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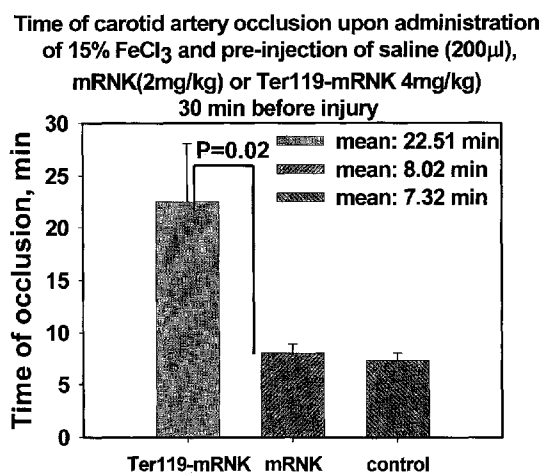


Fig. 24

(57) Abstract: Fusion proteins comprising a single chain antigen-binding domain (scFv) of a monoclonal antibody, linked to an anti-thrombotic agent, anti-inflammatory agent, or a pro-drug thereof are provided, where the polypeptide binds to a binding site (antigen) expressed on the surface of a red blood cell at a density greater than 5,000 copies per red blood cell. Pharmaceutical compositions comprising these fusion proteins, and methods of delivering an anti-thrombotic agent to the surface of a red blood cell via delivery of these fusion proteins, and methods of treating or preventing thrombosis, tissue ischemia, acute myocardial infarction (AMI), ischemic stroke, cerebrovascular disease, pulmonary embolism, or ischemic peripheral vascular disease via administration of the fusion proteins or compositions comprising same are also provided.

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TARGETING RECOMBINANT THERAPEUTICS TO CIRCULATING RED BLOOD CELLS

5 BACKGROUND OF THE INVENTION

Many pathological processes such as thrombosis and hemorrhages, systemic inflammation and cytokine shock, pathologies of transport of lipids and other biological compounds, are initiated or/and develop in the blood. Accordingly, activity of therapeutic agents for optimal interventions in these processes must be localized in this compartment. However, 10 most drugs do not circulate for a sufficient time in the bloodstream because of their uptake by liver, renal filtration and diffusion into the tissues via a vascular wall that is permeable for many therapeutic agents via active and passive transport across endothelium and peri-cellular permeability of endothelial monolayer. Current means for retention of drugs in the bloodstream, such as PEG-ylation or encapsulation into PEG-ylated drug carriers (*e.g.*, liposomes) improve 15 their blood solubility and inhibit recognition by the immune system, thus prolonging their longevity in circulation. However, all previous means for drug retention in bloodstream, including PEG-technology, employed relatively small compounds and carriers (*e.g.*, liposomes of ~100 nm diameter) that can diffuse in tissues (leading to side effects and drug elimination) and uptake by clearing organs (liver and kidney). Therefore, despite relatively easy access to many 20 pathological targets in blood, repeated administrations of high doses of a drug are needed to compensate for clearance. This complicates therapies and leads to adverse effects. The general problem of inadequate therapeutic interventions in the pathological processes in blood has not been resolved.

For example, hemostasis, the sealing of damaged blood vessels by mural clots, prevents 25 bleeding. Thrombosis, pathological intravascular occlusion by clots, can cause tissue ischemia and damage leading to acute myocardial infarction (AMI), ischemic stroke, pulmonary embolism and ischemic peripheral vascular disease, among other conditions. Thrombosis is the leading cause of mortality and disability in the United States. Thrombi are prone to recur within hours to days after an AMI or stroke and the risk is great after transient ischemic attack or pulmonary 30 embolism and in immobilized patients. Thrombosis is also a common and dangerous complication of surgery that is especially difficult to manage due to the risk of acute bleeding at the operative site. Invasive interventions (*e.g.*, angioplasty and carotid endarterectomy) may be complicated by formation of small clots that embolize to the brain and cause neurological dysfunction.

35 Therefore, situations in which patients are at highest risk for occurrence or recurrence of thrombosis, and means to identify such high-risk patients are known. Nevertheless, safety and

efficacy of current prevention and management of thrombosis attained with current anti-thrombotic agents (ATAs) and means for their delivery remain inadequate. Anti-platelet and anticoagulant agents provide only limited prophylaxis and pose considerable risk of bleeding. Emergency therapy of thrombosis employs vascular injection of plasminogen activators (PAs), proteases (MW 30-60 kD) that generate plasmin, which cleaves fibrin clots and thus restores perfusion. However, inadequate circulation time (blood clearance within <15 min), inactivation by plasma inhibitors such as PAI-1 and impermeability of occlusive clots restrict the effectiveness of therapeutic fibrinolysis by PAs. Significant pharmacological doses of a PA (*e.g.*, ~100 mg of tissue type plasminogen activator, tPA) are needed to overcome its inefficiency and achieve fibrinolysis locally. As a result, excess drug diffuses into hemostatic mural clots within minutes after infusion, causing bleeding into tissues. Bleeding into the CNS may cause cerebral hemorrhage. In addition, tPA diffusing into the CNS causes neuronal toxicity and inflammation in the brain. Due to the risk of bleeding and collateral CNS damage, fibrinolytics and anti-coagulants are not used in the post-operative period and in over 95% of stroke patients.

In addition, the only currently employed use of PA, *i.e.*, post-thrombosis, is marred by inevitable delays (time needed for diagnosis, transportation, injection and clot dissolution, slowed by clot impermeability). This delay increases the risk of ischemia-reperfusion (I/R) injury that worsens outcome. Unfortunately, available prophylactic drugs (*e.g.*, anticoagulants and thrombin inhibitors) do not provide adequate protection against thrombosis. First, they are not completely effective due to redundancy of thrombotic mechanisms (*e.g.*, anticoagulants do not prevent platelet activation and anti-platelet agents do not inhibit coagulation). Second, many prophylactic agents altering metabolism of pro-coagulant factors (*e.g.*, inhibitors of the synthesis of vitamin-K dependent coagulation factors, warfarin) require a substantial time to develop an effect (*e.g.*, approximately 36 hours after warfarin administration). Such time frames are not suitable for thromboprophylaxis in acute settings. Third, all of these drugs predispose to bleeding (in addition to other side effects), resulting in a high danger of bleeding, which limits drug utility and dosing especially in the acute settings.

PAs currently in use cannot be used for prophylaxis because of their unfavorable pharmacokinetics and therefore the need to administer potentially dangerous doses of drug for a prolonged period of time. At clinically relevant doses, these PAs cleave fibrin in both hemostatic as well as newly formed clots, which predisposes to bleeding, whereas their penetration into the surrounding tissues causes toxic effects including collateral damage in the central nervous system (CNS).

Targeting of ATAs to clot components (e.g., by chemical conjugation or recombinant fusion with antibodies or antibody fragments that bind to fibrin or activated platelets) does not obviate the failure of ATAs to distinguish between hemostatic and pathological thrombi. In addition, these ATAs with high affinity to clots less effectively permeate into the clots due to enhanced retention on the clot surface, which impedes thrombolysis.

Other research has involved the targeting of plasminogen activators to the vascular lumen (endothelial targeting) using a PECAM-1 single-chain scFv (Ding, *et al.*, Blood 2005, 106(13):4191-4198. *See also* Ding, *et al.*, Molecular Interventions April 2006, 6(2): 98-112). Targeting of ATAs, such as PAs, to the surface of endothelium by means of fusion proteins consisting of anti-cell adhesion molecule (anti-CAM) scFvs and ATAs may be employed for a prophylactic administration of PAs, which would preferentially dissolve newly formed pathological clots in the vascular area of interest, hence reduce danger of hemorrhage caused by dissolution of existing hemostatic clots. This approach seems ideal for prophylaxis of local thromboses after ischemia or reperfusion, such as in organ transplantation. Nevertheless, this approach is also not ideal for thromboprophylaxis of many other pro-thrombotic states (AMI, TIA, PE). Targeting of CAMs on endothelium is relevant only to highly vascularized organs such as lungs. Also, targeting to CAMs cannot fully prevent endocytosis or transcytosis of ATA from luminal to adventitial side of the vessels which will decrease the duration of the prophylactic effect of the drug.

By targeting ATAs, such as PAs, to red blood cells (RBCs), which can be attained by chemical conjugation of ATAs with antibodies that bind to RBCs, the half-life of the drug in circulation can be prolonged from minutes to days. ATA/RBC complexes selectively lyse newly formed clots because they are incorporated during clot development, and their size prevents penetration of pre-existing clots. This drastically diminishes the risk of bleeding. In addition, coupling to a RBC prevents drug permeation into tissues including the CNS.

However, this approach for selective lysis of newly formed clots has several disadvantages for clinical application. For example, current anti-CR1 conjugates (e.g., anti-CR1/tPA, targeted to a specific RBC determinant, CR-1), such as those described in Zaitsev, *et al.* (Blood, 108(6):1895-1902 (May 30, 2006)) are not preferred therapeutically for a number of reasons, in addition to those described in the preceding paragraph. Furthermore, the CR1 expression level in humans varies from 300 to 1,500 copies per RBC and ~15% of humans are CR1 negative. Therefore, targeting CR1 may provide insufficient dosing in some cases and will not be useful in CR1-negative patients. Targeting of a highly expressed determinant on RBC with bivalent antibodies can cause RBC aggregation. Therefore, this approach maybe useful only

for targeting ATAs to low-abundance RBC determinants, which limits dosing that may be insufficient in the cases of excessive thromboses. Also, Fc fragment of antibodies can activate complement, promote clearance or signal through Fc receptors or induce an immune response in the host. The synthetic chemistry limits yield and homogeneity of the ATA/antibody conjugates, thereby restricting their clinical utility.

In addition, thrombosis is closely intertwined with vascular inflammation. In many cases one of these conditions leads to another and both mutually propagate each other, further aggravating the outcome.

There exists a need for safe and clinically applicable compositions for effective treatment of thrombosis and associated pathological conditions including vascular inflammation.

SUMMARY OF THE INVENTION

The compositions and methods described herein provide a solution to the acute unmet medical need for design of new drug delivery systems aimed at safer and more effective management of thrombosis. These compositions and methods provide clear advantages over existing compositions and methods for treatment with anti-thrombotic agents (ATAs). These compositions and methods also provide clear advantages over existing compositions and methods for treatment with anti-inflammatory drugs or agents. Further, these compositions and methods provide a novel delivery system for the administration of pro-drugs useful in treating thrombosis and other medical conditions.

In one aspect, a fusion protein is provided that comprises a single chain antigen-binding domain (scFv) of a monoclonal antibody linked to an anti-thrombotic agent (ATA), anti-inflammatory agent, or a pro-drug thereof, where the scFv binds to a determinant (*e.g.*, binding site, antigen) expressed on the surface of a red blood cell at a density greater than 5,000 copies per red blood cell. In certain embodiments these fusion proteins also contain a cleavage site.

In another aspect, a fusion protein is provided that comprises a single chain antigen-binding domain (scFv) linked to an anti-thrombotic agent, anti-inflammatory agent, or a pro-drug thereof, wherein the scFv binds a determinant expressed on the surface of a red blood cell, and the determinant is not a specific site for recognition by host defense cells that clear microscopic objects from surface of circulating RBC without damage to the RBC.

In a further aspect, a fusion protein is provided which comprises a single chain antigen-binding domain (scFv) linked to an anti-thrombotic agent, anti-inflammatory agent, or a pro-drug thereof, wherein at least 10% of the fusion protein injected into the bloodstream is maintained on the surface of a red blood cell *in vivo* for at least 48 hours.

In another aspect, a fusion protein is provided which binds a pro-inflammatory mediator, *e.g.*, HMGB1.

In yet another aspect, pharmaceutical compositions are provided that comprise one or more of these above-described fusion proteins.

5 In yet a further aspect, methods of delivering an anti-thrombotic agent or anti-inflammatory agent to the surface of a red blood cell via delivery of a fusion protein, as described above, are also provided. Methods of treating or preventing thrombosis, tissue ischemia, acute myocardial infarction (AMI), ischemic stroke, cerebrovascular disease, pulmonary embolism, sepsis, acute lung injury (ALI) or other forms of vascular inflammation, or ischemic peripheral
10 vascular disease via administration of the fusion proteins or compositions comprising same, are similarly provided.

In another aspect, methods of delivering an anti-inflammatory agent to the surface of a red blood cell via delivery of a fusion protein, as described above, are also provided.

In still another aspect, methods of delivering a pro-drug to the surface of a red blood cell
15 via delivery of a fusion protein, as described above, are also provided.

Also provided is the use of any of these fusion proteins or compositions comprising same as a medicament.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Fig. 1A provides a general description of an antibody structure (having variable heavy and light chains, *e.g.*, V_H and V_L , and constant heavy and light chains, *e.g.*, C_H and C_L), and derived scFv molecular structure with a peptide linker between the V_H and V_L . Fig. 1B reflects RT PCR (reverse transcription polymerase chain reaction) amplification of V_H and V_L chains of the antibody from total RNA of a corresponding hybridoma cell line. The V_H , V_L , and linker
25 fragments are then fused by PCR to form the resulting scFv (Fig. 1C).

Fig. 2 is a bar graph showing the functional activity of a fusion protein comprising an anti-glycophorin A associated protein (expressed on mouse RBC) scFv derived from parental rat MAb Ter119 as described in Fig. 1, and an extracellular domain of mouse thrombomodulin (Ter119-TM). Mouse and human RBCs were incubated in serum free medium from induced and
30 non-induced S2 cells transfected with a plasmid encoding Ter119-TM. RBC were washed and incubated with protein C in the presence or absence of thrombin. The experiment demonstrated that only mRBC loaded with Ter119-TM in the presence of thrombin cause protein C activation thus confirming both determinant binding and functional activity of Ter119-TM. The columns are marked as induced murine RBC (mRBC i) with (+) or without (-) thrombin; non-induced

murine RBC (mRBC) with (+) or without (-) thrombin; induced human RBC (hRBC i) with (+) or without (-) thrombin; non-induced human RBC (hRBC) with (+) or without (-) thrombin; and control.

Fig. 3 is a bar graph showing fibrin clot lysis by mouse and human RBC loaded with Ter119-lmvUK-T and Ter119-mRNK. This fusion protein comprises an anti-glycophorin A associated protein (mouse RBC) scFv derived from parental rat MAb Ter119 as described in Fig. 1, and kringle 2 and protease domains of mouse tPA. Moreover, the protease domain contains point mutations similar to those in human Tenectase making it less sensitive to inhibition by PAI-1. The bars are labeled as shown in the figure. Mouse and human RBCs were incubated in serum free medium from induced S2 cells transfected with a plasmid encoding either Ter119-lmvUK-T (indicated in the figure as Ter119-IUK-T) or Ter119-mRNK. RBC were washed and incorporated into fibrin solution before induction of clotting by addition of Ca and thrombin. Part of RBC loaded with Ter119-lmvUK-T were pre-incubated with thrombin in order to activate fusion protein. The experiment confirmed the high selectivity of binding of both fusion proteins to mouse RBC vs human RBC and their proteolytic activity in mRBC surface bound state. Moreover, it showed that pre-incubation with thrombin accelerates subsequent dissolution of thrombi by mRBC-bound Ter119-lmvUK-T, yet even short time exposure of Ter119-lmvUK-T to thrombin during clotting is enough to activate the fusion protein.

Figs. 4A through 4D illustrate binding of Ter119-lmvUK-T or Ter119-mRNK to mouse RBC under different percentages of hematocrit. Figs 4A and 4B represent binding at 10% hematocrit, while Figs. 4C and 4D represent binding at 1% hemotocrit. Binding procedure was performed with ¹²⁵I labeled fusion proteins.

Figs. 5A and 5B are bar graphs showing the biodistribution of Ter119-mRNK *in vivo*. For these experiments, 2-5 µg of ¹²⁵I radiolabeled fusion protein or control non-targeted mRNK were injected intravenously to wild type (WT) mice. One hour later, tissue uptake was determined. Fig. 5A is a bar graph showing the percentage of injected dose per tissue (%ID). Ter119-mRNK showed a greatly higher level in blood than mRNK, due to binding to RBC. The latter statement is validated by Fig. 5B, a bar graph showing the distribution of Ter119-mRNK in blood (RBC vs plasma) at different time points. More than 90% of Ter119-mRNK are associated with RBC at 30 min and 60 min of circulation.

Fig. 6 illustrates the kinetics of scFv-mRNK binding to mouse heparinized whole blood and RBCs in PBS/BSA. Time to 50% saturation (molecules/RBC) is approximately 30 minutes.

Fig. 7 reflects Ter119-mRNK-¹²⁵I distribution in organs at time points through 48 hours (left to right 1h, 3h, 6h, 24h, and 48h).

Fig. 8 reflects blood component distributions (RBC vs. plasma) at time points (left to right) 0.5h, 1h, 3h, 6h, 24h, and 48h, corresponding to Fig. 7.

Fig. 9 shows organ distribution of Ter119-TM-¹²⁵I loaded on ⁵¹Cr-labeled mouse RBCs at 1h, 3h, and 6h, or control ⁵¹Cr-labeled RBC. mouse.

5 Fig. 10 reflects blood component distribution of isotopes at indicated time after injection of the complex in this experiment (RBC vs. plasma), corresponding to Fig. 9.

Fig. 11 reflects Ter119-TM-¹²⁵I distribution in organs at time points through 48 hours (left to right 0.5h (no sample taken), 1h, 3h, 6h, 24h, and 48h).

10 Fig. 12 reflects blood component distributions (RBC vs. plasma) at time points (left to right) 0.5h, 1h, 3h, 6h, 24h, and 48h, corresponding to Fig. 11.

Fig. 13 reflects Ter119-TM-¹²⁵I distribution in organs, when delivered by intraperitoneal (IP) injection, at time points 1h and 3h.

Fig. 14 reflects blood component distribution of injected isotopes in this experiment (RBC vs. plasma) at time points 1h and 3h, corresponding to Fig. 13.

15 Fig. 15 shows circulation of the fusion protein Ter119-IUK-T (vs. free uPA (IUK)) in blood during the 48 hours after injection.

Fig. 16 reflects organ distribution of free uPA (IUK) after one hour of circulation.

Fig. 17 reflects organ distribution of the fusion protein (Ter119-IUK-T) through 48 hours (left to right, 1h-48h) after injection.

20 Fig. 18 reflects the distribution of the fusion protein (Ter119-IUK-T) between blood plasma and cells through 48 hours (left to right, 0.5h-48h) after injection.

Fig. 19 reflects clot lysis (as a percentage) by fusion protein Ter119-IUK-T through 48 hours circulation after clot incubation (5h or 24h). Maximal fibrinolytic effect was noted from hour 1 through 48 hours after injection into the fibrin clot.

25 Fig. 20 shows in vitro fibrin clot lysis by 2μL, 5μL, and 10μL of mRBCs loaded with Ter119-mRNK at 17,000 mol/RBC (dose dependence on clot lysis % vs time following injection). Human (h)RBC-Ter119-mRNK matches negative control, as Ter119 is specific for mouse RBCs. tPA is provided as a positive control.

30 Fig. 21 illustrates that following 45 minutes circulation, scFv-mRNK retains its ability to activate plasminogen reflected by lysis of clots formed from blood obtained from mice injected with scFv-mRNK (radioactive fibrinogen used as tracer). Free mRNK matches negative control (PBS), as anticipated.

Fig. 22 reflects kinetic data (activity (U/mg) as a function of time) of the activation of Ter119-IUK-T (Ter119scFv-uPA-T) by thrombin.

Fig. 23 shows clot lysis in human platelet poor plasma by Ter119-IUK-T (bound to mouse RBC vs. free), as a function of concentration and time. Near rate limiting doses are utilized to explore effects of binding to RBC on activity of the fusion.

Fig. 24 shows the thromboprophylactic effect of Ter119scFv-mRNK manifested by
5 delayed full occlusion. Free RNK was comparable to PBS negative control, as expected.

DETAILED DESCRIPTION OF THE INVENTION

The compositions and methods described herein meet the needs in the art.

I. THE FUSION PROTEIN

10 Fusion proteins are provided which comprise a single chain antigen-binding domain (scFv) linked to an anti-thrombotic agent, anti-inflammatory agent, or a pro-drug, wherein said scFv binds to a determinant (*e.g.*, binding site, antigen) expressed on the surface of a red blood cell at a density greater than 5,000 copies per red blood cell. The scFv is linked to a native or genetically or otherwise modified polypeptide chain of an anti-thrombotic agent (ATA), anti-
15 inflammatory agent, or pro-drug thereof. For example, the recombinant fusion proteins as described herein are useful particularly for thromboprophylaxis in patients at risk of thrombus formation.

In one embodiment, the fusion protein optionally contains a cleavage site interposed between the scFv and the anti-thrombotic agent/anti-inflammatory agent to permit release of the
20 therapeutic molecule at the site of a pathological condition (pro-drug construct). In this embodiment, the cleavage site is introduced for the purpose of releasing the anti-thrombotic/anti-inflammatory agent from the RBC at a desired site. Using this modular recombinant format, one of skill in the art can design and synthesize a series of monovalent, Fc-fragment free, and homologous proteins carrying diverse anti-thrombotic/anti-inflammatory drugs directed to diverse
25 RBC determinants, which are easy to produce, maintain quality control, store and administer, as described in detail below.

A. The scFv of the Fusion Protein

The fusion proteins contain a targeting single chain antigen-binding domain
30 (scFv) that binds to a determinant expressed on the surface of a red blood cell at a density greater than 5,000 copies per red blood cell. Use of an scFv (monovalent) avoids cross-linking of binding sites or determinants, thereby avoiding potentially harmful cell membrane modification and cell aggregation.

