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(54) **METHODS AND USES OF POLYPEPTIDE LIGANDS CONTAINING LINKERS**

VERFAHREN UND VERWENDUNGEN VON LINKER ENTHALTENDEN POLYPEPTIDLIGANDEN

PROCEDES ET UTILISATIONS DE LIGANDS POLYPEPTIDIQUES CONTENANT DES LIEURS

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- **PETITJEAN A ET AL: "Dynamic devices. Shape switching and substrate binding in ion-controlled nanomechanical molecular tweezers" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY 20040602 US, vol. 126, no. 21, 2 June 2004 (2004-06-02), pages 6637-6647, XP002523373 ISSN: 0002-7863**
- **MIYAWAKI A ET AL: "FLUORESCENT INDICATORS FOR CA2+ BASED ON GREEN FLUORESCENT PROTEINS AND CALMODULIN" NATURE, NATURE PUBLISHING GROUP, LONDON, UK, vol. 388, 28 August 1997 (1997-08-28), pages 882-887, XP002940051 ISSN: 0028-0836**
- **WOOD D W ET AL: "OPTIMIZED SINGLE-STEP AFFINITY PURIFICATION WITH A SELF-CLEAVING INTEIN APPLIED TO HUMAN ACIDIC FIBROBLAST GROWTH FACTOR" BIOTECHNOLOGY PROGRESS, AMERICAN INSTITUTE OF CHEMICAL ENGINEERS, US, vol. 16, no. 6, 1 January 2000 (2000-01-01), pages 1055-1063, XP000978915 ISSN: 8756-7938**
- **DATABASE USPTO Proteins [Online] 25 September 2002 (2002-09-25), "Sequence 11 from patent US 6413730." XP002523375 retrieved from EBI accession no. USPOP: AAN21177 Database accession no. AAN21177**

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- **TOLKATCHEV D ET AL: "Transforming bivalent ligands into retractable enzyme inhibitors through polypeptide-protein interactions" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, ELSEVIER SCIENCE, GB, vol. 15, no. 23, 1 December 2005 (2005-12-01), pages 5120-5123, XP025313801 ISSN: 0960-894X [retrieved on 2005-12-01]**
- **TOLKATCHEV DMITRI ET AL: "Polypeptide ligands containing switchable flexible linkers as retractable inhibitors of protein-protein interactions" UNDERSTANDING BIOLOGY USING PEPTIDES SPRINGER, 233 SPRING STREET, NEW YORK, NY 10013, UNITED STATES, 2006, pages 669-670, XP008105149 & 19TH AMERICAN PEPTIDE SYMPOSIUM; SAN DIEGO, CA, USA; JUNE 18 -23, 2005 ISSN: 0-387-26569-4(H)**

Description**Field of the Invention**

5 [0001] The invention relates to an ex-vivo method of delivering a compound of interest for preferential release at a biological site of interest and to a molecule for use in a method of delivering a compound of interest of preferential release at a biological site of interest.

Background of the Invention

10 [0002] Strategies of linking weak-binding molecular fragments together to produce a significantly stronger ligand molecule have been implemented in drug discovery. Tweezer-like molecules have also been designed recently in the area of host-guest chemistry to control the specific complexation of artificial receptors (hosts) with small molecules (guests). In these applications, the linking bridge is normally optimized and often rigidified to achieve maximal affinity of the bivalent molecule. Bivalent and polyvalent ligands have been reported that incorporate multiple copies of a single binding moiety on a polymer backbone.

15 [0003] It is an object of the invention to provide multivalent binding molecules containing linkers through which binding can be modulated.

20 [0004] Petitjean A et al "Dynamic Devices. Shape Switching and Substrate Binding in Ion-Controlled Nanomechanical Molecular Tweezers" Journal of the American Chemical Society Vol.126, no.21, 2 June 2004, pages 6637-6647 describes two molecular conformational switches as examples of supramolecular devices performing chemical control of binding events and models of related natural systems. These display cation-controlled nanomolecular motion coupled to substrate binding and release. The substrate binding relies on donor/acceptor interactions, provided by intercalation between planar sites located at the extremities of the switching units, whereas cation complexation is responsible for conformational regulation. The terpyridine py-py-py-based receptor is activated toward substrate binding upon complexation of a zinc (II) cation and operates in a two-state process. It is described that the replacement of the central pyridine by a 4-6-disubstituted pyrimidine, as in py-pym-py, induces a state reversal and is stated to yield a new receptor which binds a substrate in the absence of cation, and releases it when copper (I) is introduced, following a three-step process.

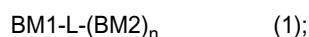
25 [0005] Miyawaki A. et al "Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin" Nature Vol.388, 28 August 1997, pages 882-887 describe the construction of fluorescent indicators for Ca²⁺ that are described as being genetically encoded without cofactors and are said to be targetable to specific intracellular locations. The indicators consist of tandem fusions of a blue- or cyan-emitting mutant of the green fluorescent protein (GFP), calmodulin, the calmodulin-binding peptide M13 and an enhanced green- or yellow-emitting GFP.

Summary of the Invention

35 [0006] There is disclosed herein an approach combining independent binding moieties in a single molecular structure, which couples binding affinity to an on/off or modulatable switch. This molecular organization provides responsiveness of the inherent ligand (effector/inhibitor) potency to an external triggering signal. A principle of such a molecular structure is the design of the ligand in a bivalent or otherwise multivalent fashion, termed "biomolecular tweezers", which contain two or more binding moieties (or "heads") linked by a structurally flexible bridge (Figure 1). Each binding moiety in isolation preferably has only low-affinity and transient interactions with an intrinsic dissociation constant preferably less than 1 M for its specific binding site on a target biomacromolecule. When linked together, the resulting bivalent or multivalent ligand makes a substantially stable complex with the target, achieving enhancement of preferably at least two (2) fold in overall binding affinity as compared to the highest affinity of the constituent monovalent ligands. To achieve control of binding, a change (normally, decrease) in the flexibility of the linker can be induced by an external trigger to disrupt the molecule's ability to bind in a bivalent or multivalent high-affinity mode. Vice versa, removal of constraints imposed on the linker would preferably restore the high-affinity binding of the freed bivalent or multivalent ligand. Where the binding sites are known to occur in a defined spatial relationship, it may in some instances be desirable to select a linker which is substantially rigid in the environment in which binding is desired and has a conformation when rigid that places the ligands in preferred positions for binding.

40 [0007] According to a first aspect of the present invention there is provided an ex-vivo method of delivering a compound of interest for preferential release at a biological site of interest, comprising an ex-vivo method of delivering a compound of interest for preferential release at a biological site of interest, comprising:

45 (a) obtaining a molecule of formula 1:



- (b) providing the compound of interest and the molecule of formula (1) at the biological site of interest; and,
(c) providing a linker-specific molecule at the biological site of interest, wherein,

n is 1 or greater,

BM1 is a first binding moiety having an affinity for site 1 on the compound,

BM2 is a second binding moiety having an affinity for a site other than site 1 on the compound,

BM1 and BM2 are the same or different, and where $n > 1$, different BM2 moieties have affinities for different binding sites on the compound,

BM1 and BM2 are selected such that in use each of the BM1 and BM2 existing separately has a lower binding affinity than the molecule of formula 1, and

L is a linker joining BM1 and BM2, said linker being a flexible polypeptide without a well-defined three-dimensional structure in a free state, said linker undergoing a loss or decrease of flexibility or change in conformation upon non-covalent binding to the linker-specific molecule at the biological site of interest thereby impeding simultaneous binding of the binding moieties, thereby producing a reversing effect on the binding affinity of the molecule of formula 1 at the biological site of interest.

[0008] According to a second aspect of the present invention there is provided a molecule of formula 1 as specified in the above first aspect for use in a method of delivering a compound of interest for preferential release at a biological site of interest as part of a method of treatment by therapy comprising:

- (a) obtaining a molecule of formula 1:

$BM1-L-(BM2)_n$ (1);

- (b) providing the compound of interest and the molecule of formula (1) at the biological site of interest; and,

- (c) providing a linker-specific molecule at the biological site of interest.

[0009] In an embodiment of the invention there is provided a molecule of formula I wherein the amino acid sequence is selected from at least one of SEQ. ID. NO. 8, 12, 17, 24, 27, 28, 37-47, 48, 49, 50-56, 57, 58, 59-60, 124-126, 127, or 128.

[0010] In an embodiment of the invention there is provided a molecule of formula I wherein BM1 comprises an amino acid sequence selected from: SEQ. ID. NO. 6, 9, 15, 19, 35, 68, 69-71, 72, 93, 92, 94-95, 116, 122 or linked sequences SEQ. ID. NO. 15 and SEQ. ID. NO. 16.

[0011] In an embodiment of the invention there is provided a molecule of formula I wherein BM2 comprises an amino acid sequence selected from SEQ. ID. NO. 1, 20, 36, 96-99.

[0012] In an embodiment of the invention there is provided a molecule of formula I comprising at least one amino acid sequence selected from SEQ. ID. NO. 2, 9, 10, 11, 13; 14, 16, 21, 22, 23, 25, 29, 32, 33, 34, 73, 74, 75, 76, 77, 78, 79, 80, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 100, 101, 102, 112, 115, 117, 119, 121, or 117.

Brief Description of the Figures

[0013]

Figure 1. Depicts an embodiment of a biomolecular tweezer structure (A) in which a ligand is designed in a bivalent fashion, containing two binding moieties ("heads"), and linked by a structurally polymeric linker. (B) Depicts a proposed thermodynamic principle of linker-mediated control of bivalent ligands.

Figure 2. Inhibition of fibrinogen clotting assays for embodiments of thrombin inhibitors of the general formula Bbs-R-(D-Pip)-linker-GDFEEIPEEYLQ. See further descriptions below:

Figure 2a. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-(GS)₂-GDFEEIPEEYLQ.

Figure 2b. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-(GS)₄-GDFEEIPEEYLQ.

Figure 2c. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-(GS)₆-GDFEEIPEEYLQ.

Figure 2d. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-(GS)₈-GDFEEIPEEYLQ.

Figure 2e. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Piop)-(GS)₁₀-GDFEEIPEEYLQ.

Figure 2f. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-(GS)₁₂-GDFEEIPEEYLQ.

5 **Figure 2g.** Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-(GS)₁₄-GDFEEIPEEYLQ.

Figure 2h. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-Gly-Cys ... Cys-(Gly-Ser)₈-Gly-DFEEIPEEYLQ.

10 **Figure 2i.** Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GTLDLNTPVDKTSN-GDFEEIPEEYLQ.

Figure 2j. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GSGSGSGSG-KGSGSGSGSGS-GDFEEIPEEYLQ.

15 **Figure 2k.** Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GSVPRPQLHND-GDFEEIPEEYLQ.

Figure 2l. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GSHAPRPQIHND-GDFEEIPEEYLQ.

Figure 2m. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GHHLGGAKQAGDV-GDFEEIPEEYLQ.

25 **Figure 2n.** Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GYMESRADR-GDFEEIPEEYLQ.

Figure 2o. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GQSHNR-GDFEEIPEEYLQ.

30 **Figure 3.** Inhibition of fibrinogen clotting assays for embodiments of thrombin inhibitors of the general formula Bbs-R-(D-Pip)-G-(SPH(B)EKVSG)_n-DFEEIPEEYLQ. See further descriptions below:

Figure 3a. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr-Glu-Lys-VaJ.-Ser-Gly)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.

35 **Figure 3b.** Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr (P)-Glu-Lys-Val-Ser-Gly)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.

Figure 3c. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly)₂-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.

40 **Figure 3d.** Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr (P)-Glu-Lys-Val-Ser-Gly)₂-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.

45 **Figure 4.** Inhibition of fibrinogen clotting assays for thrombin inhibitors of the general formula Bbs-R-(D-Pip)-G-(SPH(B)EKVSG)_n-DFEEIPEEYLQ in the presence and absence of an SH2 domain from the Grb4 adaptor protein. See further descriptions below:

Figure 4a. Inhibition of fibrinogen clotting by the thrombin inhibitors Bbs-R-(D-Pip)-G-(SPH-B-EKVSG)₂-DFEEIPEEYLQ in the presence and absence of an SH2 domain from the Grb4 adaptor protein.

Figure 4b. Inhibition of fibrinogen clotting by the thrombin inhibitors Bbs-R-(D-Pip)-G-(SPH-B-EKVSG)-DFEEIPEEYLQ in the presence and absence of an SH2 domain from the Grb4 adaptor protein.

55 **Figure 5.** Inhibition of fibrinogen clotting assays for thrombin inhibitor of the formula Bbs-R-(D-Pip)-GEQKLISEED-LG-DFEEIPEEYLQ in the presence and absence of the anti-c-myc antibody 9E10 (Sigma).

Figure 6. Effect of calcium on the NMR spectra of calcium-binding linkers. See further descriptions below:

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Figure 6a. Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-NH₂ upon the addition of CaCl₂.

5 **Figure 6b.** Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-NH₂ upon the addition of CaCl₂.

Figure 6c. Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH₂ upon the addition of CaCl₂.

10 **Figure 6d.** Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH₂ upon the addition of CaCl₂.

Figure 6e. Changes in the proton NMR spectra of Bbs-Arg-(D-Pip)-Gly-Cys...Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln upon the addition of CaCl₂.

15 **Figure 6f.** Changes in the proton NMR spectra of Bbs-Arg-(D-Pip)-Gly-Cys...Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln upon the addition of CaCl₂.

20 **Figure 7.** Effect of calcium on the inhibition of the amidolytic activity of thrombin by Bbs-Arg-(D-Pip)-Gly-Cys...Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.

Figure 8. Depicts the inhibition of fibrinogen clotting assays by mini-hirudin 1 and mini-hirudin 2. See further descriptions below:

25 **Figure 8a.** Inhibition of fibrinogen clotting by the thrombin inhibitor mini-hirudin 1.

Figure 8b. Inhibition of fibrinogen clotting by the thrombin inhibitor mini-hirudin 2.

30 **Figure 8c.** Inhibition of fibrinogen clotting by the thrombin inhibitor mini-hirudin 3.

Figure 9. Amino acid sequences of the CaM-DTI and CaM-DTI2 protein(s).

35 **Figure 10.** (A) Inhibition of the amidolytic activity of thrombin by CaM-DTI in the absence of Ca²⁺ (circle) and in the presence of 5 mM Ca²⁺ (square). (B) Inhibition of the amidolytic activity of thrombin by CaM-DTI2.

Figure 11. Depicts a summary of the CRIB-containing peptide fragments and their hybrids.

Figure 12. Depicts a bivalency model for two-site binding between extended CRIB peptides and Cdc42.

40 **Figure 13.** Depicts fluorescence titration of sNBD-labeled and GMPPCP-loaded CaCdc42 (R150K) with different CRIB peptides.

45 **Figure 14.** Figures 14A and 14B depict fluorescence titration assays of a SLAM-binding SH2 with an extended CRIB peptide containing the SLAM sequence as linker. Figures 14C, 14D and 14E depict inhibition of fibrinogen clotting assays for a bivalent thrombin inhibitor containing the SLAM peptide sequence as linker.

Figure 15. Depicts an embodiment of the preparation of conjugated stable complexes between Cdc42 and some extended CRIB peptides.

50 **Figure 15b.** Proton-15N NMR HSQC spectrum of the conjugated eCla4-CaCdc42 complex

Figure 15c. Proton-15N NMR HSQC spectrum of the conjugated eCst20-CaCdc42 complex

55 **Figure 15d.** Local dissociation of a bivalent ligand conjugated to the binding protein by a monovalent (L) molecule

Figure 16. Depicts a utility of a bivalent polypeptide with a controllable polymeric linker in the fabrication of biomolecular devices remotely activatable by radio frequency magnetic fields (RFMF).

Figure 16b. Activation of a bivalent ligand by localized heating of the conjugated binding protein

Figure 16c. Activation of a bivalent ligand by localized heating of the linker moiety

5 **Figure 17.** Is a schematic depiction of a utility of a bivalent polypeptide with a controllable polymeric linker in the dissection of cell-signaling pathways.

Figure 18. Depicts photographic data relating to the arrest of arterial bleeding facilitated by fibrin glue application.

10 Detailed Description of the Referred Embodiments

[0014] There is disclosed herein an approach combining independent binding moieties in a single molecular structure, which couples binding affinity to an on/off or modulatable switch. There is described a molecule which contains two or more binding moieties (or "heads") (Figure 1) joined by a linker. Looking at the embodiment of Figure 1, each binding moiety in isolation provides only moderate to weak binding affinity (typically up to hundreds of millimolar in dissociation constants) to its specific binding site on a biomolecular target as compared to binding affinity in a bivalent or multivalent complex. When linked together a resulting bivalent ligand binds to its target with a significantly increased affinity (in some instances at least about twice the affinity, in some instances at least about three times, in some instances at least about five times, in some instances at least about ten times.). A change in flexibility of the linker caused by its non-covalent binding to a linker-specific molecule, covalent modification of the linker, or ambient environmental change leads to a decrease or complete disruption of the molecule's ability to bind the target in a bivalent or multivalent mode (Figure 1). *Vise versa*, removal of constraints imposed on the linker can restore the high-affinity binding of the freed bivalent ligand. From a thermodynamic point of view, binding of a linker-specific well-structured protein (labelled by "N") confers on the polymeric linker a well-defined conformation enabling for the interaction, which substantially prevents the ligand from acting in a bivalent fashion (Figure 1B). Each binding moiety in isolation preferably has only low-affinity and transient interactions with an intrinsic dissociation constant up to the high millimolar range for its specific binding site on a target biomacromolecule. When linked together, the resulting bivalent or multivalent molecule makes a substantially stable complex with the target, achieving enhancement of preferably a minimum of two (2) fold in overall binding affinity as compared to the highest affinity of the constituent monovalent ligands. In the design disclosed herein, a polymeric linker is preferably used, such that on one hand, it allows both binding heads to settle freely in their binding sites on a macromolecular target, thereby improving the stability of the complex upon simultaneous occupation of the two individual binding sites. The linker can also be optimized to be selectively responsive to each or a combination of external signals. Since the specifics of the molecular structure of the polymeric linker would not be crucial for the binding association between the bivalent ligand and the target, the linkers and the pairs of binding "heads" are in principle interchangeable, allowing for a number of practical applications. It would be apparent to one skilled in the art, in light of the disclosure herein, how to select a suitable linker and binding heads for a specific purpose.

[0015] Feasibility and generality of this approach are assured by the abundance of pharmaceutically important proteins with multiple or forming large binding surface areas, e.g. thrombin and Cdc42. In addition, many of these proteins bind unfolded polypeptides, the latter becoming structured only when in complex with target proteins. These target proteins in particular are suitable for binding bivalent/multivalent ligands or serve as switching or modulating devices through binding to the polymeric linkers in tweezer-like bivalent or multivalent ligands.

[0016] As used herein the term "polymeric linker" includes an oligomeric or a polymeric linker without a well-defined three-dimensional structure in the free state of a ligand. Such linkers are capable of connecting a variety of binding moieties and have sufficient length and flexibility to allow simultaneous binding of the individual moieties, enabling a higher binding affinity to the desired molecular target than the affinity of each moiety taken alone. As used herein, the term "controllable polymeric linker" refers to a polymeric linker which allows external control of its flexibility or conformation. The loss or decrease of flexibility or change in conformation of the linker preferably impedes simultaneous binding of the binding moieties, thus producing a reversing effect on the enhanced affinity. Accordingly, the linker will generally be chosen and optimized for the affinity-reversing external signal instead of being optimized to achieve the highest affinity of binding of the ligand to its target.

[0017] The controllable linker is a flexible peptide or peptide bound to another material.

[0018] Occasionally, the linkers may exhibit some molecular interactions with the targets. In some other cases, linker-bound antidotes may produce steric hindrance of their own with the targets in conflicts with a potential bivalent mode of ligand binding. In some cases, an antidote may bind to both the polymeric linker and to a binding moiety. These influences can make additional contributions to reversing the bivalent binding upon antidote complexation. Regardless, in all these cases the linker can still be optimized according to its interaction with the antidote to achieve the desired affinity-reversing effect.

[0019] Approaches taken in the Specific Examples

[0020] Tolerance of the bivalent mode of inhibition to the properties of the linking sequences is shown in the examples by a series of inhibitors of thrombin containing an active site binding moiety Bbs-Arg-(D-Pip)-Gly [H1, Bbs=4-tert-butylbenzenesulfonyl, D-Pip=D-pipecolic acid, K_I in low μM range (Slon-Usakiewicz and others, 2000, 2384-2391)] and an exosite 1 binding moiety Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 1) derived from the C-terminal tail of hirudin (H2, K_I in low μM range). The H1 and H2 heads are linked by a variety of flexible sequences producing bivalent thrombin inhibitors with a general formula of Bbs-Arg-(D-Pip)-linker-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 2), where the linker is an amino acid sequence. With the wide range of linker lengths IC_{50} values of the bivalent inhibitors in fibrinogen clotting assays catalyzed by thrombin remained between 0.3 and 3 nM (Table 1 and Figure 2), which are sufficient for peptide-based antithrombotic agents, and much lower than the K_I values of the constituent binding moieties. In these specific examples, the C-terminal portion of the bivalent peptides consisted of only natural amino acids and included the polymeric linker plus the H2 moiety [-linker-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln] (SEQ. ID. NO. 3), which can be produced using recombinant methods. Linking of the H1 moiety, containing unnatural amino acids, with the rest of the peptide was performed using standard disulfide coupling techniques. For example, peptides with amino acid sequences of Bbs-Arg-(D-Pip)-Gly-Cys (SEQ. ID. NO. 4) and Cys-(Gly-Ser)₈-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 5) were synthesized and purified. A product of disulfide-bonded linkage between peptides Bbs-Arg-(D-Pip)-Gly-Cys (SEQ. ID. NO. 6) and Cys-(Gly-Ser)₈-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 7) was tested for IC_{50} in the fibrinogen clotting assay. It was established that the two-chain peptide was potent and therefore bivalent with an IC_{50} of 1.1 ± 0.2 nM (Figure 2). A variety of amino acid sequences with high complexity, originating from naturally-occurring proteins, or binding to naturally-occurring macromolecules, was introduced between the two binding heads producing potent inhibitors. It is noted that the use of only natural amino acids is not essential and non-natural amino acids and chemically modified amino acids (natural or non-natural) are also specifically contemplated for use in the design of controllable bivalent peptides.

[0021] Looking at the results of Figure 2, the assay employs bovine plasma fibrinogen dissolved at 0.1% in 50 mM Tris-Cl, 100 mM NaCl, 0.1% PEG-8000 at pH 7.6. Curves represent OD_{420} time course after the addition of 0.6 nM thrombin in the presence of (◆) 0 nM; (○) 0.5 nM; (□) 1 nM; (Δ) 1.5 nM; (◇) 2.5 nM; (●) 3.75 nM; (■) 6.25 nM; and (▲) 12.5 nM of the inhibitor with linker (GS)_n and n=2 (SEQ. ID. NO. 50) (a); n=4 (SEQ. ID. NO. 51) (b); n=6 (SEQ. ID. NO. 52) (c); n=8 (SEQ. ID. NO. 53) (d); n=10 (SEQ. ID. NO. 54) (e); and in the presence of (◆) 0 nM; (○) 1 nM; (□) 2 nM; (Δ) 4 nM; (◇) 6 nM; (●) 10 nM; (■) 15 nM; and (▲) 25 nM of the inhibitor with linker (GS)_n and n=12 (SEQ. ID. NO. 55) (f); n=14 (SEQ. ID. NO. 56) (g), at 37°C. The onset clotting time was determined as an intersection of the baseline and the extrapolated linear portion of the OD change curve. Extracted IC_{50} values are shown in Table 1. Curves (h) represent OD_{420} time course in the presence of (■) 0 nM; (○) 1 nM; (□) 3 nM; (Δ) 5 nM; (◇) 7 nM; and (●) 9 nM of a product of disulfide-bonded linkage between peptides Bbs-Arg-(D-Pip)-Gly-Cys and Cys-(Gly-Ser)₈-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 83), at 37°C. Curves (i) represent OD_{420} time course in the presence of (▲) 0 nM; (○) 1 nM; (□) 2 nM; (Δ) 4 nM; (◇) 6 nM; and (●) 15 nM of the inhibitor with linker GTLDLNTPVDKTSN (SEQ. ID. NO. 103), at 37°C. Curves (j) represent OD_{420} time course in the presence of (○) 0 nM; (□) 2 nM; (Δ) 4 nM; (◇) 6 nM; (●) 8 nM; (■) 10 nM; (▲) 12 nM; and (◆) 15 nM; of the inhibitor with linker GSGSGSGSGKSGSGSGSGS (SEQ. ID. NO. 58) at 25°C. Curves (k) represent OD_{420} time course in the presence of (○) 0 nM; (□) 0.5 nM; (Δ) 1 nM; (◇) 2 nM; (●) 3 nM; (■) 4 nM; (▲) 5 nM; and (◆) 6 nM; of the inhibitor with linker GSVVPRPQLHND (SEQ. ID. NO. 105) at 37°C. Curves (l) represent OD_{420} time course in the presence of (○) 0 nM; (□) 0.25 nM; (Δ) 0.5 nM; (◇) 1 nM; (●) 1.5 nM; (■) 2 nM; and (▲) 2.5 nM; of the inhibitor with linker GSHAPRPQIHND (SEQ. ID. NO. 104) at 37°C. Curves (m) represent OD_{420} time course in the presence of (○) 0 nM; (□) 2 nM; (Δ) 4 nM; (◇) 6 nM; (●) 8 nM; (■) 10 nM; and (▲) 12 nM; of the inhibitor with linker GHHLGGAKQAGDV (SEQ. ID. NO. 106) at 37°C. Curves (n) represent OD_{420} time course in the presence of (○) 0 nM; (□) 1 nM; (Δ) 2 nM; (◇) 3 nM; (●) 4 nM; (■) 5 nM; (▲) 6 nM; and (◆) 7 nM; of the inhibitor with linker GYMESRADR (SEQ. ID. NO. 107) at 37°C. Curves (o) represent OD_{420} time course in the presence of (○) 0 nM; (□) 4 nM; and (Δ) 8 nM; of the inhibitor with linker GQSHNR (SEQ. ID. NO. 108) at 37°C.

[0022] For some peptide-based linkers, modifications of amino acid side chains (such as tyrosine, serine or threonine phosphorylation by kinases, or dephosphorylation by phosphatases) will turn these peptides into binding ligands for signaling proteins and signaling protein subdomains or interrupt their specific interactions. The peptide sequence Cys-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly (SEQ. ID. NO. 8) derived from the ephrinB cytoplasmic tail (ephrinB2₃₀₁₋₃₀₉) was used to link the H1 and H2 heads. The peptide is flexible and in its tyrosine-phosphorylated state binds an SH2 domain from the Grb4 adaptor protein with an affinity of 0.2 μM (Su, Xu, and Ni, 2004b, 1725-1736). Four peptides of a general formula Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-B-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 9) were produced, wherein B was either tyrosine (Tyr) or O-phosphotyrosine (Tyr(P)), and n was 1 or 2. IC_{50} of the inhibitors in the fibrinogen clotting assay were comparable and in the vicinity of 0.5-1 nM, except for the peptide with two phosphotyrosines which had an IC_{50} of 18-20 nM (Table 1, Figure 3).

[0023] Looking at the results of Figure 3, the curves represent OD_{420} time course after the addition of 0.6 nM thrombin in the presence of (◆) 0 nM; (○) 1 nM; (□) 2 nM; (Δ) 4 nM; (◇) 6 nM; (●) 10 nM; (■) 15 nM; and (▲) 25 nM of inhibitor for n=1, B=Y (a); n=1, B=Y(P) (b); n=2, B=Y (c); n=2, B=Y(P) (d); at 25°C. Other experimental conditions were as used

for the assays described in Figure 2. Extracted IC_{50} values are shown in Table 1.

[0024] To reverse the inhibitory potency of the peptides they were brought in contact with the SH2 domain in solution. Presence of the SH2 domain reversed the inhibitory potency of the Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln peptide ((SEQ. ID. NO. 10), corresponding to (SEQ. ID. NO. 9) when B is Tyr (P)), but not that of Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln peptide ((SEQ. ID. NO. 11), corresponding to (SEQ. ID. NO. 9) when B is Tyr) (Figure 4). The change in thrombin inhibitory activity upon binding of SH2 makes it useful in an assay for protein-to-peptide binding, which in some embodiments could be implemented in a high-throughput manner.

[0025] Looking at the results of Figure 4, peptide inhibitors were designated in Table 1 as P3161 (n=2, B=Y); P3162 (n=2, B=Y(P)); P3169 (n=1, B=Y); and P3170 (n=1, B=Y(P)). The curves represent OD₄₂₀ time course after the addition of 0.6 nM thrombin in the presence of (a) (○) 2 nM P3161; (□) 2 nM P3161, 3 μM SH2; (Δ) 50 nM P3162; (◇) 50 nM P3162, 3 μM SH2; (●) no inhibitor, no SH2; (■) no inhibitor, 3 μM SH2; and (b) (○) 1 nM P3169; (○) 1 nM P3169, 3 μM SH2; (Δ) 4 nM P3170; (◇) 4 nM P3170, 3 μM SH2; (●) no inhibitor, no SH2; (■) no inhibitor, 3 μM SH2. Other experimental conditions were as used for assays described in Figure 3.

[0026] In another case a peptide linker Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu (also called the c-myc sequence, SEQ. ID NO. 12) known to bind an anti-c-myc antibody 9E10 with an affinity of approximately 0.5 μM (Hilpert et al. 2001, 803-806) was built into the bivalent thrombin inhibitor Bbs-Arg-(D-Pip)-Gly-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 13). The antibody 9E10 reversed the inhibitory potency of the inhibitor (Figure 5).

[0027] Looking at the results of Figure 5, the curves represent OD₄₂₀ time course in the presence (□,■) or absence (○,●) of 150 nM of the inhibitor. Addition of ~1.2 μM anti-c-myc antibody 9E10 (Sigma) only slightly slowed clotting of free thrombin (○), but reversed the inhibitory effect of the inhibitor (□). Other experimental conditions were as used for assays described in Figure 3.

[0028] In other cases, disulfide bonds can be formed or opened to rigidify or make the linkers more flexible. Limited specific proteolysis may turn a well-folded disulfide-bonded peptide into a polymeric linker, allowing for bivalent binding. In other instances amino acid side chain modifications producing two or more charged groups (as in the case of phosphorylation of an amino acid side chain) in the linker will generate electrostatic repulsion or attraction affecting the linker's flexibility and the end-to-end statistically average distance.

[0029] Incorporation of two phosphotyrosines in the polymeric linker of the peptide with the sequence Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly)₂-Asp-Phe-Glu-Glu-De-Pro-Glu-Glu-Tyr-Leu-Gln ((SEQ. ID. NO. 14), corresponding to (SEQ. ID. NO. 10) when n=2) produced a significant drop in inhibition potency as compared to the dephosphorylated analog (Figure 3). The potency of the bivalent inhibitor generally depends on the phosphorylation state of the linker. Thus, kinase or phosphatase activities can be converted into serine protease (thrombin) activity in a coupled enzymatic assay in light of the disclosure herein. In such an assay, the linker preferably contains tyrosine, or other residues that can be phosphorylated or dephosphorylated after phosphorylation. Therefore, reversible and irreversible posttranslational modifications of the linker can be used as another mechanism of controlling ligand (inhibitor) affinity.

[0030] Some flexible peptides will bind metal ions specifically (Figure 6). Organization of metal ion coordination sphere will change the flexibility of the peptides achieving the required affinity control. For example, a peptide Bbs-Arg-(D-Pip)-Gly-Cys...Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 91) was prepared through coupling of two peptides, Bbs-Arg-(D-Pip)-Gly-Cys (SEQ. ID. NO. 15) and Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 16) by means of a disulfide bond. The linker moiety of the bivalent peptide contains the sequence segment Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu (SEQ. ID. NO. 17) that binds calcium(II) with an affinity in the millimolar range. Calcium(II) addition altered inhibition of chromogenic substrate proteolysis by human α-thrombin observed in the presence of the peptide (Figure 7).

[0031] Looking at the results in Figure 6, panels a,b,c,d show changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-NH₂ (SEQ. ID. NO. 109) (P3230, panels a,b) and Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH₂ (SEQ. ID. NO. 110) (P3231, panels c,d) upon the addition to the initial -450 μL of the corresponding peptide in 20 mM sodium acetate-d₃ buffer, pH 5.5, containing 10% D₂O, of 1 μL (final CaCl₂ concentration ~0.22 mM), additional 2 μL (final CaCl₂ concentration ~0.66 mM), additional 10 μL (final CaCl₂ concentration ~2.8 mM) of 100 mM CaCl₂, and additional 10 μL (final CaCl₂ concentration ~23.9 mM) of 1 M CaCl₂. Panels e and f show changes in the proton NMR spectrum of a disulfide-linked peptide Bbs-Arg-(D-Pip)-Gly-Cys...Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. No. 16) upon the addition to the initial volume of ~450 μL of 1 μL (final CaCl₂ concentration ~0.22 mM), additional 2 μL (final CaCl₂ concentration ~0.66 mM) of 100 mM CaCl₂, and additional 10 μL (final CaCl₂ concentration ~22.2 mM) of 1 M CaCl₂.

[0032] Looking at the results in Figure 7, the effect was tested in the presence of two bivalent thrombin inhibitors, the calcium-binding Bbs-Arg-(D-Pip)-Gly-Cys...Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Tle-

Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. No. 16) (CaR), and control peptide Bbs-R-(D-Pip)-GSGSGSGS-GDFEEIPEEYLQ (SEQ. S10) (P3150). Curves represent OD₄₀₅ time course at 25°C after the addition of 0.6 nM thrombin to 50 µM S-3266 (Chromogenics) in the clotting buffer, and in the presence of (O) no inhibitors; (□) 150 nM CaR; (Δ) 150 nM CaR, 50 mM CaCl₂; (◇) 150 nM CaR, 100 mM CaCl₂; (●) 2 nM P3150; (■) 2 nM P3150, 50 mM CaCl₂; and (▲) 2 nM P3150, 100 mM CaCl₂.

[0033] Polypeptides containing only natural amino acids can also be used as starting points for the generation of a bivalent ligand with a controllable linker. In order to design a ligand with a controllable linker, at least two binding heads of adequate affinities to two distinct sites on a target should preferably be known. The binding heads can be discovered through *ab initio* screening or minimization of structurally or functionally characterized polypeptide interactions with its target. Outlining minimal regions of polypeptides capable of binding to their macromolecular targets ("hot spots") may produce a set of at least two peptide sequences, interacting with distinct sites on the target surface. Determination of minimal binding regions ("hot spots") can be carried out using spectroscopic (e.g. NMR spectroscopy) or recombinant (e.g. alanine scan) methods. Through minimization of hirudin two peptides were designed having sequences of Val-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Lys-Pro-Gln-Ser-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (mini-hirudin 1) (SEQ. ID. NO. 22) and Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Asn-Pro-Glu-Ser-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (mini-hirudin 2) (SEQ. ID. NO. 23) incorporating N-terminal and C-terminal moieties believed to interact with the active site and exosite I of thrombin, respectively. These peptides displayed high potencies for thrombin inhibition with IC₅₀ of 33±3 nM (mini-hirudin 1) and 14±1 nM (mini-hirudin 2), indicating a bivalent mode of binding (Figure 8). The modular character of interaction was further confirmed when a candidacidal peptide known to bind laminarin (Polonelli, L.; and others, 2003, 6205-6212), in other words, the peptide of the sequence -Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser-(SEQ. ID. NO. 24), was inserted as a linker into mini-hirudin 2 to give mini-hirudin 3 with a sequence of Ile-Arg-Phe-Thr-Asp-Gly-Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 25). The peptide was shown to preserve high affinity of binding to thrombin, with an IC₅₀ of 10±1 nM (Figure 8).

[0034] Looking at the results in Figure 8, curves (a) represent OD₄₂₀ time course in the presence of (O) 0 nM; (□) 10 nM; (Δ) 30 nM; (◇) 50 nM; (●) 70 nM; and (■) 100 nM of mini-hirudin 1 at 37°C. Curves (b) represent OD₄₂₀ time course in the presence of (O) 0 nM; (O) 4 nM; (Δ) 8 nM; (◇) 12 nM; (●) 20 nM; (■) 30 nM; (▲) 50 nM; and (◆) 100 nM of mini-hirudin 2 at 37°C. Curves (c) represent OD₄₂₀ time course in the presence of (O) 0 nM; (D) 2.15 nM; (Δ) 4.3 nM; (◇) 8.6 nM; (●) 17.2 nM; and (■) 43 nM of mini-hirudin 3 at 37°C. Other experimental conditions are as used for assays shown in Figure 2.

[0035] The peptide with a sequence of Trp-Asp-Pro-Arg-Pro-Gln-Arg-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 18) is a bivalent molecule with a K_i of ~17 nM for thrombin inhibition. The peptide can be decomposed into two moieties, an active site binding moiety, Trp-Asp-Pro-Arg-Pro-Gln-Arg-His (SEQ. ID. NO. 19), and an exosite-1 binding moiety, Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 20). A thrombin inhibitor was prepared with the sequence of Trp-Asp-Pro-Arg-Pro-Gln-Arg-His-(CamCKK)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 21), designated as CaM-DTI, where CamCKK is a protein with calcium-binding properties (see for example Truong, 2001, 1069-1073). The sequence of the CaM-DTI molecule is shown in Figure 9. As depicted in Figure 9, the sequence includes at the N-terminus a binding moiety (bold) to the thrombin active site, a binding moiety (italic bold) to the exosite-1 of thrombin at the C-terminus, and a calcium-responsive protein linker. CaM-DTI was prepared with a recombinant DNA approach. Potency of thrombin inhibition by CaM-DTI was determined by an amidolytic assay. Upon the addition of 5 mM Ca²⁺, an increase in apparent K_i from 480 nM (no calcium) to 2200 nM (Ca²⁺) was observed (Figure 10).

