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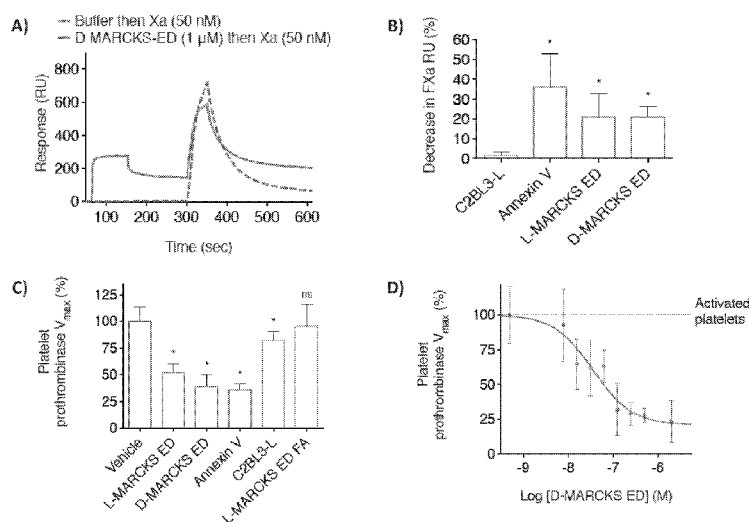
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(54) **Title:** COMPOSITIONS, METHODS AND USES FOR NOVEL ANTICOAGULANTS IN THE COAGULATION PATHWAY



FIGS. 2A-2D

(57) **Abstract:** Embodiments of the present disclosure generally relate to materials and methods for modulating blood coagulation. In certain embodiments, the present disclosure provides materials and methods for modulating assembly and function of protein complexes involved in blood coagulation processes. In other embodiments, the present disclosure provides anticoagulation agents capable of inhibiting the assembly of coagulation factor protein complexes on lipid membranes. In yet other embodiments, materials and methods described herein offer improved therapeutic measures for the treatment of various diseases involving blood coagulation, including, but not limited to, thrombosis and cancers.

COMPOSITIONS, METHODS AND USES FOR NOVEL ANTICOAGULANTS IN THE COAGULATION PATHWAY

PRIORITY

[0001] This PCT application claims priority to U.S. Provisional Patent Application Serial No. 62/025,570, filed July 17, 2014, which is incorporated herein by reference in its entirety for all purposes.

FUNDING

[0002] This invention was made with government support under grant numbers GM103843 and F30CA180249, awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD

[0003] Embodiments of the present disclosure relate generally to materials and methods for modulating blood coagulation. In certain embodiments, the present disclosure provides materials and methods for modulating assembly and function of protein complexes involved in blood coagulation processes. In still other embodiments, the present disclosure provides anticoagulation agents capable of inhibiting the assembly of coagulation factor protein complexes on lipid membranes for use as a treatment or prevention of a disease or a condition.

BACKGROUND

[0004] Blood clotting occurs in two forms, hemostasis and thrombosis. Hemostasis is considered a normal response to vascular injury and refers to the many physiologic mechanisms that halt the loss of blood. Thrombosis is the pathophysiologic counterpart to hemostasis. In the case of thrombosis, a clot occludes a vessel and disrupts blood flow in the circulatory system. Pulmonary embolisms, heart attacks, and deep vein thrombosis (DVTs), are all common examples of thrombosis as they affect humans or animals. Thrombosis can also be the result of another underlying disease, such as the concurrent development of hypercoagulability that occurs during the progression of many types of cancers (*e.g.*, Trousseau Syndrome). About 20% of all

deep vein thrombosis and pulmonary embolism cases are linked to patients with cancer, and hypercoagulability is the second most common cause of death in cancer patients. The most common types of cancer linked to deep vein thrombosis include pancreas, lung and stomach cancer, but other cancers are also linked to deep vein thrombosis.

[0005] Anticoagulation agents include different types of drugs used to treat and prevent thrombosis by reducing the formation of blood clots in arteries and veins. Activation of platelets, for example, by contact with the interrupted endothelium of a blood vessel, causes the exposure of the anionic lipid phosphatidylserine on the surface of the platelets and on platelet-derived microparticles (MPs). Generally, anticoagulants work by directly interacting with the coagulation factor proteins, inhibiting their synthesis, or blocking platelet activation.

SUMMARY

[0006] Embodiments of the present disclosure include compositions for preventing onset of or treating thrombosis in a subject. In some embodiments, compositions disclosed herein include one or more synthetic polypeptide having an amino acid sequence including one or more peptide fragments of myristoylated alanine-rich C kinase substrate effector domain (MARCKS ED). In certain embodiments, compositions disclosed herein can be in the form of a pharmaceutical and further include a pharmaceutically acceptable excipient or agent. In other embodiments, methods can include administering a pharmaceutically effective composition including one or more synthetic polypeptide having an amino acid sequence comprising one or more peptide fragments of the MARCKS ED and a pharmaceutically acceptable excipient to a subject can treat thrombosis in a subject by reducing blood coagulation.

[0007] In some embodiments, compositions can include one or more synthetic polypeptide having an amino acid sequence represented by the peptide of SEQ ID NO:2 KKKKKRFSFKKSGFSFKKNKK, or an amino acid sequence that is at least 50%, or at least 60%, or at least 70%, or at least 80% or at least 85% or at least 90% homologous to the amino acid sequence represented by the peptide of SEQ ID NO:2. In some embodiments, at least one lysine amino acid residue in the one or more synthetic polypeptide represented by SEQ ID NO:2 is substituted with an arginine amino acid residue. In other embodiments, at least one arginine amino acid residue in the one or more synthetic polypeptide represented by SEQ ID

NO:2 is substituted with a lysine amino acid residue. In yet other embodiments, at least one serine amino acid residue in the one or more synthetic polypeptide represented by SEQ ID NO:2 is substituted with a threonine, tyrosine, or histidine amino acid residue. Embodiments can include compositions having at least one synthetic polypeptide with an amino acid sequence that is 25 amino acids or less in length.

[0008] In some embodiments, compositions disclosed herein include one or more synthetic polypeptide of one or more peptide fragments of MARCKS ED and further including a myristoyl group associated with at least one peptide fragment. In certain embodiments compositions can include one or more synthetic polypeptide of one or more peptide fragments of MARCKS ED, wherein amino acid residues of the polypeptides are in an L configuration, a D configuration, or combinations thereof.

[0009] In other embodiments, a composition disclosed herein can be used to treat a blood condition. In certain embodiments, the condition can be a hypercoagulability or a prothrombotic state, for example, thrombosis. In other embodiments, compositions can include one or more synthetic polypeptide of one or more peptide fragments of MARCKS ED and a pharmaceutically acceptable excipient for administration to a subject having a blood disorder regarding hypercoagulability. In accordance with these embodiments, a composition can be used to inhibit formation of a coagulation protein complex and its enzymatic activity in a subject experiencing such a disorder. In other embodiments, compositions disclosed herein can be used to administer to a subject to prevent onset of such a condition by early intervention. In yet other embodiments, compositions disclosed here can be used to treat thrombosis in a subject having such a disorder. In accordance with these embodiments, a composition having one or more synthetic polypeptide of one or more peptide fragments of MARCKS ED administered to a subject where the composition is capable of inhibiting prothrombinase coagulation protein complex formation and prothrombinase enzymatic activity in order to treat the condition in the subject.

[00010] Certain embodiments of the present disclosure concern methods for treating thrombosis in a subject. In accordance with these embodiments, methods disclosed herein can include obtaining a composition having one or more synthetic polypeptide of one or more peptide fragments of MARCKS ED, and a pharmaceutically acceptable excipient, and

administering the composition to a subject where administering the composition to the subject treats thrombosis in the subject in the subject.

[00011] In some embodiments of the present disclosure, methods can include administering to a subject at least one synthetic polypeptide represented by SEQ ID NO:2, or a peptide having an amino acid sequence that is at least 50%, at least 60%, at least 70%, at least 80% or at least 90% homologous to the amino acid sequence represented by SEQ ID NO:2. In other embodiments, at least one lysine residue in the at least one synthetic polypeptide represented by SEQ ID NO:2 is substituted with an arginine residue. In other embodiments, at least one arginine residue in the at least one synthetic polypeptide represented by SEQ ID NO:2 is substituted with a lysine residue. In still other embodiments, at least one serine residue in the at least one synthetic polypeptide represented by SEQ ID NO:2 is substituted with a threonine, tyrosine, or histidine residue. In other embodiments, a synthetic polypeptide represented by SEQ ID NO:2 is substituted with one or more of lysine for an arginine residue; arginine for a lysine residue and/or threonine, tyrosine, or histidine for a serine residue.

[00012] In other embodiments, methods can include administering a composition of at least one synthetic polypeptide of one or more peptide fragments of MARCKS ED where the synthetic polypeptide further includes a myristoyl group. In yet other embodiments, methods can include administering a composition that includes at least one synthetic polypeptide of one or more peptide fragments of MARCKS ED, wherein amino acid residues of the peptide(s) are in an L configuration, a D configuration, or combinations thereof.

[00013] In some embodiments of the present disclosure, methods can include treating thrombosis or other hypercoagulation-related disease or condition in a subject with a composition comprising a synthetic polypeptide having an amino acid sequence comprising one or more peptide fragments of MARCKS ED. Certain conditions associated with thrombosis are deep vein thrombosis and pulmonary embolism which are life-threatening conditions. In accordance with these embodiments, compositions of the present disclosure are capable of inhibiting formation of a coagulation protein complex and its enzymatic activity in order to treat hypercoagulability or a prothrombotic state in a subject. In other embodiments, methods include treating thrombosis in a subject with a composition comprising a synthetic polypeptide

corresponding to one or more peptide fragments of MARCKS ED and inhibiting prothrombinase coagulation protein complex formation and prothrombinase enzymatic activity to treat thrombosis in the subject. In certain embodiments, methods disclosed herein can be used to treat subjects experiencing hypercoagulability and/or an acute thrombotic event. In some embodiments, treating thrombosis in a subject with a composition having a synthetic polypeptide of one or more peptide fragments of MARCKS ED prolongs survival of the subject as compared to a subject or subjects not administered the composition.

[00014] Other embodiments of the present disclosure can include methods for preventing thrombosis in a subject. In accordance with these embodiments, methods can include obtaining a composition comprising at least one synthetic polypeptide having an amino acid sequence comprising one or more peptide fragments of MARCKS ED, and a pharmaceutically acceptable excipient, and administering the composition to a subject. Administering the composition can prevent or reduce onset of thrombosis by acting directly on blood coagulation events and reducing blood coagulation in the subject. In some embodiments, treating thrombosis in a subject with a composition comprising a synthetic polypeptide corresponding to one or more peptide fragments of MARCKS ED prolongs the survival of the subject as compared to a subject or subjects not administered the composition.

[00015] In some embodiments of the present disclosure, methods can include treating subjects having cancer. In accordance with these embodiments, cancers capable of being treated by compositions disclosed herein can include, but are not limited to liver, pancreas, cervical, kidney, lung, stomach, colon, ovarian, breast, prostate, bone, skin, or brain cancer. In some embodiments, methods can include treating subjects having cancer with compositions disclosed herein, alone or in combination with an anti-cancer agent.

[00016] Embodiments of the present disclosure can include a kit for reducing coagulation of blood in a subject. In accordance with these embodiments, kits can include, but are not limited to, a composition of at least one synthetic polypeptide of one or more peptide fragments of MARCKS ED, and a pharmaceutically acceptable carrier or excipient, alone or in combination with other agents (e.g. anti-cancer agents).

Definitions and Terms

[00017] As used herein, “thrombosis” relates to coagulation or clotting of blood as a part of the circulatory system of a subject. Thrombosis can be a primary disease indication (e.g. hypercoagulability conditions etc.), and/or thrombosis can occur as part of a separate disease indication, including, but limited to cancer, immune-mediated diseases, neoplasia, systemic inflammation and sepsis, cardiac disease, protein-losing states, and infectious diseases.

[00018] As used herein, “peptide fragments,” “polypeptide fragments,” “fragments” and derivatives thereof generally refer to segments of a polypeptide or protein having at least two contiguous amino acid residues of the polypeptide or protein (*e.g.*, two amino acid residues of MARCKS ED). As would be readily recognized by one of skill in the art, one or more peptide fragments of a given polypeptide or protein can be arranged in various orders and combinations, can be separate or fused together in tandem or otherwise, as well as in combination with peptide fragments from other polypeptides or proteins, to produce a polypeptide or protein having a different amino acid sequence compared to that of the polypeptide or protein from which the peptide fragments originate. It is contemplated herein the peptide fragments can be 30 amino acids in length or less, or 25 amino acids in length or less, or 20 amino acids in length or less, or 15 amino acids in length or less or 10 amino acids in length or less.

