# CHAPTER 2

# Literature review

In this chapter compose of relative theories, principles, and literature reviews can be divided into 6 sections;

## 2. 1 Hemostatic agent and fabrication technique

### 2.1.1 Hemostatic agent

Uncontrolled hemorrhage is an important problem in operation [1,2]. During surgery, blood vessels are ruptured and increased the bleeding complication such as hematoma, loss of blood volume, hypoxia, metabolic acidosis, hypovolemic shock, multiple organ failure and cause of death [3,4,29]. Management of bleeding is the primary control in procedures [5,6]. Thus, the hemostatic agent plays an important role in stopping the bleeding. In the past, gauze product was generally used in medical treatment but its blood absorption rate was dependent on the compressive force, and not providing clot mechanism [7,29]. A previous study reported the cotton gauze was absorbed blood within 357 s, and completed blood coagulation time in 17.7 min [23]. Furthermore, the prolonged pressure at the bleeding site may injury of nerves or cell tissue [7,8]. Meanwhile, it was limited the risk of foreign-body reactions, inflammation, and they could not biocompatibility and biodegradation properties as preferring to remove from the surgical site [9]. In the last few years, the hemostatic agent plays an important role as the most effective method to stop bleeding by activated coagulation cascade proteins such as thrombin, fibrin and collagen, and providing a clot formation [5,6,9,10]. Absorbable hemostatic must be safe, rapidly absorbed, effective, non-allergic, and biocompatibility [1]. According to research by the Science and Technology Research Institute, Chiang Mai University, the commercial hemostatic agent should

quickly stop bleeding, effectively absorb, can be degraded in the body within the specific period, have no side effects, work temporary, easy to use, can be stored for long time, and low cost [12]. Hemostatic agent can be two classified [2,9];

**1) The passive hemostatic agent**. It provides physical to initial forming of platelet as lattice-matrix adhere at the bleeding site but it is not strong plug formation [2]. Passive hemostatic agent including a collagen, gelatin, and oxidized cellulose, they are not a biological activity or direct action with platelet activation mechanical and aggregation of clotting [1,9]. A previous study reported Gel was effective to control bleeding in irregular wound and surgical cavities such as the mucosa surface of sinus and nasal surgery. At the same time, applied to use in minimally invasive procedures, anorectal surgery. Gel sponge was general used in neurons and urology surgery [9]. It is well-known that Gel is one of the commercial hemostatic agents such as Gelfoam, it allow to absorb about 40 times its weight in blood and it could expand to 200% of its initial volume, which is control in minor bleeding, effective in bleeds of a small vessel, useful in oral cavity and post dental extraction, while it suitable for use in irregular wound [2]. Gel was a good swelling and promote thrombin for early accelerated platelet clot by collagen component of Gel [3,7,30]. Furthermore, some study reported the SUGIFOAM was blood absorbable in 45 s, porosity 36.39%, and water content 90.69% [10].

In addition, cellulose-derived hemostatic agent have been successfully used in general surgery, neurosurgery, cardiovascular surgery, control capillary, venous and small arterial bleeding, and oozing control [9]. Surgicel® is one of the commercial hemostatic agents from oxidizing cellulose polymer which depends on two acidic reactions for stop bleeding. The first process, carboxylic acid groups of Surgicel® was decreasing pH of tissue, and cause of artificial blood clot by acid etching. Then, the secondary platelet activates forming the temporary platelet plug and completely clotting. Thus, the acidic property is the benefit of clot mechanism. Surgicel® is achieved hemostasis with compression force mechanism of 2-4 min at surgical site, and it could not depress at the nerve cell such as inferior alveolar nerves. It must be used at dry without addition of saline. On the other hand, it should not to use in closed spaces, bone fractures, the large arteries bleeding, granuloma and neurological surgery.

In meanwhile, the acidic of Surgicel® can be irritate epithelialization and burning sensation in the nasal cavity, sinus lifting and tear sinus mucosa. Furthermore, it has limited of antimicrobial, foreign body reaction, so after surgery must be use the antibiotic treatment for prevent infection [2,31]. In the last few years, RS was a performance absorbable. Starch granule was a gelatinization property and good swelling. A previous study reported the medical grade of Thai RS 2.5% (v/w) with crosslinking agent of 1% GA has been a water absorption 40.6% and RS hydrogel 50 mg could be fast biodegradation of 20.05 min within 1% amylose solution [19].