[0036] In another case, the small GTPase Cdc42 binds with high-affinities to the ~40-residue extended CRIB domains of the *Candida* Cla4 and Ste20 kinases (K_D= 20 ~ 50 nM) (the latter also known as Cst20). When subjected to NMR relaxation dispersion analysis (Tolkatchev, Xu, and Ni, 2003b, 12432-12442), these complexes exhibit no responses, as expected for a tight binding complex. The full-length CRIB domains were decomposed into two peptide fragments (Figure 11): (i) mCla4 (mCst20) including the consensus CRIB motif, and (ii) cCla4 (cCst20) which comprises residues directly to the C-terminus of the minimal CRIB sequence. Looking at Figure 11, the extended CRIB fragments (eCRIBs) comprise the CRIB motif, plus -20 residues to the C-terminus and exhibit high-affinity binding to CaCdc42. These sequences were dissected into two fragments: the minimal CRIB (mCRIB), mCla4 and mCst20, and the C-terminal CRIB (cCRIB), cCla4 and cCst20. In order to construct a bivalent peptide ligand for *Candida* Cdc42 (CaCdc42) with a suitable linker, the binding affinities of these component peptides derived from the CRIB domains of *Candida* Cla4 and Ste20 were determined. For this purpose, a CaCdc42 expression vector encoding the R150K mutation was constructed and the sequence of the R150K CaCdc42 mutant (Table 4) was verified by DNA sequencing.

[0037] Figure 12 depicts a bivalency model for two-site binding between extended CRIB peptides and Cdc42. This dissectional strategy is used to analyse the interaction of the CRIB fragments with CaCdc42 (A). The m and c represent the mCRIB and the cCRIB fragments, respectively, as defined in Figure 11. Dissociation constants and corresponding Gibbs free energies are indicated according to the reaction coordinate. (B) depicts the bivalent binding mode of covalently-

linked CRIB sub-fragments with Cdc42. The mCRIB and cCRIB sequences are assumed to have the same "intrinsic" binding affinities after linkage. An additional factor C_{eff} is introduced together with the cooperativity factors, c_{12} and c_{21} to define the partial dissociation constants of the individual dissociation steps. The thermodynamic dissociation constant representing complete dissociation of the extended "bivalent" CRIB peptide can be deduced following microscopic equilibria from either one of the two dissociation pathways.

[0038] Figure 13 shows binding isotherms obtained following the CRIB-induced changes in the sNBD fluorescence of the CaCdc42 (R150K) protein. All the titration curves could be best fitted to a simple bimolecular binding model. The average apparent K_d values for different CRIB peptides are summarized in Table 2. As expected, the extended CRIB (eCRIB) fragments exhibited the strongest affinities to CaCdc42 in the low nanomolar range. The mCRIB fragments containing the consensus CRIB sequence, ISXPXXFXBXXHVGXD (SEQ. ID. NO. 26) (Burbelo, P. D., Drechsel, D., and Hall, A., 1995, 29071-29074), also had moderately strong binding affinities in micromolar concentrations, but clearly, as seen previously for the human PAK homologues (Rudolph, M. G., Bayer, P., Abo, A., Kuhlmann, J., Vetter, I. R., and Wittinghofer, A., 1998, 18067-18076; Thompson, G., Owen, D., Chalk, P. A., and Lowe, P. N., 1998, 7885-7891), require extra residues to retain stronger binding to Cdc42. The cCRIB peptides exhibited much weaker affinities to the CaCdc42 protein. The K_d value of cCla4 for binding to CaCdc42 is in a high micromolar concentration (275 μ M). An even weaker binding ($K_d = 1160 \mu$ M) was observed between cCst20 and CaCdc42 with the current fluorescence titration strategy.

[0039] Looking at the results of Figure 13, one micromolar concentration of sNBD-labeled, and GMPPCP-loaded CaCdc42 (R150K) was titrated with the indicated amounts of CRIB fragments shown in Figure 11. (A) eCla4 (*open circle*) and eCst20 (*open triangle*); (B) mCla4 (*open circle*) and mCst20 (*open triangle*), and (C) cCla4 (*open circle*), cCst20 (*open triangle*), cCla4 in the presence of 50 μ M mCla4 (*filled circle*) and cCst20 in the presence of 50 μ M mCst20 (*filled triangle*). Solid lines represent fits of the data to a bimolecular association model.

[0040] Fluorescence measurements were used to substantiate and quantify the effects of cross-titrations observed by NMR (Table 2). The affinity of the Cla4 peptide fragments for CaCdc42 was not significantly affected by the addition of the cognate peptide. In contrast, the affinities of the Cst20 peptide fragments preincubated with CaCdc42 exhibited a dramatic enhancement in binding for CaCdc42 by ~ 5.5 -fold, upon addition of the cognate Cst20 peptide (Table 2). Thus, upon addition of mCst20 to the cCst20/CaCdc42 complex, the affinity of cCst20 for CaCdc42 increased from a K_d of 1160 μ M to 207 μ M (Table 2 and Figure 13c). Similarly, mCst20 affinity for CaCdc42 increased from 0.43 μ M to 0.081 μ M when cCst20 was added to a preincubated mCst20/CaCdc42 complex. These results strongly suggest that the eCst20 and eCla4 peptides exhibit different mechanisms for binding CaCdc42, in which long eCst20 peptide utilizes a cooperative mechanism for high-affinity interaction while eCla4 does not.

[0041] Modular nature of interactions of m- and c- CRIB fragments is emphasized by the binding affinities of hybrid peptides incorporating m- and c- CRIBs from different molecular species. Both mCla4-cCst20 and mCst20-P-cCla4 constructs (Figure 11) displayed affinities of the same order of magnitude as the original eCRIB peptides (Table 2). Moreover, incorporation of -Ser-Gly-Ser-Gly- (SEQ. ID. NO. 27) and -Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys- (SEQ. ID. NO. 28), in other words the SLAM peptide sequence (Li et al and Pawson, Curr. Biol. 9, 1355-1362, 1999) as linkers into the eCla4 sequence preserved a bivalent mode of binding.

[0042] In addition to bivalent binding to CaCdc42, the eCla4-SLAM peptide (Figure 11 and Table 2) also preserved the binding capacity of the SLAM linker peptide to the SH2 domain derived from the SAP protein (Table 4). Figure 14A and Figure 14B depict the competition binding of SAP-SH2 and CaCdc42 to the polypeptide eCla4-SLAM. Figure 14A shows the effect of including SAP-SH2 at various concentrations on the binding affinity of eCla4-SLAM to CaCdc42 R150K. The concentration of CaCdc42 R150K is 1 μ M. Figure 14B shown that the apparent dissociation constant for the Cdc42-eCla4 complex is a function of the concentration of added SAP-SH2

[0043] The SLAM sequence, i.e. the peptide of SEQ.ID.NO.28 can also be used as a linker moiety with a bivalent thrombin molecule. Figures 14C, 14D and 14E depict inhibition of fibrinogen clotting assays by the thrombin inhibitor Bbs-Arg-dPip-Gly-Arg-Lys-Ser-Leu- Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 102) in the presence and absence of an SH2 domain from the SAP protein (SAP-SH2). This thrombin inhibitor is designated as P3291. The curves represent OD₄₂₀ time course after the addition of 0.6 nM thrombin in the presence of (C) (○) 0 nM P3291; (□) 10 nM P3291; (Δ) 15 nM P3291; (◇) 20 nM P3291; (●) 30 nM P3291; (■) 40 nM P3291; (▲) 50 nM P3291; (◆) 60 nM P3291; (D) (○) 0 nM P3291, 0 μ M SAP-SH2; (□) 0 nM P3291, 5 μ M SAP-SH2; (Δ) 25 nM P3291, 0 μ M SAP-SH2; (◇) 25 nM P3291, 5 μ M SAP-SH2; (E) (○) 0 nM P3291, 0 μ M SAP-SH2; (□) 0 nM P3291, 10 μ M SAP-SH2; (Δ) 25 nM P3291, 0 μ M SAP-SH2; (◇) 25 nM P3291, 10 μ M SAP-SH2. Other experimental conditions were as used in assays described in Figure 3.

[0044] Generally, linkers are elongate oligomeric or polymeric molecules adapted to permit strong covalent attachment or strong electrostatic binding of at least two moieties, wherein the moieties spaced apart along said linker. Linkers are preferably "modulatable linkers", in other words, linkers which undergo a change in flexibility and/or conformation in response to a defined environmental condition such as pH, temperature, proteolysis, chemical modification, magnetic field, local concentration of one or more molecules or complexes. Examples of temperature-sensitive linkers include polypeptides containing the elastin repeats (Urry, 1997, 11007-11028), specifically the (VPGVG)₁₉-VPGV (SEQ. ID.

NO. 118) peptide which is producible by recombinant DNA methods (McPherson and others, 1992, 347-352). Other examples of peptide linkers responsive to protein binding including short linear peptide motifs known for cell compartment targeting, protein-protein interactions, and regulation by post-translation modifications (Puntervoll and others, 2003, 3625-3630; Diella and others, 2004, 79).

5 **[0045]** Introduction of a long oligomeric or polymeric linker between two binding heads may reduce the affinity beyond the point where the bivalent ligand is no longer switchable for a desired application. This is particularly true if one of the binding moieties is significantly weaker than the other, as is the case for the CaM-DT1 and CaM-DT12 proteins (Figure 10) (see Table 2 for other examples). In addition, long oligomeric linkers are often more flexible, giving rise to significantly larger statistical distances spanning any given covalent spacing (Bright, Woolf and Hoh, 2001, 131-173). In this case
10 the observed affinity is approximately equal to the monovalent affinity of the stronger binding moiety, in other words, the bivalent increase in affinity is lost. One can remedy this situation by optimizing the binding affinities of the individual heads through combinatorial library selection.

[0046] A strategy to improve the binding affinity of polypeptide ligands consisting of natural amino acids is to utilize phage display optimization. If one of the binding moieties is sufficiently strong, phage-displayed peptides need to be randomized only in the vicinity of the other binding moiety. The reappearing bivalency allows strong affinity of the ligand and the corresponding polypeptide sequences will be readily selected from a medium-sized phage library. Alternatively or additionally, NMR relaxation dispersion techniques can be used to identify an appropriate candidate from a fragment library for subsequent linkage to the other binding moiety. The advantage of this new NMR-based approach lies in its ability to provide both the molecular structure (identity) and ranking of the dissociation kinetics of hit fragments. Such
15 an NMR-based screening can also be applied to molecules that are either natural polypeptides or other chemical entities available only through chemical synthesis.

[0047] In addition, if more than two distinct sites interacting with their specific ligands are known on a target surface, one can design an inhibitor containing more than two binding heads and link them with two or more controllable linkers, identical or different.

25 **[0048]** Production of polypeptides containing multiple binding moieties and controllable linkers can be achieved either through chemical peptide synthesis or using recombinant methods. Additional opportunities are provided by the possibilities to conjugate peptide fragments using thiol, primary amine or carboxyl chemistries. In light of the disclosure herein, one skilled in the art could readily produce such polypeptides. For example, thiol chemistry is particularly effective for coupling oligonucleotides to peptides (Lin and others, 1995, 11044-11048) in the fabrication of biomolecular devices containing oligonucleotides as linkers (Figure 16C).
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[0049] Molecular species and methods of the invention can be used in a number of screening methods. In some instances the recorded compound ("readout") is preferably chromogenic or fluorogenic. For example, some commercially available substrates for thrombin are based on p-nitroaniline (chromogenic) or on 6-amino-1-naphthalenesulfonamide (fluorogenic). The presence of an inhibitor impedes development of color or fluorescence in an assay that can be readily performed in a 96-well plate and recorded by a plate-reader. If the inhibitor contains a linker sensitive to a certain type
35 of specific peptide-protein molecular interaction, the presence of linker-binding protein can be identified in a 96-well plate format. Alternatively, the same 96-well plate format can be used for the identification of an enzymatic activity (e.g. performed by such enzymes as phosphatases or kinases) changing the ability of the linker to bind a known protein or altering flexibility of the linker. In this regard, binding or enzymatic activities are converted and recorded in the activities or changes in the activities of the target protein (e.g. thrombin). As such, it will be complementary to fluorescence-based methods (UK patent Application, GB 2375538) that have limitations in dynamic-range imposed by specific conformational changes (Truong and others, 2001, 1069-1073).
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[0050] Figure 15 depicts the concept of covalent conjugations between tweezer-like polypeptides and the binding proteins. Figure 15A is a model for a conjugated complex of CaCdc42 and an eCRIB via a polymeric linker. Figure 15B depicts resonance assignment of the ^1H - ^{15}N HSQC spectra for *Candida* Cla4-eCRIB in a conjugated complex with CaCdc42. The resonance peaks have the same pattern as those in the non-covalent complex. However, the conjugated complex is significantly more stable (>3 months) than the non-covalent one (<one week). Figure 15C shows the ^1H - ^{15}N HSQC spectrum for *Candida* Cst20-eCRIB in a conjugated complex with CaCdc42. Figure 15D shows the potential application of a stable conjugated complex for discovering stronger and specific binders. The dissociation of the tethered bivalent polypeptide can be detected by use of NMR relaxation and H/D exchange experiments using the assigned
45 ^1H - ^{15}N HSQC spectrum of the conjugated protein (see Figure 15B and 15C).
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[0051] Certain polypeptides are known to undergo folding or unfolding transitions upon changes of pH, ionic strength or temperature. Inhibitors incorporating these flexible peptides as linkers will be affected in their potency by the corresponding environmental changes. In an embodiment of the invention, the controllable linker is a well-folded and structured biomolecule, whose rigid three-dimensional structure prevents the binding of the bivalent ligand in the high-affinity mode. Defined three-dimensional structure of the biomolecule can be denatured by a variety of environmental effects such as changes in pH, temperature, proteolysis, chemical modifications and localized electromagnetic irradiation. Such a denaturation will render the linker moiety flexible, thereby providing the linker moiety with suitable physicochemical prop-
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erties for bivalent ligand binding to its target.

[0052] Bivalent polypeptides at the generic level are responsive to signals that modulate the physicochemical properties of the linker moiety (Figure 1). When the linker is responsive to binding, e.g. of a protein or of an oligonucleotide, or other biomolecules, the active concentration of the linker binding molecule can generally be reduced through denaturation, e.g. by use of radio-wave (or radio-frequency magnetic field, RFMF) induced biomolecular heating (Hamad-Schifferli and others, 2002, 152-155). Such a reduction in concentration of the activity-reversing protein will be accompanied by the reactivation of the inhibited target protein, a phenomenon governed by thermodynamic principles (Figure 1B).

[0053] Figure 16 depicts three scenarios by which the bivalent polypeptides can be used to fabricate biomolecular devices sensitive to electromagnetic irradiation. Figure 16A shows a generally inactive complex produced by the binding of the linker moiety of a bivalent peptide to a linker-specific protein. The linker-binding protein is in addition conjugated to a heat-transducing nanoparticle, which in the illustrated case is a gold nanoparticle as described previously (Hamad-Schifferli and others, 2002, 152-155). Upon electromagnetic (RF fields) irradiation, the linker-binding (antidote) protein will be denatured upon heating, leading to release and activation of the bivalent polypeptide. Figure 16B depicts the construction of a covalent conjugate of the bivalent polypeptide with a heat-transducing antidote protein. The bivalent polypeptide is more efficiently inactivated in a covalent conjugate as a result of intramolecular effects. RF irradiation will lead to local release of the bivalent peptide, in a way suitable for target binding through bivalent interactions (Figure 1). Figure 16C depicts an extension of the fabrication procedure illustrated in Figure 16B. In this scenario, the linker moiety itself confers the antidote effect, in that the linker can fold into a defined three-dimensional structure with geometry not suitable for bivalent binding of the attached monovalent ligands. Presence of RF fields will denature the structure of the linker moiety, thereby creating a polymeric linker conformation suitable for bivalency effects. In this regard, the CaM-DTI proteins (Figure 9) can be attached to a gold nanoparticle or a magnetic nanoparticle (MNPs) and RF irradiation is expected to enhance thrombin inhibitory activity of a MNP-conjugated CaM-DTI protein.

[0054] In an embodiment of the invention, the linker moiety is an oligonucleotide, to which is attached covalently two weak-binding monovalent ligands. The oligonucleotide linker is in addition labeled by a gold or magnetic nanoparticle for inductive coupling to and activation by an external field. Specifically, Bbs-Arg-(dPip)-Gly-Cys (SEQ.ID.NO.15) is to be coupled using thiol chemistry to the 3' or 5' end of a single-stranded DNA (e.g. the DNA-I molecule or 5'-TAGCGA-TACTGCGTGGGTTGGGGCGGGTAGGGCCAGCAGTCTCGT-3' of Lin et al and Jayasena (Lin and others, 1995, 11044-11048) or 5'-GCGCCCTAAACTGGTGGT*GGAATGCGTCATGAGGGCGC-3' of Hamad-Schifferli et al and Jacobson (Hamad-Schifferli and others, 2002, 152-155). The other end of the DNA molecule will be attached covalently with a peptide containing the sequence Asp-Phe-Glu-Gly-Ile-Pro-Glu-Glu-Tyr-Gln. Denaturation of the single-stranded DNA hairpin should activate the bivalent functionality of the attached peptides for high-affinity thrombin inhibition in the presence of an RF magnetic field (Figure 16C).

[0055] In some cases molecular species and methods of the invention can be used to specifically dissect, interrupt or initiate biological pathways. One can design a bivalent ligand with a trigger to release its target at a certain location and/or at a specific time. The ligand/target pair can be delivered together or separately using known methods of extra or intracellular delivery including protein expression from an oligonucleotide template. Alternatively, the target can occur naturally, outside or inside the cell, e.g. the GTPase of the Rho-family, Cdc42 (Figure 15). The triggering molecular device can be a delivered molecule or a naturally occurring molecule. The triggering molecular device can be a molecular process (for example, catalytic phosphorylation, dephosphorylation, or specific proteolysis). The triggering molecular device can be localized and/or produced and initiated at a certain time point. For example, a bivalent CRIB-based ligand of Cdc42 can be delivered into the cell to arrest the action of membrane-anchored Cdc42 (Figure 15). The inhibitory action of intracellularly-delivered and membrane-localized CRIB peptides can be reversed by the binding of the linker portion (i.e. the SLAM segment of eCla4-SLAM in Figure 11 and Table 2) to an SH2 domain. The affinity of the SH2-linker interaction is relatively weak, with a thermodynamic dissociation constant in the micromolar range (~1 μ M, see Figures 14A and 14B). Therefore, the SH2 domain (i.e. the antidote) is to be conjugated to the surface a nanoparticle for affinity enhancement through multivalent presentation. Vice versa, the complexes of the CRIBS peptides with the SH2 molecules conjugated to metal or magnetic nanoparticles (MNPs) can be disrupted by radio-wave induced heating of MNPs, as reported previously (Jordan and others, 1999, 413-419; Hamad-Schifferli and others, 2002, 152-155; also see Figure 16).

[0056] Figure 17 depicts schematically the use of a bivalent polypeptide with a controllable polymeric linker in the examination of cell-signaling pathways. In the embodiment of Figure 17, a bivalent CRIB polypeptide is to be delivered into the cytoplasmic space of a cell, specifically for associations with the cytoplasmic face of the cell membrane. As such, the CRIB peptide (e.g. the eCla4 peptide containing the - Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys- sequence (SEQ. ID. NO. 28), or eCla4-SLAM (Figure 11 and Table 2) will inhibit the membrane-anchored Cdc42 for its interactions with downstream effector proteins. This inhibitory action can be reversed by the delivery of an SH2 domain (i.e. the antidote or "A") with specific binding to the linker portion. For affinity enhancement, the SH2 domain antidote can be conjugated to the surface of a nanoparticle for multivalent presentation. A metal or magnetic nanoparticle (MNPs) is used here since these nanoparticles can transduce radio-frequency waves into heat (for both metal and magnetic

nanoparticles, see also Figure 16) or can be used as contrast agents (magnetic nanoparticles) in magnetic resonance imaging (MRI) applications.

[0057] In light of the disclosures provided herein, it should be apparent to ones skilled in the art that Cdc42 inhibition can be achieved by any number of suitable polypeptides (see for example Pirone, Carter and Burbelo, Trends in Genetics 17, 370-373, 2001) containing sequences homologous to the extended CRIB sequences derived from *Candida albicans* Cla4 and Cst20 proteins (Figure 11). In these applications, monovalent CRIB fragments will be identified following the same procedures as used for *Candida* proteins (Figures 11-13). Such peptide fragments will then be reassembled into bivalent polypeptides, containing as linkers either the SLAM sequence (SEQ.ID.NO.28) or other linear peptide motifs (Puntervoll and others, 2003, 3625-3630; Diella and others, 2004, 79) depending on the applications. Furthermore, all the bivalent peptides including eCla4-SLAM (Figure 11 and Table 2) are preferably prepared in palmitoylated forms, which enable intracellular delivery and localization to the cytoplasmic face of the cell membrane (Covic et al, and Kuliopulos, Proc. Natl. Acad. Sci. 99, 643, 2002).

[0058] Molecular species and methods of the invention can also be used to design new molecules for pharmaceutical intervention. Medical intervention in case of an injury to an internal organ requires a strategy to seal the wound. Fibrin sealant is found to be effective and can be used safely on vital organs. It is thus widely used as a bioactive hemostat in cases of both superficial and internal injury. The formulation that is commercially available (e.g. Tisseel VH Fibrin Sealant, Baxter) consists of two components: thrombin and fibrinogen. When both components are reconstituted and mixed thrombin catalyses the conversion of fibrinogen to fibrin, which in turn forms a fibrin scaffold or sealant. One of the limitations of the present formulation is that once reconstituted, thrombin proteolytically degrades itself. In light of the disclosure herein there is provided a new formulation, wherein the proteolytic activity of thrombin is inhibited by a stimulatory-responsive bivalent inhibitor e.g. Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P))-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (P3170) (SEQ. ID. NO. 29) with a controllable polymeric linker binding to an SH2 domain (Figure 4). In the new formulation of fibrin glue, thrombin and inhibitor can be premixed and will stay substantially inactive (and stable) for a reasonable period of time until exposed to SH2.

[0059] The middle auricular artery of eight rabbits was cut transversely with a scalpel. Two rabbits were left untreated to measure the bleeding time. The Tisseel fibrin glue (one fresh sample, one incubated overnight at 37°C) was applied to the wound of two other rabbits. The fibrin glue containing inhibitor-stabilized thrombin component, "Thrombin 4", was applied to another pair of rabbits immediately after activation with SH2. SH2-activated fibrin glue with the P3170 inhibitor was able to seal the wound at around ~5 minutes (Figure 18).

[0060] Looking at Figure 18, the top panel shows arterial bleeding from a rabbit ear 5 minutes after the transversal cut was made. Left bottom panel shows an arrest of the bleeding 1'47" after a commercial Tisseel preparation was applied to the fresh cut according to the manufacturer's procedure. Right bottom panel shows an arrest of bleeding 5 minutes after an inhibited and reactivated commercial Tisseel preparation was applied to the fresh cut according to the manufacturer's instructions. Thrombin inhibition was achieved by the addition of 8 nM of P3170 to the reconstituted "Thrombin 4" component of the Tisseel product. Thrombin activation was achieved by the inclusion of concentrated SH2 solution in the fibrinogen component of Tisseel to a final concentration of 12 µM.

[0061] In light of the disclosures provided herein, it will be apparent to one skilled in the art that other forms of fibrin sealants can be formulated. In particular, bivalent thrombin inhibitors with Ca⁺⁺-sensitive linkers can be used to inactivate (and stabilize) thrombin. The inactivated thrombin can in turn be reactivated upon contact with the bleeding wounds, wherein the fresh blood contains Ca⁺⁺ ions in millimolar concentrations. As well, the SH2-binding linker can be replaced by linker peptides with specific binding to other components of the blood, e.g. to integrin receptors on platelet surfaces (i.e. peptides P3234 and P3238 of Table 1, or SEQ. ID. NO. 88 and SEQ. ID. NO. 90), to fibrinogen itself (i.e. peptide P3236, Table 1 or SEQ. ID. NO. 89), to prothrombin (see the next section) and even to human serum albumin. One peptide sequence to use for the latter can be Leu-Ile-Glu-Asp-Ee-Cys-Leu-Pro-Arg-Trp-Gly-Cys-Leu-Trp-Glu-Asp (SEQ. ID. NO. 111), which is derived from panning a phage library against human serum albumin (Dennis and others, 2002, 35035-35043). One bivalent thrombin inhibitor containing an albumin-binding linker will have the sequence of Bbs-Arg-(D-Pip)-Gly-Leu-Ile-Glu-Asp-Be-Cys-Leu-Pro-Arg-Trp-Gly-Cys-Leu-Trp-Glu-Asp-Gly-Asp-Phe-Gln-Gm-ne-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 112). One can envision a bivalent thrombin inhibitor of the formula Bbs-Arg-dPip-Gly-(Val-Pro-Gly-Val-Gly)₂₀-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln, (SEQ. ID. NO. 119) containing as linker a temperature responsive elastin-repeat peptide Gly-(Val-Pro-Gly-Val-Gly)₁₉-Val-Pro-Gly-Val (SEQ. ID. NO. 120) (McPherson and others, 1992, 347-352). An analogue of this peptide suitable for recombinant production will have the formula of Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-(Val-Pro-Gly-Val-Gly)₂₀-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Leu-Gln (SEQ. ID. NO. 121) with the Bbs-Arg-dPip-Gly (SEQ. ID. NO. 122) moiety replaced by Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly (SEQ. ID. NO. 116) for binding to the thrombin active site. Other thrombin inhibitors can also be constructed that contain as linkers with specific binding to other blood-borne proteins. For example, these peptide sequences and binding proteins can be selected from the database of linear peptide motifs as published previously (Puntervoll and others, 2003, 3625-3630). The different means of thrombin inhibition and re-activation can be combined to address specific requirements for the properties of new fibrin sealants.

[0062] In another case bivalent thrombin inhibitors were generated, which can bind to (and be neutralized by) prothrombin. One clinical application of such inhibitors is in the formulation of new fibrin sealants using inactivated thrombin that can be reactivated by prothrombin (vide supra). Another clinical application of this type of inhibitors is to display potency of thrombin inhibition only at a location with low prothrombin concentration due to its binding to prothrombinase and rapid turnover into thrombin (e.g. localized to the site of an atherosclerotic plaque). C-termini of the inhibitors contain hirudin residues 55-65, a fragment known to bind proexosite I of prothrombin with low affinity (Ni, F., Ning, Q., Jackson, C.M., and Fenton, J.W., 1993, 16899-16902; Anderson, P.J.; Nasset, A.; Dharmawardana, K.R.; and Bock, P.E., 2000, 16428-16434; Tolkathev, Xu and Ni, 2003, JACS 12432-12442). A linker is engineered to provide additional contacts with prothrombin and confer much stronger specific affinity of the inhibitor to prothrombin. A phage-displayed peptide library was designed and constructed (preparation of the library is described in Su, Z.; Vinogradova, A.; Koutychenko, A.; Tolkathev, D.; and Ni, F., 2004a, 647-657). The library was panned against prothrombin immobilized on the bottom of a MaxiSorp plate well. Panning enhanced growth of two phage species containing displayed sequences Gly-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 30) and Gly-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 31). Discovered sequences were used to construct two bivalent thrombin inhibitors, Bbs-Arg-(D-Pip)-Gly-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 32) and Bbs-Arg-(D-Pip)-Gly-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 33). Measured IC_{50} were 1.1 and 0.6 nM, respectively, indicating the bivalent nature of inhibitor interaction with thrombin was retained (Figure 2, Table 1). Further improvement of the linker includes panning against phage-displayed peptide library with four randomized residues in the sequence Gly-Ser-Val-Val-Pro-Asn-XXX-XXX-Leu-XXX-XXX-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 34). Specifically, bio-panning of new phage libraries against prothrombin will expand the sequence hits from the two sequences shown (i.e. SEQ. ID. NO. 30 and SEQ. ID. NO. 31), leading to bivalent peptides with adequate binding affinities to prothrombin. These new prothrombin-binding polypeptides are conjugated through their N-termini to the Bbs-Arg-(D-Pip) (SEQ. ID. NO. 68) moiety to create high-affinity bivalent inhibitors of thrombin, as demonstrated with peptides Bbs-Arg-(D-Pip)-Gly-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 32) and Bbs-Arg-(D-Pip)-Gly-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 33). Ultimately, a dual-affinity polypeptide is to be selected from this process. In other words, one can generate polypeptides with high-affinity bivalent binding of and inhibition against thrombin and at the same time with suitable binding affinities to prothrombin and whose thrombin-binding potency can be neutralized by circulating concentrations of prothrombin (in the range of a few micromolar in normal plasma).

Specific Examples

[0063] *Example 1.* A tolerance of the bivalent mode of inhibition to the amino acid composition of the linker moiety on a series of bivalent inhibitors of thrombin with an active site binding moiety Bbs-Arg-(D-Pip)-Gly (HI, Bbs=4-*tert*-butylbenzenesulfonyl, D-Pip=D-pipecolic acid, K_I in low μ M range (SEQ. ID. NO. 35) (Slon-Usakiewicz and others, 2000, 2384-2391) and an exosite 1 binding moiety Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln ((SEQ. ID. NO. 36), H2, K_I in low μ M range) derived from the C-terminal tail of hirudin was demonstrated. The peptides were synthesized using standard Fmoc chemistry. Crude peptides were purified by HPLC using a reversed-phase C_{18} Vydac column and a linear 10-45% or 20-45% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). Peptides were freeze-dried and their identity was confirmed by ion-spray mass spectrometry. Clotting assays were carried out by use of the protocols described previously (DiMaio and others, 1990, 21698-21703; Witting and others, 1992, 737-743). The assay employs bovine plasma fibrinogen dissolved at 0.1% in 50 mM Tris-Cl, 100 mM NaCl, 0.1% PEG-8000 at pH 7.6 (i.e. the clotting buffer). Each assay mixture contained a certain concentration of the peptide, and the reaction was started by the addition of human thrombin to a final concentration of 0.6-1.2 nM. Optical absorbance increase at 420 nm caused by fibrin clot formation was measured at 25°C or 37°C using the Spectramax plate reader. The onset clotting time was determined as an intersection of the baseline and the extrapolated linear portion of the OD change curve. The concentration of a peptide needed to double the clotting time was defined as IC_{50} (DiMaio and others, 1990, 21698-21703). Kinetic amidolytic curves were obtained in clotting buffer at 25°C using eight inhibitor concentrations and three to five concentrations of the chromogenic substrate S-2238 (Chromogenix) (DiMaio and others, 1990, 21698-21703). Inhibition constants were extracted from Lineweaver-Burk equation by using weighted linear regression. Errors in K_I determination were estimated by using Monte-Carlo sampling with 1-3% variance of the experimental points. Peptide concentrations were determined spectrophotometrically using predicted extinction coefficients at 278 nm (Gill and von Hippel, 1989, 319-326).

[0064] With a wide range of linker lengths and compositions IC_{50} of the bivalent inhibitors in a fibrinogen clotting assay remained in low-nanomolar range (Table 1, and Figure 2), values sufficiently low for peptide-based antithrombotic pharmaceutical compounds (Witting and others, 1992, 737-743), and much lower than the K_I values of the constituent binding moieties (Slon-Usakiewicz and others, 2000, 2384-2391). In every case an improvement in IC_{50} as compared

with that of the H2 moiety confirmed the bivalent mode of the polypeptide-thrombin interaction. The C-terminal portion of the peptide consisting of only natural amino acids and including the polymeric linker plus the H2 moiety ((Gly-Ser)_n-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln) (SEQ. ID. NO. 113) can be produced using recombinant methods. Linking of the H1, containing unnatural amino acids, with the rest of the peptide can be performed using standard coupling techniques. We synthesized and purified peptides with amino acid sequences Bbs-Arg-(D-Pip)-Gly-Cys (SEQ. ID. NO. 4) and Cys-(Gly-Ser)₈-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 5). They were linked together by thiol oxidation in 2% ammonium acetate buffer, pH 8.6, over a period of 2 days. Resulting products were separated by reversed-phase HPLC and their identity was established by ion-spray mass spectroscopy. A product of disulfide bond linkage between peptides Bbs-Arg-(D-Pip)-Gly-Cys (SEQ. ID. NO. 4) and Cys-(Gly-Ser)₈-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 5) (corresponding to SEQ. ID. NO. 83) was tested for IC_{50} in the clotting assay. We established that the two-chain peptide was potent and therefore bivalent with an IC_{50} of 1.1 ± 0.2 nM (Figure 2). Another disulfide-linked bivalent thrombin inhibitor (corresponding to SEQ. ID. NO. 91) was prepared in the same fashion from two peptides Bbs-Arg-(D-Pip)-Gly-Cys (SEQ. ID. NO. 4) and Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 114).

[0065] Example 2. We made use of an amino acid sequence Cys-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly (SEQ. ID. NO. 8) derived from the cytoplasmic tail of the cell-surface anchored ligand ephrin B2 (ephrinB2₃₀₁₋₃₀₉) to link the H1 and H2 moieties. The peptide is known to be flexible and in its tyrosine-phosphorylated state to bind SH2 domain from Grb4 with an affinity of 0.2 μ M (Su, Xu, and Ni, 2004b, 1725-1736). We produced four peptides of a general formula Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-B-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 9), wherein B was either tyrosine (Tyr) or phosphotyrosine (Tyr(P)), and n was 1 or 2. The peptides were synthesized and their identity confirmed as outlined in Example 1. IC_{50} of the inhibitors in the thrombin-clotting assay were comparable and in the vicinity of 0.5-1 nM, except for the peptide with two phosphotyrosines whose IC_{50} was 18-20 nM (Table 2, Figure 3). Incorporation of two phosphotyrosines in the linker resulted in a significant drop in the inhibition potency. Given the fact that the potency of the bivalent inhibitor Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-B-Glu-Lys-Val-Ser-Gly)₂-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 115) depends on the phosphorylation state of the linker, a coupling with enzymatic assay translating activity of kinase or phosphatase into serine protease activity such as that of thrombin can be developed.

[0066] Example 3. An alternative way to reverse the inhibitory potency of the peptides with a general formula Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-B-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 9), wherein B was either tyrosine (Tyr) or phosphotyrosine (Tyr(P)), and n was 1 or 2, is to bring them in contact with SH2 domain in solution. SH2 domain of Grb4 was prepared as follows. The DNA sequences encoding the Grb4 SH2 domain was deduced from the amino acid sequences of murine Grb4 protein using the codon preference of *Escherichia coli*. The synthetic gene was amplified by PCR from six pairs of overlapping synthetic primers containing the two restriction sites of *Nco*I and *Bam*HI for the SH2 domain at its two ends. The double-digested DNA fragment of SH2 was subcloned into the pET3215 expression vector, which was modified from pET32 and pET15 vectors (Novagen, Madison, WI, USA), removing the original fusion carrier in the pET32 vector. In order to facilitate protein purification, a His-tag with six histidine residues was placed at the N-terminus of the SH2 domain linked with a thrombin cleavage sequence. The expression construct was confirmed by DNA sequencing and transformed into the *E. coli* BL21(DE3) expression host. The SH2 protein was expressed at 37°C. The cells were harvested four hours after induction with isopropyl thio- β -D-galactoside at OD₆₀₀=0.8. Protein purification was performed under denaturing conditions with Ni-nitriloacetic acid agarose beads (Qiagen) in the presence of 20 mM 2-mercaptoethanol at pH values of 8.0, 6.3, 5.9 and 4.5 for the binding, two washing, and eluting steps, respectively. Protein fractions were analyzed using SDS PAGE. Fractions containing SH2 domain were collected and refolded by dialyzing 2-3 times against a large volume of 50 mM sodium phosphate buffer containing 20 mM 2-mercaptoethanol (pH 6.8) at 4°C. The pellet was removed by centrifugation and the supernatant was concentrated by ultrafiltration (Millipore, Bedford, MA, USA). Protein concentration was determined spectrophotometrically at 280 nm with a calculated extinction coefficient of 12210 M⁻¹cm⁻¹.

[0067] Influence of SH2 on inhibitory potency of the four peptides was tested in the clotting assay. Clotting time in the presence or absence of each of the inhibitors, presence and absence of 3 μ M SH2 (inhibitor antidote), and equal amount of thrombin (0.6 nM) was measured at 22°C. The peptide Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 80) was used at a concentration of 1 nM, the peptide Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 82) was used at a concentration of 4 nM, the peptide Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly)₂-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 81) was used at a concentration of 2 nM, and the peptide Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly)₂-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 14) was used at a concentration of 50 nM. Interaction of the SH2 domain with phosphotyrosine-containing inhibitors reversed the inhibitory potency of the Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 29) (n=1,2) peptides, but not that of Bbs-R-(D-Pip)-Gly-(Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly)_n-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 11) (n=1,2) peptides (Figure 4).

The change in thrombin activity upon binding of SH2 is a basis for developing an assay for protein-to-peptide binding, which can be realized in a high-throughput manner.

[0068] A linker known to bind to a specific antibody may be used to perform as a switchable polymeric linker if the antibody is introduced into the activity assay. A peptide with a formula Bbs-R-(D-Pip)-Gly-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 13) was prepared and tested for its ability to inhibit thrombin and be neutralized by a commercially available anti-c-myc antibody, known to bind to the peptide with a sequence Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu (SEQ. ID. NO. 12). The peptide was present in the clotting assay at a concentration of 150 nM, and thrombin - at a concentration of 0.6 nM. In the absence of the neutralizing antibody clotting onset time was delayed from approximately 100 s to approximately 530 s (Figure 5). Addition of anti-c-myc antibody 9E10 (Sigma) in the stock buffer provided by the supplier to the final concentration of 1.2 μ M reversed the inhibitory effect of the inhibitor to the clotting onset time of approximately 230 s. The effect of the control on thrombin activity in the absence of the inhibitor was very small (Figure 5).

