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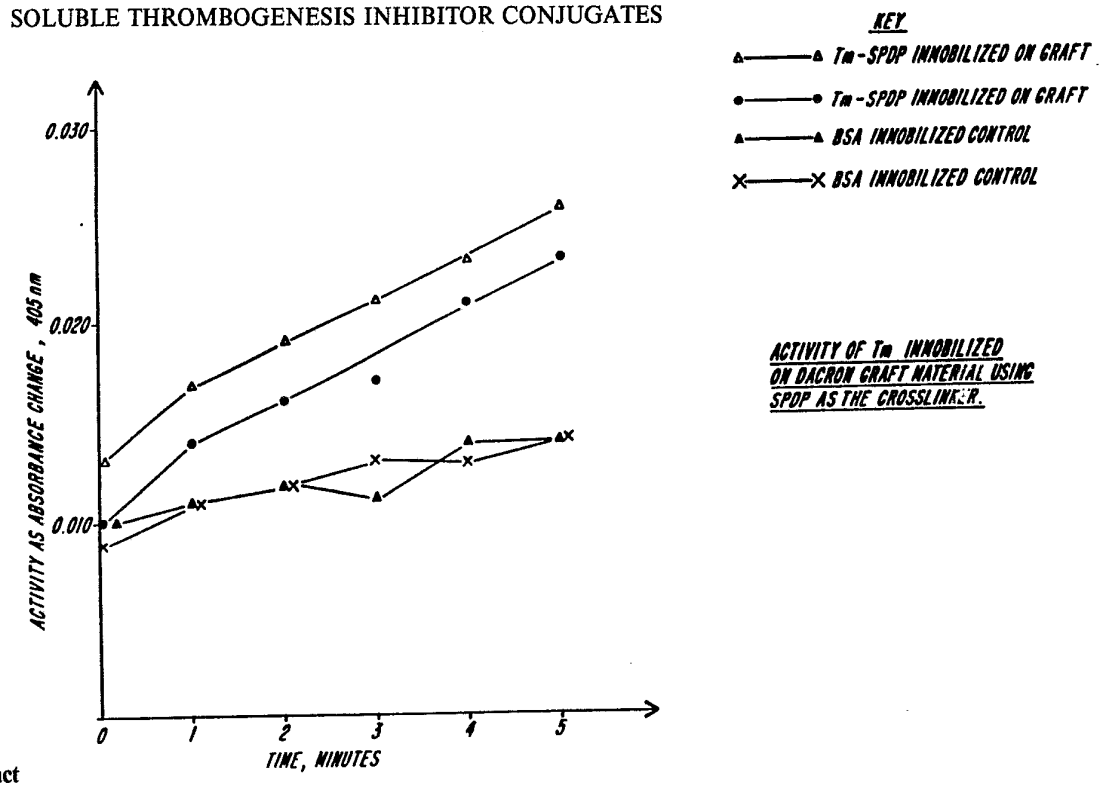

ACTIVITY OF Tm IMMOBILIZED ON DACRON GRAFT MATERIAL USING SPDP AS THE CROSSLINKER.

(57) Abstract

Disclosed is a soluble, biocompatible, pharmacological agent for inhibiting thrombin generation and thrombus formation, and methods for producing the same. The pharmacological agent or conjugate includes a soluble, biocompatible carrier and a thrombogenesis inhibitor immobilized thereto via a component of the carrier which binds the inhibitor. The thrombogenesis inhibitor is other than hirudin, or an active analog or active fragment of the inhibitor. The inhibitor may be bound to the component of the carrier via a bifunctional cross-linking reagent.

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SOLUBLE THROMBOGENESIS INHIBITOR CONJUGATES

Background of the Invention

The technical field of the present invention is thrombogenesis inhibitors, and more specifically involves soluble, pharmacologic agents useful for inhibiting thrombin generation and thrombin-mediated aggregation, and methods of their preparation and use.

Exposure of blood to artificial surfaces usually leads to deposition of a layer of adherent platelets, accompanied by activation of the intrinsic coagulation system, and ultimately to the formation of a thrombus. In fact, significant blood/materials interaction can occur on a single pass through a prosthetic arterial graft. The types of blood proteins initially adsorbed or bound to synthetic surfaces may include proteins involved in contact coagulation. Contact coagulation or the extrinsic pathway of coagulation is a complex pathway of

biochemical events that induces fibrin formation, platelet and complement activation, chemotaxis, kinin generation, and activation of fibrinolytic components. In addition, each of these events augments subsequent biochemical pathways often controlled by positive and negative feedback loops. Thus, thrombosis induced by contact with artificial materials is a major obstacle in the development and use of internal prostheses and extracorporeal devices such as artificial vessels and organs, and cardiopulmonary bypass and hemodialysis equipment.

Materials having varying degrees of thromboresistance have been utilized in vascular prostheses with limited success. These materials include corroding (self-cleaning) metals, synthetic polymers such as polydimethyl siloxane, Teflon, acrylates and methacrylates such as polyethylene terphthalate, electrets, anionic copolymers, and hydrogels (for a review see Salzman et al. (1987) in Hemostasis and Thrombosis, Basic Principles and Clinical Practice (Colman et al., eds.) J. B. Lippincott Co., Phila. PA, pp. 1335-1347).

To decrease the chances of thrombosis due to extended periods of contact with such artificial materials, patients have been treated with systemically administered anti-coagulant, anti-platelet, and thrombolytic drugs. These include any compound which selectively inhibits thromboxane synthetase without affecting prostacycline synthetase, affects platelet adherence as well as aggregation and release, enhances vascular PGI2 production, and/or inhibits both thrombin- and

thromboxane-mediated platelet aggregation. Such compounds include aspirin, sulfinpyrazone, dipyridamole, ticlopidine, and suloctidil. However, treatment with these drugs often elicits unwanted side effects including systemic hemorrhaging and the inability to initiate and complete desired clotting elsewhere in the body.

To improve on the thromboresistance of artificial materials, biologically active molecules having thrombolytic, anticoagulating, thrombogenesis-inhibiting, and/or platelet inhibiting abilities have been linked thereto. For example, heparin has been bound to artificial surfaces to reduce coagulation by activating various inhibitors of the intrinsic clotting system (Salzman et al. (1987) in Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 2nd Ed., (Colman et al., eds.), Lippincott Co., Phila., PA, pp. 1335-1347). However, heparin enhances platelet responses to stimuli such as ADP or collagen, and promotes two adverse primary blood responses towards synthetic surfaces: platelet adhesion and aggregation. In addition, although surface-bound heparin/antithrombin complex may be passive towards platelets, the wide variety of effects it has on interactions with endothelial cell growth factor, inhibition of smooth muscle proliferation, and activation of lipoprotein lipase raises questions as to what adverse effects it may induce over time.

Anti-platelet agents such as PGE_1, PGI_2 (experimental use only), cyclic AMP, and aspirin have also been attached to solid polymer surfaces. These

agents discourage the release of platelet factors that stimulate adverse healing responses in the vicinity of a vascular graft. They may also reduce platelet-aided thrombus formation by inhibiting platelet adhesion.

The exposure of many artificial surfaces to albumin prior to vascular contact results in reduced reactivity with platelets (NIH Publication No. 85-2185, September, 1985, pp. 19-63). Therefore, albumin has been used to coat extracorporeal surfaces before cardiopulmonary by-pass surgery. However, long-term thromboresistance has not been achieved by this procedure.

Fibrinolytically active streptokinase and urokinase, alone or in combination with heparin have been attached to artificial surfaces by Kusserow et al (Trans. Am. Soc. Artif. Intern. Organs (1971) 17:1). These enzymes reduce excessive fibrin deposition and/or thrombotic occlusions. However, the long term assessment of their ability to confer thromboresistance to a synthetic surface has not been determined.

Surface active agents such as Pluronic F-68 have also been immobilized on artificial surfaces, but do not appear to offer long term blood compatibility (Salyer et al. (1971) Medical Applications of Plastics, Biomed. Materials Res. Sym. (Gregor, ed.) No. 1 pp. 105).

Therefore, what is needed are better biocompatible materials which are thromboresistant in

the long term and whose active components do not cause detrimental side affects.

An object of the present invention is to provide a synthetic, biocompatible, thromboresistant material useful for implantable and extracorporeal devices in contact with bodily fluids.

Another object is to provide an immobilized thrombogenesis inhibitor which is biologically active, and a method of preparing the same.

Yet another object is to provide a stabilized, soluble form of a thrombogenesis inhibitor.

Still another object of this invention is to provide a method of inhibiting platelet aggregation, the release of platelet factors, and thrombogenesis at the localized site of the graft or prosthesis-blood interface, thus avoiding the systemic effect of antiplatelet and antithrombosis drugs.

Summary of the Invention

Materials and methods are disclosed herein for the provision of biocompatible, thromboresistant substances useful as a component of implantable or extracorporeal devices in contact with the blood. Also disclosed are soluble, biocompatible pharmacologic agents which inhibit thrombin generation and thrombus formation.

It has been discovered that a synthetic, biocompatible material can be made into a thromboresistant substance by immobilizing to it, by way of a base coat layer, a thrombogenesis inhibitor other than hirudin, or an active analog or active fragment of that thrombogenesis inhibitor, in such a way that does not compromise the inhibitor's thrombogenesis inhibiting activity.

It has also been discovered that the base coat material can act as a soluble, biocompatible, carrier of the thrombogenesis inhibitor to which it is immobilized, and by which it is stabilized. This thrombogenesis inhibitor-carrier conjugate is useful as a pharmacologic agent having an increased half-life and/or improved pharmacologic effectiveness.

The term "thrombogenesis inhibitor" is used herein to describe molecules, other than hirudin, which interfere with, or inhibit, the formation of a thrombus. Such molecules include those which interfere or inhibit the intrinsic and extrinsic coagulation system, platelet adherence, aggregation,

or factor release or activity, or the release or active of tissue factors. Included are native, synthetic, or recombinant proteins, active analogs, active fragments, active derivatives, or active fusion products thereof, and mixtures thereof which can interfere and inhibit the formation of a thrombus. Thrombogensis inhibitors useful for imparting thromboresistance to the substance include proteins which are membrane-bound in their native state (e.g., adenosine triphosphatase (ATPase), adenosine diphosphatase (ADPase), 3'-nucleotidase (AMPase), and thrombomodulin). Thrombomodulin, for example, modulates the coagulation pathway by behaving as a cofactor in the activation of Protein C by thrombin. Activated Protein C in the presence of Protein S degrades active Factors V and VIII, cofactors which are necessary for coagulation, thereby turning off the coagulation pathway. Other useful thrombogenesis inhibitors include those which are normally soluble in vivo (e.g., tissue plasminogen activator (tPA), urokinase (UK), and streptokinase (SK)). Other molecules which inhibit or interfere with the activity of other thrombogenesis inhibitors are useful as well.

Synthetic materials contemplated by the instant invention are preferably polymers such as polyethylene terphthalate, nylon, polyurethane, cross-linked collagen, polytetrafluoroethylene, polyglycolic acid, and mixtures thereof, the most preferred polymeric material being polyethylene terphthalate. Other synthetic materials might also be used.

