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(54) Title: LYOPHILIZED COLLAGEN–BASED BIOMATERIALS, PROCESS OF PREPARATION AND USES THEREOF

(57) Abstract

This invention pertains to methods of lyophilizing collagen–based biomaterials, which lyophilates, when reconstituted, retain hemostatic activity and can be extruded. This invention also pertains to methods of radiation–sterilization of lyophilized collagen–based biomaterials.

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LYOPHILIZED COLLAGEN-BASED BIOMATERIALS, PROCESS OF PREPARATION AND USES THEREOF

TECHNICAL FIELD

This invention is in the general field of bioadhesive materials. More specifically, this invention is directed to lyophilized collagen-based materials and methods for their preparation and use. Optionally, the lyophilized collagen-based materials can be radiation sterilized. The lyophilized collagen-based materials of the invention are useful in forming tissue sealants.

BACKGROUND OF THE INVENTION

Biomaterials have been used for implantation into the human body to act as supports for wound and solid tissue healing. A surgical adhesive fibrin sealant has historically been designed as a two-component system, analogous to a two-component epoxy adhesive. The first component consists of fibrinogen and Factor XIII; thrombin and a calcium chloride solution are in the second component. The components may be applied sequentially or simultaneously by a syringe or by spraying. Fibrin sealants have been used for hemostasis as well as tissue sealing in patients being treated with heparin or with coagulation deficiencies. They promote wound healing by decreasing oozing and control air leaks by producing a fluid tight seal at wound sites. Fibrin glues can partially or totally preclude the use of sutures and thereby avoid inflammatory reactions. (DePalma, L. *et al.* (1993) *Transfusion* **33**(9):717-720). For a detailed review of the history and use of fibrin sealant adhesive systems, see Sierra, D.H. (1993) *J. Biomaterials Appl.* 7:309-352.

Early surgical adhesives contained a high content of fibrinogen (about 8-10%) which could only be prepared with difficulty from fibrinogen lyophilates. They were generally unstable and therefore required storage at -20°C to 5°C until use. Examples of these early adhesives include compositions marketed under the tradenames "Tisseel"® or "Tissucol"® (Immuno AG of Vienna, Austria), Beriplast® (Behringwerke AG, Marburg, Germany) and Biocoll® (LFB, Lille, France). These were prepared from large quantities of screened pooled donor sourced human plasma.

Patient autologous and single-donor sourced fibrin sealants were developed in the United States in response to concerns over viral disease transmission of the commercial,

pooled donor sourced fibrin sealants. These efforts were focused on producing concentrated fibrinogen as bovine thrombin was readily available.

In 1983, Gestring and Lerner described a cryoprecipitation production method which utilized small amounts of patient autologous blood. (Gestring and Lerner (1983) *Vasc. Surg.* 294-304). These compositions and methods of use are limited by availability of large amounts of patient blood, preparation time (which can range from an hour to overnight) and the equipment and expertise of trained hospital personnel. This method was modified for large-scale production. (Dresdale and Rose, U.S. Patent 4,627,879).

Adhesive hemostatic or other biomaterial compositions incorporating other blood factor components have been described. U.S. Patent No. 4,061,731 describes a composition comprising patient autologous plasma and finely-divided collagen and/or gelatin in combination with endogenous thrombin. U.S. Patent No. 5,290,552 describes a dual-component system comprising fibrinogen, Factor XIII, collagen, thrombin, and a source of calcium ions which are mixed together just prior to use. U.S. Patent No. 4,600,574 describes a surgical adhesive comprising a flat web-like collagen, gelatin or polysaccharide coated with a solution of fibrinogen and Factor XIII which is lyophilized to form a matrix. U.S. Patent No. 4,453,939 describes a composition for the healing of wounds which comprises a "web-like" carrier comprised of collagen which is coated on one side with a mixture of: (1) a fibrinogen component which contains fibrinogen and Factor XIII; and (2) a thrombin-containing component. Coagulation is initiated upon insertion of the "web" into the patient.

Thromboplastin (also referred to as tissue factor protein (TF)) as a therapeutic or diagnostic agent for coagulation disorders has been described. U.S. Patent No. 5,091,363 describes a composition and method for the treatment of hemophilia A. The composition of Factor VIII, antithrombin III, a phospholipid, a source of calcium ions and Factor IX is mixed and maintained for a period of time until a partial thromboplastin time (PT) of 15 to 30 seconds is obtained. A polyol is added to complete the composition.

Renewed efforts to develop novel tissue sealants are currently underway, and it has recently been shown by Applicants that a composite adhesive of fibrinogen and collagen has both hemostatic efficacy and mechanical strength. (Jackson (1996) *Nature Medicine* **2**(5):637-638).

U.S. Patent No. 4,515,637 to Cioca describes a collagen-thrombin sponge formed by mixing collagen and thrombin at basic pH and lyophilizing the mixture. The collagen-thrombin mixture is lyophilized to form a sponge and is not reconstituted into a flowable slurry prior to application to a wound. Once wet, the lyophilized collagen-thrombin mixture remains stable as a sponge.

U.S. Patent No. 4,948,540 to Nigam describes a mechanically stable, conformable collagen wound dressing sheet material. The collagen is made by lyophilizing soluble and native collagen fibers and compressing the resulting porous pad at high pressure. The sheet material may be impregnated with thrombin after lyophilization.

To minimize the risk of infection, tissue sealants must be sterile prior to use. The FDA has indicated that terminal sterilization to achieve a Sterility Assurance Level of 10^{-6} would be desirable in biomaterials including tissue adhesives. Terminal sterilization is also practical from a manufacturing standpoint, as it reduces the need for aseptic techniques during production. Liu *et al.* (1989) *J. Biomedical Materials Research* **23**:833-844 investigated gamma radiation dosages from 2.5 to 25 KGy on pepsin-extracted human amnion collagen and found that the solubility in neutral PBS decreased with increasing radiation dosage. Other attempts to radiation-sterilize collagen with gamma radiation have been unsuccessful because doses as low as 10 KGy have been shown to cause cleavage and denaturation of collagen (Cheung *et al.* (1990) *J. Biomedical Materials Research,* **24**:581-589). In addition, collagen which is dried prior to irradiation shows greater structural damage than collagen irradiated in the presence of water (Grant *et al.* (1973) *J. Anat.* **115**(1):29-43). Similarly, soluble collagen has been shown to be more resistant to gamma-radiation than insoluble collagen (Ramanathan *et al.* (1965) *Biochim. Biophys. Acta* **102**:533-541). At high doses, gamma irradiation of collagen results in gelation (Labout (1972) *Int. J. Radiat. Biol.* **21**(5):483-492).