[0069] *Example 4.* An inhibitor with a linker known to bind specific metal ions will be affected by the presence of these ions in solution. Two peptides homologous to the calcium-binding loop of troponin C were designed and established that they bind calcium ions in solution. The peptides have the following sequences - Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-NH₂ (P3230) (SEQ. ID. NO. 109) and Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH₂ (P3231) (SEQ. ID. NO. 110). The peptides were synthesized, purified and their identity was confirmed as described in Example 1. They were tested for calcium binding by use of NMR. For this both freeze-dried peptides were reconstituted at a concentration of approximately 0.5 mM in 20 mM sodium acetate-d₃ buffer, pH 5.5, containing 10% D₂O. Proton spectra of the peptides were recorded at 800 MHz, 15°C, before and after addition of increasing amounts of 0.1. and 1 M stock solutions of CaCl₂ in the same buffer. Figures 6a,b,c,d show changes in the proton NMR spectra of these two peptides upon the addition to the initial volume of ~450 μ L of 1 μ L (final CaCl₂ concentration ~0.22 mM), additional 2 μ L (final CaCl₂ concentration ~0.66 mM), additional 10 μ L (final CaCl₂ concentration ~2.8 mM) of 100 mM CaCl₂, and additional 10 μ L (final CaCl₂ concentration ~23.9 mM) of 1 M CaCl₂. The changes in the spectra confirm binding of calcium with affinity in mM range.

[0070] One of the two designed peptides was used to construct a calcium-responsive bivalent thrombin inhibitor. The disulfide-linked bivalent thrombin inhibitor (corresponding to SEQ. ID. NO. 91) prepared by cross-oxidation of cysteine thiol groups from two peptides Bbs-Arg-(D-Pip)-Gly-Cys (SEQ. ID. NO. 4) and Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 114) (preparation described in Example 1) was tested for its ability to bind calcium and inhibit amidolytic reaction in the presence and absence of calcium. Figures 6e,f show changes in the proton NMR spectrum of this peptide upon the addition to the initial volume of ~450 μ L of 1 μ L (final CaCl₂ concentration ~0.22 mM), additional 2 μ L (final CaCl₂ concentration ~0.66 mM) of 100 mM CaCl₂, and additional 10 μ L (final CaCl₂ concentration ~22.2 mM) of 1 M CaCl₂. The changes in the spectra confirm binding of calcium and the peptide with affinity in mM range.

[0071] Samples tested for inhibition potency contained in the clotting buffer 0.6 nM thrombin, 50 μ M chromogenic substrate S-3266 (Chromogenix), and either no inhibitors or 2 nM of P3150, or 150 nM of the calcium-responsive disulfide-linked bivalent thrombin inhibitor. The time course of reactions is displayed in Figure 7. Upon addition of increasing concentrations of calcium (50 and 100 mM) to the inhibitor incorporating calcium-binding linker, the potency of the latter is decreased. The same amounts of calcium produce no visible effect on the potency of control peptide P3150.

[0072] *Example 5.* Two peptides with sequences Val-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Lys-Pro-Gln-Ser-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 22) (mini-hirudin 1) and Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Asn-Pro-Glu-Ser-His-Asn-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 23) (mini-hirudin 2) were designed incorporating N-terminal and C-terminal moieties presumably interacting with the active site and exosite I of thrombin. We found that they displayed high affinity to thrombin with IC_{50} of 33 ± 3 nM (mini-hirudin 1) and 14 ± 1 nM (mini-hirudin 2) indicating a bivalent mode of binding (Figure 8). The modular character of interaction was further implied when a candidacidal peptide known to bind laminarin (Polonelli, L.; and others, 2003, 6205-6212), or -Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser- (SEQ. ID. NO. 24), was inserted as a linker into the mini-hirudin-2 to give minihirudin-3 with a sequence of Ile-Arg-Phe-Thr-Asp-Gly-Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 25). The peptide exhibited high affinity of binding to thrombin, with IC_{50} of 10 ± 1 nM (Figure 8), confirming the presence of bivalent interactions.

[0073] *Example 6.* A peptide with a sequence of Trp-Asp-Pro-Arg-Pro-Gln-Arg-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 18) is a bivalent inhibitor of thrombin with a K_i of 17 nM (subject of another patent application). The peptide is built of two moieties, an active site binding moiety, Trp-Asp-Pro-Arg-Pro-Gln-Arg-His (SEQ. ID. NO. 19), and an exosite-1 binding moiety, Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 20). We prepared a bivalent thrombin inhibitor with the sequence Trp-Asp-Pro-Arg-Pro-Gln-Arg-His-(CamCKK)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 21), designated as CaM-DTI, where CamCKK is a protein linker with a calcium-responsive property (Truong and others, 2001, 1069-1073). Another potentially bivalent thrombin inhibitor was derived from CaM-DTI, where the active-site targeting moiety Trp-Asp-Pro-Arg-Pro-Asn-Arg-His (SEQ. ID. NO. 18)

of CaM-DTI was replaced by the sequence Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly (SEQ. ID. NO. 116) in mini-hirudins 1 and 3. In other words, this bivalent peptide incorporating the CamCKK linker was built from an N-terminal module, Ile-Arg-Phe-Thr-Asp-(SEQ. ID. NO. 72), and the exosite-1 binding moiety, Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 20). CaM-DTI2 has the sequence Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-(CamCKK)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 117). This new generation of CaM-DTI was named Cam-DTI2. The sequences of the thrombin inhibitors CaM-DTI and CaM-DTI2 are shown in Figure 9. Both CaM-DTI and CaM-DTI2 were prepared by use of a recombinant DNA approach. Typically, the proteins were expressed and purified by a standard procedure using Ni-NTA agarose affinity chromatographic column (Qiagen). The N-terminal tag was removed by digesting the sample with enterokinase with a subsequent passage through a Ni-NTA agarose affinity chromatographic column. 20 mM EDTA was added into the flow-through and the sample was desalted on a PD-10 column. The final purification was carried out with ion-exchange chromatography on a Mono-S column. Purity was confirmed by SDS-PAGE. Final samples were essentially Ca^{2+} - free.

[0074] Thrombin inhibition potencies of CaM-DTI and CaM-DTI2 were determined by an amidolytic assay. Kinetics of thrombin-catalyzed hydrolysis of the chromogenic substrates S-2238 or S-2366 (Chromogenix) was followed by absorbance at 405 nm on a SpectraMax plate reader thermostated at 37°C. The concentration of the substrate was 400 μM . Inhibition assays were performed in the clotting buffer with a certain fixed concentration of α -thrombin (~ 0.3 nM) such that linear progress curves were observed within at least 15 min in the absence of the inhibition. The total volume of the reaction mixture was 200 μl . Reactions were initiated by addition of the chromogenic substrate to the wells containing thrombin and a certain concentration of CaM-DTI premixed for less than 2 min. The concentration of CaM-DTI ranged from 25 nM to 2.5 μM . Kinetics data from initial rate experiments were used to construct Lineweaver-Burke plot; i.e. the relationship of (substrate concentration)⁻¹ versus (initial velocity)⁻¹ which were analysed by linear regression with MicroCal Origin 6.0 program (MicroCal, MD). The K_i values of the inhibitors were determined using the equation $K_i = [I] / \{ (SL_0 / SL_1) - 1 \}$, where [I] is the inhibitor concentration, SL_0 is the slope of the reaction in the absence of inhibitors, and SL_1 is the slope of the reaction in the presence of the inhibitor.

[0075] Upon the addition of 5 mM Ca^{2+} an increase in inhibition constant for CaM-DTI was observed from 480 nM (calcium-free sample) to 2200 nM (calcium-loaded sample) (Figure 10A). The CaM-DTI2 protein also inhibited the thrombin active site (Figure 10B), but this inhibition was not affected by the presence of Ca^{2+} upon concentration of 5 mM.

[0076] Figure 10B depicts the kinetics of thrombin-catalyzed hydrolysis of the chromogenic S-2366 (Chromogenix). Thrombin inhibition potency of CaM-DTI2 was determined by amidolytic assay. Kinetics of thrombin-catalyzed hydrolysis of the chromogenic substrate S-2366 (Chromogenix) was followed by absorbance at 405 nm on a SpectraMax plate reader at 25°C. The concentration of the substrate S-2366 was 50 μM . Inhibition assays were performed in the clotting buffer with a certain fixed concentration of α -thrombin (~ 0.6 nM). The total volume of the reaction mixture was 200 μl . Reactions were initiated by the addition of the chromogenic substrate to the wells containing thrombin in the presence of 4.2 μM and 8.4 μM CaM-DTI2. Curves represent OD_{405} time course after the addition of 0.6 nM thrombin in the presence of (O) 0 nM; (Δ) 4.2 μM ; and (\square) 8.4 μM CaM-DTI2. Inhibition of the amidolytic reaction confirming the bivalent mode of binding as shown for CaM-DTI in Figure 10A.

[0077] *Example 7.* Cdc42 binds tightly to the -40-residue extended CRIB domains of *Candida* Cla4 and Ste20. When subjected to NMR relaxation dispersion analysis (Tolkatchev, Xu, and Ni, 2003b, 12432-12442), these complexes exhibit no responses, as expected for a tight binding complex.

[0078] We over-expressed two peptide fragments of the extended CRIB regions from the *Candida* Cla4 and *Candida* Ste20 (or Cst20) kinases (Figure 11): (i) mCla4 (mCst20) including the consensus CRIB motif, and (ii) cCla4 (cCst20) which comprises residues directly to the C-terminus of the minimal CRIB sequence. All the peptides described in the example were prepared *via* a recombinant technique as described previously (Gizachew, D. and Oswald, R. E., 2001, 14368-14375; Osborne, M. J., and others, 2003, 317-326). The identity of the final products was verified by mass spectrometry.

[0079] Cdc42 constructs were prepared as follows. DNA fragments encoding the Cdc42 protein (residues 1-178) of *Candida albicans* SC5314 were amplified from the genomic DNA by a standard PCR reaction using the *pfu* polymerase. Through PCR reactions, two restriction sites, *Nde I* and *BamH I*, were generated in the 5'-end and 3'-end, respectively. A stop codon, TAG, was placed immediately after the codon for residue 178. The PCR fragment was subcloned into pET-15b (Novagen, Madison, WI) and the resulting construct was defined as pCaCdc42 Δ 13 (Stevens & Ni, unpublished data). A CaCdc42 expression vector encoding the R150K mutation was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The sequences of the wild-type and R150K mutant CaCdc42 (Table 4) vectors were verified by DNA sequencing.

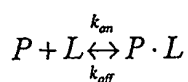
[0080] Wild type and mutant CaCdc42 proteins were expressed in the *E. coli* BL21 strain as hexa-histidine fusion proteins. Cells expressing CaCdc42 were grown in LB media. Cells were harvested from 1 L culture by centrifugation at 8000 g for 30 min and re-suspended in 50 mL of lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 5 mM MgCl_2 , 100 μM GDP, 2 $\mu\text{g/ml}$ aprotinin, leupeptin and pepstatin, and 10 $\mu\text{g/ml}$ benzamidin and PMSF). The collected cells were treated with lysozyme (1 mg/mL) for 30 min on ice, followed by sonication for 4 min and subsequent

addition of DNase at 2 (μg /ml. The insoluble fraction was removed by centrifugation at 10,000 g for 30 min. The supernatant was mixed with Ni-NTA agarose beads (Qiagen, Mississauga, ON) by rocking for one hour and then washed extensively in a column with a washing buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 15 mM imidazole, 5 mM MgCl_2). The fusion protein was eluted with the wash buffer (50mL) except that the concentration of imidazole was 200 mM. The protein sample was buffer-exchanged extensively using CentriPrep YM10 to remove imidazole.

[0081] The non-hydrolyzable GTP analogues, GMPPNP or GMPPCP (Sigma, St-Louis, MI) were used to produce the activated, but stable nucleotide-loaded form of CaCdc42. In this work, no differences were observed for the two GTP analogues-loaded forms of Cdc42 in NMR and fluorescence experiments except that the lifetime of the complex with GMPPCP is longer than that with GMPPNP. Nucleotide exchange was facilitated by incubating CaCdc42 with a 5- to 10-fold molar excess of the non-hydrolyzable GTP analogue in the presence of 10 mM EDTA. To this mixture, 100 units of alkaline phosphatase beads were added and the mixture was gently shaken on ice for 3 hrs. The alkaline phosphatase beads were removed by filtration, followed by the addition of MgCl_2 to a final concentration of 15 mM. The excess unbound nucleotides were removed using a PD-10 gel filtration column (Amersham Bioscience, Piscataway, NJ).

[0082] In order to construct a bivalent peptide ligand for *Candida* Cdc42 (CaCdc42) (with a suitable linker) (Figure 12), the binding affinities of component peptides derived from the CRIB domains of *Candida* Cla4 and Ste20 were determined. Residue K150 of the R150K CaCdc42 mutant was covalently modified with the fluorescent probe, sNBD (Molecular Probes, Eugene, OR), essentially as described by Nomanbhoy and Cerione (Nomanbhoy, T. and Cerione, R. A., 1999, 15878-15884.). The stoichiometry of the fluorescent probe per protein molecule was estimated at 1.13, based on protein concentration determined with $\epsilon_{280\text{nm}} = 13,610 \text{ M}^{-1} \text{ cm}^{-1}$ (Gill, S. C. and von Hippel, P. H., 1989, 319-326), and using the absorbance of the sNBD moiety of $\epsilon_{463\text{nm}} = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$. Interaction of the CRIB peptides with sNBD-labeled CaCdc42 was monitored using extrinsic fluorescence measurements with a Hitachi F-2500 fluorescence spectrophotometer. Samples of sNBD-labeled, activated CaCdc42 were added in the assay buffer (50 mM phosphate, pH 6.8, 50 mM NaCl and 5 mM MgCl_2) to a cuvette being continuously stirred. The protein concentration was 1 μM . Individual CRIB peptide dissolved in the same assay buffer was added drop-wise to the cuvette. The mixture was excited at 488 nm with an excitation slit width of 5 nm. The emission spectra were scanned from 510 nm to 590 nm. The fluorescence emission intensity at the emission maximum 545 nm was determined from each spectrum and the final value was obtained by averaging the values from five scans of the same sample. Control titration experiments were performed by adding the same volume of buffer instead of peptide. Each set of the titration data was repeated three times.

[0083] Figure 13 shows binding isotherms obtained following the CRIB-induced changes in the sNBD fluorescence of the CaCdc42 (R150K) protein. The K_d values for the binding of the CRIB peptides to sNBD-labeled activated CaCdc42 were determined by fitting the fluorescence titration data to a simple bimolecular association model as described by Leonard *et al* (Leonard, D. A., and others, 1997, 1173-1180). The association between CaCdc42 (P) and a CRIB peptide (L) can be described by the following equation



The fluorescence intensity (F) is related to the dissociation constant, K_d as follows,

$$F = F_0 + (F_i - F_0) \left[\frac{(K_d + L_T + P_T) - \sqrt{(K_d + L_T + P_T)^2 - 4P_T L_T}}{2P_T} \right]$$

where F_0 and F_i are the fluorescence intensities at the starting and end points of the titration, respectively. P_T is the total concentration of sNBD-labeled activated CaCdc42 and L_T is the total concentration of the CRIB peptide at any point in the titration. Fitting of the data was carried out using the computer program Microcal Origin™ 6.0 (Northampton, MA). Average K_d values were determined from multiple independent measurements.

[0084] The average apparent K_d values for different CRIB peptides are summarized in Table 2. As expected, the extended CRIB (eCRIB) fragments exhibited the strongest affinities of binding to CaCdc42 in the low nanomolar range. The mCRIB fragments containing the consensus CRIB sequence, ISXPXXFXHXXHVGXD (SEQ. ID. NO. 26) (Burbelo, P. D., Drechsel, D., and Hall, A., 1995, 29071-29074), also had moderately strong binding affinities in micromolar concentrations, but clearly, as seen previously for the human PAK homologues (Rudolph, M. G., Bayer, P., Abo, A., Kuhlmann, J., Vetter, I. R., and Wittinghofer, A., 1998, 18067-18076; Thompson, G., Owen, D., Chalk, P. A., and Lowe, P. N., 1998, 7885-7891), require extra residues to retain stronger binding to Cdc42. The cCRIB peptides exhibited much

weaker affinities to the CaCdc42 protein. The K_d value of cCla4 for binding to CaCdc42 is in a high micromolar concentration (275 μ M). An even weaker binding ($K_d = 1160 \mu$ M) was observed between cCst20 and CaCdc42 with the current fluorescence titration strategy.

[0085] Fluorescence measurements of cross-titrations were used to quantify allosteric effects (Table 2). The affinity of the Cla4 peptide fragments for CaCdc42 was not significantly affected by the addition of the cognate peptide. In contrast, the affinities of the Cst20 peptide fragments preincubated with CaCdc42 exhibited a dramatic enhancement in binding for CaCdc42 by - 5.5-fold, upon addition of the cognate Cst20 peptide (Table 2). Thus, upon addition of mCst20 to the cCst20/CaCdc42 complex, the affinity of cCst20 for CaCdc42 increased from a K_d of 1160 μ M to 207 μ M (Table 2 and Figure 13c). Similarly, mCst20 affinity for CaCdc42 increased from 0.43 μ M to 0.081 μ M when cCst20 was added to a preincubated mCst20/CaCdc42 complex. These results strongly suggest that the eCst20 and eCla4 peptides exhibit different mechanisms for binding CaCdc42, in which long eCst20 peptide utilizes a cooperative mechanism for high-affinity interaction while eCla4 does not.

[0086] Modular nature of interactions of m- and c- CRIB fragments is confirmed by the binding affinities of hybrid peptides incorporating m- and c- CRIBs from different molecular species. Both mCla4-cCst20 and mCst20-P-cCla4 constructs (Figure 11) displayed affinities of the same order of magnitude as the original eCRIB peptides (Table 2). Moreover, incorporation of -Ser-Gly-Ser-Gly- (SEQ. ID. NO. 27) and -Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys- (SEQ. ID. NO. 28) linkers (Figure 11) into the eCla4 sequence preserved a bivalent mode of binding, since the affinity of the chimeric peptide was significantly stronger than those of H1 and H2 heads (Table 2).

[0087] Example 8. The dissociation constant (K_i) for the interaction between SAP-SH2 and the eCla4-SLAM peptide was obtained by fitting fluorescence titration data (Figures 14A and 14B) using the following equation

$$K_d^{app} = K_d + \frac{K_d}{K_i} [SH_2]$$

where, K_d^{app} , K_d are the apparent dissociation constants between CaCdc42 and eCla4-SLAM in the presence or absence of SAP-SH2, respectively. K_i is the dissociation constant for the binding interaction between SAP-SH2 and the linker portion (i.e. the SLAM sequence of eCla4-SLAM). The value of K_i determined from these experiments is 362 μ M, indicating that the SLAM sequence in the eCla4-SLAM peptide preserved the binding affinity to SAP-SH2 (Li et al and Pawson, Curr. Biol. 9, 1355-1362, 1999).

[0088] A peptide of the sequence Bbs-Arg-dPip-Gly-Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ. ID. NO. 102), was synthesized and purified which contains as linker the SLAM sequence with specific binding to SAP-SH2 in the absence of tyrosine phosphorylation (Li et al and Pawson, Curr. Biol. 9, 1355-1362, 1999). The peptide was added at the concentrations of 10, 15, 20, 30, 40, 50 and 60 nM to 0.6 nM thrombin in the clotting buffer. Optical absorbance increase at 420 nm caused by fibrin clot formation was measured at 25°C using the Spectramax plate reader. The onset clotting time was determined as an intersection of the baseline and the extrapolated linear portion of the OD change curve. The concentration of the peptide needed to double the clotting time was defined as IC_{50} . The peptide is found to be a potent inhibitor of thrombin with $IC_{50} = 7 \pm 1$ nM (Figure 14C). Also, to the clotting buffer containing 0.6 nM thrombin and 25 nM of the inhibitor were added 5 and 10 μ M SAP-SH2 from the stock solution of 116 μ M SAP-SH2 in 10 mM 2-[N-Morpholino] ethanesulfonic buffer (MES) at pH 5.0. The clotting assays serving as control experiments included thrombin+inhibitor, thrombin+SAPSH2, and thrombin alone. Figure 14D and 14E shows the course of the optical absorbance changes at 420 nm, and at 25°C, demonstrating the reversal of thrombin inhibition by SAP-SH2.

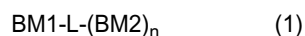
[0089] Example 9. The tweezer-like bivalent ligands can be attached to the protein target, either chemically or through recombinant techniques. We used the recombinant approach to conjugate *Candida albicans* Cdc42 (CaCdc42) with the full-length CRIB peptides from *Candida* Cla4 and Ste20 (Figure 11). A model for a conjugated complex of CaCdc42 and the eCRIBs via a polymeric linker is displayed in Figure 15a. Resonance assignments of the 1H - ^{15}N HSQC spectra for *Candida* Cla4-eCRIB in a conjugated complex with CaCdc42 are displayed in Figure 15b. The resonance peaks of the Cla4-eCRIB have the same pattern as those in the non-covalent complex. However, the conjugated complex is more stable (>3 months) than the non-covalent one (<one week). The 1H - ^{15}N HSQC spectrum of *Candida* Cst20-eCRIB in a conjugated complex with CaCdc42 is shown in Figure 15c. One potential application of the stably-conjugated complex is for discovering stronger and specific binding molecules to Cdc42 is outlined in Figure 15d. More specifically, NMR techniques such as relaxation and H/D exchange can be used to detect the dissociation of a conjugated bivalent ligand by competing monovalent small molecules.

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	Left ear	Right ear	
5	Group 1- R1	Bleeding rabbit (no sealant)	
	Group 1- R2	Bleeding rabbit (no sealant)	
10	Group 2- R1	Syringe (1) - 250 μ L Commercial Tisseel solution, prepared fresh Syringe (2) - 250 μ L Tisseel "Thrombin 4" solution, prepared fresh	Syringe (1) - 250 μ L Commercial Tisseel solution, prepared fresh Syringe (2) - 250 μ L Tisseel "Thrombin 4" solution, prepared the night before and incubated o/n at 37 C
	Group 2- R2	Same sample as above	Same sample as above
15	Group 3- R1	Syringe (1) - 250 μ L Commercial Tisseel solution, prepared fresh + SH2 to 12 μ M concentration. Syringe (2) - 250 μ L Tisseel "Thrombin 4" solution + P3170 to 8 nM concentration, prepared the night before and incubated o/n at 37 C	Syringe (1) - 250 μ L Commercial Tisseel solution, prepared fresh + SH2 to 12 μ M concentration. Syringe (2) - 250 μ L Tisseel "Thrombin 4" solution, prepared the night before and incubated o/n at 37 C
	Group 3- R2	Same sample as above	Same sample as above
20	Group 4- R1	Syringe (1) - 250 μ L Commercial Tisseel solution, prepared fresh + SH2 to 12 μ M concentration. Syringe (2) - 250 μ L " α -thrombin (10^{-5})" solution + P3170 to 8 nM concentration, prepared the night before and incubated o/n at 37 C	Syringe (1) - 250 μ L Commercial Tisseel solution, prepared fresh + SH2 to 12 μ M concentration. Syringe (2) - 250 μ L " α -thrombin (10^{-5})" solution, prepared the night before and incubated o/n at 37 C
25	Group 4- R2	Same sample as above	Same sample as above

[0090] SH2-activated fibrin glue with P3170 inhibitor was able to seal the wound after -5 minutes (Figure 18).

[0091] In an embodiment of the invention there is provided a multivalent binding molecule and uses thereof. The molecule is useful in a method of delivering a compound of interest for preferential release at a biological site of interest as part of a method of treatment by therapy. The molecule has a general formula (1) of



wherein,

BM1 is a first binding moiety having an affinity for site 1 on the target,

BM2 is a second binding moiety having an affinity for a site other than site 1 on the target, n is 1 or greater, and

L is a linker joining BM1 and BM2, said linker being adapted to respond to a change in its environment with a change in conformation and/or flexibility,

wherein BM1 and BM2 may be the same or different, and when $n > 1$, different BM2 moieties have affinities for different binding sites on the target. BM1 and BM 2 are selected such that in use each of the BM1 and BM2 existing separately has a lower binding affinity than the complex of BM1 and BM2 does when they are linked to form the molecule. In some instances the ligand is a polypeptide. In some instances the ligand is covalently attached to its target. In some instances the target is a protein, and the ligand is attached to its protein target by means of recombinant conjugation. In some instances the linkers are modified by means of binding to a biomolecule. In some instances the linkers are modified by means of covalent modification. In some instances the linkers are modified by means of a local environment change. In some instances the linker binds to an antibody. In some instances the linker binds to an SH2 domain. In some instances the linker binds to Cdc42. In some instances the linker binds to prothrombin. In some instances the linker binds to metal ion. In some instances the linker binds to calcium. In some instances the linker binds to a cell surface. In some instances the linker sequence contains at least two residues, selected from the group of tyrosine; serine; threonine; histidine; phosphotyrosine; phosphoserine; phosphothreonine; phosphohistidine.

[0092] In some instances the linker sequence is selected from the group consisting of

-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-;

5
-Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-
His-Lys-Val-His-Val-Gly-Phe-Asp-Pro-Ala-Ser-Gly-Asn-
Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-Leu-Gln-
His-Ser-Lys-Ile-Thr-;

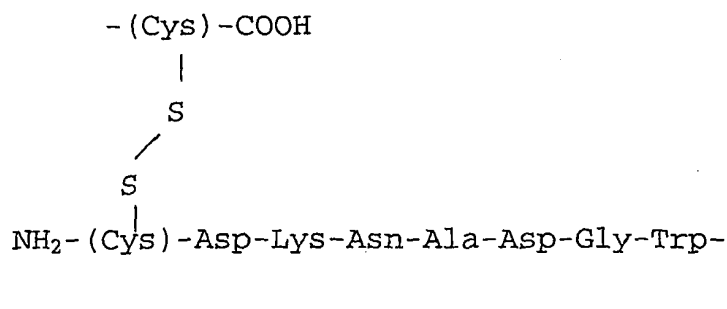
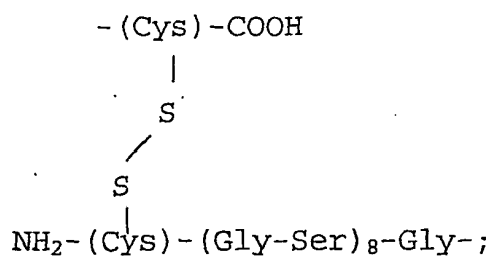
10
-Glu-Val-Asn-Ile-Lys-Ile-Ser-Thr-Pro-Phe-Asn-Ala-Lys-
15 His-Leu-Ala-His-Val-Gly-Ile-Asp-Asp-Asn-Gly-Ser-Tyr-
Thr-Gly-Leu-Pro-Ile-Glu-Trp-Glu-Arg-Leu-Leu-Ser-Ala-
Ser-Gly-Ile-Thr-;

20 -Thr-Leu-Asp-Leu-Asn-Thr-Pro-Val-Asp-Lys-Thr-Ser-Asn-;
-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-;
-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-;

25 -Asn-Gly-Arg-Lys-Ile-Cys-Leu-Asp-Leu-Gln-Ala-Pro-Leu-
Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser-;

30 -Asn-Gly-Arg-Lys-Ile-Cys-Leu-Glu-Leu-Arg-Ala-Pro-Leu-
Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser-;

35 -His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val-;
-Tyr-Met-Glu-Ser-Arg-Ala-Asp-Arg-;
-Gln-Ser-His-Asn-Arg-;



-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-
Glu-;

5

-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-
Glu-;

10

-Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser-;
-Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys-;

In some instances the FL sequence is selected from the group consisting of

15

- (Gly-Ser)₂-;
- (Gly-Ser)₄-;
- (Gly-Ser)₆-;
- (Gly-Ser)₈-;
- 20 - (Gly-Ser)₁₀-;
- (Gly-Ser)₁₂-;
- (Gly-Ser)₁₄-;
- Gly-Cys...Cys- (Gly-Ser)₈-;
- (Gly-Ser)₄-Gly-Lys-(Gly-Ser)₅-
- 25 - Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly-;
- (Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly)₂-;
- Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly-;
- (Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly)₂-;
- Pro-His-Tyr-Glu-Lys-Val-Ser-;

30

-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly-Ser-Pro-His-Tyr-Glu-
Lys-Val-Ser-;

35

- Pro-His-Tyr(P)-Glu-Lys-Val-Ser-;

-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly-Ser-Pro-His-Tyr(P)-
Glu-Lys-Val-Ser-;

40

wherein Tyr(P) is O-phosphotyrosine;

In some instances the FL sequence is selected from the group consisting of

45

-Ser-Val-Val-Pro-Asn-Aaa-Bbb-Leu-Ccc-Ddd-Asp-;

wherein Aaa, Bbb, Ccc, and Ddd - natural amino acids;

[0093] In some instances the molecule is a thrombin inhibitor;

50 **[0094]** In some instances the BM1 sequence is selected from the group consisting of:

Bbs-Arg-(D-Pip);
Bbs-Arg-(D-Pip)-Gly;

55 where Bbs is 4-tert-butylbenzenesulfonyl, D-Pip is D-pipecolic acid;

[0095] In some instances the BM1 sequence is a subsequence from an amino acid sequence selected from the group consisting of

Val-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Lys;

Val-Arg-Phe-Thr-Asp;

5

Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Asn;

Ile-Arg-Phe-Thr-Asp;

10

Trp-Asp-Pro-Arg-Pro-Gln-Arg-His;

[0096] In some instances the BM2 amino acid sequence is selected from the group consisting of:

Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

15

Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

In some instances the molecule is selected from the group consisting of:

20

Bbs-Arg- (D-Pip) - (Gly-Ser)₂-Gly-Asp-Phe-Glu-Glu-Ile-Pro-
Glu-Glu-Tyr-Leu-Gln;

25

Bbs-Arg- (D-Pip) - (Gly-Ser)₄-Gly-Asp-Phe-Glu-Glu-Ile-Pro-
Glu-Glu-Tyr-Leu-Gln;

30

Bbs-Arg- (D-Pip) - (Gly-Ser)₆-Gly-Asp-Phe-Glu-Glu-Ile-Pro-
Glu-Glu-Tyr-Leu-Gln;

35

Bbs-Arg- (D-Pip) - (Gly-Ser)₈-Gly-Asp-Phe-Glu-Glu-Ile-Pro-
Glu-Glu-Tyr-Leu-Gln;

40

Bbs-Arg- (D-Pip) - (Gly-Ser)₁₀-Gly-Asp-Phe-Glu-Glu-Ile-
Pro-Glu-Glu-Tyr-Leu-Gln;

45

Bbs-Arg- (D-Pip) - (Gly-Ser)₁₂-Gly-Asp-Phe-Glu-Glu-Ile-
Pro-Glu-Glu-Tyr-Leu-Gln;

50

Bbs-Arg- (D-Pip) - (Gly-Ser)₁₄-Gly-Asp-Phe-Glu-Glu-Ile-
Pro-Glu-Glu-Tyr-Leu-Gln;

55

Bbs-Arg- (D-Pip) -Gly-Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-
Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

Bbs-Arg- (D-Pip) -Gly- (Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-
 Gly)₂-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

5

Bbs-Arg- (D-Pip) -Gly-Ser-Pro-His-Tyr (P) -Glu-Lys-Val-Ser-
 Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

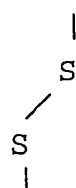
10

Bbs-Arg- (D-Pip) -Gly- (Ser-Pro-His-Tyr (P) -Glu-Lys-Val-Ser-
 Gly)₂-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

15

Bbs-Arg- (D-Pip) -Gly- (Cys) -COOH

20



25

NH₂- (Cys) - (Gly-Ser)₈-Gly-Asp-Phe-Glu-
 Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

30

Bbs-Arg- (D-Pip) - (Gly-Ser)₄-Gly-Lys- (Gly-Ser)₅-Gly-Asp-
 Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

35

Bbs-Arg- (D-Pip) -Gly-Thr-Leu-Asp-Leu-Asn-Thr-Pro-Val-
 Asp-Lys-Thr-Ser-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-
 Glu-Tyr-Leu-Gln;

40

Bbs-Arg- (D-Pip) -Gly-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-
 Asp-Leu-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-
 Gln;

45

50

Bbs-Arg- (D-Pip) -Gly-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-
 His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-
 Leu-Gln;

55

5 Bbs-Arg- (D-Pip) -Gly-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-
His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-
Leu-Gln;

10 Bbs-Arg- (D-Pip) -Gly-Asn-Gly-Arg-Lys-Ile-Cys-Leu-Asp-
Leu-Gln-Ala-Pro-Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-
Leu-Glu-Ser-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-
Leu-Gln;

20 Bbs-Arg- (D-Pip) -Gly-Asn-Gly-Arg-Lys-Ile-Cys-Leu-Glu-
Leu-Arg-Ala-Pro-Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-
Leu-Glu-Ser-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-
Leu-Gln;

25 Bbs-Arg- (D-Pip) -Gly-His-His-Leu-Gly-Gly-Ala-Lys-Gln-
Ala-Gly-Asp-Val-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-
Tyr-Leu-Gln;

35 Bbs-Arg- (D-Pip) -Gly-Tyr-Met-Glu-Ser-Arg-Ala-Asp-Arg-
Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

40 Bbs-Arg- (D-Pip) -Gly-Gln-Ser-His-Asn-Arg-Gly-Asp-Phe-
Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

45 Bbs-Arg- (D-Pip) -Gly- (Cys) -COOH
|

50 S
/ S
|
NH₂- (Cys) -Asp-Lys-Asn-Ala-Asp-Gly-Trp-
55 Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-
Leu-Gln;

Ile-Arg-Phe-Thr-Asp-Gly-Ala-Lys-Val-Thr-Met-Thr-Cys-
 Ser-Ala-Ser-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-
 5 Leu-Gln;

where Bbs is 4-*tert*-butylbenzenesulfonyl, D-Pip is D-pipecolic acid; Tyr(P) is O-phosphorylated tyrosine;

[0097] In some instances the molecule is

10 W DPRPQRHADQLTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRS LGQNPTEAE
 LQDMINEVDADGNGTIDFPEFLTMMARKMKDTGGVKLIPSWTTVILVKSMLRKRS
 15 FGNPFGGDSEEEIREFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIRE
 ADIDGDGQVNYEEFVQMMTAKDFEEIPEEYLQ;

20 [0098] In some instances the molecule is a ligand of Cdc42;

[0099] In some instances the BM1 sequence is a subsequence from an amino acid sequence selected from the group consisting of

25 Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-
 His-Lys-Val-His-Val-Gly-Phe-Asp-Pro-Ala-Ser;

30 Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-
 His-Lys-Val-His-Val-Gly-Phe-Asp;

35 Glu-Val-Asn-Ile-Lys-Ile-Ser-Thr-Pro-Phe-Asn-Ala-Lys-
 His-Leu-Ala-His-Val-Gly-Ile-Asp-Asp-Asn-Gly;

40 Glu-Val-Asn-Ile-Lys-Ile-Ser-Thr-Pro-Phe-Asn-Ala-Lys-
 His-Leu-Ala-His-Val-Gly-Ile-Asp;

45 [0100] In some instances the BM2 sequence is a subsequence from an amino acid sequence selected from the group consisting of

50 Gly-Asn-Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-
 Leu-Gln-His-Ser-Lys-Ile-Thr;

55 Asn-Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-Leu-
 Gln-His-Ser-Lys-Ile-Thr;

Gly-Ser-Tyr-Thr-Gly-Leu-Pro-Ile-Glu-Trp-Glu-Arg-Leu-
Leu-Ser-Ala-Ser-Gly-Ile-Thr;

5

Ser-Tyr-Thr-Gly-Leu-Pro-Ile-Glu-Trp-Glu-Arg-Leu-Leu-
Ser-Ala-Ser-Gly-Ile-Thr;

10

[0101] In some instances the molecule is selected from the group consisting of:

15

Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-
His-Lys-Val-His-Val-Gly-Phe-Asp-Ser-Gly-Ser-Gly-Asn-
Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-Leu-Gln-
His-Ser-Lys-Ile-Thr;

20

25

Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-
His-Lys-Val-His-Val-Gly-Phe-Asp-Arg-Lys-Ser-Leu-Thr-
Ile-Tyr-Ala-Gln-Val-Gln-Lys-Asn-Phe-Thr-Gly-Leu-Pro-
Asp-Thr-Trp-Lys-Ser-Leu-Leu-Gln-His-Ser-Lys-Ile-Thr;

30

[0102] Thus, it will be apparent that there has been provided herein multivalent binding molecules containing linkers through which binding can be modulated.