At least one layer of biocompatible base coat layers is adhered to at least one surface of the synthetic material. This base coat layer or carrier contains a component which binds the thrombogenesis inhibitor. Examples of such base coat or carrier components include proteins, polypeptides, peptides, lipoproteins, glycoproteins, glycosaminoglycans, hydrogels, synthetic polymers, dyes, antibiotics, and mixtures thereof. In preferred aspects of the invention, the base coat layer or carrier includes a polypeptide component such as serum albumin or fibronectin, or plasminogen activators, or active analogs or active fragments thereof, or mixtures thereof from human, bovine, bacterial, or other sources for example. Alternatively, other materials may be used to form the base coat layer or carrier.

In accordance with one aspect of the invention, the thrombogenesis inhibitor is immobilized on the synthetic material via a base coat layer which is adhered to least one surface of the synthetic material. The base coat layer contains a component capable of binding the thrombogenesis inhibitor without compromising the biological activity of the inhibitor.

In exemplary aspects of the invention, the synthetic material is activated prior to having the base coat layer adhered thereto so as to enhances its ability to bind the base coat base layer. For example, in one preferred aspect, the synthetic material is contacted with a solution which makes available at least one chemically active group (e.g., a carboxylic acid group) in the material for binding

to a bifunctional cross-linking reagent (e.g., carbodiimide). The material so treated is then put into contact with a solution containing the cross-linking carbodiimide reagent for a time sufficient to allow the chemically active group to bind thereto.

In another embodiment, the synthetic material may be contacted with a solution which removes impurities therein and/or thereon prior to the activation step described above.

The immobilization step may be carried out by initially contacting the thrombogenesis inhibitor with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow linkage of the reagent to the inhibitor, and then binding the thrombogenesis inhibitor-linked reagent to the base coat layer or carrier. The bound thrombogenesis inhibitor retains its thrombogenesis inhibiting activity. A bifunctional cross-linking reagent useful for such an immobilization step may be heterobifunctional (e.g., N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)), homobifunctional (e.g., ethylene glycolbis (succinimidylsuccinate), (EGS)), or a mixture of both.

The term "bifunctional cross-linking reagent" is defined herein as a molecule having the ability to bind to, and therefore link, two reactive groups on, for example, one molecule or two separate molecules. If the bifunctional cross-linking reagent binds two different types of groups, it is a "heterobifunctional" cross-linking reagent. However,

if the bifunctional cross-linking reagent binds only to two similar groups, it is "homobifunctional".

Prior to the binding step, the thrombogenesis-linked reagent may be subjected to chromatographic procedures to remove impurities mixed in with it.

In an alternative aspect of the invention, the base coat adhered to the synthetic material may be linked at the same time to at least one molecule of a bifunctional cross-linking reagent. In this embodiment, the method further includes binding the thrombogenesis inhibitor-linked reagent to the base coat-linked reagent, thereby linking the thrombogenesis inhibitor to the material-adhered base coat layer.

In another aspect of the invention, the base coat is linked to the thrombogenesis inhibitor before it is linked to the synthetic, biocompatible material.

In yet another aspect of the invention, the soluble, biocompatible, pharmacologic agent (or thrombogenesis inhibitor-carrier conjugate) is produced by immobilizing the thrombogenesis inhibitor to a soluble, biocompatible carrier. The thrombogenesis inhibitor may be an active analog or active fragment of that inhibitor. In preferred aspects of the invention immobilization includes contacting the inhibitor with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow linkage of the reagent to the inhibitor. The carrier is then bound to the

inhibitor-linked reagent. Alternatively, or in addition, the carrier is contacted with a cross-linking reagent and then is bound via the reagent to the inhibitor or to a reagent-linked inhibitor.

In another aspect of the invention, the base coat-linked or carrier-linked reagent is reduced prior to the binding step. The resulting exposed sulfhydryl group is then contacted with the inhibitor-linked reagent. Reduction results in the formation of sulfhydryl groups on the base coat or carrier which can react with the inhibitor-linked, bifunctional cross-linking reagent via a substitution reaction to form a disulfide bond, thereby covalently linking the thrombogenesis inhibitor to the base coat layer or carrier.

The invention will next be described in connection with certain illustrated embodiments. However, it should be clear that various modifications, additions, and deletions can be made without departing from the spirit or scope of the invention.

Brief Description of the Drawing

The foregoing and other objects of the present invention, the various features thereof, as well as the inventions thereof may be more fully understood from the following description when read together with the accompanying drawings in which:

FIGURE 1 is a diagrammatic representation of the pathways involved in thrombogenesis;

FIGURE 2 is a diagrammatic representation of pathways involved in Protein C activation and expression;

FIGURE 3 is a diagrammatic representation of platelet involvement in thrombogenesis;

FIGURE 4 is a schematic representation of the amino acid sequence of native thrombomodulin;

FIGURE 5 is a graphic representation of the activity of thrombomodulin derivatized with SPDP;

FIGURE 6 is a graphic representation of the activities of grafts including immobilized thrombomodulin or BSA; and

FIGURE 7 is a graphic representation of the activities of TM- or BSA-immobilized grafts.

Description of the Invention

This invention provides biocompatible, thromboresistant substances useful for implantable and extracorporeal devices in contact with the vascular system, and methods for their fabrication.

The substances provided by this invention include a synthetic biocompatible substance having a thrombogenesis-inhibiting reagent linked thereto via a biocompatible base coat adhered to the material's surface.

The material useful in a prosthetic extracorporeal or implantable device may be composed of any biocompatible, synthetic, preferably polymeric material having enough tensile strength to withstand the rigors of blood circulation, and having groups onto which a base coat can be directly or indirectly bound. Examples of such synthetic materials are polytetrafluoroethylene, polyethylene terphthalate, nylon, and the like. The material may have any dimensions suitable for the purpose for which it is being used. For example, it may be an integral part of an implanted heart valve or of an extracorporeal device used for hemodialysis or cardiopulmonary by-pass surgery, or it may be used to coat catheters or to line the interior of a vascular graft.

The synthetic material, when obtained, may be coated with or contain various noncovalently adhered impurities whose removal may be prerequisite for the adherence of a base coat thereto. For example, lubricants on commercial quality

polyethylene terphthalate can be removed by contacting the polyethylene terphthalate with a solution containing, for example, various detergents, solvents, or salts, which loosen and/or solubilize these impurities.

TABLEs 1 and 2 outline representative methods of preparing the biocompatible, thromboresistant substance, where "Da" refers to a synthetic material composed of woven polyethylene terphthalate fibers, and "HSA" refers to human serum albumin, "EDC" refers to carbodiionide, and "P-2-T" refers to pyridine-2-thione.

TABLE 1

STEP #	PROCESS
1)	Da + NaOH ----> Da-COOH
2)	Da-COOH + EDC ----> Da-EDC
3)	Da-EDC + HSA ----> Da-HSA + urea (EDC by-product)
4)	Da-HSA + SPDP ----> Da-HSA-SPDP
5)	Da-HSA-SPDP + DTT ----> Da-HSA-SH + P-2-T
6)	Inhibitor + SPDP ----> Inhibitor-SPDP
7)	Da-HSA-SH + Inhibitor-SPDP ----> Da-HSA-S-S-Inhibitor + P-2-T

TABLE 2

STEP #	PROCESS
1)	HSA + SPDP ----> HSA-SPDP
2)	HSA-SPDP + DTT ----> HSA-SH + P-2-T
3)	Inhibitor + SPDP ----> Inhibitor-SPDP
4)	HSA-SH + Inhibitor-SPDP --> HSA-S-S-Inhibitor + P-2-T
5)	Da + NaOH ----> Da-COOH
6)	Da-COOH + EDC ----> Da-EDC
7)	Da-EDC + HSA-S-S-Inhibitor -->Da-HSA-S-S-Inhibitor + urea (EDC by-product)

Initially, the material may be activated so as to enhance the binding of the base coat layer. This activating step increases the number of chemically active groups in the material. For example, alkaline hydrolysis may be performed to increase the number of reactive carboxylic acid groups in the polyethylene terphthalate to which a bifunctional cross-linking reagent such as carbodiimide may be bound. Ultimately, the base coat will adhere to the bound carbodiimide groups on the material. However, this method must be performed with care, as alkaline hydrolysis partially degrades

the polyethylene terphthalate, resulting in a fraying of the material's fibers.

At least one base coat layer is adhered to at least one surface of the synthetic material.

This layer, either adhered to the material or unbound, provides components for attachment of the thrombogenesis inhibitor. Such components provide more binding sites for the inhibitor than the synthetic material, alone, thereby amplifying the amount of inhibitor which may be bound. Useful components include proteins, peptides, lipoproteins, glycoproteins, glycosaminoglycans, synthetic polymers, dyes, antibiotics, and mixtures thereof.

Proteins such as serum albumin and fibronectin are particularly useful for this purpose as they are known to have anti-thrombogenic properties, themselves, are very desirable as base coat components (Lyman et al. (1965) Trans. Am. Soc. Artif. Intern. Organs 11:301; Falb et al. (1971) Fed. Proc. 30:1688). An HSA molecule, for example, has 65 amino groups available as binding sites.

Alternatively, the thrombogenesis inhibitor may be cross-linked to a second proteinous thrombogenesis inhibitor to enhance the thrombolytic activities of the conjugate. This second thrombogenesis inhibitor my be different from, or the same as the first thrombogenesis inhibitor.

Useful dyes and antibiotics include those that dissolve into the material surface, thus

providing functional groups for attachment of the thrombogenesis inhibitors. These include, for example, those chemicals classified as dispersed dyes such as anthraquinone-dispersed dyes (e.g., Dispersed Blue I (CAS Registry Number 2475-45-8) and related structures (Kirk-Othmer Encyclopedia of Chem. Tech., 3rd Ed. (1978-1984) (Grayson et al., eds.) Wiley Publishers, New York), and azo-type dyes such as Dispersed Orange 15 (Chemical Index Number CI #26080) and Dispersed Blue 11 (Chemical Index Number CI #11260). Useful antibiotics include quinolones having a chemical structure similar to the structure of anthraquinones, for example, Ciprofloxacin (Wood, (1988) British J. Clin. Prac. 42:469-472). These antibiotics are also useful for providing infection resistance.

Attachment of the base coat to the artificial surface may be covalent in nature. Methods to covalently bind proteins to polyethylene terphthalate involve attack of the free reactive succinimide ester group of the cross-linking reagent to primary amino groups on a protein. As shown in the example in TABLE 1, to covalently adhere the base coat to polyethylene terphthalate, the polyethylene terphthalate is initially treated with 0.5 N NaOH and reacted with carbodiimide before it is coated with HSA (base coat) in phosphate buffered saline (PBS).

A thrombogenesis inhibitor useful as a coating for surfaces in contact with blood, bodily fluids, or tissues, is then covalently adhered to the base coat via the component. Inhibitor-coated substances are ideal for implantable use in devices

which are in direct contact with blood. For example, by-pass grafts used to replace blood vessels often become filled with blood clots or thrombi, resulting in restricted blood flow. Since the inhibitor-coated substance is resistant to formation of blood clots, thrombosis and subsequent blockage of the bypass graft will be prevented. Likewise when catheters are placed into the vascular system for a diagnostic or therapeutic purposes, a blood clot often forms on the outside of the catheter. The clot may be washed off the catheter by flowing blood, or be jarred loose by manipulation of the catheter, increasing the possibility of embolism and blockage of the circulation to vital organs. Inhibitor-coated substances provide similar advantages for artificial or prosthetic heart valves, intraaortic balloon pumps, total or artificial heart or heart assist devices, intracaval devices, and any device in contact with the bloodstream. In addition, inhibitor-coated devices provide advantages for intracavity devices such as intraperitoneal dialysis catheters and subcutaneous implants where the thrombogenesis-induced inflammatory reactions would be diminished.