None of the above-described references describes a method of forming a lyophilized collagen/thrombin composition which, when reconstituted, is hemostatic, can be extruded from a needle or other narrow gauge orifice ($\leq$ 1mm). The reconstituted collagen-based composition may also be combined with fibrinogen to form a tissue sealant. Methods of radiation-sterilizing a lyophilized collagen-based biomaterial, and compositions of lyophilized collagen-based biomaterials are also not described in the

literature. The lyophilized mixture may be stored in its final delivery device or container, greatly improving convenience and decreasing preparation time.

DISCLOSURE OF THE INVENTION

Accordingly, one aspect of the present invention provides a process for the preparation of a hemostatic, collagen-based biomaterial useful in wound healing and tissue sealing produced by mixing an effective amount of processed collagen and an effective amount of thrombin under suitable conditions to achieve a homogenous dispersion of from about 1 mg/mL to about 10 mg/mL of collagen, and preferably between 5 and 9, and most preferably about 6.5 to 8.5 mL collagen, and drying the concentrated mixture under suitable conditions. The collagen optionally can be concentrated by centrifugation or dialysis, for example, before the combining of collagen and thrombin. The dried mixture can be reconstituted under conditions to obtain the collagen-based biomaterial, wherein the biomaterial is hemostatic and capable of extrusion through an orifice having a diameter of ≤ 1 mm. After lyophilization, reconstitution is made such that the collagen and thrombin concentrations are higher than that of the original dispersion.

In one embodiment, the homogeneous dispersion has a final concentration of greater than 10 mg/mL of collagen and about 6 to about 2000 U/mL of thrombin and the pH of the homogenous dispersion is maintained at a range from about pH 5.5 to about pH 8.0. In other embodiments, the collagen dispersion is concentrated by centrifugation or dialysis, thrombin added, and the mixture is dried by any one or a combination of lyophilization, freeze-drying or drying under a vacuum. In yet another embodiment, the mixing step further comprises adding an effective amount of an agent selected from the group consisting of a therapeutic agent, a cytokine, a growth factor or an analog or mixture of any of the agents. Alternatively, when the agent is sensitive to the various mechanical and/or heat-processing steps, it can be added to the biomaterial after reconstitution. In another preferred embodiment, the reconstitution step comprises admixing the dried mixture with an effective amount of an aqueous source of a calcium ion, for example, aqueous $CaCl_2$ at a concentration of about 10 mM or greater.

In another aspect the present invention provides process for the preparation of a sterilized, hemostatic collagen-based biomaterial useful in wound healing and tissue

sealing, by mixing an effective amount of processed collagen and an effective amount of thrombin under suitable conditions to achieve a homogenous dispersion, optionally concentrating the homogeneous solution by centrifugation or dialysis, for example, to collect the solid collagen, then admixing the collagen mixture to obtain a solution of from about 1 mg/mL to about 10 mg/mL collagen and preferably from about 5 to about 9, and most preferably from about 6.5 to 8.5 mg/mL collagen. The mixture is dried under suitable conditions, and then exposed to a sterilization process to obtain a composition which upon reconstitution, is hemostatic, flowable and capable of extrusion through an orifice. The composition can be reconstituted under conditions to obtain the collagen-based biomaterial, wherein the biomaterial is hemostatic and capable of extrusion through an orifice of ≤ 1 mm diameter.

In one embodiment, the homogeneous dispersion has a final concentration of greater than 10 mg/mL of collagen and about 6 to about 2000 U/mL of thrombin and the pH of the homogenous dispersion is maintained at a range from about pH 5.5 to about pH 8.0. In other embodiments, the homogeneous dispersion is concentrated by centrifugation or dialysis and dried by any one or a combination of lyophilization, freeze-drying or drying under a vacuum. In yet other embodiment, the mixing step further comprises adding an effective amount of an agent selected from the group consisting of an anti-oxidant, a therapeutic agent, a cytokine, a growth factor or an analog or mixture of any of these agents to the initial collagen/thrombin mixture. If the agent is subject to degradation and/or inactivation by the subsequent processing steps, it may be added when the biomaterial is reconstituted and prior to extrusion. The reconstitution step can include admixing the dried mixture with an effective amount of an aqueous source of a calcium ion, for example, ≥ 5 mM $CaCl_2$. In one embodiment, the sterilization process comprises electron beam radiation or gamma radiation in doses ranging from about 10 KGy to about 40 KGy. Optionally, the mixture can be sterilized at temperatures ranging from about -80°C to about 10°C. This material may also be gamma irradiated at low dose rate or on dry ice at standard dose rate to 40 KGy.

In another aspect, the invention provides for biomaterials produced according to the processes described herein.

In yet another aspect, the invention provides a process for the preparation of a hemostatic biomaterial by admixing a suitable amount of the biomaterial produced

according to the processes described herein with an effective amount of fibrinogen-based solution prior to application to the wound. In an embodiment, the fibrinogen-based solution is fibrinogen, a fibrinogen analog or a derivative thereof isolated from whole blood or obtained by recombinant technology. Coagulation factor XIII may be present optionally to stabilize the fibrin network.

In another aspect, the invention provides a method of treating a wound comprising contacting the wound with an effective amount of any of the biomaterials produced according to the processes described herein under conditions to treat the wound. These conditions are well known to those of skill in the art. The lyophilate may be prepared and then transferred to its final container or be co-lyophilized directly in the container. Different final containers may be the delivery device itself, such as a syringe. Diluent may be stored in a separate syringe and attached to the lyophilate syringe via a two-way stopcock, or in a two-chamber syringe.

As will become apparent, preferred features and characteristics of one aspect of the invention are applicable to any other aspect of the invention.

MODES OF CARRYING OUT THE INVENTION

Definitions

As used herein, certain terms will be used which have defined meanings.

As used herein, the term "hemostasis," as it relates to the collagen-based biomaterial, is intended to mean the arrest or decrease in bleeding or promotion of coagulation. "Hemostat" refers to any compound or material which serves to check bleeding or promote coagulation.