35 **Table 1.** IC₅₀ and K_i values of thrombin inhibitors of the series Bbs-R-(D-Pip)-linker-GDFEEIPEEYLQ (SEQ. ID. NO: 2).

	linker	K _i , nM (25°C)	IC ₅₀ , nM (37°C)	Fig.
40 P3149, SEQ. ID. NO. 73	GSGS (SEQ. ID. NO. 50)		9.7±0.7	2a
P3150, SEQ. ID. NO. 74	GSGSGSGS (SEQ. ID. NO. 51)	0.5±0.2	0.5±0.1	2b
45 P3151, SEQ. ID. NO. 75	GSGSGSGSGSGS (SEQ. ID. NO. 52)	0.6±0.1	0.5±0.1	2c
P3152, SEQ. ID. NO. 76	GSGSGSGSGSGSGSGS (SEQ. ID. NO. 53)	1.3±0.3	0.7±0.1	2d
50 P3153, SEQ. ID. NO. 77	GSGSGSGSGSGSGSGSGS (SEQ. ID. NO. 54)	2.0±0.3	1.0±0.1	2e
P3160, SEQ. ID. NO. 78	GSGSGSGSGSGSGSGSGSGSGS (SEQ. ID. NO. 55)	4.6±0.8	2.8±0.1	2f
55 P3159, SEQ. ID. NO. 79	GSGSGSGSGSGSGSGSGSGSGSGS (SEQ. ID. NO. 56)	6.7±1.9	3.5±0.2	2g
P3172-P3165, SEQ. ID. NO. 83	Gly-Cys...Cys-GSGSGSGSGSGSGSGS (SEQ. ID. NO. 57)		1.1±0.2	2h

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(continued)

	linker	K _i , nM (25°C)	IC ₅₀ , nM (37°C)	Fig.
5	P3169, SEQ. ID. NO. 80 GSPHYEKVS (SEQ. ID. NO. 123) Ligand of an SH2 domain from Grb4, dephosphorylated	1.0±0.2	0.4 (25°C)	3a
10	P3170, SEQ. ID. NO. 82 GSPH (Y(P)) EKVS (SEQ. ID. NO. 124) Ligand of an SH2 domain from Grb4, phosphorylated	1.5±0.4	0.7 (25°C)	3b
15	P3161, SEQ. ID. NO. 81 GSPHYEKVSGSPHYEKVS (SEQ. ID. NO. 125) Tandem of two peptide ligands to SH2 from Grb4, dephosphorylated		0.7 (25°C)	3c
20	P3162, SEQ. ID. NO. 14 GSPH (Y(P)) EKVSGSPH (Y(P)) EKVS (SEQ. ID. NO. 126) Tandem of two peptide ligands to SH2 from Grb4, phosphorylated		19±1 (25°C)	3d
25	P3174, SEQ. ID. NO. 84 GSGSGSGSGKGS GSGSGSGS (SEQ. ID. NO. 58) Lys in the middle of a long GS repeat linker		1.6±0.9 (25°C)	2j
30	P3181, SEQ. ID. NO. 85 GTLDLNTPVDKTSN (SEQ. ID. NO. 103) C5a receptor peptide		1.9±0.2	2i
35	P3182, SEQ. ID. NO. 13 GEQKLISEEDL (SEQ. ID. NO. 127) c-myc peptide	66±13	~25	
40	P3209, SEQ. ID. NO. 32 GSWPRPQLHND (SEQ. ID. NO. 105) Prothrombin-binding linker 1 (VV)		1.1	2k
45	P3210, SEQ. ID. NO. 33 GSHAPRPQIHND (SEQ. ID. NO. 104) Prothrombin-binding linker 2 (HA)		0.6	2l
	P3234, SEQ. ID. NO. 88 GHHLGAKQAGDV (SEQ. ID. NO. 106) Fibrinogen γ-chain 400-411, integrin specific		3.9±0.8	2m
	P3236, SEQ. ID. NO. 89 GYMESRADR (SEQ. ID. NO. 107) Fibrinogen antagonist, also targets the fibrinogen-integrin interaction		1.0±0.5	2n
	P3238, SEQ. ID. NO. 90 GQSHNR (SEQ. ID. NO. 108) Linkers conferring a RGDF sequence, with potential binding to an integrin receptor		2±1	2o
	3243-3255, SEQ. ID. NO. 91 Gly-Cys...Cys-DKNADGWTDN (SEQ. ID. NO. 48) Calcium-binding linker			7
	P3291, SEQ. ID. NO. 102 GRKSLTIYAQVQK (SEQ. ID. NO. 128) SLAM peptide (ligand for SAP-SH2)		7±1	

50 **Table 2. Dissociation constants for binding of *Candida* CRIB fragments to CaCdc42 measured by fluorescence titration.**

Peptide	mCla4	mCst20	cCla4	cCst20	eCla4	eCst20
55 K_d^m (μM)	4.2 ± 0.15	0.43 ± 0.03	275 ± 9	1160 ± 106	0.025 ± 0.002	0.046 ± 0.002

(continued)

5	Peptide	mCla4 (+mCla4)	mCst20 (+cSt20)	cCla4 (+mCla4)	cCst20 (+mCst20)	
	K_d^m (μM)	4.1 \pm 0.13	0.081 \pm 0.002	311 \pm 12	207 \pm 10	
10	Peptide	mCla4- cCst20	mCst20- cCla4	mCst20-P- cCla4	eCla4-SG	eCla4-SLAM
15	K_d^m (μM)	0.031 \pm 0.002	2.64 \pm 0.20	0.093 \pm 0.01	0.067 \pm 0.008	0.127 \pm 0.07

Table 3

	<u>SEQ. ID. NO.</u>	<u>SEQUENCE</u>
20	SEQ. ID. NO. 1	Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
25	SEQ. ID. NO. 2	Bbs-Arg-(D-Pip)-linker-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
30	SEQ. ID. NO. 3	[-linker-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln]
35	SEQ. ID. NO. 4	Bbs-Arg-(D-Pip)-Gly-Cys
40	SEQ. ID. NO. 5	Cys-(Gly-Ser) ₈ -Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
45	SEQ. ID. NO. 6	Bbs-Arg-(D-Pip)-Gly-Cys
50	SEQ. ID. NO. 7	Cys-(Gly-Ser) ₈ -Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
55	SEQ. ID. NO. 8	Cys-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly
	SEQ. ID. NO. 9	Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-B-Glu-Lys-Val-Ser-Gly) _n -Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
	SEQ. ID. NO. 10	Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly) _n -Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
	SEQ. ID. NO. 11	Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly) _n -Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
	SEQ. ID. NO. 12	Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu

(continued)

SEQ. ID. NO.	SEQUENCE
5 SEQ. ID. NO. 13	Bbs-Arg-(D-Pip)-Gly-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
10 SEQ. ID. NO. 14	Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly) ₂ -Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
15 SEQ. ID. NO. 15	Bbs-Arg-(D-Pip)-Gly-Cys...
15 SEQ. ID. NO. 16	Bbs-Arg-(D-Pip)-Gly-Cys...Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
20 SEQ. ID. NO. 17	Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu
25 SEQ. ID. NO. 18	Trp-Asp-Pro-Arg-Pro-Gln-Arg-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
30 SEQ. ID. NO. 19	Trp-Asp-Pro-Arg-Pro-Gln-Arg-His
30 SEQ. ID. NO. 20	Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
35 SEQ. ID. NO. 21	Trp-Asp-Pro-Arg-Pro-Gln-Arg-His-(CamCKK)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
40 SEQ. ID. NO. 22	Val-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Lys-Pro-Gln-Ser-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (mini-hirudin 1)
45 SEQ. ID. NO. 23	Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-Thr-Pro-Asn-Pro-Glu-Ser-His-Asn-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (mini-hirudin 2)
50 SEQ. ID. NO. 24	Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser-
50 SEQ. ID. NO. 25	Ile-Arg-Phe-Thr-Asp-Gly-Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
55 SEQ. ID. NO. 26	ISXPXXFXHXXHVGXD
SEQ. ID. NO. 27	-Ser-Gly-Ser-Gly-

(continued)

SEQ. ID. NO.	SEQUENCE
SEQ. ID. NO. 28	-Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys-
SEQ. ID. NO. 29	Bbs-Arg-(D-Pip)-Gly-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly) _n -Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 30	Gly-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 31	Gly-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 32	Bbs-Arg-(D-Pip)-Gly-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 33	Bbs-Arg-(D-Pip)-Gly-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 34	Gly-Ser-Val-Val-Pro-Asn-Xxx-Xxx-Leu-Xxx-Xxx-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 35	Bbs-Arg-(D-Pip)-Gly (H1, Bbs=4- <i>tert</i> -butyl-benzenesulfonyl, D-Pip=D-pipecolic acid, K_I in low μ M range
SEQ. ID. NO. 36	Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 37	-Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-His-Lys-Val-His-Val-Gly-Phe-Asp-Pro-Ala-Ser-Gly-Asn-Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-Leu-Gln-His-Ser-Lys-Ile-Thr-

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SEQ. ID. NO.	SEQUENCE
5 10 15 20 25	SEQ. ID. NO. 38 -Glu-Val-Asn-Ile-Lys-Ile- Ser-Thr-Pro-Phe-Asn-Ala-Lys- His-Leu-Ala-His-Val-Gly-Ile- Asp-Asp-Asn-Gly-Ser-Tyr-Thr- Gly-Leu-Pro-Ile-Glu-Trp-Glu- Arg-Leu-Leu-Ser-Ala-Ser-Gly- Ile-Thr-;
15	SEQ. ID. NO. 39 -Thr-Leu-Asp-Leu-Asn-Thr- Pro-Val-Asp-Lys-Thr-Ser-Asn-
20	SEQ. TD. NO. 40 -Ser-Val-Val-Pro-Arg-Pro- Gln-Leu-His-Asn-Asp-
25	SEQ. ID. NO. 41 -Ser-His-Ala-Pro-Arg-Pro- Gln-Ile-His-Asn-Asp-
30	SEQ. ID. NO. 42 -Asn-Gly-Arg-Lys-Ile-Cys- Leu-Asp-Leu-Gln-Ala-Pro-Leu- Tyr-Lys-Lys-Ile-Ile-Lys-Lys- Leu-Leu-Glu-Ser-
35	SEQ. ID. NO. 43 -Asn-Gly-Arg-Lys-Ile-Cys- Leu-Glu-Leu-Arg-Ala-Pro-Leu- Tyr-Lys-Lys-Ile-Ile-Lys-Lys- Leu-Leu-Glu-Ser-
40	SEQ. ID. NO. 44 -His-His-Leu-Gly-Gly-Ala- Lys-Gln-Ala-Gly-Asp-Val-
45	SEQ. ID. NO. 45 -Tyr-Met-Glu-Ser-Arg-Ala- Asp-Arg-
50	SEQ. ID. NO. 46 -Gln-Ser-His-Asn-Arg-
55	SEQ. ID. NO. 47 $ \begin{array}{c} \text{-(Cys)-COOH} \\ \\ \text{S} \\ / \\ \text{S} \\ \\ \text{NH}_2\text{-(Cys)-(Gly-Ser)}_8\text{-Gly-} \end{array} $
55	SEQ. ID. NO. 48 $ \begin{array}{c} \text{-(Cys)-COOH} \\ \\ \text{S} \\ / \end{array} $

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SEQ. ID. NO.	SEQUENCE
	$\begin{array}{c} \text{S} \\ \\ \text{NH}_2 - (\text{Cys}) - \text{Asp} - \text{Lys} - \\ \text{Asn} - \text{Ala} - \text{Asp} - \text{Gly} - \text{Trp} - \text{Ile} - \text{Asp} - \\ \text{Asn} - \end{array}$
SEQ. ID. NO. 49	-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-
SEQ. ID. NO. 50	-(Gly-Ser) ₂ -
SEQ. ID. NO. 51	-(Gly-Ser) ₄ -
SEQ. ID. NO. 52	-(Gly-Ser) ₆ -
SEQ. ID. NO. 53	-(Gly-Ser) ₈ -
SEQ. ID. NO. 54	-(Gly-Ser) ₁₀ -
SEQ. ID. NO. 55	-(Gly-Ser) ₁₂ -
SEQ. ID. NO. 56	-(Gly-Ser) ₁₄ -
SEQ. ID. NO. 57	-Gly-Cys...Cys-(Gly-Ser) ₈ -
SEQ. ID. NO. 58	-(Gly-Ser) ₄ -Gly-Lys-(Gly-Ser) ₅ -
SEQ. ID. NO. 59	-Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly-
SEQ. ID. NO. 60	-(Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly) ₂ -
SEQ. ID. NO. 61	-Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly-
SEQ. ID. NO. 62	-(Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly) ₂ -
SEQ. ID. NO. 63	-Pro-His-Tyr-Glu-Lys-Val-Ser-
SEQ. ID. NO. 64	-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly-Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-
SEQ. ID. NO. 65	-Pro-His-Tyr(P)-Glu-Lys-Val-

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SEQ. ID. NO.	SEQUENCE
	Ser-
SEQ. ID. NO. 66	-Pro-His-Tyr (P) -Glu-Lys-Val- Ser-Gly-Ser-Pro-His-Tyr (P) - Glu-Lys-Val-Ser-
SEQ. ID. NO. 67	-Ser-Val-Val-Pro-Asn-Aaa- Bbb-Leu-Ccc-Ddd-Asp-
SEQ. ID. NO. 68	Bbs-Arg-(D-Pip)
SEQ. ID. NO. 69	Val-Arg-Phe-Thr-Asp-Gly-Glu- Gly-Thr-Pro-Lys
SEQ. ID. NO. 70	Val-Arg-Phe-Thr-Asp
SEQ. ID. NO. 71	Ile-Arg-Phe-Thr-Asp-Gly-Glu- Gly-Thr-Pro-Asn
SEQ. ID. NO. 72	Ile-Arg-Phe-Thr-Asp
SEQ. ID. NO. 73	Bbs-Arg- (D-Pip) - (Gly-Ser) ₂ - Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 74	Bbs-Arg- (D-Pip) - (Gly-Ser) ₄ - Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 75	Bbs-Arg- (D-Pip) - (Gly-Ser) ₆ - Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 76	Bbs-Arg- (D-Pip) - (Gly-Ser) ₈ - Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 77	Bbs-Arg- (D-Pip) - (Gly-Ser) ₁₀ - Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 78	Bbs-Arg- (D-Pip) - (Gly-Ser) ₁₂ - Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln

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SEQ. ID. NO.	SEQUENCE
5 SEQ. ID. NO. 79	Bbs-Arg- (D-Pip) - (Gly-Ser) ₁₄ - Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
10 SEQ. ID. NO. 80	Bbs-Arg- (D-Pip) -Gly-Ser-Pro- His-Tyr-Glu-Lys-Val-Ser-Gly- Asp-Phe-Glu-Glu-Ile-Pro-Glu- Glu-Tyr-Leu-Gln
15 SEQ. ID. NO. 81	Bbs-Arg- (D-Pip) -Gly- (Ser- Pro-His-Tyr-Glu-Lys-Val-Ser- Gly) ₂ -Asp-Phe-Glu-Glu-Ile- Pro-Glu-Glu-Tyr-Leu-Gln
20 SEQ. ID. NO. 82	Bbs-Arg- (D-Pip) -Gly-Ser-Pro- His-Tyr (P) -Glu-Lys-Val-Ser- Gly-Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
25 SEQ. ID. NO. 83	<p>Bbs-Arg- (D-Pip) -Gly-</p> <p>(Cys) -COOH</p> <p style="text-align: center;"> </p> <p style="text-align: center;">S</p> <p style="text-align: center;">/</p> <p style="text-align: center;">S</p> <p style="text-align: center;"> </p> <p>NH₂- (Cys) - (Gly-Ser)₈-Gly-</p> <p>Asp-Phe-Glu-Glu-Ile-Pro-Glu-</p> <p>Glu-Tyr-Leu-Gln;</p>
30 SEQ. ID. NO. 84	Bbs-Arg- (D-Pip) - (Gly-Ser) ₄ - Gly-Lys- (Gly-Ser) ₅ -Gly-Asp- Phe-Glu-Glu-Ile-Pro-Glu- Glu-Tyr-Leu-Gln
35 SEQ. ID. NO. 85	Bbs-Arg- (D-Pip) -Gly-Thr- Leu-Asp-Leu-Asn-Thr-Pro- Val-Asp-Lys-Thr-Ser-Asn- Gly-Asp-Phe-Glu-Glu-Ile-

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SEQ. ID. NO.	SEQUENCE
	Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 86	Bbs-Arg-(D-Pip)-Gly-Asn- Gly-Arg-Lys-Ile-Cys-Leu- Asp-Leu-Gln-Ala-Pro-Leu- Tyr-Lys-Lys-Ile-Ile-Lys- Lys-Leu-Leu-Glu-Ser-Gly- Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 87	Bbs-Arg-(D-Pip)-Gly-Asn- Gly-Arg-Lys-Ile-Cys-Leu- Glu-Leu-Arg-Ala-Pro-Leu- Tyr-Lys-Lys-Ile-Ile-Lys- Lys-Leu-Leu-Glu-Ser-Gly- Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 88	Bbs-Arg-(D-Pip)-Gly-His- His-Leu-Gly-Gly-Ala-Lys- Gln-Ala-Gly-Asp-Val-Gly- Asp-Phe-Glu-Glu-Ile-Pro- Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 89	Bbs-Arg-(D-Pip)-Gly-Tyr- Met-Glu-Ser-Arg-Ala-Asp- Arg-Gly-Asp-Phe-Glu-Glu- Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 90	Bbs-Arg-(D-Pip)-Gly-Gln- Ser-His-Asn-Arg-Gly-Asp- Phe-Glu-Glu-Ile-Pro-Glu- Glu-Tyr-Leu-Gln

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SEQ. ID. NO.	SEQUENCE
SEQ. ID. NO. 91	Bbs-Arg- (D-Pip) -Gly- (Cys) - COOH <div style="text-align: right; margin-right: 50px;"> S </div>
	<div style="text-align: center; margin-bottom: 10px;"> $\begin{array}{c} \diagup \\ S \\ \\ \text{NH}_2 - (\text{Cys}) - \end{array}$ </div> Asp-Lys-Asn-Ala-Asp-Gly- Trp-Ile-Asp-Asn-Gly-Asp- Phe-Glu-Glu-Ile-Pro-Glu- Glu-Tyr-Leu-Gln
SEQ. ID. NO. 92	Gly-Gly-Asn-Ser-Gly-Val-Ser- Gly-Pro-Ile-Asn-Phe-Thr-His- Lys-Val-His-Val-Gly-Phe-Asp- Pro-Ala-Ser
SEQ. ID. NO. 93	Gly-Gly-Asn-Ser-Gly-Val-Ser- Gly-Pro-Ile-Asn-Phe-Thr-His- Lys-Val-His-Val-Gly-Phe-Asp
SEQ. ID. NO. 94	Glu-Val-Asn-Ile-Lys-Ile-Ser- Thr-Pro-Phe-Asn-Ala-Lys-His- Leu-Ala-His-Val-Gly-Ile-Asp- Asp-Asn-Gly
SEQ. ID. NO. 95	Glu-Val-Asn-Ile-Lys-Ile-Ser- Thr-Pro-Phe-Asn-Ala-Lys-His- Leu-Ala-His-Val-Gly-Ile-Asp
SEQ. ID. NO. 96	Gly-Asn-Phe-Thr-Gly-Leu-Pro- Asp-Thr-Trp-Lys-Ser-Leu-Leu- Gln-His-Ser-Lys-Ile-Thr
SEQ. ID. NO. 97	Asn-Phe-Thr-Gly-Leu-Pro-Asp- Thr-Trp-Lys-Ser-Leu-Leu-Gln- His-Ser-Lys-Ile-Thr

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SEQ. ID. NO.	SEQUENCE
SEQ. ID. NO. 98	Gly-Ser-Tyr-Thr-Gly-Leu-Pro-Ile-Glu-Trp-Glu-Arg-Leu-Leu-Ser-Ala-Ser-Gly-Ile-Thr
SEQ. ID. NO. 99	Ser-Tyr-Thr-Gly-Leu-Pro-Ile-Glu-Trp-Glu-Arg-Leu-Leu-Ser-Ala-Ser-Gly-Ile-Thr
SEQ. ID. NO. 100	Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-His-Lys-Val-His-Val-Gly-Phe-Asp-Ser-Gly-Ser-Gly-Asn-Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-Leu-Gln-His-Ser-Lys-Ile-Thr
SEQ. ID. NO. 101	Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-His-Lys-Val-His-Val-Gly-Phe-Asp-Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys-Asn-Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-Leu-Gln-His-Ser-Lys-Ile-Thr
SEQ. ID. NO. 102	Ebs-R-dPip-Gly-Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 103	Gly-Thr-Leu-Asp-Leu-Asn-Thr-Pro-Val-Asp-Lys-Thr-Ser-Asn-
SEQ. ID. NO. 104	Gly-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-
SEQ. ID. NO. 105	Gly-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-

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<u>SEQ. ID. NO.</u>	<u>SEQUENCE</u>
SEQ. ID. NO. 106	Gly-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val-
SEQ. ID. NO. 107	Gly-Tyr-Met-Glu-Ser-Arg-Ala-Asp-Arg-
SEQ. ID. NO. 108	Gly-Gln-Ser-His-Asn-Arg-
SEQ. ID. NO. 109	Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-NH ₂
SEQ. ID. NO. 110	Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH ₂
SEQ. ID. NO. 111	Leu-Ile-Glu-Asp-Ile-Cys-Leu-Pro-Arg-Trp-Gly-Cys-Leu-Trp-Glu-Asp
SEQ. ID. NO. 112	Bbs-Arg- (D-Pip) -Gly-Leu-Ile-Glu-Asp-Ile-Cys-Leu-Pro-Arg-Trp-Gly-Cys-Leu-Trp-Glu-Asp-Gly-Asp-Phe-Gln-Gln-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 113	((Gly-Ser) _n -Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln)
SEQ. ID. NO. 114	Cys-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 115	Bbs-R- (D-Pip) -Gly- (Ser-Pro-His-B-Glu-Lys-Val-Ser-Gly) ₂ -Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln

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<u>SEQ. ID. NO.</u>	<u>SEQUENCE</u>
SEQ. ID. NO. 116	Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly
SEQ. ID. NO. 117	Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-(CamCKK)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 118	(Val-Pro-Gly-Val-Gly) 19-Val-Pro-Gly-Val
SEQ. ID. NO. 119	Bbs-Arg-dPip-Gly-(Val-Pro-Gly-Val-Gly) 20-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 120	Gly-(Val-Pro-Gly-Val-Gly) 19-Val-Pro-Gly-Val
SEQ. ID. NO. 121	Ile-Arg-Phe-Thr-Asp-Gly-Glu-Gly-(Val-Pro-Gly-Val-Gly) 20-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Leu-Gln
SEQ. ID. NO. 122	Bbs-Arg-dPip-Gly
SEQ. ID. NO. 123	Gly-Ser-Pro-His-Tyr-Glu-Lys-Val-Ser
SEQ. ID. NO. 124	Gly-Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser
SEQ. ID. NO. 125	Gly-Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly-Ser-Pro-His-Tyr-Glu-Lys-Val-Ser
SEQ. ID. NO. 126	Gly-Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly-Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser
SEQ. ID. NO. 127	Gly-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu
SEQ. ID. NO. 128	Gly-Arg-Lys-Ser-Leu-Thr-Ile-Tyr-Ala-Gln-Val-Gln-Lys
SEQ. ID. NO. 129	-Ser-Pro-His-Tyr(P)-Glu-Lys-Val-Ser-Gly-

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SEQ. ID. NO.	SEQUENCE
5 SEQ. ID. NO. 130	GGNSGFPGPI NFTHKVHVG DRKSLTIYAQ VQKNFTGLPD TWKSLQHSK IT
10 SEQ. ID. NO. 131	YGRKKRRQRR RGGNSGFPGP INFTHKVHVG FDRKSLTIYA QVQKNFTGLP
15 SEQ. ID. NO. 132	GSSHHHHHSSFNPRGSWYY GNVTRHQAEALNERGVEGDFLIRDSE SSPSDFSVSLKASGRNKHFKVQL VDSVYCIGQRRFHSMDDELVEHYKKAP IFTSEHGKLYLVRALQ
20 SEQ. ID. NO. 133	GSSHHHHHSSFNPRGSD AVAVYHGKISRETGEKLLLATGLDG SYLLRDSSEVPGVYCLCVLYHGYI
25	YTYRVSQTETGSWSAETAPGVHKRYF RKIKNLISAFQKPDQGIVIPVLPVEK
30 SEQ. ID. NO. 134	GSSHHHHHSSGLVPRGSHMQTIKCVV VGDGAVGKTCLLISYTTSKFPA DYVPTVFDNYAVTVMIGDEPFTLGLF DTAGQEDYDRLRPLSYPSTDV FLVCFSVISPAFENVKEKWFPEVHHH CPGVPIIIVGTQTDLRNDDVI LQRLHRQKLSPI TQEQGEKLAKELKA 35 VKYVECSALTQRGLKTVFDEAIVAALE

Table 4. Full sequences of recombinant peptides and proteins

40 1. eCla4-SLAM

GGNSGFPGPI NFTHKVHVG DRKSLTIYAQ VQKNFTGLPD TWKSLQHSK IT (SEQ. ID. NO. 130)

45 2. TAT-eCla4-SLAM:

YGRKKRRQRR RGGNSGFPGP INFTHKVHVG FDRKSLTIYA QVQKNFTGLP
DTWKSLQHS KIT (SEQ. ID. NO. 131)

50 3. Grb4-SH2

55 GSSHHHHHSSFNPRGSWYYGNVTRHQAEALNERGVEGDFLIRDSESSPSDFSVSL
KASGRNKHFKVQLVDSVYCIGQRRFHSMDDELVEHYKKAP IFTSEHGKLYLVRALQ
(SEQ. ID. NO. 132)

(continued)

4. SAP-SH2:

5 GSSHHHHHH SSFNPRGSD AVAVYHGKISR ETGEKLLLATGLDG
 SYLLRDSESVPGVYCLC VLYHGYI YTYRVSQT ETGSWSAE TAPGVHKRYF
 RKIKNLI SAFQ KPDQGI VIPLQYPVEK
 (SEQ. ID. NO. 133)

10 5. CaCdc42 (R150K)

15 GSSHHHHHHS SGLVPRGSH MQTIKCVV VGDGAVG KTCLLISY TTSKFPA
 DYVPTVF DNYAVT VMIGDE PFTLGLF DTAGQED YDRLRPL SYPSTDV
 FLVCFSV ISPASF ENVKEKW FPEVHHH CPGVPPII IVGTQTD LRNDVVI
 LQRLHRQ KLSPIIT QEQGEKLA KELKA VKYVEC SALTQRGLKT VFDEA
 IVAALE
 (SEQ. ID. NO. 134)

20 6. CaM-DTI:

25 W DPRPQRHADQLTTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRS LGQNPTAEALQ
 DMINEVDADGNGTIDFPEFLTMMARKMKDTGGVKLIPSWTTVILVKSMLRKR SFGNP
 FGGDSEEEI REAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGD
 GQVNYEEFVQMMTAKDFEEIPEEYLQ (SEQ. ID. NO. 21)

30 7. CaM-DTI2:

35 IRFTDGE GADQLTTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRS LGQNPTAEALQ
 DMINEVDADGNGTIDFPEFLTMMARKMKDNGGVKLI PSWTTVILVKSMLRKR SFGNP
 FGGDSEEEI REAFRVFDKDGNGYIRAAELRHVMTNLGEKLTDEEVDEMIREADIDGD
 GQVNYEEFVQMMTAKDFEEIPEEYLQ (SEQ. ID. NO. 117)

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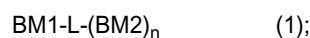
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Claims

1. An ex-vivo method of delivering a compound of interest for preferential release at a biological site of interest, comprising:

(a) obtaining a molecule of formula 1:



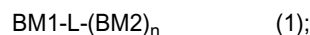
(b) providing the compound of interest and the molecule of formula (1) at the biological site of interest; and,
(c) providing a linker-specific molecule at the biological site of interest,

wherein,

n is 1 or greater,
BM1 is a first binding moiety having an affinity for site 1 on the compound,
BM2 is a second binding moiety having an affinity for a site other than site 1 on the compound,
BM1 and BM2 are the same or different, and where n>1, different BM2 moieties have affinities for different binding sites on the compound,
BM1 and BM2 are selected such that in use each of the BM1 and BM2 existing separately has a lower binding affinity than the molecule of formula 1, and
L is a linker joining BM1 and BM2, said linker being a flexible polypeptide without a well-defined three-dimensional structure in a free state, said linker undergoing a loss or decrease of flexibility or change in conformation upon non-covalent binding to the linker-specific molecule at the biological site of interest thereby impeding simultaneous binding of the binding moieties, thereby producing a reversing effect on the binding affinity of the molecule of formula 1 at the biological site of interest.

2. A molecule of formula 1 as specified in Claim 1 for use in a method of delivering a compound of interest for preferential release at a biological site of interest as part of a method of treatment by therapy comprising:

(a) obtaining a molecule of formula 1:



(b) providing the compound of interest and the molecule of formula (1) at the biological site of interest; and,
(c) providing a linker-specific molecule at the biological site of interest.

3. The method of Claim 1 or the molecule of Claim 2 wherein the binding affinity of BM1 or BM2 alone is no more than 1/2 the binding affinity of the molecule of formula 1.
4. The method of Claim 1 or 3 or the molecule of Claim 2 or 3 wherein L comprises an amino acid sequence as set forth in SEQ. ID. NO. 8, 12, 17, 24, 27, 28, 37-47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 124, 125, 126, 127 or 128.
5. The method or molecule of any one of Claims 1 to 4, wherein BM1 comprises the amino acid sequence as set forth in SEQ. ID. NO. 6, 9, 15, 19, 35, 68, 69, 70, 71, 72, 93, 92, 94, 95, 116, 122 or linked sequences SEQ. ID. NO. 15 and SEQ. ID. NO. 16.

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6. The method or molecule of any one of Claims 1 to 5, wherein BM2 comprises the amino acid sequence as set forth in SEQ. ID. NO. 1, 20, 36, 96, 97, 98 or 99.
7. The method of Claim 1 or the molecule of Claim 2, wherein the molecule of formula 1 comprises at least one amino acid sequence selected from the group consisting of SEQ. ID. NO. 2, 9, 10, 11, 13, 14, 16, 21, 22, 23, 25, 29, 32, 33, 34, 73, 74, 75, 76, 77, 78, 79, 80, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 100, 101, 102, 112, 115, 117, 119, 121, 130, 131 and 117.
8. The method or molecule of any one of Claims 1 to 7, wherein the linker contains at least one amino acid which is a substrate for a kinase or phosphatase.
9. The method or molecule of any one of Claims 1 to 7, wherein the linker contains a specific binding site for an SH2 protein and linker conformation is modulated by SH2 binding.
10. The method or molecule of any one of Claims 1 to 7, wherein the linker contains a specific binding site for an antibody and the linker-specific molecule is an antibody.
11. The method or molecule of Claim 10, wherein the antibody is a monoclonal antibody.
12. The method or molecule of Claim 11, wherein the specific binding site is SEQ. ID. NO. 12 and the antibody is anti-c-myc antibody 9E10.
13. The method or molecule of any one of Claims 1 to 7, wherein the linker-specific molecule is a metal ion and the linker has an affinity for the metal ion.
14. The method or molecule of Claim 13, wherein the linker comprises SEQ. ID. NO. 17 and the metal ion is a calcium ion.
15. The method or molecule of Claim 13, wherein the linker comprises SEQ. ID. NO. 21 and the metal ion is a calcium ion.
16. The method or molecule of any one of Claims 1 to 7, wherein the linker-specific molecule is prothrombin and the linker has an affinity for prothrombin.
17. The method or molecule of any one of Claims 1 to 16, wherein the compound of interest is thrombin.
18. The method or molecule of any one of Claims 1 to 16, wherein the compound of interest is a polypeptide having the amino acid sequence as set forth in SEQ ID NO: 134 (Cdc42).
19. The method or molecule of any one of Claims 1 to 16, wherein the compound of interest is at least one of a chemotherapeutic, an antifungal agent, an anticoagulant, an antibiotic, an antioxidant, a neurotransmitter or mimetic thereof, an antiviral agent, a degradatory enzyme, a kinase, a phosphatase, an anaesthetic, an analgesic, a transacting promoter of gene expression, an antigen, an antibody or fragment thereof, a retrovirus, a chelator, a hormone, a cytokine, an ionophore, or an inhibitor or antagonist of such a material.

Patentansprüche

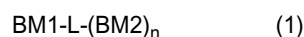
1. ex-vivo-Verfahren der Abgabe einer Verbindung von Interesse zur bevorzugten Freisetzung an einem biologischen Ort von Interesse, bei dem man
- (a) ein Molekül der Formel 1
- $$\text{BM1-L-(BM2)}_n \quad (1)$$
- beschafft,
- (b) die Verbindung von Interesse und das Molekül der Formel (1) an dem biologischen Ort des Interesses bereitstellt, und
- (c) ein Linker-spezifisches Molekül an dem biologischen Ort des Interesses bereitstellt, wobei

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n 1 oder größer ist,
BM1 ein erster Bindungsteil mit einer Affinität für Ort 1 auf der Verbindung ist,
BM2 ein zweiter Bindungsteil mit einer Affinität für einen anderen Ort als Ort 1 auf der Verbindung ist,
BM1 und BM2 gleich oder verschieden sind und, wenn $n > 1$, unterschiedliche BM2-Teile Affinitäten für
5 unterschiedliche Bindungsstellen auf der Verbindung haben,
BM1 und BM2 so ausgewählt sind, daß im Gebrauch jedes der getrennt existierenden BM1 und BM2 eine
geringere Bindungsaffinität als das Molekül der Formel 1 hat, und
L ein BM1 und BM2 verbindender Linker ist, der ein flexibles Polypeptid ohne eine gut definierte, dreidi-
10 mensionale Struktur in einem freien Zustand ist und der einen Verlust oder eine Abnahme der Flexibilität
oder eine Konformationsänderung bei nicht-kovalenter Bindung an das Linker-spezifische Molekül an dem
biologischen Ort des Interesses erfährt, wodurch eine gleichzeitige Bindung der Bindungsteile behindert
wird und **dadurch** an dem biologischen Ort des Interesses eine Umkehrwirkung auf die Bindungsaffinität
des Moleküls der Formel 1 erzeugt wird.

- 15 **2.** Molekül der Formel 1 wie in Anspruch 1 beschrieben zum Gebrauch bei einem Verfahren der Abgabe einer Ver-
bindung von Interesse zur bevorzugten Freisetzung an einem biologischen Ort von Interesse als Teil einer Therapie-
Behandlungsmethode, bei dem man

20 (a) ein Molekül der Formel 1



beschafft,

25 (b) die Verbindung von Interesse und das Molekül der Formel (1) an dem biologischen Ort von Interesse
bereitstellt, und

(c) ein Linker-spezifisches Molekül an dem biologischen Ort des Interesses bereitstellt.

- 30 **3.** Verfahren des Anspruchs 1 oder Molekül des Anspruchs 2, bei dem die Bindungsaffinität von BM1 oder BM2 alleine
nicht mehr als 1/2 der Bindungsaffinität des Moleküls der Formel 1 ist.

- 4.** Verfahren des Anspruchs 1 oder 3 oder Molekül des Anspruchs 2 oder 3, bei dem L eine Aminosäuresequenz
aufweist gemäß Angabe in SEQ. ID. NR. 8, 12, 17, 24, 27, 28, 37-47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59,
60, 124, 125, 126, 127 oder 128.

- 35 **5.** Verfahren oder Molekül eines der Ansprüche 1 bis 4, bei dem BM1 die Aminosäuresequenz aufweist gemäß Angabe
in SEQ. ID. NR. 6, 9, 15, 19, 35, 68, 69, 70, 71, 72, 92, 93, 94, 95, 116, 122 oder verlinkten Sequenzen SEQ. ID.
NR. 15 und SEQ. ID. NR. 16.

- 40 **6.** Verfahren oder Molekül eines der Ansprüche 1 bis 5, bei dem BM2 die Aminosäuresequenz aufweist gemäß Angabe
in SEQ. ID. NR. 1, 20, 36, 96, 97, 98 oder 99.

- 7.** Verfahren des Anspruchs 1 oder Molekül des Anspruchs 2, bei dem das Molekül der Formel 1 wenigstens eine
Aminosäuresequenz aufweist, die aus der Gruppe ausgewählt ist, bestehend aus SEQ. ID. NR. 2, 9, 10, 11, 13,
45 14, 16, 21, 22, 23, 25, 29, 32, 33, 34, 73, 74, 75, 76, 77, 78, 79, 80, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 100, 101,
102, 112, 115, 117, 119, 121, 130, 131 und 117.

- 8.** Verfahren oder Molekül eines der Ansprüche 1 bis 7, bei dem der Linker wenigstens eine Aminosäure enthält, die
ein Substrat für eine Kinase oder Phosphatase ist.

- 50 **9.** Verfahren oder Molekül eines der Ansprüche 1 bis 7, bei dem der Linker eine spezifische Bindungsstelle für ein
SH2-Protein enthält und die Linkerkonformation durch SH2-Bindung moduliert wird.

- 10.** Verfahren oder Molekül eines der Ansprüche 1 bis 7, bei dem der Linker eine spezifische Bindungsstelle für einen
Antikörper enthält und das Linker-spezifische Molekül ein Antikörper ist.

- 55 **11.** Verfahren oder Molekül des Anspruchs 10, bei dem der Antikörper ein monoklonaler Antikörper ist.

- 12.** Verfahren oder Molekül des Anspruchs 11, bei dem die spezifische Bindungsstelle SEQ. ID. NR. 12 ist und der

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Antikörper anti-c-myc-Antikörper 9E10 ist.

- 5
13. Verfahren oder Molekül eines der Ansprüche 1 bis 7, bei dem das Linker-spezifische Molekül ein Metallion ist und der Linker eine Affinität für das Metallion hat.
14. Verfahren oder Molekül des Anspruchs 13, bei dem der Linker SEQ. ID. NR. 17 umfaßt und das Metallion ein Calciumion ist.
- 10
15. Verfahren oder Molekül des Anspruchs 13, bei dem der Linker SEQ. ID. NR. 21 umfaßt und das Metallion ein Calciumion ist.
16. Verfahren oder Molekül eines der Ansprüche 1 bis 7, bei dem das Linker-spezifische Molekül Prothrombin ist und der Linker eine Affinität für Prothrombin hat.
- 15
17. Verfahren oder Molekül eines der Ansprüche 1 bis 16, bei dem die Verbindung von Interesse Thrombin ist.
18. Verfahren oder Molekül eines der Ansprüche 1 bis 16, bei dem die Verbindung von Interesse ein Polypeptid mit einer Aminosäuresequenz gemäß Angabe in SEQ. ID. NR. 134 (Cdc42) ist.
- 20
19. Verfahren oder Molekül eines der Ansprüche 1 bis 16, bei dem die Verbindung von Interesse wenigstens ein Chemotherapeutikum, ein antifungales Mittel, ein Antikoagulans, ein Antibiotikum, ein Antioxidans, ein Neurotransmitter oder dessen Mimetikum, ein antivirales Mittel, ein Degratory-Enzym, eine Kinase, eine Phosphatase, ein Anästhetikum, ein Analgetikum, ein transaktivierender Promotor der Gen-Expression, ein Antigen, ein Antikörper oder sein Fragment, ein Retrovirus, ein Chelatbildner, ein Hormon, ein Cytokin, ein Ionophor oder ein Hemmer oder Antagonist eines solchen Materials ist.
- 25

Revendications

- 30
1. Procédé *ex vivo* pour délivrer un composé d'intérêt et obtenir une libération préférentielle au niveau d'un site biologique d'intérêt, consistant à :

(a) obtenir une molécule de formule 1 :



(b) fournir le composé d'intérêt et la molécule de formule (1) au niveau du site biologique d'intérêt ; et,

(c) fournir une molécule spécifique à un segment de liaison au niveau du site biologique d'intérêt,

40 où,

n est 1 ou plus,

BM1 est un premier fragment de liaison ayant une affinité pour un site 1 sur le composé,

BM2 est un second fragment de liaison ayant une affinité pour un site autre que le site 1 sur le composé,

45 BM1 et BM2 sont identiques ou différents, et où $n > 1$, et différents fragments BM2 possèdent des affinités pour différents sites de liaison sur le composé,

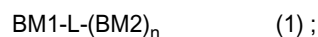
BM1 et BM2 sont sélectionnés de sorte que lorsqu'ils sont utilisés, chacun de BM1 et BM2 existant séparément possède une affinité de liaison plus faible que la molécule de formule 1, et

50 L est un segment de liaison qui joint BM1 et BM2, ledit segment de liaison étant un polypeptide flexible ne comportant pas de structure tridimensionnelle bien définie dans un état libre, ledit segment de liaison subissant une perte ou une réduction de flexibilité, ou un chargement de conformation, suite à une liaison non covalente à la molécule spécifique un segment de liaison au niveau du site biologique d'intérêt, ce qui empêche la liaison simultanée des fragments de liaison, produit ainsi un effet d'inversion sur l'affinité de liaison de la molécule de formule 1 au niveau du site biologique d'intérêt.

