

HEMOSTATIC DRESSING

I. Field Of The Invention

The present invention relates to a hemostatic dressing that comprises a plurality of layers that contain resorbable materials and/or coagulation proteins.

5 The hemostatic dressing is useful for the treatment of wounded tissue.

II. Background Of The Invention

The control of hemorrhage (bleeding) is a critical step in first aid and field trauma care. Unfortunately, the occurrence of excessive bleeding or fatal
10 hemorrhage from an accessible site is not uncommon. J.M. Rocko *et al.*, *J. Trauma* 22:635 (1982). Mortality data from Vietnam indicates that 10% of combat deaths were due to uncontrolled extremity hemorrhage. *SAS/STAT Users Guide*, 4th ed. (Cary, NC: SAS Institute Inc; 1990). Up to one third of the deaths from exsanguination during the Vietnam War could have been prevented by the
15 use of effective field hemorrhage control methods. *SAS/STAT Users Guide*, 4th ed. (Cary, NC: SAS Institute Inc; 1990).

Although civilian trauma mortality statistics do not provide exact numbers for prehospital deaths from extremity hemorrhage, case and anecdotal reports indicate similar occurrences. J.M. Rocko *et al.*, *J. Trauma* 22:635 (1982). These
20 data suggest that a substantial increase in survival can be effected by the prehospital use of a simple and effective method of hemorrhage control.

Liquid fibrin sealants have been used as an operating room adjunct to hemorrhage control. J.L. Garza *et al.*, *J. Trauma* 30:512-513 (1990); H.B. Kram
25 *et al.*, *J. Trauma* 30:97-101 (1990); M.G. Ochsner *et al.*, *J. Trauma* 30:884-887 (1990); T.L. Matthew *et al.*, *Ann. Thorac. Surg.* 50:40-44 (1990); H. Jakob *et al.*, *J. Vasc. Surg.*, 1:171-180 (1984). The widespread use of fibrinogen and thrombin was common in the last year of World War II, but was abandoned because of the transmission of hepatitis. D.B. Kendrick, *Blood Program in WWII* (Washington, DC: Office of the Surgeon General, Department of Army; 1989), 363-368.

30 Single donor fibrin sealants have been widely used clinically, not only for hemorrhage control but in various surgical situations. W.D. Spotnitz, *Thromb. Haemost.* 74:482-485 (1995); R. Lerner *et al.*, *J. Surg. Res.* 48:165-181 (1990).

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The American Red Cross and others have developed plasma protein purification methods that seem to eliminate the hepatitis risk. Reiss *et al.*, *Trans. Med. Rev.* 10:85-92 (1996).

5 A dry fibrinogen-thrombin dressing (TACHOCOMB™, Hafslund Nycomed Pharma, Linz, Austria) is also available for operating room use in many European countries. Schiele *et al.*, *Clin. Materials* 9:169-177 (1992). Present formulations of this dressing use bovine thrombin. While this fibrinogen-thrombin dressing requires no premixing and is easy to use, its utility is limited by a requirement for storage at 4°C and the necessity for prewetting with saline solution prior to application to the wound.

10 A hemostatic sandwich dressing has been described, which contains a layer of thrombin sandwiched between layers of fibrinogen (see, e.g., PCT/US99/10952, which is incorporated herein by reference). Although such dressings can be used in methods for treating wounded tissue, such conventional sandwich dressings can become delaminated, whereby the edges of the layers of the dressing no longer adhere to each other. Such delamination can result in reduced interaction of the dressing components layers, with decreased effectiveness of the dressing in preventing hemorrhage.

20 III. Summary Of The Invention

The present invention provides a hemostatic dressing (e.g., a bandage) that includes a layer of thrombin sandwiched between a first and a second layer of fibrinogen, wherein the thrombin layer is noncoextensive with the first and/or second fibrinogen layer. Such a hemostatic dressing is useful for treating wounds and offers the unexpected advantage of inhibiting delamination of the layers, as compared with dressings in which the thrombin layer is coextensive with the entire first and second fibrinogen layers.

25 Thus, the present invention provides a hemostatic dressing which comprises: (i) a first fibrinogen layer; (ii) a thrombin layer adjacent to the first fibrinogen layer; and (iii) a second fibrinogen layer adjacent to the thrombin

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layer, wherein the thrombin layer is non-coextensive with the first and/or second fibrinogen layers of the hemostatic dressing.

5 A related dressing of the invention comprises: (i) a resorbable material layer; (ii) a first fibrinogen layer adjacent to the resorbable material layer; (iii) a thrombin layer adjacent to the first fibrinogen layer; and (iv) a second fibrinogen layer adjacent to the thrombin layer, wherein the thrombin layer is noncoextensive with the first and/or second fibrinogen layers of the hemostatic dressing.

10 In another embodiment, the hemostatic dressing comprises: (i) a first fibrinogen layer; (ii) a resorbable material layer adjacent to the first fibrinogen layer; (iii) a thrombin layer adjacent to the resorbable material layer; and (iv) a second fibrinogen layer adjacent to the thrombin layer, wherein the thrombin layer is non-coextensive with the first and/or second fibrinogen layers of the hemostatic dressing.