Use of recombinant proteins, which are synthesized as an scFv, has an advantage over chemical conjugation with respect to each of the above-noted problems in the art. For example, recent research has included the use of scFv-mediated targeting of human complement regulatory protein (CRP) decay-accelerating factor (DAF) to a red blood cell restricted surface antigen of the mouse (Spitzer, *et al.*, Mol. Immunol. 2003, 40:911-919).

scFvs may be generated conventionally, *e.g.*, by the method of Spitzer, *et al.* (Mol. Immunol. 2003, 40:911-919). Total RNA of a hybridoma cell line is isolated (*e.g.*, by RNeasy, Qiagen, Velencia, CA), followed by reverse transcription, *e.g.*, using the SMARTTM technology (Clontech, Palo Alto, CA) employing known primers (*e.g.*, those of Dübel, *et al.* (J. Immunol. Methods 1994, 175:89-95)). The resulting heavy (V_H) and light (V_L) chain variable cDNA fragments are then subcloned into a suitable plasmid, *e.g.*, pCR®2.1-TOPO® (Invitrogen, Carlsbad, CA). The materials utilized are not a limitation of these embodiments. The V_H and V_L chains generated are combined with a suitable linker, resulting in the desired scFv (*see, e.g.*, Example 1). In one embodiment, an scFv is prepared as illustrated in Fig 1. Since scFv fusion proteins are monovalent, they are not anticipated to cross-link RBC determinants, which is the main safety concern for using most monoclonal antibodies. Lack of Fc-fragment and deletion of unnecessary domains in the protein drug entity enhances specificity and safety of interventions.

The determinant to which the single chain antigen-binding domain (scFv) of the fusion protein binds is any determinant expressed on the surface of a red blood cell, preferably a human RBC, in a sufficient density. In one embodiment, the cell surface determinant is expressed across the human population at a density greater than 5,000 molecules per red blood cell. In another embodiment, the cell surface determinant is expressed across the human population at a density greater than 10,000 molecules per red blood cell. In another embodiment, the cell surface determinant is expressed across the human population at a density greater than 20,000 molecules per red blood cell. In still another embodiment, the cell surface determinant is expressed across the human population at a density greater than 50,000 molecules per red blood cell. In another embodiment, the cell surface determinant is expressed on the red blood cell at greater than 100,000 molecules per red blood cell. In another embodiment, the cell surface determinant is expressed on the red blood cell at greater than 500,000 molecules per red blood cell. In still further embodiments, the determinant is expressed on the surface of a red blood cell at a density greater than 1,000,000 molecules per red blood cell. In still further embodiments, the determinant is expressed on the surface of a red blood cell at a density greater than 2,000,000 molecules per red blood cell.

In a specific embodiment, the determinant is glycophorin A associated protein (GPA). This is the mouse-specific analog of human glycophorin A, recognized by a rat monoclonal antibody Ter119, and is thus the GPA used in the mouse models provided within the Examples, *infra*. One of skill in the art will recognize that 'GPA' is often used in the literature to specifically refer to the glycophorin A antigen. Accordingly, for clarity, the specific antigen human glycophorin A is designated hGPA herein.

Preferably the anti-GPA antibody from which the scFv is obtained is an anti-human GPA antibody. The level of GPA expression in humans is $\sim 10^6$ copies per RBC across the population. Thus, the use of anti-GPA scFv in the fusion proteins described herein provides a viable therapeutic composition due to its wider range of RBC loading. This enables strict dosage control of the anti-thrombotic agent, which is not available for constructs for which determinant density is lower. The anti-thrombotic and anti-inflammatory protein thrombomodulin is also demonstrated in such fusion proteins.

In another embodiment, the determinant is an ABO blood group antigen. Thus, suitable scFvs for use in the fusion proteins described herein include scFv to GPA or ABO blood group antigens. In another embodiment, the scFv is to RBC band 3 antigen. Still other appropriate determinants meeting the above density requirements may be selected by one of ordinary skill in the art.

In another embodiment, the binding site or cell surface determinant is not a specific site for recognition by host defense cells that clear microscopic objects from the surface of a red blood cell without damage to the red blood cell. Both the GPA and ABO blood group antigens meet this requirement. In contrast, CR1 antigen does not meet this requirement.

However, while scFvs to the determinants GPA or ABO blood group antigens are one example of scFvs useful in the fusion proteins, other scFvs capable of binding to the designed determinant on a RBC may be used in place thereof. One of skill in the art provided with the teachings of this specification and publically available information can readily design an scFv useful in the fusion proteins described herein.

B. The Anti-Thrombotic Agent, Anti-Inflammatory Agent, or Pro-Drug thereof of the Fusion Protein

Fusion proteins described herein may contain any anti-thrombotic agent (molecule), anti-inflammatory agent, or pro-drug thereof for which targeting to a red blood cell is desired for purposes of systemic delivery, or alternatively, for delivery to the site of a pathological condition including conditions characterized by the production or presence of an

enzyme that can cleave the anti-thrombotic agent, anti-inflammatory agent, or the pro-drug, from the fusion protein.

As used herein, the term "pro-drug" or "prodrug" encompasses any polypeptide encoding an anti-thrombotic or anti-inflammatory agent and a cleavage site for activation of the agent. The pro-drug is inactive (or significantly less active) upon administration, and is metabolized *in vivo* into an active form. In further embodiments, the pro-drug is a pro-drug of an anti-thrombotic or anti-inflammatory agent.

In one embodiment, the anti-thrombotic agent is one that is capable of producing its therapeutic effect when attached to the RBC, i.e., an active anti-thrombotic agent. In another embodiment, the anti-thrombotic agent is a pro-drug which contains a native or synthetic cleavage site and which produces an active anti-thrombotic effect only upon cleavage from its pro-drug state.

Among such anti-thrombotic agents include without limitation, plasminogen activators. In still a further embodiment, the plasminogen activator is tPA, urokinase, tenectase, retavase, streptokinase, staphylokinase, or a plasminogen activator from venoms and saliva of bats, insects, and other animals. In another embodiment, the plasminogen activator is anistreplase, pro-urokinase (pUK), or a hybrid plasminogen activator (e.g., as described in US Pat. No. 4,916,071).

In a further embodiment the anti-thrombotic agent is the low molecular weight single chain urokinase-like plasminogen activator described in the examples below (also termed uPA (as the exemplary plasminogen activator), IUK, lmwUK, and lmw scuPA within the examples). Also included are mutants or variants thereof, which retain plasminogen activator activity, such as variants which have been chemically modified or in which one or more amino acids have been added, deleted or substituted or in which one or more functional domains have been added, deleted or altered such as by combining the active site of one plasminogen activator or fibrin binding domain of another plasminogen activator or fibrin binding molecule. In a further embodiment, the anti-thrombotic agent contains a moiety presented by a protease domain of a plasminogen activator. Naturally-occurring pro-drugs of these agents may be employed. Synthetically designed prodrugs based on these agents may also be employed in the fusion proteins. Prodrugs containing modified cleavage sites may also be employed as part of the fusion protein.

In one embodiment, the therapeutic molecule or pro-drug is a therapeutic protein or pro-drug of an anti-inflammatory agent. In one embodiment, the anti-inflammatory agent is an antibody against a cytokine or other pro-inflammatory mediator. In a further embodiment, the

anti-inflammatory agent may comprise a moiety presented by thrombomodulin or a domain thereof. Among other anti-inflammatory agents for use in the fusion proteins described herein are, without limitation, somatostatin, adiponectin, cortistatin, corticotrophin releasing factor, sauvagine, nocifensins, as well as the anti-inflammatory cytokines, IL-1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10, and IL-13 and the soluble receptors sTNFR_I, sTNFR_{p55}, sTNFR_{II}, sTNFR_{p75}, sIL-1R_{II}, mIL-1R_{II}, and IL-18BP, among others. Anti-inflammatory proteins may be native or mutated proteins. Similarly, native, mutated or synthetic anti-inflammatory peptides, including without limitation, peptides described in US Patent Nos. 5480869; 7816449 and 5229367, among other known peptides may also form part of the fusion proteins described herein.

One of skill in the art may select or design an appropriate anti-inflammatory agent or prodrug depending on the pathological condition being treated.

In still another embodiment, the therapeutic molecule is molecule which binds a pro-inflammatory mediator. In one embodiment, the pro-inflammatory mediator is the HMGB1 cytokine. In this embodiment, signaling by HMGB1 is disrupted by binding of the lectin-like domain of thrombomodulin (abbreviated herewith as TM). In other embodiments, the pro-inflammatory cytokine is IL-1- α , IL-1- β , IL-6, TNF- α , TGF- β , LIF, IFN- γ , OSM, CNTF, GM-CSF, IL-8, IL-11, IL-12, IL-17, and IL-18.

In one embodiment, a fusion protein may contain a therapeutically-active site, domain or moiety of any of the anti-thrombotic agents, anti-inflammatory agents, or pro-drugs listed herein or known to the art to be suitable for direct targeted administration to the site of a thrombus. Other useful pro-drugs known to one of skill in the art may be used herein.

In still other embodiments, mutations in protein sequence of the anti-thrombotic agent or anti-inflammatory agent, therapeutically-active site, domain, or moiety thereof allows its conversion into a pro-drug activated and/or released locally at a desired pathological sites (e.g., pathological nascent intravascular thrombi) using specific activity of pathological factors that exist only in these pathological sites, such as protease thrombin. Such mutations in the amino acid sequences or nucleotide sequences encoding the therapeutic protein can be employed to insert a desired cleavage, enzymatic or activation site into the therapeutic molecule, or into or adjacent the linker between the scFv and the therapeutic molecule in the fusion protein.

Alternatively, such mutations can change a native cleavage site to another desired cleavage site, or to insert a cleavage site where none naturally existed into or adjacent to a therapeutic molecule.

In one embodiment, the therapeutic pro-drug molecule is activated or the mature drug molecule released from the fusion protein by an enzyme, which level is locally elevated under pathological conditions. In a further embodiment, the enzyme is a protease. In still further

embodiments, the protease is a leukocyte protease (*e.g.*, cathepsin), an activated protease in the coagulation cascade (*e.g.*, activated Factor Xa), or an activated protease in the complement cascade. In other embodiments, the protease's activity is elevated locally in tissue. In still other embodiments, the protease is a metalloproteinase, elastase, or collagenase.

5 In still other embodiments of fusion proteins containing therapeutic pro-drug molecules, the enzyme is a pathological mediator. In further embodiments, the pathological mediator is involved in coagulation or fibrinolysis. In another embodiment, the pathological mediator is thrombin or plasmin. In a further embodiment, the pathological mediator is thrombin. Thus, for example, in one embodiment, the therapeutic pro-drug molecule is the thrombin
10 activatable low molecular weight single chain urokinase-like plasmin activator, described in the examples below. In another embodiment, the therapeutic pro-drug molecule is thrombin-activatable thrombomodulin, or thrombin-activatable tPA (or its mouse analog, mRNK-T).

In other embodiments, the pathological mediator is a pro-inflammatory mediator, such as a pro-inflammatory cytokine (IL-1- α , IL-1- β , IL-6, TNF- α , TGF- β , LIF, IFN- γ , OSM, CNTF, GM-CSF, IL-8, IL-11, IL-12, IL-17, or IL-18). In other embodiments, the pro-
15 inflammatory mediator is a complement protein or prostaglandin. Still other pro-inflammatory mediators are known in the art and may be used.

C. The Linker Between the scFv and Anti-Thrombotic Agent, Anti-Inflammatory 20 Agent, or Pro-Drug Thereof

The fusion protein as described herein is prepared by linking (fusing) the above-described scFv capable of binding a determinant expressed on the surface of a red blood cell to the above-described anti-thrombotic agent, anti-inflammatory agent, or pro-drug molecule. Moreover, genetic engineering allows the design and synthesis of targeted pro-drugs which can
25 be cleaved by pathophysiologically relevant enzymes that are generated at the site of disease that cannot be attained using chemical conjugation.

As noted above, linkers may also be utilized to join variable heavy and variable light chain fragments. A linker as used herein refers to a chain of as short as about 1 amino acid to as long as about 100 amino acids, or longer. In a further embodiment, the linker is 3, 4, 5, 6, 7,
30 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids in length. In one embodiment, the linker is 13 amino acids in length.

In one embodiment, the linker is -Ser-Ser-Ser-Ser-Gly-Ser-Ser-Ser-Ser-Gly-Ala-Ala-Ala-, *i.e.*, "(S₄G)₂AAA" (SEQ ID NO: 1). In another embodiment, the linker is (G₄S)₃, *i.e.*, -Gly-Gly-Gly-Gly-S-Gly-Gly-Gly-Gly-S-Gly-Gly-Gly-Gly-S- (SEQ ID NO: 2)(see Böldicke, *et*

al. (Stem Cells 2001, 19:24-36)). However, as will be understood by one of skill in the art, other linkers can be utilized.

Further, a cleavage sequence, such as the thrombin-sensitive cleavage sequence or other enzyme cleavage sequence, can be inserted in the linker to provide for release of the drug when the RBC to which it is targeted encounters the appropriate cleaving enzyme at the site of the pathological condition, *e.g.*, upon active thrombosis. This cleavage sequence may be located within a linker or at a terminus thereof. In one embodiment, a thrombin cleavage site -Met-Tyr-Pro-Arg-Gly-Asn- (SEQ ID NO: 3) may be inserted in, or appended to, the linker between the scFv and the therapeutic molecule or pro-drug. In another embodiment, the thrombin cleavage site is Pro-Arg. In still a further embodiment, lack of the native Phe-Lys plasmin cleavage site prevents single chain (sc) uPA activation (into fully active two-chain plasminogen activator (tcuPA)) via plasmin.

In another embodiment, antibody-derived scFv with a thrombin releasing site can be cloned by an upstream primer, which anneals to the carboxy terminus and introduces the sequence including a short peptide linker with the thrombin cleavage site. In still another embodiment, the cleavage site is internal to the pro-drug itself.

II. METHODS OF PREPARATION AND SPECIFIC EMBODIMENTS

The sequences, proteins, and fragments of the fusion proteins described herein may be produced by any suitable means, including recombinant production, chemical synthesis, or other synthetic means. Suitable production techniques are well known to those of skill in the art. See, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (Cold Spring Harbor, NY). Alternatively, peptides can also be synthesized by the well known solid phase peptide synthesis methods (Merrifield, *J. Am. Chem. Soc.*, 85:2149 (1962); Stewart and Young, *Solid Phase Peptide Synthesis* (Freeman, San Francisco, 1969) pp. 27-62). Polymerase chain reaction (PCR) and related techniques are described in Derbyshire, *et al.* (*Immunochemistry 1: A practical approach*. M. Turner, A. Johnston eds., Oxford University Press 1997, *e.g.*, at pp. 239-273). Plasmids useful herein have been described in Derbyshire, *et al.* (cited above), as well as Gottstein, *et al.* (*Biotechniques* 30: 190-200, 2001). Cloning techniques are also described in these and other suitable production methods are within the knowledge of those of skill in the art and are not a limitation of the compositions and methods described herein. Generation of recombinant proteins provides flexibility in design, rapid production, large-scale production and uniform composition.

III. PHARMACEUTICAL COMPOSITIONS AND METHODS OF ADMINISTRATION

Pharmaceutical compositions containing a fusion protein described herein and a pharmaceutically acceptable carrier or vehicle as described herein are useful for the treatment of a variety of diseases and disorders, depending upon the selection and identity of the anti-thrombosis agent, anti-inflammatory agent, or pro-drug, and the optional enzymatic cleavage site of the fusion protein. In one embodiment, a composition comprises a pharmaceutically acceptable vehicle for intravenous administration. In another embodiment, a composition comprises a pharmaceutically acceptable vehicle for administration via other vascular routes, including but not limited to, intra-arterial and intra-ventricular administration, as well as routes providing slower delivery of drugs to the bloodstream such as intramuscular administration to an animal in need thereof. As used herein, the terms "animal" and "patient" include any mammal. In a further embodiment, the terms "animal" and "patient" refer to a human.

In one embodiment, the fusion proteins described herein may be delivered intraperitoneally. Stable and prolonged loading of fusion protein on red blood cells is demonstrated in Example 14C.

Pharmaceutically acceptable vehicles/carriers include any of those conventionally used in the art, *e.g.*, saline, phosphate buffered saline (PBS), or other liquid sterile vehicles accepted for intravenous injections in clinical practice. Pharmaceutical compositions may also include buffers, pH adjusting agents, and other additives conventionally used in medicine. In one embodiment, compositions described herein are administered systemically as a bolus intravenous injection of a single therapeutic dose of the fusion protein. In a further embodiment, the dose is 0.1-5.0 mg/kg. In another embodiment, the dose is 0.01-0.5 mg/kg.

In other embodiments, methods of delivering an anti-thrombotic agent to the surface of a red blood cell are provided comprising delivering a fusion protein as described herein, or a pharmaceutical composition described herein, to a blood vessel. In one embodiment, fusion proteins are administered via a systemic intravascular route, *e.g.*, a vascular catheter. In some embodiments, rapid targeting of an organ or system may be accomplished by delivery via coronary artery (*e.g.*, for prophylaxis of acute myocardial infarction (AMI)) or the cerebral artery (*e.g.*, for prophylaxis of stroke and other cerebrovascular thrombotic events). Further, the fusion proteins described herein may be administered prophylactically, *i.e.*, in patients predisposed to thrombosis. In a further embodiment, the fusions proteins may be administered to an organ donor, utilized with an isolated organ transplant (*e.g.*, via perfusion), or used with vascular stents.

Intravenous administration of these fusion proteins results in: i) rapid binding of the drug to circulating RBC without altering its biocompatibility; ii) drastically prolonged circulation of

the drug as a blood cell-bound complex, iii) limited drug penetration into the blood vessel or surrounding tissues, minimizing attendant side effects and, iv) unique features of local activation in the site of pathology. In one embodiment, at least 10% of a fusion protein described herein is maintained on the surface of a red blood cell *in vivo* for at least 24 to 48 hours following administration. In another embodiment at least 20% of a fusion protein described herein is maintained on the surface of a red blood cell *in vivo* for at least 24 to 48 hours following administration. In another embodiment at least 30% of a fusion protein described herein is maintained on the surface of a red blood cell *in vivo* for at least 24 to 48 hours following administration. In another embodiment at least 40% of a fusion protein described herein is maintained on the surface of a red blood cell *in vivo* for at least 24 to 48 hours following administration. In another embodiment at least 50% of a fusion protein described herein is maintained on the surface of a red blood cell *in vivo* for at least 24 to 48 hours following administration. In another embodiment at least 60% of a fusion protein described herein is maintained on the surface of a red blood cell *in vivo* for at least 24 to 48 hours following administration. In another embodiment at least 70% of a fusion protein described herein is maintained on the surface of a red blood cell *in vivo* for at least 24 to 48 hours following administration.

Thus, in one embodiment, methods of treating or preventing a cardiovascular disorder, such as thrombosis, tissue ischemia, AMI, ischemic stroke, pulmonary embolism, sepsis, acute lung injury (ALI) or other type of vascular inflammation, or ischemic peripheral vascular disease, involves administering a fusion protein as described herein, or a pharmaceutical composition as described herein, to a blood vessel in a mammal in need thereof. In such disorders, the anti-thrombotic or anti-inflammatory agent and its dosage in delivery (*i.e.*, the amount fused to an individual RBC may be selected and adjusted by an attending physician with regard to the nature of the disorder, the physical condition of the patient, and other such factors). The selection of the cleavage site in the fusion protein may also be selected to match the disorder, *e.g.*, a thrombin cleavage site suitable for most cardiovascular disorders. Loading red blood cells (RBC) *in vivo* with anti-thrombotic agents (ATAs) constitutes a new approach to thromboprophylaxis that holds promise for improving the management of patients at high risk of thrombosis for a defined period of time in whom anticoagulation poses an unacceptable risk. Delivery of plasminogen activators (PAs) and thrombomodulin (TM) via RBCs markedly prolongs intravascular lifespan and restricts vascular and tissue damage.

In one embodiment, the compositions described herein are effective in the treatment or prevention of cerebrovascular thrombi. In a further embodiment, the compositions described

herein are effective in the treatment or prevention of cerebrovascular disease, such as transient ischemic attack and stroke.

Similarly, in another embodiment, methods of treating or preventing disseminated intravascular coagulation (DIC), sepsis, acute lung injury (ALI/ARDS), aseptic systemic inflammation, and other inflammatory conditions are provided by administering the appropriately designed fusion protein, according to the teachings of this specification.

In the same manner, novel fusion proteins containing a selected pro-drug can be designed according to the teachings herein and employed to treat a variety of disorders in addition to those identified above.

Also provided is the use of a fusion protein or a pharmaceutical composition as described herein as a medicament. The use of a fusion protein or a pharmaceutical composition as described herein to treat any of the above conditions.

IV. EXAMPLES

The examples that follow do not limit the scope of the embodiments described herein. One skilled in the art will appreciate that modifications can be made in the following examples which are intended to be encompassed by the spirit and scope of the invention.

EXAMPLE 1 - GENERATION OF A SINGLE CHAIN ANTIGEN-BINDING DOMAIN (scFv) MOLECULE

Generation of a single chain antigen-binding domain (scFv) molecule is illustrated in Fig. 1. scFvs are generated in accordance with the teachings herein, as well as those of Spitzer, *et al.* (Mol. Immunol. 2003, 40:911-919). Total RNA of a hybridoma cell line is isolated (*e.g.*, by RNeasy, Qiagen, Velencia, CA). RNA is reverse transcribed, *e.g.*, using the SMART™ technology (Clontech, Palo Alto, CA) employing known primers (*e.g.*, those of Dübel, *et al.* (J. Immunol. Methods 1994, 175:89-95)). The resulting heavy (V_H) and light (V_L) chain variable cDNA fragments are subcloned into a suitable plasmid, *e.g.*, the pCR®2.1-TOPO® (Invitrogen, Carlsbad, CA). Plasmids are then transfected into E. coli. V_H and V_L chains are then isolated by conventional techniques, *e.g.*, agarose gel column or gel electrophoresis.