- 55
2. Molécule de formule 1 telle que spécifiée dans la revendication 1, destinée à être utilisée dans un procédé pour délivrer un composé d'intérêt et obtenir une libération préférentielle au niveau d'un site biologique d'intérêt, en tant qu'élément d'un procédé de traitement thérapeutique consistant à :

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(a) obtenir une molécule de formule 1 :



- 5 (b) fournir le composé d'intérêt et la molécule de formule (1) au niveau du site biologique d'intérêt ; et,
(c) fournir une molécule spécifique à un segment de liaison au niveau du site biologique d'intérêt.
- 10 3. Procédé selon la revendication 1 ou molécule selon la revendication 2, où l'affinité de liaison de BM1 ou BM2 seul n'excède pas la moitié ($\frac{1}{2}$) de l'affinité de liaison de la molécule de formule 1.
- 15 4. Procédé selon la revendication 1 ou 3, ou molécule selon la revendication 2 ou 3, où L comprend une séquence d'acides aminés telle que décrite dans SEQ ID NO: 8, 12, 17, 24, 27, 28, 37-47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 124, 125, 126, 127 ou 128.
- 20 5. Procédé ou molécule selon l'une quelconque des revendications 1 à 4, où BM1 comprend la séquence d'acides aminés telle que décrite dans SEQ ID NO: 6, 9, 15, 19, 35, 68, 69, 70, 71, 72, 93, 92, 94, 95, 116, 122 ou les séquences liées SEQ ID NO: 15 et SEQ ID NO: 16.
- 25 6. Procédé ou molécule selon l'une quelconque des revendications 1 à 5, où BM2 comprend la séquence d'acides aminés telle que décrite dans SEQ ID NO: 1, 20, 36, 96, 97, 98 ou 99.
7. Procédé selon la revendication 1 ou molécule selon la revendication 2, où la molécule de formule 1 comprend au moins une séquence d'acides aminés sélectionnée dans le groupe consistant en SEQ ID NO: 2, 9, 10, 11, 13, 14, 16, 21, 22, 23, 25, 29, 32, 33, 34, 73, 74, 75, 76, 77, 78, 79, 80, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 100, 101, 102, 112, 115, 117, 119, 121, 130, 131 et 117.
- 30 8. Procédé ou molécule selon l'une quelconque des revendications 1 à 7, où le segment de liaison contient au moins un acide aminé qui est un substrat pour une kinase ou une phosphatase.
9. Procédé ou molécule selon l'une quelconque des revendications 1 à 7, où le segment de liaison contient un site de liaison spécifique pour une protéine SH2 et la conformation du segment de liaison est modulée par la liaison de SH2.
- 35 10. Procédé ou molécule selon l'une quelconque des revendications 1 à 7, où le segment de liaison contient un site de liaison spécifique pour un anticorps et la molécule spécifique à un segment de liaison est un anticorps.
- 40 11. Procédé ou molécule selon la revendication 10, où l'anticorps est un anticorps monoclonal.
12. Procédé ou molécule selon la revendication 11, où le site de liaison spécifique est SEQ ID NO: 12 et l'anticorps est un anticorps 9E10 anti-c-myc.
- 45 13. Procédé ou molécule selon l'une quelconque des revendications 1 à 7, où la molécule spécifique à un segment de liaison est un ion métallique et le segment de liaison possède une affinité pour l'ion métallique.
14. Procédé ou molécule selon la revendication 13, où le segment de liaison comprend SEQ ID NO: 17 et l'ion métallique est un ion calcium.
- 50 15. Procédé ou molécule selon la revendication 13, où le segment de liaison comprend SEQ ID NO: 21 et l'ion métallique est un ion calcium.
16. Procédé ou molécule selon l'une quelconque des revendications 1 à 7, où la molécule spécifique à un segment de liaison est la prothrombine et le segment de liaison possède une affinité pour la prothrombine.
17. Procédé ou molécule selon l'une quelconque des revendications 1 à 16, où le composé d'intérêt est la thrombine.
- 55 18. Procédé ou molécule selon l'une quelconque des revendications 1 à 16, où le composé d'intérêt est un polypeptide ayant la séquence d'acides aminés telle que décrite dans SEQ ID NO: 134 (Cdc42).
19. Procédé ou molécule selon l'une quelconque des revendications 1 à 16, où le composé d'intérêt est au moins l'un

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5 parmi un agent de chimiothérapie, un agent antifongique, un anticoagulant, un antibiotique, un antioxydant, un neurotransmetteur ou un mimétique de celui-ci, un agent antiviral, une enzyme de dégradation, une kinase, une phosphatase, un anesthésique, un analgésique, un prompteur de l'expression génique agissant en trans, un anti-gène, un anticorps ou un fragment de celui-ci, un rétrovirus, un chélateur, une hormone, une cytokine, un ionophore, ou un inhibiteur ou un antagoniste d'un tel matériel.

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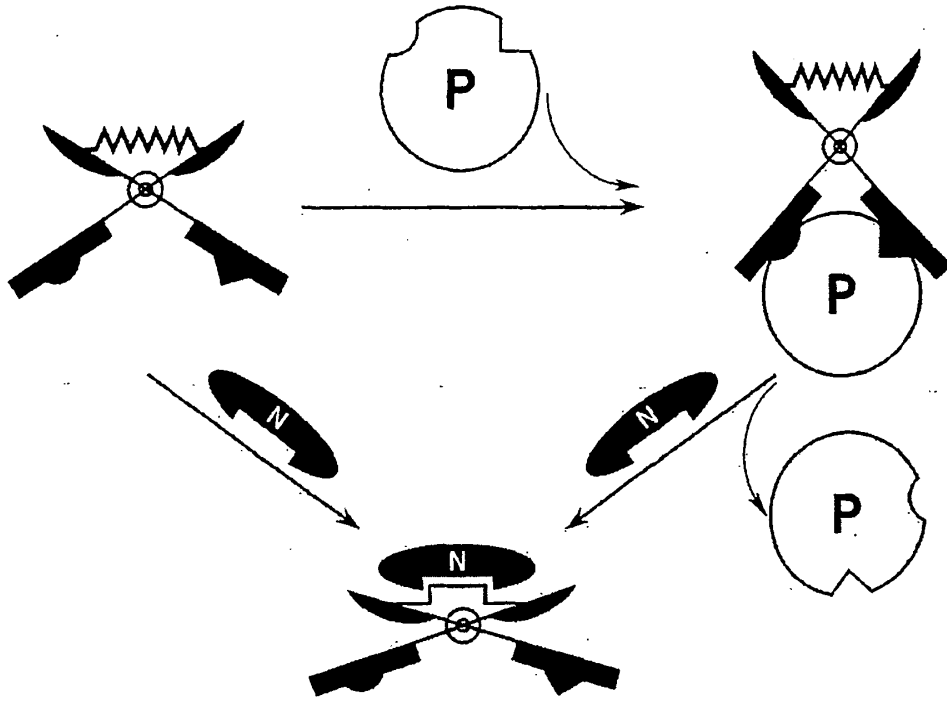


Figure 1A.

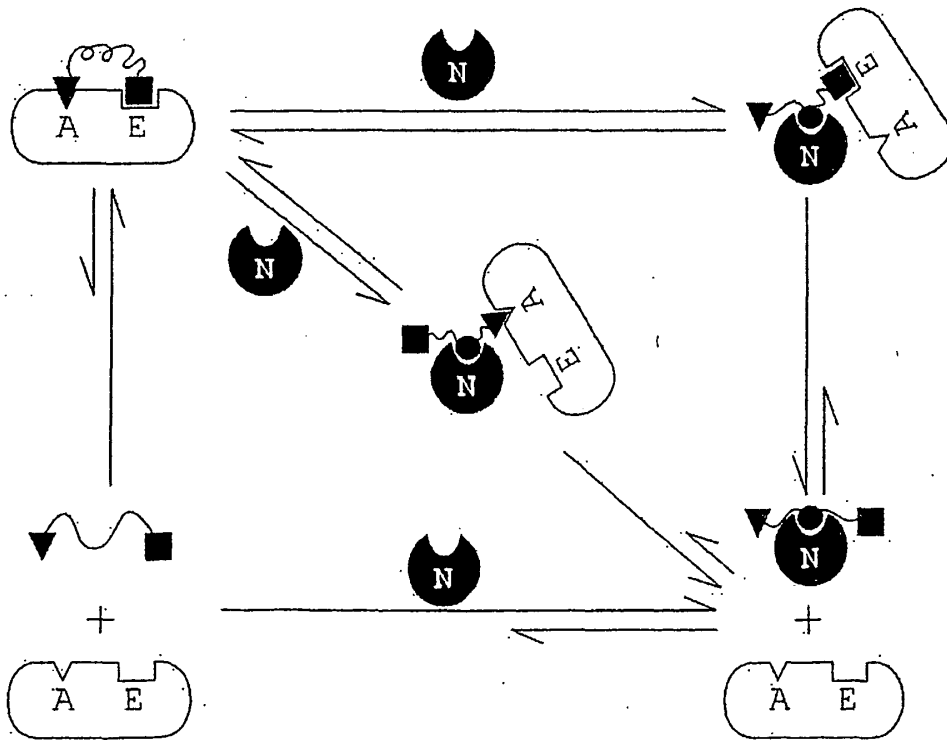


Figure 1B.

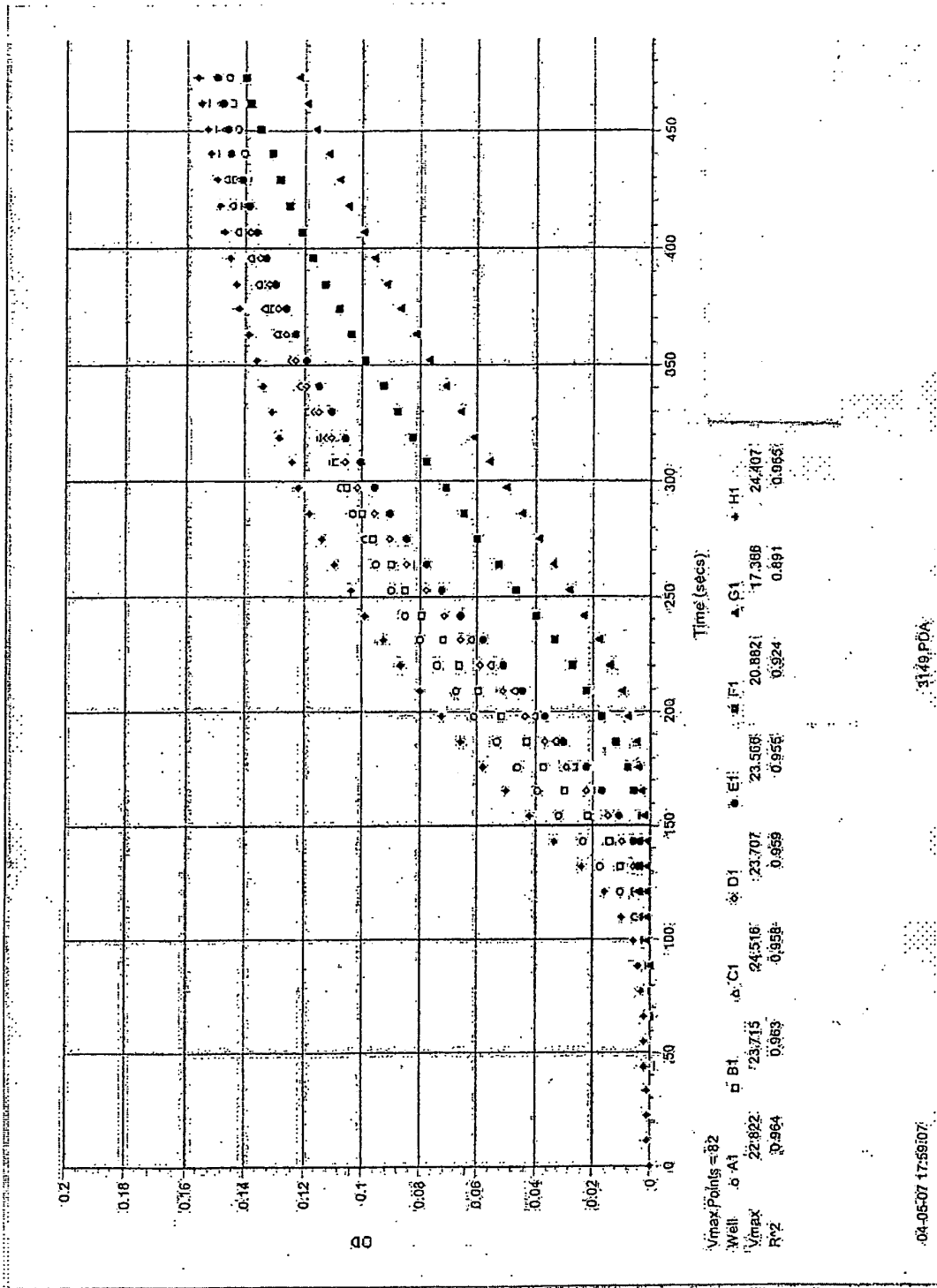


Figure 2a.

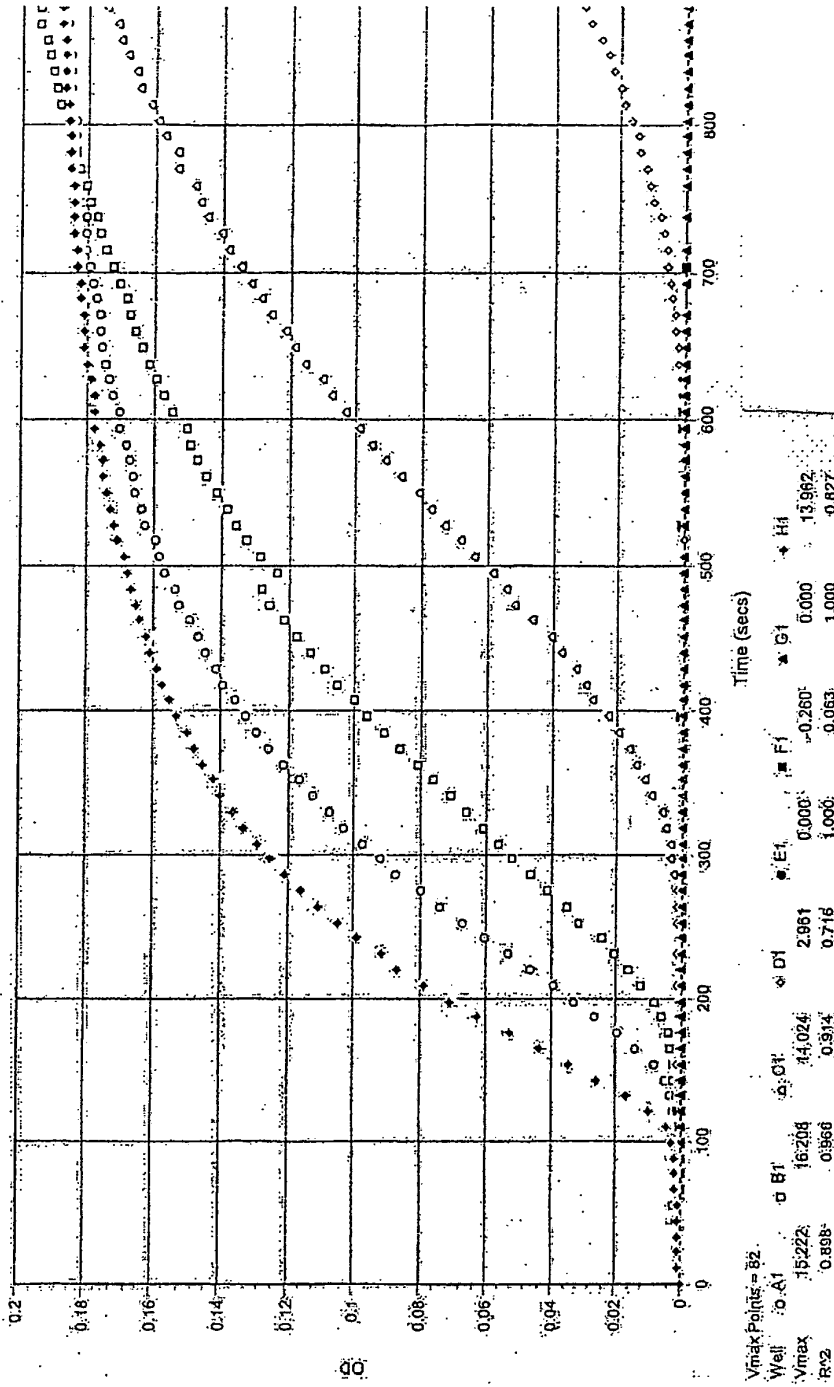


Figure 2b.

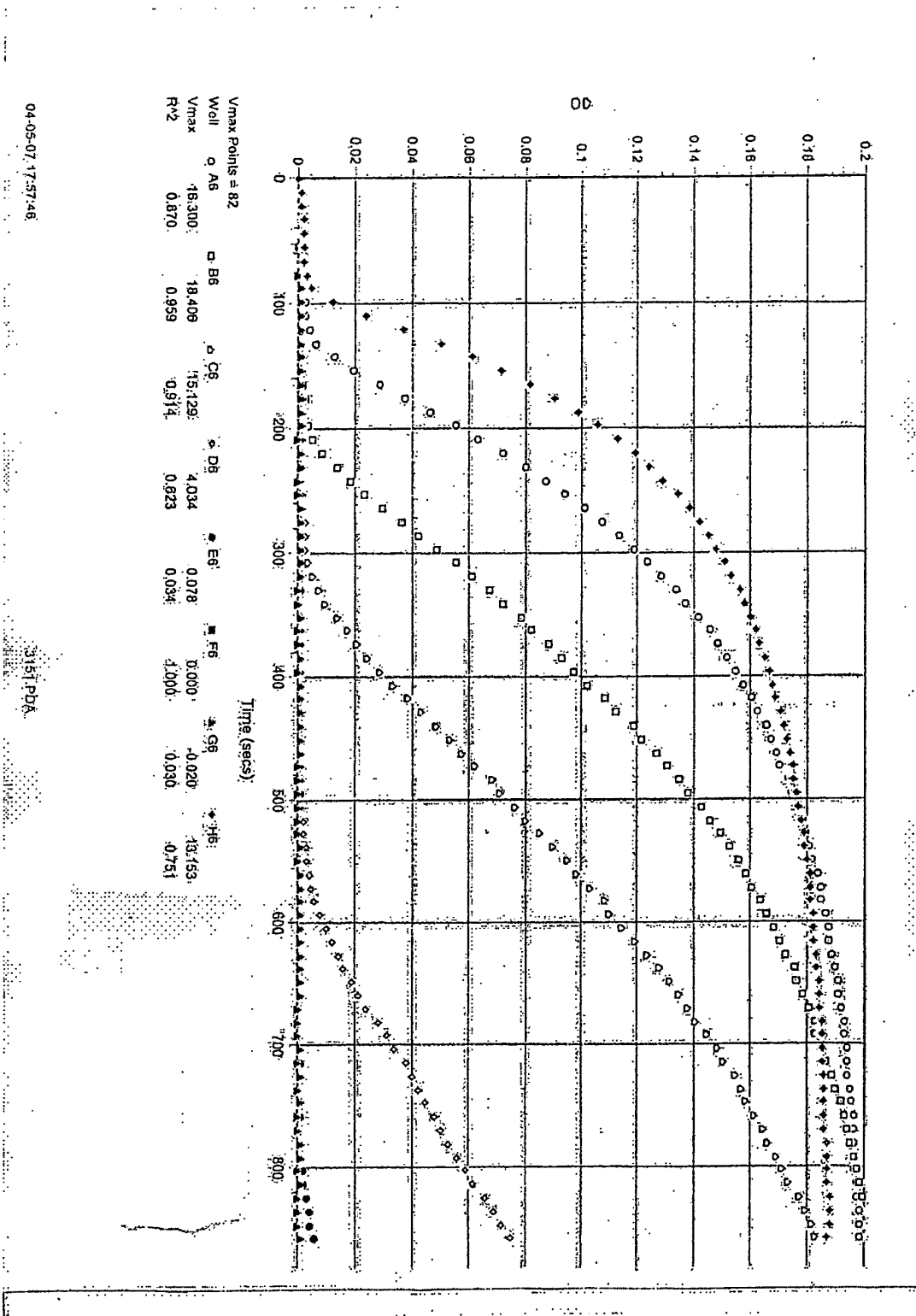


Figure 2c.

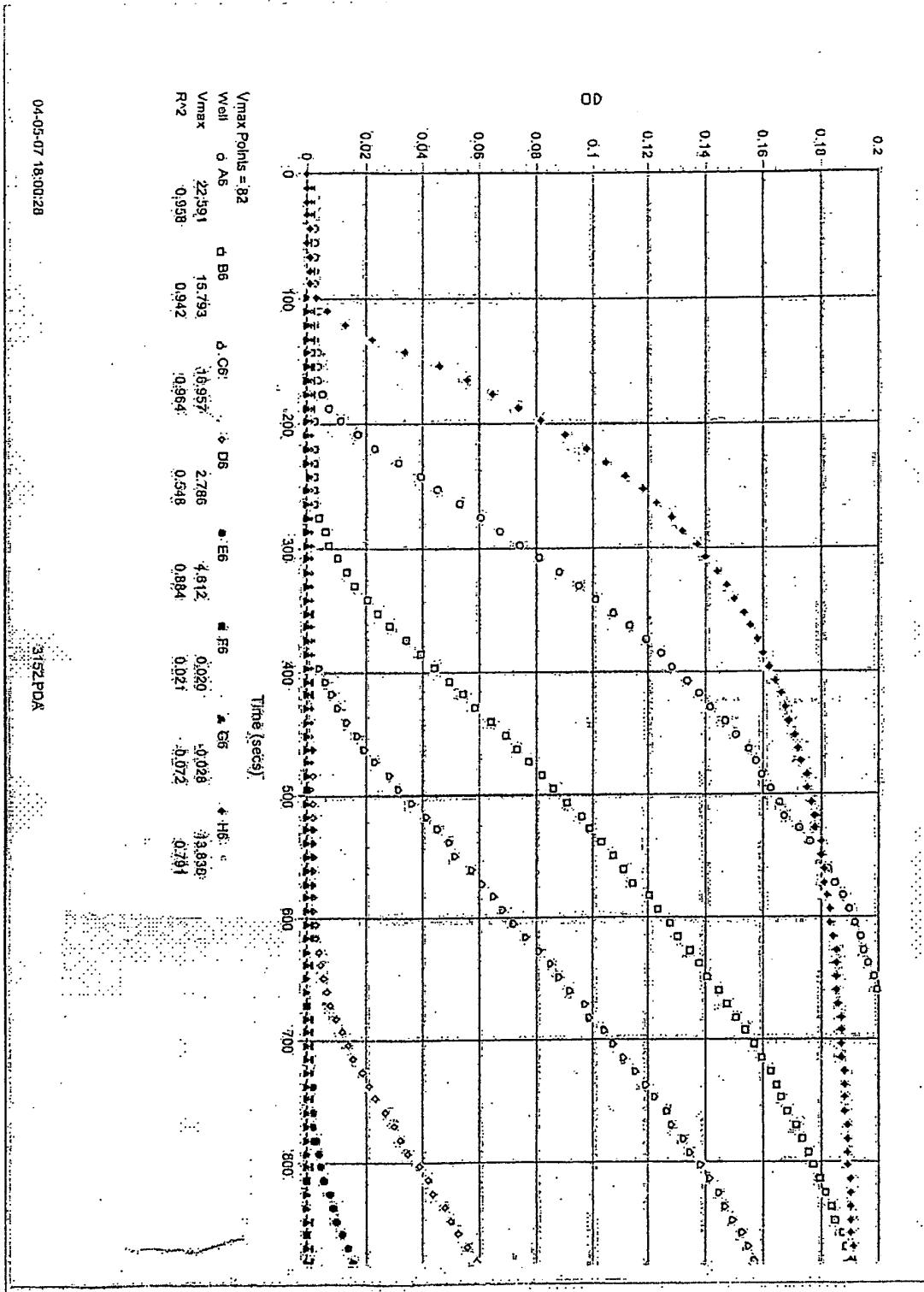


Figure 2d.

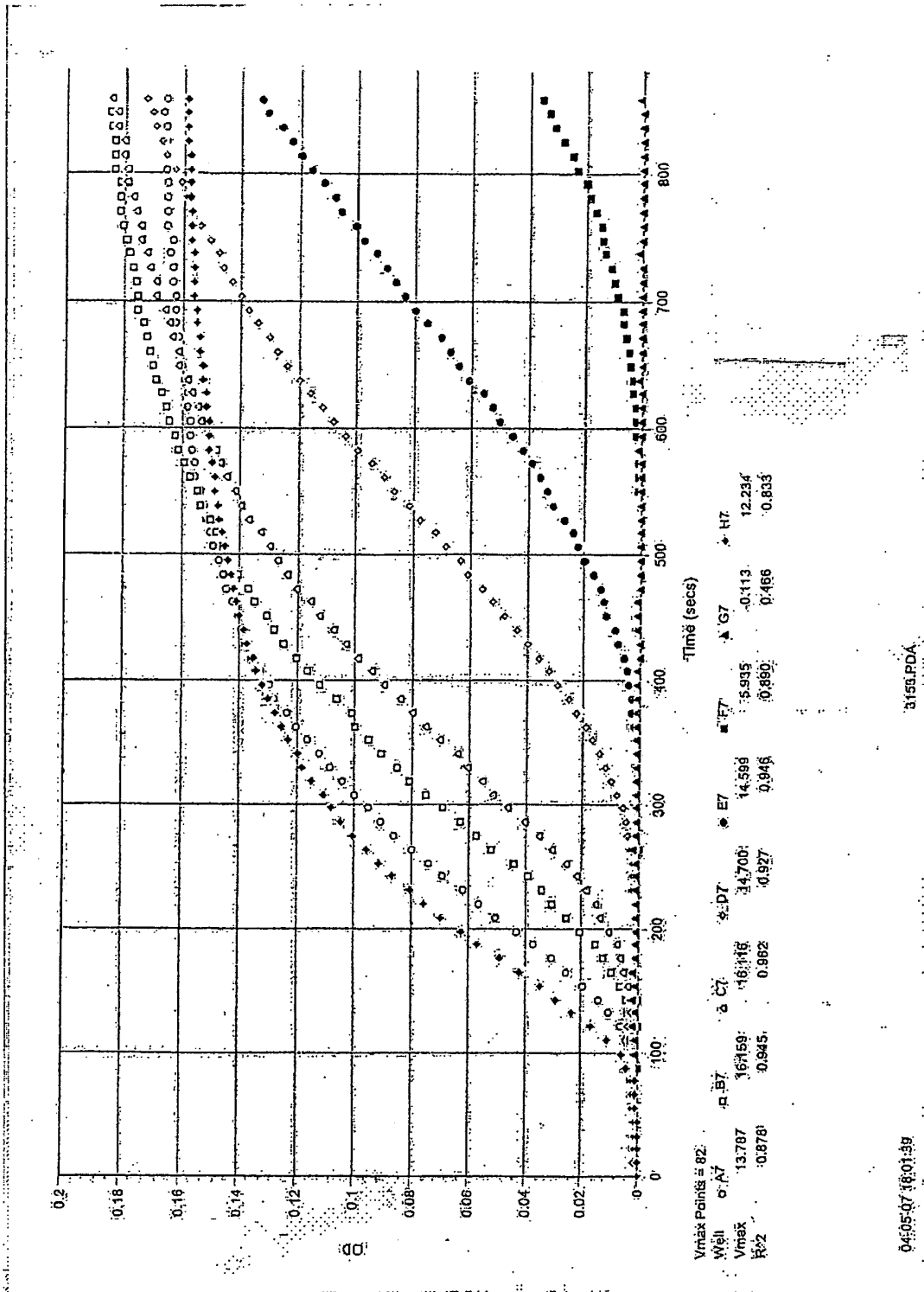
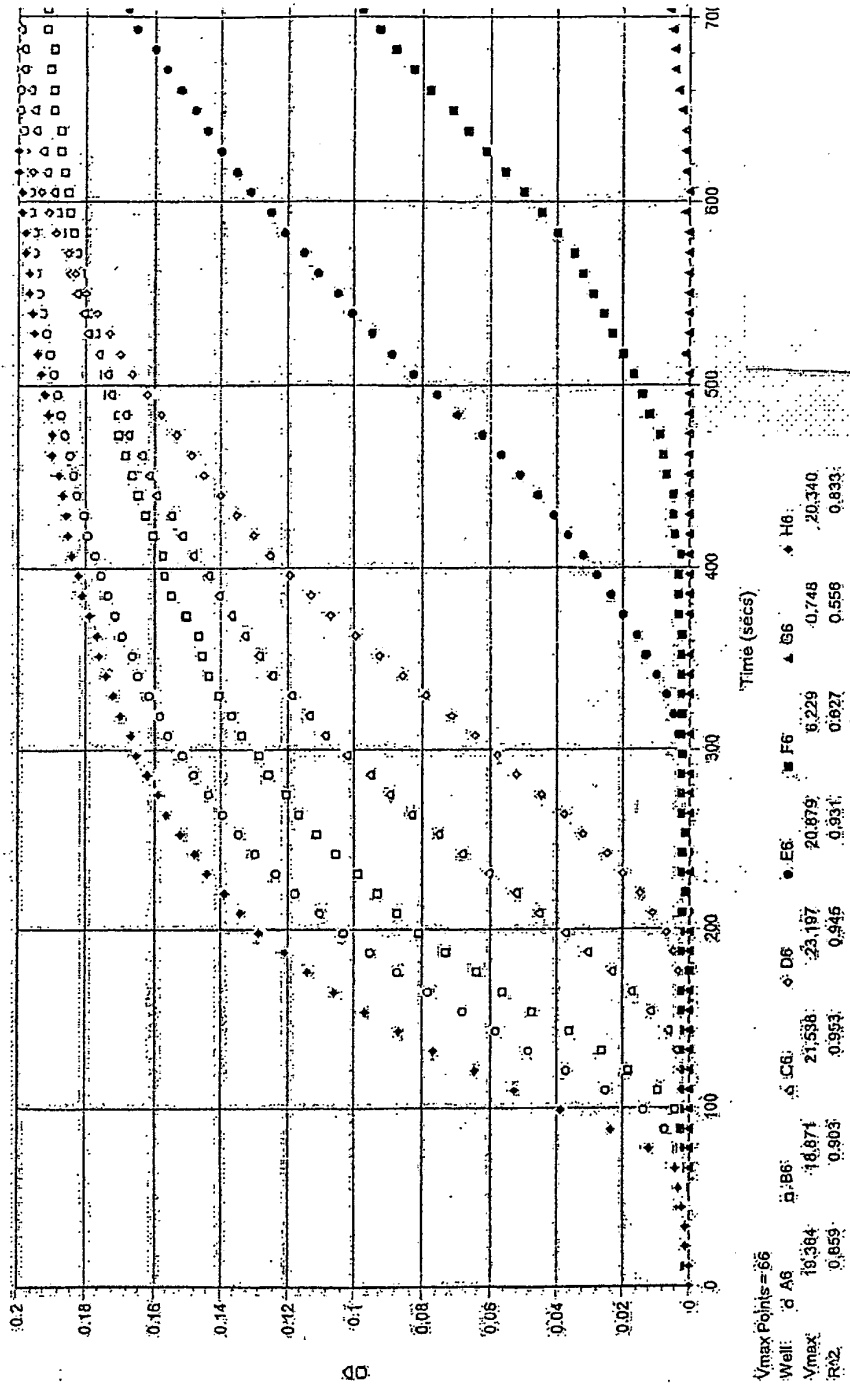


Figure 2e.



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Figure 2f.

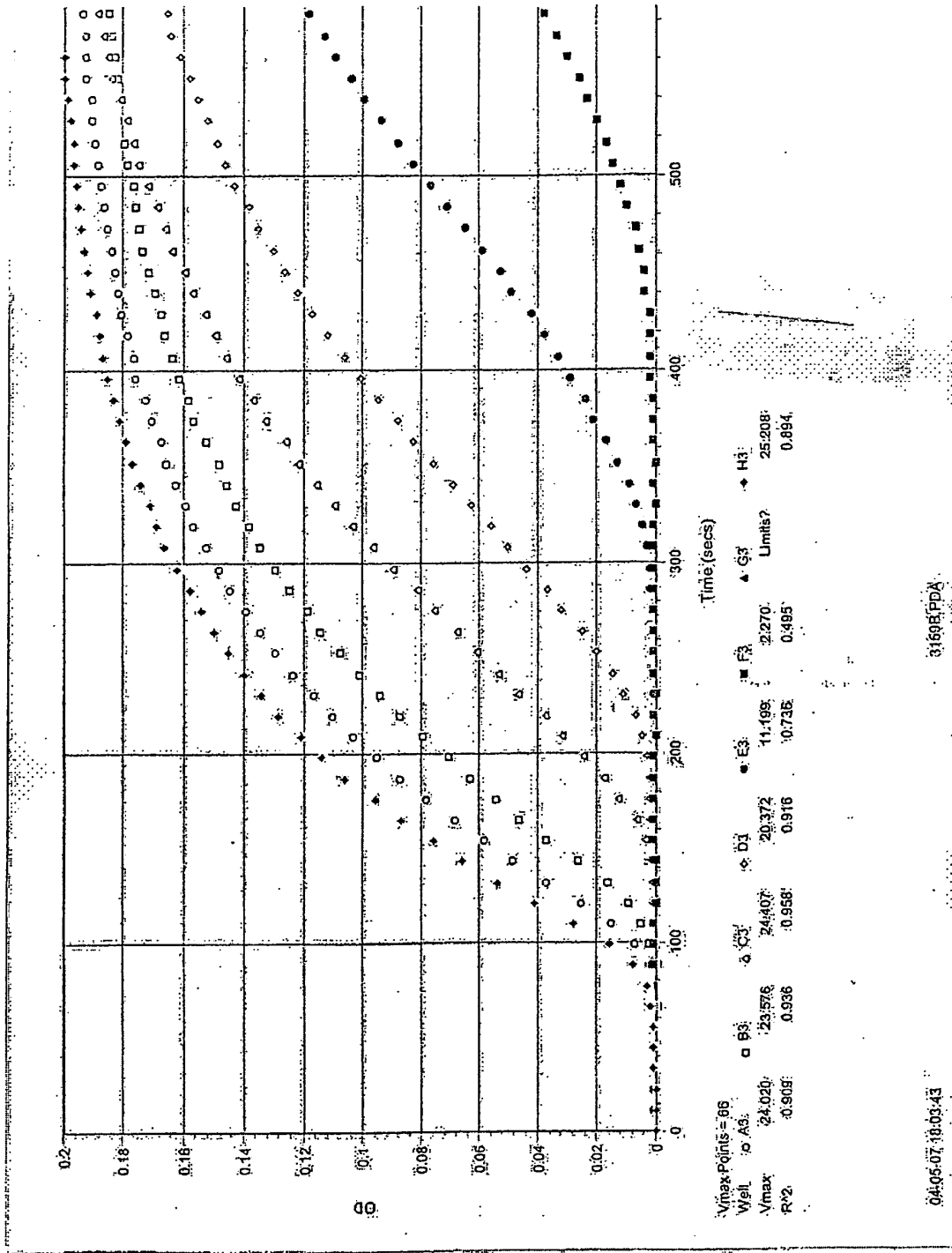


Figure 2g.