15 The invention also includes methods for treating wounded tissue in a patient, which comprise applying any of the novel hemostatic dressings described herein to wounded tissue. In such methods, the hemostatic dressing can be hydrated with liquids that are exogenous to the wounded tissue, or they can be hydrated with liquids that are endogenous to the wounded tissue.

20 Also included within the invention are methods for preparing a hemostatic dressing by providing a first layer of fibrinogen, applying a layer of thrombin to the first layer of fibrinogen, and applying a second layer of fibrinogen to the layer of thrombin, wherein the layer of thrombin is noncoextensive with the first fibrinogen layer and/or noncoextensive with the second fibrinogen layer.

25 Similarly, the invention includes a method for preparing a hemostatic dressing by providing a resorbable or nonresorbable backing layer having attached thereto a first layer of fibrinogen; applying a layer of thrombin to said first layer of fibrinogen on a side of the fibrinogen layer that is opposite of the side to which the resorbable or nonresorbable backing layer is attached; and
30 applying a second layer of fibrinogen to the layer of thrombin, wherein the layer

of thrombin is noncoextensive with the first fibrinogen layer and/or noncoextensive with the second fibrinogen layer.

In various embodiments, the thrombin layer is coextensive with 5% to 95% (e.g., 20 to 50%) of the first and second fibrinogen layers, independently.

5 The thrombin layer can be configured in any of a variety of shapes and patterns. For example, and without limitation, the thrombin layer can be configured as an array of spots comprising thrombin, or as a single spot comprising thrombin. Alternatively, the thrombin layer can be configured as a plurality of lines comprising thrombin.

10 Each layer of the hemostatic dressings can also optionally contain one or more suitable fillers, binding agents and/or solubilizing agents. In addition, each of the hemostatic dressings can also optionally further comprise a release layer which contains a release agent and/or a backing material.

15 It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents, patent applications, and publications
20 mentioned herein are incorporated herein by reference.

As used herein, a thrombin layer that is said to be “noncoextensive” with a fibrinogen layer is one in which the spatial boundaries of the thrombin layer in two dimensions are smaller than the spatial boundaries of one or both fibrinogen layers such that the thrombin layer is coextensive with only about 5% to about
25 95% of the surface area of the first fibrinogen layer of the hemostatic dressing and/or coextensive with only about 5% to about 95% of the surface layer of the second fibrinogen layer of the hemostatic dressing, independently. For example, the thrombin layer can be coextensive with about 10, 20, 30, 40, 50, 60, 70, 75, 80, or 90 % of the surface area of each of the first and second fibrinogen layers,
30 independently. A thrombin layer that is “coextensive” with a fibrinogen layer

provides full coverage of the fibrinogen layer and is coextensive with 100% of the surface area of the fibrinogen layer. A thrombin layer can be noncoextensive with the first fibrinogen layer and yet be coextensive with the second fibrinogen layer, or *vice versa*, e.g., by employing fibrinogen layers having different total surface areas or shapes.

"Patient" as used herein refers to human or animal individuals in need of medical care and/or treatment.

"Wound" as used herein refers to any damage to any tissue of a patient that results in the loss of blood from the circulatory system. The tissue can be an internal tissue, such as an organ or blood vessel, or an external tissue, such as the skin. The loss of blood can be internal, such as from a ruptured organ, or external, such as from a laceration. A wound can be in a soft tissue, such as an organ, or in hard tissue, such as bone. The damage may have been caused by any agent or source, including traumatic injury, infection or surgical intervention. The damage can be life-threatening or non-life-threatening.

"Resorbable material" as used herein refers to a material that is broken down spontaneously and/or by the mammalian body into components which are consumed or eliminated in such a manner as not to interfere significantly with wound healing and/or tissue regeneration, and without causing any significant metabolic disturbance.

"Stability" as used herein refers to the retention of those characteristics of a material that determine activity and/or function.

"Binding agent" as used herein refers to a compound or mixture of compounds that improves the adherence of one layer of the hemostatic dressing to one or more different layers and/or the adherence of the components of a given layer to other components of that layer.

"Solubilizing agent" as used herein refers to a compound or mixture of compounds that improves the dissolution of a protein or proteins in aqueous solvent.

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"Filler" as used herein refers to a compound or mixture of compounds that provide bulk and/or porosity to one or more layers of the hemostatic dressings.

5 "Release agent" as used herein refers to a compound or mixture of compounds that facilitates removal of an hemostatic dressing from a manufacturing mold.

"Foaming agent" as used herein refers to a compound or mixture of compounds that produces gas when hydrated under suitable conditions.

As used herein, "about" means plus or minus approximately ten percent of the indicated value.

10 The hemostatic dressing of the invention offers various advantages as compared with conventional dressings. By using a thrombin layer that is noncoextensive with one or both fibrinogen layers, the dressings of the invention are less likely to become delaminated at their edges, thus rendering the dressings more durable and easier to handle than conventional dressings. In addition, such
15 dressings are more amenable to large-scale manufacturing and provide for better control of the amount of thrombin dispensed in the dressing.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DETAILED DESCRIPTION OF THE INVENTION

20 An exemplary embodiment of the present invention is directed to a hemostatic dressing, e.g., for treating wounded tissue in a patient, which comprises: (i) a first fibrinogen layer; (ii) a thrombin layer adjacent to the first fibrinogen layer; and (iii) a second fibrinogen layer adjacent to the thrombin layer, wherein the thrombin layer is noncoextensive with the first and/or second
25 fibrinogen layers.