As used herein, the term "co-lyophilization" refers to the controlled freezing and drying of the mixed constituents in any combination or formulation of fibrinogen, thrombin, Factor XIII, Ca^{2+}, collagen, thromboplastin or other coagulation factors, derivatives, hybrids or analogs.

As used herein, the term "single-component" is intended to mean that the surgical adhesive composition does not require mixing with an activating agent, mixture or component prior to administration. In contrast, conventional "dual-component" systems provided as two elements, require contacting prior to or simultaneous with administration for activation of the coagulation cascade and *in situ* fibrin formation.

A material which can be "extruded" is intended to mean a material that will pass through an orifice of less than 1 mm diameter, for instance, a 20 gauge or smaller needle or spray orifice.

As used herein, the term "analog" is intended to mean materials having similar chemical or physical entities of the same material as naturally occurring in nature or purified from a native source.

As used herein, the "radiation sterilized" is intended to mean a material which has been exposed to radiation to significantly reduce the possibility of carrying microorganisms. The term "radiation" includes electromagnetic radiation, for example, x-rays, ultraviolet rays, gamma rays and other forms such as alpha particles, beta particles, and atomic particles such as helium nuclei or electrons.

As used herein, the terms "reconstituted" and "rehydrated" are intended to mean restoring a substance to a condition similar its former condition. Thus, a reconstituted lyophilized material is one which has been restored to liquid or fluid form. Aqueous solutions appropriate for reconstitution are known to those of skill in the art and include, for example, water, buffered saline or aqueous $CaCl_2$.

As used herein, the term "vacuum" is intended to mean a gas pressure which is less in magnitude than atmospheric pressure, more preferably less than about 500 mTorr. A 1 Torr vacuum is equivalent to 1 mm of mercury or 133.322 Pascals (Pa).

As used herein, the term "comprising" is intended to mean that the biomaterials, processes and methods include the recited elements, but do not exclude other elements. "Consisting essentially of" when used to define biomaterials, processes and methods, shall mean excluding other elements of any essential significance to the combination. Thus, an implant consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method or pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial process or method steps for using the biomaterials of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

Hemostatic Collagen/Thrombin Compounds

A collagen-based surgical tissue formulation is described herein, which is available as a single-component system. The single-component tissue adhesive composition contains as its essential elements an effective amount of collagen; thrombin, thromboplastin or a thromboplastin equivalent; and calcium. Additionally, fibrinogen, Factor II, Factor V, Factor VII, Factor VIII, Factor IX, Factor XIII, lipids, analogs, hybrids or conjugates thereof, can be provided in the composition.

In one aspect, the single-component system is obtained by mixing the collagen and thrombin in an aqueous calcium chloride solution. The adhesive remains flowable until it contacts tissue and/or blood. The fibrinogen and platelets in the blood react with the collagen, thrombin and Ca(II) to effect formation of fibrin, thus stemming the blood flow at the site of application. Alternatively, various factors in the blood found at the application or administration site will start the reaction.

Collagen, lyophilized collagen, recombinant collagen, a collagen analog, modified collagen, derivatized collagen or a collagen-containing conjugate may be utilized. The collagen may be atelopeptide collagen or telopeptide collagen. Animal or human-based collagen is suitably used and can be purified using methods well known to those of skill in the art and described in, for example, U.S. Patent Nos. 4,233,360. These collagen preparations also are available commercially from a supplier such as Collagen Corp. (Palo Alto, CA) under the tradename Zyderm®. An analog can consist of hybridized or conjugated proteins, as described in published PCT International Publication No. WO 94/16085 to Inani, M.H., incorporated herein by reference. Modified or derivitized collagen can be any chemically modified collagen such as methylated, succinylated or guanidinated collagen as described in U.S. Patent Nos. 4,164,559 and 4,271,070 by Miyata *et al.*

Thrombin, thromboplastin or thrombin analogs may be used in this invention. Thrombin acts as a catalyst for fibrinogen to yield fibrin, an insoluble polymer. Thrombin may be isolated from a variety of host animal sources, including bovine, porcine, equine or human or may be derived by recombinant means such as cell expression techniques or transgenic animals. The thrombin may be prepared by methods well known to those skilled in the art and is typically prepared from prothrombin. Medical grade thrombin approved for medical use is known as "topical thrombin USP." Thrombin is commercially

available from a variety of sources including Thrombin-JMI™, usually lyophilized with buffer salts and stabilizers in vials. Each vial of Thrombin-JMI™ thrombin typically contains 1,000, 5,000 or 50,000 units of thrombin.

To produce the adhesive, the collagen and thrombin are initially produced in soluble form having the highest possible activity, and where appropriate, having been virally deactivated. As is known to those of skill in the art, when the components are purified from a native or natural source they are provided in purified or substantially purified form. "Purified" shall mean that protein or factor of interest is substantially free of cellular and other biological components normally associated with the protein or factor in its native or natural environment in the cell or body fluid. In the case of thrombin, purity is defined by units per mg of protein. For collagen, purity is defined as being significantly (>95%) free of collagen types (other than Type I) or other contaminating proteins, proteoglycans or glycosamino-glycans. Thus, the term "purified" can be used to describe proteins and factors isolated from their native environment or isolated from a biological, non-naturally occurring environment such as when they are recombinantly produced in a host cell such as a Chinese Hamster Ovary cell which is commercially available from the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD. 20852. As used herein, processed collagen is intended to include collagen obtained from either animal or recombinant sources and treated to form fibrils which may be dispersed in an aqueous media. This dispersion is flowable through a narrow diameter orifice ($\leq$ 1 mm) or may be sprayed. The processed collagen may be prepared by a variety of methods including precipitating fibrils from dilute collagen solution by altering the pH, temperature or solvent conditions by adding alcohol or acetone. Alternatively, corium, tendon or another collagen-rich tissue may be comminuted. Examples of these materials can be found in U.S. Patent Nos. 4,374,121 and 4,412,947. An "isolated" protein or factor means that the substance is devoid of at least some other component that is naturally present with the protein or factor. Thus, an isolated factor can be prepared by using a purification technique to enrich it from a source mixture. Enrichment can be measured on an absolute basis, such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture.