[00019] As used herein, “D configuration” and “L configuration” refer to designations for chemical compounds that are chiral (*e.g.*, mirror images of each other). For example, a single amino acid that is chiral has two enantiomers (*e.g.*, optical isomers), often designated as a having a D configuration (*e.g.*, “right-handed”) and an L configuration (*e.g.*, “left-handed”). Generally, amino acids having a D configuration are not commonly found in nature, while amino acids having an L configuration are commonly found in nature within a naturally-occurring protein.

[00020] As used herein, the term “pharmaceutically acceptable excipient” and derivatives thereof generally refers to any natural or synthetic substance formulated with an active pharmaceutical or biological agent.

[00021] As used herein, “modulate” can mean an increase, a decrease, an induction, a change in encoded activity, a change in activity or the like.

[00022] As used herein, the terms “subject,” “user,” and/or “patient” include humans and other living species that are in need of treatment and capable of using the devices and systems as described herein. Additionally, the terms “subject,” “user,” and/or “patient” includes humans and other mammals treated in any type of environment such as a clinical setting, non-clinical setting, experimental setting, etc.

[00023] As used herein, “determine,” “calculate,” and “compute,” and variations thereof, as used herein, are used interchangeably and include any type of methodology, process, mathematical operation or technique.

[00024] It is to be noted that the term “a” or “an” entity refers to one or more of that entity. As such, the terms “a” (or “an”), “one or more” and “at least one” can be used interchangeably herein. It is also to be noted that the terms “comprising,” “including,” and “having” can be used interchangeably.

[00025] As used herein, “at least one,” “one or more,” and “and/or” are open-ended expressions that are both conjunctive and disjunctive in operation. For example, each of the expressions “at least one of A, B and C,” “at least one of A, B, or C,” “one or more of A, B, and C,” “one or more of A, B, or C,” and “A, B, and/or C” means A alone, B alone, C alone, A and B together, A and C together, B and C together, or A, B and C together. When each one of A, B, and C in the above expressions refers to an element, such as X, Y, and Z, or class of elements, such as X1-Xn, Y1-Ym, and Z1-Zo, the phrase is intended to refer to a single element selected from X, Y, and Z, a combination of elements selected from the same class (e.g., X1 and X2) as well as a combination of elements selected from two or more classes (e.g., Y1 and Zo).

[00026] The term “means” as used herein shall be given its broadest possible interpretation in accordance with 35 U.S.C. § 112(f). Accordingly, a claim incorporating the term “means” shall cover all structures, materials, or acts set forth herein, and all of the equivalents thereof. Further, the structures, materials or acts and the equivalents thereof shall include all those described in the summary, brief description of the drawings, detailed description, abstract, and claims themselves.

[00027] It should be understood that every maximum numerical limitation given throughout this disclosure is deemed to include each and every lower numerical limitation as an alternative, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this disclosure is deemed to include each and every higher numerical limitation as an alternative, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this disclosure is deemed to include each and every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[00028] The following drawings form part of the present specification and are included to further demonstrate certain embodiments. Some embodiments may be better understood by reference to one or more of these drawings alone or in combination with the detailed description of specific embodiments presented.

[00029] FIGS. 1A-1D are graphical representations of surface plasmon resonance (SPR) sensograms demonstrating the ability of Annexin V (FIG. 1A; positive control), C2BL3-L (FIG. 1B; negative control), L-MARCKS (FIG. 1C), and D-MARCKS (FIG. 1D) to affect the binding of coagulation factor Xa to monolayer lipid surfaces, according to some embodiments of the present disclosure.

[00030] FIGS. 2A-2D are histogram and graphical representations of SPR sensograms (FIGS. 2A-2B) and prothrombinase assays (FIGS. 2C-2D), according to some embodiments of the present disclosure. FIG. 2A represents the effects of pre-treatment with D-MARCKS on the binding of coagulation factor Xa to microparticle (MP)-like lipid surfaces. FIG. 2B is a representative summary of experiments testing the effects of Annexin V, C2BL3-L, L-MARCKS, and D-MARCKS on coagulation factor Xa binding. FIG. 2C is a representative summary of experiments testing the effects of L-MARCKS, D-MARCKS, Annexin V, C2BL3-L, and L-MARCKS Mutant FA on prothrombinase activity using activated platelets. FIG. 2D is a representative summary of a prothrombinase dose response curve using D-MARCKS.

[00031] FIGS. 3A-3B are representative histogram summaries of multiple experiments testing effects of L-MARCKS, D-MARCKS, Annexin V, and C2BL3-L on prothrombinase

activity in MPs (FIG. 3A) and synthetic liposomes (FIG. 3B), according to some embodiments of the present disclosure.

[00032] FIG. 4 is a graphical representation of the relative stability of L-MARCKS and D-MARCKS in a serum stability assay, according to some embodiments of the present disclosure.

[00033] FIG. 5A is a schematic representation of a microfluidic flow assay using whole human blood to test the effects of L-MARCKS, D-MARCKS, C2BL3-L, L-MARCKS Mutant FA, and Annexin V on fibrin formation, published previously. FIG. 5B includes final epifluorescence images from the microfluidic flow assays of FIG. 5A, according to some embodiments of the present disclosure.

[00034] FIGS. 6A-6C are graphical representations of fluorescence intensity values in microfluidic flow assays using whole human blood, according to some embodiments of the present disclosure. FIG. 6A is a representative time course experiment of fluorescence intensity values of D-MARCKS. FIGS. 6B-6C illustrate peak value of fluorescence intensity for fibrinogen (FIG. 6B) and CD41 surface area coverage for platelets (FIG. 6C) for experiments using L-MARCKS, D-MARCKS, Annexin V, C2BL3-L and L-MARCKS Mutant FA.

[00035] FIGS. 7A-7B are representative scanning electron microscope (SEM) images of control (FIG. 7A) and D-MARCKS-treated (FIG. 7B) samples from microfluidic flow assays, according to some embodiments of the present disclosure.

DETAILED DESCRIPTION

[00036] In the following sections, various exemplary compositions and methods are described in order to detail various embodiments. It will be obvious to one skilled in the art that practicing the various embodiments does not require the employment of all or even some of the details outlined herein, but rather that concentrations, times and other details may be modified through routine experimentation. In some cases, well-known methods or components have not been included in the description.

[00037] Hypercoagulability (e.g. thrombophilia or having a prothrombotic state) involves an abnormality in the process of blood coagulation that increases the risk of thrombosis (e.g. blood clots in blood vessels including the legs and lungs etc.). In some cases, these abnormalities

can be identified in 50% of people who have an episode of thrombosis (e.g. deep vein thrombosis in the leg), which is not linked to a separate disease indication or condition. In other cases, this condition is directly linked to a separate disease indication or condition. A significant proportion of the human population has a detectable abnormality that can lead to thrombosis, but many of these people only develop thrombosis in the presence of an additional risk factor, for example, risk factors present in cancer, immune-mediated diseases, neoplasia, systemic inflammation and sepsis, cardiac disease, protein-losing states, and infectious diseases or other related conditions.

[00038] Common conditions that can be associated with thrombophilia are deep vein thrombosis (DVT) and pulmonary embolism. These combined conditions are often referred to as venous thromboembolism (VTE). Deep vein thrombosis usually occurs in the legs, and is characterized by pain, swelling and redness of the limb. It may lead to long-term swelling and heaviness due to damage to valves in the veins. There is also a risk of that the clot can break off and migrate (embolize) to arteries in the lungs or even, the heart or brain. Depending on the size and the location of the clot, this may lead to sudden-onset shortness of breath, chest pain, palpitations and may be complicated by collapse, shock and can lead to cardiac arrest and often death. There are few treatments known to treat such a condition.

[00039] In addition, venous thrombosis can also occur in other places such as in the veins of the brain, liver (portal vein thrombosis and hepatic vein thrombosis), mesenteric vein, kidney (renal vein thrombosis) and even in veins of the arms. Thrombophilia may also increase the risk of arterial thrombosis, an underlying cause of heart attacks and strokes. Further, thrombophilia has been linked to recurrent miscarriage, and various complications of pregnancy such as intrauterine growth restriction, stillbirth, severe pre-eclampsia and other conditions. Another condition, referred to as protein C deficiency can cause purpura fulminans, which is a severe clotting disorder in the newborn that can lead to necrosis of tissue as well as bleeding into the skin and other organs. It has also been observed in adults. Protein C and protein S deficiency have also been linked with an increased risk of skin necrosis after starting anticoagulant treatment with warfarin or related drugs.

[00040] Some embodiments of the present disclosure relate to materials and methods for modulating blood coagulation in a subject. In certain embodiments, the present disclosure

provides materials and methods for modulating the assembly and function of protein complexes involved in blood coagulation processes. In yet other embodiments, the present disclosure provides anticoagulation agents capable of inhibiting the assembly of coagulation factor protein complexes on lipid membranes. Embodiments of the present disclose involve alternative compositions and methods for treating and/or preventing hypercoagulability in a subject in need thereof. In other embodiments, compositions disclosed herein can be used as anticoagulants in various other conditions as appropriate to treat or prevent the condition. In certain embodiments, compositions disclosed herein can be used to treat an acute thrombotic event, for example, as a result of an accident, a heart condition (e.g. heart attack or other condition) or during pregnancy or other condition presented above.

[00041] Anticoagulants are a group of agents that can reduce formation of blood clots in arteries and veins. These agents are commonly used for conditions related to the heart (*e.g.*, a subject having atrial fibrillation or mechanical valve), following surgery, pulmonary embolisms, heart attacks, and deep vein thrombosis, among others. Anticoagulants, as known in the art work by either, directly interacting with the coagulation factor proteins, inhibiting their synthesis, or blocking platelet activation.

[00042] Certain embodiments of the present disclosure include peptides capable of inhibiting coagulation through a mechanism involving inhibiting or preventing assembly of coagulation factor protein complexes on lipid membranes, a critical event in the coagulation cascade. Peptides of the present disclosure can act to reduce or prevent the coagulation cascade at an early juncture in the coagulation pathway, which then can prevent or reduce downstream events that can lead to a hypercoagulable state in a subject. Lipid membranes play an essential role in the coagulation pathway by providing surfaces for coagulation factor complexes to assemble. For example, activation of platelets leads to the exposure of the anionic lipid phosphatidylserine on the surface of the platelets and on platelet-derived microparticles (MPs). Phosphatidylserine can promote coagulation by directly interacting with and allowing the assembly of coagulation protein complexes, such as an initiating complex (*e.g.*, tissue factor and factor VIIa), tenase (*e.g.*, factor VIIIa and factor IXa), and prothrombinase (*e.g.*, factor Va: factor Xa).

[00043] It has been previously demonstrated that phosphatidylserine-bearing MPs can be released at high levels from many cancers. This condition can lead to a hypercoaguable state in a subject having cancer which is linked to a significant cause of cancer-related mortalities. Certain embodiments of the present disclosure concern treating a subject having cancer in order to reduce and/or prevent mortality related to hypercoagulability.

[00044] Other embodiments concern using, for example, biologic assays (*e.g.*, assays to measure enzymatic activity) and surface plasmon resonance (SPR) to assess the ability of peptides to sense and bind to the synthetic curvature of various lipid surfaces to inhibit or prevent the assembly of coagulation protein complexes and/or to inhibit or prevent enzymatic activity. In accordance with these embodiments, inhibition and/or prevention of the formation of these complexes can using these peptides can inhibit blood coagulation. In certain embodiments, compositions of the present disclosure concern myristoylated alanine-rich C kinase substrate (MARCKS) protein or peptides derivatives and fragments thereof (*e.g.*, such as the effector domain).

[00045] In some embodiments, compositions can include the entire MARCKS protein or peptide fragments derived or segments of domains of the MARCKS protein. In other embodiments, peptides can include or be derived from the effector domain (ED) of MARCKS (MARCKS ED), which is illustrated below and identified as SEQ ID NO:2.

[00046] MARCKS ED: KKKKKRFSFKKSFKLSGFSFKKNKK (SEQ ID NO:2).