Another embodiment of the present invention is directed to a hemostatic dressing which comprises: (i) a resorbable material layer; (ii) a first fibrinogen layer adjacent to the resorbable material layer; (iii) a thrombin layer adjacent to the first fibrinogen layer; and (iv) a second fibrinogen layer adjacent to the

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thrombin layer, wherein the thrombin layer is noncoextensive with the first and/or second fibrinogen layers.

Yet another embodiment of the present invention is directed to a hemostatic dressing for treating wounded tissue in a patient which comprises: (i)
5 a first fibrinogen layer; (ii) a resorbable material layer adjacent to the first fibrinogen layer; (iii) a thrombin layer adjacent to the resorbable material layer; and (iv) a second fibrinogen layer adjacent to the thrombin layer, wherein the thrombin layer is noncoextensive with the first and/or second fibrinogen layers.

Each layer of the hemostatic dressings can also optionally contain one or
10 more suitable fillers, such as sucrose.

Each layer of the hemostatic dressings can also optionally contain one or more suitable binding agents, such as sucrose.

Each layer of the hemostatic dressings can also optionally contain one or more suitable solubilizing agents, such as sucrose.

15 Each layer of the hemostatic dressings can also optionally contain one or more suitable foaming agents, such as a mixture of citric acid and sodium bicarbonate.

Each of the hemostatic dressings can also optionally further comprise a release layer which contains a release agent. An exemplary release agent is
20 sucrose.

Each of the hemostatic dressings can also further comprise a backing material on the side of the dressing opposite the wound-facing side when the dressing is in use. The backing material can be affixed with a physiologically-acceptable adhesive or can be self-adhering (*e.g.* by having a surface static
25 charge). The backing material can be a resorbable material or a non-resorbable material, such as a silicone patch or plastic.

The fibrinogen employed in the hemostatic dressing can be a fibrinogen complex or any fibrinogen, or a derivative or metabolite thereof (such as fibrinopeptide A and fibrinopeptide B) can be employed as desired. The
30 fibrinogen can also contain Factor XIII.

The fibrinogen complex can be a mixture of human plasma proteins which has been purified and virally inactivated. An exemplary aqueous solution of fibrinogen complex contains 100-130 mg/mL total protein, of which at least 80% is fibrinogen. Other constituents of the fibrinogen complex can include albumin (generally about 5-25 mg/mL); plasminogen (generally less than about 5 µg/mL); Factor XIII (generally about 10-40 Units/mL); and polysorbate 80 (generally less than 3%). The pH of the fibrinogen complex is generally in the range of 7.1-7.5. Suitable fibrinogen complexes can also contain fibronectin.

The fibrinogen applied to form a layer of the dressing typically has a concentration of 1 mg/cm² to 60 mg/cm², e.g., at least 5, 10, 15, 20, 30, 40, 50 mg/cm². The first and second fibrinogen layers can be the same size, e.g., such that the second fibrinogen layer generally is coextensive with the first fibrinogen layer. Alternatively, the first fibrinogen layer can be noncoextensive with the second fibrinogen layer; or the second fibrinogen layer can be noncoextensive with the first fibrinogen layer. Thus, the first fibrinogen layer can be up to 100% of the size of the second fibrinogen layer, or the second fibrinogen layer can be up to 100% of the size of the first fibrinogen layer.

The thrombin employed in the hemostatic dressing can be a lyophilized mixture of human plasma proteins which have been purified and virally inactivated. The dressings of the invention typically contain thrombin at a potency of about 1 to 160 International Units (IU)/cm², e.g., at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 100, 125, 150 IU/cm². Optional constituents include albumin (generally about 5 mg/mL) and glycine (generally about 0.3 M ± 0.05M). The pH of the thrombin is generally in the range of 6.5-7.1.

The thrombin layer is applied to the first fibrinogen layer such that it is noncoextensive with the first fibrinogen layer and/or will be noncoextensive with the second fibrinogen layer upon application of the second fibrinogen layer. For example, the thrombin layer can occupy about 5% to about 95% of the surface area of the first fibrinogen layer and/or about 5% to about 95% of the surface area of the second fibrinogen layer. The thrombin can be applied to the fibrinogen

layer in a single spot or as a series of spots on the fibrinogen layer such that the total surface area of the thrombin spots occupies about 5% to about 95% of the surface area of the first fibrinogen layer and/or about 5% to about 95% of the surface area of the second fibrinogen layer.