**2) The active hemostatic agent** has a biological activity [9] and directly induced the coagulation cascade to form a fibrin clot by thrombin. They were combined with a passive agent to improve hemostasis mechanism. [2]. For example, CS hemostatic agents were achieved fast absorbable the serum proteins while directly activate platelet, integrin complex and calcium signaling binding with materials surface. Moreover, the hemostatic agent of CS can be related positive charge of the amino backbone which interacts with a negative charge of RBCs membrane by electrostatic mechanism [5,11,32] The commercial of CS was widely used, such as HemCon dressing was fabricated from shellfish CS that received the approval of FDA 2003, it is a fast absorbance less than 30 s [2,5,29,33]. In meanwhile, Celox is a commercial hemostatic agent of a various polymer CS compounds. It achieved control bleeding with compression force in 3 min, and which is received FDA approval in 2006 [4,5,7,30]. A previous study reported CS 2.0% (v/w), molecular weight 70 kDa, and 83% of deacetylation degree, it was fabricated with freeze drying process, that presented the fast of blood absorption less than 5 s, and accelerated early blood clotting after 30 s, while a high water content 95.12%, good porosity 85.57% and ESR 36% [11]. Meanwhile, some study achieved CS 2.0% (v/w): RS 2.5% (v/w) as 1:1 ratio, presented the rapid blood absorption in 10 s, a high water content 97.33%, good porosity 86.84% and ESR 76.64% [10].

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### 2.1.2 Fabrication technique

A previous study reported sponge and sheet forming are appropriate to fabricate the hemostatic agent, while they can be apply in general surgery and several procedure such as hepatic, orthopedic, and vascular surgery [9]. Some study reported the CS sponges (3D) were fabricated by lyophilization or freeze-dry technique, this exhibited high porosity, smaller pore size, high surface roughness, high absorbable properties, and good swelling [1,21,31]. Meanwhile, freeze dry process in the low temperature of hydrogel that presented the large pore size of Gel sponges [5]. Freeze drying is a removing process of the water from sensitive products, biological materials, without damage the structure of the material, and can be preserved easily. This process as shown in Fig. 1. and it can be divided into three steps as;

1) **Freezing process** is a change solid phase to the gaseous phase, material to be freeze-dried must first be adequately pre-frozen. The product to be dried is frozen under atmospheric pressure.

2) **The primary drying process** is an initial water solid state (ice) to change the vapor state without passing through the liquid state under vacuum, pressures, and temperature below triple point. The high vacuum to heat, frozen liquid sublimes leaving only solid, dried components of the original liquid. This process requires very careful control of the temperature, and pressure involved in the freeze-drying system.

3) **The secondary drying process** is an isothermal desorption. After primary freeze-drying is complete, and all ice has sublimed. The sample was dry, but the residual moisture content that still present. The continued warmer temperature was necessary to reduce the moisture from the sample and the bound water is desorbed from the product [34].

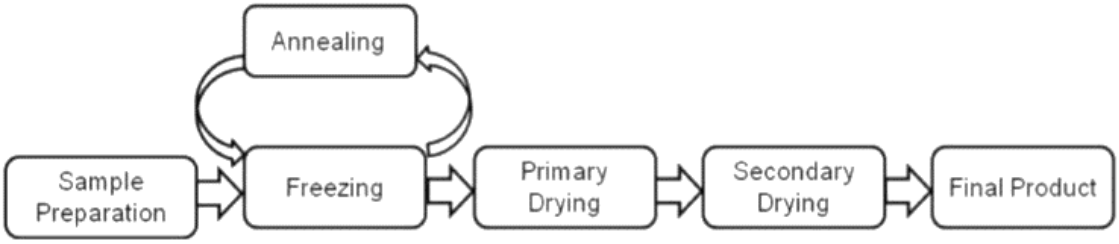


Fig. 1. Lyophilization or freeze drying process [34]

**2.2 Biomaterials**

The most accepted definition of biomaterials is currently the one employed by the American National Institute of Health that describes biomaterial as ‘‘any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual’’ Based on the reaction of the tissue to the biomaterial, these are classified into three distinct categories, The first is a biotolerant materials, they separated from the bone tissue by a layer of fibrous tissue. The second is a bioactive materials, they have the property of establishing chemical bonds with bone tissue, known as osseointegration, such as the collagen, and mineral phase of the adjacent bone that deposited directly on the implant surface. The third is a bioinert materials, they have direct contact with the adjacent bone tissue, and no chemical reactions between the implant, and the tissue. An active interface between biomaterials with biological systems, leading to several important basic ideas about biocompatibility. The interactions at the material-tissue interface which is a response from the body and the body elicit a response from the material. Furthermore, all of the materials will be changed with biological environment-either via corrosion, chemical modification, deposition of substance, degradation, or another mechanism. This exchange of responses leads to the dynamic of the material-tissue interface. Thus, the interface is not static, and always changing in a lifetime by aging. Biomaterials are foreign bodies and biological responses to biodegradability [14]. Biomaterials were widely used in several