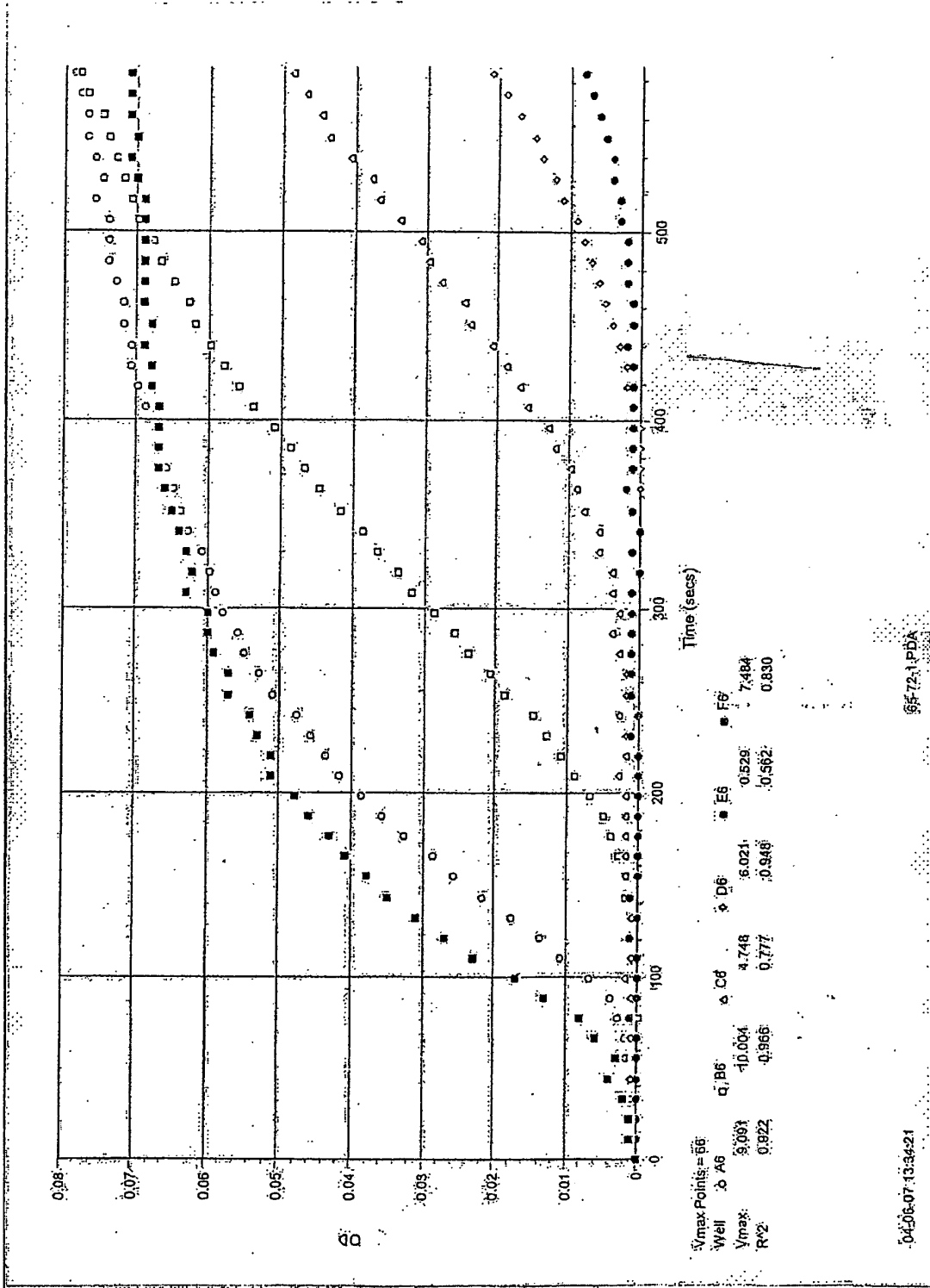


Figure 2h

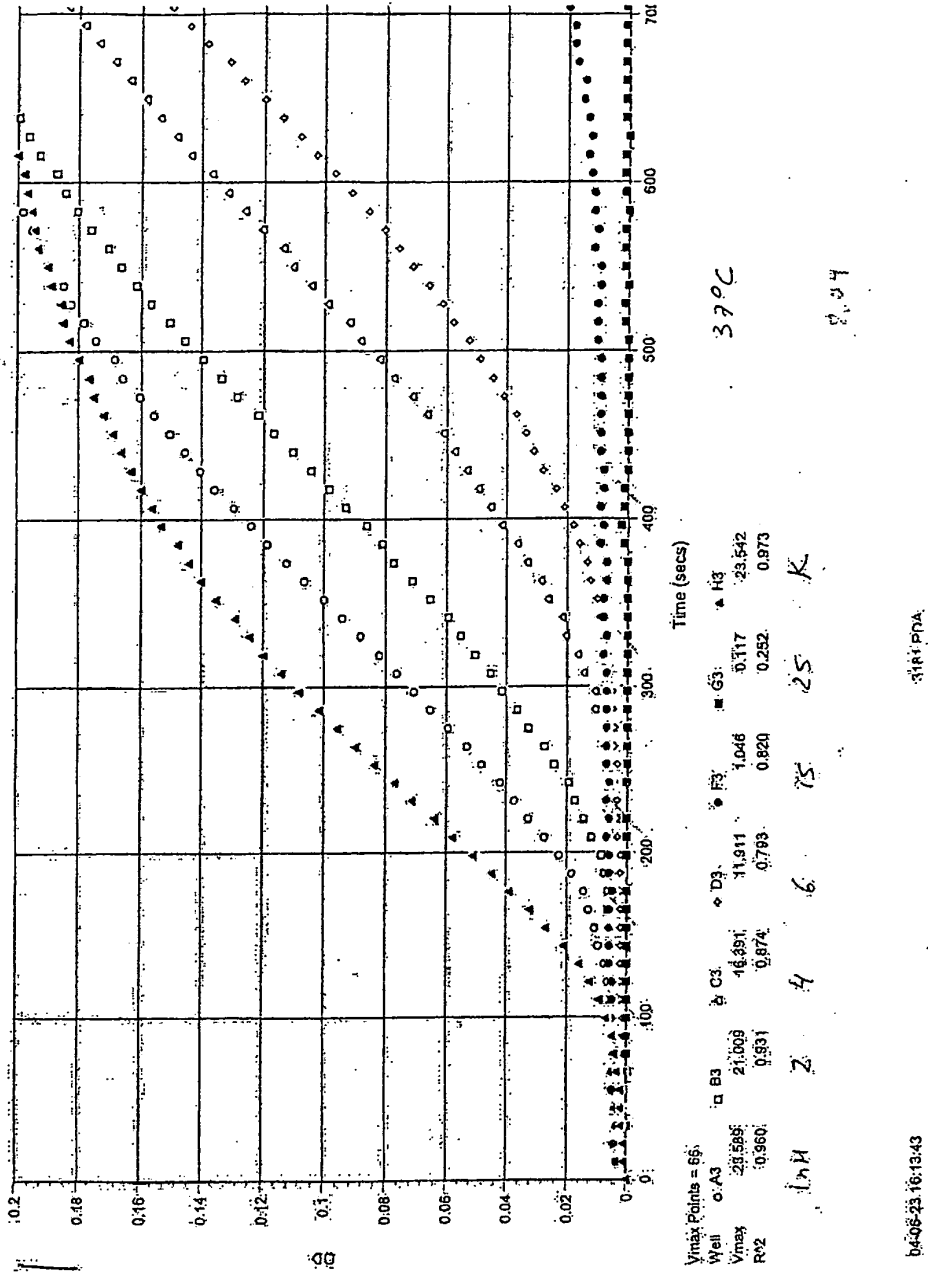


Figure 2i

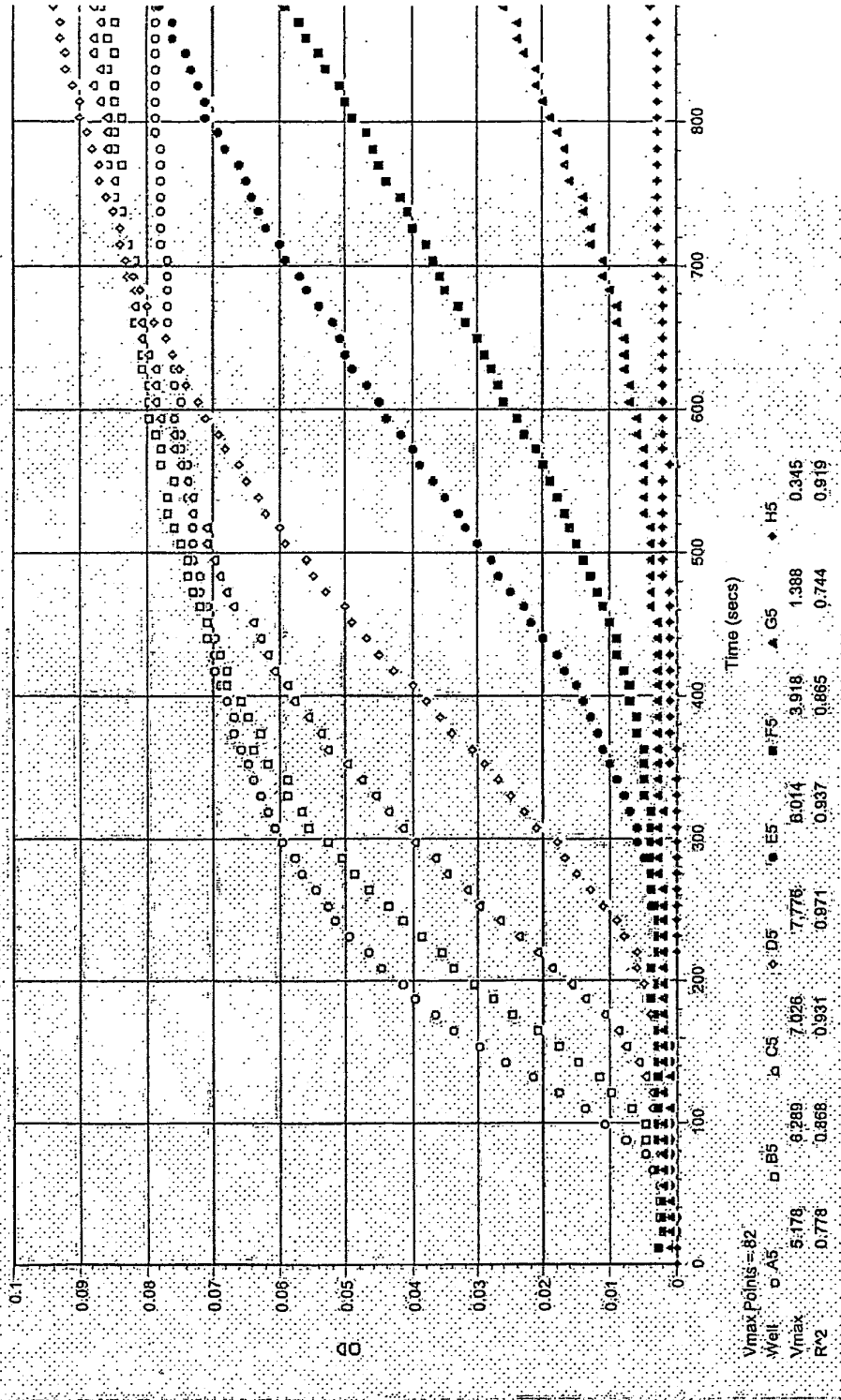


Figure 2j

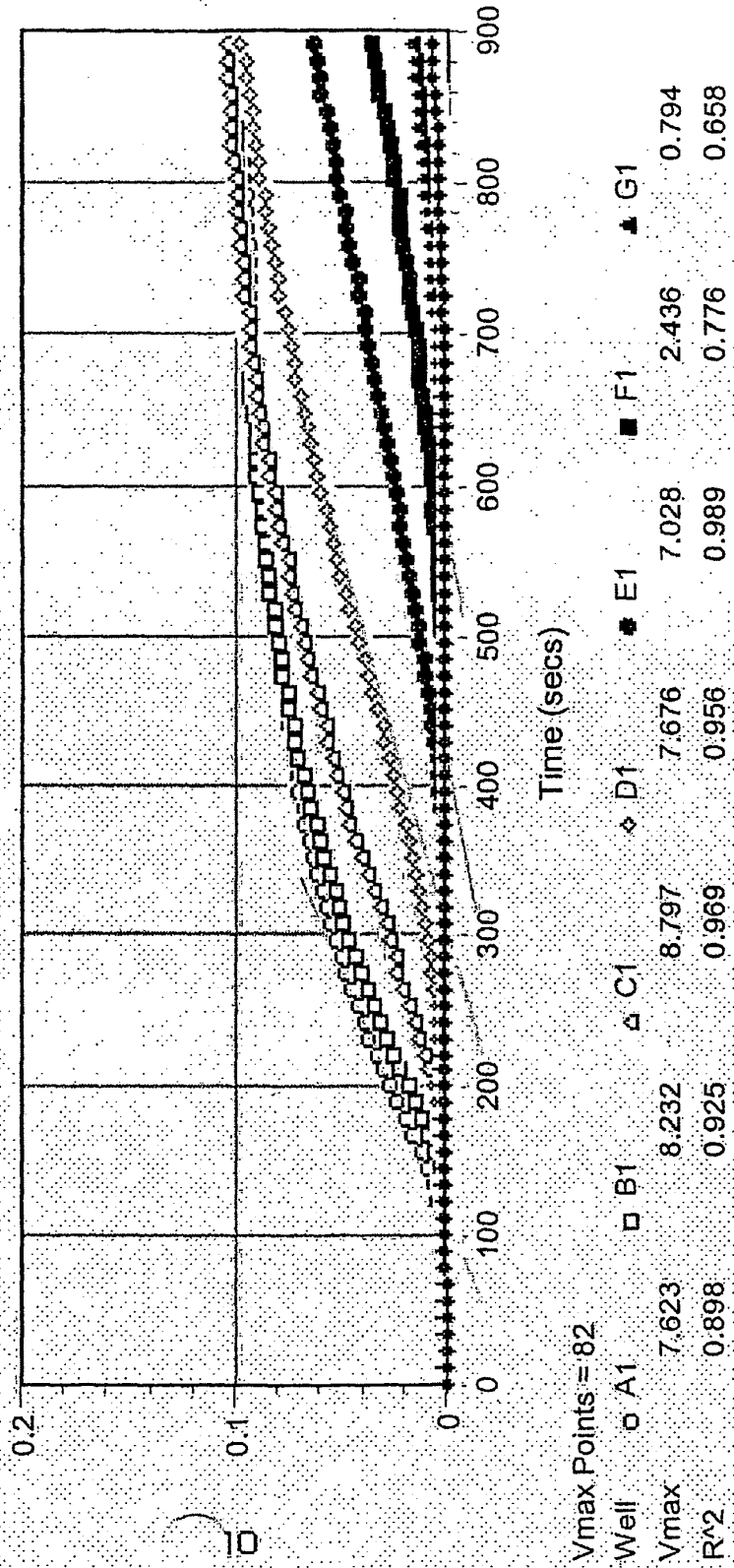


Figure 2k

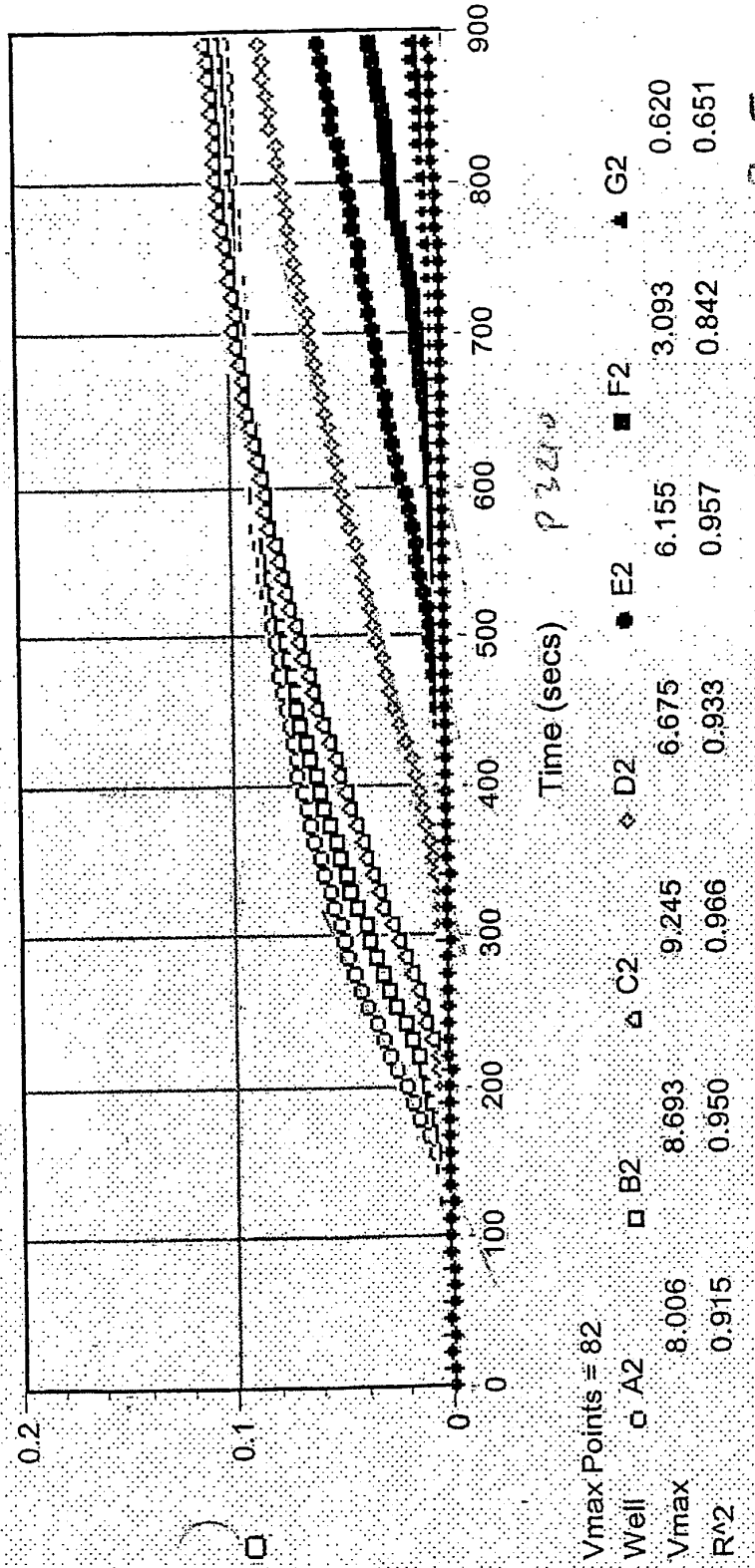


Figure 21

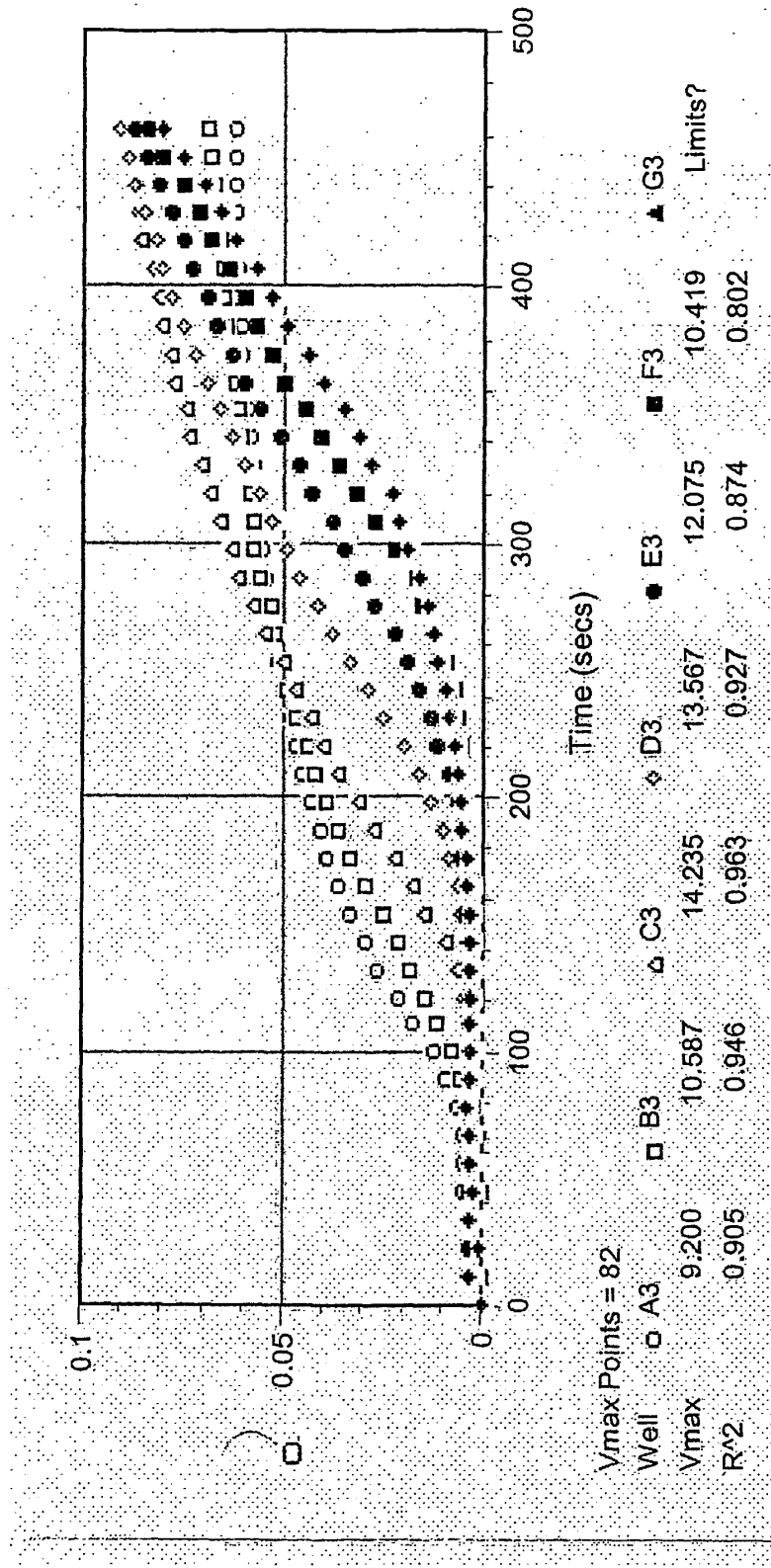


Figure 2m

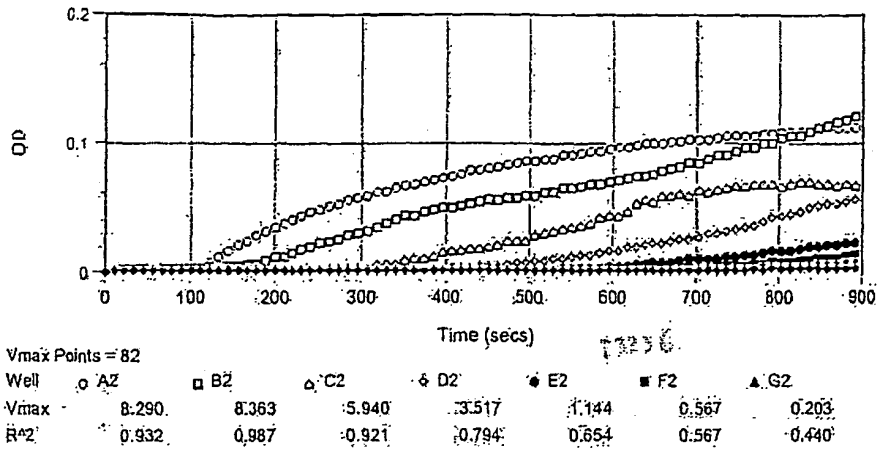


Figure 2n

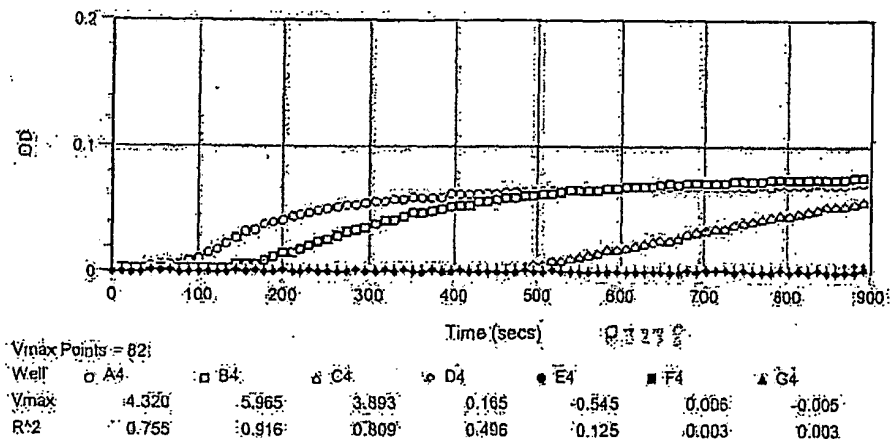
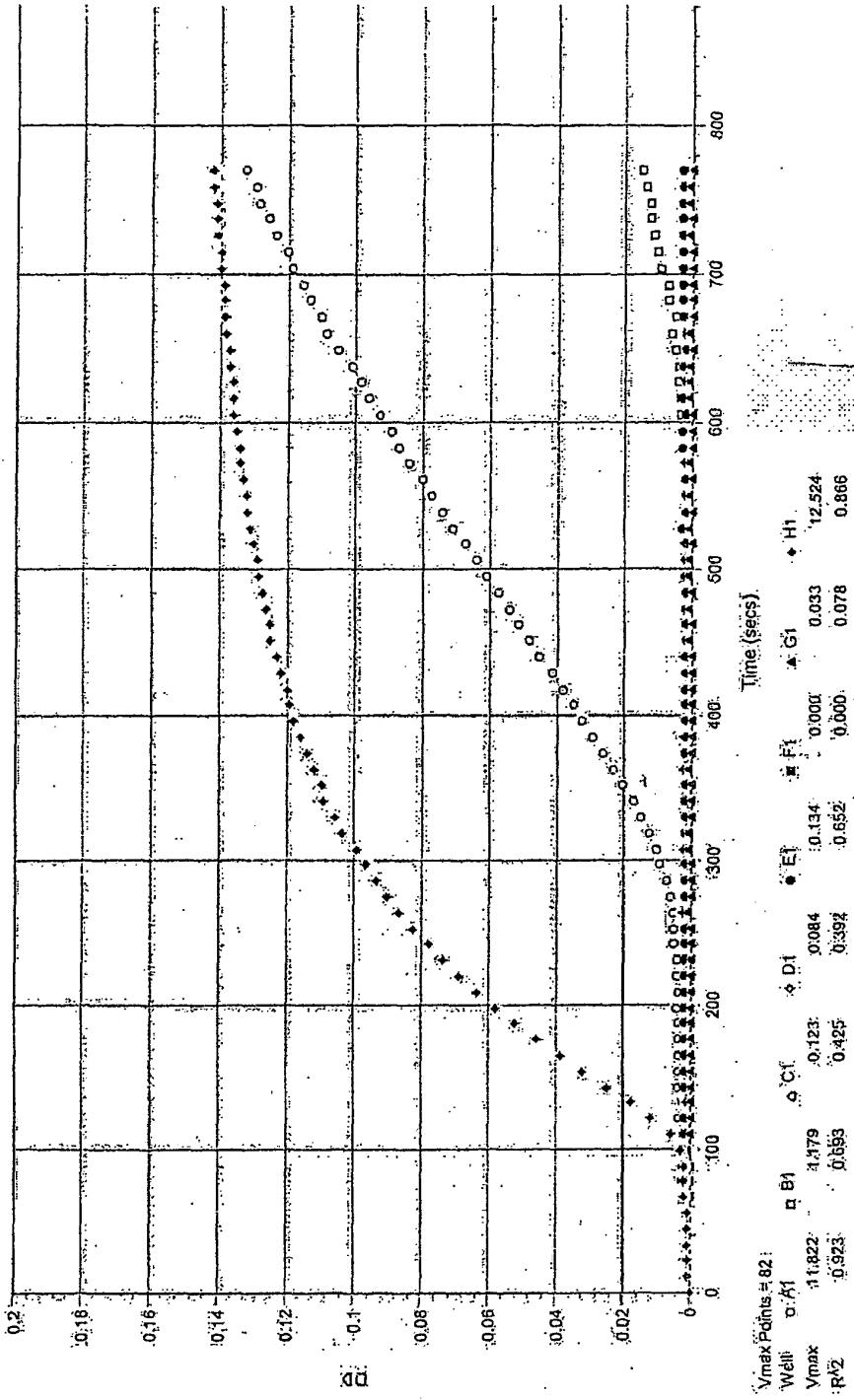


Figure 2o



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Figure 3a

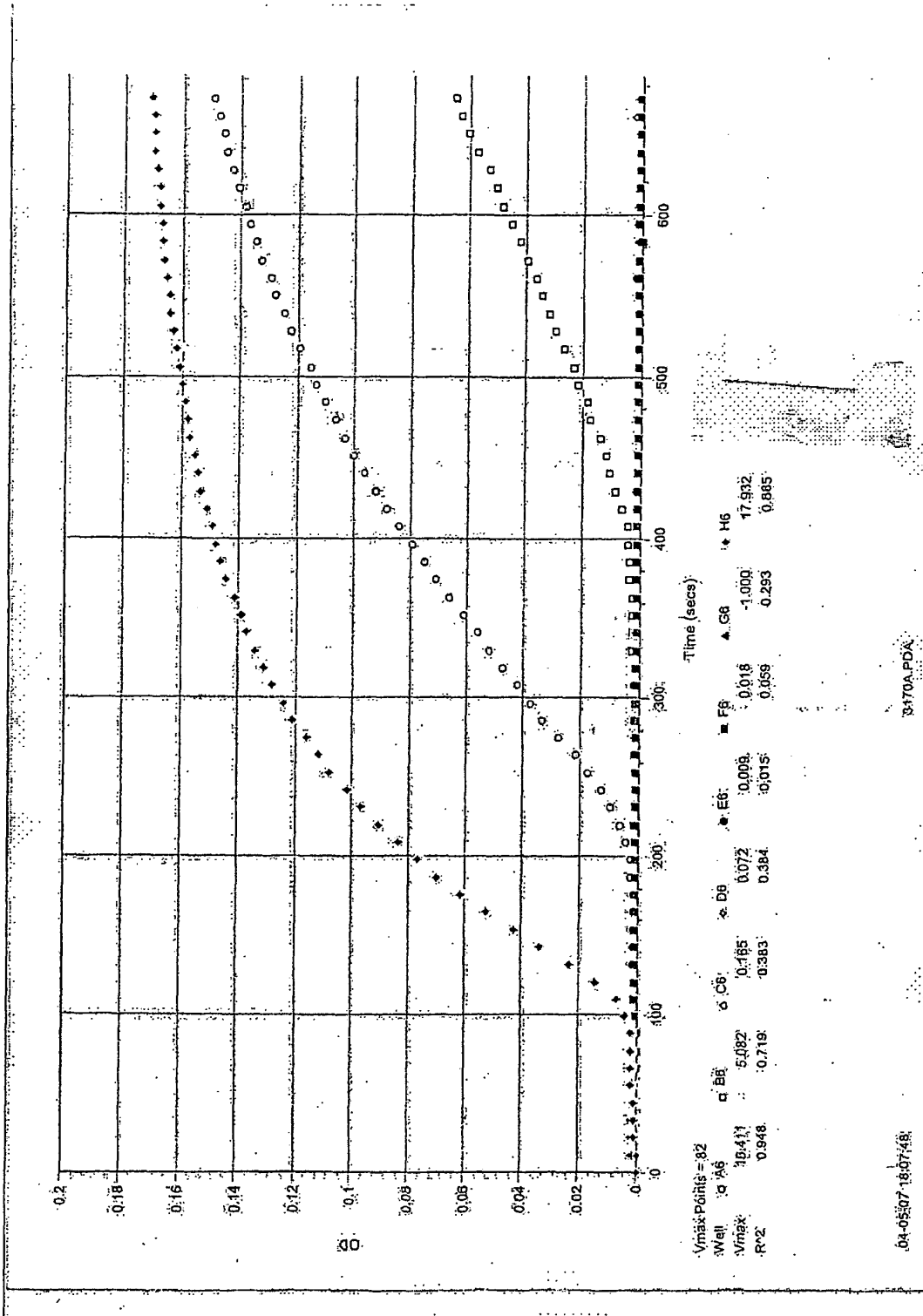


Figure 3b

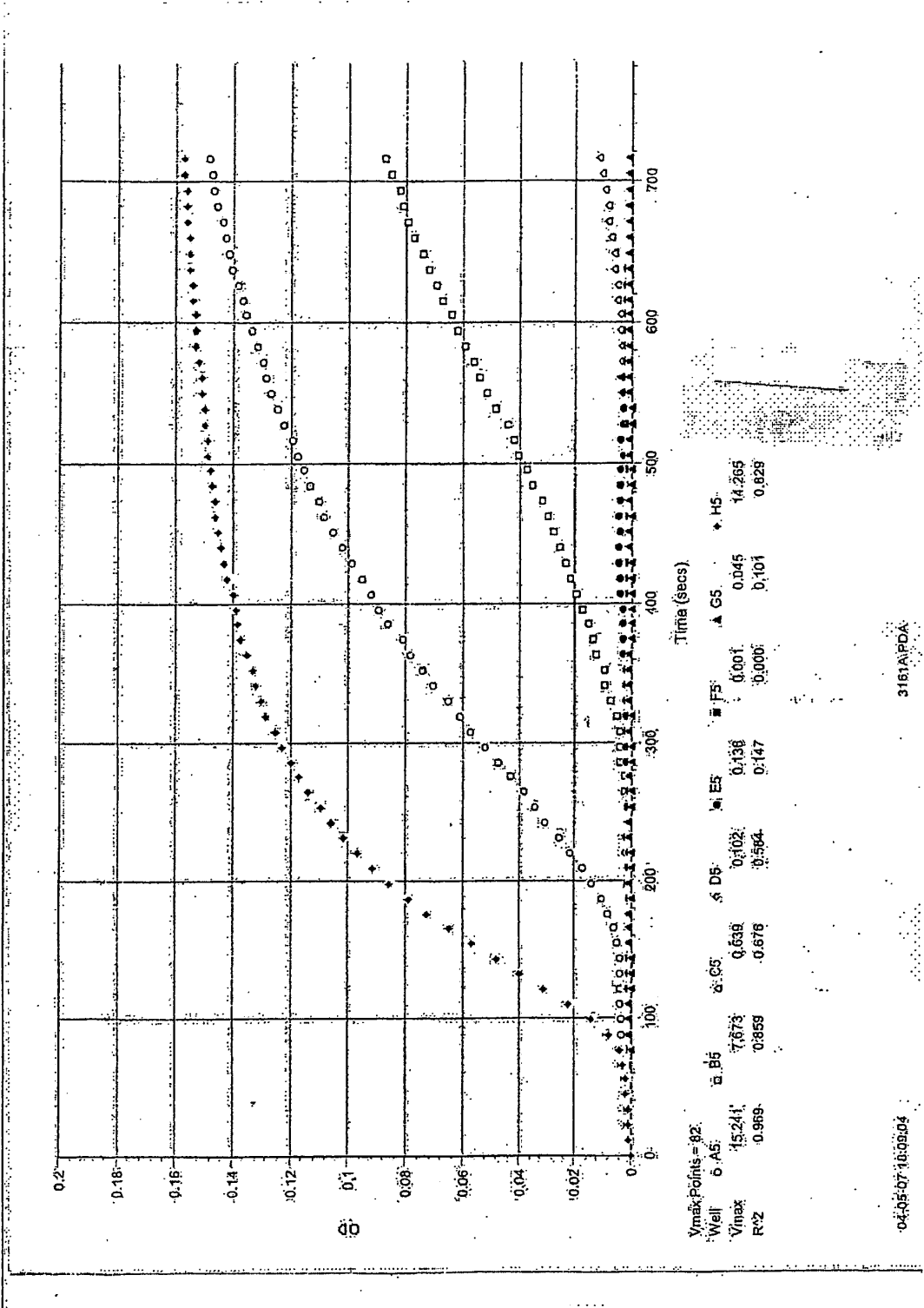
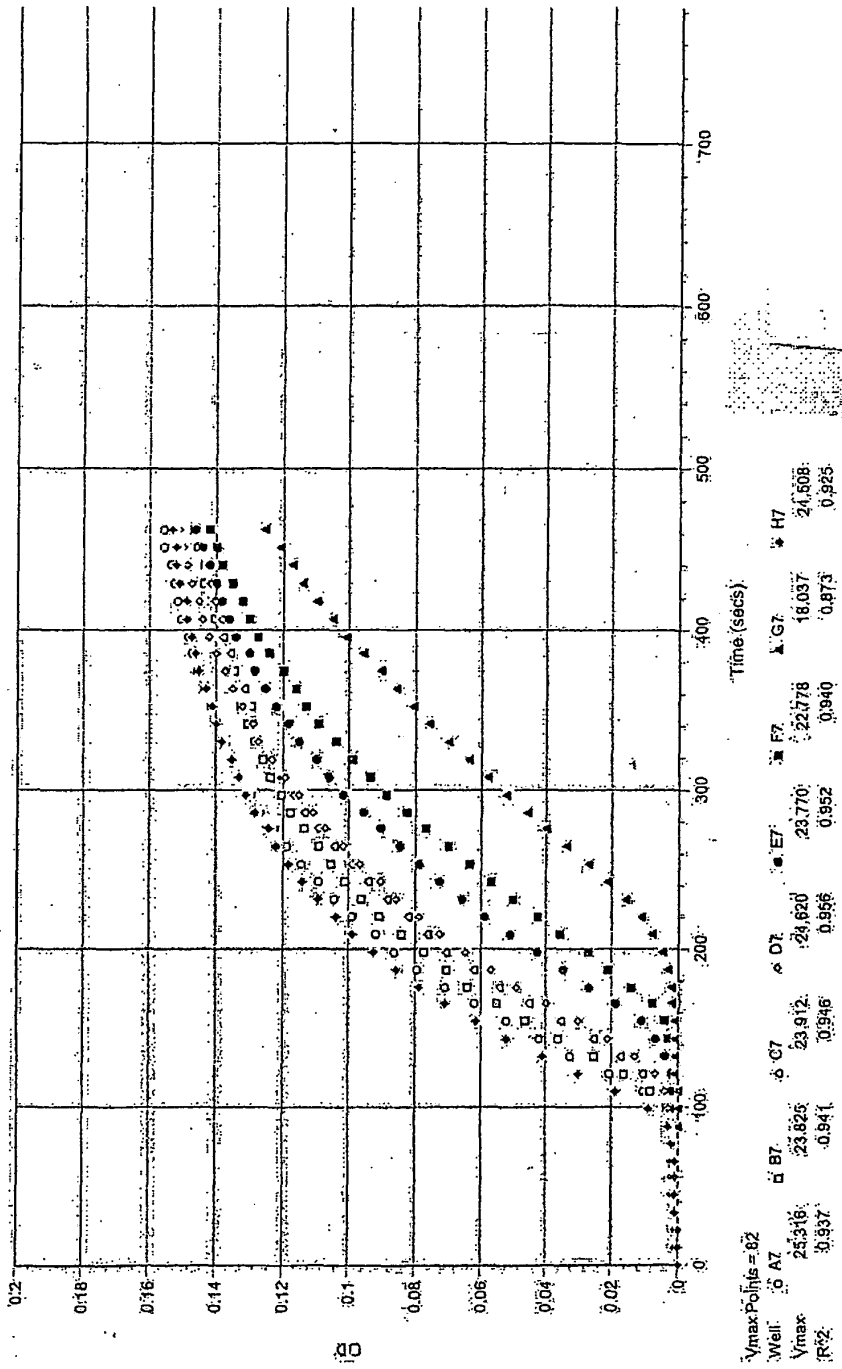