Likewise, TABLES 3 and 4 outline exemplary methods of preparing the soluble, pharmacological agent, wherein "SMCC" refers to "succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-caroboxylate."

TABLE 3

STEP #	PROCESS
1)	HSA + Traunt's ----> HSA-SH
2)	Inhibitor + SMCC ----> Inhibitor-SMCC
3)	HSA-SH + Inhibitor-SMCC ----> Inhibitor-SMCC-HSA

TABLE 4

STEP #	PROCESS
1)	Inhibitor + Traunt's ----> Inhibitor-SH
2)	HSA + SMCC ----> HSA-SMCC
3)	Inhibitor-SH + HSA-SMCC ----> Inhibitor-SMCC-HSA

Thrombogenesis inhibitors useful for these purposes include molecules which interfere with, or inhibit thrombogenesis. These include, but are not limited to, ATPase, ADPase, 5'-nucleotidase, streptokinase, urokinase, tissue plasminogen activator, thrombomodulin, anticoagulants, and platelet inhibit : (e.g., prostacycline and aspirin).

Active fragments of these thrombogenesis inhibitors may be provided by enzymatic digestion using any number of know proteolytic enzymes (e.g. trypsin, chymotrypsin) whose exact locus of cleavage along an amino acid sequence is well known in the art. In addition, active analogs, active fragments, active derivatives, and fusion products of these thrombogenesis inhibitors, and mixtures thereof may be provided by recombinant DNA techniques. See, e.g., Phillips et at. Fibrinolysis In Experimental Stroke; and Fears et al. (1990) Biochem. J. 266:693-696 (tPA analogs); Cassels et al. (1987) Biochem. J. 247:395-400 (tPA, UK, and SK analogs); Banton et al. (1987) Drugs 33:93-96; and Jackson et al. (1986) Biochem. J. 25:108-114 (SK analogs); and Lijnen et al. (1988) J. Biolog. Chem. 263:5594-5598 (UK analogs); and Bode et al. (1990) Circulation 81:1974-1980 (UK fragments).

ADPase reduces platelet aggregation by degrading ADP. ADP is stored in the dense granules of platelets and can be released by thrombin, epinephrine, collagen, and other stimulants. When released, ADP promotes platelet receptor binding to fibrinogen and to von Willebrand Factor, two proteins essential for aggregation, and then promotes thromboxane synthesis, platelet aggregation, more ADP release, and thus, self-enhances platelet aggregation.

ATPase catalyzes the conversion of ATP to ADP, which can then be acted upon by ADPase, as described above.

5'-nucleotidase (or AMPase), like ADPase, degrades ADP. It also degrades AMP, a competitive antagonist for ADP receptor binding, to adenosine, a platelet inhibitor. Adenosine inhibits platelet aggregation by increasing intracellular levels of cyclic AMP. High levels of cyclic AMP inhibit mobilization of calcium ions from platelet storage pools. Free calcium ions within the platelet stimulates release of additional ADP from platelet dense granules, and is necessary for platelet aggregation, hence thrombogenesis (see FIG. 2).

Thrombomodulin (TM) is a receptor protein found surface of endothelial and other cells which is involved in the regulation of coagulation, the various pathways of which are shown in FIG. 1. Thrombomodulin is a glycoprotein of about 60.3 kD molecular weight and approximately 575 amino acids (Esmon (1989) Prog. Hemost. Thromb. 9:29-55). Thrombomodulin binds thrombin, and in doing so, acts as a cofactor in the activation of Protein C by thrombin; it accelerates the binding of thrombin to the inactive form of Protein C (FIG. 2), thereby forming activated Protein C. Activated Protein C exhibits both anticoagulant and thrombolytic activities: it inhibits the clotting cascade at the levels of Factors V and VIII by the enzymatic cleavage of the activated forms of these clotting factors, and it takes part in the production of plasminogen activator, a protein with thrombolytic activity. Thrombomodulin also inhibits blood coagulation by inhibiting the unbound thrombin-catalyzed cleavage of inactive fibrinogen to fibrin (see e.g., Esmon et al. (1982) J. Biol. Chem.

257:7944-7947), and by the inhibiting platelet aggregation by blocking the ability of thrombin to activate platelets (see e.g., Murata et al. (1988) Thrombosis Res. 50:647-656 and Esmon et al. (1983) J. Biol. Chem. 20:12238-12242).

Native thrombomodulin can be obtained in active form from human lung and placenta, the isolation procedures of which are known to those skilled in the art (see e.g., EP 0239644; and Salem et al. (1984) J. Biol. Chem. 259:12246-12251). Thrombomodulin may also be obtained from cultured endothelial cells such as cultured human umbilical vein endothelial cells (Murata et al. (1988) Thrombosis Res. 50:647-656). Alternatively, since its amino acid sequence is known (FIG. 3), synthetic and recombinant forms of thrombomodulin may be produced by known procedures (see e.g., WO 88/09811 and EP 0290419).

The thrombogenesis inhibitor is directly or indirectly immobilized to the base coat or carrier via the use of a bifunctional cross-linking reagent. In particular, a heterobifunctional cross-linking reagent which has two different reactive groups at each end of a linear molecule, and can therefore bind two different reactive groups or other molecules or on a different region of the same molecule, is most useful as a bifunctional cross-linking agent. For example, photoreactive cross-linkers, such as sulfosuccinimidyl 2-(m-azodo-o-nitro-benzamido)-ethyl-1, 3'-dithio-propionate (SAND), or N-succinimidyl-6-(4-azoido-2'-nitrophenyl-amino) hexanoate (SANPAH) have a photoreactive group that

can directly insert into C-H bonds of the base coat by photochemical coupling, while the other group remains free to bind to proteins.

Other useful and preferable cross-linking reagents such as N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and succinimidyl 4-(N-maleimido methyl)-cyclohexane-1-carboxylate (SMCC) and their characteristics are found in TABLE 5. The "Double-Agent Number" listed for each reagent is the commercial designation for the reagent as made available by Pierce Chemical Co. (Rockford, Illinois). However, these reagents may be obtained from other commercial sources, as well.

TABLE 5

CROSS-LINKING REAGENTS (part A)

Double-Agent Number	Double-Agent Acronym	Bifunctionality		Reactive towards:		
		Homo	Hetero	NH_2	SH	Photo-Reactive
21551	ANB-NOS		X	X		X
20106	APB		X		X	X
20107	APG		X			X
21559	APTP		X		X	X
21579	BS^3	X		X		
22319	BMH	X			X	
21554	BSOCOES	X		X		
21524	DFDNB	X		X		
20047	DIDS	X		X		
20664	DMA	X		X		
20666	DMP	X		X		
20668	DMS	X		X		
22585	DSP	X		X		
21555	DSS	X		X		
20590	DST	X		X		
20665	DTBP	X		X		
22590	DTBPA	X				X
21577	DTSSP	X		X		
21550	EADB		X	X		X
21565	EGS	X		X		
23700	FNPA		X	X		X
21560	HSAB		X	X		X

TABLE 5 (part A, Cont'd)

Double-Agent Number	Double-Agent Acronym	Bifunctionality		Reactive towards:		
		Homo	Hetero	NH_2	SH	Photo-Reactive
26095	MABI		X	X		X
22310	MBS		X	X	X	
27715	NHS-ASA		X	X		X
20669	PNP-DTP		X	X		X
21552	SADP		X	X		X
21549	SAND		X	X		X
22588	SANPAH		X	X		X
27716	SASD		X	X		X
22325	SIAB		X	X	X	X
22320	SMCC		X	X	X	
22315	SMPB		X	X	X	
21557	SPDP		X	X	X	
21556	Sulfo-BSOCOES	X		X		
20591	Sulfo-DST	X		X		
21556	Sulfo-EGS	X		X		
22312	Sulfo-MBS		X	X	X	
21553	Sulfo-SADP		X	X		X
22589	Sulfo-SANPAH		X	X		X

TABLE 5 (part A, Cont'd)

Double-Agent Number	Double-Agent Acronym	Bifunctionality Homo	Bifunctionality Hetero	Reactive towards: NH_2	Reactive towards: SH	Reactive towards: Photo-Reactive
22327	Sulfo-SIAB		X	X	X	
22322	Sulfo-SMCC		X	X	X	
22317	Sulfo-SMPB		X	X	X	
26101	TRAUNT'S	X		X		

CROSS-LINKING REAGENTS (part B)

Agent Acronym	Chemical Name
ANB-NOS	N-5-azido-2-nitrobenzoyloxysuccinimide
APB	p-azidophenacyl bromide
APG	p-azidophenyl glyoxal
APTP	n-4-(azidophenylthio)phthalimide
BS^3	bis(sulfosuccinimidyl) suberate
BMH	bis maleimidohexane
BSOCOES	bis[2-(succinimidooxycarbonyloxy)-ethyl]sulfone
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DIDS	4,4'-diisothiocyano-2,2'-disulfonic acid stilbene

TABLE 5 (part B, Cont'd)

Agent Acronym	Chemical Name
DMA	dimethyl adipimidate-2 HCl
DMP	dimethyl pimelimidate-2 HCl
DMS	dimethyl suberimidate-2 HCl
DSP	dithiobis(succinimidylpropionate)
DSS	disuccinimidyl suberate
DST	disuccinimidyl tartarate
DTBP	dimethyl 3,3'-dithiobispropionimidate-2-HCl
DTBPA	4,4'-diothiobisphenylazide
DTSSP	3,3-dithiobis(sulfosuccinimidyl-propionate)
EADB	ethyl-4-azidophenyl 1,4-dithio-butyrimidate
EGS	ete glycolbis(succinimidyl-succinate)
FNPA	1-azido-4-fluoro-3-nitobenzene
HSAB	N-hydroxysuccinimidyl-4-azidobenzoate
MABI	methyl-4-azidobenzoimidate
MBS	m-maleimidobenzoyl-N-hydroxysulfo-succinimide ester
NHS-ASA	N-hydroxysuccinimidyl-4-azidosalicylic acid
PNP-DTP	p-nitrophenyl-2-diazo-3,3,3-trifluoro-propionate
SADP	N-succinimidyl(4-axidophenyl)-1,3'-di-thiopropionate

TABLE 5 (part B, Cont'd)

Agent Acronym	Chemical Name
SAND	sulfosuccinimidyl 2-(m-azido-o-nitro-benzamido)-ethyl-1,3'-dithiopropionate
SANPAH	N-succinimidyl-6(4'-azido-2'-nitro-phenyl-amino)hexanoate
SASD	sulfosuccinimidyl 2-(p-azidosalicyl-amido)ethyl-1,3'-dithio-propionate
SIAB	N-succinimidyl(4-iodoacetyl)amino-benzoate
SMCC	succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate
SMPB	succinimidyl 4-(p-maleimidophenyl)-butyrate
SPDP	N-succinimidyl 3-(2-pyridyldithio) propionate
Sulfo-BSOCOES	bis[2-(sulfosuccinimidooxy-carbonyl-oxy)ethyl]sulfone
Sulfo-DST	disulfosuccinimidyl tartarate
Sulfo-EGS	ethylene glycolbis(sulfosuccinimidyl-succinate)
Sulfo-MBS	m-maleimidobenzoyl-N-hydro-xysulfo-succinimide ester
Sulfo-SADP	sulfosuccinimidyl(4-azidophenyldithio)-propionate
Sulfo-SANPAH	sulfosuccinimidyl 6-(4'azido-2'-nithro-phenylamino)hexanoate

TABLE 5 (part B, Cont'd)

Agent Acronym	Chemical Name
Sulfo-SIAB	sulfosuccinimidyl(4-iodoacetyl)amino-benzoate
Sulfo-SMCC	sulfosuccinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate
Sulfo-SMPB	sulfosuccinimidyl 4-(p-maleimido-phenyl)butyrate
TRAUNT'S	2-iminothiolane-HCl

The cross-linking reagent maybe applied to the base coat in amounts such that the desired binding site density is achieved. Binding site density is that amount of cross-linking reagent, in terms of moles/gram synthetic material, to bind to the base coat while providing confluent coverage of the surface.