application in medical, which are safe, effective, good biocompatibility, and biodegradation. The biocompatibility includes the natural materials coat with polysaccharides, absorption of the protein-energy surface, and covalent immobilized of the hemocompatibility membranes, such as collagen, gelatin, and chitosan surface. Furthermore, the polar groups of biomaterial such as carboxyl, hydroxyl, and amine group achieved the hydrophilic surface [35]. The trends of biodegradable are one of the indicated new development materials [23]. The hemostatic agent of biomaterials was not damaged the RBCs or interrupted the protein activity [5].

### 2.2.1 Chitosan (CS)

Chitosan is an unique biopolymer. It was exhibited both of physicochemical, and biological properties such as good swelling, biocompatibility, non-toxicity, biodegradability, and antimicrobial activity [1,3,23,36]. CS is a natural polymer derived from crustacean shell, crab exoskeleton, and squid pen [1,11,37]. CS is compose of a linear polysaccharide of N-acetyl and D-glucosamine units (Fig. 2.), which are derived from the deacetylation of chitin. A previous study reported the shell CS hemostatic agent of DD 96% could be induced RBCs aggregation than CS with DD 74% [21]. The deacetylation degree (DD) presented the number of amino groups, the cause of chemical hydrolysis (hydrogen bonding), and polycation from ionic complexes such as the negative charge of erythrocyte on RBCs membranes, proteins, DNA, and lipids. Meanwhile, polysaccharide of CS can stabilize a covalent bonding and it is non-specific binding with other particles. Thus, high DD also increase the number of positive charges, permeability, and the electrostatic force [1,2,23,32]. Furhtermore, the polar groups of CS are react between amine ($NH_2$) and hydroxyl (OH) group, and the hydrophilic property was mainly affected by $NH_2$ group [18,20,22,32]. The swelling property of CS was leading to the high absorb the serum proteins or fluids in RBCs, it was accelerated platelets and combined with CS surface receptor, and it increased the rapid of clot formation [21]. As a consequence, CS is relevant in the field of biomaterials. For example, CS in 3D-scaffold, gel, film, and sponge form. The hydrogels of CS were interested biomaterials study, it is a high water component, and compatible of living tissue. Moreover, the hydrogel can activate with non-covalent, non-ionic, and hydrophobic materials. Some study reported the thin film layer technique

could not polycation effects of CS [6,22,23,32]. CS could dissolve in water, alkali, inorganic acid, aqueous acetic, and formic acids by contributed with amino groups [38,39].

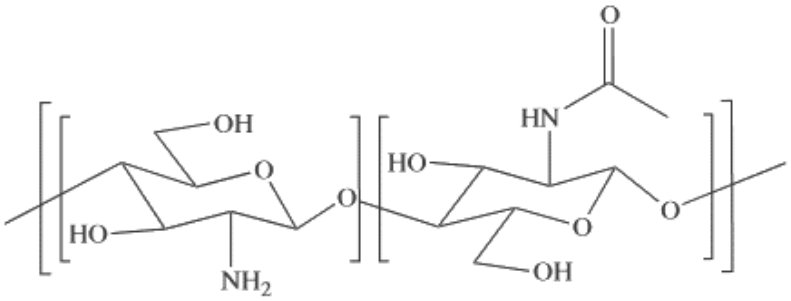


Fig. 2. Chemical structure of chitosan [31]

### 2.2.2 Gelatin (Gel)

Gelatin was prepared from pork skin or bovine [9] which is relevant in the many fields of powder, and sponge of sheet and able adjust to irregular wounds [3]. Gel is compose of collagen and the amine group, and it is a natural polyanion category, which is activated the platelet plug factors [19,32,40]. The protein chains of Gel have immobilized the water by gelatinization properties. A previous study reported the freeze-drying of Gel sponge was create a big pore size and allow quickly permeability the high volume of blood flow [1]. Thus, Gel hemostatic agent was exhibited early physical reaction of hemostasis mechanism, hydrolysis properties, and the rapid swelling by protein chains immobilized a large amount of fluid, and blood plasma serum. In the meantime, it combined with thrombin and release clot factors, this mechanism can leading to a high concentration of blood clotting, and instantly stop bleeding [9,16,19]. Furthermore, it was activated with macrophage and enhanced the wound healing [5,41,42].