[00047] MARCKS is an intracellular protein that is the predominate substrate for protein kinase C (PKC). MARCKS has been implicated in the regulation of brain development, macrophage activation, neuro-secretion, growth factor-dependent mitogenesis, among other cellular processes. The N-terminal glycine of the MARCKS protein is the site of myristoylation, which allows interaction of the MARCKS protein with the intracellular surface of the plasma membrane, where it co-localizes with PKC. MARCKS binds calmodulin in a calcium-dependent manner; the region responsible for calcium-binding is highly basic, a domain of about 25 amino acids known as the PSD or effector domain (ED), which also contains the PKC phosphorylation sites and has been shown to contribute to membrane binding. When not phosphorylated, the effector domain can bind to filamentous actin. MARCKS may be a regulatory interface between

actin and the plasma membrane. Modulation of the actin cross-linking activity of MARCKS by calmodulin and phosphorylation may be a potential convergence of the calcium-calmodulin and PKC signal transduction pathways in regulation of the actin cytoskeleton.

[00048] In contrast to the previously described structure and functions of the full-length MARCKS protein, the MARCKS ED peptides and peptide fragments of the present disclosure do not bind the intracellular surfaces of the plasma membrane of cells or act as regulatory interfaces for various cellular processes involving PKC and calcium-calmodulin signaling. The MARCKS ED peptides and peptide fragments of the present disclosure generally function extracellularly (*e.g.*, at the site of platelet activation) to inhibit and/or prevent the association of coagulation factors on lipid membranes, and to reduce prothrombinase coagulation protein complex formation and enzymatic activity.

[00049] In accordance with these embodiments, peptide fragments or polypeptide fragments generally refer to portions of a polypeptide or protein comprising at least two contiguous amino acid residues of the polypeptide or protein (*e.g.*, two amino acid residues of MARCKS ED). As would be readily recognized by one of skill in the art based on the present disclosure, one or more peptide fragments of a given polypeptide or protein can be arranged in various orders and combinations, as well as with peptide fragments from other polypeptides or proteins, to produce a polypeptide or protein having a different amino acid sequence as that of the polypeptide or protein from which the peptide fragments originate. For example, compositions of the present disclosure can include synthetic polypeptides including the entire full-length MARCKS ED peptide, and/or peptide fragments of the MARCKS ED peptide. In some embodiments, compositions disclose herein can include one or more fragments of the MARCKS ED peptide, wherein the fragments are about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or all 25 amino acids of the MARCKS ED peptide. In some cases, the compositions comprise a peptide fragment of MARCKS ED 25 amino acids or less, for example, the MARCKS ED peptide represented in SEQ ID NO:2 alone or fused to another peptide. In other embodiments, dimeric or trimeric MARCKS EDs can create stronger binding affinities to reduce or prevent coagulation when administered to a subject in need thereof. In other embodiments, pegylation or other modification to a peptide contemplated herein can

increase the half-life in circulation. In yet other embodiments, conjugation to a platelet antibody (e.g., CD41) might help specifically target platelets.

[00050] In accordance with the embodiments of the present disclosure, compositions can include one or more MARCKS ED polypeptide or fragments thereof. In certain embodiments, MARCKS ED derived peptides having amino acid sequences that are at least about 50%, to about 60%, to about 70%, to about 80%, to about 90% homologous to the amino acid sequence represented by SEQ ID NO:2. In certain embodiments, compositions can include one or more MARCKS ED peptide fragments of at least about 60%, of at least 70%, of at least 80%, of at least 85%, of at least 90% homologous to the amino acid sequence represented by SEQ ID NO:2. In certain embodiments, compositions can include one or more MARCKS ED peptide fragments having an amino acid sequences that are at least about 95% homologous to the amino acid sequence represented by SEQ ID NO:2.

MARCKS Peptide

[00051] The MARCKS peptide can operate as a membrane curvature sensor by recognizing membrane bilayers in an extended conformation, which is driven at least in part by electrostatic interaction with negatively charged lipids. The MARCKS protein is approximately 87 kDa, and therefore, it is contemplated that smaller peptide fragments may be more useful in certain applications as disclosed herein. In certain contexts, the MARCKS ED demonstrated anticoagulation capabilities. Certain embodiments of the present disclosure concern compositions of polypeptides having MARCKS ED peptide fragments. In some embodiments, the polypeptides including MARCKS ED peptide fragments are between about 5 to about 50; or about 5 to about 45; or about 5 to about 40; or about 5 to about 35; or about 5 to about 30; or about 5 to about 25; or about 5 to about 20; or about 5 to about 15; or about 5 to about 10 amino acids in length. In other embodiments, polypeptides comprising MARCKS ED can be monomeric polypeptides, or they can be joined in dimeric or trimeric or other multimeric configurations (e.g. as fusion molecules). As one of skill in the art would readily recognize based on the present disclosure, it is contemplated that any peptide derived from MARCKS ED can be used to inhibit coagulation in compositions disclosed herein.

[00052] As presented above, MARCKS ED principally includes positively charged basic amino acids (arginine (R) and lysine (K)), but also includes serine amino acid residues (S) that are phosphorylatable (e.g., by PCK and ROCK). Generally, unphosphorylated MARCKS ED has

a substantially positive charge and can interact electrostatically with for example, negative phosphatidylserines in a lipid membrane (see, for example, FIGS. 1A-1D). Given that the activation of platelets can lead to, for example, exposure of the anionic lipid phosphatidylserine on the surface of the platelets and on platelet-derived microparticles (MPs), polypeptides comprising MARCKS ED can interact with the exposed phosphatidylserine and inhibit or prevent downstream events, such as prothrombinase complex formation and/or prothrombinase enzyme activation that are required for blood coagulation (see, for example, FIGS. 2A-2D). If in certain embodiments contemplated herein, serine amino acid residues in MARCKS ED are phosphorylated, a negative charge conferred by the phosphorylation can interrupt interaction of MARCKS ED with the phosphatidylserines in the lipid membrane.

[00053] In other embodiments, MARCKS ED can include a myristoyl group at its N-terminus (*e.g.*, myristoylated). Myristoylation is a lipidation modification where a myristoyl group, derived from myristic acid, is covalently attached by an amide bond to the alpha-amino group of an N-terminal amino acid residue. Myristic acid is a 14-carbon saturated fatty acid (14:0) with the systematic name of n-Tetradecanoic acid. Myristoylation allows for weak protein-protein and protein-lipid interactions and can play an essential role in membrane targeting, protein-protein interactions and functions widely in a variety of signal transduction pathways. In some embodiments, MARCKS ED peptides having a myristoyl group can interact with a lipid membrane via the myristoyl group and facilitate inhibition and/or prevention of downstream events, such as prothrombinase complex formation and/or prothrombinase enzyme activation that are required for blood coagulation (see, for example, FIGS. 5A-5B and FIGS. 6A-6C).

[00054] In accordance with some embodiments of the present disclosure, compositions can include one or more MARCKS ED peptide fragments having amino acid substitutions that do not compromise and may increase the ability of the peptide reduce or prevent coagulation. For example, in certain embodiments, one or more basic amino acids in a MARCKS ED peptide fragment can be substituted with another basic amino acid (*e.g.*, an arginine residues can be substituted with a lysine residue). In other embodiments, amino acid residues that are phosphorylatable can be substituted for each other. For example, in some embodiments, a serine amino acid residue in a MARCKS ED peptide fragment can be substituted with a threonine (T),

tyrosine (Y), or histidine (H) amino acid residue. It is also contemplated that MARCKS ED peptide fragments of the present disclosure may have a combination of substitutions or modifications as provided herein (e.g. basic amino acid substitutions; myristoyl group addition etc.)

[00055] Certain embodiments concern compositions that include one or more MARCKS ED peptides, fragments and/or derivative thereof where the amino acids that make up the one or more peptides or fragments or derivatives thereof have either L or D configurations, or a mixture of both. In other embodiments, amino acids having an L configuration are typically found in nature, while amino acids having a D configuration are not typically found in nature and are therefore not naturally-occurring. In some embodiments, presence of amino acids having a D configuration in a MARCKS ED peptide fragment can reduce the susceptibility of the MARCKS ED peptide fragment to proteolytic degradation, as compared to a MARCKS ED peptide derivative having amino acids with an L configuration (see, for example, FIG. 4). In certain embodiments, D amino acids can be used to increase half-life of the MARCKS ED peptide fragments *in vivo*. In other embodiments, a MARCKS ED peptide of the present invention can include a peptide with amino acids having L configurations or a mixture of D and L configurations.

[00056] Peptides and polypeptides, as well as fragments and derivatives thereof of the present disclosure can be generated by any methods known in the art. In some embodiments, peptides and polypeptides of the present disclosure can be generated using SPPS synthesis with or without cyclization with solid phase chemistry. Certain solid phase chemistry techniques include Click chemistry. Peptides of the present disclosure can include, but are not limited to, peptides of about 5 to about 50 amino acids in length. Certain embodiments include generating cyclic peptides from linear peptides immobilized on a solid substrate. Any other methods of generating proteins and peptides disclosed herein are be readily identifiable and understood by one of ordinary skill in the art. In certain embodiments, a larger peptide may be generated in order to obtain a fragment of the larger peptide. In other embodiments, mutagenesis can be used to change amino acids in a synthetic peptide of the present disclosure. In yet other embodiments, any systems or methods known in the art to generate synthetic peptides, fusion polypeptides or

multiple copies of synthetic peptides can be used to generate the peptides of the present disclosure.

[00057] In certain embodiments, compositions of the present disclosure can be used in methods to treat and/or prevent hypercoagulability in a subject. In certain embodiments, thrombosis can be treated and/or prevented (e.g. reducing the risk of onset of thrombotic-related conditions) in a subject in need thereof. For example, compositions comprising MARCKS ED peptide fragments can be administered prophylactically to prevent or reduce onset of a disease condition having hypercoagulability as a risk factor (e.g., a subject who is susceptible to hypercoagulability), and/or therapeutically to treat a subject that has one or more symptoms of hypercoagulability. Certain conditions of the present disclosure include, but are not limited to, cancers such as liver, pancreas, ovarian, cervical, kidney, lung, stomach, colon, breast, ovarian, prostate, bone, skin, or brain cancer (see, e.g., FIGS. 3A-3B), as well as other disease conditions, including but not limited to, immune-mediated diseases, neoplasia, systemic inflammation and sepsis, cardiac disease, protein-losing states, and infectious diseases. In some embodiments, administration of compositions having one or more MARCKS ED peptide fragments and a pharmaceutically effective excipient can prolong the survival of a subject, as compared to a subject that has not been administered compositions having one or more MARCKS ED peptide fragments.

[00058] In some embodiments, a subject can be administered a composition disclosed herein wherein one or more peptides are administered at about 0.1 mg/kg-10.0 mg/kg (body weight); or 0.2 mg/kg-5.0 mg/kg; or 0.5mg/kg-5.0 mg/kg; or 0.5mg/kg-1.5 mg/kg or similar range as needed depending on the subject and condition of the subject. In other embodiments, a subject can be administered a composition disclosed herein by immediate intervention, bi-daily; daily; bi-weekly; bi-monthly or as needed and prescribed by a healthcare professional.

[00059] In other embodiments, compositions having MARCKS ED peptide fragments can be administered as part of a dosing regimen as well as alongside other active pharmaceutical agents known to be used for particular conditions (e.g., anti-cancer agents). In other embodiments, compositions of the present disclosure can also include various other pharmaceutically acceptable excipients and adjuvants, which can be contained in a kit. In yet

other embodiments, compositions disclosed herein can be administered by any method known in the art including, but not limited to, orally, subcutaneously, intranasally, intravenously, intramuscularly, intradermally, as a suppository, intraperitoneally, as well as via other modes of administration.

[00060] Embodiments of the present disclosure can include kits for treating a hypercoagulability-related condition in a subject. In accordance with these embodiments, kits can include, but are not limited to, a composition of at least one synthetic polypeptide of one or more peptide fragments of MARCKS ED, and a pharmaceutically acceptable carrier or excipient, alone or in combination with other agents (e.g. anti-cancer agents). In addition, kits can include an appropriate applicator for administering the one or more agents as well as one or more container to hold the components of a kit.