Collagen and thrombin are combined to form a mixture. In a preferred embodiment, the ratio of collagen to thrombin is approximately 25 mg of collagen to at

least 250 units of thrombin per mL of solution. Although the precise ratio is not believed to be critical, thrombin must be present in an amount sufficient to convert fibrinogen into fibrin and in a ratio as clinically advantageous in varying applications requiring rapid or slow polymerization.

The collagen-based mixture may be used in aqueous form, or lyophilized for storage as described below. In one embodiment, the collagen/thrombin mixture is provided in aqueous $CaCl_2$. The collagen/thrombin/$CaCl_2$ composition can be applied to a wound site by any method known in the art including, for example, syringes, sprays or the like. The mixture may be sprayed onto a tissue site with oozing blood or may be combined with a fibrinogen preparation to obtain a two component tissue adhesive. The co-lyophilate may also be used either "as-is" or reconstituted for sealing venous or arterial access sites after procedures such as percutaneous transluminal coronary angioplasty (PTCA) and the like.

"An effective amount" of the individual components is an amount that, when combined as formulated herein, will induce the formation of fibrin or a fibrin clot. Suitable concentrations for most of the factors correspond to a range present in normal human plasma and as provided herein. It should be assumed, although always explicitly stated, that "effective amounts" of the components are used and incorporated into the compositions of this invention.

The thrombin, collagen, fibrinogen or other protein defined herein and used in the formulations that are the subject of this invention can be substituted by other naturally occurring or synthetic compounds or compositions which fulfill the same function.

It should be understood, although not always explicitly stated, that the compositions of this invention can include, in addition to the factors in forms as they appear in nature, i.e., in a "purified" state, analogs, muteins, conjugates, and homologues of the proteins or factors, provided that the biological activity of the factor is not substantially impaired. The biological activity of a protein or factor includes any feature of the polypeptide determined by suitable experimental investigation, including, but not limited to the experiments set forth herein relating to coagulation time and the ability to promote the formation of fibrin *in situ.* "Substantially impaired" would include a greater than 50% reduction in the biological activity of the analog, homologue or mutein, as compared to native or natural protein or factor.

As is apparent to those of skill in the art to which this invention pertains, the compositions of this invention can be combined with standard carriers and preservatives. These pharmaceutical compositions are within the scope of this invention. Accordingly, the use of these components to prepare medicaments for promoting and/or inducing the formation of fibrin *in situ*, is further within the scope of this invention.

There are advantages the single-component system over conventional two-component fibrin sealant systems now in place. No exogenous fibrinogen of either human or other source is required, especially in large quantities. This eliminates concerns over virally transmitted adventitious agents such as HIV or hepatitis. Another important advantage of the composition and its use is its that it eliminates the need for uniform mixing of the catalysis to ensure curing and subsequent strength of the material. The convenience is improved in that only one delivery device or dosage unit is required to prepare and apply the material.

This invention also provides compositions comprising a single tissue adhesive in combination with other constituents, such as stabilizers, preservatives, therapeutics, collagen, collagen analogs and collagen conjugates. Any stabilizer that functions to maintain the activity of the tissue adhesive upon administration to the patient can be used in practicing the invention. Examples of such stabilizers include, but are not limited to Tris (trishydroxymethylaminomethane), PIPES (piperazine-N,N-bis(2-ethane-sulfonic acid, 1.5 sodium salt), imidazole, and MOPS (3-(N-morpholine) propanesulfonic acid). Suitable preservatives include sodium azide, thimerosal, BHA, BHT. Other preservatives that function to prevent the growth of microorganisms that would damage the component system is suitably added to the adhesive components.

Therapeutic additives can be added and the biomaterials can serve as a vehicle for these components. Growth factors such as EGF, TGF-α, TGF-ß, FGF, PDGF can be added. Cytokines such as interleukin or stem cell factor also can be suitably added. Antibiotics can be added and are particularly useful when the adhesive is applied to exposed wound sites such as mouth sores and burns. The surgical adhesive compositions also can be mixed with cells, autologous, cultured or modified, allogeneic or xenogeneic. Other biomaterials including, but not limited to, hydroxyapatite, demineralized bone and hylauronic acid may be added to modify the materials mechanical or biological properties. These materials may be added during the course of producing the material, or they may be

added during application. This material may be particularly advantageous because collagen is a commonly used cell growth support matrix. Various other therapeutic agents and biological modifiers such as radioisotopes, anti-hyperproliferative drugs, calmodulins and the like can also be incorporated. As is apparent to those skilled in the art, the amount of an added component will vary with the use of the adhesive and the recipient but is easily determined by the treating physician.

The invention also provides for the use of a collagen/thrombin biomaterial in a dual-component system with fibrinogen. The collagen-based composition is prepared as described herein and may be reconstituted after lyophilization. Fibrinogen is available from commercial sources. Alternatively, human or animal plasma can be used as is after removal of the cellular components of blood by centrifugation. Alternatively, it can be further processed to prepare a plasma cryoprecipitate by freezing, thawing and further centrifugation. Methods of obtaining these constituent fractions are described in U.S. Patent No. 5,290,552, which is incorporated herein by reference. It can be employed in crystalline or amorphous form, or as a lyophilate. For example, bovine fibrinogen is commercially available from Sigma Chemical Co. (product #4754) or it can be obtained from a fibrin sealant preparation such as Tisseel®, from Immuno AG, Vienna, Austria. Fibrinogen and the Factor XIII components can be obtained from allogeneic, autologous plasma preparations or by recombinant means.

Lyophilizing Collagen/Thrombin Mixtures

This invention also pertains to a process of lyophilizing a hemostatic collagen-based biomaterial such that the reconstituted lyophilized material retains hemostatic properties and can be extruded from a orifice of less than 1 mm in diameter. Thus, the invention provides a process for producing a storage-stable collagen compound which can be reconstituted and can be used as a tissue sealant or hemostat.

In one aspect, the collagen-based biomaterial is comprised of collagen and thrombin. The collagen/thrombin mixture can be placed in an appropriate container, such as a mold, tray or dish and co-lyophilized. Lyophilization of the mixture in the final delivery container (*e.g.* syringe) is also possible. Also, the mixture may be lyophilized in bulk and shredded. It also may be frozen by dropping the mixture through a blanket of liquid N_2, forming granules and freeze-drying in bulk. General lyophilization procedures

are known to those skilled in the art. The lyophilized collagen/thrombin mixture can be stored at room temperature or subjected to further manipulations.