V_H and V_L chains are combined with a suitable linker, *e.g.*, a $(G_4S)_3$ linker (SEQ ID NO: 2)(Böldicke, *et al.* (Stem Cells 2001, 19:24-36)) resulting in the desired scFv. PCR-derived sequences are verified by DNA sequencing. The amino acid primary sequence may be analyzed to determine complementarity determining regions (CDRs) by application of the rules described at: Antibody Structure and Sequence Information V2.0 (<http://www.rubic.rdg.ac.uk>).

EXAMPLE 2 - GENERATION OF A PRO-DRUG SCFV FUSION PROTEIN

An scFv (RBC epitope binding) is prepared as described in Example 1. A thrombin cleavage site (PR, *i.e.*, Pro-Arg) is cloned into a (S₄G)₂AAA (SEQ ID NO: 1) linker peptide (internally, or at its N- or C-terminus), and the resulting peptide is used to link the V_L domain of the scFv to the low molecular weight single chain urokinase-like plasmin activator. The thrombin cleavage site (PR) cloned in the linker peptide allows the release of single chain urokinase-like plasmin activator from RBC/fusion protein complex during clot formation.

EXAMPLE 3 - GENERATION OF A NON-RELEASABLE SCFV-PLASMINOGEN ACTIVATOR (UPA) FUSION PROTEIN

Recombinant anti-Ter119scFv [anti-GPA (glycophorin A associated protein)] was produced as described in Example 1. A plasmid (pcDNA3, (Dr. Atkinson, Washington University, St. Louis) encoding Ter119-scFv (SEQ ID NO: 4) was utilized. Primers were designed via conventional techniques, and two PCR amplifications were performed. The first amplification was of DNA encoding the scFv, adding DNA encoding the (S₄G)₂AAA (SEQ ID NO: 1) linker to its C terminus. The second amplification was of DNA encoding uPA (SEQ ID NO: 5). Both amplified segments were ligated together into the pMT/Bip/V5HisA plasmid (Invitrogen), with the Ter-119scFv portion N-terminal to the uPA sequence.

Ter119scFv-uPA (SEQ ID NO: 6) was expressed in pMT/Bip/V5HisA plasmid in *Drosophila* S2 cells (*Drosophila* DS2 expression System (Invitrogen)).

EXAMPLE 4 - MOUSE ANTI-GLYCOPHORIN A ASSOCIATED PROTEIN SCFV THROMBIN-RELEASABLE TER119-LMWUK-T (UPA-T)

A fusion protein was prepared according by the method of Examples 2 and 3 comprising an anti-glycophorin A associated protein (mouse RBC) scFv derived from parental rat MAb Ter119 as illustrated in Fig. 1, and thrombin activatable low molecular weight single chain urokinase-like plasmin activator (Ter119-lmwUK-T). Casein zymography indicated that fusion protein by itself had very low plasminogen activation capacity. Thrombin activation lead to a dramatic increase of plasminogen conversion activity. Binding capacity of the fusion protein was confirmed by capture of the fusion protein from the induced S2 cell serum free medium on RBC ghosts from different species. Only mouse RBC ghosts (membranes) were able to pull Ter119-lmwUK-T from the medium (lane 2), which confirmed the specificity of binding.

EXAMPLE 5 - GENERATION OF A NON-RELEASABLE SCFV-PLASMINOGEN ACTIVATOR (MRNK) FUSION PROTEIN

A ~70 kDa Ter119scFv/mRNK fusion protein was expressed in Drosophila S2 cells (Drosophila DS2 expression System, (Invitrogen)). Recombinant anti-Ter119scFv [anti-GPA (glycophorin A associated protein)] was produced as described in Example 1. A plasmid (pcDNA3, (Dr. Atkinson, Washington University, St. Louis) encoding Ter119-scFv (SEQ ID NO: 4) was utilized. Primers were designed via conventional techniques, and two PCR amplifications were performed. The first amplification was of DNA encoding the scFv, adding DNA encoding the (S₄G)₂AAA (SEQ ID NO: 1) linker to its C terminus. The second amplification was of DNA encoding mRNK (SEQ ID NO: 7). Both amplified segments were ligated together into the pMT/Bip/V5HisA plasmid (Invitrogen), with the Ter-119scFv portion N-terminal to the uPA sequence.

Ter119scFv-mRNK (SEQ ID NO: 8) was expressed in pMT/Bip/V5HisA plasmid in Drosophila S2 cells (Drosophila DS2 expression System (Invitrogen)). Expression of the Ter119-mRNK fusion protein was confirmed via Western Blot with anti-mRNK. mRNK control was run against the Ter119-mRNK protein, and predicted ~40 kDa and 70 kDa bands were identified, respectively.

EXAMPLE 6 - GENERATION OF AN EXPRESSED THROMBIN-RELEASABLE SCFV-MRNK FUSION PROTEIN

An scFv-mRNK is prepared as described in Example 5. However, a thrombin cleavage site (PR) is encoded within the (S₄G)₂AAA linker peptide (internally, or at its N- or C-terminus) that links the scFv to mRNK (*see* Example 2). The thrombin cleavage site cloned in the linker peptide allows the release of mRNK from the RBC/fusion protein complex during clot formation.

EXAMPLE 7 - GENERATION OF AN EXPRESSED SCFV-TM (THROMBOMODULIN) FUSION PROTEIN

Recombinant anti-Ter119scFv [anti-GPA (glycophorin A associated protein)] was produced as described in Example 1. A plasmid (pcDNA3 (Dr. Atkinson, Washington University, St. Louis)) encoding Ter119-scFv (SEQ ID NO: 4) was utilized. Primers were designed via conventional techniques, and two PCR amplifications were performed. The first amplification was of DNA encoding the scFv, adding DNA encoding the (S₄G)₂AAA (SEQ ID NO: 1) linker to its C terminus. The second amplification was of DNA encoding TM (thrombomodulin) (SEQ ID NO: 9). Both amplified segments were ligated together into the pMT/Bip/V5HisA plasmid (Invitrogen), with the Ter-119scFv portion N-terminal to the uPA sequence.

Ter119scFv-TM (SEQ ID NO: 10) was expressed in pMT/Bip/V5HisA plasmid in *Drosophila* S2 cells (*Drosophila* DS2 expression System (Invitrogen)).

EXAMPLE 8 - MOUSE ANTI-GLYCOPHORIN A ASSOCIATED PROTEIN SCFV THROMBIN-
5 RELEASABLE TER119-TM

An scFv-mRNK is prepared as described in Example 7. However, a thrombin cleavage site (PR) is encoded within the (S₄G)₂AAA linker peptide (internally, or at its N- or C-terminus) that links the scFv to TM (*see* Example 2). The thrombin cleavage site cloned in the linker peptide allows the release of mRNK from the RBC/fusion protein complex during clot formation.

10 Mouse and human RBCs were incubated in serum free medium from induced and non-induced S2 cells transfected with a plasmid encoding Ter119-TM. RBC were washed and incubated with protein C in the presence or absence of thrombin. The experiment demonstrated that only mRBC loaded with Ter119-TM in the presence of thrombin cause protein C activation, thus confirming both determinant binding and functional activity of Ter119-TM.

15 EXAMPLE 9 - BINDING OF THE SCFV/ FUSION TO TER-119

A. Ter119scFv-mRNK was prepared according to Example 5. mRNK was radiolabeled with ¹²⁵I-Na (Perkin Elmer, Wellesley, MA) using Iodogen (Pierce, Rockford, IL). Mouse and human red blood cells (mRBC and hRBC, suspended to 10% hematocrit) were loaded
20 at the same concentration with Ter119scFv-mRNK by incubation for one hour at 37°C. Unbound ligand was removed via centrifugation with PBS-BSA (phosphate buffered saline - bovine serum albumin) and RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

Specific binding to mRBC was revealed (~20,000 molecules/mRBC vs. ~120 molecules/hRBC).

25 B. Ter119scFv-TM fusion protein was prepared according to Example 7.

1. Agglutination (aggregation) Assay

Mouse and human RBCs were washed in PBS/3%BSA buffer, resuspended in the same buffer to 10% hematocrit and incubated with Ter119-TM (final
30 concentration 40 mg/ μ l) for 1h. Binding experiments show that such conditions of incubation provide loading of mRBC with ~20000 molecules of fusion protein/mRBC. Then cells were washed 3 times with PBS/BSA buffer and equimolar amount of mouse HMGB1 (produced in *E.coli* (Sigma)) was added to the assay for 30 min at room temperature. Then RBC were washed with PBS/BSA and brought to the final hematocrit of 1% and the monoclonal Ab to mouse

HMGB1 (Sigma, dilution as per manufacturer) was added to the assay placed into a V-shaped plate (total volume – 100µl). After 40 min the V-shaped plate aggregation picture was documented by photography: only mRBC loaded with Ter119-TM and incubated with HMGB1 and appropriate ABs showed the tendency for aggregation thus confirming the late cytokine (HMGB1) scavenging capacity of Ter119-TM fusion protein in RBC-bound state.

Only Ter119scFv-TM loaded RBCs incubated with HMGB1 and then polyclonal anti-HMGB1 antibody aggregated. This result confirms that the thrombomodulin domain of the scFv-TM fusion binds the pro-inflammatory mediator HMGB1.

2. Western Blot confirmed Ter119scFv-TM's binding to HMGB1, as well as Ter119's specificity for mouse red blood cells. Mouse and human RBCs isolated from the fresh heparinated blood were washed with PBS/BSA buffer as described above and brought to the final 10% hematocrit. Both types of cells were incubated in the medium from Drosophila S2 cells expressing Ter119-TM for 1h (the protein production was confirmed by WB (data not shown), then washed 3 times with PBS/BSA and incubated with equimolar concentration of HMGB1 (E.coli produced protein, Sigma) for 1h at room temperature. Then cells were washed 3 times with PBS and lysed in isotonic buffer in order to obtain RBC ghosts. RBC ghosts were washed 3 times by centrifugation in 5mM phosphate buffer to remove the hemoglobin and finally lysed in a lysis buffer. The lysates from experimental mRBC and hRBC as well as the lysates from intact mRBC and hRBC in equal amount of total loaded protein were run on SDS-PAGE and transferred to Nitrocellulose membrane for WB analyses (The sample of recombinant HMGB1 (Sigma) served as a positive control). The membrane was probed with the monoclonal Abs to HMGB1 followed with the detection with HRP-conjugated anti-mouse-mAbs. The experiment clearly shows that only mRBCs loaded with Ter119-TM were able to pull HMGB1 from the experimental essay.

EXAMPLE 10 - ACTIVITY OF EXPRESSED SCFV-TM (THROMBOMODULIN) FUSION PROTEIN

A Ter119scFv-TM (SEQ ID NO: 10) was prepared according to Example 7. Mouse and human red blood cells (mRBC and hRBC, suspended to 10% hematocrit) were loaded with Ter119scFv-TM by incubation for one hour at 37°C. Unbound ligand was removed via centrifugation with PBS-BSA (phosphate buffered saline - bovine serum albumin). Loaded and intact (non-loaded) RBC were incubated with thrombin and protein C. Activation of protein C was measured by spectrazyme assay at 4450.

Only mRBC incubated with Ter119-TM caused activation of protein C in the presence of thrombin. This result confirms the binding specificity of Ter119-TM and the functional activity of Ter119-TM when bound to the red blood cell.

5 EXAMPLE 11 - EFFECT OF CONCENTRATION (AT VARYING HEMATOCRIT PERCENTAGES) ON SCFV BINDING

 A. Ter119-mRNK (prepared according to Example 5) or Ter119-lmwUK-T (prepared according to Example 4) ¹²⁵I labeled fusion proteins (*see* Example 9 for labeling method) were bound to mouse red blood cells (mRBCs) at 10% hematocrit [Figs. 4A (mRNK) and 10 4B (lmwUK-T)] and 1% hematocrit [Figs. 4C (mRNK) and 4D (lmwUK-T)]. Fig. 4C reflects the negative control (free mRNK).

 Approximate B_{max} (~80,000 molecules/RBC) and K_d (~77.9 nM) was calculated from the data reflected in Fig. 4C (mRNK, 1% hematocrit). This reflects high-affinity, high capacity binding to RBC. More accurate B_{max} and K_d values may be obtained with more diluted 15 suspensions (lower hematocrit), but would be less physiologically relevant, given normal blood hematocrit levels in humans.

 B. Using the same protocol as 'A' (above), Ter119scFv-TM (prepared according to Example 7) was tested. The resulting data corresponds to that reflected in Fig. 4. 20

EXAMPLE 12 - KINETICS OF SCFV/MRNK BINDING TO RBC

 Ter119scFv-mRNK was prepared according to Example 5, Freshly isolated mRBCs were resuspended in PBS/BSA or previously obtained heparinated mouse plasma at equal 25 physiological hematocrit (determined previously, ~50%) and incubated with 40µg/ml of ¹²⁵I-labeled Ter119-TM at 37°C. At the indicated time points the aliquots of the RBC suspension were taken, washed 3 times with PBS/BSA and the residual radioactivity in the pellet indicating the extent of Ter119-TM binding was determined in a γ-counter (Perkin Elmer).

 Both whole and heparinized blood yielded similar binding, with a 50% saturation time of 30 30 minutes. Results are shown in Figure 6.

EXAMPLE 13 - BIODISTRIBUTION OF SCFV-mRNK *IN VIVO*

A. Tracer doses (2-5 μ g) of ^{125} I radiolabeled fusion protein (prepared as indicated in Example 9) or mRNK were injected intravenously in wild type (WT) mice. One hour later, tissue uptake was determined. RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

Figs. 5A and 5B are bar graphs showing the biodistribution of Ter119-mRNK *in vivo*. Ter119-mRNK, but not mRNK, showed preferential accumulation in blood (Fig. 5A). More than 90% of Ter119-mRNK is associated with RBC at 30 min of circulation (Fig. 5B). Parallel levels of mRNK to Ter119-mRNK (Fig. 5A) within organs reflects that targeting to RBCs does not alter non-specific metabolism of the fusion.

B. Double-label tracing data was obtained parallel to the method described in section 'A' above. ^{51} Cr labeled RBCs loaded with Ter119scFv-mRNK and RBCs loaded with Ter119scFv-mRNK- ^{125} I were prepared. The biodistribution and percentage of RBC-bound fusion was comparable to that noted in 'A', above at one hour circulation. Similar levels of iodine and chromium isotopes in blood and of the organs reflect only minor detachment of the fusion from preloaded red blood cells (90% and 75% ID at one and three hours, respectively, of iodinated fusion in the bloodstream is associated with RBCs).

Minor reticuloendothelial system uptake of the fusion in the liver reflects a minor fraction of the fusion detached from the RBC. No uptake in the spleen was noted, reflecting no harm to RBCs. Further, no aggregation of the loaded RBCs is noted based on lack of lung clearance (within pulmonary capillaries).

Tracing of pre-loaded ^{51} Cr-labeled complexes reflects a major fraction of injected fusion protein *in vivo* binds to RBCs stably and rapidly, with unbound or reversibly bound material taken up by the liver parallel to untargeted mRNK.

C. Long-Term Biodistribution of Ter119(anti-GPA)-mRNK *in vivo*

Tracer doses (2-5 μ g) of ^{125} I radiolabeled fusion protein (prepared as indicated in Example 11) were injected intravenously in wild type (WT) mice. At time points 1h, 3h, 6h, 24h, and 48h, tissue uptake was determined (Figure 7). At time points 0.5h, 1h, 3h, 6h, 24h, and 48h, blood components distribution was determined (Figure 8). RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

The resulting data reflects that the fusion circulates for several hours, and that a fraction of the fusion detaches from RBC within hours after injection.

EXAMPLE 14 - BIODISTRIBUTION OF SCFV-TM *IN VIVO*

A. ^{51}Cr labeled RBCs loaded with Ter119scFv-TM (prepared according to Example 7) and RBCs loaded with Ter119scFv-TM- ^{125}I were prepared and injected intravenously in wild type (WT) mice. At one hour, three hours, and six hours of circulation, tissue uptake was determined. RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

Figure 9 shows a lack of accumulation of loaded RBCs in the lungs, reflecting the absence of aggregation, and lack of accumulation in the spleen reflects the absence of damage to the RBCs. Figure 10 reflects no significant detachment of fusion from RBCs within 6 hours.

B. Long-Term Biodistribution of scFv-TM *in vivo*

RBCs loaded with Ter119scFv-TM- ^{125}I (prepared according to Example 7; see Example 9 for labeling method) were injected intravenously in wild type (WT) mice. At one-half hour, one hour, three hours, six hours, and twenty-four hours of circulation, tissue uptake was determined. RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

Figure 11 shows a lack of accumulation of loaded RBCs in the lungs, reflecting the absence of aggregation, and lack of accumulation in the spleen reflects the absence of damage to the RBCs. Figure 12 reflects no significant detachment of fusion from RBCs within 6 hours.

C. Stable and Prolonged Loading via Intraperitoneal Delivery

RBCs loaded with Ter119scFv-TM- ^{125}I (prepared according to Example 7) were injected intraperitoneal (IP) in wild type (WT) mice. At one hour and three hours of circulation, tissue uptake was determined. RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

Figures 13 and 14 reflect that intraperitoneal (IP) delivery offers stable and prolonged loading of RBCs. IP delivery provides for chronic and repetitive use.

EXAMPLE 15 - BIODISTRIBUTION OF SCFV-UPA-T *IN VIVO*

A. ^{51}Cr labeled RBCs loaded with Ter119scFv-uPA-T (prepared according to Example 4) were prepared and injected intravenously in wild type (WT) mice. At one hour, three hours and six hours of circulation, tissue uptake was determined. RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

The resulting data shows a lack of accumulation of loaded RBCs in the lungs, reflecting the absence of aggregation, and lack of accumulation in the spleen reflects the absence

of damage to the RBCs. The data also reflects that there was no significant detachment of fusion from RBCs within 6 hours.

B. Long-Term Biodistribution of Ter119-uPA-T (IUK-T) *in vivo*

RBCs loaded with Ter119scFv-uPA-T-¹²⁵I (*see* Example 9 for labeling method) were injected intravenously in wild type (WT) mice. At time points through 48 hours of circulation, tissue uptake was determined. RBC-bound radioactivity was measured in a γ -counter (Perkin Elmer).

Figure 15 shows circulation of the fusion protein in blood during the 48 hours after injection. Figures 16 and 17 reflect organ distribution of free uPA (IUK) and the fusion through 48 hours after injection. Figure 18 reflects the distribution of the fusion protein between blood plasma and cells through 48 hours after injection. Non-targeted protein is IUK (uPA).

The Ter119scFv-uPA fusion bound to RBCs circulates longer than the scFv-mRNK fusion bound to RBCs. While not wishing to be bound by theory, one rational for this difference may be higher affinity of the uPA fusion due to protein folding (enhanced binding properties). Another rational is that given the RBC-bound scFv-uPA-T pro-drug does not bind PAI-1 and other inhibitors until thrombin releases uPA from the fusion. PAI-1, which is known to accelerate clearance of plasminogen activators from blood via hepatic receptors, may more rapidly clear the constitutively active Ter119scFv-mRNK fusion.

EXAMPLE 16 - FIBRIN CLOT LYSIS BY MOUSE AND HUMAN LOADED RED BLOOD CELLS

Fusion proteins were prepared comprising an anti-glycophorin A associated protein (mouse RBC) scFv derived from parental rat mAb Ter119 as described in Example 4, and kringle 2 and protease domains of mouse tPA. Moreover, the protease domain contains point mutations similar to those in human tenectase making it more resistant to inhibition by PAI-1.

A. Mouse and human RBCs were incubated in serum free medium from induced *Drosophila* S2 cells transfected with a plasmid encoding either Ter119-lmwUK-T (prepared according to Example 4) or Ter119-mRNK (SEQ ID NO: 8)(prepared according to Example 5). RBCs were washed and incorporated into fibrin solution before induction of clotting by addition of calcium and thrombin. RBCs loaded with Ter119-lmvUK-T were pre-incubated with thrombin in order to activate the fusion protein. The experiment confirmed the high selectivity of binding of both fusion proteins to mouse RBC vs. human RBC and their proteolytic activity in the RBC surface bound state (Fig. 3). Moreover, it showed that even short time exposure of Ter119-

lmwUK-T to thrombin during clotting is enough to activate the fusion protein. Human and mouse RBC loaded with either Ter119-lmwUK-T (activated (act) and non-activated) or Ter119-mRNK were injected into preformed fibrin clots (10 μ L of 50% Hc RBC suspension). Again, only mRBC caused lysis and in the case of Ter119-lmwUK-T the necessity of thrombin activation of fusion protein was confirmed (Fig. 3).

B. Mouse RBCs were incubated in serum free medium from induced *Drosophila* S2 cells transfected with a plasmid encoding scFv-uPA-T (prepared according to Example 4). RBCs were washed, pre-incubated with thrombin in order to activate the fusion protein, washed, and incorporated into fibrin solution. Clotting was induced by addition of calcium and thrombin, and allowed to incubate for 5 or 24 hours.

Figure 19 reflects clot lysis through 48 hours after clot incubation. Maximal fibrinolytic effect was noted from hour 1 through 24 hours after injection into the fibrin clot. Based on these results, the construct is suited to prolonged thromboprophylaxis.