### 2.2.3 Rice starch (RS)

RS is the most widely of basic food in Asia, which is easy to find in Thailand, low cost, and several filed of biomedical application [10]. The RS is a polysaccharide polymer with carbohydrate compositions, it is a compose of amylose

and amylopectin [17,18]. Heat up the starch granule of RS induced the gelatinization properties, protein denaturation, broke crystalline, stimulated amylose granule, and increased the swelling properties, and water absorption. Meanwhile, amylopectin of RS enhanced the anion exchange, and allow the water permeability [16,43]. On the other hand, RS is uniform and poor mechanism, whereas the disadvantage of RS exhibited a good biodegradation [15].

### 2.2.4 Glutaraldehyde (GA)

GA is common widely crosslink-agent for biomaterials, include free amino group and hydroxyl group [44]. GA is a linear 5-carbon dialdehyde, clear liquid, pale straw-color, and it could solute in water, alcohol, and organic solvent [45]. A previous study reported the chemically cross-linked of CS hydrogel is depends on GA, which is obtained, stabilized, and fast formed the hydrogel properties. GA has induced a rapid polymerization but they cloud not activate hemostatic mechanism [9,32,39]. Some previous study reported, the higher GA may decrease blood absorption rate property [16].

## 2.3 Plasma technology

Plasma is the fourth stage of matter from gas discharge, it is activated high energy, excited ionized gas, and mixtured of ions, free electron, and neutral gas atoms [35,37,46,47]. Plasma technology is an easy performance, low cost, and it is widely emerging filed in medicine such as blood clotting, wound healing, improve cell adhere in tissue engineering, against bacteria in dentistry, drug delivery, or sterilization of equipment [20,25,27,48,49]. Meanwhile, indirect or non-thermal plasma is one of an technique for modified or treated many surfaces of medical materials such as metals, polymer, and ceramics because it was effect with the physical or chemical of the surface morphology, but it not interrupted structure of materials [24,50,51]. A previous study reported plasma treatment could chemical modified the CS scaffold, and it was ability to activate cell adhere with molecules of collagen, fibronectin, fibronectin, laminin, and integrins for nerves cell repair [52].

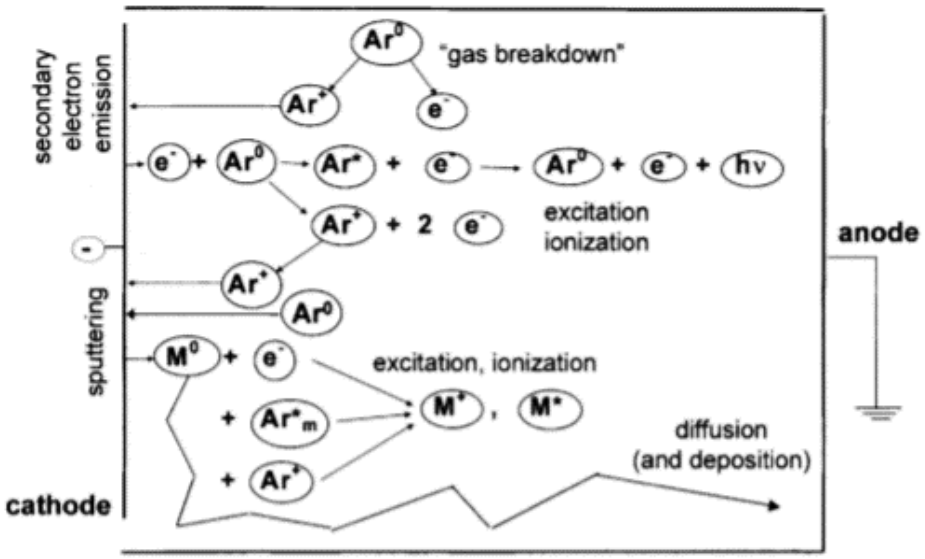


Fig. 3. Schematic of the basic plasma processes [36]

The collisions energy transfers of plasma can be classified elastic, and inelastic collisions. Elastic electron-molecule was increased the kinetic energy, which is the energy transfer less than the ionized potential, bound electron, and jump to a high level in the excitation stage. Afterward, electron return to the lower level energy stage or relaxation stage, the cause of the inelastic collision, lead to dissociation, ionization of molecules, and formation of plasma species (free radical, excited metastable, and ions) as shown in Fig. 3. [37,47,53].