This invention also provides any of the biomaterials described herein in a container suitable for lyophilization of the biomaterial and instructions for preparation and use thereof.

The lyophilized collagen/thrombin composition may be reconstituted into an aqueous form prior to use. In one embodiment, the lyophilate is reconstituted in water, saline or PBS. In a preferred embodiment, 40 mM aqueous $CaCl_2$ is used to rehydrate the lyophilate. Any other pharmaceutically buffered solution may be used as well with the pH ranging from about 5.0 to about 8.5. The osmolarity may be hypotonic, isotonic or hypertonic. Other suitable aqueous solutions will be known to those skilled in the art. The lyophilate retains thrombin activity which may be measured, for example, by ability to convert fibrinogen into fibrin.

The lyophilized collagen-based biomaterial of the invention is useful as a tissue sealant on its own or when combined with a source of fibrinogen. Accordingly, it is an aspect of the invention that, when reconstituted, the lyophilized material can be extruded through a needle having a diameter of less than about 1 mm. As is known in the art, tissue sealants are often applied to the wound site using a needle or spray nozzle attached to a syringe(s) containing the component(s) of the sealant. Accordingly, the rehydrated lyophilate of the invention can be extruded through an orifice having a diameter less than 1 mm, for example, a spray nozzle or a 30 gauge or larger needle, catheter or delivery port in an endoscopic device.

Radiation-Sterilizing Lyophilized Collagen/Thrombin Compounds

The invention also provides a method of radiation-sterilizing a lyophilized collagen-based biomaterial. An important aspect of the this invention is that the sterilized lyophilized compound may then be reconstituted into an aqueous form for use, for example, as a tissue sealant. The lyophilized collagen-based material may be processed aseptically, thus remaining sterile and eliminating the need for terminal sterilization. The reconstituted sterilized compound can also be combined with a source of fibrinogen to form a tissue sealant. Further, the colyophilate may be admixed prior to administration with other two component therapeutic agents chemically incompatible with collagen

and/or thrombin. A cross-linking agent may also be incorporated in a manner analogous to the fibrinogen.

Electron beam radiation (e-beam) radiation may be performed as is described in the literature. Although the type of radiation used is not believed to be critical, in a preferred embodiment, the lyophilized material is exposed to e-beam radiation at a certified facility. In one embodiment, irradiation may be performed at between 0°C and ambient (room) temperature. A build-up of heat in the product can be prevented by, for example, placing the product on dry ice during the irradiation process. In another embodiment, the lyophilized material is kept cool during the irradiation process (*i.e.* below ambient or room temperature). The lyophilized collagen-based material is exposed to between 10 KGy to 40 KGy, preferably between 10 KGy and 25 KGy, and more preferably less than 20 KGy. As used herein, a "Grey (Gy)" is a unit of absorbed radiation dose, where one grey equals 10 ergs (10^{-5} Joules) per gram of absorbing material.

Stabilization of collagen and thrombin to radiation can be improved by addition of anti-oxidant compounds which may interfere with propagation of free radicals. Such materials are well-known and may include tyrosine, tryptophane, ascorbic acid, citric acid, cysteine methionin, Vitamin E, BHA and BHT. These excipients preferably are used together with low temperature during irradiation. (See Negre-Salvayre, A. et al. Biochem. Pharma. 42:450-453 (1991) and Taylor and Richardson, J. Food Sci. 45:1223-1227 (1980).)

Thus, terminal sterilization of tissue adhesive had the advantage of reducing manufacturing costs and increasing efficiency because it eliminates the need for aseptic techniques in the manufacturing process. The radiation-sterilization method of the subject invention also fulfills the FDA standards suggesting a Sterility Assurance Level of 10^{-6}.

It is apparent to those skilled in the art that the compositions described herein are useful for the preparation of medicaments for any suitable use, for example, tissue repair and/or for the release of therapeutic agents.

It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the foregoing description as well as the examples that follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the biomedical implant art.

EXAMPLES

Example 1 - Co-Lyophilization of Processed Collagen and Thrombin; Precipitated Collagen

To evaluate the hemostatic effect, with or without terminal sterilization, of co-lyophilized formulations of collagen and topical thrombin, collagen was isolated as follows: 4.5 liters of a solubilized collagen solution available from Collagen Corp. ("CSF") were sterile filtered through a 0.45 micron filter at room temperature and poured into a 8 liter Nalgene beaker. The CSF was titrated to pH 7.4 with 475 mL of 100 mM sodium phosphate buffer at pH 11.2. The CSF was mixed for 15 hours at room temperature.

The CSF was then centrifuged at 9,000 rpm for 30 minutes to remove the precipitate and the supernatant was poured off. The collagen pellets were collected into one centrifuge jar and re-dispersed into an aqueous solution. The protein concentration was 13.1 mg/mL and the yield was 96%. The collagen material was transferred to a 2 L Nalgene container.

The collagen was diluted to 7.0 mg/mL with a combination of purified water and reconstituted thrombin. To reconstitute the thrombin, a bottle of 50,000 units of thrombin was diluted with 50 mL of purified water. The mixture was agitated at approximately 50 rpm with a 3-inch agitator (7.5 cm) blade. Aliquots of the collagen/thrombin homogenate were taken at intervals throughout the fill to demonstrate that the thrombin activity was sustained. Table 1 shows that thrombin retains its activity while mixing prior to lyophilization.

Table 1: Thrombin activity of Homogenate

Time (minutes)	Thrombin Activity (units/mL)
1	608
5	703
15	606
45	544
65	604
110	647
1200	654

The material was dispensed into molds using a Filmatic filler. The molds were placed in stainless steel lyophilization pans and transferred to a -80°C freezer.

The pans were frozen at -80°C for 2 hours and then transferred to a standard pharmaceutical lyophilizer and processed using a standard freeze/drying program. The lyophilization chamber was purged with an inert gas and the samples packaged in a room having 50% humidity. The samples appeared well formed and dry. The lyophilate was then packaged in a variety of ways, including loading it into syringes in measured amounts, irradiating it with 15 KGy of e-beam radiation and testing it in an *in vivo* hemostatis model. The normal concentrations of collagen when reconstituted with a $CaCl_2$ solution was 25 mg/mL and thrombin concentration was 500 U/mL.