EXAMPLE 17 - KINETICS OF FIBRIN CLOT LYSIS BY SCFV-MRNK DOSE

Ter119 scFv-mRNK was prepared according to Example 5. mRBCs were loaded to 17,000 mol/RBC, and RBCs were washed to remove unbound fusion. 2, 5, and 10 μ L of 50% RBC suspension were added to a fibrinogen solution prior to clotting. The resulting RBC bound mRNK concentrations were 1.5 nM, 3.74 nM, and 7.48 nM, respectively. Resulting 50% fibrinolysis time was 130, 45, and 15 minutes, respectively, which is similar to 60 minutes obtained with 5 nM tPA (positive control). Figure 20 reflects these results in graphical form.

EXAMPLE 18 - EX VIVO FIBRINOLYSIS BY INJECTED SCFV-MRNK

Ter119scFv-mRNK was prepared according to Example 5. PBS (control), non-targeted mRNK, or the fusion was injected into mice via jugular vein at equimolar doses. Blood was collected from animals 45 minutes post-injection (without anticoagulant), and blood clots were formed with addition of tracer dose of radioactive fibrinogen. Clots were matured for 20 minutes, covered with PBS, and brought to 37°C. Probes of supernatants were used to detect radioactivity, which indicated dissolution of blood clots.

The resulting data reflects that following 45 minutes circulation, the scFv-mRNK retains its ability to activate plasminogen, which was reflected by clot lysis. Figure 21 reflects the results in graphical form. RBC-bound scFv-mRNK is functionally active for significantly longer than non-targeted mRNK.

EXAMPLE 19 - KINETICS OF SCFV-uPA-T ACTIVATION (CLEAVAGE) BY THROMBIN

Ter119scFv-uPA-T was prepared according to Example 4.

5 A. Anti-GPA-scFv/uPA-T (Ter119-IUK-T) was brought to final concentration of 150nM in PBS containing Ca^{2+} and incubated with 25nM thrombin for indicated times in order to test the kinetic of anti-GPA-scFv/uPA-T activation (conversion into 2 chain form). At the indicated time points the reaction was stopped by addition of hirudin (final concentration in the assay 33U/ml). The activity of anti-GPA-scFv/uPA-T was tested by Spectrozyme-uPA Kit
10 (American diagnostica) as per manufacturer. The results are reflected in Fig. 22.

 B. Using the protocol of 'A', above, near rate-limiting doses of scFv-uPA-T RBC-bound fusion (2 - 4 nM), the RBC-bound fusion caused significantly faster and more effective fibrinolysis of clots formed from human platelet poor plasma (PPP), as reflected in Figure 23. Without wishing to be bound by theory, it is believed that this effect is due to the masking of
15 plasma inhibitors including PAI-1 by the RBC glycocalyx. Further, as reflected in Figure 23, at concentrations exceeding PAI-1's capacity to inhibit plasminogen activators via irreversible binding, the free fusion was slightly more effective than RBC-bound fusion. This is believed to be due to a less impeded diffusion into the fibrin network.

20 EXAMPLE 20 - IN VIVO THROMBOLYSIS IN A MODEL OF INTRAVASCULAR THROMBOLYSIS IN MICE - SCFV-uPA-T

 The method of dissolution of carotid arterial thrombi according to J. Murciano, *Nature Biotechnology*, 21(8): 891-896, 895 (August 2003) was used. Ter119scFv-uPA-T fusion (prepared according to Example 4), PBS, and uPA (equimolar) were administered via injection
25 via jugular vein. 30 minutes after administration, acute vascular trauma is induced by administration of 15% FeCl_3 (2 minutes flow).

 Injection of non-targeted uPA did not affect rapid thrombotic occlusion of the artery comparing with mice pre-treated with placebo (PBS). On average, in animals from PBS or uPA, a complete arterial occlusion occurred within ten minutes of vessel damage and no significant
30 reperfusion occurred in the ensuing 45 minutes after insult. In contrast, pre-injection of the fusion provided either complete prevention of occlusion, or rapid reperfusion after a brief period of intermittent reduction of perfusion.

 These data indicate that the scFv-uPA-T fusion provides thromboprophylaxis in animal models.

EXAMPLE 21- IN VIVO THROMBOLYSIS IN A MODEL OF INTRAVASCULAR THROMBOLYSIS IN MICE - scFv-mRNK

The method of dissolution of carotid arterial thrombi according to J. Murciano, *et al.*,
5 *Nature Biotechnology*, 21(8): 891-896, 895 (August 2003) was used. Ter119scFv-mRNK fusion (prepared according to Example 5), PBS, and mRNK (equimolar - 4 mg/kg scFv/mRNK vs. 2 mg/kg mRNK) were administered via injection via jugular vein. 30 minutes after administration, acute vascular trauma is induced by administration of 15% FeCl₃ (2 minutes flow).

Injection of non-targeted RNK did not affect rapid thrombotic occlusion of the artery
10 comparing with mice pre-treated with placebo (PBS). mRNK did not attenuate nor delay artery occlusion (defined as complete cessation of blood perfusion by Doppler ultrasound). However, the equimolar dose of scFv-mRNK fusion significantly delayed occlusion (~11 minutes vs. ~ 6 minutes)

The data (Figure 24) indicates that the scFv-mRNK fusion provides thromboprophylaxis
15 in animal models.

EXAMPLE 22 - PROPHYLACTIC THROMBOLYSIS OF CEREBROVASCULAR THROMBI

The protocol of K. Danielyan, *et al.*, *J. Pharm. and Exp. Therapeutics*, 321(3): 947-952, 948 (June 2007) is used.

20 Ter119scFv-mRNK fusion (prepared according to Example 5), mRNK alone (equimolar), and PBS, are administered in a standard 120-μL volume of PBS via catheter inserted into the right femoral vein of anesthetized mice. 30 minutes after administration, a suspension of ¹²⁵I-fibrin emboli is injected via the right middle cerebral artery. 30 minutes later, mice are sacrificed and ¹²⁵I content of the brain are measured to determine the extent of cerebrovascular thrombolysis
25 based on the amount of residually radiolabeled clots residing in the brain.

Residual activity for scFv-mRNK fusion mice is anticipated to be significantly lower than that for PBS mice alone. scFv-mRNK fusion is thus anticipated to provide thromboprophylaxis of cerebrovascular thrombi in animal models.

30 EXAMPLE 23 - BINDING OF SCFV-MRNK FUSION TO GLYCOPHORIN A

A. Generation of human anti-glycophorin A (hGPA) scFv

The scFv with specificity for hGPA is generated essentially as described (Spitzer, *et al.*, *Mol. Immunol.*, 40:911-919 (2004)) from the mouse hybridoma cell line BRIC 256 (Anstee, *et al.*, *Eur. J. Immunol.*, 12:228-232 (1982). This line secretes an IgG1 mAb that recognizes a

blood group-independent epitope on human GPA (Gardner, *et al*, *Immunology*, 68:283-289 (1989)). Total RNA is isolated (RNeasy™; Qiagen). Reverse transcription, followed by PCR (RT-PCR), is conducted using the SMART™ technology (BD Clontech) using primer combinations described previously (Dubel, *et al.*, *J. Immunol. Methods*, 175:89-95 (1994)). The
5 resulting H and L chain variable cDNA fragments are subcloned into pCR2.1-TOPO (Invitrogen Life Technologies). After introducing suitable flanking restriction sites via PCR, the V_H and V_L chains are combined with a (G₄S)₃ linker resulting in the scFv Bric-256. Analysis of the amino acid primary sequence to determine the complementarity determining regions of the scFv Bric-256 is performed by applying the rules described at Ab Structure and Sequence Information version
10 2.0 (www.rubic.rdg.ac.uk).

B. Generation of the human anti-hGPAscFv-mRNK fusion

A restriction fragment containing the scFv Bric-256 sequence described in 'A', above, is inserted into the plasmid described in Example 14 (in place of the sequence encoding
15 Ter119 scFv). The remaining techniques of Example 14 are described.

C. hGPAscFv-mRNK is prepared as described in 'B', above. mRNK is radiolabeled with ¹²⁵I-Na (Perkin Elmer, Wellesley, MA) using Iodogen (Pierce, Rockford, IL). Mouse and human red blood cells (mRBC and hRBC, suspended to 10% hematocrit) are loaded at the same
20 concentration with hGPA-mRNK by incubation for one hour at 37°C. Unbound ligand is removed via centrifugation with PBS-BSA (phosphate buffered saline - bovine serum albumin) and RBC-bound radioactivity is measured in a γ-counter (Perkin Elmer).

Specific binding to hRBC is revealed vs. mRBC.

All documents listed in this specification are incorporated herein by reference. While the invention has been described with reference to specific embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A fusion protein comprising a single chain antigen-binding domain (scFv) linked to an anti-thrombotic agent or anti-inflammatory agent, wherein said scFv binds to a determinant expressed on the surface of a red blood cell at a density greater than 5,000 copies per red blood cell.
2. A fusion protein comprising a single chain antigen-binding domain (scFv) linked to a pro-drug of an anti-thrombotic agent or anti-inflammatory agent, wherein said scFv binds to a determinant expressed on the surface of a red blood cell at a density greater than 5,000 copies per red blood cell.
3. The fusion protein according to claim 2, wherein the pro-drug is an anti-thrombotic agent.
4. The fusion protein according to claim 2, wherein the pro-drug is an anti-inflammatory agent.
5. The fusion protein according to claim 1 or claim 2, wherein said determinant is expressed on the surface of a red blood cell at a density greater than 10,000 molecules per red blood cell.
6. The fusion protein according to claim 1 or claim 2, wherein said determinant is expressed on the surface of a red blood cell at a density greater than 50,000 molecules per red blood cell.
7. The fusion protein according to claim 1 or claim 2, wherein said determinant is expressed on the surface of a red blood cell at a density greater than 500,000 molecules per red blood cell.
8. The fusion protein according to claim 1 or claim 2, wherein said determinant is expressed on the surface of a red blood cell at a density greater than 2,000,000 molecules per red blood cell.

9. A fusion protein comprising a single chain antigen-binding domain (scFv) linked to an anti-thrombotic agent or anti-inflammatory agent, wherein said scFv binds to a determinant expressed on the surface of a red blood cell, and said determinant is not a specific site for recognition by host defense cells that clear microscopic objects from the surface of a circulating red blood cell without damage to the red blood cell.

10. A fusion protein comprising a single chain antigen-binding domain (scFv) linked to a pro-drug of an anti-thrombotic agent or anti-inflammatory agent, wherein said scFv binds to a determinant expressed on the surface of a red blood cell, and said determinant is not a specific site for recognition by host defense cells that clear microscopic objects from the surface of a circulating red blood cell without damage to the red blood cell.

11. A fusion protein comprising a scFv linked to an anti-thrombotic agent or anti-inflammatory agent, wherein at least 10% of the fusion protein injected into the bloodstream is maintained on the surface of a red blood cell in vivo for at least 48 hours.

12. A fusion protein comprising a scFv linked to a pro-drug of an anti-thrombotic or anti-inflammatory agent, wherein at least 10% of the fusion protein injected into the bloodstream is maintained on the surface of a red blood cell in vivo for at least 48 hours.

13. The fusion protein according to claim 11 or claim 12, wherein at least 30% of the fusion protein injected into the bloodstream is maintained on the surface of a red blood cell in vivo for at least 48 hours.

14. The fusion protein according to claim 11 or claim 12, wherein at least 50% of the fusion protein injected into the bloodstream is maintained on the surface of a red blood cell in vivo for at least 48 hours.

15. The fusion protein according to claim 11 or claim 12, wherein at least 70% of the fusion protein injected into the bloodstream is maintained on the surface of a red blood cell in vivo for at least 48 hours.

16. The fusion protein according to any of claims 1-15, wherein said determinant is a glycophorin A associated protein (GPA).

17. The fusion protein according to any of claims 1-15, wherein said determinant is glycophorin A.
18. The fusion protein according to any of claims 1-15, wherein said determinant is an ABO blood group antigen.
19. The fusion protein according to any of claims 1-18, wherein the anti-thrombotic agent comprises a moiety presented by a protease domain of a plasminogen activator.
20. The fusion protein according to claim 19, wherein the plasminogen activator is tissue-type plasminogen activator (tPA), urokinase, tenectase, retavase, streptokinase, staphylokinase, or a plasminogen activator from venoms and saliva of bats, insects and other animals.
21. The fusion protein according to any of claims 1-18, wherein the anti-thrombotic agent comprises a moiety presented by an anti-coagulant.
22. The fusion protein according to any of claims 1-18, wherein the anti-thrombotic agent comprises a moiety presented by thrombomodulin or a domain thereof.
23. The fusion protein according to any of claims 1-18, wherein the anti-inflammatory agent comprises a moiety presented by thrombomodulin or a domain thereof.
24. The fusion protein according to any of claims 1-10, wherein the anti-inflammatory agent is a pro-drug.
25. The fusion protein according to any of claims 1-18, wherein the anti-thrombotic agent is protein C.
26. The fusion protein according to any of claims 1-10, wherein the anti-thrombotic agent is a pro-drug.

27. The fusion protein according to claim 26, wherein an anti-thrombotic agent, therapeutically-active site, domain, or moiety is released by cleavage of the anti-thrombotic agent pro-drug.
28. The fusion protein according to claim 26, wherein the anti-thrombotic agent pro-drug is activated by an enzyme, which level is locally elevated under pathological conditions.
29. The fusion protein according to claim 28, wherein the enzyme is a pathological mediator.
30. The fusion protein according to claim 28, wherein the enzyme is a protease.
31. The fusion protein according to claim 30, wherein the protease is a leukocyte protease.
32. The fusion protein according to claim 31, leukocyte protease is cathepsin.
33. The fusion protein according to claim 30, wherein the protease is an activated protease in the coagulation cascade.
34. The fusion protein according to claim 33, wherein the protease is activated Factor Xa.
35. The fusion protein according to claim 30, wherein the protease is an activated protease in the complement cascade.
36. The fusion protein according to claim 30, wherein the protease's activity is elevated locally in tissue.
37. The fusion protein according to claim 36, wherein the protease is a metalloproteinase, elastase, or collagenase.
38. The fusion protein according to claim 29, wherein the pathological mediator is involved in coagulation or fibrinolysis.

39. The fusion protein according to claim 29, wherein the pathological mediator is thrombin or plasmin.
40. The fusion protein according to claim 39, wherein the pathological mediator is thrombin.
41. The fusion protein according to claim 1, wherein the fusion protein comprises an anti-glycophorin A associated protein scFv and a urokinase-type plasminogen activator or low molecular weight single chain urokinase-type plasminogen activator.
42. The fusion protein according to claim 1, wherein the fusion protein comprises an anti-glycophorin A associated protein scFv and tissue-type plasminogen activator.
43. The fusion protein according to claim 1, wherein the fusion protein comprises an anti-glycophorin A associated protein scFv and thrombomodulin.
44. The fusion protein according to any one of claims 41-43, wherein the fusion comprises a thrombin cleavage site.
45. The fusion protein according to claim 1 or claim 2, wherein the anti-inflammatory agent is a molecule binding a pro-inflammatory mediator.
46. The fusion protein according to claim 45, wherein the pro-inflammatory mediator is HMGB1.
47. A pharmaceutical composition comprising the fusion protein of any of claims 1-45 and a pharmaceutically acceptable carrier.
48. A method of delivering an anti-thrombotic agent, an anti-inflammatory agent, or a pro-drug thereof to the surface of a red blood cell comprising delivering a fusion protein according to any of claims 1-46, or the pharmaceutical composition of claim 47, to a blood vessel.

49. A method of treating or preventing thrombosis, tissue ischemia, acute myocardial infarction (AMI), non-segmented elevated AMI, deep vein thrombosis, ischemic stroke, hyperoxic injury, transient ischemic attack (TIA), cerebrovascular disease, disseminated intravascular coagulation (DIC), pulmonary embolism, ischemic peripheral vascular disease, inflammation, pulmonary edema, sepsis, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and aseptic systemic inflammation, comprising administering a fusion protein according to any of claims 1-46, or the pharmaceutical composition of claim 47, to a blood vessel in a mammal in need thereof.

50. The method according to claim 48 or claim 49, wherein the fusion protein or composition is delivered intraperitoneally.

51. Use of a fusion protein according to any of claims 1-46, or the pharmaceutical composition of claim 47, as a medicament.