5 Such a spot or spots of thrombin can have any geometric shape, e.g., filled or unfilled circles, rectangles, triangles, lines, amorphous shapes, or combinations thereof. Such spots can be applied to the first fibrinogen layer in an ordered or random pattern. A plurality of spots can form any of a variety of shapes and patterns, such as an array, a grid, a series of concentric spots (e.g., concentric
10 circles or squares), an overlapping series of spots (e.g., overlapping circles), spokes emanating from an axis, or any other configuration, provided that the total surface area of the thrombin is about 5% to about 95% of the surface area of the first fibrinogen layer and/or about 5% to about 95% of the surface area of the second fibrinogen layer. In general, a large number of small spots is preferred
15 over a small number of large spots. For example, a 20 x 20 array of spots generally is preferable over a 10 x 10 array of spots occupying the same total surface area. However, the spots can be of any size provided that the total surface area of the thrombin is about 5% to about 95% of the surface area of the first fibrinogen layer and/or about 5% to about 95% of the surface area of the second
20 fibrinogen layer. For example, depending upon the overall size of the dressing, the spots can be, without limitation, at least about 0.01, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mm or more in diameter, width, or length. In one embodiment, for example, 4 circular spots having a diameter of 2-3 mm each can occupy a square centimeter of a dressing. A variety of other configurations are within the scope of
25 the invention and can readily be utilized by those skilled in the art.

 The dressing can be prepared as any of a variety of sizes and shapes. Typically, the dressings are of a size and shape that can readily be handled by those skilled in the art, typically less than 12" in length along any side, e.g., 1"x1", 1"x2", 4"x4", etc. The moisture level of the dressing typically is less than
30 8% (e.g., less than 7, 6, 5, 4, 3, 2, or 1%).

Any of a variety of resorbable material known to those skilled in the art can be employed in the present invention. For example, the resorbable material can be a proteinaceous substance, such as fibrin, keratin, collagen and/or gelatin, or a carbohydrate substances, such as alginates, chitin, cellulose, proteoglycans
5 (e.g. poly-N-acetyl glucosamine), glycolic acid polymers, lactic acid polymers, or glycolic acid/lactic acid co-polymers. For example, the resorbable material can be a carbohydrate substance. Illustrative examples of resorbable materials are sold under the tradenames VICRYL™ and DEXON™.

Generally, the various layers of the hemostatic dressing can be affixed to
10 one another by any means known and available to those skilled in the art. Typically, the fibrinogen layer(s) and/or the thrombin layer(s) is (are) applied as a series of quick-frozen aqueous solution layers and subsequently lyophilized or freeze-dried, e.g., after application of each layer, and upon assembly of the entire dressing. The layers can be applied by any of a variety of techniques, including
15 spraying, pipetting (e.g., with a multi-channel pipettor), sprinkling, using a mask, electrostatic deposition, using a microsyringe array system, or dispensing using a dispensing manifold that contains ports for producing a high density array.

In certain embodiments of the present invention, when the dressings are prepared using a mold, a release agent, such as sucrose, is applied to the mold
20 before the first layer of the dressing is applied. In such embodiments, the hemostatic dressing further comprises a release layer, which contains said release agent.

Alternatively, a physiologically-acceptable adhesive can be applied to the resorbable material and/or the backing material (when present) and the fibrinogen
25 layer(s) and/or the thrombin layer(s) subsequently affixed thereto.

In one embodiment of the dressing, the physiologically-acceptable adhesive has a shear strength and/or structure such that the resorbable material and/or backing material can be separated from the fibrinogen layer after application of the dressing to wounded tissue. In another embodiment, the
30 physiologically-acceptable adhesive has a shear strength such that the resorbable

material and/or backing material cannot be separated from the fibrinogen layer after application of the dressing to wounded tissue.

Suitable fibrinogen and thrombin can be obtained from human or mammalian plasma by any of the purification methods known and available to those skilled in the art; from supernatants or pastes of recombinant tissue culture, viruses, yeast, bacteria, or the like that contain a gene that expresses a human or mammalian plasma protein which has been introduced according to standard recombinant DNA techniques; or from the fluids (*e.g.*, blood, milk, lymph, urine or the like) of transgenic animals that contain a gene that expresses human fibrinogen and/or human thrombin which has been introduced according to standard transgenic techniques.

In general, the purity of the fibrinogen and/or the thrombin for use in the hemostatic dressing will be of an appropriate purity known to one of ordinary skill in the relevant art to lead to efficacy and stability of the protein. The fibrinogen and/or the thrombin can be subjected to multiple chromatographic purification steps, such as affinity chromatography and immunoaffinity chromatography, to remove substances which may cause fragmentation, activation and/or degradation of the fibrinogen and/or the thrombin during manufacture, storage and/or use. Illustrative examples of such substances that can be removed by purification include protein contaminants, such as plasminogen, inter-alpha trypsin inhibitor and pre-alpha trypsin inhibitor; non-protein contaminants, such as lipids; and mixtures of protein and non-protein contaminants, such as lipoproteins.

During use of the hemostatic dressing, the fibrinogen and the thrombin can be activated at the time the dressing is applied to the wounded tissue by the endogenous fluids (*e.g.*, blood) of the patient escaping from the hemorrhaging wound. Alternatively, in situations where fluid loss from the wounded tissue is insufficient to provide adequate hydration of the protein layers, the fibrinogen and or the thrombin can be activated by a application of a physiologically-acceptable liquid (*e.g.*, water, buffer, saline), optionally containing any necessary co-factors

and/or enzymes, prior to or upon application of the hemostatic dressing to the wounded tissue.

