as the thrombin concentration increases from 0.5U/mL to 1.0U/mL and then to 4.0U/mL, the clotting time decreases significantly. This is because more fibrinopeptides were formed per unit time.

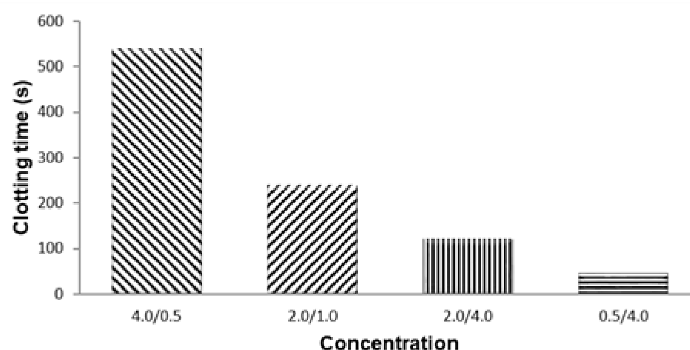

Figure 1. Clotting time versus concentration

Meanwhile, when the fibrinogen concentration increases, the changes in the clotting time is not very significant. The clotting time decreases with the increase of concentration of thrombin. The clotting time is independent of the fibrinogen concentration. Fibrinopeptides which are the monomers of fibrin gel play an important role in the clotting time. The concentration of fibrinopeptide is dependent on the ratio of fibrinogen to thrombin. With the increase of thrombin concentration, more fibrinopeptides per unit time will be produced by the cleavage of fibrinogen leading to shorter clotting time [13,15].

The fibrin gel was soaked in PBS at room temperature for different times. 1000 μL of PBS is soaked in the fibrin gel and the volume of PBS which does not diffuse to the gel is measured after 30 minutes. Higher thrombin concentration results in better water uptake property of the fibrin gel. At a higher thrombin concentration, the cross-linked fibrin gel should have a more complete cross linking structure, which has stronger ability to resist the shrinkage, therefore more water can be absorbed compared to lower concentration of thrombin. From the graph of Figure 2, The concentration 0.5/4.0 has the highest water uptake property followed by concentration of 2.0/4.0, 0.5/4.0, and 4.0/0.5. The shrinkage process is slower in concentration of 0.5/4.0 therefore more water could be uptake. The concentration of 4.0/0.5 has the lowest water uptake property due to the faster shrinkage process of the hydrogel. The water uptake property of fibrin gel is not significantly influenced by concentration of fibrinogen [16].

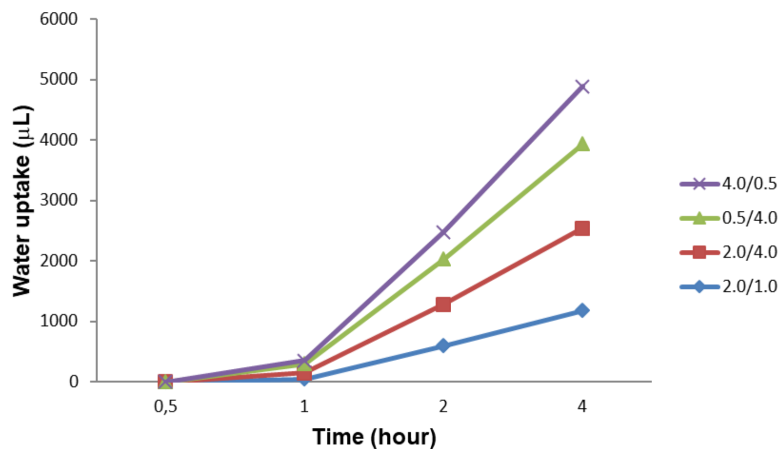

Figure 2. Water uptake property versus time

A change of turbidity measured for different time intervals using UV-VIS Spectrophotometer with the wavelength of 550 nm. Turbidity is defined as a decrease in the transparency of a solution due to the presence of suspended and some dissolved substances, which causes incident light to be scattered, reflected, and attenuated rather than transmitted in straight lines. The higher the intensity of the scattered or attenuated light, the higher the value of turbidity. A spectrophotometer directly measures the amount of light of a particular wavelength transmitted by a substance, and therefore indirectly measures the amount of light of a particular wavelength absorbed by a substance.