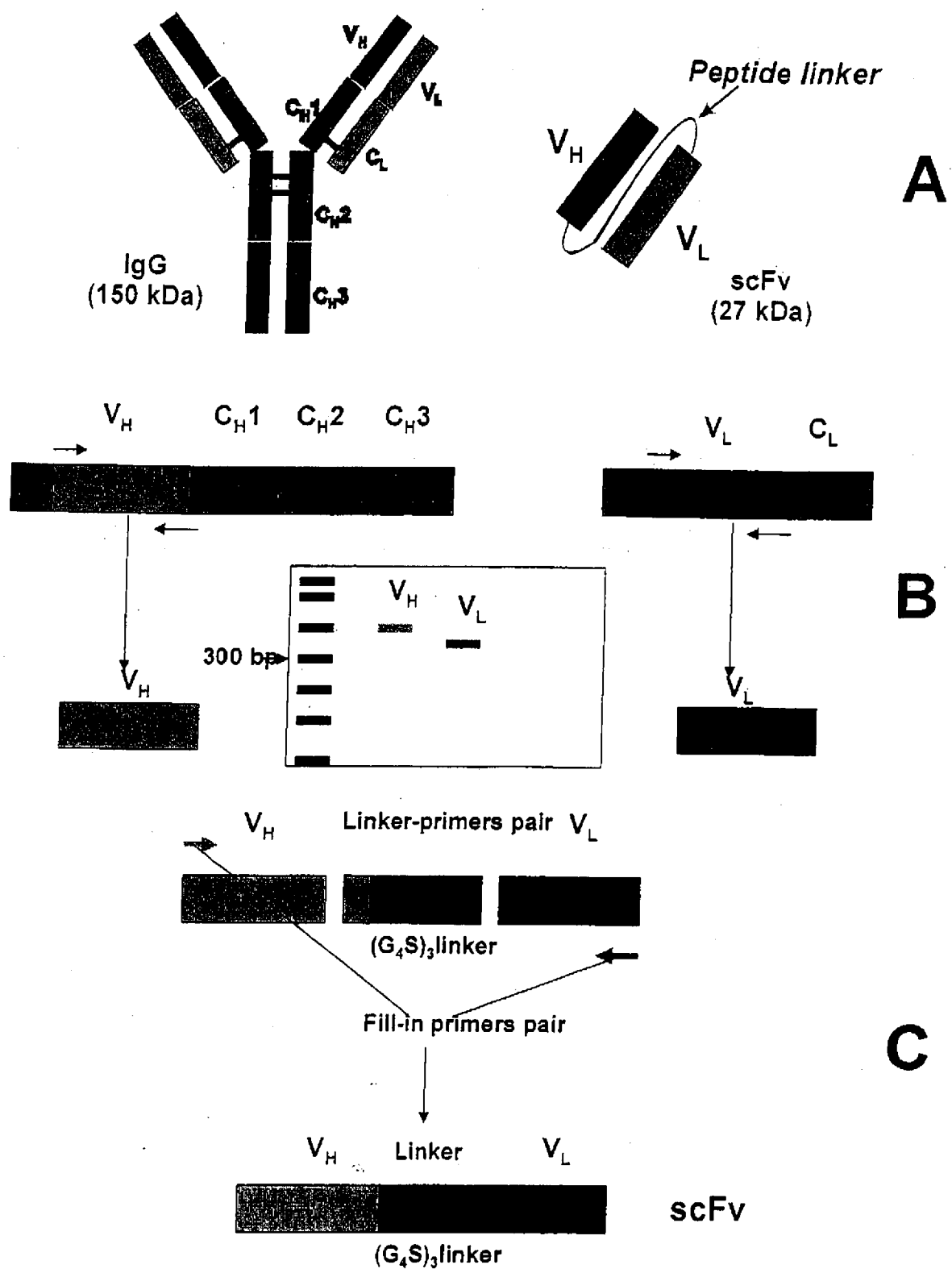
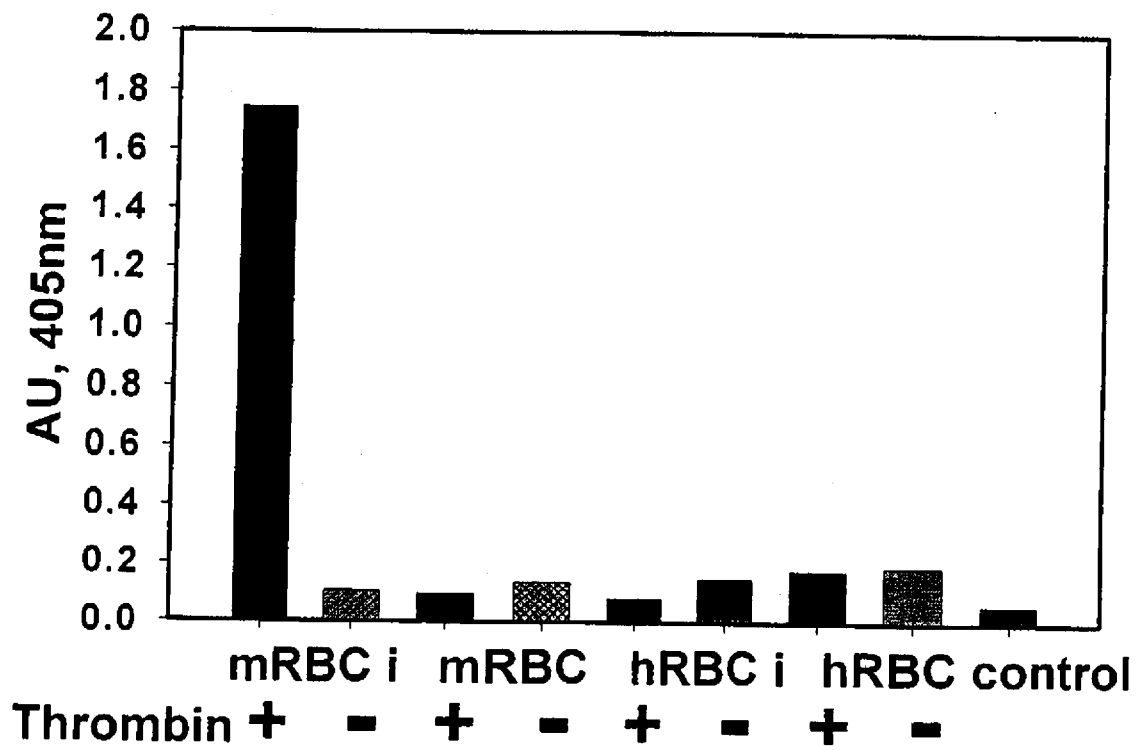
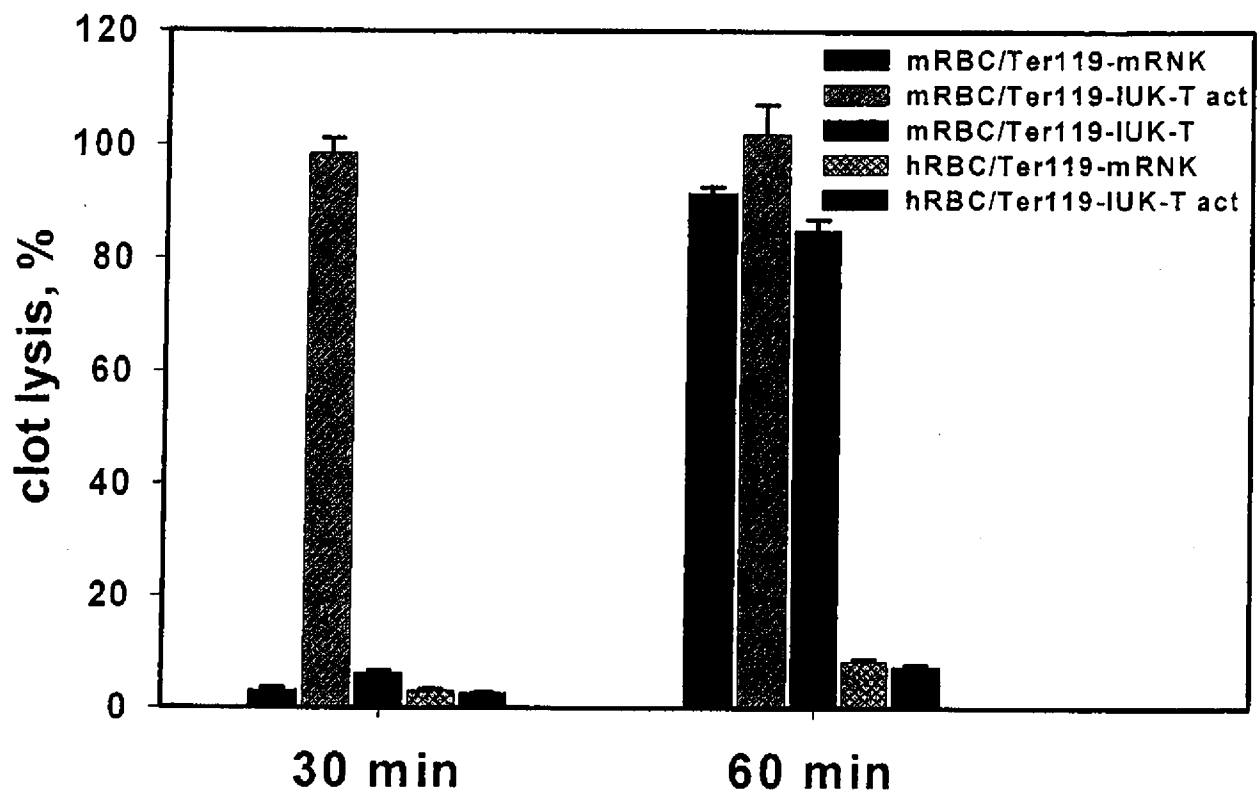
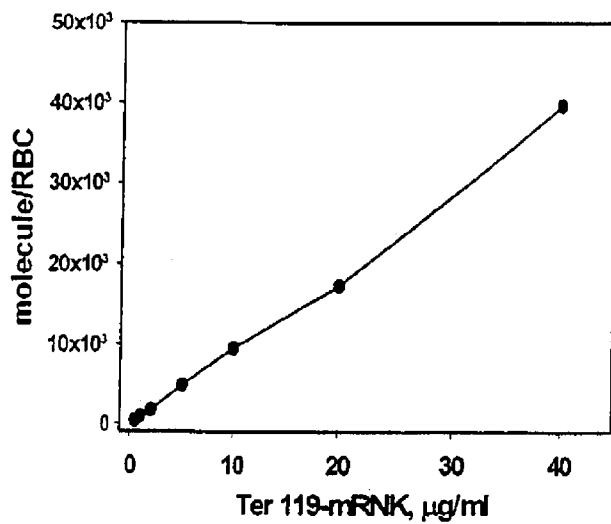
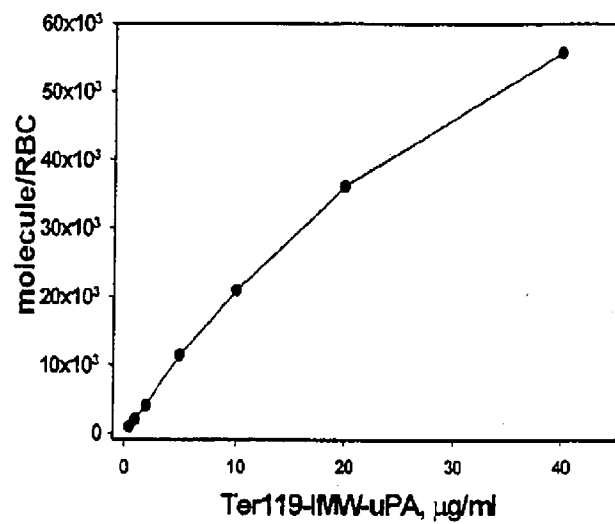
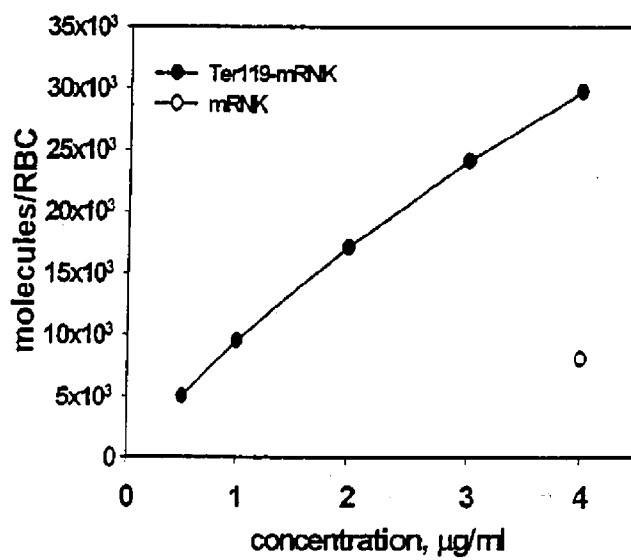
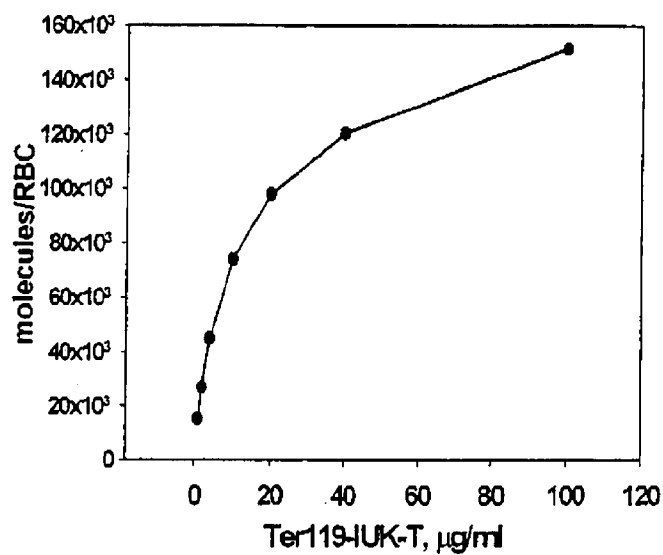


Fig. 1

**Fig. 2**

**Fig. 3**

A**B****C****D****Fig. 4**

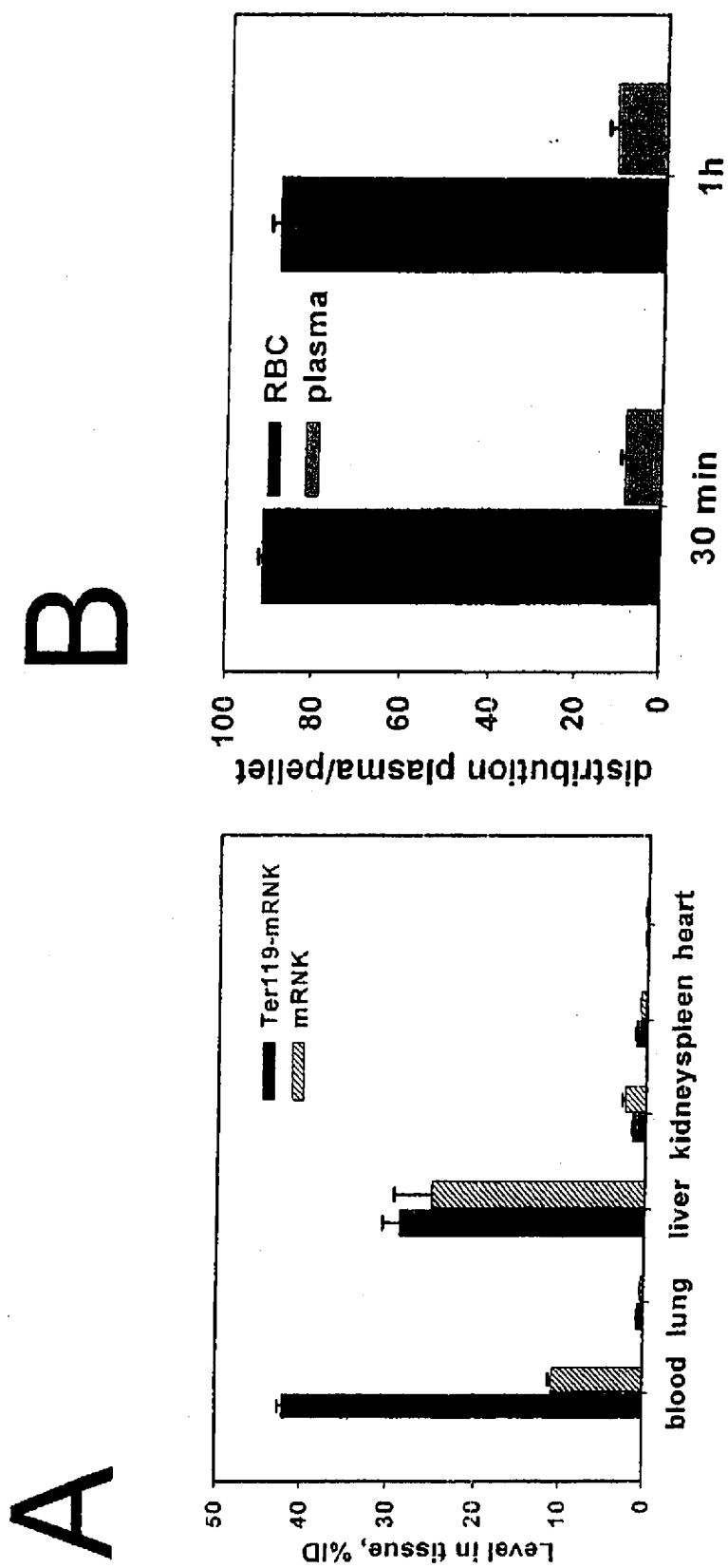


Fig. 5

Binding kinetics of Ter119-mRNK to mRBC in whole
blood vs PBS/BSA RBC suspension (equal hematocrit)

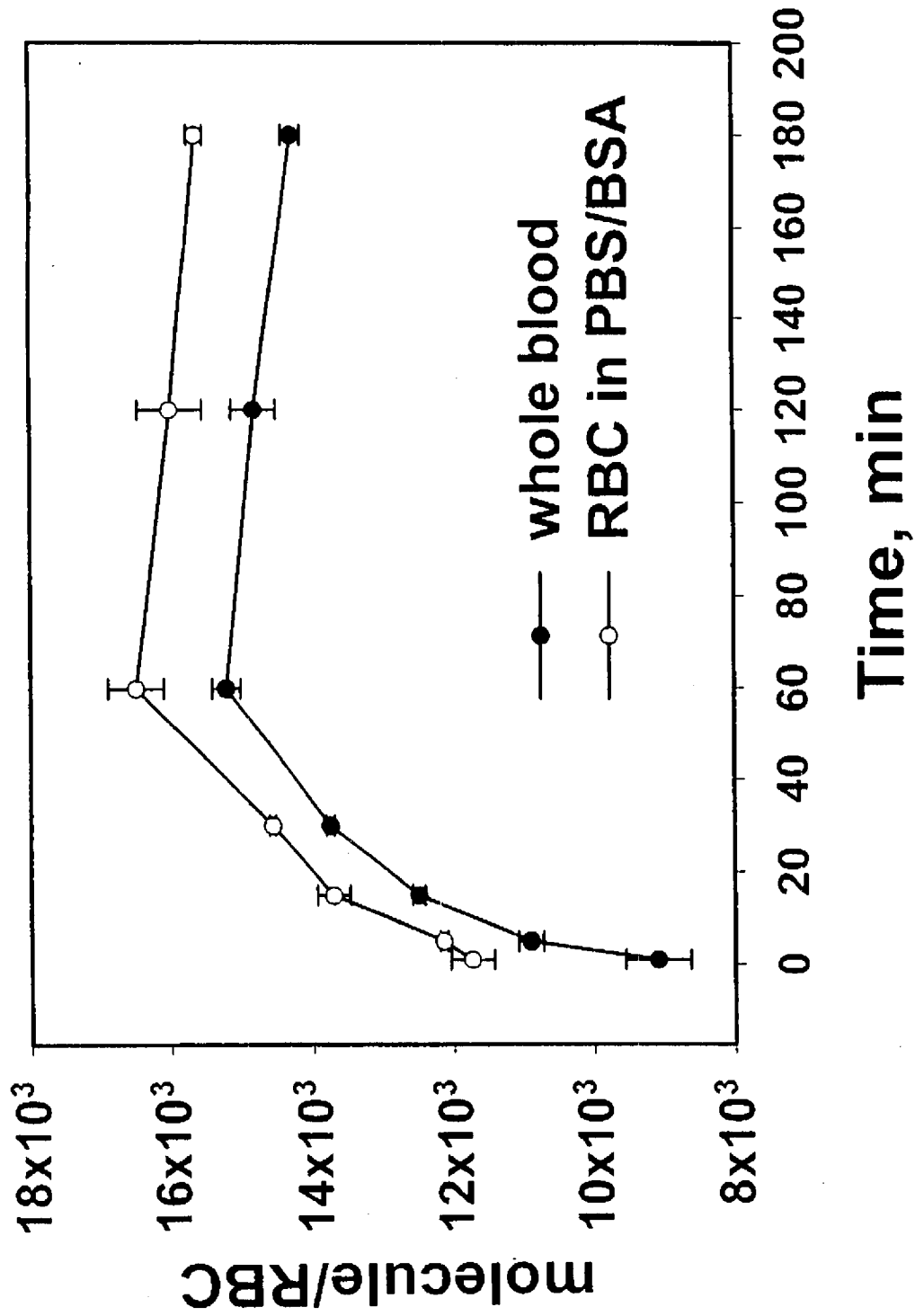


Fig. 6

Organ distribution of Ter119-mRNK during 1h-48h circulation in mice

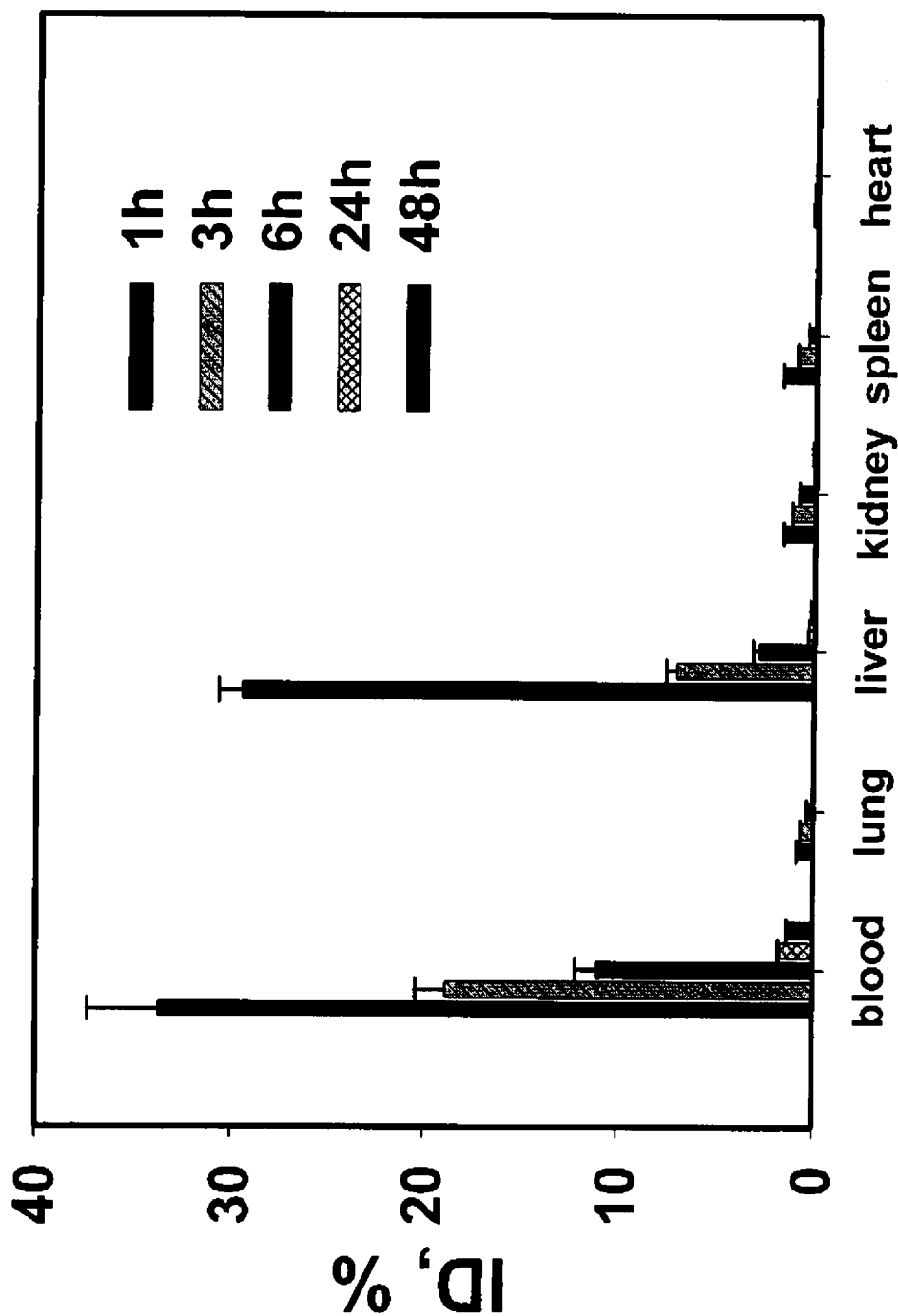


Fig. 7

Blood components distribution of Ter119-mRNK during 1h-48h circulation in mice

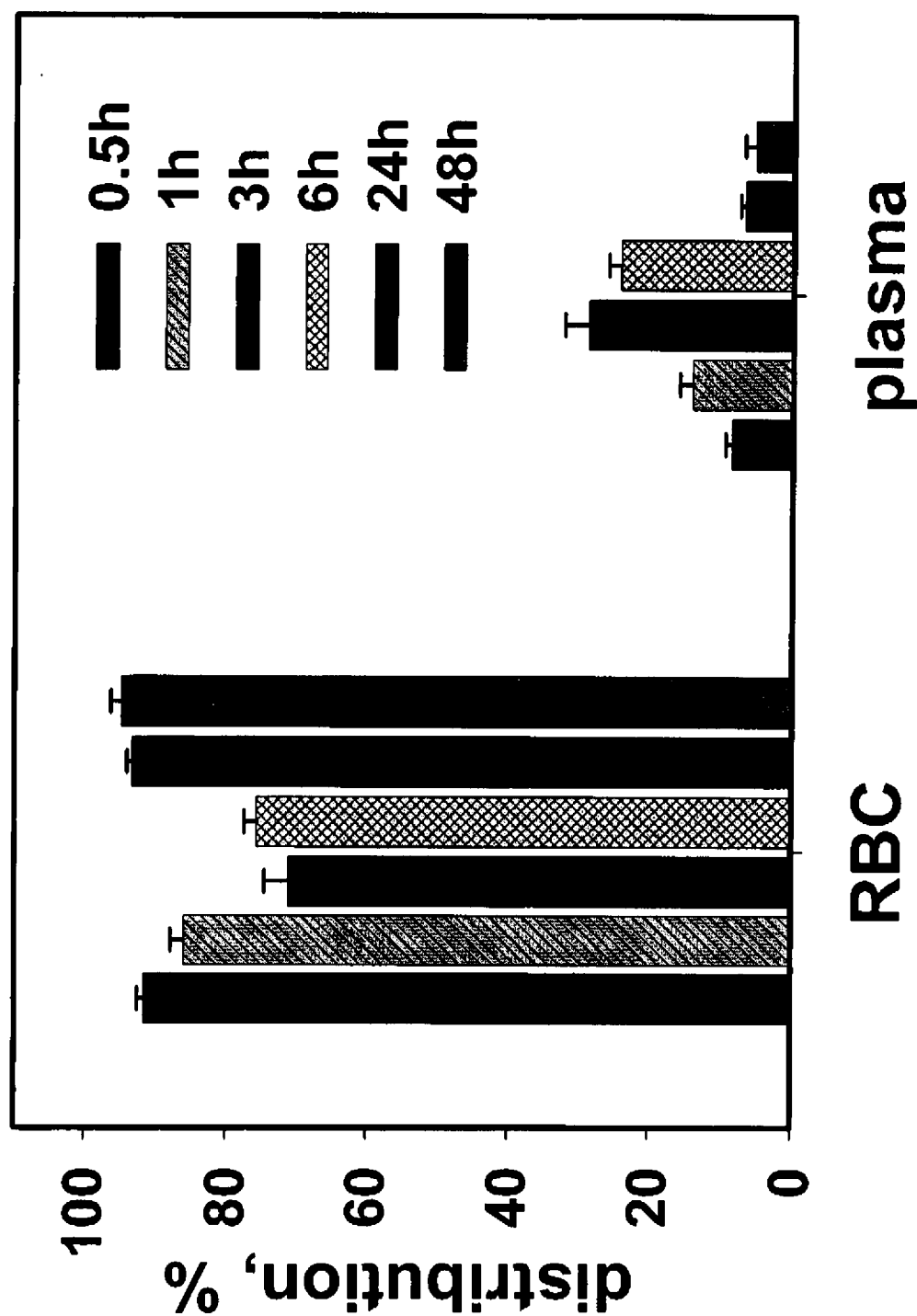


Fig. 8

Organ distribution of ⁵¹Cr labeled and ¹²⁵I-Ter119-TM loaded mouse RBC after 1-6h of circulation in mice