EXAMPLES

[00061] The following examples are included to illustrate various embodiments. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered to function well in the practice of the claimed methods, compositions and apparatus. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes may be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

[00062] FIGS. 1A-1D are graphical representations of one exemplary methods of surface plasmon resonance (SPR) sensograms demonstrating the ability of Annexin V (FIG. 1A; positive control), C2BL3-L (FIG. 1B; negative control), L-MARCKS (FIG. 1C), and D-MARCKS (FIG. 1D) to affect the binding of coagulation factor Xa to monolayer lipid surfaces, according to one embodiment of the present disclosure. SPR is a label-free method to measure protein-membrane interactions. Using a novel surface plasmon resonance (SPR) method, effects of MARCKS ED on coagulation factor/lipid membrane binding were evaluated. This method involves the elution of a ligand over immobilized receptors on a metal surface. When the surface is excited with polarized light, the total internal reflection of the plasmon wave can be measured. The plasmon wave interacts with the bound material on the metal surface and produces a corresponding

change in the angle of the reflected light. These experiments were performed using a protein interaction analyzer (Biacore 3000 and BiOptix 404pi SPR instruments). HPA sensor chips were used to form monolayer lipid membrane surfaces, which are representative of an activated platelet or microparticle (50% PS (phosphatidylserine), 20% PE (phosphatidylethanolamine), 30% PC (phosphatidylcholine)).

[00063] As illustrated in FIGS. 1A-1D, addition of 1 μ M of either L-MARCKS (FIG. 1C) or D-MARCKS (FIG. 1D) reduced the binding of coagulation factor Xa (50 nM) to the monolayer lipid surface (dotted line) as compared to Xa (50 nM) alone (solid line). Annexin V (50 μ M) was used as a positive control (FIG. 1A; dotted line), while C2BL3-L was used as a negative control peptide (FIG. 1B; dotted line).

[00064] FIGS. 2A-2D are graphical representations of SPR sensograms (FIGS. 2A-2B) and prothrombinase assays (FIGS. 2C-2D), according to some embodiments of the present disclosure. FIG. 2A represents effects of pre-treatment with D-MARCKS on the binding of coagulation factor Xa to microparticle (MP)-like lipid surfaces. FIG. 2B is a representative summary of experiments testing the effects of Annexin V, C2BL3-L, L-MARCKS, and D-MARCKS on coagulation factor Xa binding. FIG. 2C is a representative summary of experiments testing the effects of L-MARCKS, D-MARCKS, Annexin V, C2BL3-L, and L-MARCKS Mutant FA on prothrombinase activity using activated platelets. FIG. 2D is a representative summary of a prothrombinase dose response curve using D-MARCKS. These experiments were performed using a protein interaction analyzer (Biacore 3000 and BiOptix 404pi SPR instruments). HPA sensor chips were used to form monolayer lipid membrane surfaces, which are representative of an activated platelet or microparticle (50% PS, 20% PE, 30% PC).

[00065] As illustrated in FIG. 2A, pre-treatment of the membrane surface with 1 μ M D-MARCKS ED (solid line) reduced the association of coagulation factor Xa (50 nM) to the membrane surface, as compared to Xa alone (50 nM; dotted line). D-MARCKS ED is a peptide with the same sequence as the L-MARCKS ED peptide, but D-MARCKS ED is synthesized using D amino acids (e.g. not naturally occurring). D-MARCKS ED demonstrated similar inhibitory activity as L-MARCKS ED peptide, as illustrated by similar decreases in Xa response

units (RU) in FIG. 2B. As both MARCKS and PS are chiral molecules, preservation of anticoagulant activity in the D-MARCKS peptide was a surprising result, and suggests that D-MARCKS ED may potentially be advantageous for more complex *in vivo* biologic applications.

[00066] In another example, effects of MARCKS peptides on *in vitro* coagulation protein complex enzymatic activity were examined using a modified prothrombinase assay. This assay measures the enzymatic activity of the coagulation protein complex XaVa (prothrombinase) in the presence of activated human platelets with exposed phosphatidylserine. Human platelets were isolated, washed, and activated using a combination of thrombin (0.5 U/mL) and convulxin (250ng/mL). Following the addition of coagulation factors Va (6 nM) and Xa (3 nM), prothrombin (4 μ M) was added to the solution and the reaction was sub-sampled into a reaction stopping cuvette buffer. Thrombin activity was then measured using S-2238 (3 mM), a thrombin chromogenic substrate, and microplate spectrophotometer. When the prothrombinase assay was performed in the presence of L- or D-MARCKS ED at 1 μ M concentration, prothrombinase enzymatic activity was reduced by approximately 50% (FIG. 2C). Additionally, inhibition of prothrombinase activity was observed as a dose-dependent response and was observed to be surprisingly potent (FIG. 2D; D-MARCKS ED IC₅₀ = 30.6 nM, 95% CI 14.7 to 63.6 nM).

[00067] In yet another example, to further define the anticoagulant activity of MARCKS ED, effects of L- and D-MARCKS peptides on the assembly of prothrombinase on the membranes of biologic microparticles and synthetic liposomes were examined. FIGS. 3A-3B are representative summaries of experiments testing the effects of L-MARCKS, D-MARCKS, Annexin V, and C2BL3-L on prothrombinase activity in MPs (FIG. 3A) and synthetic liposomes (FIG. 3B), according to one embodiment of the present disclosure. Release of PS-positive microparticles (MPs) from tumors leads, in part, to the hypercoagulable state commonly associated with progressing cancers (*e.g.*, Trousseau Syndrome). MPs were isolated from MDA-MB-231 cells, a metastatic breast cancer cell line, and were used in place of the activated platelets in the prothrombinase assays of FIGS. 2A-2D. As illustrated in FIG. 3A, L- and D-MARCKS inhibited assembly and activity of prothrombinase in the presence of biologic MPs. This data suggests that MARCKS ED may be used to reduce or prevent pro-coagulant activity of tumor-derived MPs.

[00068] In another example, the prothrombinase assay was repeated using synthetic liposomes, as illustrated in FIG. 3B. Compared to platelets and biologic MPs, synthetic liposomes bear no surface-tethered or transmembrane proteins with which MARCKS ED could potentially interact. The liposomes were composed of 95% phosphatidylcholine (PC) and 5% phosphatidylserine (PS) lipids, and the absolute lipid concentration was optimized to mimic the prothrombinase response of the platelets. As illustrated in FIG. 3B, L- and D-MARCKS inhibited prothrombinase assembly and activity on synthetic liposomes. These data provide evidence that MARCKS ED may inhibit prothrombinase by blocking the binding and assembly of the coagulation protein complex on exposed PS lipids.

[00069] It has been observed that peptides synthesized with non-natural D amino acids are more resistant to proteases, including, for example, proteases in human serum. However, peptides synthesized with non-natural D amino acids do not always retain the biological and/or chemical functions of their L amino acid counterparts. As illustrated in the preceding figures and data, D-MARCKS exhibited similar functionality as L-MARCKS. In FIG. 4, the relative stability of L- and D-MARCKS in a serum stability assay was also tested. Briefly, 100 $\mu\text{g ml}^{-1}$ L- or D-MARCKS was incubated at 37 °C in RPMI 1640 medium supplemented with 25% (v/v) pooled normal human serum. At certain time points, the reaction was sub-sampled, and a trichloroacetic acid (TCA) solution was added to a final concentration of 5% (w/v). Each sub-sample mixture was cooled for 15 min at 4 °C, then centrifuged at 16,000g for 4 min to precipitate the serum proteins. The resulting supernatant was analyzed with reverse phase HPLC. Non-degraded peptide was quantified by setting the UV-visible HPLC detection to 480 nm and integrating the chromatogram peaks with retention times matching that of the whole peptide (39 to 40 min). As illustrated in FIG. 4, it was observed that the D form of MARCKS ED (squares) was approximately 6 times more stable than the L form (circles) (D-MARCKS $t_{1/2}$ = 356 min (95% CI 252 to 606 min), L-MARCKS ED $t_{1/2}$ = 57 min (95% CI 46 to 79 min)).

[00070] In another exemplary method, as illustrated in FIGS. 5A-5B, effects of MARCKS ED on whole human blood platelet activation and accumulation, as well as fibrin formation, were examined using a modification of a previously described microfluidic flow assay, represented schematically in FIG. 5A. Briefly, a custom polydimethylsiloxane microfluidic flow device containing four channels, each with a height of 100 μm and width of 500 μm , was vacuum

mounted to a glass slide patterned with a type I fibrillar collagen strip. The microfluidic flow device channels were oriented perpendicular to the patterned collagen strip, resulting in a 50 μm patch of collagen across the width of each channel. Whole blood was labeled with a Pacific Blue anti-human CD41 antibody for 10 min, followed by the addition of 30 $\mu\text{g ml}^{-1}$ Alexa Fluor 647 human plasma fibrinogen conjugate and either 1 μM NBD-labeled investigational MARCKS ED peptide, 1 μM Annexin V, or 15 USP ml^{-1} heparin. Immediately before the assay, the whole blood mixture was recalcified to 7.5 mM CaCl_2 . The whole blood was then pulled through the device channels for 10 min at wall shear rate equivalent to that of the venous system (100 s^{-1}) using a PhD Ultra syringe pump. Platelet aggregation, fibrin formation, and peptide accumulation were captured in real time by epifluorescence microscopy using an IX81 inverted microscope with a 40x objective. Fluorescence intensities and platelet surface area coverages were measured using ImageJ. As observed in FIG. 5B, neither L- or D-MARCKS, or the positive and negative controls, significantly altered platelet surface area coverage (second row labeled "Platelet"). However, both L and D-MARCKS both significantly inhibited fibrin formation (third row labeled "Fibrin(ogen)") and also completely co-localized with the platelet clumps (top row labeled "Merge").

[00071] FIGS. 6A-6C are graphical representations of fluorescence intensity values taken using the microfluidic flow assays using whole human blood described in FIGS. 5A-5B. FIG. 6A is a representative time course experiment of fluorescence intensity values of D-MARCKS and the vehicle control. NBD was used to label the peptides (top panel), CD41 was used a marker for platelets (middle panel), and Alexa Fluor 647 human plasma fibrinogen conjugate was used to visualize fibrinogen (bottom panel). FIGS. 6B-6C are graphical representations of final fluorescence intensity values for fibrinogen (FIG. 6B) and CD41 for platelets (FIG. 6C) in experiments testing L-MARCKS, D-MARCKS, Annexin V, C2BL3-L and L-MARCKS Mutant FA. As shown, these data demonstrate a significant quantitative difference in the ability of L- and D-MARCKS to inhibit fibrin formation at the site of platelet aggregation, as compared to controls.

[00072] FIGS. 7A-7B are representative scanning electron microscope (SEM) images (2000x magnification) of control (FIG. 7A) and D-MARCKS-treated (FIG. 7B) samples taken using the microfluidic flow assays described above. As illustrated, SEM images of whole blood

microfluidic flow assay clots demonstrate that there was no fibrin formation (dense mesh network) in samples treated with D-MARCKS.

[00073] In another example, given the enhanced stability of D-MARCKS over L-MARCKS, with no concomitant loss of function, as described above, the *in vivo* effects of D-MARCKS were tested using an acceptable mouse model representative of a fatal pulmonary embolism. Briefly, anesthetized mice were given retro-orbital venous injections of collagen (0.28 mg/kg) and epinephrine (0.029 mg/kg). This combination generally induces a fatal pulmonary embolism in the mouse. As illustrated in Table 1 (below), when D-MARCKS at 3.0, 5.0, and 7.5 mg/kg was administered via retro-orbital venous injection 30 minutes prior to the collagen/epinephrine, the peptide was able to delay the time point at which the last breath was taken by the injected mice. D-MARCKS administered at a dose of 7.5 mg/kg was even able to prevent death of a mouse while other doses prolonged survival compared to the mouse not receiving any of the D-MARCKS peptide.

Table 1: The effects of D-MARCKS on a mouse model of fatal pulmonary embolism (n = 1 for each D-MARCKS dosage).

D-MARCKS Dosage (mg/kg)	Initial Stoppage of Breathing	Agonal Breath	Last Breath
0	0:20	1:27	1:41
3.0	0:15	0:59	2:30
5.0	0:39	0:57	4:40
7.5	0:28	0:42	Survived

[00074] Taken together, these results demonstrate that both L- and D-MARCKS ED or combinations of L and D can block the association of coagulation factors to lipid membranes, and that this activity reduces prothrombinase coagulation protein complex formation and

enzymatic activity. From these exemplary methods, it appears that D-MARCKS is more stable (*e.g.*, protease resistant) than L-MARCKS. In addition, D-MARCKS administration is able to increase survival in a mouse model demonstrating its *in vivo* efficacy. Because prothrombinase is one of three coagulation protein complexes in the coagulation protein cascade that depend on PS to assemble, it is likely that MARCKS ED may also inhibit the assembly of initiating complex (tissue factor: factor VIIa) and tenase (factor VIIIa: factor IXa), and therefore may have even greater inhibitory activity *in vivo* (see, *e.g.*, FIGS. 7A-7B).