In addition, one or more supplements can also be contained in one or more layers of the hemostatic dressing, e.g., drugs such as growth factors, polyclonal and monoclonal antibodies and other compounds. Illustrative examples of such supplements include, but are not limited to: antibiotics, such as tetracycline and ciprofloxacin, amoxicillin, and metronidazole; anticoagulants, such as activated protein C, heparin, prostracyclin (PGI₂), prostaglandins, leukotrienes, antithrombin III, ADPase, and plasminogen activator; steroids, such as dexamethasone, inhibitors of prostacyclin, prostaglandins, leukotrienes and/or kinins to inhibit inflammation; cardiovascular drugs, such as calcium channel blockers, vasodilators and vasoconstrictors; chemoattractants; local anesthetics such as bupivacaine; and antiproliferative/antitumor drugs such as 5-fluorouracil (5-FU), taxol and/or taxotere; antivirals, such as gangcyclovir, zidovudine, amantidine, vidarabine, ribaravin, trifluridine, acyclovir, dideoxyuridine and antibodies to viral components or gene products; cytokines, such as α - or β - or γ -Interferon, α - or β -tumor necrosis factor, and interleukins; colony stimulating factors; erythropoietin; antifungals, such as diflucan, ketaconazole and nystatin; antiparasitic agents, such as pentamidine; anti-inflammatory agents, such as α -1-anti-trypsin and α -1-antichymotrypsin; anesthetics, such as bupivacaine; analgesics; antiseptics; and hormones. Other illustrative supplements include, but are not limited to: vitamins and other nutritional supplements; glycoproteins; fibronectin; peptides and proteins; carbohydrates (both simple and/or complex); proteoglycans; antiangiogenins; antigens; lipids or liposomes; and oligonucleotides (sense and/or antisense DNA and/or RNA).

The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the

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modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

All patents and publications referred to herein are expressly incorporated by reference.

5

EXAMPLES

EXAMPLE 1

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The example set forth below demonstrates that delamination of the dressings can be decreased by preparing a dressing in which the thrombin layer is not coextensive with the first and second fibrinogen layers. In this example, thrombin was dispensed onto the first fibrinogen layer in either of two configurations. In the conventional dressings, the thrombin fully covered the first fibrinogen layer (this configuration is referred to herein as “full” coverage). In the dressings of the invention, the thrombin was configured on top of the first fibrinogen layer as a single circle that was not coextensive with the first fibrinogen layer (this configuration is referred to herein as “circle” coverage).

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To prepare the dressings, fibrinogen was formulated in a conventional manner: 35 mg/ml of total protein (TP) in Buffer D (100 mM NaCl, 1.1mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 10 mM TrisHCl, 10 mM Sodium Citrate, 2% Sucrose, 2.8 mg/ml albumin, 0.52 mg/ml TWEEN-80, pH 7.2) containing albumin at 80 mg/g of TP and polysorbate at 15 mg/g of TP. Thrombin concentrate, having a potency of 4745 IU/ml, was formulated in each of four different buffers to obtain a thrombin solution containing 2000 IU/ml. The four buffers are described in Table 1. The dressings were manufactured manually, and 18 dressings were produced for each of the four thrombin formulations and coverage types to produce a total of 144 dressings. The dressings were freeze-dried, packaged with desiccant, then tested in *in vitro* assays for appearance, moisture content, gamma dimerization, and percent clottable protein, and in an *ex vivo* pig arteriotomy assay.

Appearance

All dressings were tested for delamination in an appearance assay. Dressings were considered to be passing if the fibrinogen layers of the dressing were attached to each other along all four edges. Delamination of the dressing layers makes the dressings difficult to handle and renders the dressings susceptible to fragmentation if surface cracks in the layers are also present. As shown in Table 1, dressings in which the thrombin layer was applied with circle coverage (groups 2, 4, 6, and 8) had a higher pass rate in the delamination test (i.e., appearance test) than did dressings in which the thrombin layer was applied with full coverage (groups 1, 3, 5, and 7). While dressings made with thrombin applied with full coverage tend to fail the appearance test, nearly 100% of the dressings made with thrombin applied with circle coverage passed the appearance test.

Table 1

Group	Thrombin Formulation	Coverage	Appearance Pass	Moisture Content	% Dimerization clotted/ unclotted	% Clottable Protein	EVPA
1	150 mM NaCl 10 mM Tris 40 mM CaCl ₂ 100 mM Lysine 2000 IU thrombin pH 7.5	full	7/18	0.96	95/7	86	12/12
2		circle	18/18	1.03	90/8	78	11/12
3	150 mM NaCl 10 mM Tris 100 mM Lysine 2000 IU thrombin pH 7.5	full	4/6	1.16	83/8	99	not tested
4		circle	18/18	1.00	89/3	93	12/12
5	150 mM NaCl 10 mM Tris 40 mM CaCl ₂ 200 mM Lysine 2000 IU thrombin pH 7.5	full	0/6	0.90	90/10	83	not tested
6		circle	17/18	1.07	94/7	80	10/12
7	150 mM NaCl 10 mM Tris 200 mM Lysine 2000 IU thrombin pH 7.5	full	3/6	0.89	92/3	80	not tested
8		circle	18/18	0.80	90/7	71	12/12

The improved resistance to delamination was observed irrespective of the buffer in which the thrombin was formulated. Thus, the removal of CaCl_2 from certain of the buffer formulations did not affect clot formation, even though
5 CaCl_2 is required by thrombin for activation. These results indicate that there was sufficient CaCl_2 present in the fibrinogen formulation (1.1mM) to promote thrombin activation. Similarly, the increase in solids content (Lysine) did not have a significant effect.