To put the inhibitor in condition for linkage to the base coat or carrier, the cross-linking reagent may be initially coupled separately to both the base coat or carrier and to the inhibitor. The kinetic constants of the inhibitors are compared before and after coupling to evaluate effects of the procedure on their kinetic constants. The inhibitor should remain biologically active after being coupled. Therefore, standard activity assays specific for the inhibitor to be immobilized are performed using a standard thrombin solution to evaluate this capacity.

As an alternative, the component of the base coat may be bound to the thrombogenesis inhibitor forming a conjugate prior to its adherence to the synthetic material, and the conjugate bound to the synthetic material as shown in TABLE 2. The thrombogenesis inhibitor conjugate, itself, retains biological activity, and can be used as an agent to increase inhibitor half-life in the circulation as it is not easily cleared by the kidney.

In the special case of SPDP derivatization, linkage of certain groups on the thrombogenesis inhibitor to SPDP may destroy some inhibitor activity because at least some of these groups may be required for such activity. However, by adjusting the reaction ratio of thrombogenesis inhibitor to SPDP, and running the reaction at near physiological pH, SPDP becomes somewhat selective for epsilon amino groups. The result of these conditions favor a 1:1 (mole:mole) conjugation ratio of inhibitor to SPDP covalently bound without destroying biological activity.

SPDP will react with terminal as well as epsilon amino groups, Since derivatization of a terminal amino group can inactivate a biologically active protein, T-BLOCK (Pierce Chemical Co., Rockford, Illinois) may be used to block that group during SPDP-derivatization. The T-BLOCK is then removed after derivatization to restore biological activity.

The invention will be further understood from the following, non-limiting examples.

EXAMPLE 1: Preparation of Graft Materials

A. Pretreatment and Activation of Polyethylene Terphthalate

Polyethylene terphthalate (DuPont) is sectioned into 1.0 cm lengths. The lubricant on and in the woven surface is removed by washing once for 1 hr with carbon tetrachloride, and twice with 100% CH_3OH. The methanol is removed by multiple water washes, followed by one wash in phosphate buffered saline (PBS), pH 7.4.

The graft material is then subjected to alkaline hydrolysis to increase available COOH groups. The material is treated with 0.5 M NaOH at 50°C for 1 hr. It is then washed with H_2O repeatedly, and the following steps initiated immediately.

B. Carbodiimide Derivatization of Activated Polyethylene Terphthalate

The activated material is placed into 100.0 ml of 10 mM water-soluble carbodiimide (EDC) in deionized water, pH 4.6-5.0, for 1 hour at RT with constant stirring. The material is removed and washed in PBS to remove excess unbound EDC.

C. Base Coat Layer Formation

The base coat is applied to the lumen of the polyethylene terphthalate graft material. The derivatized polyethylene terphthalate material is incubated in a 5% HSA solution in PBS at 1 ml/cm^2

graft material for 24 hr at RT with constant stirring. The graft is removed and washed in PBS to remove nonspecifically bound HSA. Approximately 2 µg protein/mg polyethylene terphthalate is covalently bound.

D. Linkage of SPDP to the Base Coat

The HSA-bound polyethylene terphthalate material is incubated in a 1.0 mM solution of SPDP in PBS, pH 7.4, to bind SPDP to the HSA (100 mM SPDP/cm^2 base coat). Incubation is terminated after 30-40 min at RT. The graft is washed in PBS to remove nonspecifically bound SPDP.

E. Activation of SPDP on Base Coat and Measurement of Binding Site Density

The SPDP-linked material is dried and weighed to obtain its absolute weight. It is then placed in a 50 mM solution of dithiotreitol (DTT) for 5 min at RT. This reaction releases P-2-T from the bound SPDP, and simultaneously forms free sulfhydryl (SH) groups on the base coat. The released P-2-T is quantitated by absorption spectrophotometry at 343 nm using its extinction coefficient ($E = 8.08 \times 10^3$), and is directly proportional to the quantity of bound SPDP or binding sites. The number of binding sites are calculated and expressed as moles of sites/g of polyethylene terphthalate.

The material is then washed 5 times in PBS and 4 times in dH_2O.

EXAMPLE 2: Streptokinase (SK) Immobilization

A.-E. (same as described in EXAMPLE 1.)

F. Linkage of SK to Cross-linker

Albumin-free SK (KabiVitrum, Stockholm, Sweden) is filter-transferred with use of a PD-10 column (Pharmacia, Piscataway, NJ) to remove contaminants and amino acid preservatives, and to transfer SK into 0.1 M PBS buffer, pH 7.5. 0.5 ml fractions are collected, absorbance values at 280 nm recorded, and desired fractions pooled. The molar concentration of pooled SK is determined using its absorptivity coefficient at 280 nm (A_{280} 1.0%/1.0 cm = 7.5). The concentration of pooled SK is then set to approximately 0.1 mM in PBS buffer. A 20 mM SPDP solution is prepared in absolute ethanol just prior to use and mixed with SK in various mole to mole ratios (i.e., 1:10). The mixture is allowed to incubate for 30 min at 23°C. It is then filter-transferred into PBS buffer, pH 7.5 using a PD-10 column equilibrated with PBS buffer, pH 7.5. 0.5 ml fractions are collected, absorbance measurements at 280 nm recorded, and desired fractions (i.e., A_{280} greater than 1.8) pooled. The pool contains SK linked to 2-pyridine disulphide (SK-2-PD) in PBS buffer, pH 7.5.

G. Measurement of SPDP Bound to SK

The binding of SPDP to SK can be quantitated by the addition of DTT which liberates pyridine-2-thione (P-2-T) from SPDP bound to SK, and which can be measured spectrophotometrically at 343

nm. From this measurement, the moles of SPDP bound to SK can be calculated. Each P-2-T released represents one covalent attachment of SPDP to SK. One mole of SK binds per 1.2 moles SPDP in the present studies.

H. Linkage of Derivatized SK to Basecoat

The reduced SPDP-linked base coat (having free SH groups) is washed with PBS to remove the DTT. SPDP-linked SK is then added to the graft at 50.0 mg/cm^2 polyethylene terphthalate. The solution is incubated overnight at RT to allow the binding of SPDP-SK to SH groups on the polyethylene terphthalate graft. The polyethylene terphthalate material with SK covalently immobilized thereto is then washed and stored in PBS.

I. Immobilized SK Activity Analysis

SK, when mixed with human plasminogen forms an active proteolytic complex that can be quantitated using the chromogenic substrate, S-2251 (KabiVitrum A.B., Stockholm, Sweden). A standard curve is constructed using known concentrations of SK (500-10,000 units/ml). A 60 ml aliquot of each standard is added to 120 ml of 0.2 mM human plasminogen (American Diagnostica, Greenwich, CT), and incubated at 37°C for 10 min. SK-plasminogen complex is formed as well as free plasma which interferes with the analysis. This interference is eliminated by the addition of 60 ml of 2.0 mg/ml soybean trypsin inhibitor which inhibits all free plasma. The quantitative analysis of SK-plasminogen is accomplished by addition of 420 ml of 0.86 mM S-2251

in 50 mM Tris-HCl, 12 mM NaCl, pH 7.4, and monitoring the change in absorbance per min at 405 nM at 37°C. The immobilized SK is substituted for the standard solution in the assay. The measured activity of the material on S-2251 is then equated to the standard curve.

EXAMPLE 3: Urokinase (UK) Immobilization

A.-H. (Same as described for EXAMPLE 1 except that UK (Abbokinase, Abbot Chemical Co., Chicago, IL) is substituted for SK.)

I. Immobilized UK Activity Analysis

The activity of immobilized UK is evaluated against a standard curve generated using soluble UK in concentrations of from 10 to 1000 units of UK (referred here as CTA units). The chromogenic substrate, S-2444 (KabiVitrum AB, Stockholm, Sweden), is used at 0.3 mM to measure activity of UK standards by monitoring change in absorbance at 405 nm in 50.0 mM Tris, 12.0 mM NaCl, pH 8.8 at 37°C. A standard curve is thus generated. A section of material with immobilized or bonded UK is placed into the substrate under the same conditions, and the change in absorbance is recorded. The activity of the immobilized UK is evaluated by comparison to the activity of the standard curve.

EXAMPLE 4: Thrombomodulin (TM) Immobilization

A.-E. (same as described in EXAMPLE 1)

F. Linkage of SPDP to Thrombomodulin

Lyophilized thrombomodulin (American Bioproduct Co., Parsippany, N.J.) is resuspended in deionized H_2O at 10 µg/ml (or 1 U/ml). SPDP (Pharmacia, Piscataway, NJ) is dissolved in 100% EtOH to 10 mM. One part thrombomodulin is mixed with four parts SPDP (mole:mole), and incubated for 30 min at RT. SPDP-bound thrombomodulin is separated from free SPDP and reaction by-products by chromatography on a G-25 column, the derivatized thrombomodulin being eluted first.

The binding of SPDP to thrombomodulin can be quantitated by the addition of DTT which liberates pyridine-2-thione (P-2-T) from SPDP bound to thrombomodulin, and which can be measured spectrophotometrically at 343 nm. From this measurement, the moles of SPDP bound to thrombomodulin can be calculated. The amount of P-2-T released is directly proportional to the number of SPDP substitution reactions (covalent linkages) that have occurred between the base coat SH groups and SPDP-thrombomodulin. One mole of thrombomodulin appears to bind greater than 1.0 moles of SPDP in the present study. The mole:mole ratio of TM:SPDP derivatization is only an estimate, however, results suggest that TM biochemically interferes with the spectrophotometric means of P-2-T quantitation, an anomaly seemingly peculiar to TM derivatization with SPDP.

H. Linkage of Derivatized Thrombomodulin to Base Coat

The base coat (having free SH groups available due either to reduction with DTT or to treatment with Traunt's reagent) is washed with PBS (to remove the DTT or Traunt's reagent). SPDP-linked thrombomodulin is then added to the graft at approximately 4.0 $\mu g/cm^2$ polyethylene terphthalate. The solution is incubated overnight at RT to allow the binding of SPDP-thrombomodulin to SH groups on the polyethylene terphthalate graft. The polyethylene terphthalate material with thrombomodulin covalently immobilized thereto is then washed and stored in PBS.