All of the COMPOSITIONS and METHODS disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods have been described in terms of preferred embodiments, it is apparent to those of skill in the art that variations may be applied to the COMPOSITIONS and METHODS and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope herein. More specifically, certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept as defined by the appended claims.

What is claimed is:

1. A composition for treating thrombosis in a subject, the composition comprising:
at least one synthetic polypeptide comprising an amino acid sequence comprising one or more peptide fragments or modifications thereof of myristoylated alanine-rich C kinase substrate effector domain (MARCKS ED); and
a pharmaceutically acceptable excipient.
2. The composition of claim 1, wherein the at least one synthetic polypeptide comprises an amino acid sequence that is at least 50% homologous to the amino acid sequence represented by SEQ ID NO:2.
3. The composition of any of claims 1 or 2, wherein at least one of the at least one synthetic polypeptide comprises an amino acid sequence of 25 amino acid residues in length or less.
4. The composition of any of claims 1-3, wherein at least one of the at least one synthetic polypeptide comprises the amino acid sequence represented by SEQ ID NO:2.
5. The composition of any of claims 1-4, wherein at least one lysine residue in the at least one synthetic peptide fragments is substituted with an arginine residue.
6. The composition any of claims 1-5, wherein at least one arginine residue in the at least one synthetic peptide fragments is substituted with a lysine residue.
7. The composition of any of claims 1-6, wherein at least one serine residue in the at least one synthetic polypeptide represented by SEQ ID NO:2 is substituted with a threonine, tyrosine, or histidine residue.
8. The composition of any of claims 1-7, wherein at least one of the at least one synthetic polypeptide comprises a myristoyl group.

9. The composition of any of claims 1-8, wherein at least one of the at least one synthetic polypeptide comprises amino acid residues having an L configuration.
10. The composition of any of claims 1-9, wherein at least one of the at least one synthetic polypeptide comprises amino acid residues having a D configuration.
11. The composition of any of claims 1-10, wherein at least one of the at least one synthetic polypeptide comprises a combination of amino acid residues having an L configuration and a D configuration.
12. The composition of any of claims 1-11, wherein reducing blood coagulation in the subject comprises inhibiting formation of a coagulation protein complex and its enzymatic activity.
13. The composition of any of claims 1-12, wherein reducing blood coagulation in the subject comprises inhibiting prothrombinase coagulation protein complex formation and its enzymatic activity.
14. A method for treating thrombosis in a subject, the method comprising: administering a composition of claim 1 to a subject, wherein administering the composition treats thrombosis in the subject.
15. The method of claim 14, wherein at least one of the at least one synthetic polypeptide comprises an amino acid sequence that is at least 50% homologous to the amino acid sequence represented by SEQ ID NO:2.
16. The method of any of claims 14-15, wherein at least one of the at least one synthetic polypeptide comprises an amino acid sequence that is no more than 25 amino acid residues.

17. The method of any of claims 14-16, wherein at least one of the at least one synthetic polypeptide comprises the amino acid sequence represented by SEQ ID NO:2.

18. The method of any of claims 14-17, wherein at least one lysine residue in the at least one synthetic polypeptide represented by SEQ ID NO:2 is substituted with an arginine amino acid residue.

19. The method of claim 17, wherein at least one arginine residue in the at least one synthetic polypeptide represented by SEQ ID NO:2 is substituted with a lysine residue.

20. The method of claim 17, wherein at least one serine residue in the at least one synthetic polypeptide represented by SEQ ID NO:2 is substituted with a threonine, tyrosine, or histidine residue.

21. The method of claim 14, wherein at least one of the at least one synthetic polypeptide further comprises a myristoyl group associated with the at least one synthetic polypeptide.

22. The method of claim 14, wherein at least one of the at least one synthetic polypeptide comprises amino acid residues having an L configuration.

23. The method of claim 14, wherein at least one of the at least one synthetic polypeptide comprises amino acid residues having a D configuration.

24. The method of claim 14, wherein at least one of the at least one synthetic polypeptide comprises a combination of amino acid residues having an L configuration and a D configuration.

25. The method of claim 14, wherein administering the composition to the subject reduces blood coagulation in the subject by inhibiting formation of a coagulation protein complex.

26. The method of claim 14, wherein administering the composition to the subject reduces blood coagulation in the subject by inhibiting prothrombinase coagulation protein complex formation.
27. The method of claim 14, wherein the subject is experiencing hypercoagulability.
28. The method of claim 14, wherein the subject has experienced an acute thrombotic event.
29. The method of claim 14, wherein administering the composition to the subject prolongs survival of the subject compared to a subject not administered the composition.
30. A method of preventing thrombosis in a subject, the method comprising: administering a composition of claim 1 to a subject, wherein administering the composition reduces onset of thrombosis in the subject.
31. The method of claim 30, wherein the subject has cancer.
32. The method of claim 31, wherein the cancer comprises liver, pancreas, ovarian, cervical, kidney, lung, stomach, colon, breast, prostate, bone, skin, or brain cancer.
33. The method of any of claims 30-32, wherein the composition further comprises an anti-cancer agent.
34. The method of any of claims 30-33, wherein administering the composition to the subject prolongs survival of the subject as compared to a subject not administered the composition.
35. A kit for reducing blood coagulation, the kit comprising:
a composition of any of claims 1-11;
and a suitable container.

- Buffer then FXa (50 nM)
- Annexin V (0.5 μ M) then FXa (50 nM)

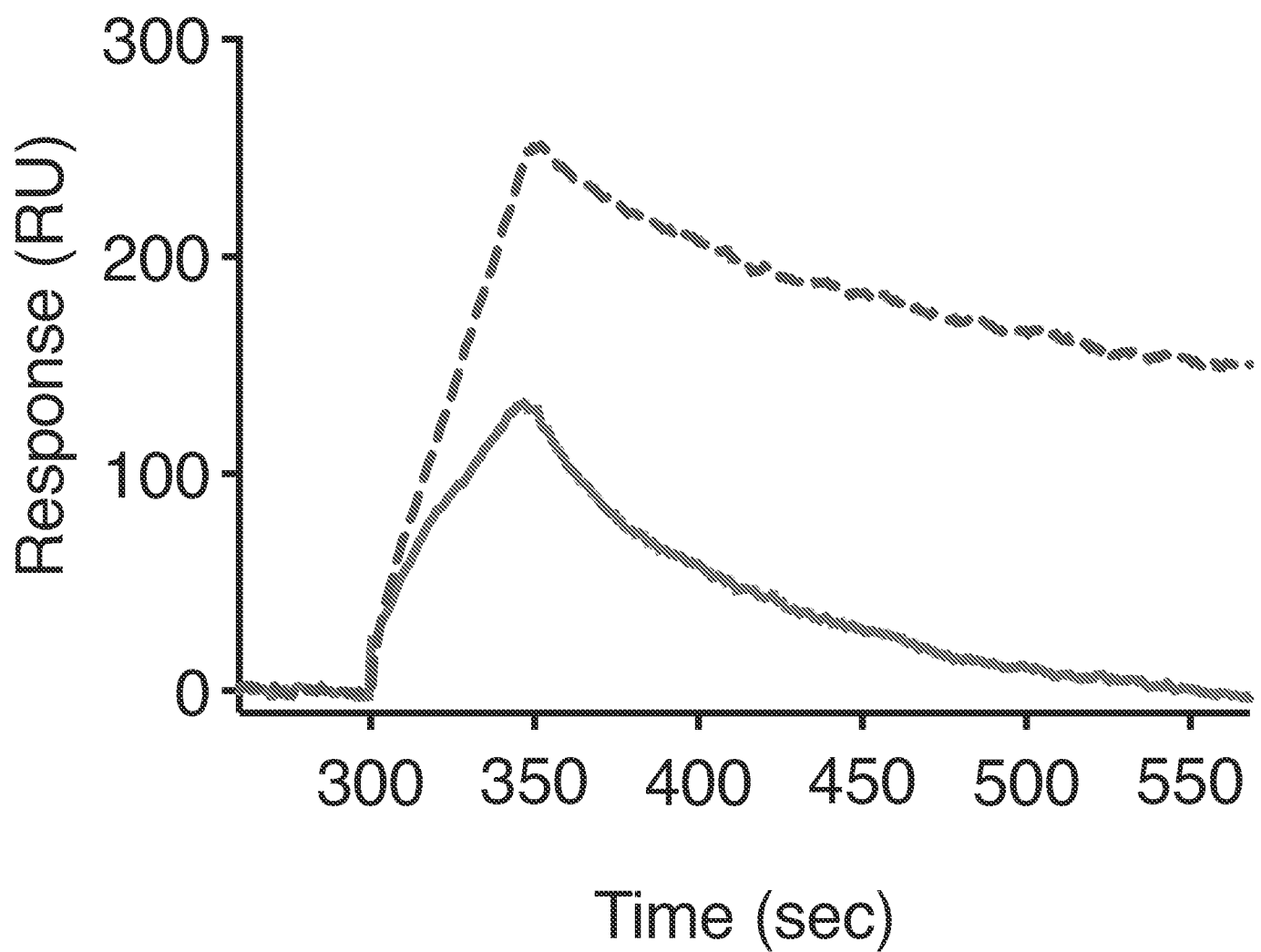


FIG. 1A

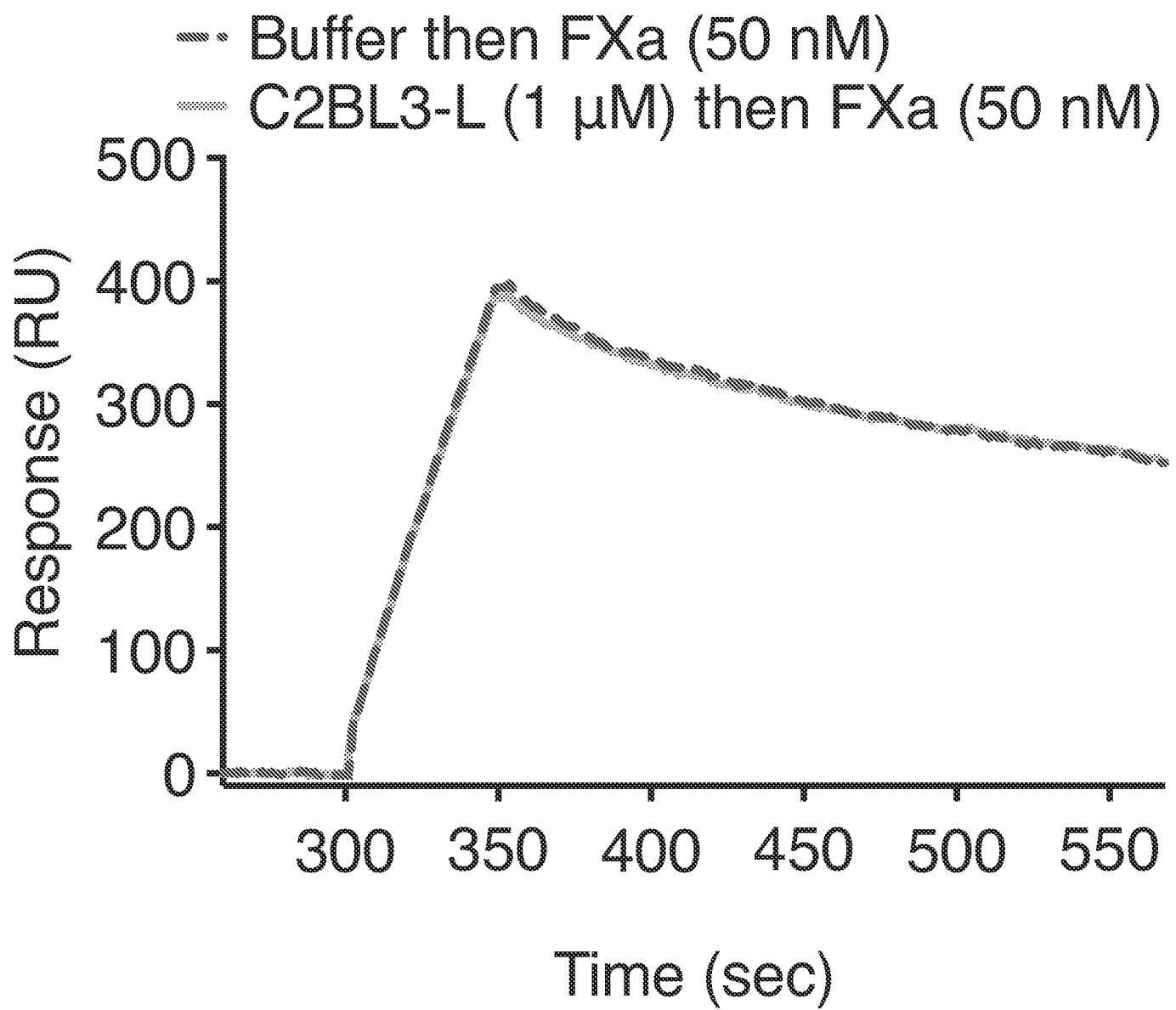


FIG. 1B

- Buffer then FXa (50 nM)
- D-MARCKS ED (1 μ M) then FXa (50 nM)