The moisture content was measured for one of the dressings from each
10 group. The moisture content of the dressings averaged 0.98% and was very consistent throughout the groups, ranging from 0.80 to 1.16%. This moisture content level is typical for such dressings.

One dressing from each group was used to measure gamma-gamma dimerization in a conventional assay. As described by Okude et al., *Biol. Pharm. Bull.* 16: 448-452, a gamma-gamma dimer assay measures the ability of thrombin to cross-link the gamma chains of fibrinogen, thus forming a clot. Generally, in order to perform this assay, each dressing is ground into a uniform, fine powder in a low moisture chamber and divided into two equal portions. 500 μL 0.9% saline is added to one portion and 500 μL of Okude Dissolving Solution (ODS) (10 M
15 Urea, 1% SDS, 0.1% β -Mercaptoethanol, 0.01% Bromophenol Blue) is added to the other. Both are vortexed briefly and incubated at 40°C for 5 minutes. This allows clot formation in the saline sample, while the denaturing agents in ODS prevent clot formation. Both samples are suspended in 5 volumes of ODS incubated for 1 h at 40°C, dissolved in Laemmli sample buffer with β -
20 Mercaptoethanol, and electrophoresed on SDS-PAGE gels. The gels are stained with Coomassie Blue to visualize proteins. As shown in Table 1, all dressings showed a high percentage of dimerization after clotting and a low percentage of dimerization in the non-clotted dressings, indicating that no interaction between the thrombin and fibrinogen took place during the manufacture of the dressing.

Clottable Protein

Two dressings from each group also were tested to measure the percentage of clottable protein in the dressing. In this assay, the dressing was wet and the clotted protein was sedimented by centrifugation. The elimination of CaCl_2 from certain of the thrombin formulations did not significantly affect clot formation. As with the dimerization assay described above, these results indicate that there was sufficient CaCl_2 present in the fibrinogen formulation (1.1 mM) to promote thrombin activation.

Ex Vivo Porcine Arteriotomy Dressing Performance Test

Twelve of the hemostatic dressings from certain of the groups were tested in an *ex vivo* porcine arteriotomy assay to determine whether the dressings can maintain adequate pressure in a simulated injury. As shown in Table 1, dressings manufactured with circle coverage, such that the thrombin layer is not coextensive with the fibrinogen layers, were able to maintain adequate pressure in this assay.

A standard *ex vivo* porcine arteriotomy assay can be performed as follows. Obtain frozen porcine aorta and thaw. Aortas can be thawed overnight at 4°C, or individually wrapped in the water bath at 37°C. Dissect excess connective tissue from approximately first 11 cm of the aorta. Usually, the first 5-5.5 cm are free from collateral vessels. The next 5-5.5 cm should not have more than 1-2 collaterals. These can be easily sealed or patched with cyanoacrylate glue.

Cut the aorta into two 5.5 cm pieces. Invert aorta exposing the interior using a hemostat or blunt forceps. Wash both the interior and exterior of the vessel with 1-5 mL of PBS at 37°C. Stretch an O-ring over a 20cc syringe with an approximately 0.6 cm (0.25 in) hole drilled into one side. Using fingers or hemostats pull the vessel onto the syringe. Fit another O-ring of the same size onto the bottom.

Using curved hemostats, carefully secure both O-rings over the top of the vessel. The distance between both O-rings should be 3.5 cm. The artery should be

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snug fitting and held securely in place. Position the secured vessel such that the hole in the syringe lies in the middle of the distance between the O-rings.

5 Fill the syringe with PBS at 37°C and place the screw through the outside of the syringe and into the plunger, so that the plunger is held in a stationary position. Wash the artery on the syringe with 1-2ml of PBS at 37°C. Using a 16-gauge needle, make a hole in the center (approximately 1.75 cm from either O-ring) over the syringe hole. The 16-gauge needle should be replaced after every 12 uses.

10 Open the sealed bag containing the dressing and immediately place the dressing over the incision (approximately 0.5 cm from each O-ring, so as not to touch either O-ring). All dressings should be individually packaged prior to use.

15 Using a P-1000 Pipetman, wet the dressing with PBS at 37°C. For 15 mg/cm² dressings use 800µL, and for 8 mg/cm² dressings use 500µL. Immediately place the syringe shield on top of the dressing, so as not to touch either O-ring. Press lightly to secure.