G. Thrombomodulin Activity Assay

The following reagents were prepared: (1) thrombomodulin (TM): 10 µg (1 U vial, American Bioproducts Co., Parsippany, NJ) was reconstituted with 1 ml dH_2O; (2) Protein C (PC): 100 µg protein (10 PEU/vial, American Bioproducts Co.) was reconstituted with 1 ml dH_2O (= 0.1 µg/µl PC stock solution), and 10 µl of stock solution was diluted into 190 µl TM buffer (20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 10 mM $CaCl_2$, 0.1% BSA) for use in the assay; (3) thrombin (T): a 25 U/ml solution was prepared from a 1:4 dilution of a 100 U/ml stock solution with TM buffer; (4) hirudin (H): 2 mg (Ciba-Geigy, Summit, NJ) was reconstituted 1.913 ml TM buffer (11500 U/ml); (5) S-2266: a 4 mM solution was prepared by mixing 2.318 mg in 1 ml dH_2O; (6) assay buffer is 25 mM Tris-HCl, pH 8.0, 0.15 M NaCl).

200 µl of the diluted PC solution, 5 µl of 25 U/ml T, and 50 µl of stock TM solution were mixed and incubated at 37°C for 30 min. Five control samples not containing TM were also made and incubated at 37°C for 30 min.

After the incubation, 10 µl of stock H solution and 740 µl of assay buffer was then added to inhibit excess thrombin. The samples are shown in TABLE 6.

TABLE 6

sample no.	diluted PC	T	stock TM	TM buffer	H
1*	200 µl	5 µl	--	50 µl	10 µl
2*	200 µl	5 µl	--	60 µl	--
3*	--	5 µl	--	250 µl	10 µl
4*	--	5 µl	--	260 µl	--
5*	200 µl	--	--	65 µl	--
6**	200 µl	5 µl	50 µl	--	10 µl

* control sample

** test sample

These solutions were incubated at 37°C for 3 min. They were then transferred to cuvettes, and 50 µl of 4 mM S-2266 was added to each. The absorbance at A_{405} was measured for 5 min., with readings taken every 15 sec. The ΔA/min. was also calculated.

The change in absorbance A_{405}/min. of the TM test sample (#6) was higher than in any of the control samples, indicative of TM activity. The

ΔA/min. of the TM test sample was stable and consistent; 50 µl of TM stock solution (1U/ml) gave a ΔA/min. of about 0.06, or 0.05 units of TM expresses a ΔA/min. of 0.06 at 405 nm using the chromogenic substrate S-2266 under these conditions.

The test mechanism is:

a. TM + T ----> TM-T complex

b. Inactive PC $\xrightarrow{\text{TM-T complex}}$ Activated PC

c. S-2266 + Activated PC ----> increased A_{405}

I. Activity of SPDP-Derivatized Thrombomodulin

200 µl of stock TM solution was put into a cuvette. 10 µl of 11.0 µM SPDP (in TM buffer and EtOH) was added, and the A_{343} before and after SPDP addition was measured. The solution was incubated at RT for about 30 min. The TM solution was allowed to stand for about 30 min. before 50 µl was assayed for activity using the same procedure described in EXAMPLE 1.

From the ΔA/min. values and as shown in FIG. 5, the TM-SPDP sample (#6) demonstrated activity (i.e., the ability to activate PC in the presence of thrombin).

J. Activity of Immobilized Derivatized TM (#1)

6 polyethylene terphthalate graft patches that were previously prepared as described in EXAMPLES 1 and 2, and stored in phosphate buffered

saline (PBS) + Na azide, were washed 2 times in PBS and 1 time with PBS + sodium dodecyl sulfate (SDS). They were then sonicated and washed 3 times in PBS. The grafts were put into clean test tubes and incubated with 20 mM Traunt's reagent in PBS buffer for about 2 hr at RT.

A solution of TM-SPDP was made by mixing 500 µl of stock TM (1 U/ml) with 10 µl of 22.0 µM SPDP. The solution was incubated at RT for about 30 min. It was then purified on a G-25 column (Pharmacia, Piscataway, NJ) to separate TM-SPDP from free SPDP.

Bovine serum albumin (BSA)-SPDP was prepared as a control in the evaluation of TM-bound polyethylene terphthalate grafts. A solution of BSA-SPDP was made by mixing 2 ml of 1% BSA with 62 µl of 20 mM SPDP. The solution was incubated at RT for 30 min. before being purified on a PD-10 column (Pharmacia, Piscataway, NJ). The first peak of each sample was collected. The A_{343} of a 1:5 dilution was measured before and 5 min. after the addition of 50 µl of 100 mM DTT to each ml of solution.

The grafts were washed 5 times in PBS after treatment with Traunt's reagent. 0.5 µl of TM-SPDP solution was added to grafts #1 and #2, and 0.5 µl of BSA-SPDP solution was added to grafts #3 and #4 (see TABLE 7). The grafts were allowed to incubate overnight at RT to immobilize TM on the test grafts and BSA on the control grafts.

TABLE 7

graft #	graft type	treatment
1	immobilized TM	PC + T + H
2	immobilized TM	PC + T + H
3	immobilized BSA	PC + T + H
4	immobilized BSA	PC + T + H

The grafts were then washed 2 times in PBS and 3 times in TM buffer. They were put into clean polyproplyene tubes and assayed for activity. 200 µl of PC (0.1 µg/µl) was added to each graft and mixed, followed by 5 µl of thrombin (25 U/ml). They were incubated at 37°C for 30 min. 10 µl of 11,500 U/ml H and 740 µl of assay buffer were added to each tube, which was then incubated for 3 min. at 37°C. Each sample was then put into a cuvette. 50 µl of 4 mM S-2266 was added, and the A_{405} was measured for 5 min.

As shown in FIG. 6, the TM-immobilized grafts demonstrated greater activity in the assay when compared with the BSA-immobilized grafts as controls. The approximate ΔA/min. for the TM grafts was 0.004, and for BSA grafts, was 0.001. The ΔA/5 min. for the TM grafts was 0.013, and for the BSA grafts, was 0.005. The TM grafts demonstrated an increase in activity in the PC assay over the activity of the BSA grafts, which shows that TM has been immobilized to the graft and retains its activity.

K. Activity of Derivitized, Immobilized TM (#2)

400 µl TM was derivitized with 10 µl of 22 µM SPDP in EtOH. The solution was run through a G-25 column to purify and arrest the run after 30 min. The first peak fractions were collected and not pooled. 1.0 ml of 1% BSA in PBS was mixed with 31 µl of 22 µM SPDP in EtOH to form a control graft. After 30 min. the solution was run through a G-25 column. The first peak fractions were collected and pooled. 5 grafts were washed 5 times with PBS, and then sonicated in PBS + 0.1% SDS to insure removal of noncovalently bound albumin. Each graft was incubated at RT for about 2 hr in 1 ml of 20 mM Traunt's reagent in PBST (PBS buffer + 0.1% Tween 20). The grafts were washed 2 times in PBST and 3 times in PBS. As shown in TABLE 8, graft #1 was incubated with 0.5 ml of the first fraction of the TM-SPDP peak (A_{280} = 0.142); graft #2 was incubated with about 0.4 ml of the TM-SPDP solution (A_{280} = 0.036) of the second fraction; and grafts #3 and #4 were incubated with 0.5 ml of BSA-SPDP solution.

TABLE 8

graft #	graft type	treatment
1	immobilized TM	PC + T + H
2	immobilized TM	PC + T + H
3	immobilized BSA	PC + T + H
4	immobilized BSA + TM solution	TM + PC + T + H

The A_{343} of each solution was measured at t = 0 using PBS as the blank. The grafts were then allowed to incubate overnight at RT. The A_{343} of each solution was measured as an attempt to estimate the degree of TM immobilization. The grafts were washed 2 times in PBS and 3 times in TM buffer to remove noncovalently bound TM. The grafts were assayed for activity as described in EXAMPLE 8 except that PC (50 µl + 950 µl TM buffer) was added before T (30 µl + 210 µl TM buffer). The A_{405} was measured over a 10 min. period.

As shown in FIG. 7, the TM-immobilized grafts (#1 and #2) showed more activity than the BSA-immobilized graft (#3). Graft #4 with free TM in solution as a positive control did not show significant activity. TM graft #1 was incubated in a solution with approximately three times the TM-SPDP than graft #2. Immobilized TM graft #1 demonstrated two times greater activity than did immobilized TM graft #2, indicating that there is a relationship of proportionally greater TM bound to that graft. TM grafts #1 and #2 had 5 times and 2 times the ΔA (respectively) as BSA-blocked graft #3.

These results indicate that TM can be successfully immobilized to the surface of polyethylene terphthalate graft material, that immobilized TM retains thrombogenesis inhibiting activity, that added T can be bound by the immobilized TM, and that TM-bound T is capable of activating Protein C. Immobilized TM serves to enhance thromboresistance, as activated Protein C

degrades Factor Va and VIIIa, thus inhibiting thrombus formation.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

We claim:

1. A soluble, biocompatible, pharmacological agent for inhibiting thrombin generation and thrombus formation comprising:

(a) a soluble, biocompatible carrier; and

(b) a thrombogenesis inhibitor immobilized on said carrier, said inhibitor being other than hirudin, or an active analog or active fragment of said inhibitor,

wherein said carrier includes a component which binds said thrombogenesis inhibitor.

2. The agent of claim 1 wherein said component of said carrier is selected from the group consisting of a protein, polypeptide, peptide, lipoprotein, glycoprotein, glycosaminoglycan, hydrogel, synthetic polymer, dyes, antibiotics, and mixtures thereof.

3. The agent of claim 2 wherein said component comprises a polypeptide.

4. The agent of claim 3 wherein said polypeptide is selected from the group consisting of serum albumin, fibronectin, and mixtures thereof.

5. The agent of claim 1 wherein said inhibitor is selected from the group consisting of streptokinase, urokinase, tissue plasminogen activator, ATPase, ADPase, 5'-nucleotidase, thrombomodulin, active fragments and active analogs thereof, and mixtures thereof.

6. The agent of claim 1 further comprising a bifunctional cross-linking reagent linking said thrombogenesis inhibitor to said carrier.

7. The agent of claim 6 wherein said bifunctional cross-linking reagent comprises a heterobifunctional cross-linking reagent.

8. The agent of claim 6 wherein said heterobifunctional cross-linking reagent comprises 3-(2-pyridyldithio)propionate.

9. The agent of claim 6 wherein said bifunctional cross-linking reagent comprises a homobifunctional cross-linking reagent.

10. A method of producing a soluble, biocompatible, pharmacological agent useful for inhibiting thrombin generation and thrombus formation,

said method comprising the step of immobilizing a thrombogenesis inhibitor on a soluble, biocompatible carrier, said inhibitor being other than hirudin, or an active analog or active fragment of said inhibitor, and said carrier including a component that binds said thrombogenesis inhibitor.

11. The method of claim 10 wherein said immobi zing step comprises the steps of:

(a) contacting said thrombogenesis inhibitor with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow li..age of said reagent to said thrombogenesis inhibitor; and

(b) binding said thrombogenesis inhibitor-linked reagent to said carrier.