### 2.3.1 Atmospheric pressure plasma jet (APPJ)

APPJ is one of a non-thermal plasma which is common widely modified surface and treated of the heat-sensitive materials, it is a flexible, reliable, non-expensive, and easy implement [38,46,54,55]. Non-thermal plasma can discharge an ion more than a thermal plasma technique. The high energy of electron was induced the molecule to excitation, ionization, dissociation stage, and breaking of chemical bonding [56]. APPJ was specific target point, penetrate of a milliliter to few centimeter on the surface, and it was ability to treat with the irregular surface of 3D shape. A previous study reported APPJ exposure with Ar was immediate increased the high atomic

oxygen, OH radical, and they incorporated with a polar groups (such as hydrogen bonding), leading to plasma cross-linking radicals and chemical etching on the surface, In the meantime, they were increased the surface roughness, and the hydrophilic property [36,55,57]. Moreover, these technique was used to decrease the surface tension and remove unwanted particles for the cleansing material process. Meanwhile, a low temperature of APPJ was achieved the degradability of polymer [28,35]. The atmospheric pressure can be divided in several sources, such as direct current (DC) discharge, alternating current (AC) discharge, radio frequency (RF), and microwave (MW) [57]. A previous study reported APPJ that well-known to perform with various gas species, and widely driven with RF power supply because RF source was ability to discharge a variety of input power level, and high volume, while this source was produced the stable of ion plasma. Furthermore, it that can be modified polymer surface, the cause of hydrophilicity, and cell adhesion [35,56]. Plasma jet is an easy device, it is compose of gas nozzle with electrodes, and driven by a general power supply [24,51,57].

Inert gas (such as helium, neon, and argon) were used in an energy transfer plasma process and modified the physical surface properties. Argon is a mostly to use in a gas discharge, which is effective to induce the polar groups on the surface [24,26,58]. Ar plasma is an effective better energy transfer than helium (He) while the lower cost in RF supply [38]. A previous study found reported the Ar plasma treatment was affected with $NH_3$ of CS membranes, it increased the film surface roughness, ionic permeability, improve cell adhesion, and blood clotting properties. In the same time, OH bonding was indicated absorbable of the water molecule, and ionic complex on the CS surface [20,32,36]. The plasma treatment of argon would not create the new functional groups, it was changed of the physical property of surface, but the Ar gas plasma could discharged free radical on the surface and then, free radical react with $O_2$ leading to increase the concentration of $O_2$ from the atmosphere [35,36].

In addition, Oxygen is a commonly of gas discharge, this effect of oxygen could be induced active site to etch reaction, and influence surface wettability [54]. The oxygen plasma could be discharge the different oxygen-containing group such as -OH, -C-O, -C=O, -O-C=O, -COOH [35,50,52,59]. Moreover, the oxygen functional

groups increased the reinforce with $H_2O$ molecule in the air surrounding, and leading to the strong hydrophilicity reaction [49,60]. A previous study reported the $Ar/O_2$ plasma was grafting of polar groups, they increased the surface energy, decreased the surface tension and provided the hydrophilicity [28].

### 2.3.2 Modification of surface

Surface modification is an easy method for changing the physical or chemical properties of a covalent bonding and react with the functional groups onto the surface. A previous study reported the inert gas of plasma treatment was direct reactive with $O_2$ plasma, OH, COOH, and free radicals onto $NH_3$ of the CS surface [35]. Etching is the methods of remove unessential surface materials, surface modification, roughness mechanism, and it could be increase wettability [26,28,54]. Some study reported Ar plasma modification was exhibited physical etching, it was indicate significant difference to increase surface roughness of the CS wound dressing [59,61]. In the meantime, hydroxyl, and carboxyl groups were catalyzed of the main chain, and oxygen radical created the chemical etching, the surface roughness, and they were increased the hydrophilic properties or wettability [35,61]. Plasma etching can be divided in 4 classified as:

**1) Sputtering-etching** is a physical changed, and non-selective process. It was efficient to remove the particles of materials from the target, and increased surface roughness which are rising the angle more than 60-80º from the normal surface.

**2) Chemical etching** is an atom, and radical from the plasma discharge, that are projectile forming, and react with the surface material in the gas phase.

**3) Ion-enhanced energetic etching** is a combination of etching particle and energetic ions that much more effective mechanism than the separate of sputtering or chemical etching.

**4) Ion-enhanced inhibitor etching** was provided etching particle, energetic ions, and inhibitor precursor molecules from plasma discharge [37].

### 2.2.3 Optical emission spectroscopy (OES)

OES is the equipment of radical spectrum analysis software in the spectrum range of 200-1000 nm [54,62]. The plasma emission occurred from the energy transfer to the inner electrons, and it was enough for the exciting, and unstable stage. Meanwhile, the electron released the photons and returned to the lower energy stage, which is cause of the of the spectrum presentation, whereas the low-pressure plasma could not observe the broader emission energy [47]. The excited of argon that found in 696-1000 nm. Spectrum detected the emission of OH radicals (OH•) at 280-309 nm, and active atomic oxygen radical (O•) at 777-844 nm [23,25,60]. Furthermore, the high power input was effective to relate the high energy range 2-5 eV and increase the intensity of atomic oxygen [38].