In this work, the particular wavelength absorbed by fibrin gel is 550 nm. As the value of absorbance is higher, the intensity of the scattered or attenuated light becomes higher which increases the value of turbidity. Therefore, the concentration of 0.5/4.0 has the highest turbidity followed by the concentration of 2.0/4.0, 2.0/1.0, and 4.0/0.5. With the increase of thrombin concentration, more fibrinopeptides per unit time will be produced by the cleavage of fibrinogen which prevents the light from moving in a straight line. Therefore, more light will be scattered, attenuated, reflected and absorbed by the fibrin gel which increases the value of absorbance and thus increasing the value of turbidity [17]. The concentration of 0.5/4.0 has the highest number of fibrinopeptides, where the gel is more turbid. The lowest number of fibrinopeptides in concentration of 4.0/0.5 makes the gel less

turbid. Turbidity is almost independent of fibrinogen concentration. According to the graph of Figure 3, all the values of the turbidity increases and becomes almost constant as the polymerization process of fibrin gel completes.

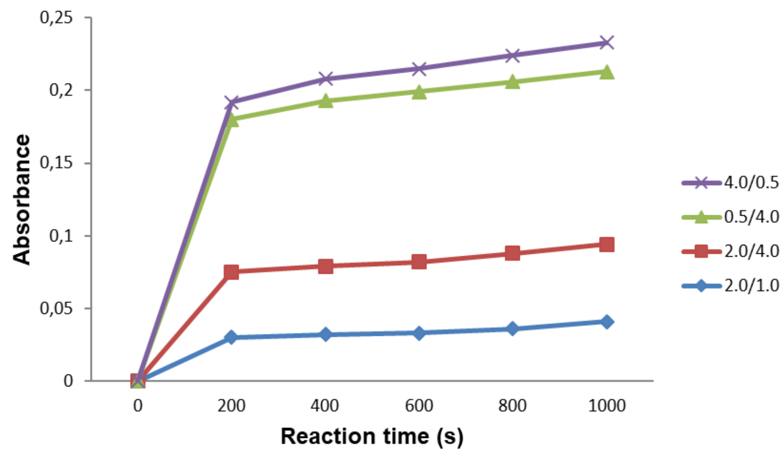

Figure 3. Absorbance versus time

The fibrin gel is fixed with 4% formaldehyde and rinsed with PBS three times. Then, the fibrin gel is freeze dried, coated and observed under FESEM as shown in Figure 4. The fibrin hydrogels are highly hydrated after freeze drying, but they still show characteristics that are affected by fibrinogen and thrombin concentration. The formed fibrin gel, with the concentration of 0.5/4.0, has a very dense structure with thick fibers. It is hardly distinguished from each other because of the low concentration of fibrinogen (0.5mg/mL), as shown in Figure 4(a). As the fibrinogen concentration increases to 2 mg/mL, a slightly loose and porous structure was obtained (Figure 4(b)). At 4 mg/ml, the fibers become thinner and become looser correspondingly (Figure 4(c)). Along with the increase of thrombin concentration, the fibers become thinner but the change is not significant. Slight difference shown by thrombin concentration of 1U/mL (Figure 4(b)) and thrombin concentration of 4U/mL (Figure 4(d)).

The turbidity of the fibrin gel also largely depends on the concentration of thrombin but less dependent on the concentration of fibrinogen. With the increase of thrombin concentration, more fibrinopeptides per unit time will be produced by the cleavage of fibrinogen which prevents the light from moving in a straight line. Therefore, more light will be scattered, attenuated, reflected and absorbed by the fibrin gel which increases the value of absorbance and thus increasing the value of turbidity. Microstructure observation proved that fibrin hydrogels show fibrous structure which is less sensitive to thrombin concentration [18]. As the fibrinogen concentration increases, looser and thinner networks of fibrin gel are formed which can be observed under FESEM. However, the fibrin gel produced in this study has several limitations that become challenges for further research. The fibrin gel produced is not suitable to be used for tissue culture purposes since all materials and instruments used in this study were not sterilized and can only maintain its gel properties for a maximum of two days, as also suggested by others [16-18].