12. The method of claim 10 wherein said immobilizing step comprises immobilizing on a soluble, biocompatible carrier a thrombogenesis inhibitor selected from the group consisting of streptokinase, u okinase, tissue plasminigen activator, ATPase, ADPase, 5'-nucleotidase, thrombomodulin, active fragments and active analogs thereof, and mixtures thereof.

13. The method of claim 10 wherein said immobilizing step comprises immobilizing a thrombogenesis inhibitor to a soluble, biocompatible carrier selected from the group consisting of a protein, polypeptide, peptide, lipoprotein, glycoprotein, glycosaminoglycan, hydrogel, synthetic polymer, dyes, antibiotics, and mixtures thereof.

14. The method of claim 10 wherein said contacting step comprises:

(a) contacting said carrier with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow linkage of said reagent to said carrier; and

(b) binding said carrier-linked reagent to said thrombogenesis inhibitor.

15. The method of claim 14 wherein said contacting step further comprising contacting said thrombogenesis inhibitor with at least one molecule of said bifunctional cross-linking reagent for a time sufficient to allow linkage thereto,

and said binding step further comprises binding said carrier-linked reagent to said thrombogenesis inhibitor-linked reagent.

16. The method of claim 11 wherein said contacting step comprises contacting said thrombogenesis inhibitor with a bifunctional cross-linking reagent selected from the group consisting of heterobifunctional cross-linking reagents, homobifuntional cross-linking reagents, and mixtures thereof.

17. The method of claim 14 wherein said contacting step includes contacting said carrier with a bifunctional cross-linking reagent selected from the group consisting of heterobifunctional cross-linking reagents, homobifuntional cross-linking reagents, and mixtures thereof.

18. The method of claim 16 wherein said contacting step includes contacting said thrombogenesis inhibitor with the heterobifunctional cross-linking reagent, 3-(2-pyridyldithio)propionate.

19. The method of claim 17 wherein said contacting step includes contacting said thrombogenesis inhibitor with the heterobifunctional cross-linking reagent, 3-(2-pyridyldithio)propionate.

20. The method of claim 11 further comprising the steps of:

(a) reducing said carrier to expose a sulfhydryl group thereon;

(b) contacting said exposed sulfhydryl group with said inhibitor-linked reagent; and

(c) inducing a substitution reaction between said sulfhydryl group and said inhibitor-linked reagent,

said reaction resulting in linkage of said carrier to said inhibitor.

AMENDED CLAIMS

[received by the International Bureau on 19 December 1991 (19.12.91); original claims 1-20 replaced by amended claims 1-12 (3 pages)]

1. A soluble, biocompatible, pharmacological agent for inhibiting thrombin generation and thrombus formation comprising:

(a) a soluble, biocompatible carrier selected from the group consisting of a protein, polypeptide, peptide, lipoprotein, glycoprotein, glycosaminoglycan, synthetic polymer, dye, antibiotic, and mixtures thereof; and

(b) a thrombogenesis inhibitor covalently immobilized on said carrier, said inhibitor being other than hirudin, or an active analog or active fragment of said inhibitor,

wherein said carrier binds said thrombogenesis inhibitor.

3. The agent of claim 1 wherein said component comprises a polypeptide.

4. The agent of claim 3 wherein said polypeptide is selected from the group consisting of serum albumin, fibronectin, and mixtures thereof.

5. The agent of claim 1 wherein said inhibitor is selected from the group consisting of streptokinase, urokinase, tissue plasminogen activator, ATPase, ADPase, 5'-nucleotidase, thrombomodulin, active fragments and active analogs thereof, and mixtures thereof.

6. The agent of claim 1 further comprising a bifunctional cross-linking reagent linking said thrombogenesis inhibitor to said carrier.

7. The agent of claim 6 wherein said bifunctional cross-linking reagent comprises a heterobifunctional cross-linking reagent.

8. The agent of claim 6 wherein said heterobifunctional cross-linking reagent comprises 3-(2-pyridyldithio)propionate.

9. The agent of claim 6 wherein said bifunctional cross-linking reagent comprises a homobifunctional cross-linking reagent.

10. A method of producing a soluble, biocompatible, pharmacological agent useful for inhibiting thrombin generation and thrombus formation,

said method comprising the step of covalently immobilizing a thrombogenesis inhibitor on a soluble, biocompatible carrier selected from the group consisting of a protein, peptide, lipoprotein, glycoprotein, dye, antibiotic, and mixtures thereof, said inhibitor being other than hirudin, or an active analog or active fragment of said inhibitor, and said carrier binding said thrombogenesis inhibitor.

11. The method of claim 10 wherein said immobilizing step comprises the steps of:

(a) contacting said thrombogenesis inhibitor with at least one molecule of a bifunctional cross-linking reagent for a time sufficient to allow linkage of said reagent to said thrombogenesis inhibitor; and

(b) binding said thrombogenesis inhibitor-linked reagent to said carrier.

12. The method of claim 10 wherein said immobilizing step comprises immobilizing on a soluble, biocompatible carrier a thrombogenesis inhibitor selected from the group consisting of streptokinase, urokinase, tissue plasminigen activator, ATPase, ADPase, 5'-nucleotidase, thrombomodulin, active fragments and active analogs thereof, and mixtures thereof.

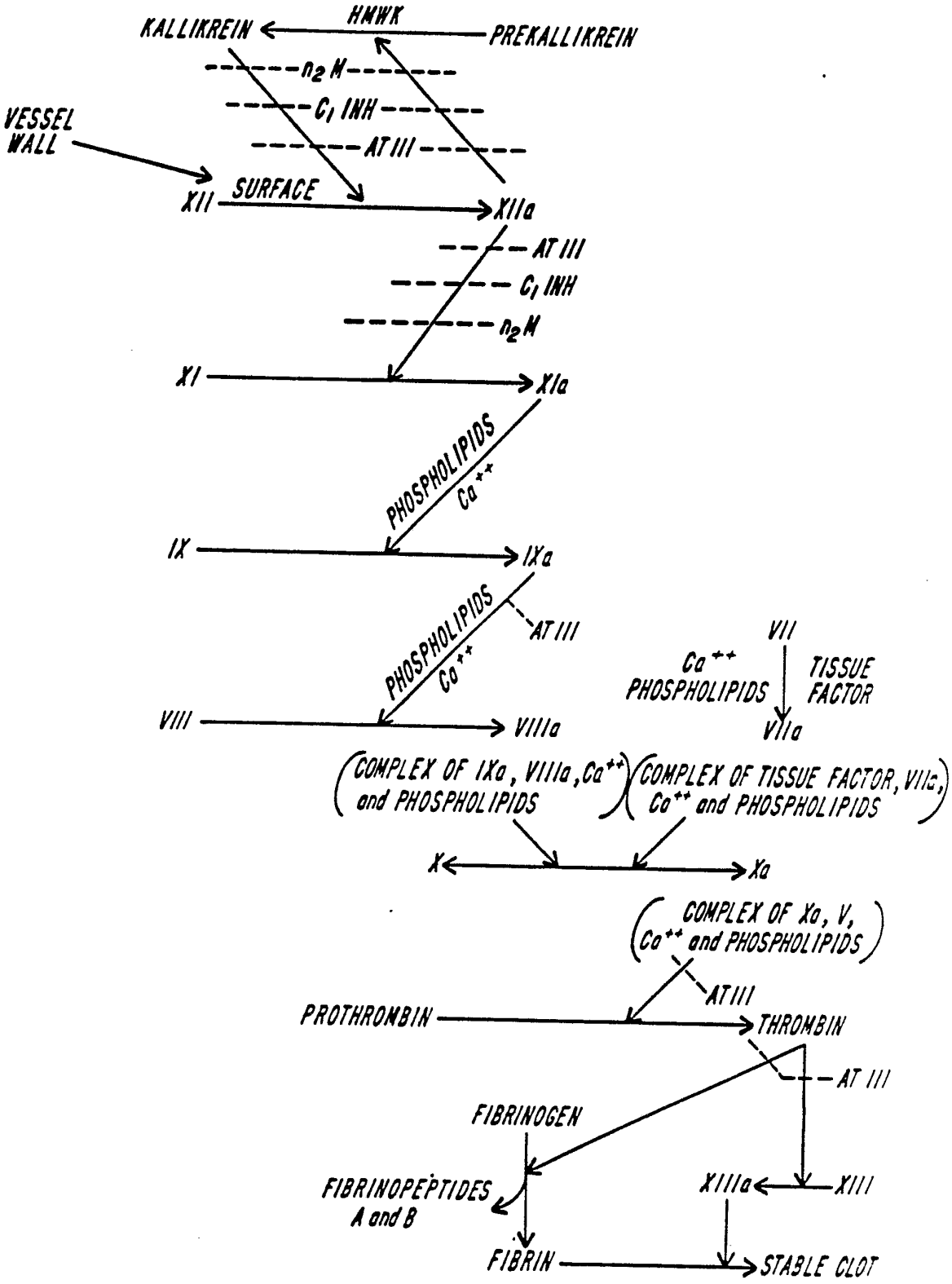
HMWK
KALLIKREIN
PREKALLIKREIN
α_2M
C_1 INH
AT III
VESSEL WALL
SURFACE
XII
XIIa
AT III
C_1 INH
α_2M
XI
XIa
PHOSPHOLIPIDS
Ca^{++}
IX
IXa
PHOSPHOLIPIDS
Ca^{++}
AT III
VIII
VIIIa
VII
Ca^{++}
PHOSPHOLIPIDS
TISSUE FACTOR
VIIa
(COMPLEX OF IXa, VIIIa, Ca^{++} and PHOSPHOLIPIDS)
(COMPLEX OF TISSUE FACTOR, VIIa, Ca^{++} and PHOSPHOLIPIDS)
X
Xa
(COMPLEX OF Xa, V, Ca^{++} and PHOSPHOLIPIDS)
AT III
PROTHROMBIN
THROMBIN
AT III
FIBRINOGEN
FIBRINOPEPTIDES A and B
XIIIa
XIII
FIBRIN
STABLE CLOT

FIG. 1

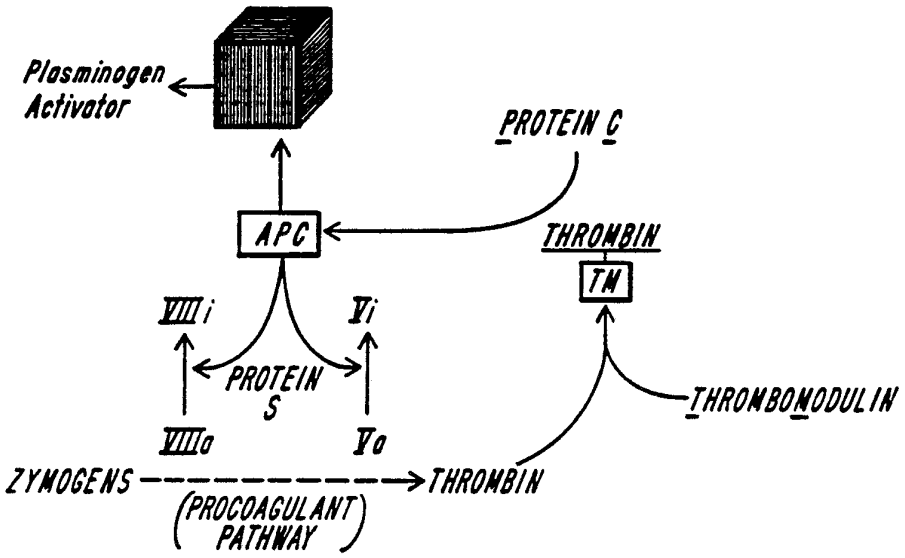
Plasminogen Activator
PROTEIN C
APC
THROMBIN
TM
VIIIi
Vi
PROTEIN S
THROMBOMODULIN
VIIIa
Va
ZYMOGENS
THROMBIN
(PROCOAGULANT PATHWAY)