## 2.4 Hemostatic mechanism

Hemostasis mechanism is a natural clotting process, initial cellular repair, and wound healing. Hemostasis mechanism can be divided in three processes [2,3];

### 2.4.1 Vasoconstriction

The first step of immediate blood vessel constriction by endothelium and that released paracrine within damage site, and then they are decrease blood volume.

### 2.4.2 Platelet plug formation

The second step is a platelet plug formation. This process is depend on collagen release cytokines, and platelet factors such as adenosine diphosphate, fibronectin, thrombospondin, fibrinogen, and growth factor into the area of injury site, and they activates platelet aggregation.

### 2.4.3 Coagulation mechanism

The third step which accelerated fibrin, and other coagulation cascade, and stabilizes platelet clot formation. The platelet plug is an important role to increase adhesion, and reduce blood loss [5]. In addition, in this mechanism compose of the clotting cascade pathway (Fig. 4.);

**1) Intrinsic pathway** is initiate after surface injury, and that are contact blood as converted prekallikrein and activates factor XII to XIIa, XI to XIa. Afterward, Ca and factor XIa activated factor IX to IXa, and factor X to Xa [5].

**2) Extrinsic pathway** is release the tissue factors, and it is initial the forming factor III complex with Factor VIIa, and then they activated factor X to Xa [2].

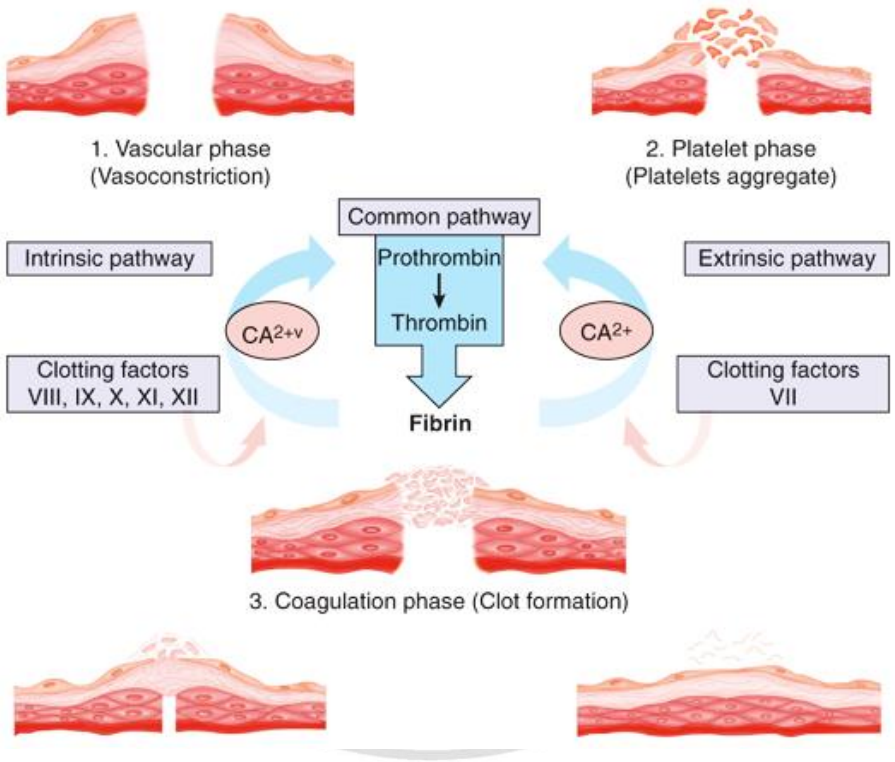


Fig. 4. Coagulation cascade composes of the intrinsic and extrinsic pathway [14]



Both intrinsic, and extrinsic coagulation pathway activated thrombin and converts fibrinogen to fibrin (clotting cascade), while factor XIII, and platelet enhance the fibrin clot formation [9]. The platelet is an early healing process after injury 24 h, the platelets adhesive reacted with integrin receptors and stimulates the migration and adhesion of fibroblasts, keratinocytes, and endothelial cells. In the same time, the aggregation of platelets were released the α granules, and numerous growth factors, such as PDGF, TGF-α, TGF-β, bFGF, IGF-1, and VEGF, which they initiated the inflammatory response step, and wound healing process. Then, the proliferation phase is an initial after 2-3 days. The first step, fibroblasts secrete IGF-1, bFGF, TGF-β, PDGF,

and EGF. Afterward, the endothelial cells synthesize VEGF, bFGF, and PDGF. Meanwhile, keratinocytes synthesize TGF-α, TGF-β, and KDAF (an autocrine factor that derives from keratinocytes). In the same time, the fibrin and fibronectin network are replace the temporary matrix by collagen matrix, enriched in proteoglycans, glycosaminoglycan, and noncollagenous glycoproteins, leading to tissue structure [42]