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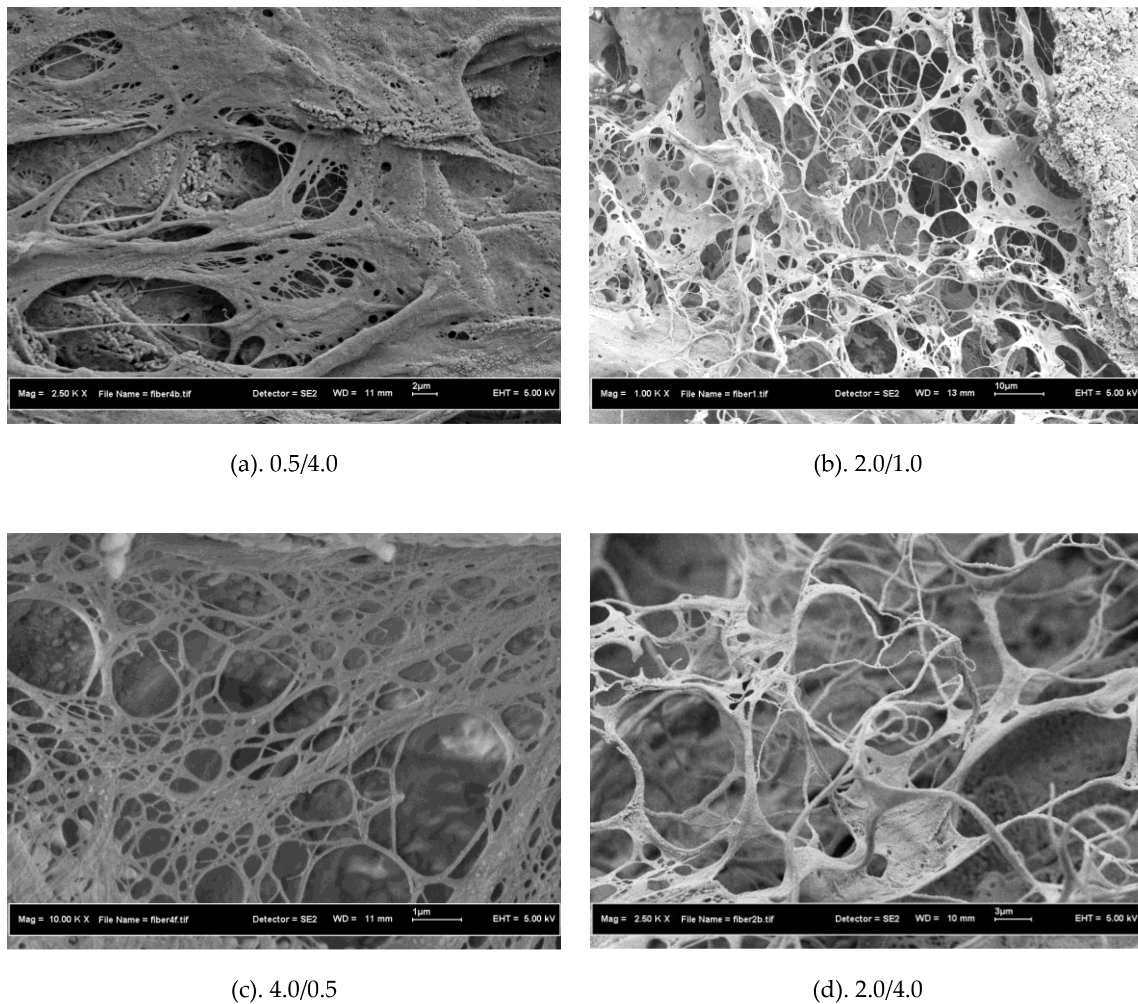

(a). 0.5/4.0

(b). 2.0/1.0

(c). 4.0/0.5

(d). 2.0/4.0

Figure 4. FESEM images of fibrin gel

4. Conclusions

Fibrin gel was successfully made by mixing different concentrations of fibrinogen and thrombin. Clotting time highly affects the microstructure and handling properties of fibrin gel which is mostly controlled by the concentration of fibrinogen and thrombin. The higher the thrombin composition, the faster the fibrin glue polymerizes. Thrombin concentration significantly influenced the clotting time of fibrin gel but less influenced by fibrinogen concentration. The concentration of fibrinopeptide is dependent on the ratio of fibrinogen to thrombin. With the increase of thrombin concentration, more fibrinopeptides per unit time will be produced by the cleavage of fibrinogen leading to shorter clotting time. With higher thrombin concentration, the fibrin gel can maintain its macroscopic shape and therefore has a larger water uptake property compared to gel with less thrombin concentration. At a higher thrombin concentration, the cross-linked fibrin gel should have a more complete cross linking structure, which has stronger ability to resist the shrinkage;. Therefore, more water can be absorbed compared to lower concentration of thrombin. The concentration of fibrinogen does not influence the water uptake property of fibrin gel significantly.

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