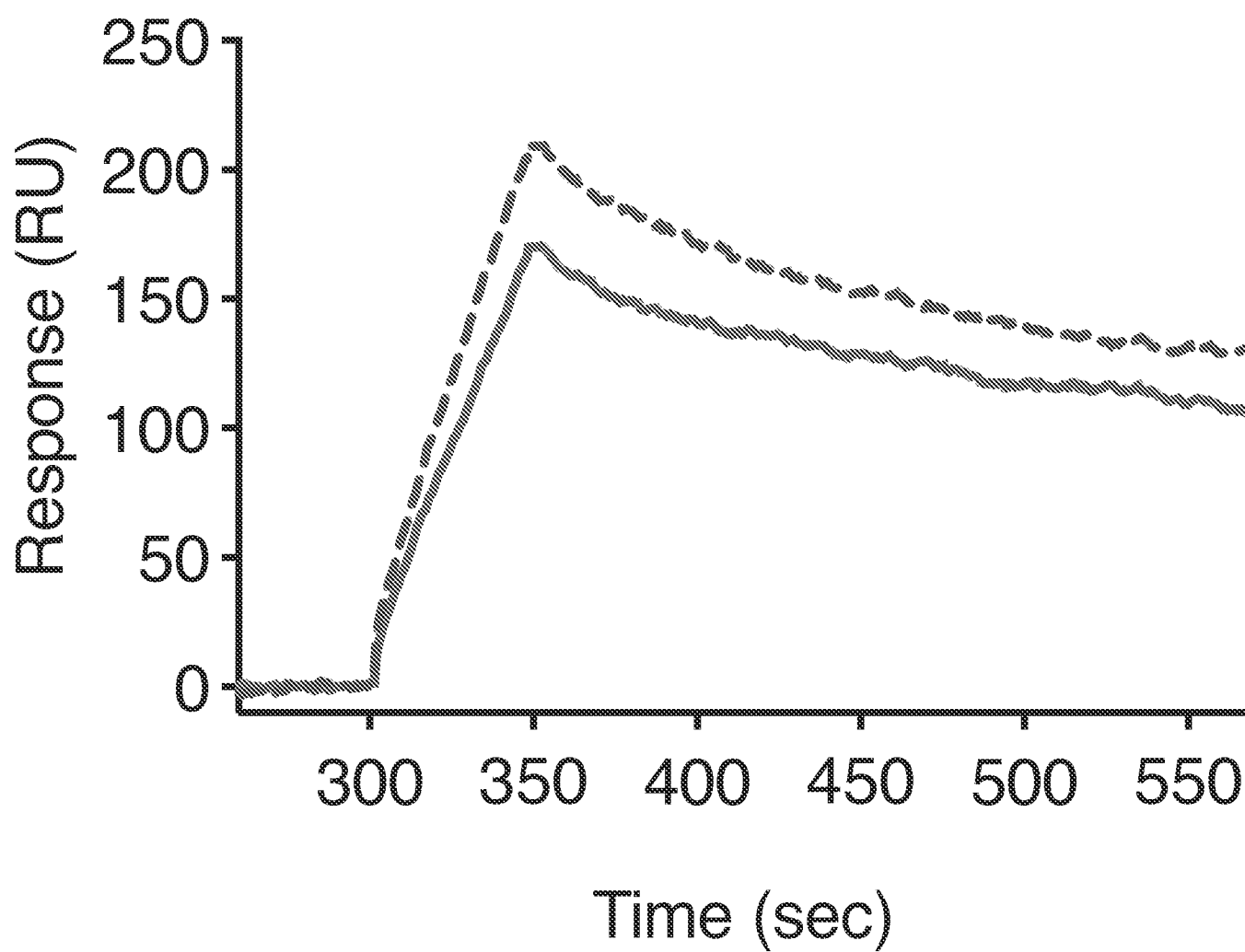


FIG. 1C

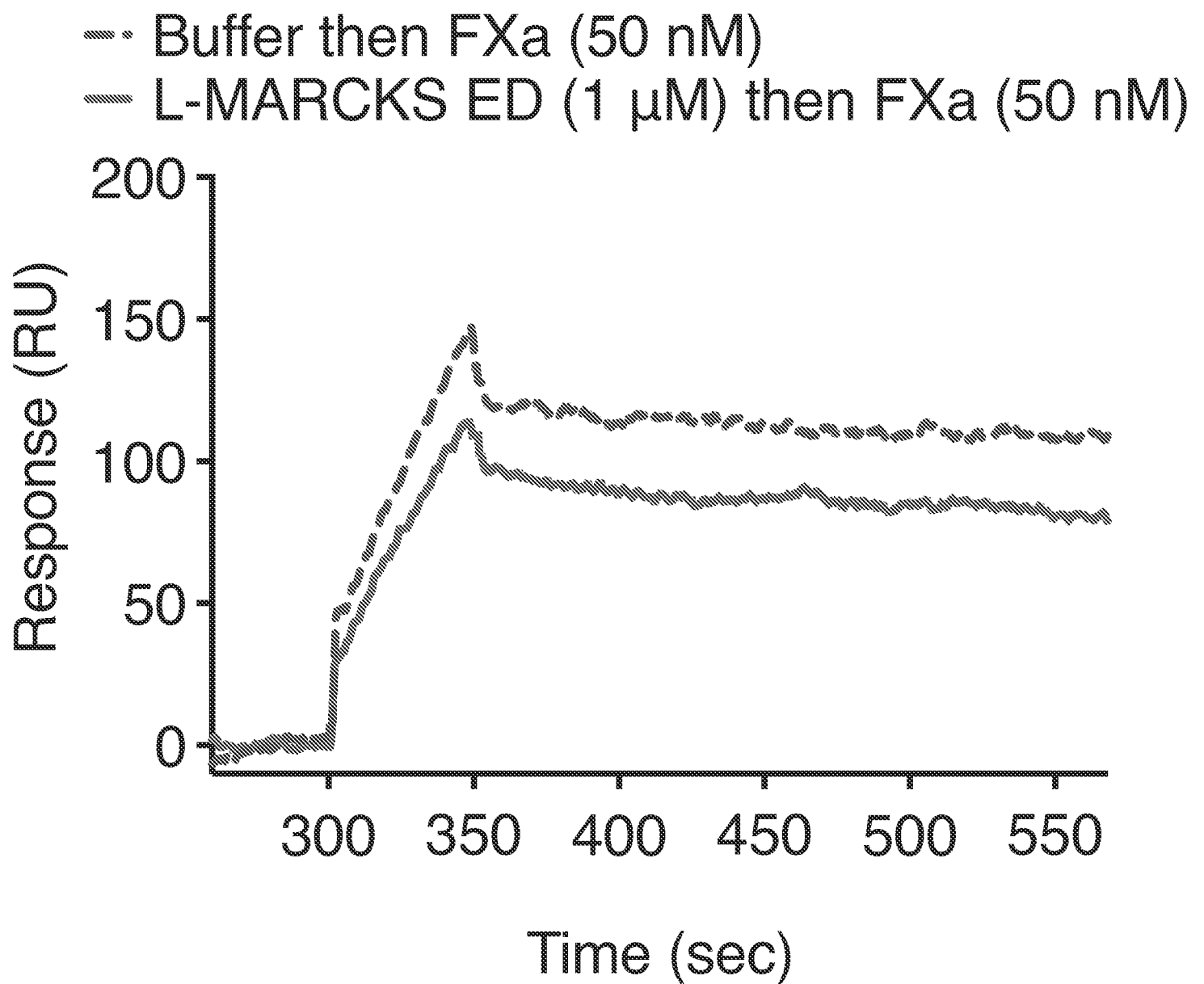
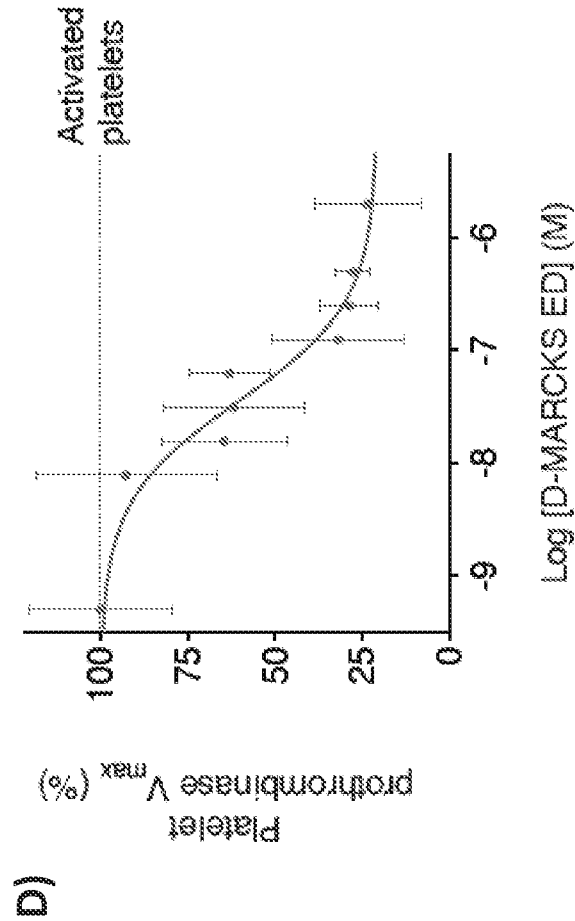
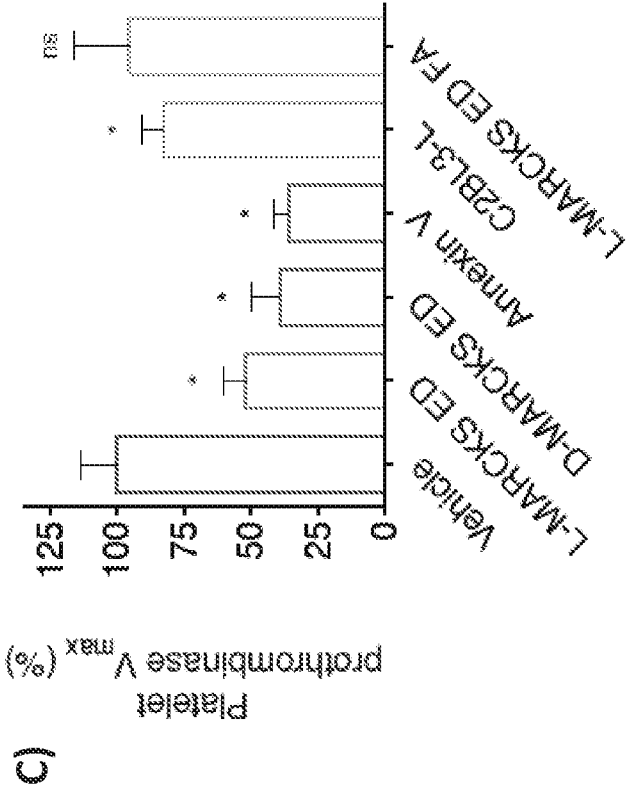
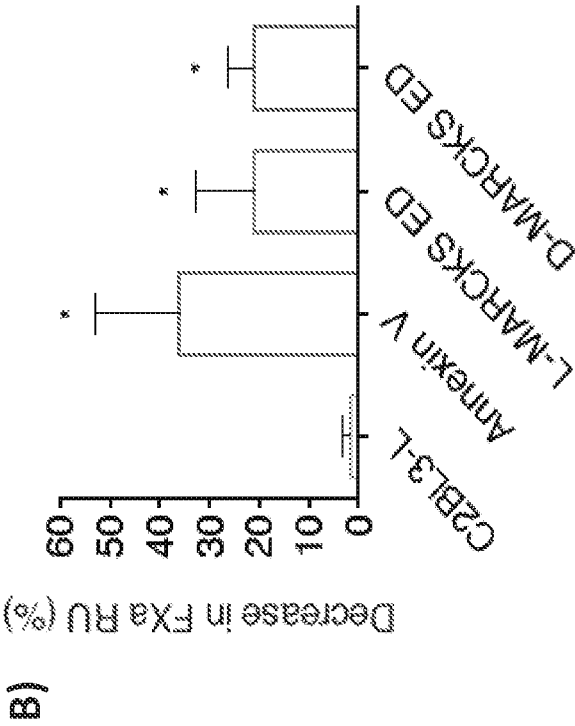
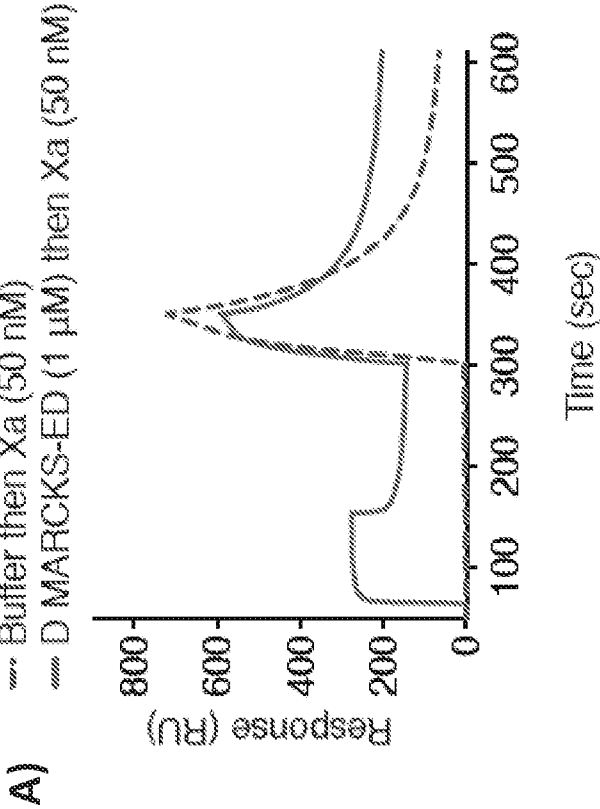
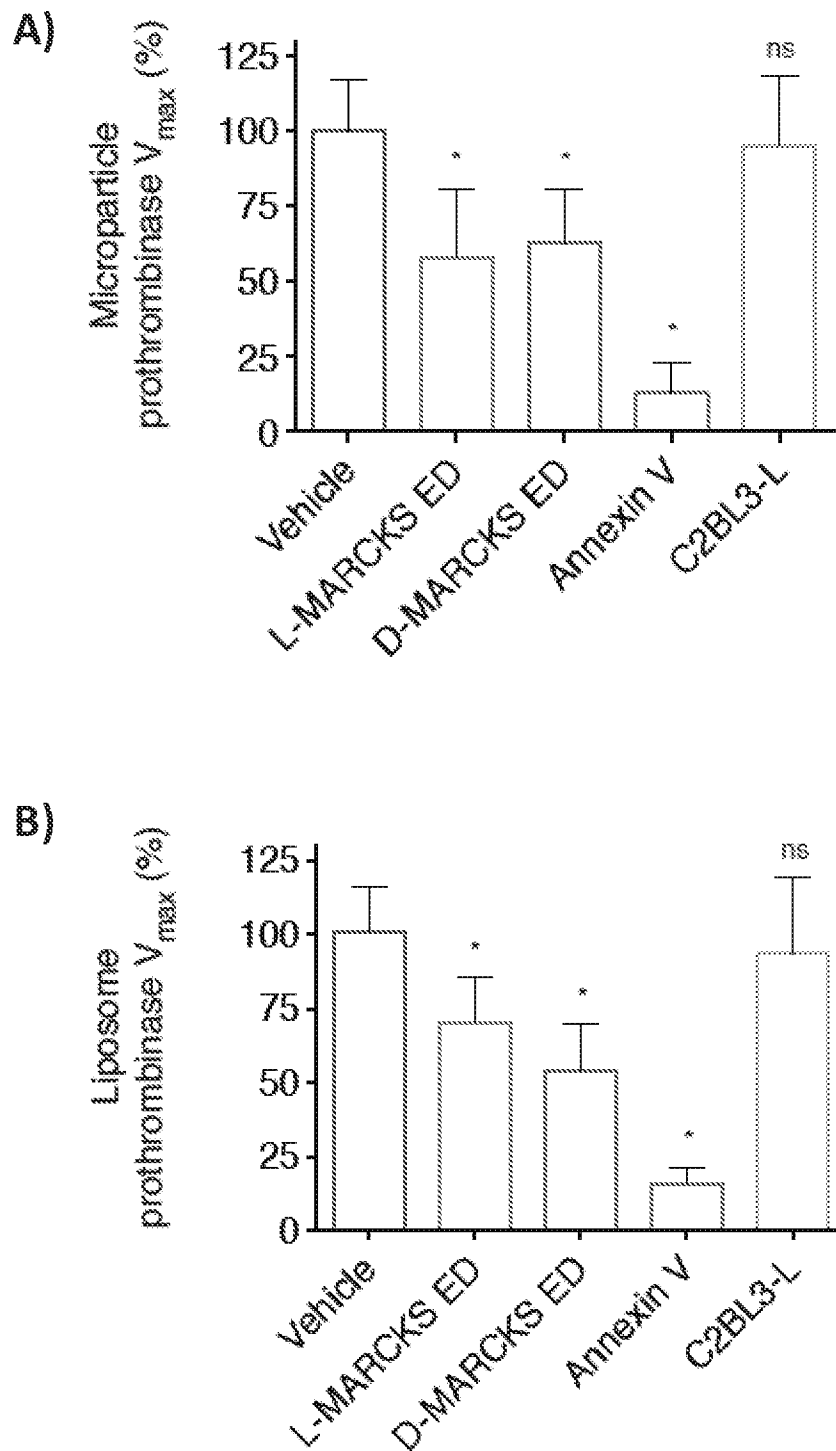