A previous study, CS hemostatic agent enhances the wound healing process by activated platelet to release growth factor-AB and transforming growth factor-β1 [7]. Some study reported CS enhances the functions of inflammatory cells, growth factors, promote granulation, and remodeling of damaged tissues of wounds. CS hydrogel interacts with fibroblast growth factor (FGF-2) on an open wound surface. These interaction accelerates in a contraction of the wound, formation of granulation tissue, closure, and healing of the wound. Furthermore, CS film layer attached to an inner layer of porous membrane, enhance the proliferation of fibroblasts and forming a monolayer to cover the wound surface. Meanwhile, CS has supported the adhesion, accelerate the serum plasma, and extracellular matrix proteins (ECM), and it activated the platelets [14]. On the other hand, a previous study reported the collagen is one of the component of Gel, and it activates clot factors at injury site on the early stage of hemostatic mechanism, and a glycoproteins from α granules of platelet (fibrinogen, fibronectin, thrombospondin, vitronectin, etc), while the prothrombin creates active thrombin, transform fibrinogen to fibrin, and clot formation [41,42].

## 2.5 Characterization of the hemostatic agent

### 2.5.1 Blood absorption properties

Several previous study attend to blood absorption rate [10,11] and according to research by the Science and Technology Research Institute, Chiang Mai University, the hemostatic materials should quickly stop bleeding, and effectively absorbed [12]. Thus, the volume of blood absorption is a necessary measurement for the effective hemostatic agent. This study was implemented the maximum volume of blood absorption testing.

### 2.5.2 Swelling property

The water absorption capability or swelling properties are important to evaluate the effect of biomedical material [1,21]. A previous study reported CS exhibited the water uptake, the swelling property stabilized the hydrogel form of materials, the hydrogen bonding allows the permeability and polyanion [32]. In the meantime, the hydrophilicity of CS was fast absorbed the serum proteins [5]. The swelling properties were changed to hydrogel forming, and which are mechanism quickly to stop blood flow [6]. Swelling property was determined by the percentage of water absorption after immersed the samples in PBS solution [6,10,11]. The equilibrium swelling ratio of the hemostatic agent was measured by the equation [21]:

$$ESR\ (\%) = \frac{Ww}{Wd}\ x\ 100 \qquad (1)$$

$W_d$ is the weight of the sample before immersed in PBS solution.

$W_w$ is the weight of the sample after immersed in PBS solution.

### 2.5.3 Determine of porosity

The porosity testing was determined based on Archimedes' principle. This method is measurement the void spaces of material by using the hexane permeate into pore structure [10,11]. The tissue engineering application should the high porosity more than 85%, and it appropriated of cell growth [21]. A previous study reported Gel sponge has a big pore structure, and it allows the high flow of fluids, and quickly absorbed, whereas CS hemostatic agent has a smaller pore size, and slower absorbed than Gel sponge, but the advantage of small pore that could be trap the RBCs particle, and induced the clot formation [1]. The percentage of porosity was calculated by the equation [22]:

$$Porosity\ (\%) = \frac{V1-V2}{Vs}\ x\ 100 \quad (2)$$

$V_1$ is the volume of hexane before immersed in the sample.

$V_2$ is the volume of hexane after immersed in the sample.

$V_s$ is the exterior volume of the sample.

### 2.5.4 Hemoglobin leak testing

The effectiveness of the hemostatic agent should be rapid the clotting acceleration. The hemoglobin leak testing modified from a previous study. The clotting ability of the hemostatic agent was observed by the leakage of hemoglobin from the sample after immersed onto the DI water. If the sample was not accelerated clotting ability, RBCs would not entrapped and they hemolyzed onto the DI water. The soaking water of each sample has measured the absorbance at 540 nm (UV-VIS spectrophotometer). A previous study reported the CS hemostatic agent exhibited the faster clot formation than the Gel hemostatic agent [5,11].