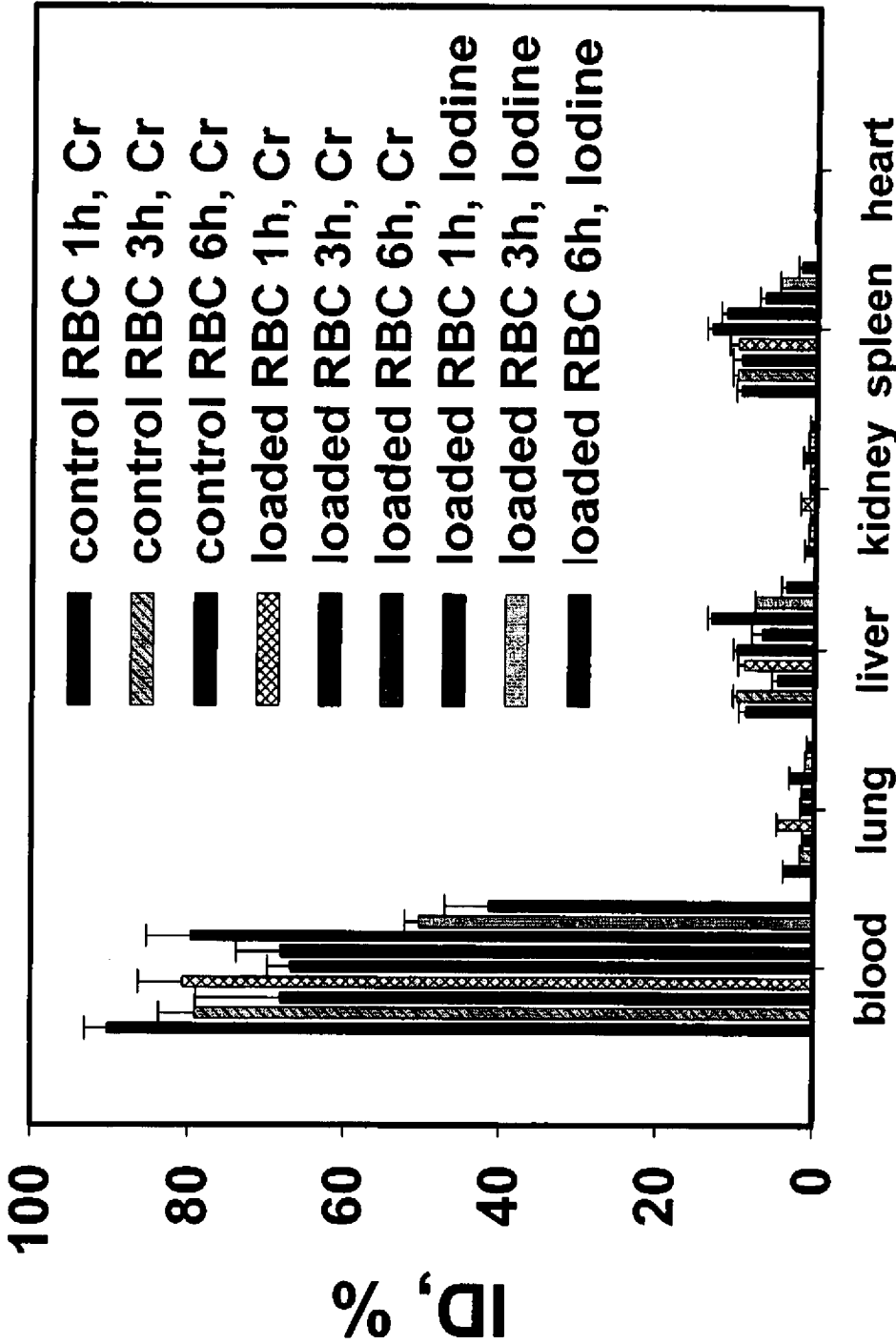


Fig. 9

Blood components distribution of ^{125}I and ^{51}Cr after 1-6 h of circulation of ^{51}Cr labeled and ^{125}I -Ter119-Tm loaded mouse RBC

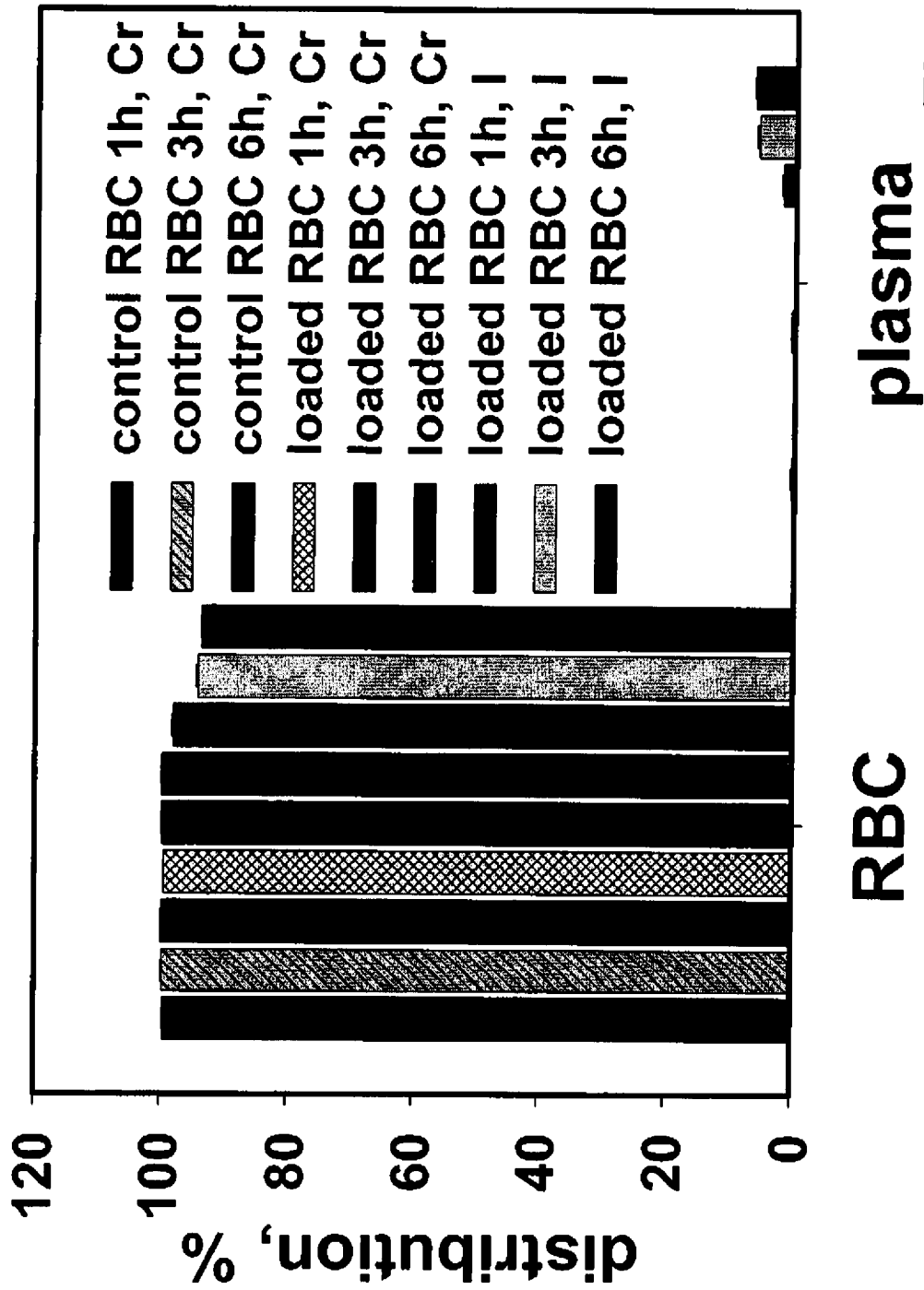


Fig. 10

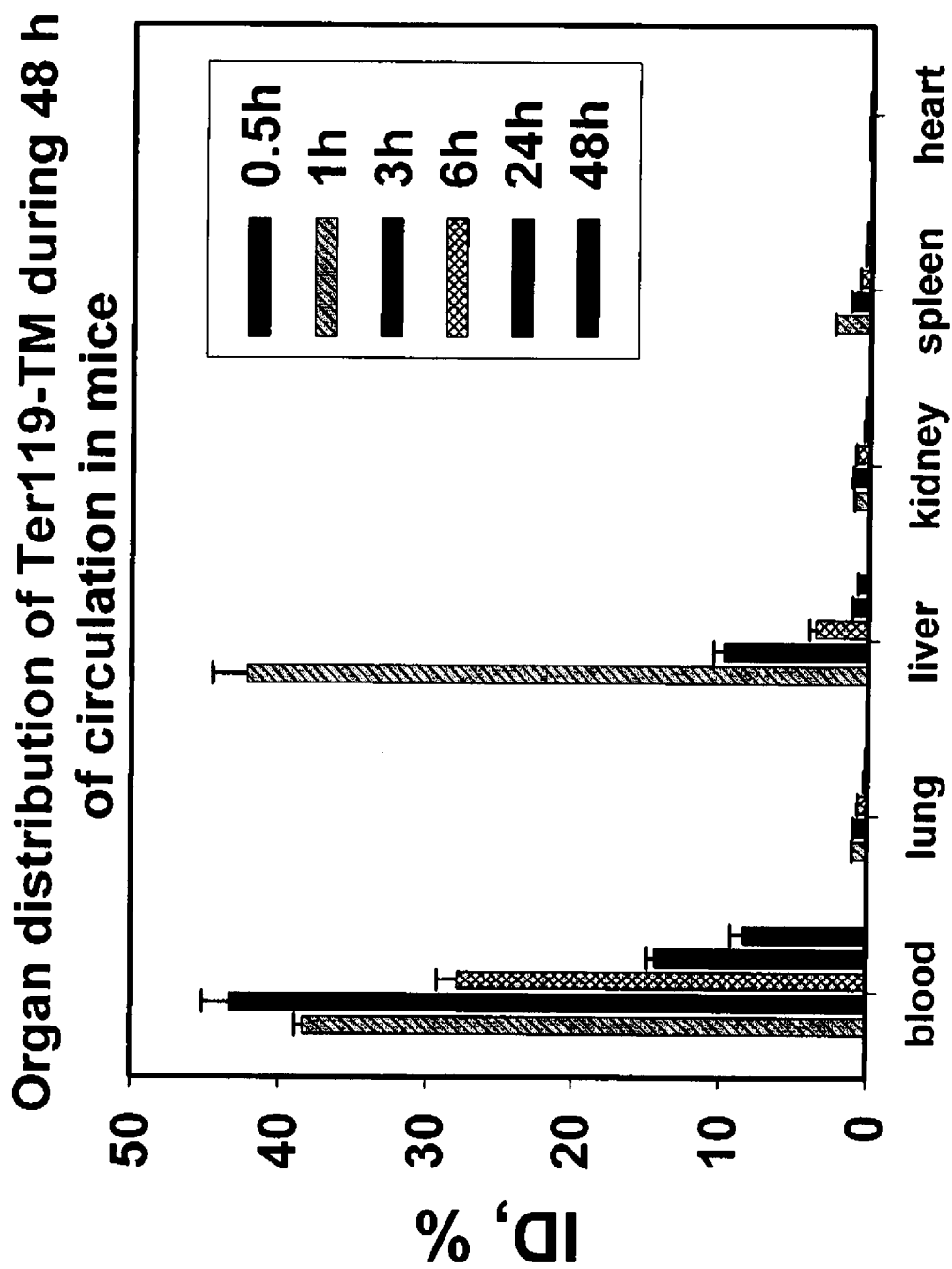


Fig. 11

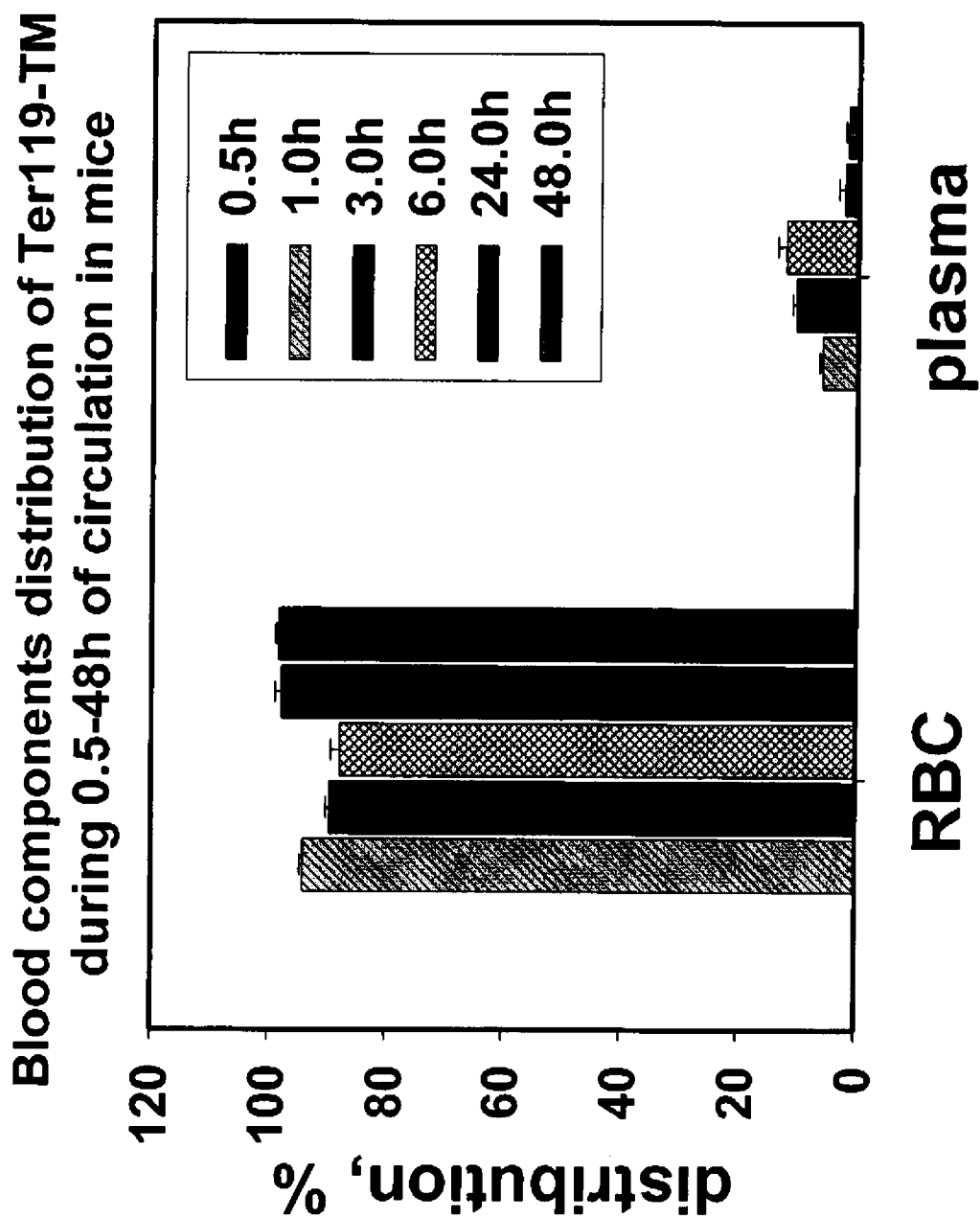


Fig. 12

Organ distribution of Ter119-TM in mice after
1h and 3h of circulation (IP injection)

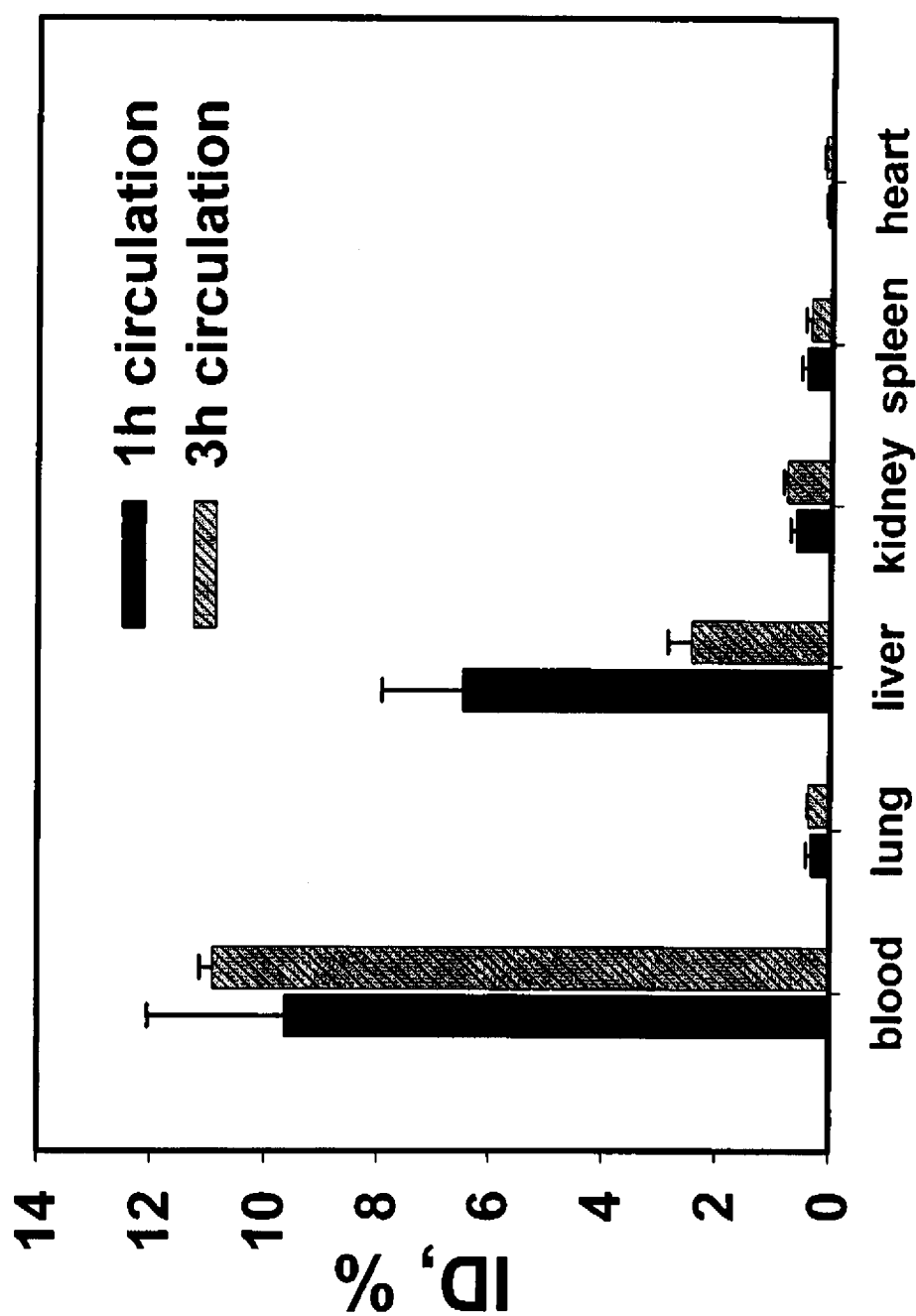
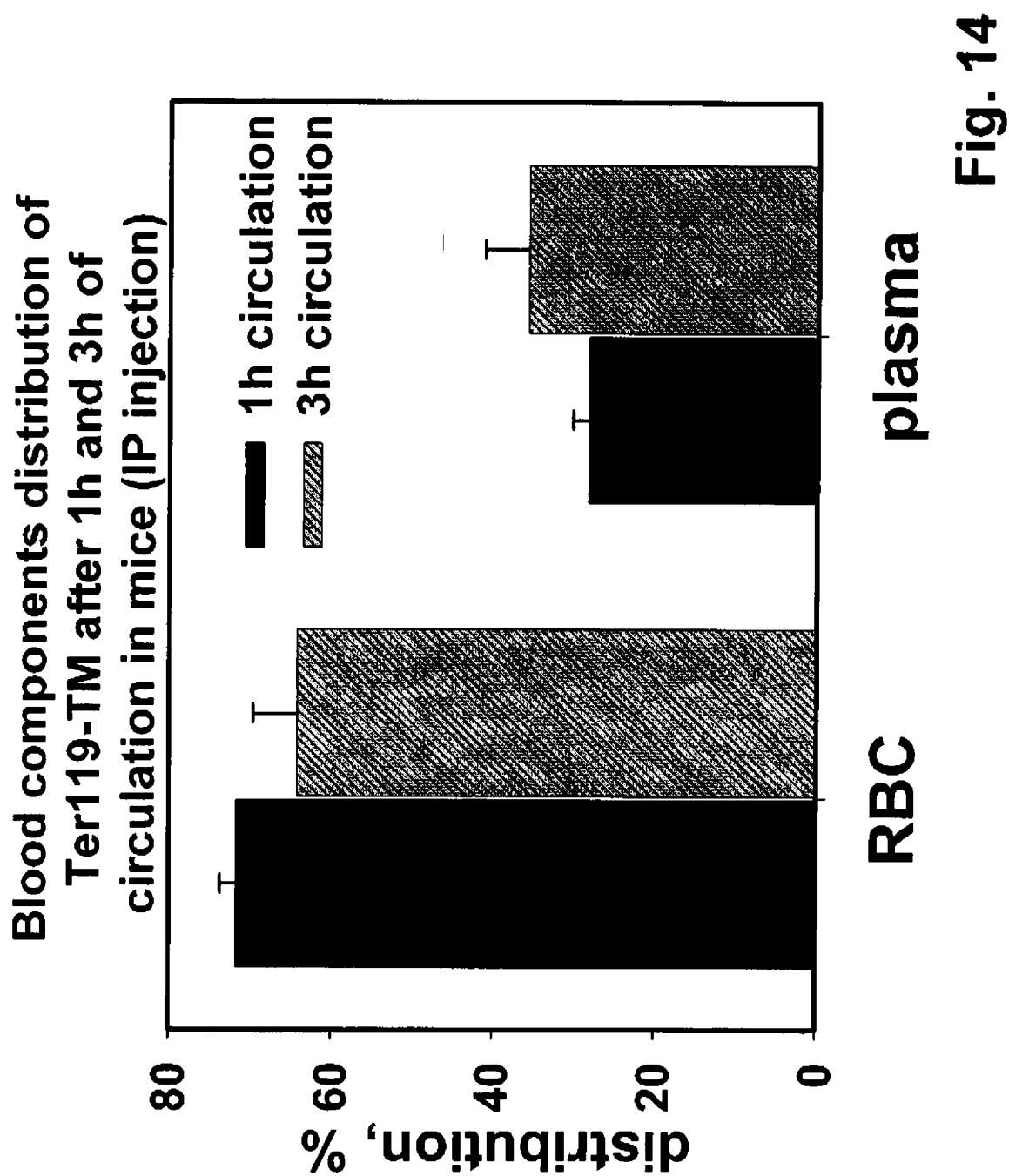