Place the syringe into the incubator at 37°C using the holding box in order to keep the syringe and all its components stationary. Cover with the plastic cover, placing a 200g weight securely over top. Assure even distribution of weight. Allow it to incubate for 5 minutes at 37°C.

20 Remove the syringe from the incubator. Carefully remove the shield covering the dressing. Attach the syringe to the tubing connected to a peristaltic pump. The tubing should be arranged so that it runs through the pump and is connected to a Y-junction on the opposite side. The Y-junction creates two outlets, allowing the PBS to be pumped into the syringe at one site as back pressure is being generated in the other. This back pressure is directly measured using an in-line pressure transducer-analyzer, and recorded using DMSI-200/1 software.

25 Pump the PBS at 37°C into the syringe, and immediately start monitoring the pressure generated. Initiate a 30-second slow ramp (setting 4 at 1x speed for

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pump in E229, setting 7.5 at 1x speed for pump in E132), such that the initial flow rate is approximately 0.3 mL/min.

After the first 30 seconds, the flow rate is escalated to approximately 3 mL/min (10x speed, both settings). This should be done until a pressure of 200 mm Hg is obtained. Once 200 mm Hg is achieved, start the timer for 2 minutes.

Stop the pump once 200 mm Hg is obtained. Monitor the pressure generated. If pressure starts to drop, turn pump back on until adequate pressure is obtained. This may be done as often as necessary throughout the two-minute interval (under normal conditions, the pressure should be maintained between 200 and 215 mm Hg.). In addition, note any leakage and its location. If a leak occurs, note the maximum time and burst pressure at the moment of leakage. Judge dressing performance based on the following pass / fail criteria.

A dressing is considered passing if it maintains a fairly consistent pressure of 200 mm Hg for two minutes with absolutely no leakage. A dressing is also considered passing if it maintains a fairly consistent pressure of 200 mm Hg for two minutes with only minimal leakage (e.g., slow seeping or a leak that has resealed itself).

A dressing is considered failing if it cannot maintain adequate pressure due to severe leakage. This includes leakage caused by poor adhesion, as well as leakage due to manufacturing flaws. Dressings that may be considered as failing in this assay can nonetheless be used to treat less severe wounds.

EXAMPLE 2

The data set forth below in Table 2 demonstrate that delamination can be attributed to full coverage of the thrombin layer (or buffer) on the first fibrinogen layer. Dressings that were produced with thrombin applied such that the thrombin layer was not coextensive with the first fibrinogen layer generally passed the delamination appearance test (groups 9-11 and 13-16 in Table 2). Similarly, dressings that were produced with no middle layer, i.e., having only

two fibrinogen layers, generally did not become delaminated, and the fibrinogen layers adhered tightly to each other.

In this example, fibrinogen was formulated as described above, and thrombin was formulated as described in Table 2. The fibrinogen layers (approx
5 1.2 mL) were applied using a programmable pipette, and the dressings were manufactured manually as described herein. The middle layer of the dressing (i.e., thrombin or fibrinogen) was applied either by spraying using an air brush or by pipetting. For each formulation and method of application, four different volumes of the middle layer were applied, with 120 μ l considered to be a
10 "standard" volume. When the middle layer was applied by spraying, the spraying time was increased to increase the volume applied. Full coverage of the first fibrinogen layer was achieved with all groups in which the thrombin was sprayed.

When the middle layer was applied by pipetting, full coverage of the first fibrinogen layer was obtained only when 1000 μ l of the middle layer was applied.

15 Smaller volumes of the middle layer were used to demonstrate delamination is inhibited by application of the thrombin layer such that it is not coextensive with the fibrinogen layers. Six dressings were tested in each group. Group 1 was used as a control and was assessed for moisture content, gamma-gamma dimerization, percentage of clottable protein, and appearance (i.e., delamination). The control
20 dressing demonstrated a moisture level of 1.3%, good dimerization on clotting (82%), low dimerization of the non-clotted sample (1%), and a low, but acceptable, percentage of clottable protein (68%). All of the dressings were assessed for delamination, and the number of passing dressings is shown in Table 2.

25 The data set forth in Table 2 show that, when the middle layer is applied with full coverage of the fibrinogen layer (groups 1-8 and 12), all of the dressings became delaminated at their edges. When partial coverage was achieved, the dressings generally passed appearance. The observation that increasing volumes of the thrombin middle layer, applied by pipette, increased the delamination of the
30 dressing indicates that delamination is more a function of the coverage and

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composition of the middle layer than it is a function of the rate at which the middle layer freezes during manufacture of the dressing. Thus, dressings having a thrombin layer that is noncoextensive with the fibrinogen layers inhibit delamination.