FIG. 1D



FIGS. 2A-2D



FIGS. 3A-3B

- ※ D-MARCKS ED
 $t_{1/2} = 356$ min (95% CI 252 to 606 min)
- ※ L-MARCKS ED
 $t_{1/2} = 57$ min (95% CI 46 to 79 min)

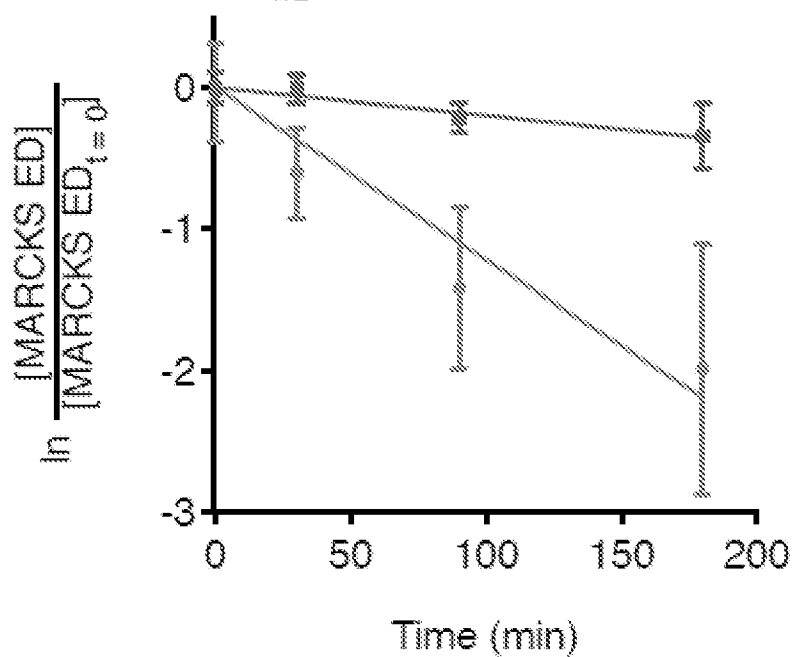
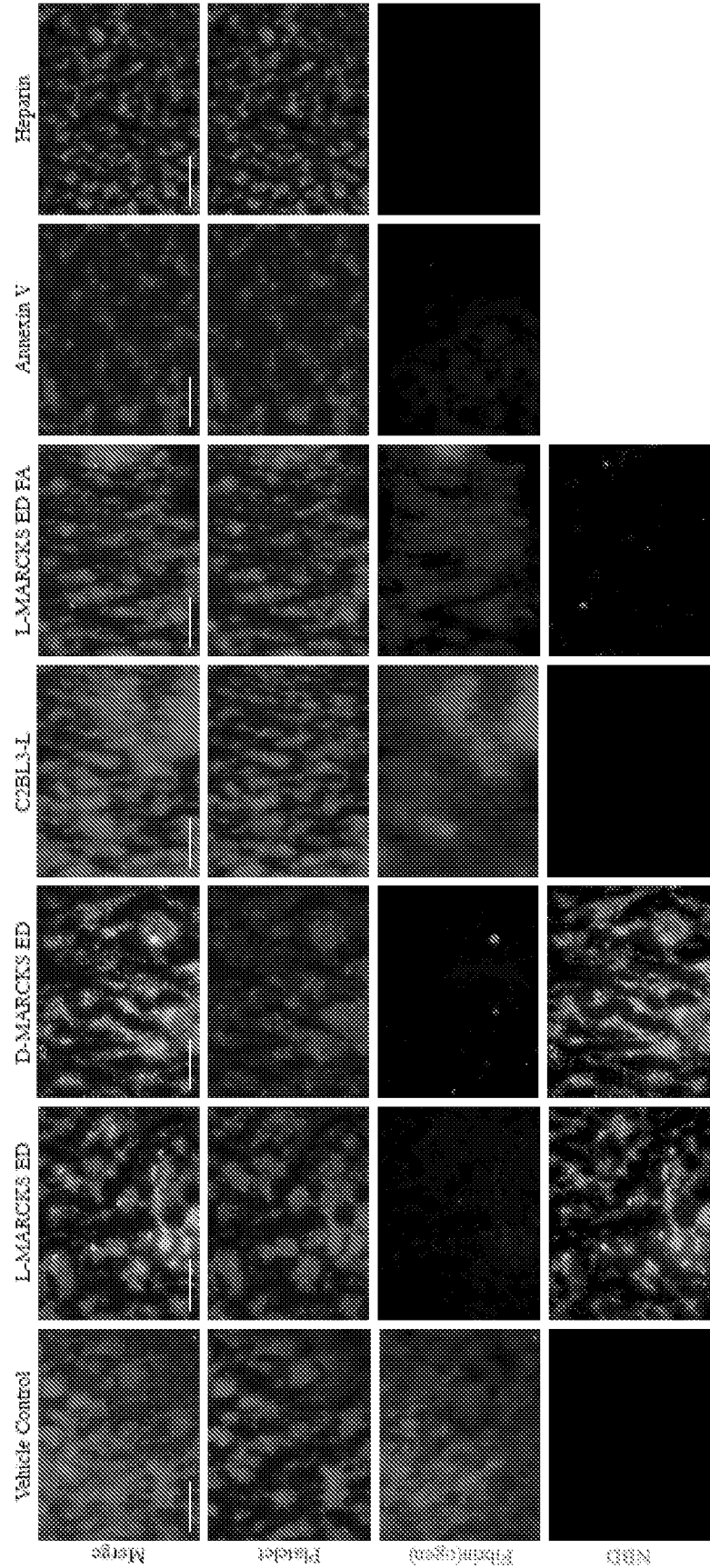
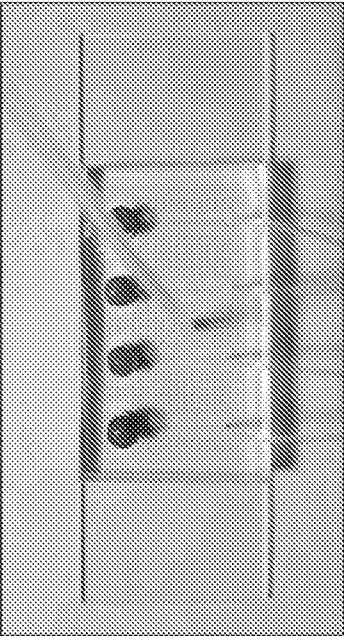
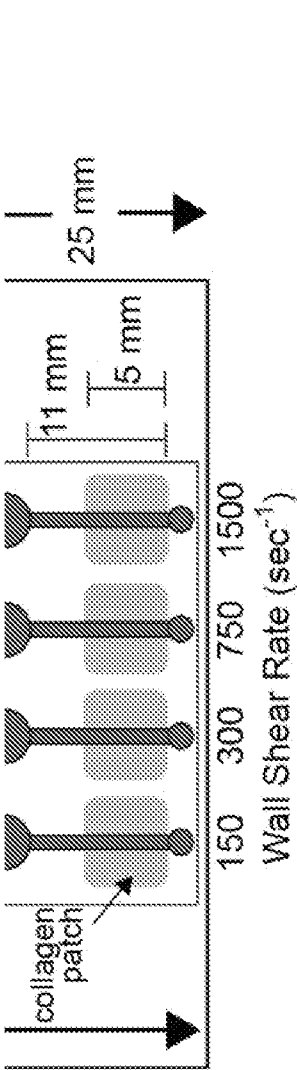
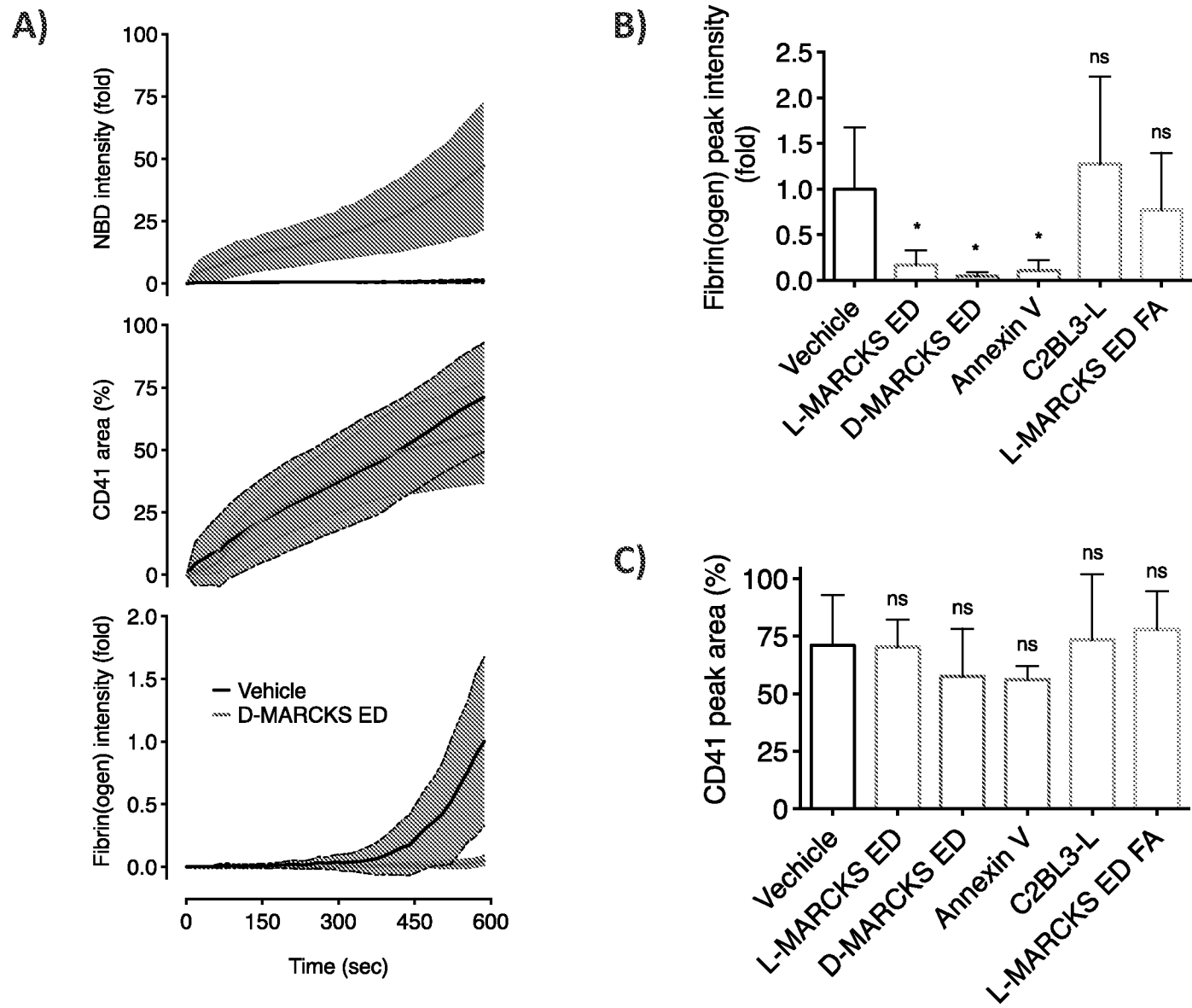