Fig. 13



Ter119-IUK-T vs IUK 0.5-48h circulation in mice (blood level)

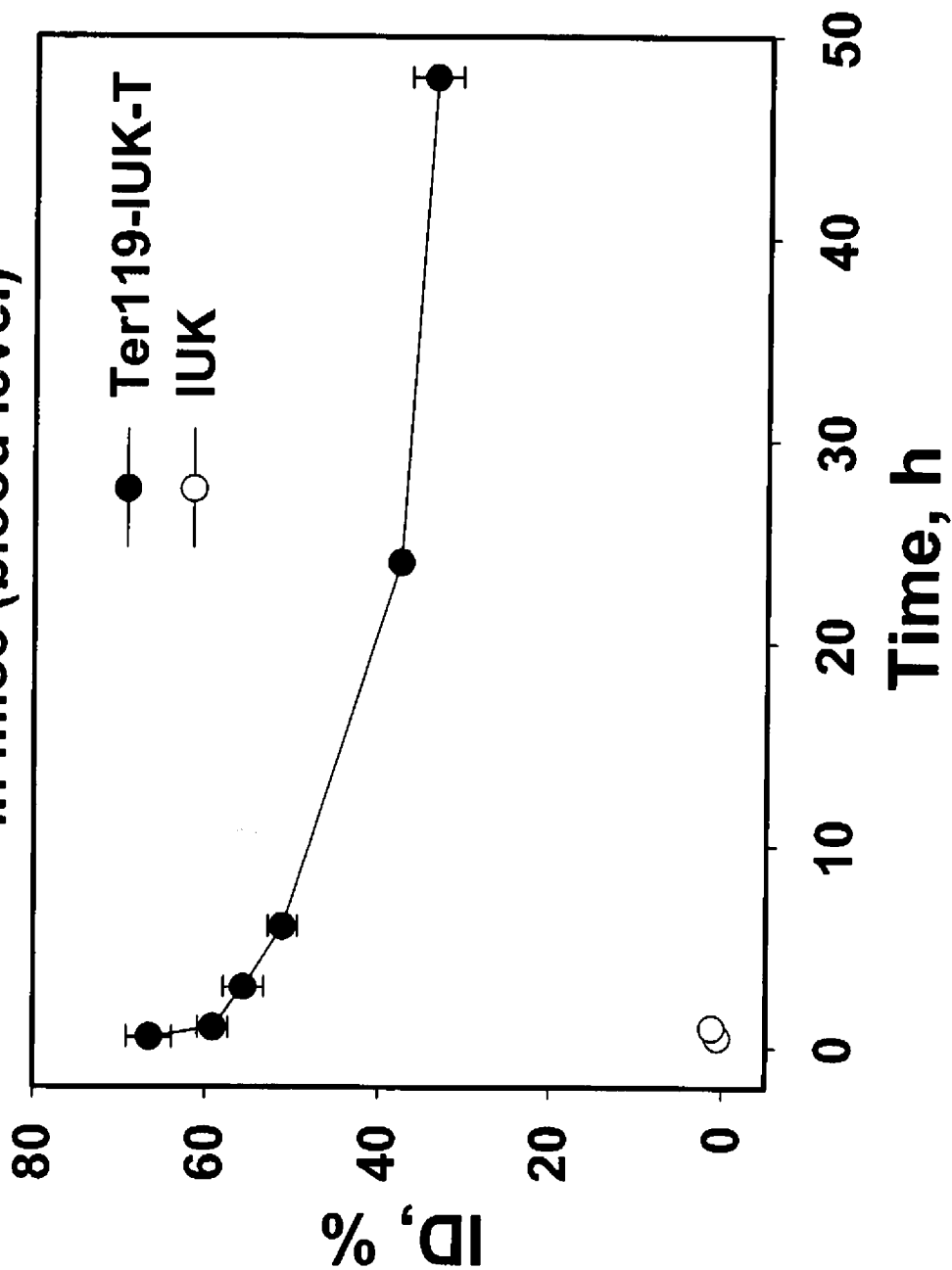


Fig. 15

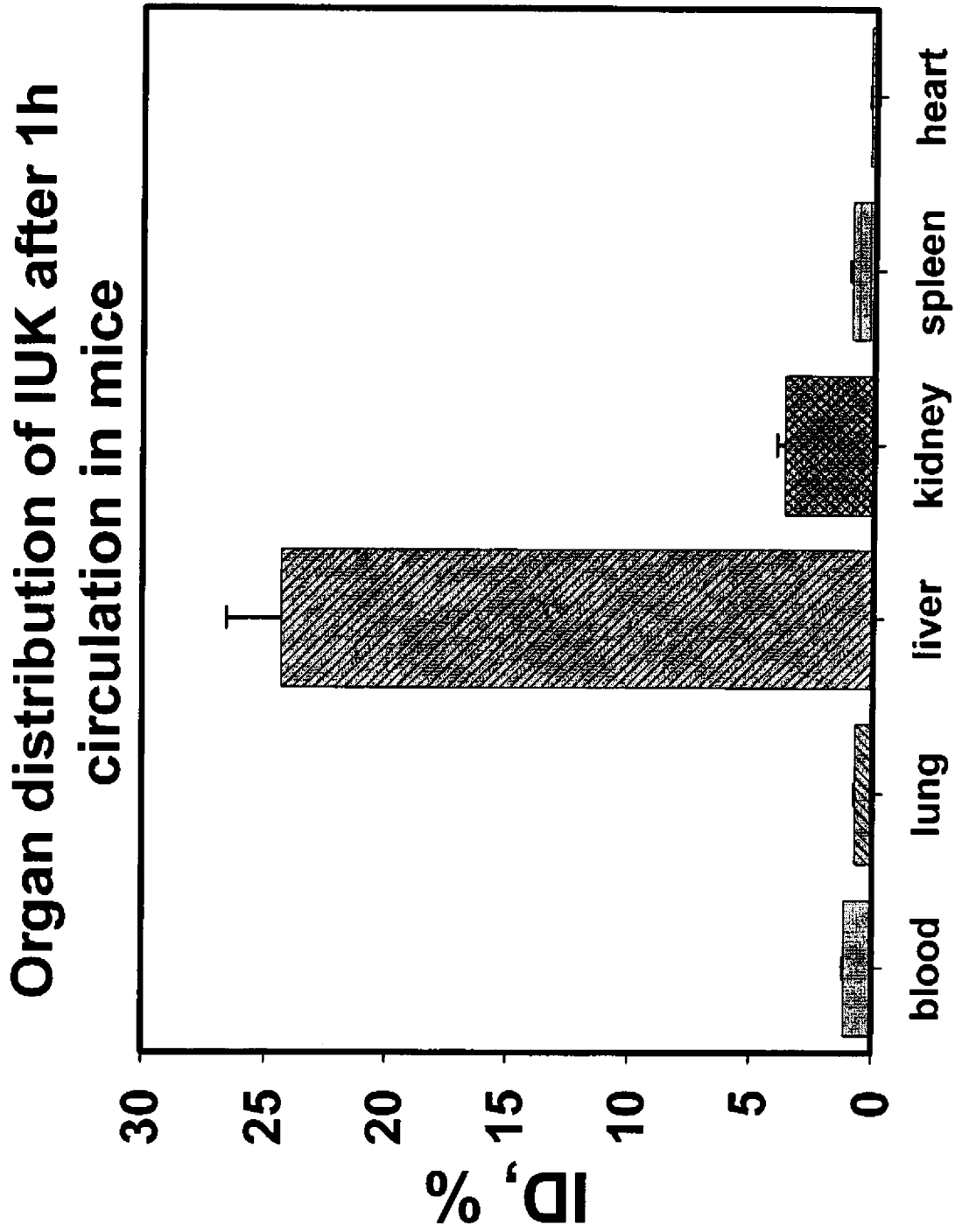


Fig. 16

Organ distribution of Ter119-IUK-T during 1h-48h circulation in mice

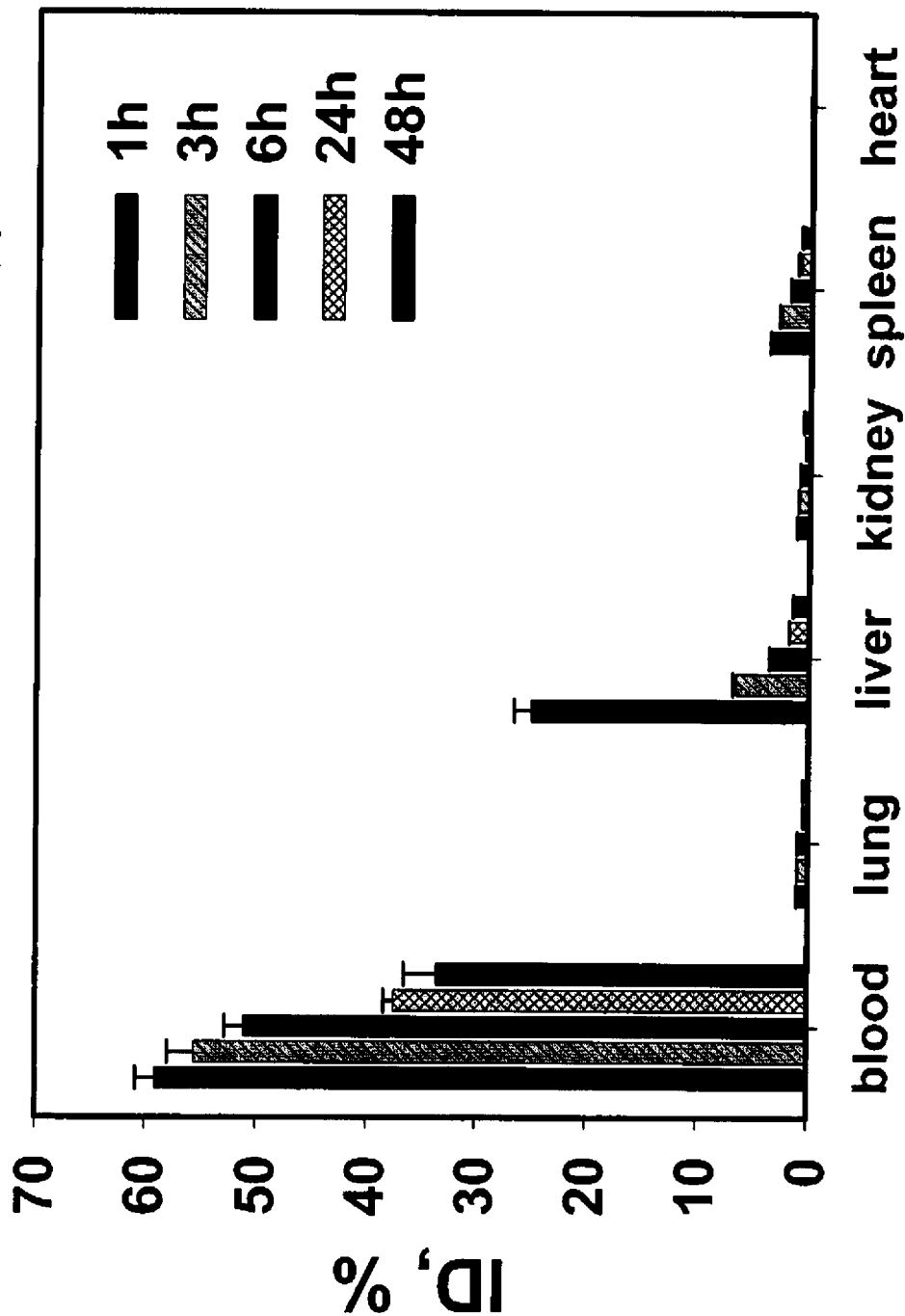


Fig. 17

Blood components distribution of Ter119-IUK-T during 1h-48h of circulation in mice