### 2.5.5 Biodegradation

Actually, biodegradability is important properties of tissue engineering. The trends of biodegradable are one of the indicated new development materials. A previous study reported the proteases and lysozyme can also break the glycosidic bonds of the polysaccharide of CS and could degrade the particle of CS to oligosaccharides, and then they combine with the metabolic pathway, and excreted, whereas the several study reported Gel sponge with the high concentration of glutaraldehyde cross-linked could be increase the mechanical property, but it may be reduce slowly degradation property [1,5,22,32]. In addition, plasma treatment was achieved to increase biodegradable of polymer surface [35]. Degradation of polymer surfaces mainly occurs when a rapid interaction with radicals or ions takes place. Plasma treatment of polymer surface causes not only a modification during the plasma exposure, but also leaves active sites at the surface also call aging [61]. Biodegradation was performed to confirm the degradation ability in the human mimic condition of the fabricated hemostatic agent in every ratio. The rate of degradation was compared

between the final weights of the sample after freeze dry ($W_o$), and the initial weight of the sample ($W_t$). The percentage of biodegradation was calculated by the equation [22]:

$$Degradation\ (\%) = \frac{Wt - Wo}{Wt} x\ 100 \quad (3)$$

$W_o$ is final weight of the sample after freeze dry.

$W_t$ is initial weight of the sample before immersed in PBS with lysozyme.

### 2.5.6 Cell culture, biocompatibility testing, and cytotoxicity assay

The toxicity of biomedical material is a serious consideration [5]. Residual proteins may be induce the allergy effect. Thus, biocompatibility is necessary proving in vitro assay study. A previous study reported the high proteins or DD values of CS could be increase biocompatible. On the other hand, some chemical modification techniques, such as an acidic solvent, and chemically cross-linked with the high concentration of the glutaraldehyde solution may be increase the toxicity. Fibroblast cell with 3-(4,5-dimethylyhaizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is widely method for cytotoxic evaluation [22,32]. The percentage of cell viability was calculated by the equation [23]:

$$Cell\ viability\ (\%) = \frac{ABS\ sample}{ABS\ control} x\ 100 \quad (4)$$

$ABS_{sample}$ is the absorbance of cells test with the sample.

$ABS_{control}$ is the absorbance of reference cells or negative control.



## 2.6 Design of experiment (DOE)

Design of experiment is widely used in many application such as design the fractional factorial, central composite design, influence problem of process factors, predict product properties, monitored, and optimized. DOE is the statistical experimental design, the planning, and execution of the experiment. In addition, DOE application was implemented in preparation and modification of mixtures. Mixture design use to predict, and change the properties effect of the mixture.

Nowadays, many commercial products are manufactured, and mixture such as pharmaceuticals, gasoline, plastics, paints, and many types of food and dairy products. In mixtures, the number of various ingredients, or constituents more than 10 important components. However, the experimenter that change the composition, and properties of a mixture may beneficially use DOE, the application takes place in the laboratory and the full-scale production. The sum of all components adds up to 100%. This means that these components, mixture factors, cannot be completed independently of one another and that their proportions must lie somewhere between 0 and 1. Some problems could not present in conventional process design applications in terms of squares, cubes, or hypercubes. Fig. 5. shows the region of two-dimensional simplex, a triangular domain, the midpoint of edges, the interior part, and at the overall centroid. The simplex-shaped is a regular experimental region, which is no lower or upper that bounds other than 0 and 1 on the proportions of the three excipients, and 2 there is only one external constraint, and the sum of all constituents must be 100%. This design is a linear model, useful in the experimental objective for screening testing, whereas the designs supporting optimization of quadratic or special cubic models. Screening is used in the beginning of the experimental procedure for investigating large numbers of factors. Optimization used for finding a factor combination corresponding to the last test before the release of a product, and ensure that one stays within specifications [64].

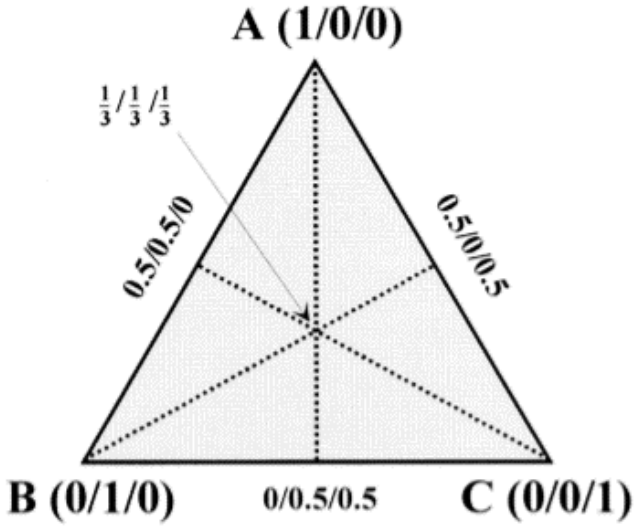


Fig. 5. The simplex-shaped mixture region for a three-component mixture