FIG. 2

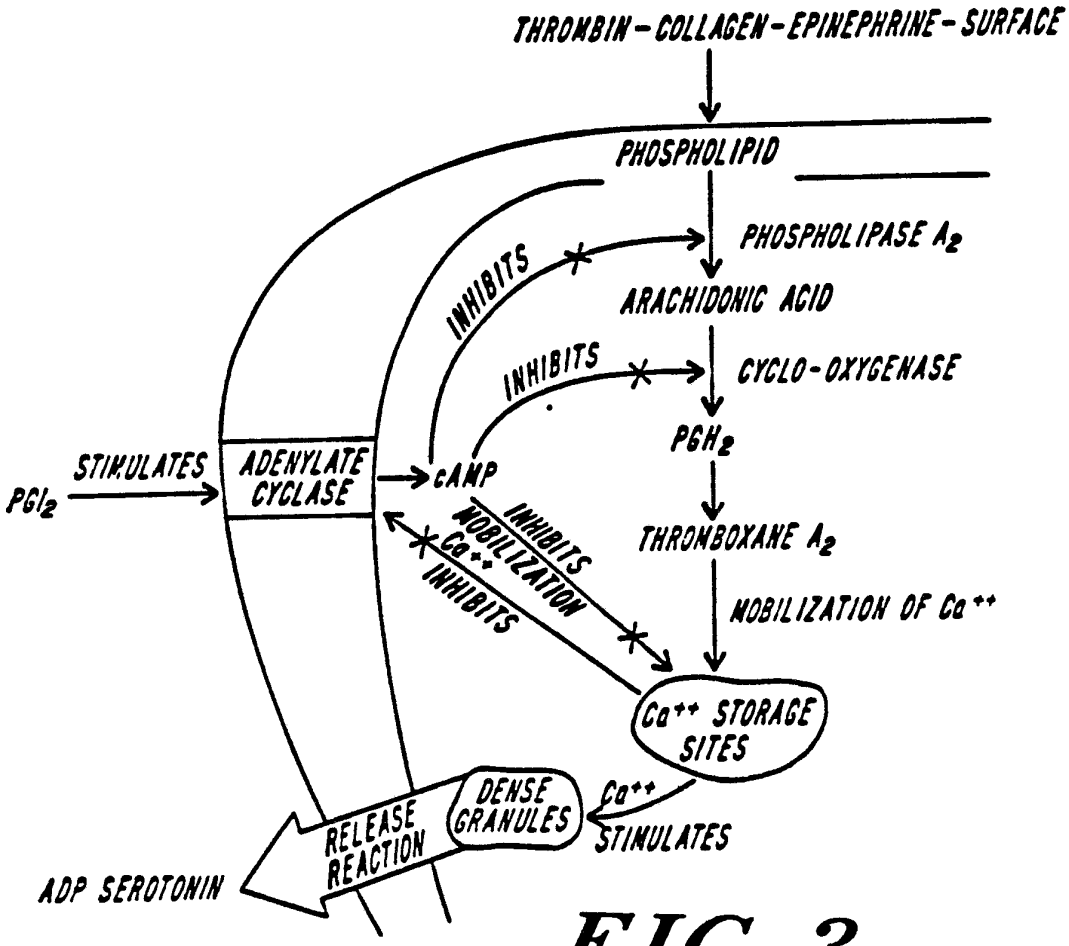
THROMBIN - COLLAGEN - EPINEPHRINE - SURFACE
PHOSPHOLIPID
PHOSPHOLIPASE A_2
INHIBITS
ARACHIDONIC ACID
INHIBITS
CYCLO-OXYGENASE
PGH_2
STIMULATES
PGI_2
ADENYLATE CYCLASE
cAMP
THROMBOXANE A_2
INHIBITS MOBILIZATION Ca^{++}
INHIBITS
MOBILIZATION OF Ca^{++}
Ca^{++} STORAGE SITES
DENSE GRANULES
Ca^{++}
STIMULATES
RELEASE REACTION
ADP SEROTONIN

FIG. 3

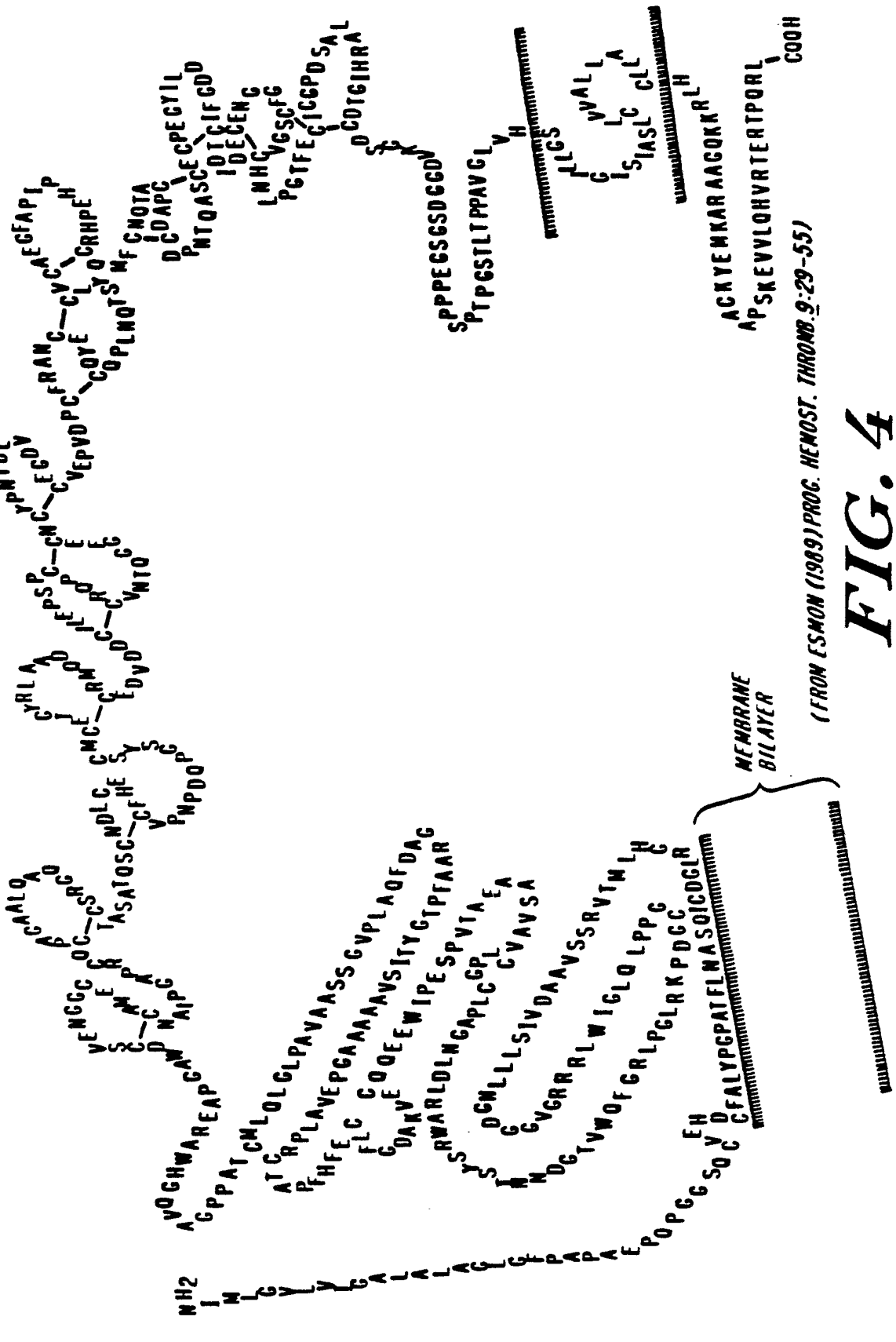

FIG. 4

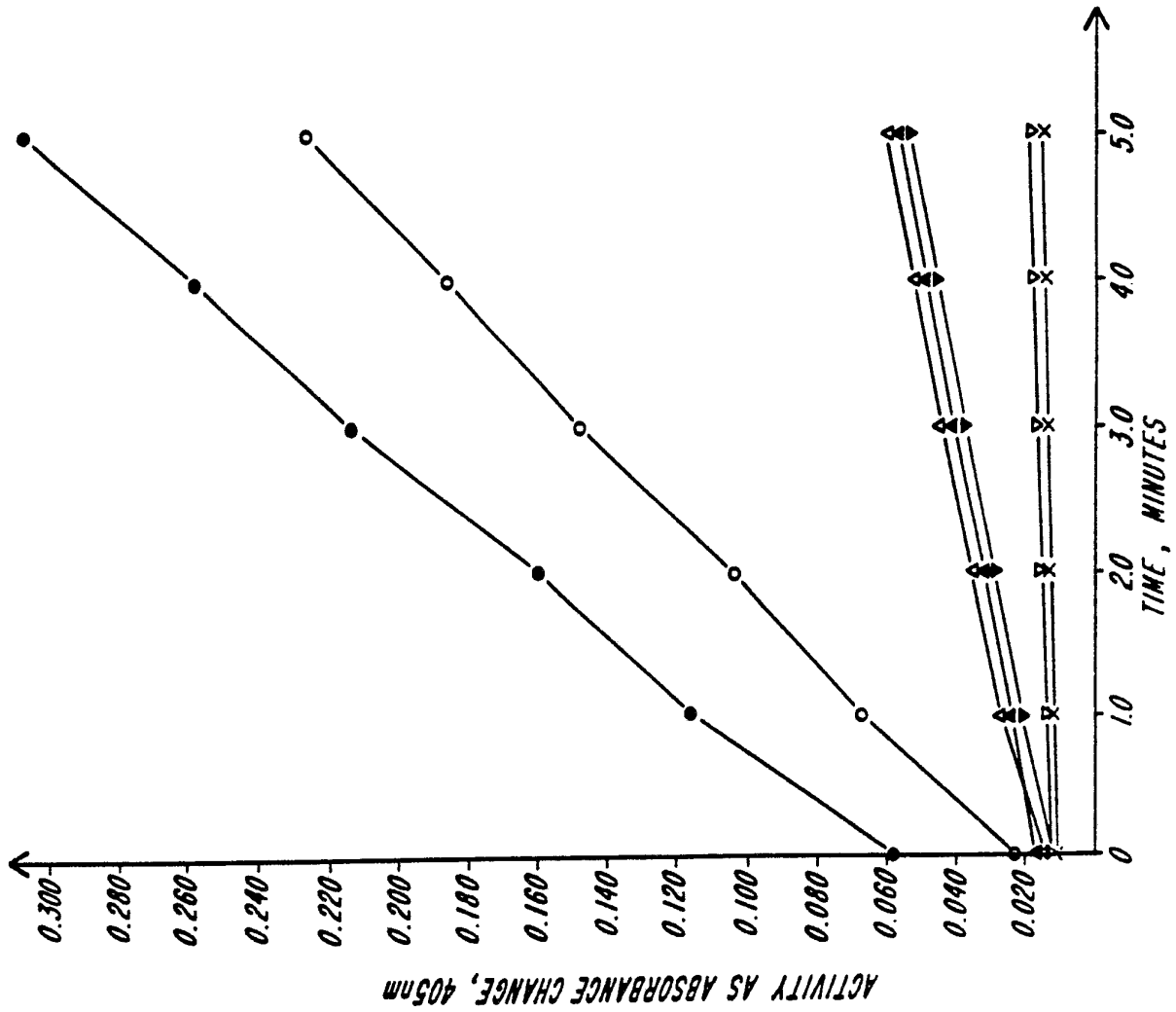
KEY
Tm, T, PC, H
Tm-SPDP, T, PC, H
PC
PC, T
T
T, H
T, PC, H
TESTS
CONTROLS
Tm - THRONBOMODULIN
Tm-SPDP - DERIVATIZED Tm
T - THROMBIN
PC - PROTEIN C
H - HIRUDIN
ACTIVITY OF DERIVATIZED Tm (Tm-SPDP) COMPARED TO Tm-ACTIVATED PC ANIOLYTIC ACTIVITY DEMONSTRATED USING CHROMOGENIC SUBSTRATE S-2266
ACTIVITY AS ABSORBANCE CHANGE, 405nm
0.300
0.280
0.260
0.240
0.220
0.200
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0
1.0
2.0
3.0
4.0
5.0
TIME, MINUTES