Example 2 - Lyophilization of Collagen-based materials in Centrifuge Tubes

As an alternative to filling molds, a collagen-thrombin mixture was prepared as described in Example 1. Centrifuge tubes, having similar diameter to syringes, were filled with the collagen-thrombin mixture (7.5 mg/ml). The material in the centrifuge tubes was lyophilized and the resulting product was resuspended in an aqueous 40 mM $CaCl_2$ solution. The product resuspended rapidly and uniformly and sprayed through a small diameter nozzle spray head.

Example 3 - The Effect of E-beam Irradiation on Lyophilized Collagen/Thrombin

Collagen-thrombin was lyophilized as described in Example 1, loaded into 3 mL syringes, closed with positive closures, labeled, radiation dots applied, placed in polyethylene bags and heat sealed. The materials were irradiated with e-beams at 10, 12.5, 15, 17.5 or 20 KGy at room temperature or at 15 or 20 KGy on samples frozen by placing the syringes on dry ice (about -78°C).

The Effect of Lyophilization on DSC peak melting temperature

The irradiated product was re-hydrated by adding 2 mL of purified water to the composite through the syringe and mixing by 40 passes through an adjunct mixer. A portion of the sample was weighed into a pan and the peak melting temperature measured, using PBS as a reference. The peak melting temperature measured on a differential scanning calorimeter is shown in Table 2.

Table 2: Effect of E-beam on Peak DSC Temperature

Sample Description exposure (KGy)	Peak DSC (°C)
Homogenate	61.0
Lyophilized	57.6
10 KGy (room temperature)	53.5
12.5 KGy (room temperature)	51.4
15 KGy (room temperature)	52.9
17.5 KGy (room temperature)	51.1
20 KGy (room temperature)	50.4
15 KGy (sample on dry ice)	57.2
20 KGy (sample on dry ice)	52.2

These results show that peak DSC melting temperature decreased with increasing exposure to e-beam radiation. The decrease in DSC temperature can be tempered by placing the material on a bed of dry ice during exposure to e-beam radiation. Thus, the native helicity of the collagen may be more readily maintained.

The Effect of Lyophilization on Thrombin Activity

To test the effect of lyophilization and e-beam on thrombin activity, the samples were rehydrated with water and diluted to approximately 5 units/mL. Thrombin activity is assayed using a Fibrometer® according to the manufacturer's instructions (BBL). Each assay was calibrated to a commercial lot of thrombin. The thrombin activity of e-beam irradiated samples is shown in Table 3.

Table 3: Thrombin Activity of Lyophilized Sample

Process Step	Thrombin Activity (units/mL)
Homogenate	608 (average from Table 1)
Lyophilized	676
10 KGy (room temperature)	779
12.5 KGy (room temperature)	729
15 KGy (room temperature)	732
17.5 KGy (room temperature)	710
20 KGy (room temperature)	581
15 KGy (frozen)	729
20 KGy (frozen)	734

These results indicate that e-beam irradiation had little to no effect on thrombin concentration or thrombin activity. E-beam radiation may be used to terminally sterilize the lyophilized composite.

The Effect of Lyophilization of Collagen-based Materials on Hemostatis

Whole blood was collected from a donor on the day of the assay. The blood was spun down in a centrifuge in vacutainer tubes at 1000g for 5 minutes to produce platelet-rich plasma. The plasma was collected in a 3 cc syringe. The lyophilized collagen/thrombin composite was rehydrated with 40 mM aqueous $CaCl_2$ and mixed using adjunct mixer mixing approximately 40 times. A custom-designed mixer was attached to the joiner and a commercially available spray head attached to the mixer. The hemostasis qualities of the material were evaluated using a diffuse organ bleeding model involving

incisions of defined length and depth to the kidney and spleen of New Zealand White rabbits. The rabbits were anesthetized, the spleen and kidney were exposed, and a 15 mm long incision of 2 mm depth was made to both organs. Results of the *in vivo* hemostasis tests are shown in Table 4. Hemostasis is superior when the collagen/thrombin co-lyophilate is exposed to less than 20 KGy of radiation. The effect of the exposure to 15 KGy e-beam can be mitigated by reducing the temperature of the product during irradiation. A positive correlation between maintaining melting temperature and *in vivo* hemostasis was observed. It appears that maintaining the native helical structure of the collagen is important in hemostasis outcome.

Hemostasis Results

The hemostasis qualities were evaluated in rabbits as described above. The time to achieve hemostasis was measured in a minimum of 6 sites for each test material. The following results were observed as shown in Table 4.

Table 4

e-beam KGy	% sites Hemostasis
0	80
15 (room temperature)	25
20 (room temperature)	17
15 (frozen)	50
20 (frozen)	17

An increase in e-beam dose correlated with a decrease in hemostasis efficiency. This effect was minimized by irradiating the co-lyophilate frozen on dry ice. When the process was scaled up, the lyophilate irradiated at 15 KGy improved in hemostasis (75% of sites).

Example 4: *In vitro* Testing of Lyophilized Samples

Two separate batches of material prepared as described in Example 1 were evaluated in a series of *in vitro* tests designed to characterize key biochemical and

performance parameters. The results of these tests are presented in Tables 5 and 6 for each batch, respectively.

Re-Suspension Testing

Lyophilized samples from Examples 1 and 2 were rehydrated with 1.5 mL purified water and shaken 30 seconds. The syringe labels were covered to prevent bias in interpretation of data, and the contents were visually inspected for homogeneity.

Extrusion

Lyophilized samples were hydrated and transferred to 1 mL BD syringes and 22, 27 and 30 gauge needles attached. The syringes were mounted on an extrusion tester where the plunger was depressed at a constant rate (10 cm/minute) and the force necessary to maintain the constant rate was recorded. The material was considered to be extruded if the force spikes did not exceed 40 Newtons. These results were compared with those obtained with a control of a collagen slurry prepared in a similar manner, but without thrombin and lyophilization. The control is referred to throughout these Examples as "wet."

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were rehydrated in distilled water. SDS-PAGE was performed according to the method of Laemmli, Nature 227:680-685 (1970), incorporated herein by reference. The gel was stained using Coomassie Blue.

Protein Concentration

Samples were testing using the Pierce BCA Protein Assay using a collagen protein standard.