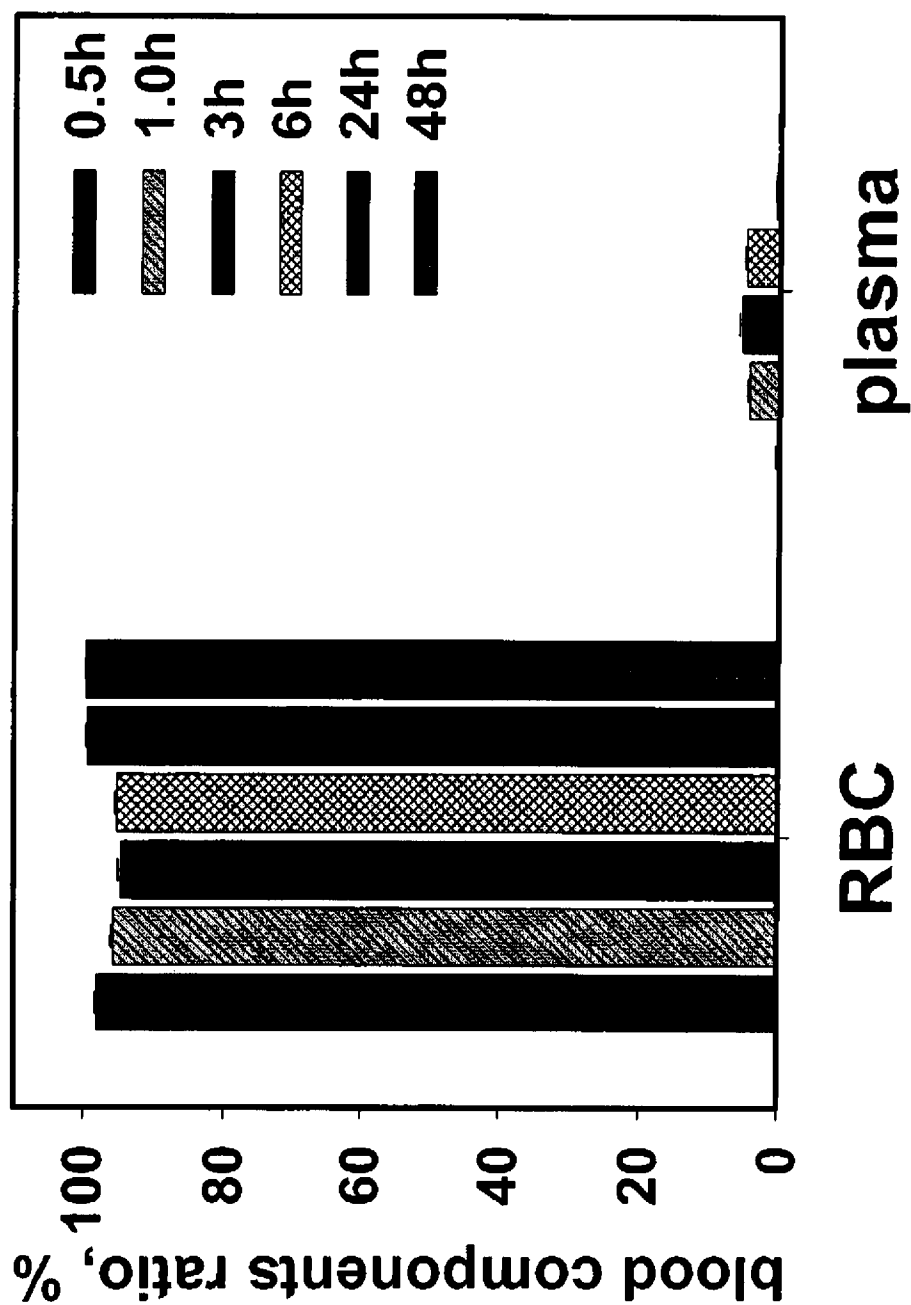


Fig. 18

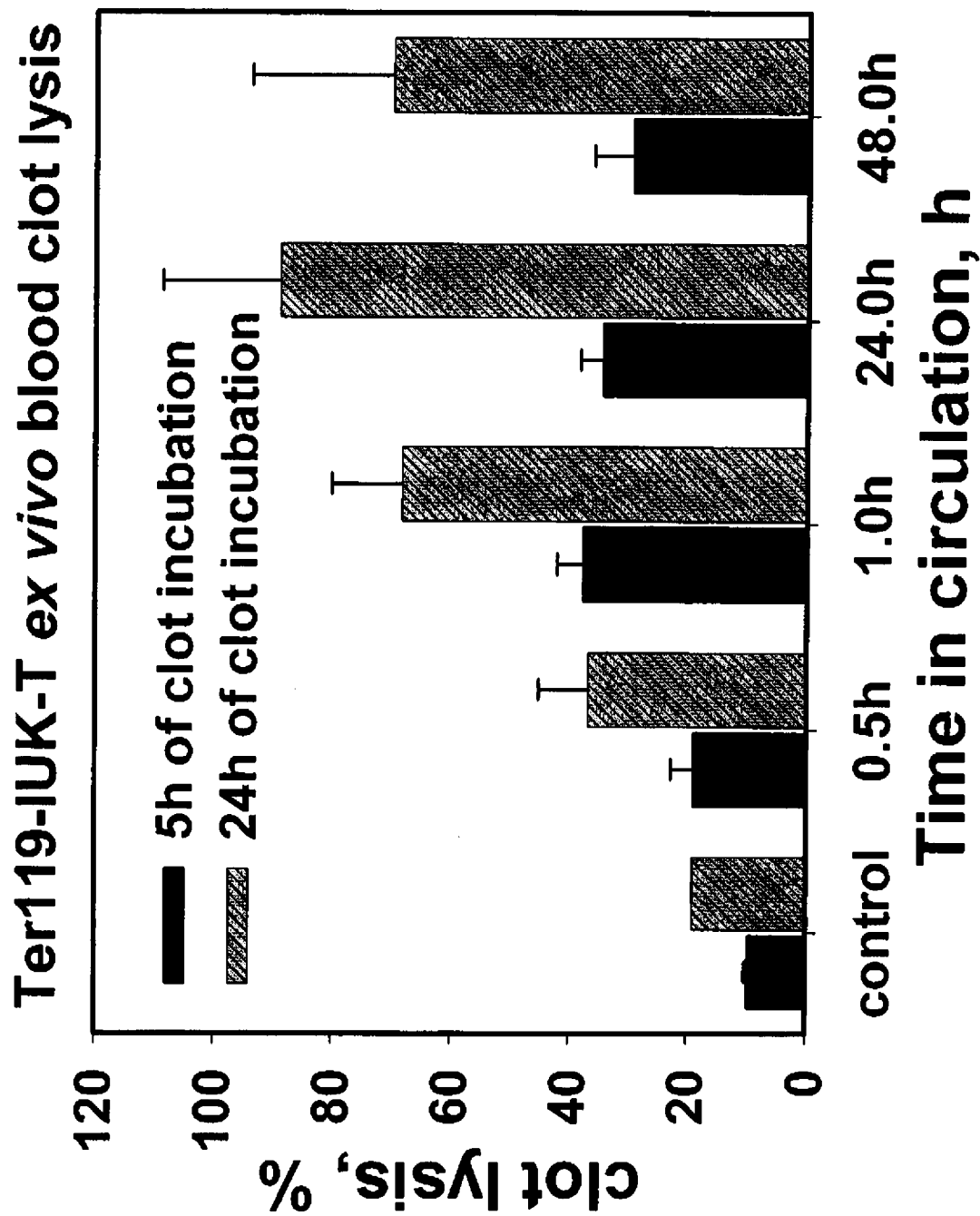
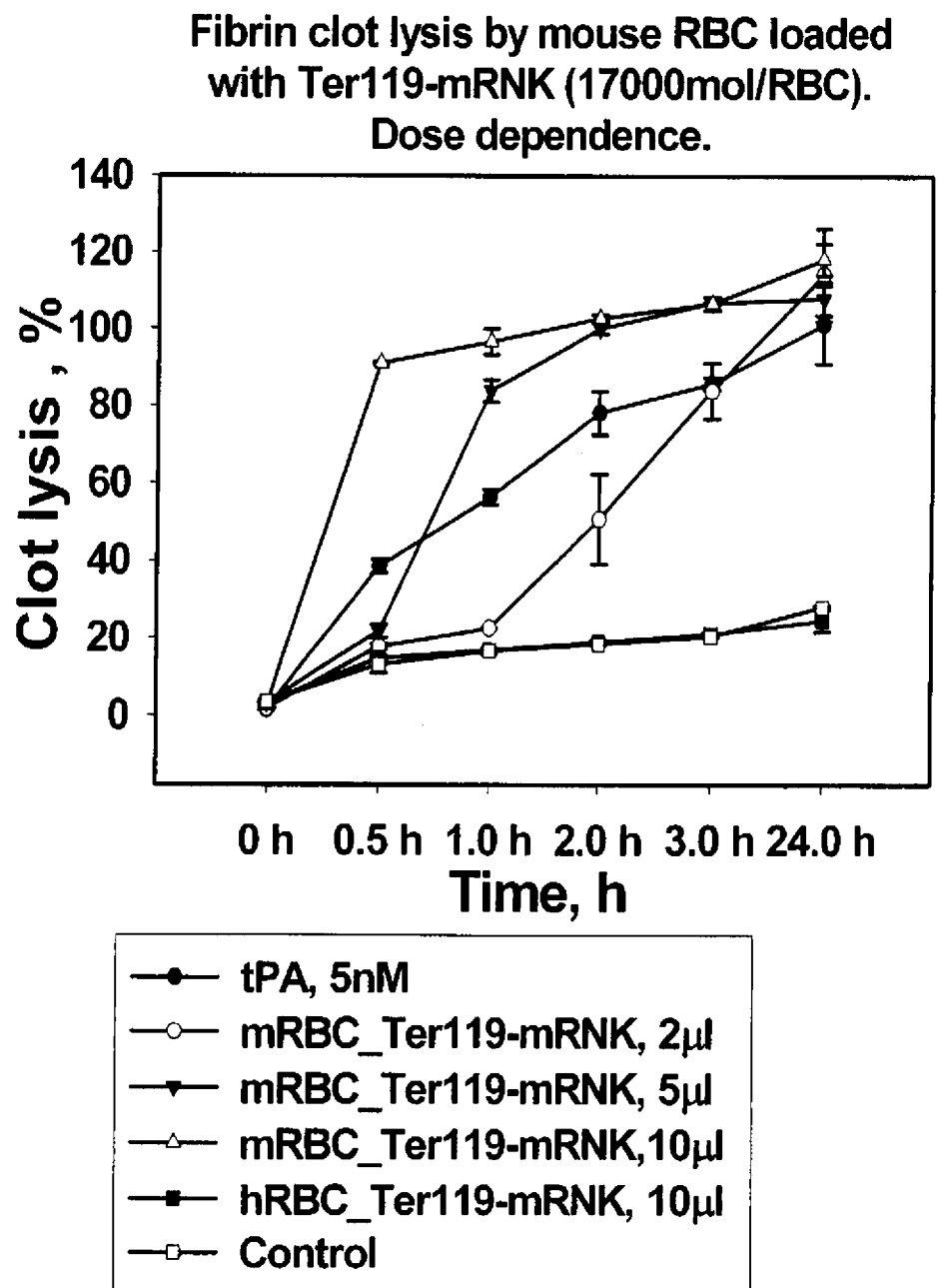


Fig. 19

**Fig. 20**

Whole blood clot lysis by Ter119-mRNK or
mRNK after 45 min postinjection in
mouse blood stream

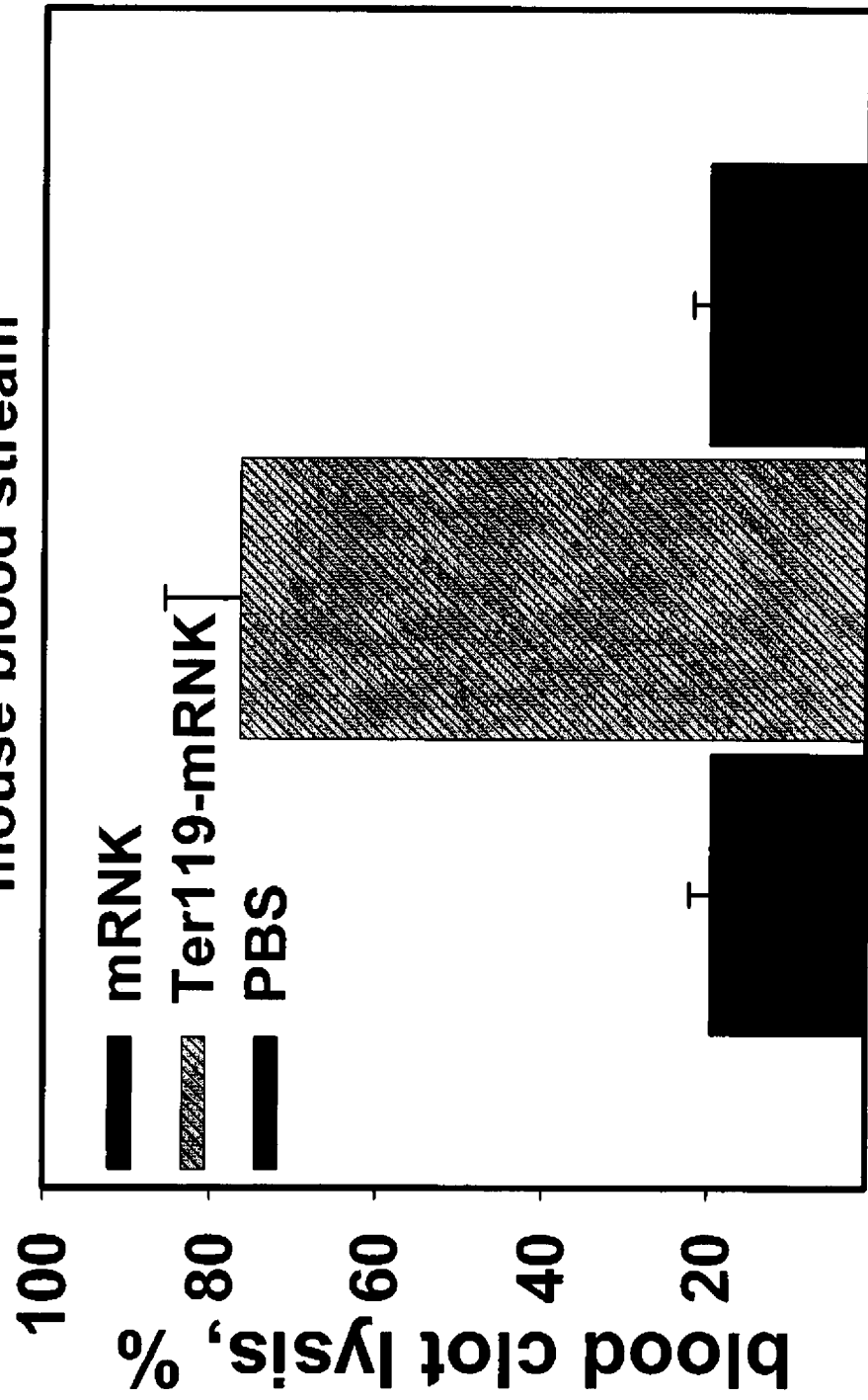


Fig. 21

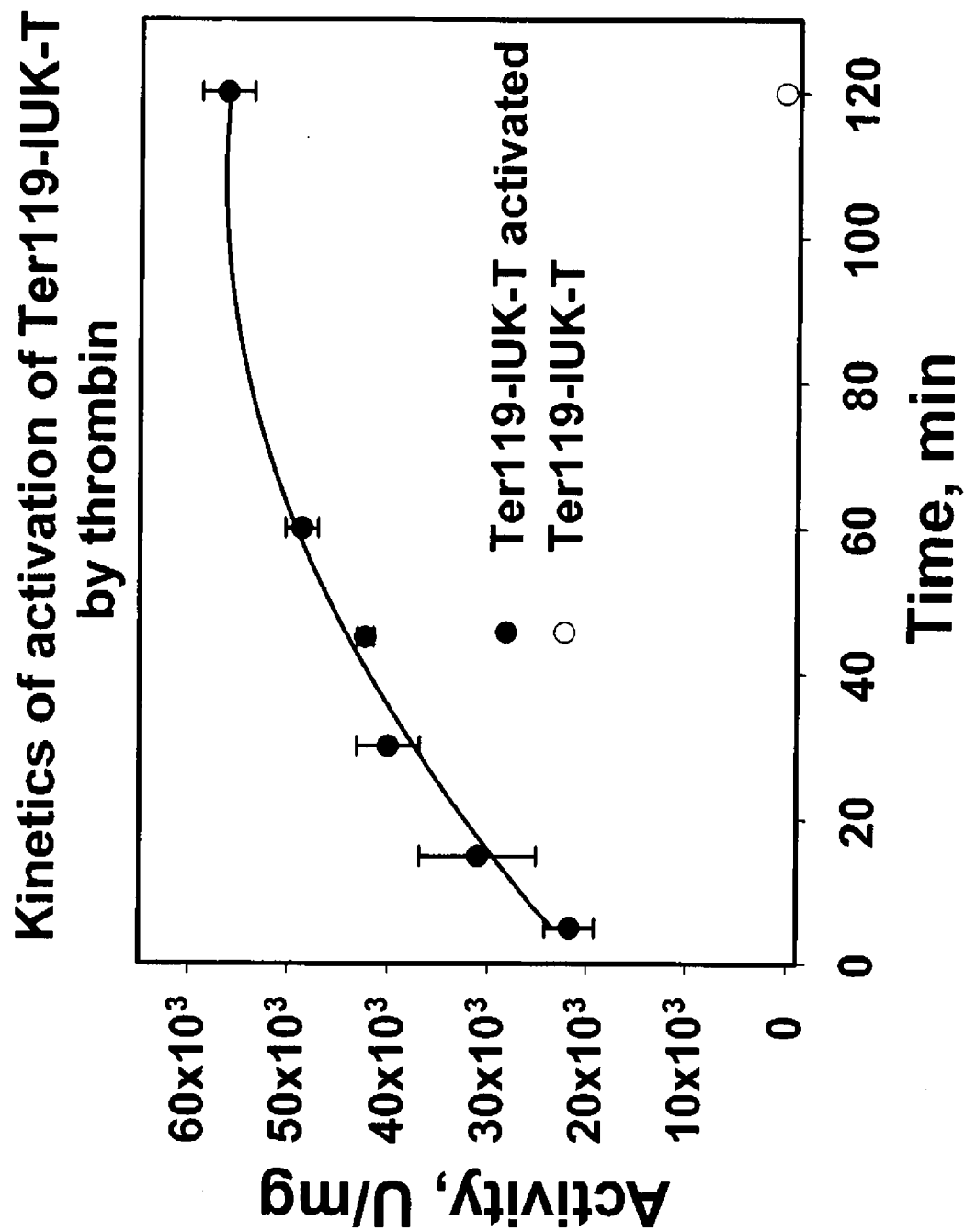
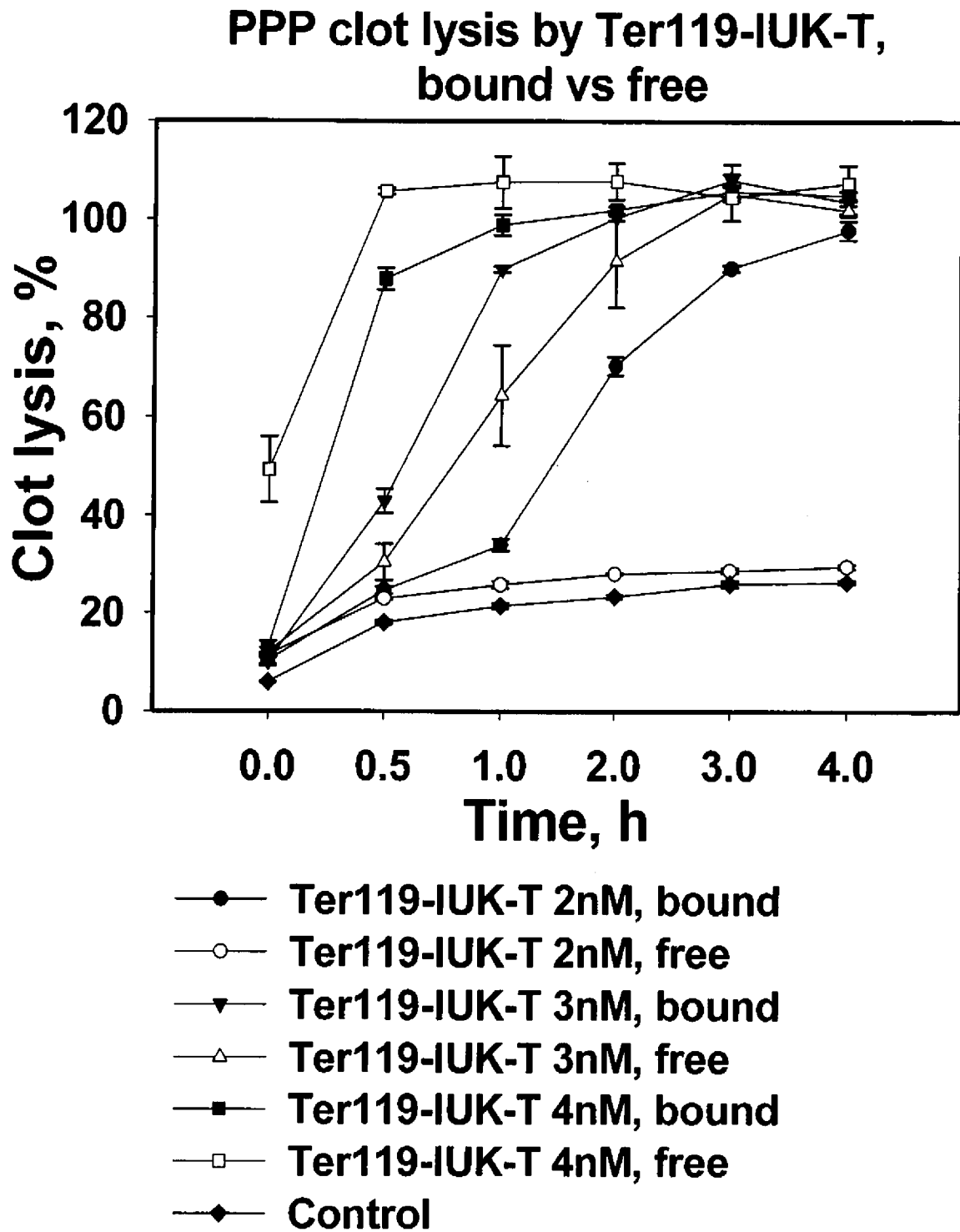


Fig. 22

**Fig. 23**

Time of carotid artery occlusion upon administration
of 15% FeCl₃ and pre-injection of saline (200μl),
mRNK(2mg/kg) or Ter119-mRNK 4mg/kg)
30 min before injury

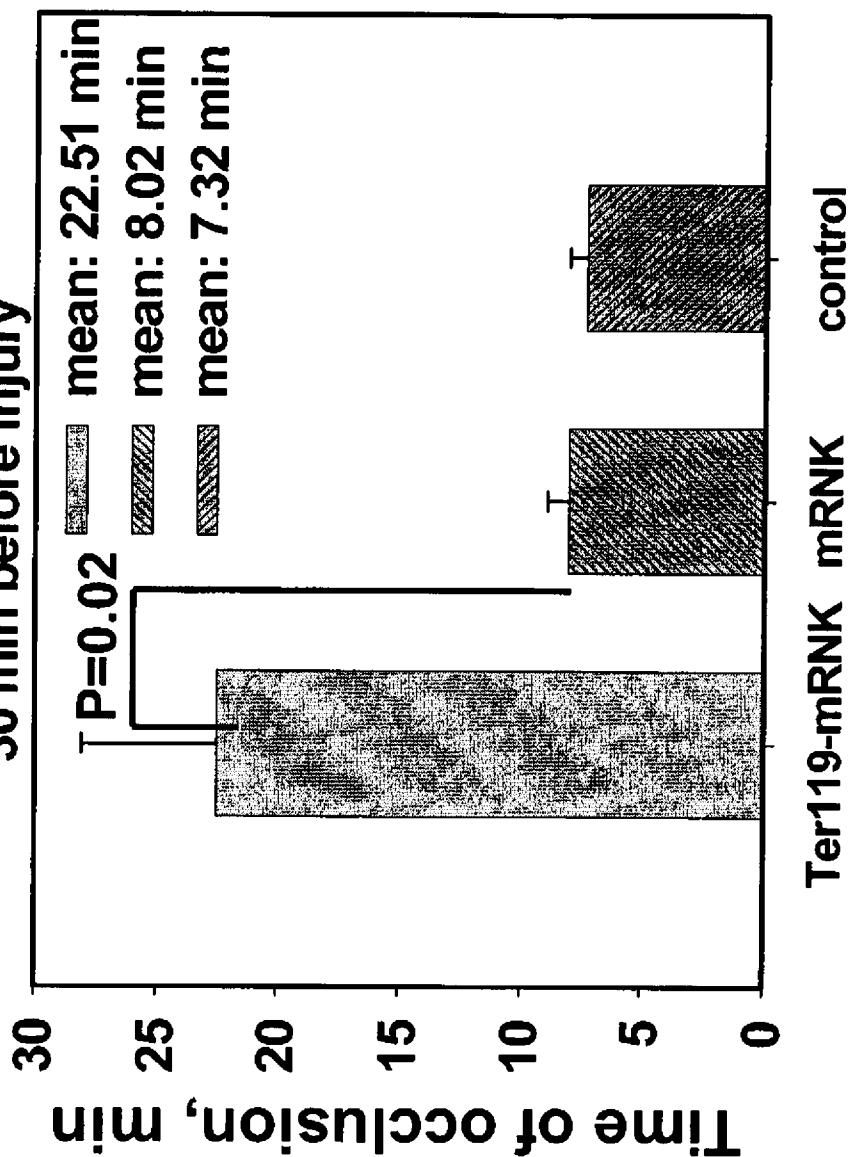


Fig. 24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/88632

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00 (2009.01)

USPC - 424/134.1

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)

USPC - 424/134.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/135.1, 809; 530/387.3Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(DB=PGPB,USPT,USOC,EPAB,JPAB), Google(CR1, glycoprotein A associated protein, platelet-endothelial cell adhesion molecule, IL-6, TNF, decay-accelerating factor), Google Scholar(anti-glycoprotein A associated protein scFv thrombotic, anti-glycoprotein A scFv thrombotic, glycoprotein A scFv plasminogen activator)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SPITZER et al, In Vivo Correction of Complement Regulatory Protein Deficiency with an Inhibitor Targeting the Red Blood Cell Membrane. The Journal of Immunology, 2005, vol 175, pp 7763-7770; Abstract; pg 7764, right col, para 5, ln 1-6; pg 7765, fig 2B	1-15, 41-46
Y	US 2003/0152563 A1 (MUZYKANTOV et al); 14 August 2003 (14.08.2003); Abstract; para [0016]-[0017], [0037]	1, 5-9, 11, 13-15, 41-44
Y	US 2006/0140917 A1 (MUZYKANTOV et al), 29 June 2006 (29.06.2006); Abstract; para [0021]-[0024], [0040], [0058]-[0059]	2-4, 10, 12, 45-46
Y	ABEYAMA et al, The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. The Journal of Clinical Investigation, May 2005, vol 115, pp1267-1274; Abstract; pg 1267, left col, para 1, ln 1-5	43, 45-46
Y	JENNY et al, A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. Protein Expression and Purification 2003, vol 31, pp 1-11; Abstract; pg 4, Table 2	44

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

* Special categories of cited documents:

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

"E" earlier application or patent 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 when the document is taken alone

"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

Date of the actual completion of the international search

19 February 2009 (19.02.2009)

Date of mailing of the international search report

02 MAR 2009

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/88632

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 16-40 and 47-51
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.