Figure 3c



3162A.PDA

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Figure 3d

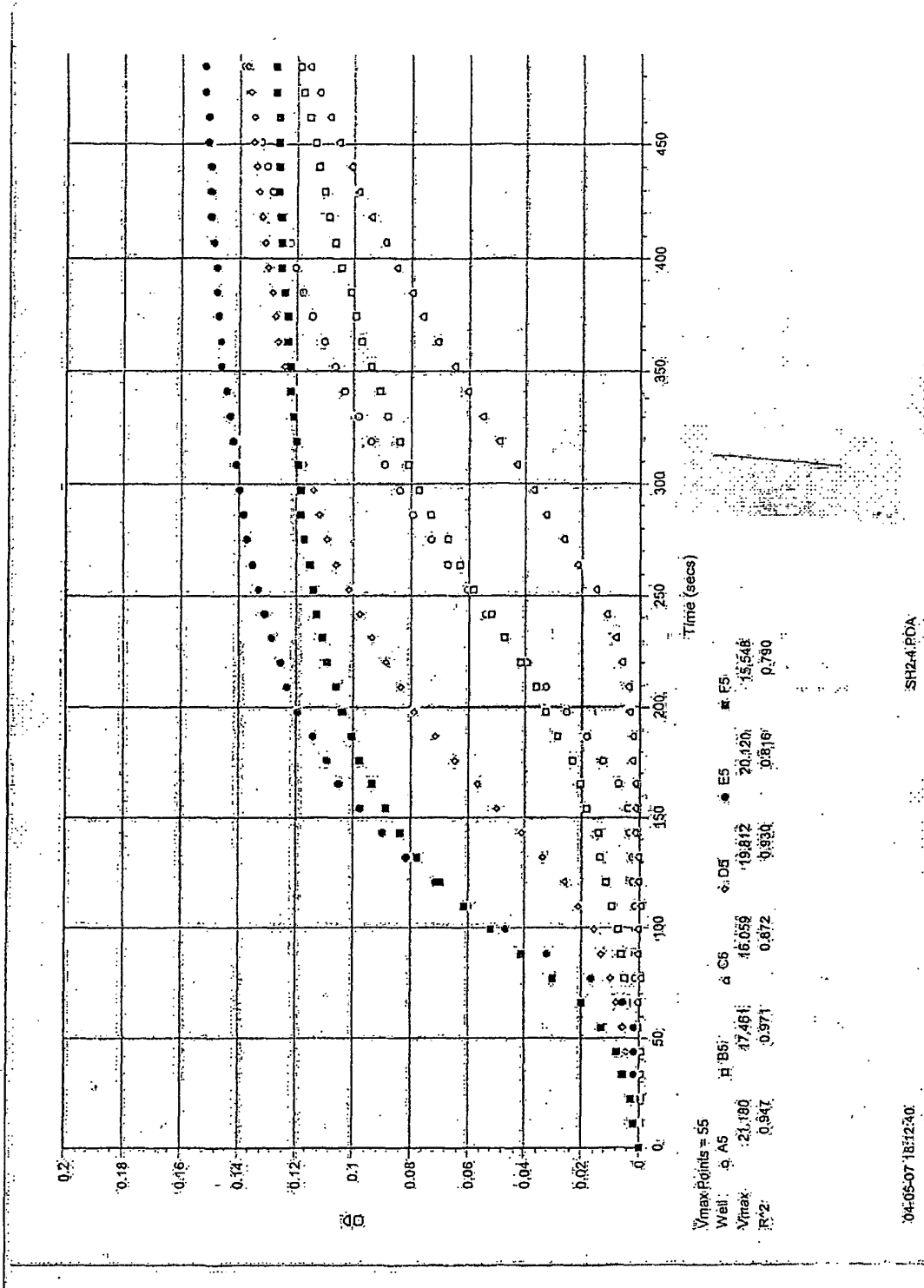


Figure 4a

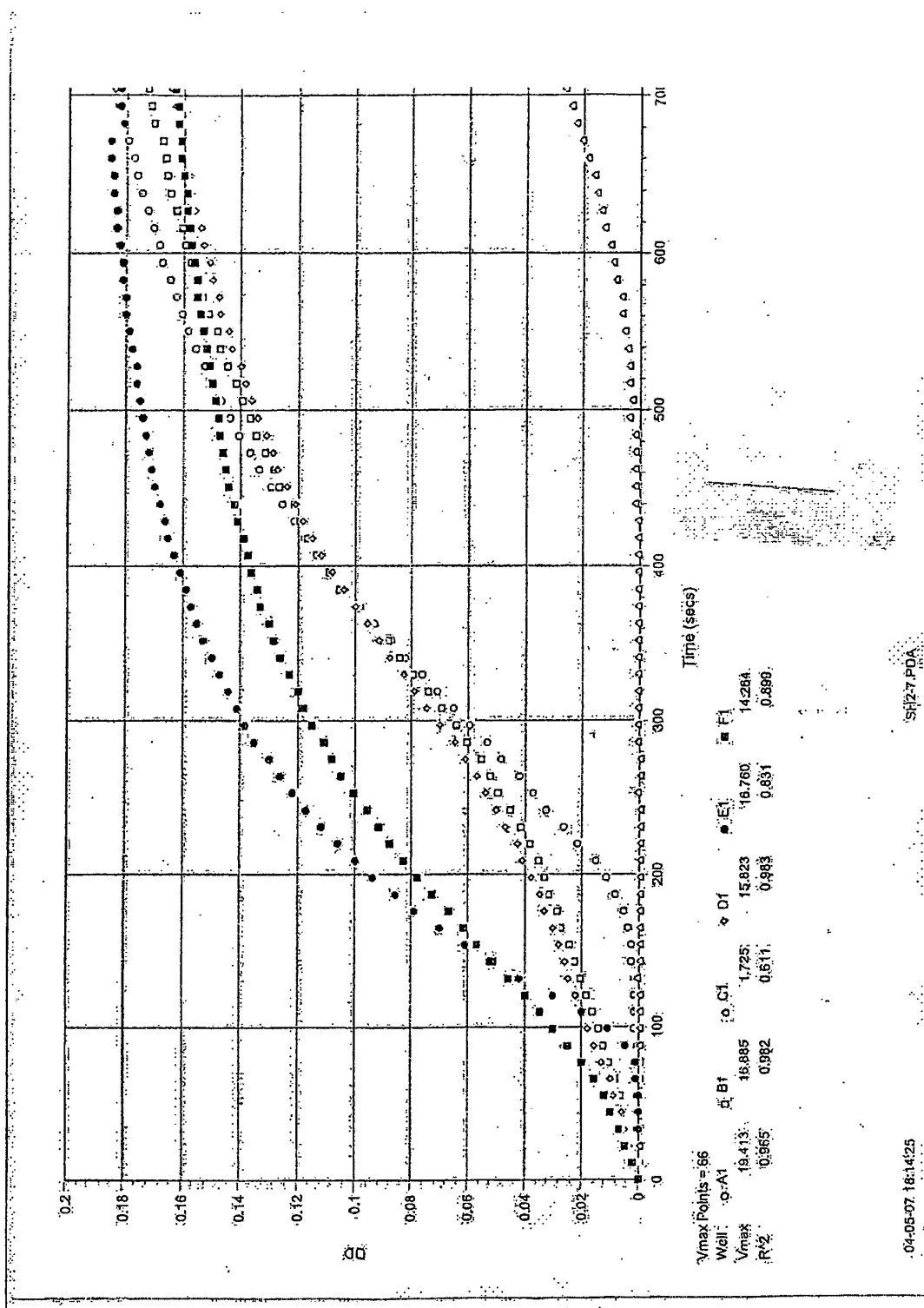


Figure 4b

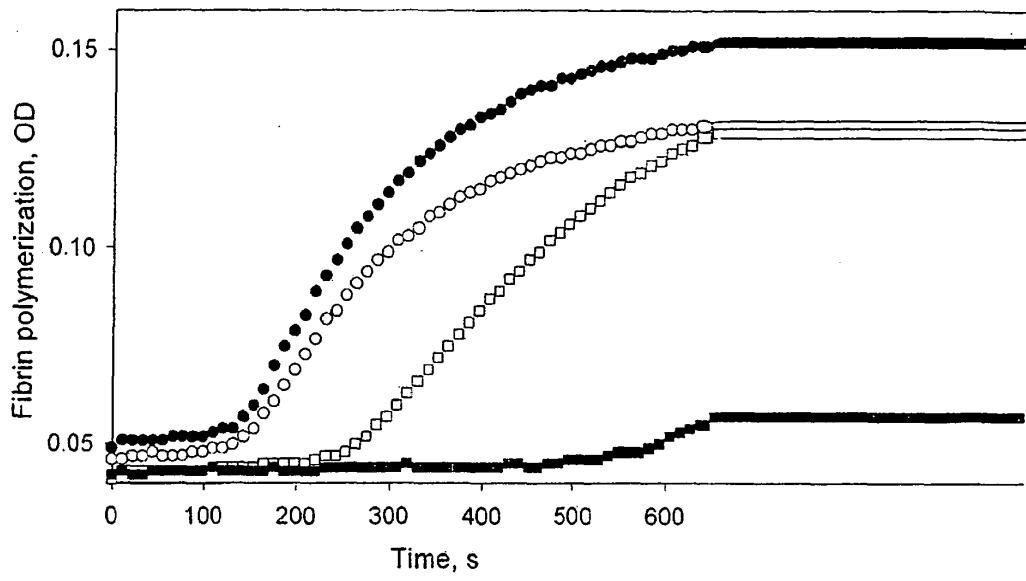


Figure 5

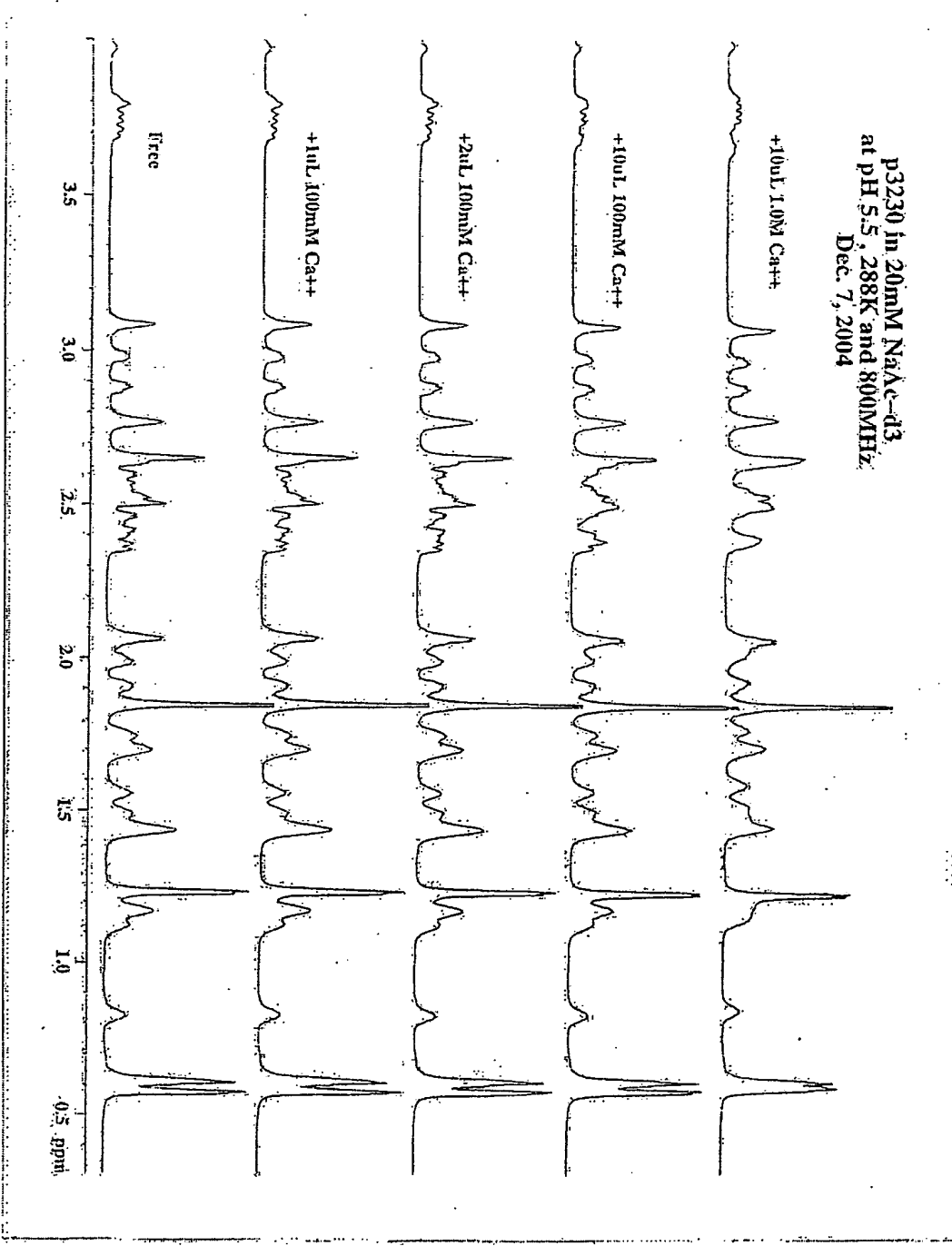


Figure 6a

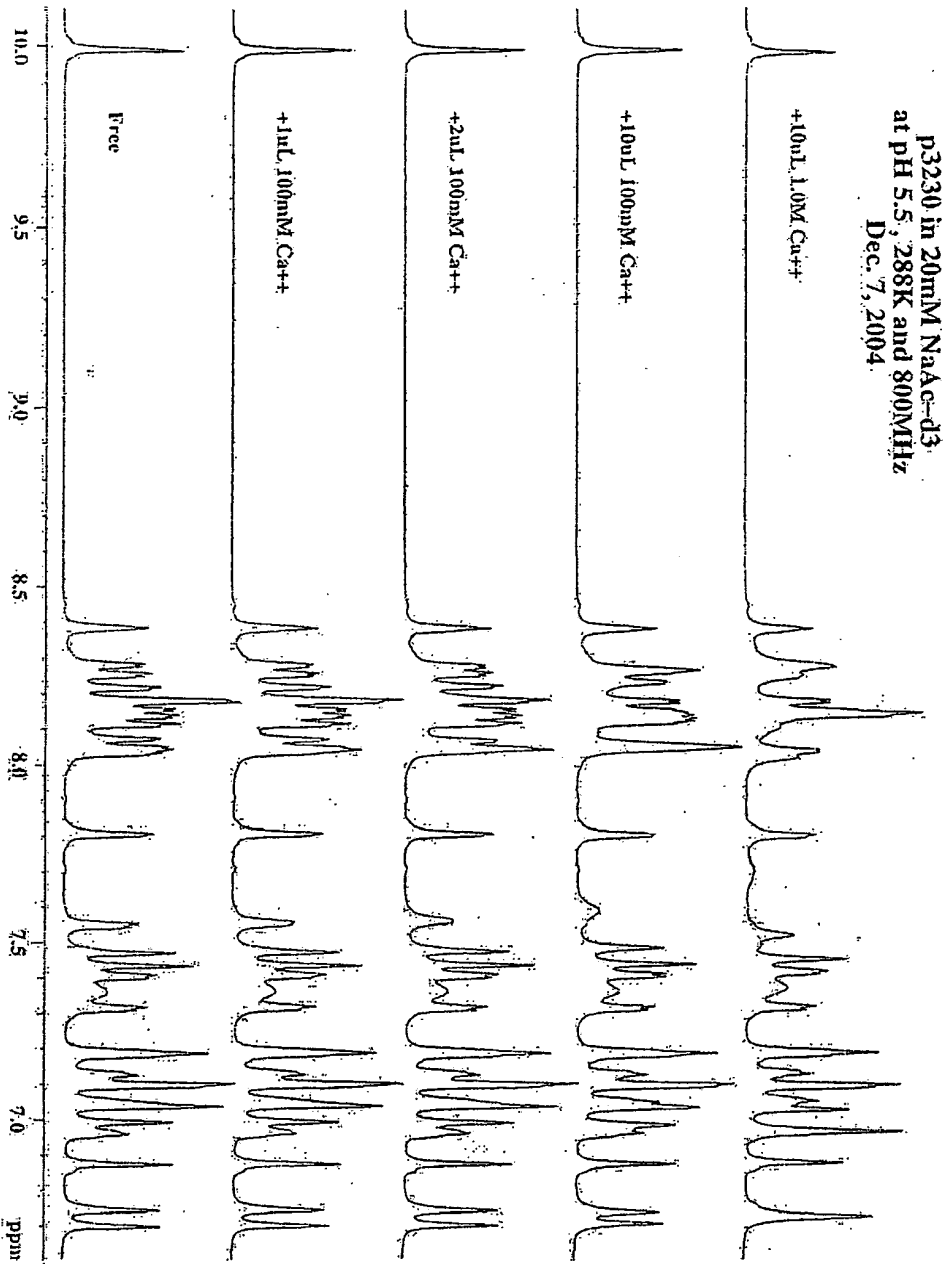


Figure 6b

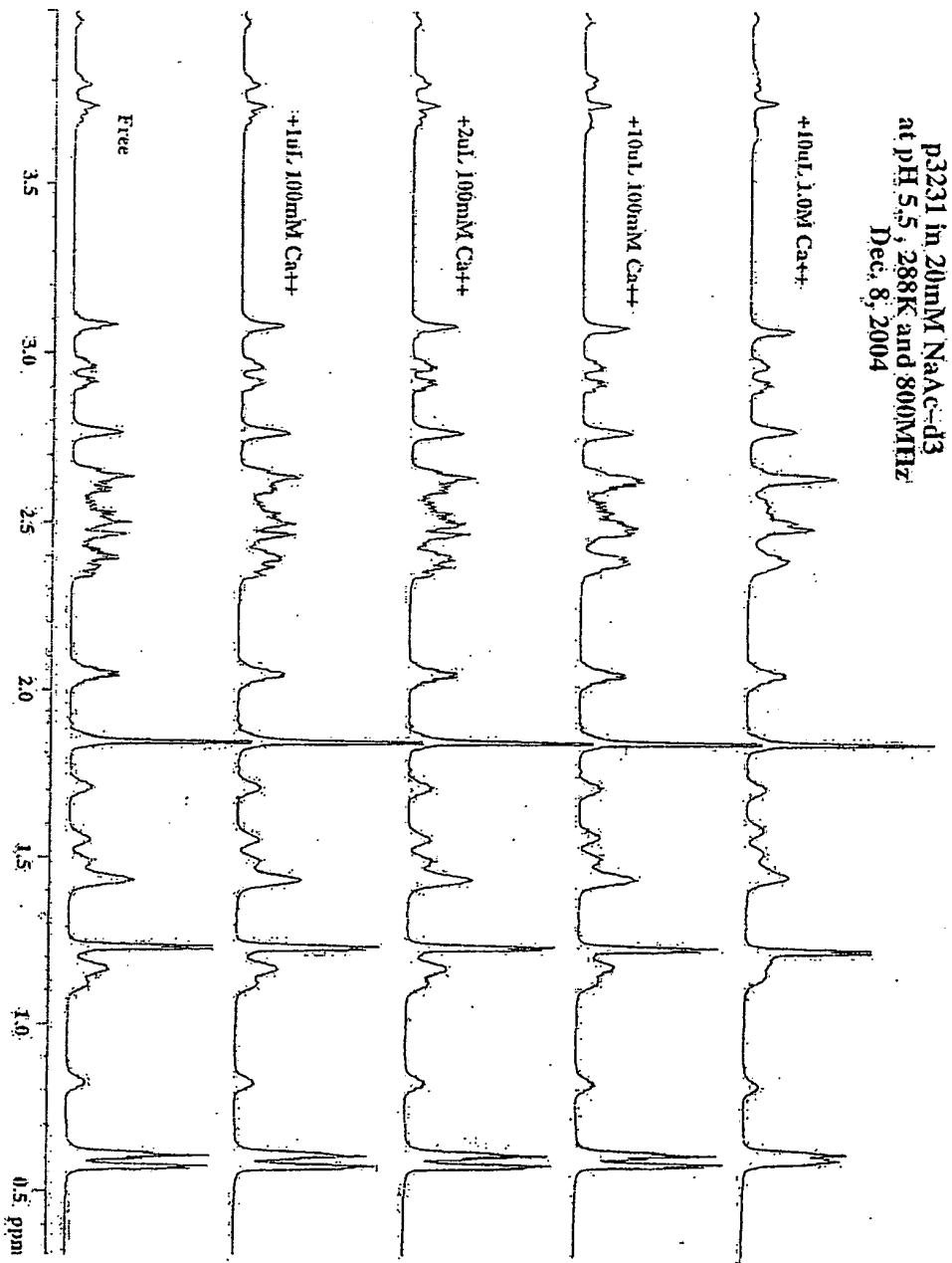


Figure 6c

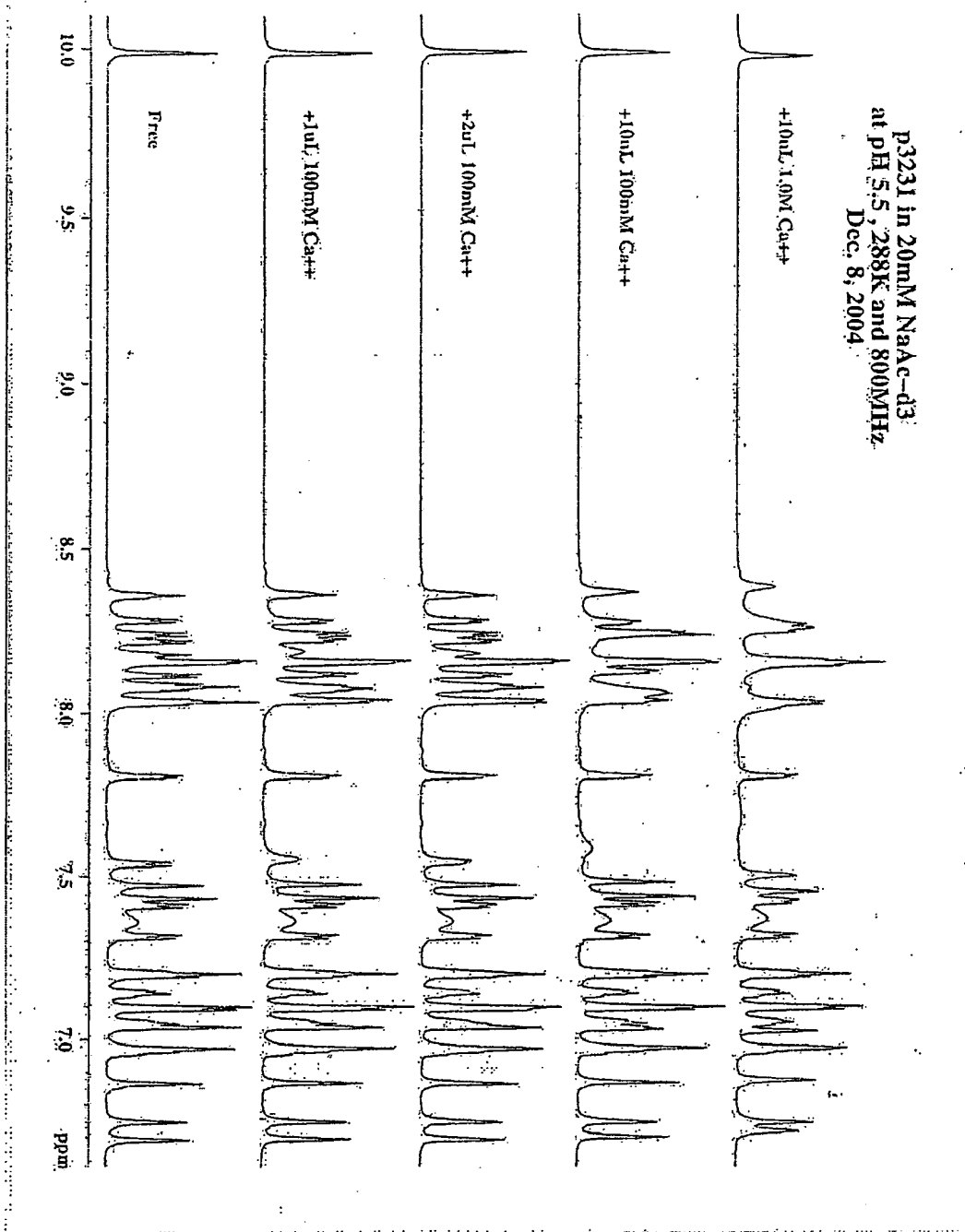


Figure 6d

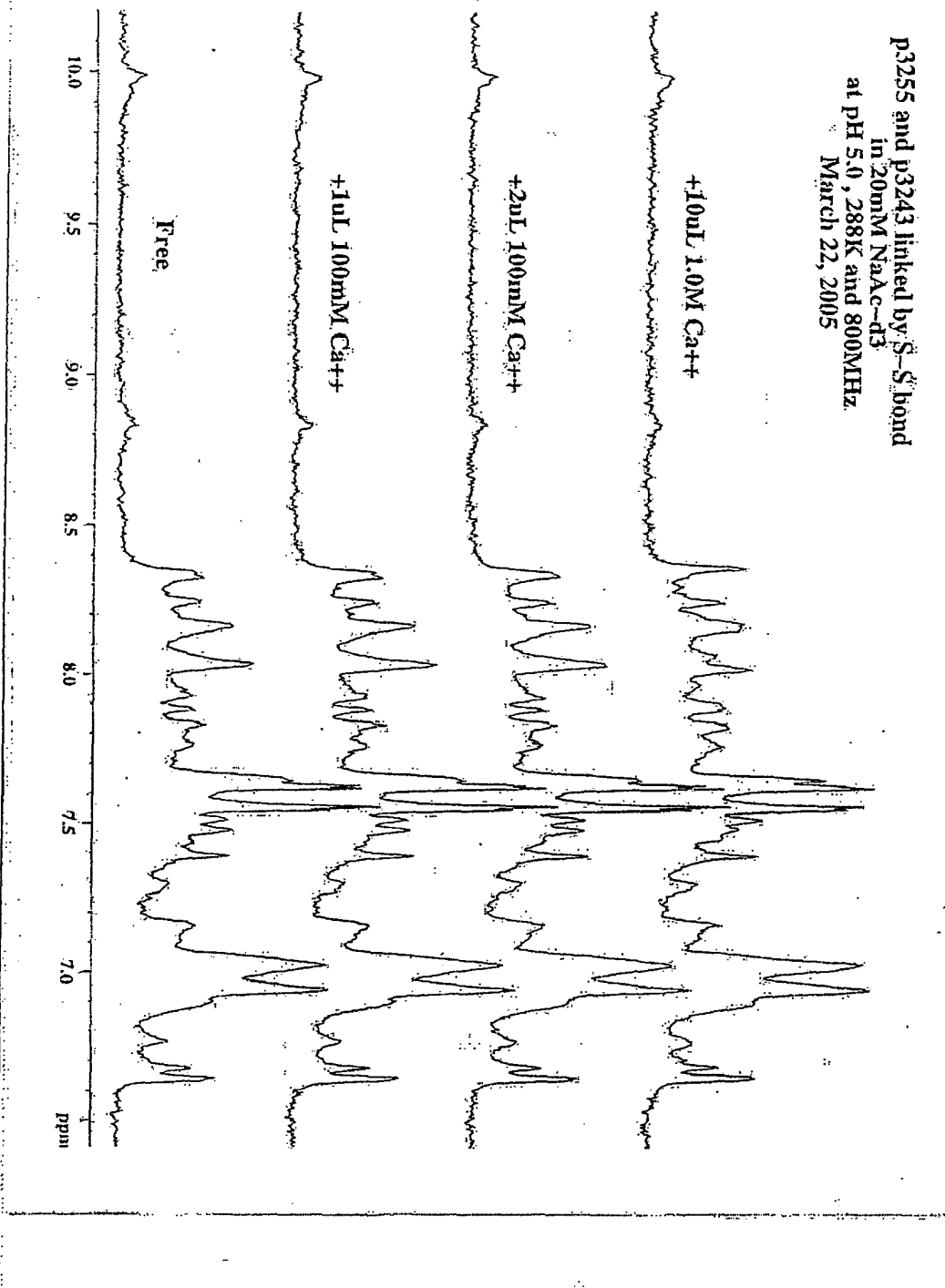


Figure 6e

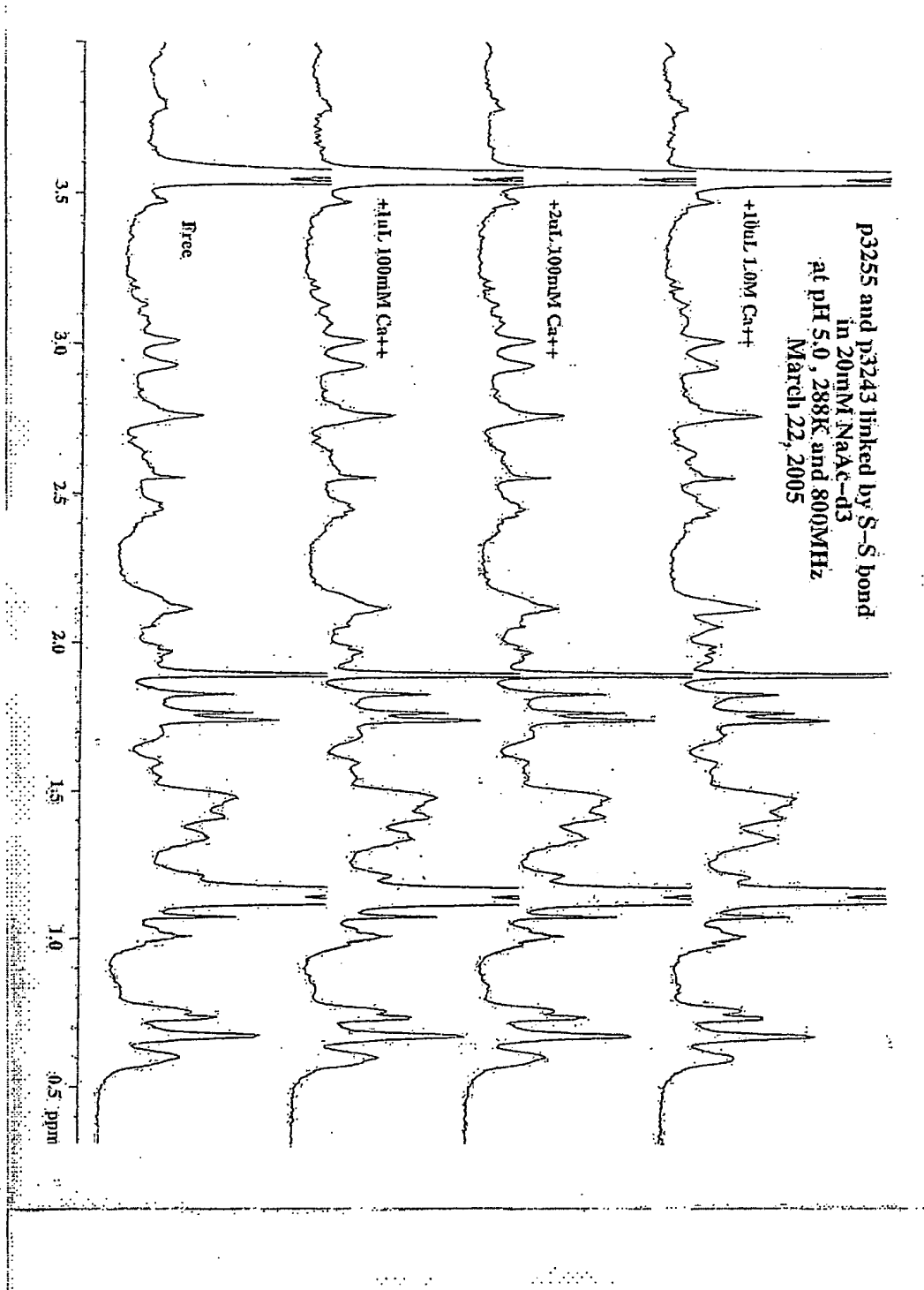


Figure 6f

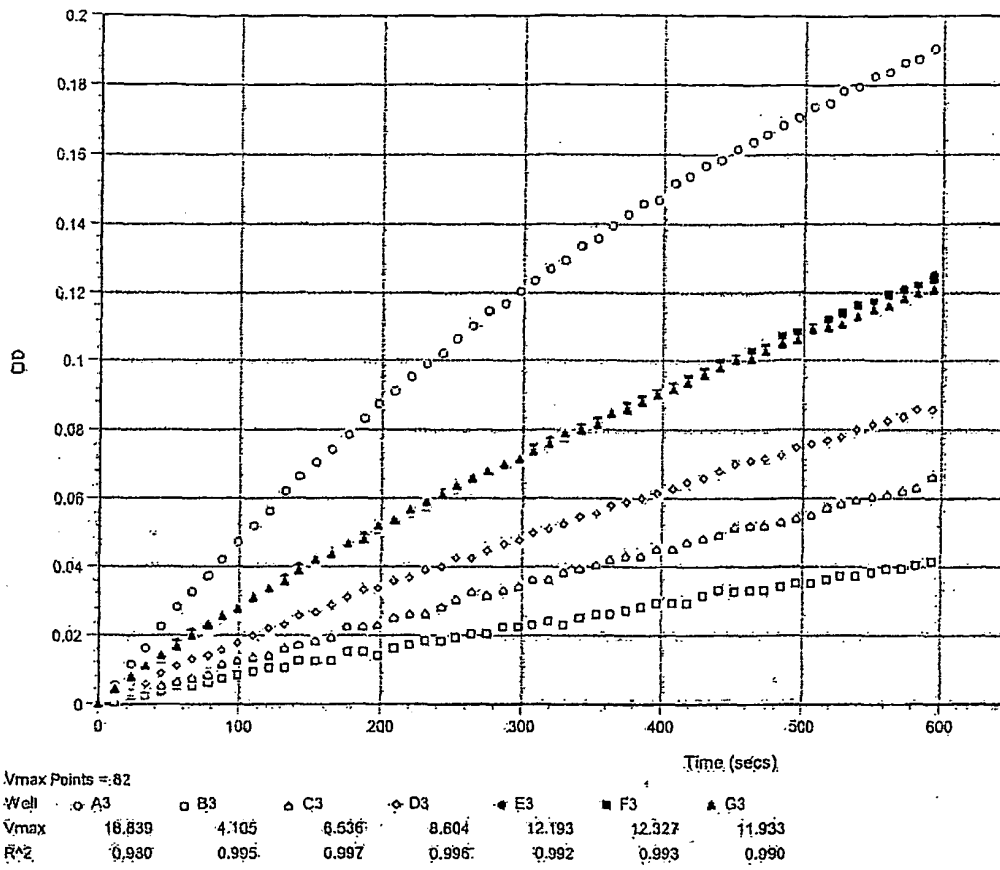


Figure 7.

Figure 8a.

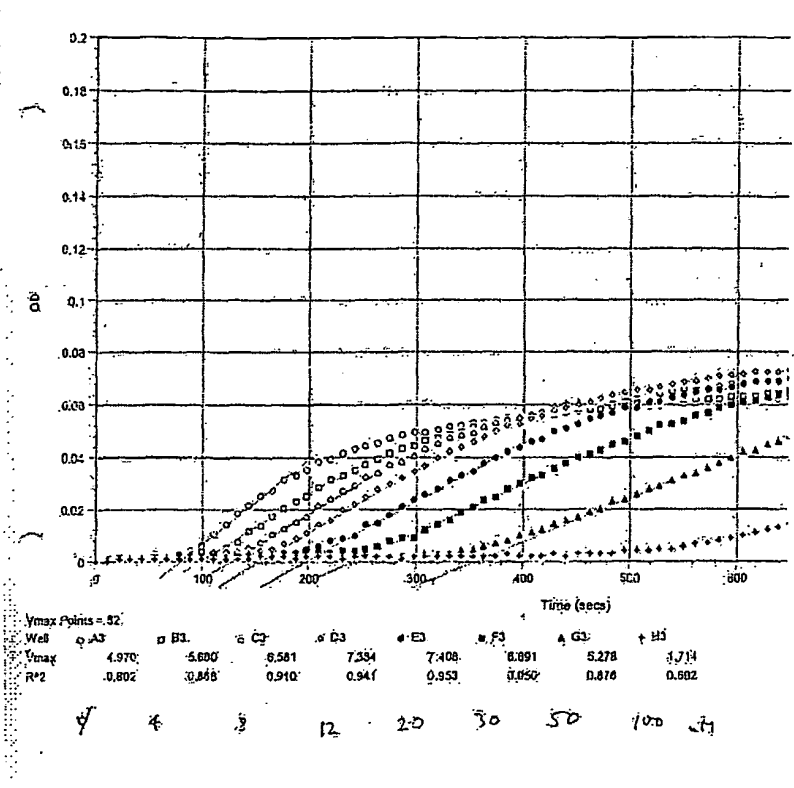
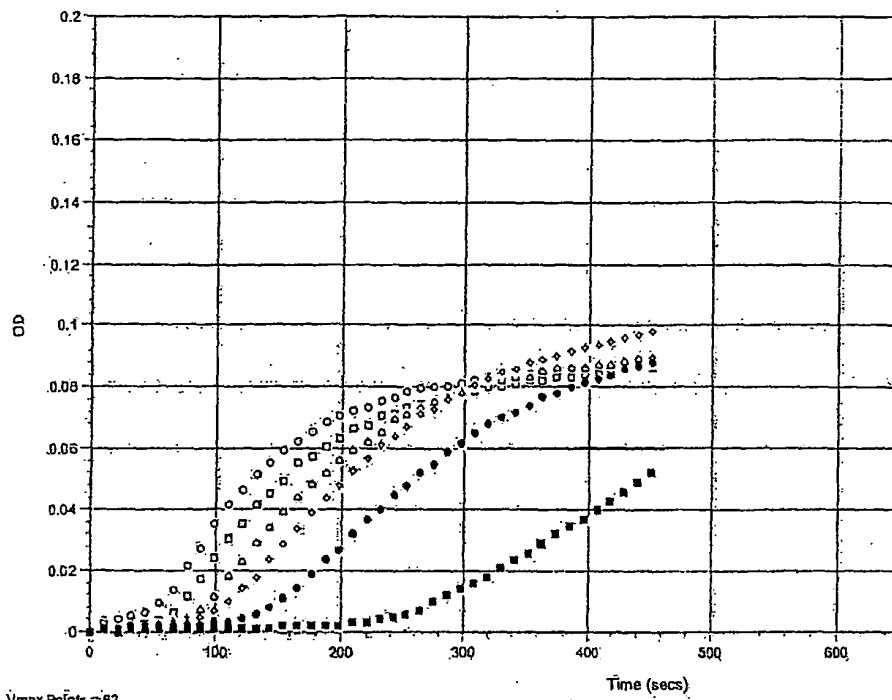


Figure 8b.