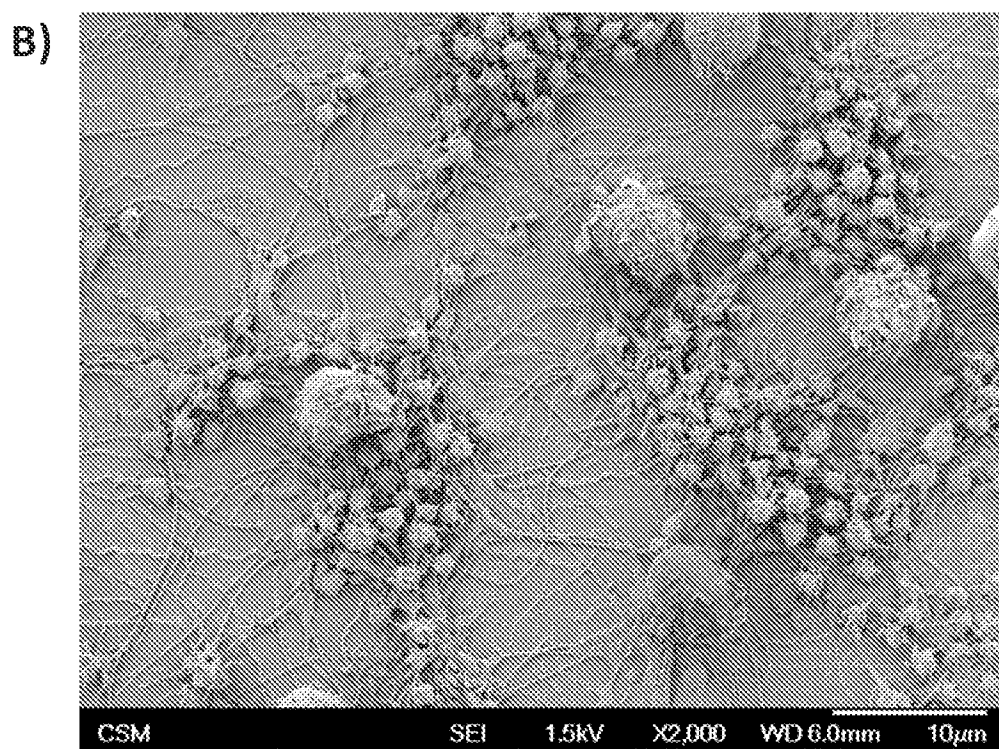
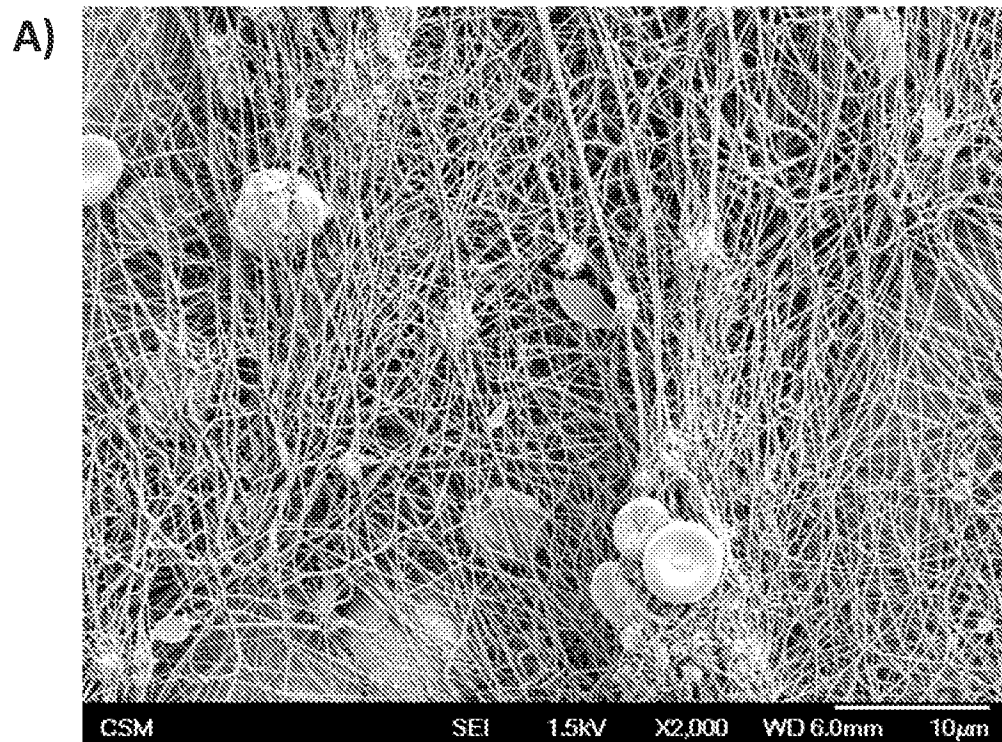
FIG. 4



FIGS. 5A-5B



FIGS. 6A-6C



FIGS. 7A-7B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/040816

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61P7/02
ADD.

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)
A61K

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 practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 602 852 B1 (ZIV ILAN [IL] ET AL) 5 August 2003 (2003-08-05)	1-6,8,9, 12-15, 17-19, 21,22, 25,26, 29,30, 34,35
Y	column 7, lines 8-15; sequence 2 ----- -/--	7,10,11, 16,20, 23,24, 27,28, 31-33

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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

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Date of the actual completion of the international search

23 September 2015

Date of mailing of the international search report

06/10/2015

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Authorized officer

Bochelen, Damien

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International application No
PCT/US2015/040816

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ARTHUR J. CHU ET AL: "Possible role of Marcks in the cellular modulation of monocytic tissue factor-initiated hypercoagulation", BRITISH JOURNAL OF HAEMATOLOGY, vol. 118, no. 2, 1 August 2002 (2002-08-01), pages 569-576, XP055213327, ISSN: 0007-1048, DOI: 10.1046/j.1365-2141.2002.03611.x pages 572,574	1-34
X	----- WO 00/50062 A2 (UNIV NORTH CAROLINA STATE [US]; LI YUEHUA [US]; MARTIN LINDA D [US]; A) 31 August 2000 (2000-08-31)	35
Y	pages 20-21; sequence 2 -----	1-34

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2015/040816

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
US 6602852	B1	05-08-2003	AU	5384699 A		14-03-2000
			EP	1107985 A1		20-06-2001
			IL	125908 A		17-05-2005
			US	6602852 B1		05-08-2003
			WO	0011026 A1		02-03-2000

WO 0050062	A2	31-08-2000	AT	279206 T		15-10-2004
			AT	553121 T		15-04-2012
			AU	766800 B2		23-10-2003
			CA	2366951 A1		31-08-2000
			DE	60014856 D1		18-11-2004
			DE	60014856 T2		02-02-2006
			DK	1154786 T3		14-02-2005
			DK	1538162 T3		23-07-2012
			EP	1154786 A2		21-11-2001
			EP	1538162 A2		08-06-2005
			ES	2230078 T3		01-05-2005
			ES	2391225 T3		22-11-2012
			HK	1041654 A1		22-04-2005
			JP	5620037 B2		05-11-2014
			JP	2002538783 A		19-11-2002
			JP	2015007065 A		15-01-2015
			PT	1154786 E		28-02-2005
			WO	0050062 A2		31-08-2000