5 Table 2

Group	Formulation of Middle Layer	Application Method	Volume (μ l)	Coverage	Appearance
1	150 mM NaCl 10 mM Tris 40 mM CaCl ₂ 100 mM Lysine 2000 IU thrombin pH 7.5	spray	120	full	0/6
2			250	full	0/6
3			500	full	0/6
4			1000	full	0/6
5	150 mM NaCl 10 mM Tris 200 mM Lysine 2000 IU thrombin pH 7.5	spray	120	full	0/6
6			1250	full	0/6
7			500	full	0/6
8			1000	full	0/6
9	150 mM NaCl 10 mM Tris 40 mM CaCl ₂ 100 mM Lysine 2000 IU thrombin pH 7.5	pipette	120	partial	6/6
10			250	partial	6/6
11			500	partial	3/6
12			1000	full	0/6
13	fibrinogen	pipette	120	partial	6/6
14			250	partial	6/6
15			500	partial	6/6
16			1000	partial	6/6

Other Embodiments

While the invention has been described with reference to the foregoing detailed description thereof and preferred embodiments, the foregoing description is intended to illustrate and not limit the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A hemostatic dressing which comprises:
 - (i) a first fibrinogen layer;
 - (ii) a thrombin layer adjacent to said first fibrinogen layer; and
 - 5 (iii) a second fibrinogen layer adjacent to said thrombin layer, wherein the thrombin layer is noncoextensive with the first fibrinogen layer and/or the second fibrinogen layer.
- 10 2. A hemostatic dressing which comprises:
 - (i) a resorbable material layer;
 - (ii) a first fibrinogen layer adjacent to said resorbable material layer;
 - (iii) a thrombin layer adjacent to said first fibrinogen layer; and
 - (iv) a second fibrinogen layer adjacent to said thrombin layer, wherein
 - 15 the thrombin layer is noncoextensive with the first fibrinogen layer and/or the second fibrinogen layer.
- 20 3. A hemostatic dressing which comprises:
 - (i) a first fibrinogen layer;
 - (ii) a resorbable material layer adjacent to said first fibrinogen layer;
 - (iii) a thrombin layer adjacent to said resorbable material layer; and
 - (iv) a second fibrinogen layer adjacent to said thrombin layer, wherein
 - the thrombin layer is noncoextensive with the first fibrinogen layer and/or the second fibrinogen layer.
- 25 4. The hemostatic dressing according to any one of claims 1-3, wherein the thrombin layer is coextensive with 5% to 95% of the first fibrinogen layer.
- 30 5. The hemostatic dressing of claim 4, wherein the thrombin layer is coextensive with 20 to 50% of the first fibrinogen layer.

6. The hemostatic dressing according to any one of claims 1-3, wherein the thrombin layer is coextensive with 5% to 95% of the second fibrinogen layer.

5

7. The hemostatic dressing of claim 6, wherein the thrombin layer is coextensive with 20 to 50% of the first fibrinogen layer.

10

8. The hemostatic dressing according to any one of claims 1-3, wherein the thrombin layer is configured as an array of spots comprising thrombin.

15

9. The hemostatic dressing according to any one of claims 1-3, wherein the thrombin layer is configured as a single spot comprising thrombin.

10. The hemostatic dressing according to any one of claims 1-3, wherein the thrombin layer is configured as a plurality of lines comprising thrombin.

20

11. The hemostatic dressing according to any one of claims 1-3, further comprising a backing material.

25

12. The hemostatic dressing of claim 2, wherein said resorbable material is selected from the group consisting of glycolic acid polymers, lactic acid polymers and glycolic acid/lactic acid co-polymers.

13. The hemostatic dressing according to any one of claims 1-3, wherein one or more of said layers comprises a solubilizing agent.

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14. The hemostatic dressing according to any one of claims 1-3, wherein one or more of said layers comprises a filler.

5 15. The hemostatic dressing according to any one of claims 1-3, wherein one or more of said layers comprises a binding agent.

16. A method of treating wounded tissue, which comprises applying to said wounded tissue a hemostatic dressing according to any one of claims 1-3.

10 17. The method of claim 16, further comprises hydrating said hemostatic dressing with a liquid that is exogenous to the wounded tissue.

15 18. The method of claim 16, further comprising hydrating said hemostatic dressing with a liquid that is endogenous to the wounded tissue.

19. A method for preparing a hemostatic dressing, the method comprising:
providing a first layer of fibrinogen;
applying a layer of thrombin to said first layer of fibrinogen; and
20 applying a second layer of fibrinogen to the layer of thrombin, wherein the layer of thrombin is noncoextensive with the first fibrinogen layer and/or the second fibrinogen layer.

25 20. The method of claim 19, wherein the thrombin layer is coextensive with 5% to 95% of the first fibrinogen layer.

21. The method of claim 19, wherein the thrombin layer is coextensive with 5% to 95% of the second fibrinogen layer.

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22. A method for preparing a hemostatic dressing, the method comprising:

providing a resorbable or nonresorbable backing layer having attached thereto a first layer of fibrinogen;

5 applying a layer of thrombin to said first layer of fibrinogen on a side of the fibrinogen layer that is opposite of the side to which the resorbable or nonresorbable backing layer is attached; and

10 applying a second layer of fibrinogen to the layer of thrombin, wherein the layer of thrombin is noncoextensive with the first fibrinogen layer and/or the second fibrinogen layer.

23. The method of claim 22, wherein the thrombin layer is coextensive with 5% to 95% of the first fibrinogen layer.

15 24. The method of claim 22, wherein the thrombin layer is coextensive with 5% to 95% of the second fibrinogen layer.