DSC

Samples were rehydrated with distilled water to approximately 30 mg/mL and run on a Mettler Differential Scanning Calorimeter (DSC) at a scan rate of 10°C/minute, from 0°C to 80°C. Melting point temperature was recorded.

Physical, Chemical and In Vivo Analysis:

Table 5: Sample Preparation 1

Parameter	WET	0 KGy	10 KGy	20 KGy	30 KGy
Extrusion w/30 gauge needle	Doesn't Extrude	Doesn't Extrude	Doesn't Extrude	Doesn't Extrude	Doesn't Extrude
Extrusion w/27 gauge needle	Spikes <40N	Spikes <40N	Spikes <40N	Spikes <40N	Spikes <40N
Extrusion w/22 gauge needle	No Spikes	No Spikes	No Spikes	No Spikes	No Spikes
Peak DSC T_m*	59.1	54.2	48.5	46.9	45.5
Re-suspension	N/A	good	good	good	good

Table 6: Sample Preparation 2

Parameter	WET	0 KGy	10 KGy	20 KGy	30 KGy
Extrusion w/30 gauge needle	Doesn't Extrude	Doesn't Extrude	Doesn't Extrude	Doesn't Extrude	Doesn't Extrude
Extrusion w/27 gauge needle	Spikes <40N	Spikes <40N	Spikes <40N	Spikes <40N	Spikes <40N
Extrusion w/22 gauge needle	No Spikes	No Spikes	No Spikes	No Spikes	No Spikes
Peak DSC T_m*	58.3	54.7	50.7	50.0	45.8
Re-suspension	N/A	good	good	good	good

*The shape of each DSC integration curve was also evaluated. The "wet" (non-lyophilized, non-irradiated) material had a very well defined sharp peak. The peak of the lyophilized material was also sharp and well defined, but had a shoulder at approximately 45°C. At 10 KGy, the peak started spreading out. The dispersion increased at 20 KGy, and by 30 KGy the DSC curve was dispersed and poorly defined. A spread in the DSC curve is generally indicative of denaturation taking place within the sample, as well as crosslinking if the broadening occurs above the peak temperature.

SDS-PAGE Results

Alpha and beta bands from "wet" and lyophilized (non-irradiated collagen) were clearly defined on the Coomassie blue stain at MW of 100 kilodaltons (KD) and 200 KD respectively. The irradiated samples lost the definition at both markers with the samples smearing along the length of the lane. At 10 KGy of exposure, both bands were visible but very light. At 20 KGy, the bands were barely discernible. At 30 KGy, none of the bands were visible, and the sample appeared to be dispersed along the entire molecular weight range. None of the protein was observed at the start of the band, therefore it was presumed that a minimal amount of crosslinking had occurred and that all of the protein had migrated into the gel.

Results presented in Tables 5 and 6 demonstrate that peak melting temperature T_m decreases with lyophilization and exposure to increasing doses of radiation. This decrease in T_m may correspond to chain scission of fibers as described by Liu et al. (1989). The DSC scans also show broadening of the peaks with increasing radiation. This increase in the heterogeneity of the samples may correspond to denaturation of the fibers. It has been proposed that densely cross-linked fibrils, and larger diameter fibrils have higher peak melting values. Lower peak melting temperatures may correspond to fragmented or smaller diameter fibrils. The decrease in T_m indicates that the primary effect of the radiation on the lyophilized collagen is chain scission rather than crosslinking.

The extrusion characteristics of the collagen composite did not change with lyophilization or increasing radiation. Nor was there a change in the resuspension characteristics of the lyophilized strips with increased radiation dosage. These results are consistent with chain scission.

The hemostasis as shown in the rabbit diffuse organ bleeding model decreased with increasing radiation. 80%, 25%, and 17% hemostasis were observed at 0, 15, and 20 KGy respectively.

Based on these results, it is apparent that even 10 KGy of radiation is sufficient to alter the physical characteristics of the collagen composite. However, the physical changes do not appear to significantly affect the re-suspension extrusion characteristics or hemostatis properties of the material. This implies that the radiation of the product primarily causes chain scission rather than crosslinking. This feature may prove of value in decreasing the persistence of the implant *in vivo*.

Example 5: Co-Lyophilization of Processed Collagen and Thrombin: Comminuted Collagen

Fibrous collagen (Semed F, Kensey-Nash Corporation, Exton, PA, U.S. Patent No. 4,374,121) is suspended in distilled water at between 3 to 5% solids (w/w). The pH of the fibrous suspension is adjusted to pH 2 ± 0.2 using HCl. The collagen dispersion is incubated for 2 to 3 days at 5°C, with occasional mixing. Additional HCl is added, as necessary, to maintain the pH near 2.

The dispersion is then comminuted in any of several ways: 1) mixing and pumping the dispersion between syringes, using connectors of varying diameters, for example 1 mm orifices; 2) wet milling, for example in a Waring blender. The sample is at less than about 15°C during these operations to avoid denaturing the collagen. The comminution and swelling in acid are continued until the dispersion could be extruded through a fine gauge needle, as described above in Example 3. Dilution of the dispersion with additional pH 2 HCl can be employed to facilitate dispersion, but the minimum percent solids should be kept above 0.5% (w/w). Dispersions which can be extruded through a #27 gauge needle with less than 40 Newtons of force (through a 1 mL syringe) are considered sufficiently comminuted. U.S. Patent No. 3,628,974 to Battista describes that mechanical disintegration of collagen yields microcrystalline collagen consisting of bundles of aggregated tropocollagen units which vary in length under 1 micron and in diameter from about 2.5 angstroms to some hundreds of angstrom units. This method may not render all of the collagen microcrystalline and separation of the finely comminuted collagen from larger fibers may be necessary to obtain a preparation which can be extruded from a fine gauge needle.