FIG. 5

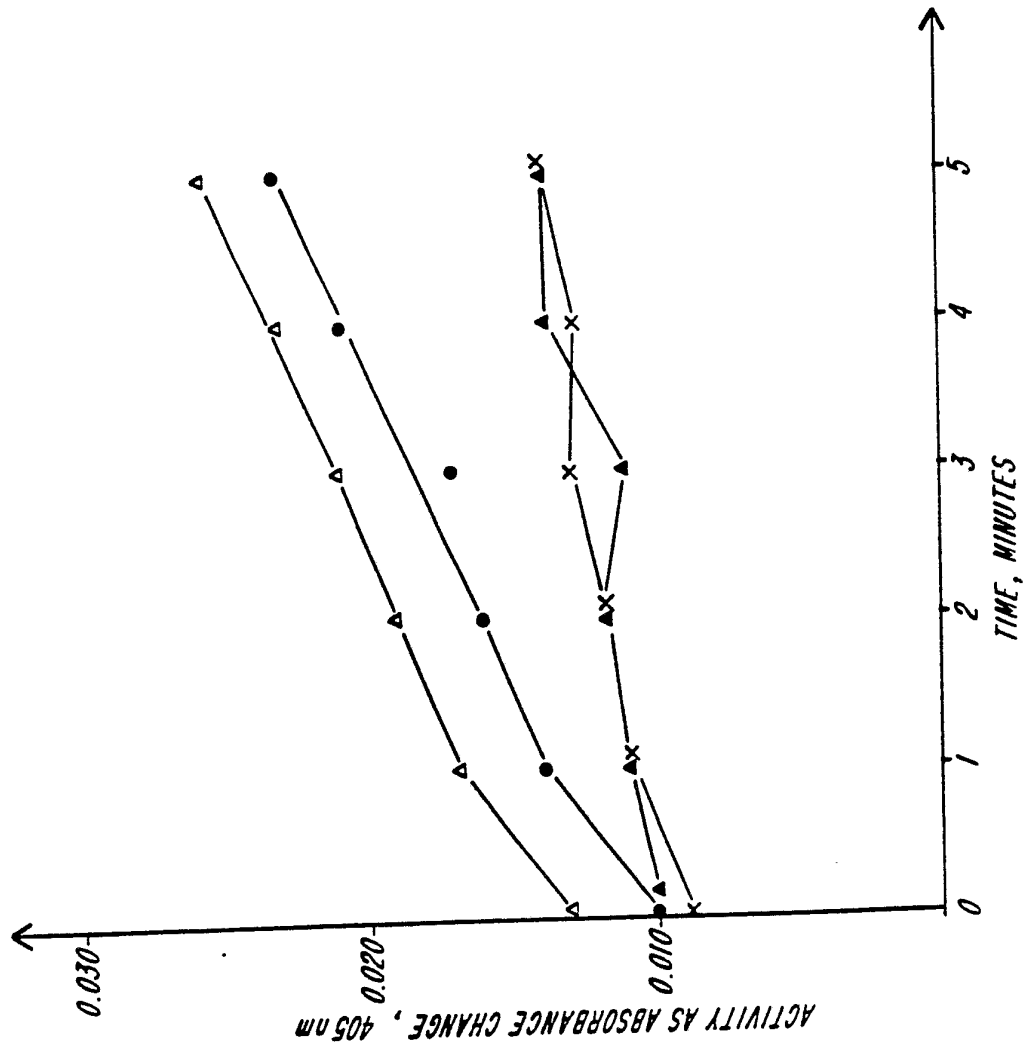
KEY
Tm-SPDP INMOBILIZED ON GRAFT
Tm-SPDP INMOBILLIZED ON GRAFT
BSA INMOBILIZED CONTROL
BSA INMOBILIZED CONTROL
ACTIVITY OF Tm INMOBILLIZED ON DACRON GRAFT MATERIAL USING SPDP AS THE CROSSLINKER.
ACTIVITY AS ABSORBANCE CHANGE, 405 nm
0.030
0.020
0.010
0
1
2
3
4
5
TIME, MINUTES

FIG. 6

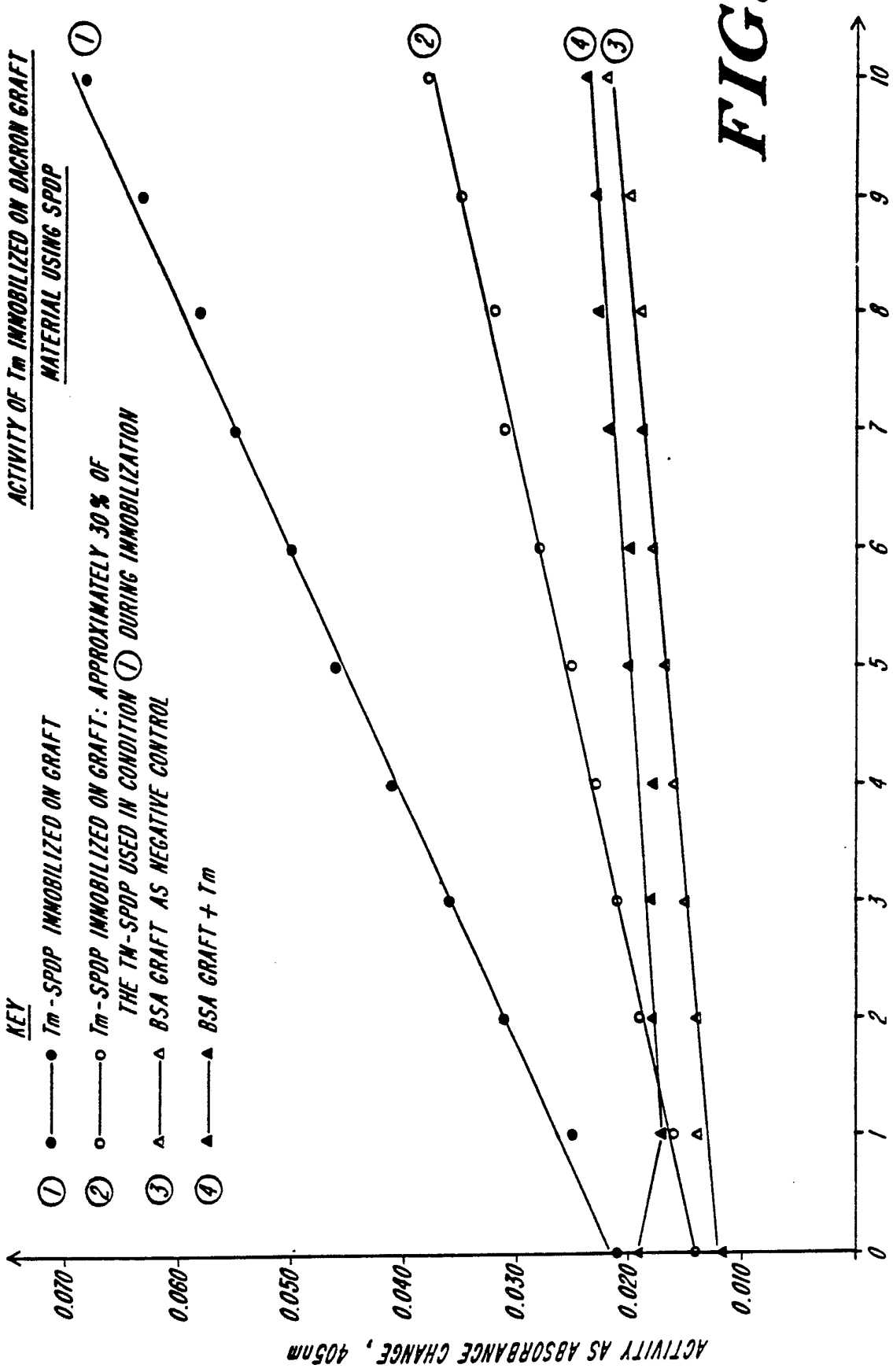
ACTIVITY OF Tm IMMOBILIZED ON DACRON GRAFT MATERIAL USING SPDP
KEY
① Tm-SPDP IMMOBILIZED ON GRAFT
② Tm-SPDP IMMOBILIZED ON GRAFT: APPROXIMATELY 30% OF THE TM-SPDP USED IN CONDITION ① DURING IMMOBILIZATION
③ BSA GRAFT AS NEGATIVE CONTROL
④ BSA GRAFT + Tm
ACTIVITY AS ABSORBANCE CHANGE, 405nm
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0
1
2
3
4
5
6
7
8
9
10
TIME, MINUTES

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05353

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61F 2/00; A61K 31/00; C07K 3/00

U.S. CL: 424/426; 514/2; 530/382

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/422, 426; 514/2, 8; 523/112, 113; 530/300, 382, 395, 810, 812, 813

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	J. LAB. CLIN. MED., VOLUME 91, JANUARY 1978 (LINDON) "Interaction of human platelets with heparinized agarose gel", pages 47-59. See abstract; materials and methods on page 48.	1-20
Y	WO, A, WO 79/00638 (THIN CONDUCTIVE COATING OSTERMALM), 06 SEPTEMBER 1979. See abstract; page 2, line 33.	1-20
Y	US, A, 4,273,873 (SUGITACHI) 16 JUNE 1981. See abstract; column 7, lines 49-68.	1-20
P,X P,Y	US, A, 5,002,582 (GUIRE) 26 MARCH 1991. See abstract, column 3, lines 35, 42; column 7, lines 44-56.	1-3, 6, 10, 11, 13, 14 4, 5, 7-9, 12, 15-19

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 SEPTEMBER 1991	28 OCT 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Gollamudi S. Kishore

Form PCT/ISA/210 (second sheet) (Rev.11-87)