The pH is raised to 7.2 ± 0.2, the per cent solids is adjusted to 0.5 to 1.0%, and comminution in the cold was continued, if necessary, to maintain extrudability through a #27 gauge needle. Thrombin, reconstituted in water as described in Example 1, is added to the suspension to a final concentration of 20 units of thrombin activity per mg of collagen solid. Higher or lower thrombin concentrations can be added depending upon the desired hemostatic potential or set time when used as part of a two-component sealant. The dispersion is frozen, lyophilized, and sterilized by irradiation, as in Example 1. The lyophilized, sponge-form collagen is reconstituted at 2.5% solids (w/w) with 40 mM aqueous $CaCl_2$ and fully dispersed by mixing between syringes. Such dispersions are then

optionally mixed with plasma or other, fibrinogen solutions and sprayed or extruded onto a bleeding wound. Alternatively, the dispersion alone is sprayed onto the wound and when the blood mixes with the formulation, fibrinogen in the blood reacts with the thrombin to form a clot. Clotting is also effected by contact of platelets and other blood clotting factors with the collagen dispersion.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in the fields of chemistry, materials science, medicine and related fields are intended to be within the scope of the following claims.

CLAIMS

What is claimed is:

1. A process for the preparation of a hemostatic collagen-based biomaterial useful in wound healing and tissue sealing comprising the steps of:

(a) mixing an effective amount of processed collagen and an effective amount of thrombin under suitable conditions to achieve a homogenous dispersion; from about 1 mg/mL to about 10 mg/mL collagen;

(b) drying the dispersion under suitable conditions; and

(c) reconstituting the dried dispersion of step (b) under conditions to obtain the collagen-based biomaterial, wherein the biomaterial is hemostatic and capable of extrusion through an orifice.

2. The process of claim 1, wherein the homogeneous dispersion has a final collagen concentration of about 5 mg/mL to about 9 mg/mL.

3. The process of claim 1, wherein the suitable conditions of step (a) comprise titrating the pH of the mixture from about pH 5.5 to about pH 8.0.

4. The process of claim 1, wherein the drying step (b) is by lyophilization, freeze-drying, or drying under a vacuum.

5. The process of claim 1, wherein the mixing step (a) further comprises adding an effective amount of an agent selected from the group consisting of a therapeutic agent, a cytokine, a growth factor or an analog or mixture of any of the agents.

6. The process of claim 1, wherein the reconstitution step (c) comprises admixing the dried mixture with an effective amount of an aqueous source of calcium ion.

7. The process of claim 6, wherein the aqueous source of calcium ion is a $CaCl_2$ solution.

8. A process for the preparation a sterilized, hemostatic collagen-based biomaterial useful in wound healing and tissue sealing, the process comprising:

(a) mixing an effective amount of processed collagen and an effective amount of thrombin under suitable conditions to achieve a homogenous dispersion of from about 1 mg/mL to about 10 mg/mL collagen;

(b) drying the dispersion under suitable conditions;

(c) exposing the dried, concentrated mixture of step (b) to a sterilization process; and

(d) reconstituting the dried solution of step (c) under conditions to obtain the collagen-based biomaterial, wherein the biomaterial is hemostatic and capable of extrusion through an orifice.

9. The process of claim 8, wherein the homogeneous solution has a final collagen concentration of about 5 mg/mL to about 9 mg/mL.

10. The process of claim 8, wherein the suitable conditions of step (a) comprise titrating the pH of the mixture from about pH 5.5 to about pH 8.0.

11. The process of claim 8, wherein the drying step (b) is by lyophilization, freeze-drying, or drying under a vacuum.

12. The process of claim 8, wherein the mixing step (a) further comprises adding an effective amount of an agent selected from the group consisting of an anti-oxidant, therapeutic agent, a cytokine, a growth factor or an analog or mixture of any of the agents.

13. The process of claim 8, wherein the reconstitution step (d) comprises admixing the dried mixture with an effective amount of an aqueous source of calcium ion.

14. The process of claim 13, wherein the aqueous source of calcium ion is a $CaCl_2$ solution.

15. The process of claim 8, wherein step (c) comprises exposing the dried, concentrated mixture of step (b) to a sterilization process comprising electron beam radiation or gamma radiation under conditions to obtain a composition which upon reconstitution, is hemostatic, flowable and capable of extrusion through an orifice.

16. The process of claim 15, wherein the sterilization process is electron beam radiation and the conditions comprise from about 10 KGy to about 40 KGy.

17. The process of claim 15, wherein the sterilization process is from about 10 KGy to about 20 KGy.

18. The process of claim 16, wherein the conditions comprise stabilization of the mixture at a low temperature prior to the sterilization process.

19. The process of claim 16, wherein the low temperature comprises from about -40°C to about 10°C.

20. The process of claim 8 or 15, wherein the sterilization process is a low dose rate of gamma irradiation.

21. The biomaterial produced by the process of claim 1.

22. The biomaterial produced by the process of claim 5.

23. The biomaterial produced by the process of claim 8.

24. The biomaterial produced by the process of claim 12.

25. A process for the preparation of biomaterial comprising a hemostatic wound dressing, the process comprising admixing a suitable amount of the biomaterial of any of

claims 21 to 24 with an effective amount of fibrinogen-based solution prior to application to the wound.

26. The process of claim 25, wherein the fibrinogen-based solution is fibrinogen, a fibrinogen analog or a derivative thereof.

27. The process of claim 25, wherein the fibrinogen-based solution is plasma isolated from whole blood.

28. The biomaterial produced by the process of claim 25.

29. A method of treatment of a wound comprising contacting the wound with an effective amount of the biomaterial of any of claims 21 to 24, under conditions to treat the wound.

30. A method of treatment of a wound comprising contacting the wound with an effective amount of the biomaterial of claims 28, under conditions to treat the wound.

31. A kit for preparing a wound healing or tissue sealant, comprising an effective amount of the biomaterial of any of claims 21 to 24, provided in a container suitable for lyophilization of the biomaterial and instructions for preparation and use of the biomaterial.

32. A kit for preparing a wound healing or tissue sealant, comprising an effective amount of the biomaterial of claim 28, provided in a container suitable for lyophilization of the biomaterial and instructions for preparation and use of the biomaterial.

33. Use of the biomaterial of any of claims 21 to 24 or 28 for the preparation of a medicament for the treatment of a wound or for tissue repair.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/00447

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L25/00 A61L2/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	DATABASE WPI Section Ch, Week 7820 Derwent Publications Ltd., London, GB; Class B04, AN 78-36236A XP002041985 & SU 561 564 A (ISTRANOV L P) , 18 July 1977 see abstract	1,4,5, 21,33

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cousins-Van Steen, G

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INTERNATIONAL SEARCH REPORT

Int. .ional Application No
PCT/US 97/00447

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