Vmax Points = 82.

Well	A7	B7	C7	D7	E7	G7
Vmax	12.255	12.967	14.395	15.873	14.315	8.388
R ²	0.847	0.888	0.929	0.962	0.958	0.790

K 2.15 nM 4.3 nM 8.6 nM 17.2 nM 43 nM

p: 3.276 fraction 50-52

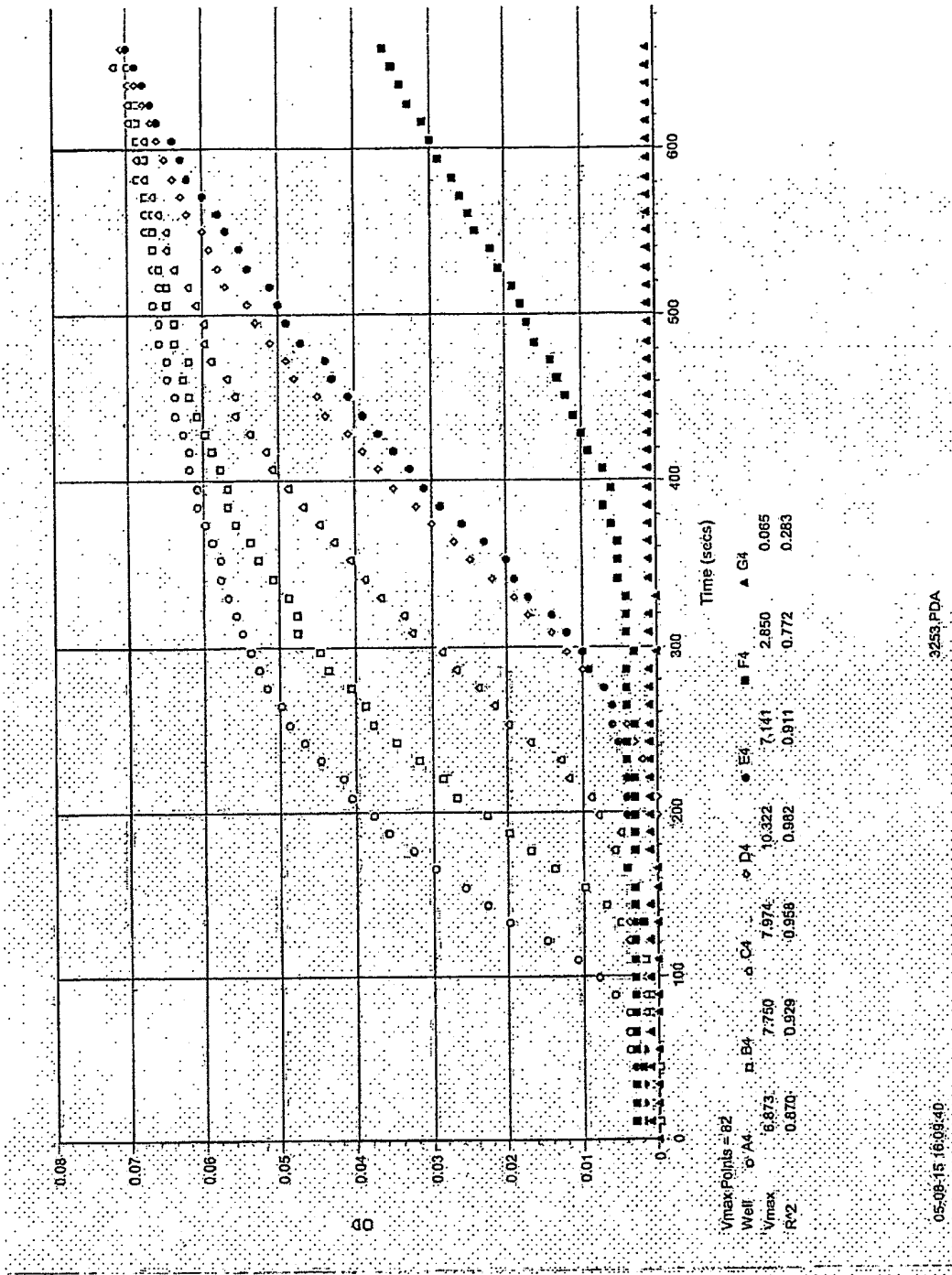


Figure 8c.

CaM-DTI:

WDPRPQRHADQLTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQN
PTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTGGVKLIPSWTTVI
LVKSMLRKRSGNPFGGDSEEEIREAFRVFDKDGNGYISAELRHVMTNL
GEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAKDFEEIPEEYLO

CaM-DTI2:

IRFTDGEGADQLTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNP
TEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDNGGVKLIPSWTTVIL
VKSMLRKRSGNPFGGDSEEEIREAFRVFDKDGNGYIRAAELRHVMTNLG
EKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAKDFEEIPEEYLO

Figure 9.

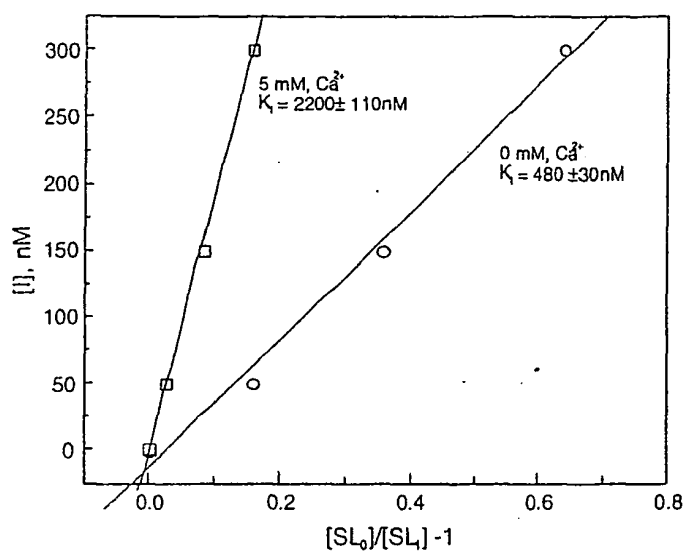


Figure 10A.

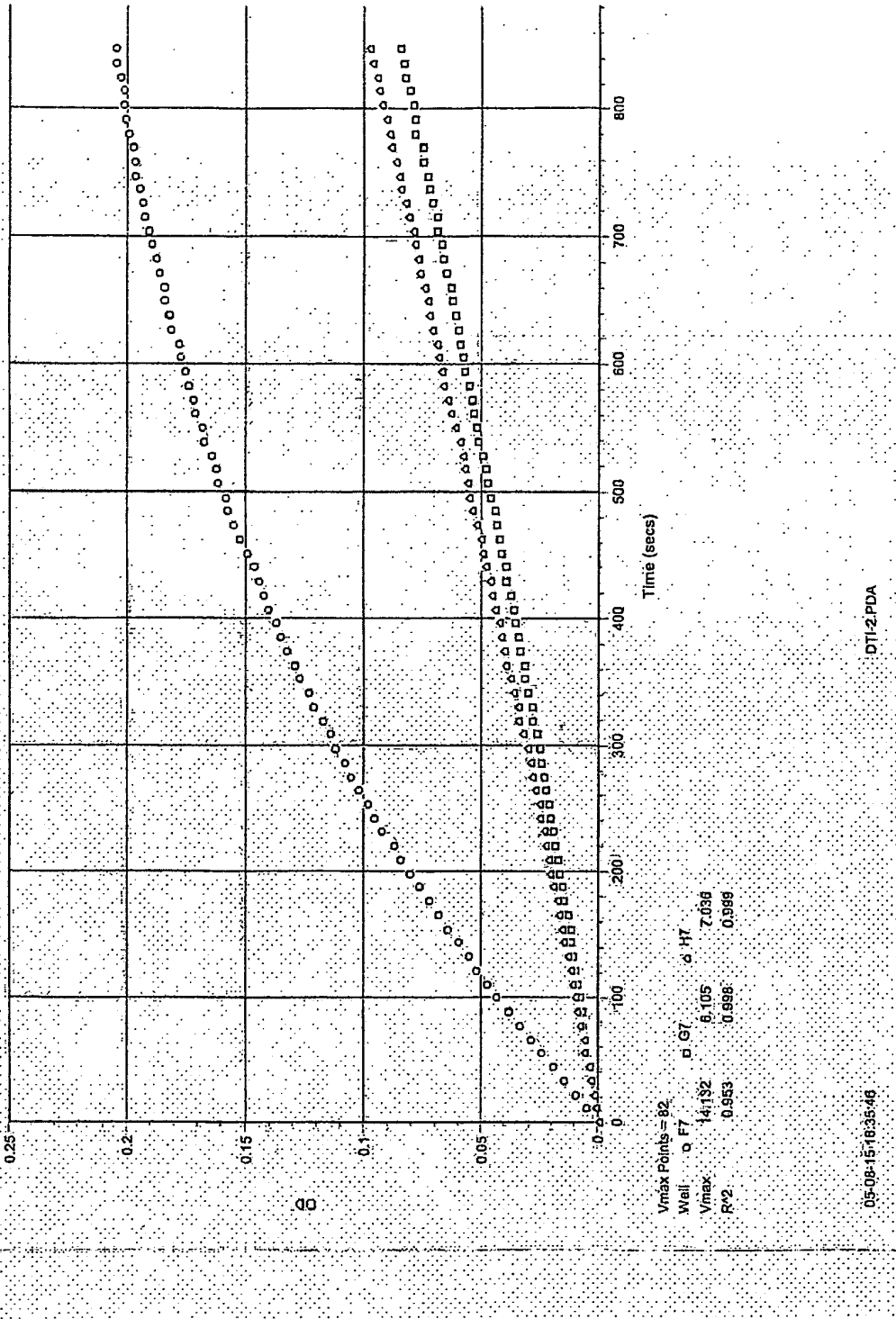
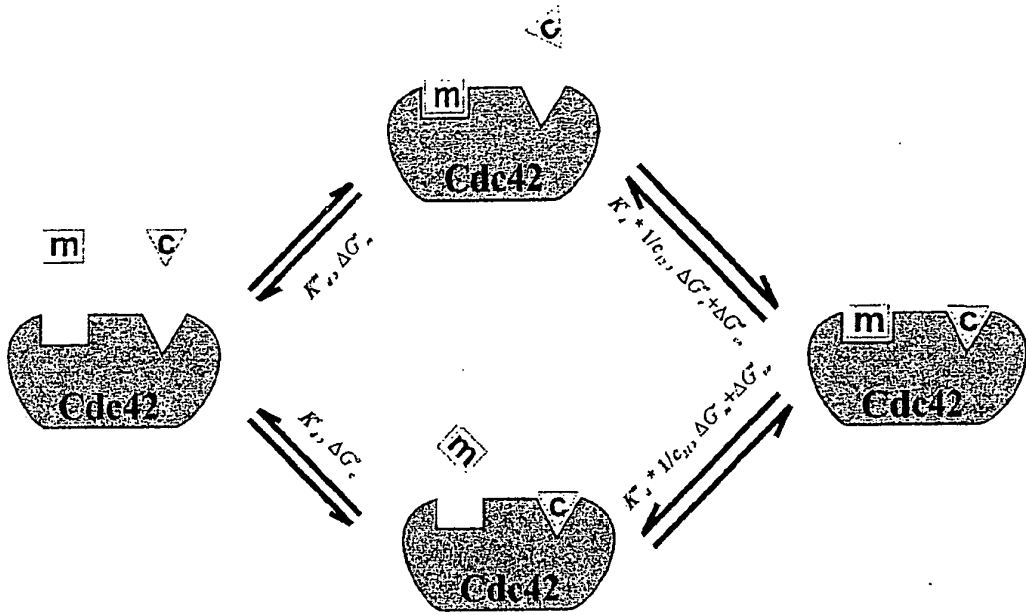


Figure 10B

A



B

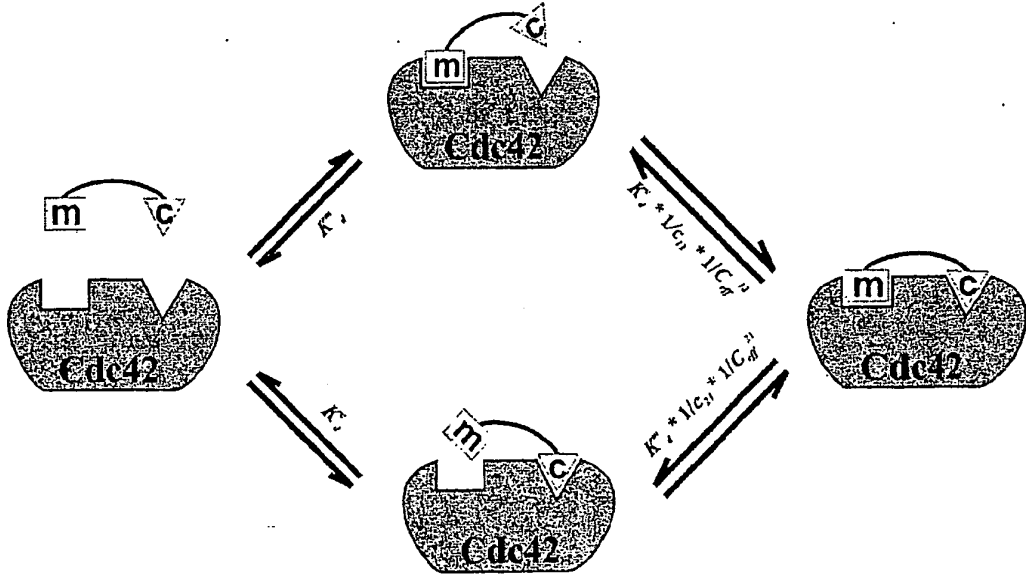


Figure 12.

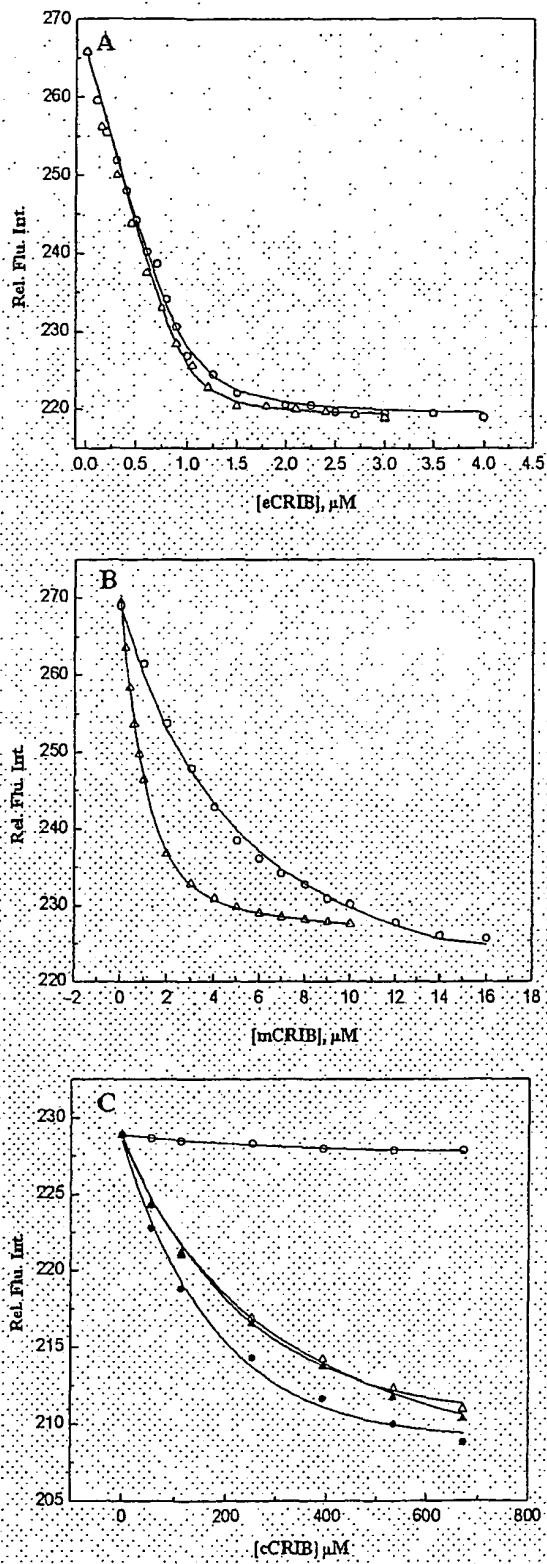


Figure 13

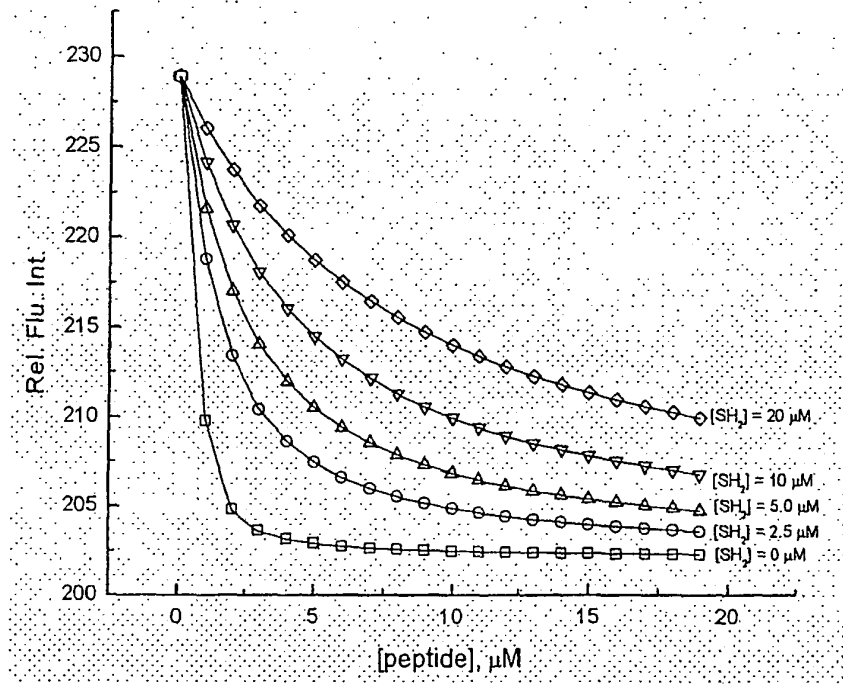


Figure 14A

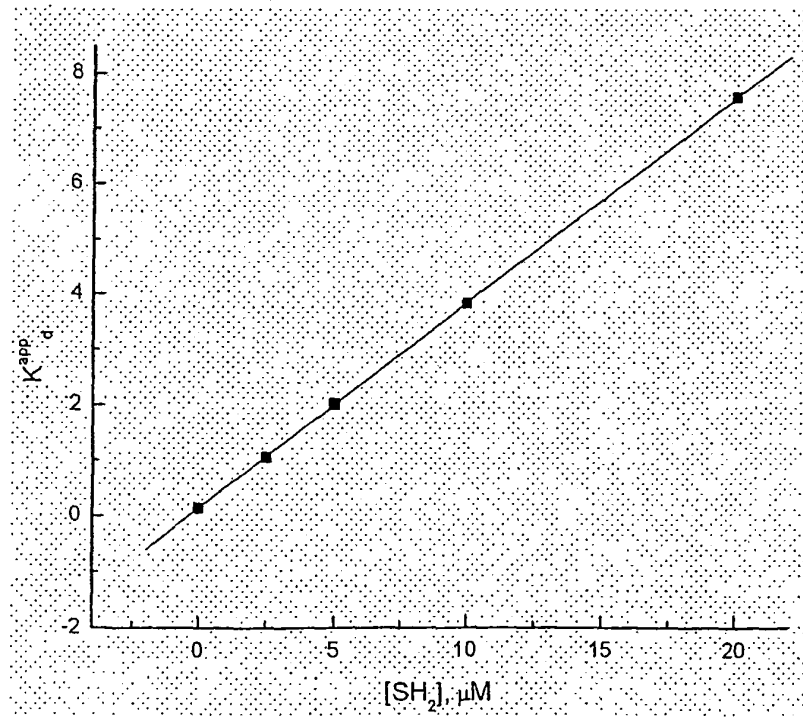
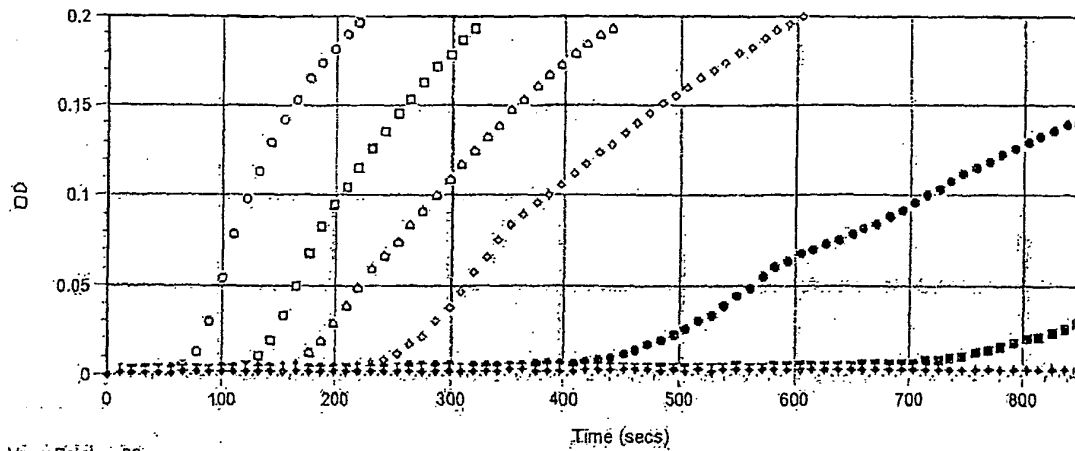


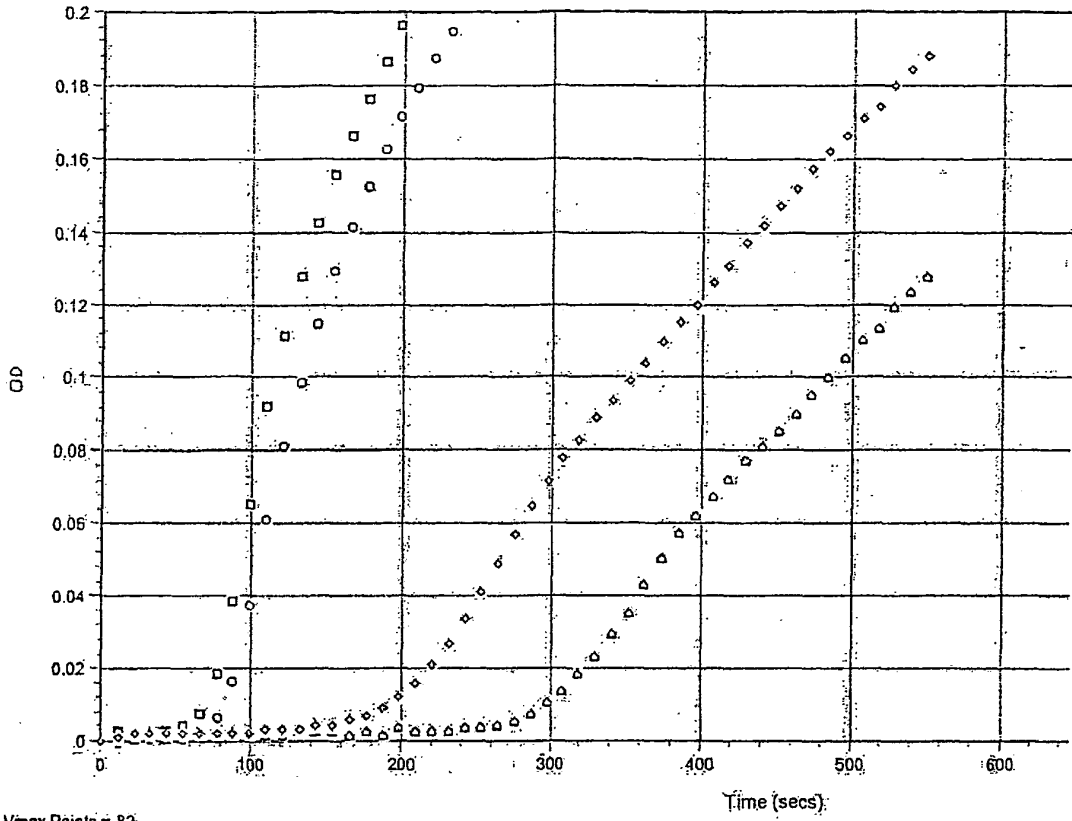
Figure 14B



Well	A7	B7	C7	D7	E7	F7	G7	H7
Vmax	64.888	42.174	30.584	22.764	10.582	1.390	0.109	0.132
R ²	0.944	0.909	0.918	0.906	0.828	0.502	0.384	0.703
K		10 nM	15	20	30	40	50	60

RT

Figure 14C



Vmax Points = 82

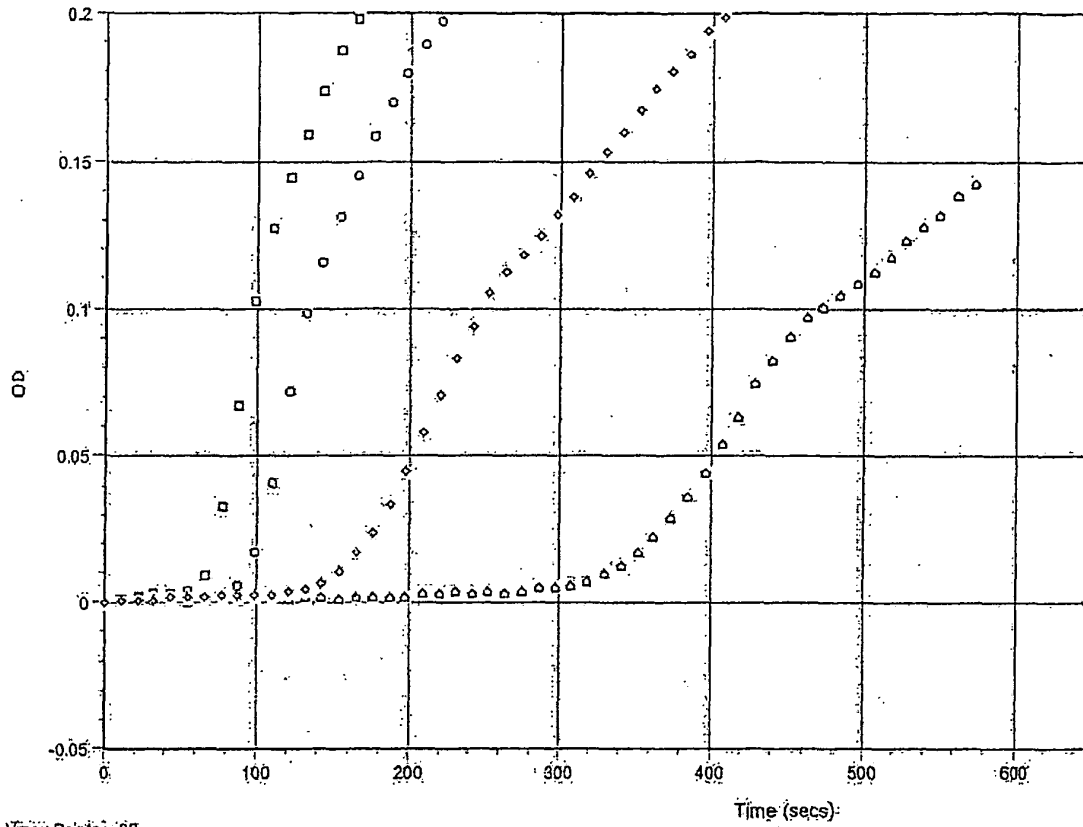
Well	A5	B5	C5	D5
Vmax	61.189	70.536	14.156	23.443
R ²	0.939	0.934	0.808	0.938
	-SH2	SH2 SMH	-SH2	SH2, SMH
	control		250M _p 329.1	

RT

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3291-2.PDA

Figure 14D



Vmax Points = 82

Well	AA	BB	CC	DD
Vmax:	64.066	85.051	14.795	33.793
R ²	0.901	0.911	0.757	0.924

-SH2 10 μ M SH2 +SH2 10 μ M SH2
control R3291, 25 μ M

Figure 14E

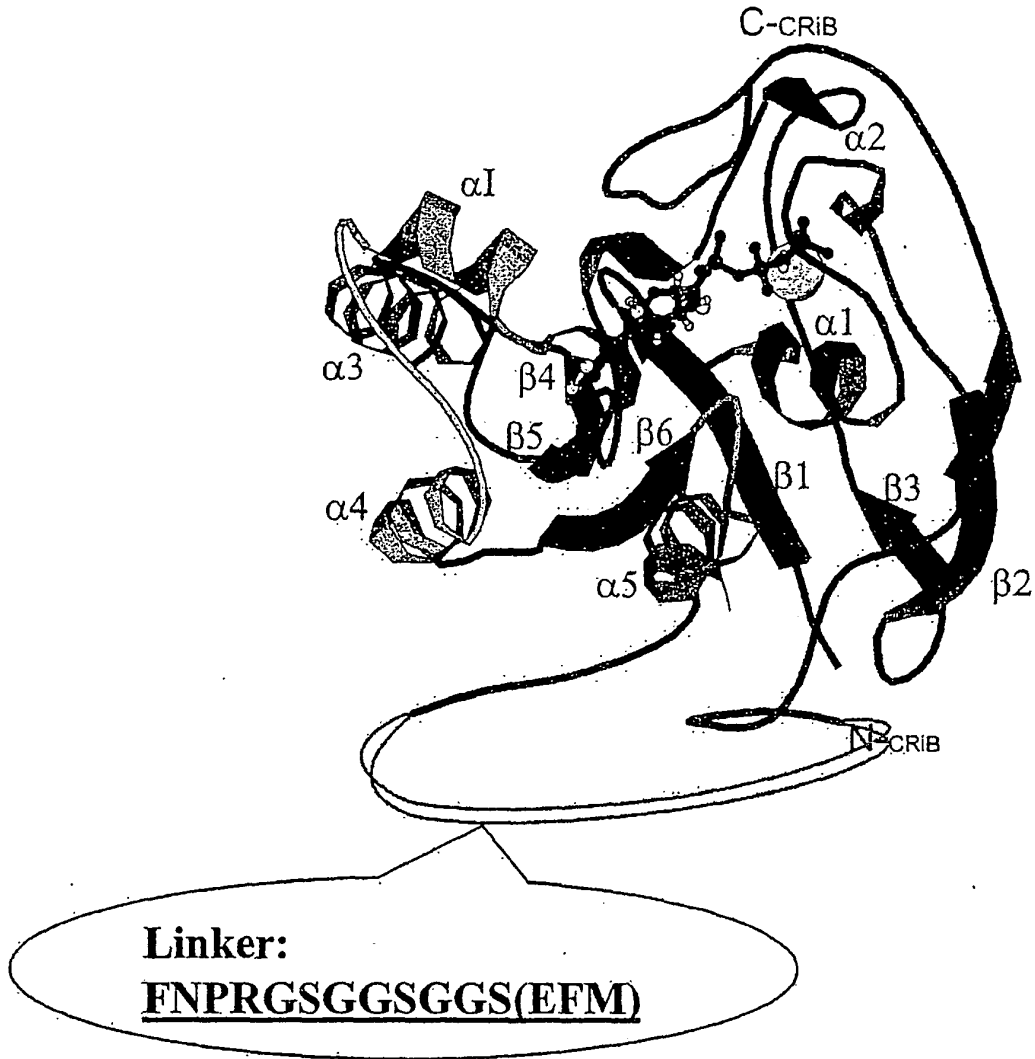


Figure 15a

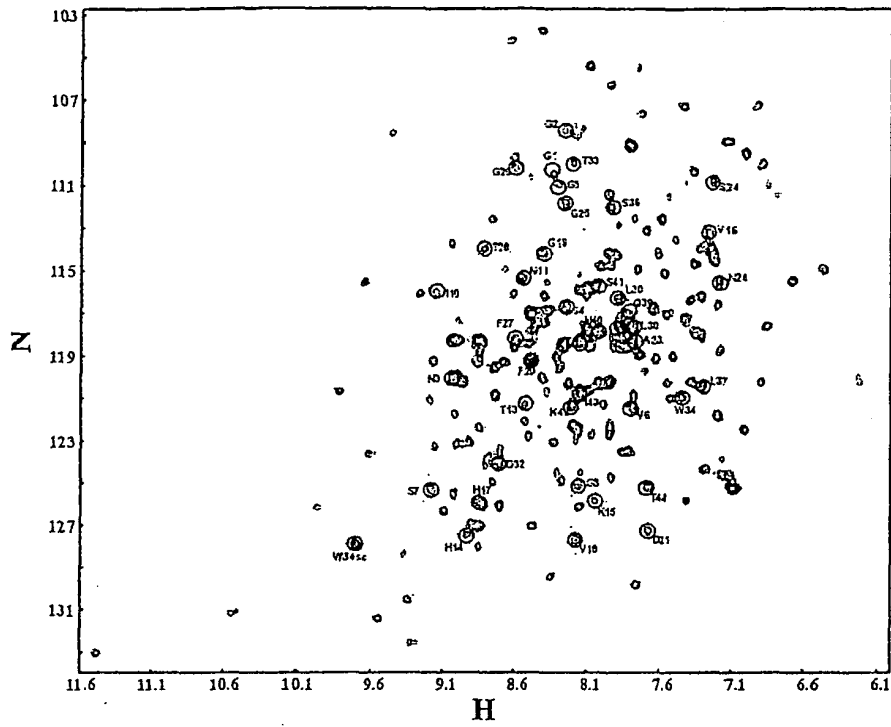


Figure 15b

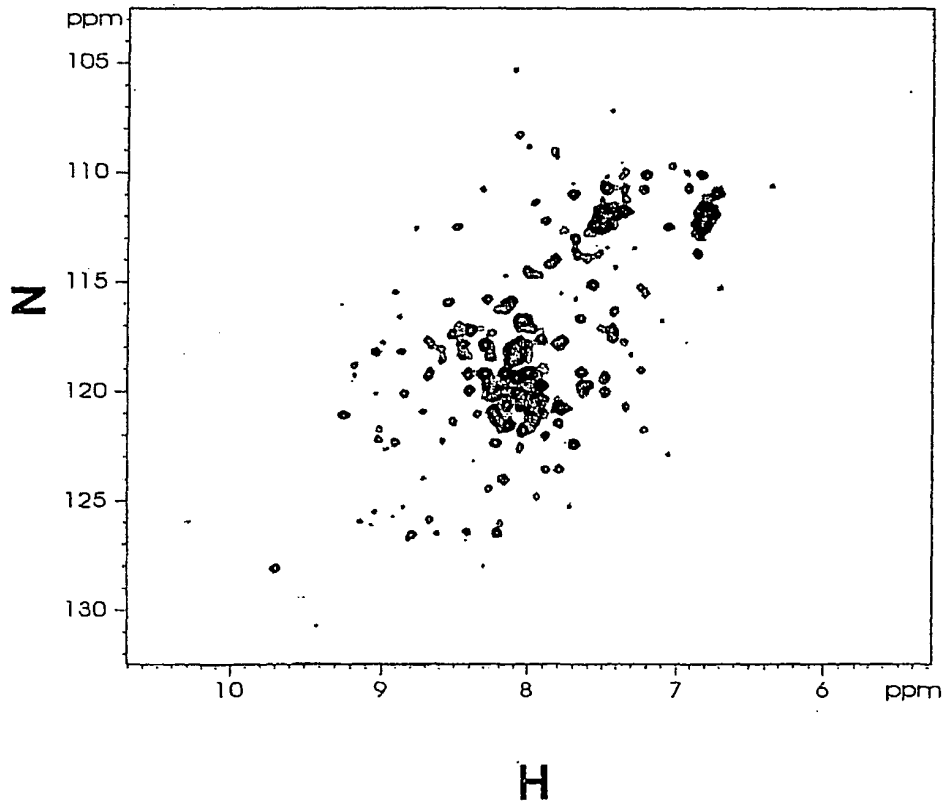
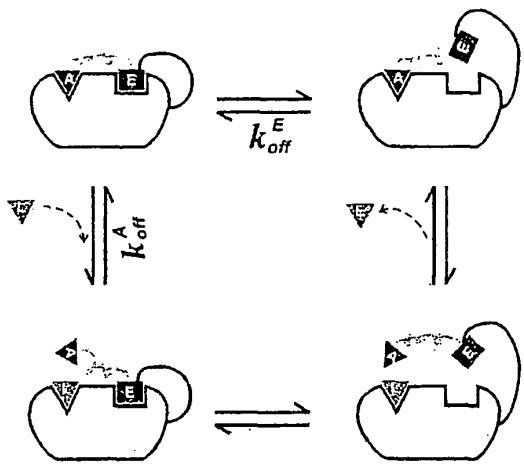


Figure 15c



If [L] is finite and $K_d^L \rightarrow 0$,
 (when L is a stronger and
 specific binder than A)

then

$$k_{off}^{obs} = k_{off}^E$$

$$k_{off}^{obs}([L], K_d^L) = \frac{k_{off}^A \cdot K_d^E}{C_E + K_d^E} \cdot \frac{K_d^L(1 + C_A/K_d^A)}{[L] + K_d^L(1 + C_A/K_d^A)} + \frac{k_{off}^E \cdot K_d^A(1 + [L]/K_d^L)}{C_A + K_d^A(1 + [L]/K_d^L)}$$

Figure 15d.

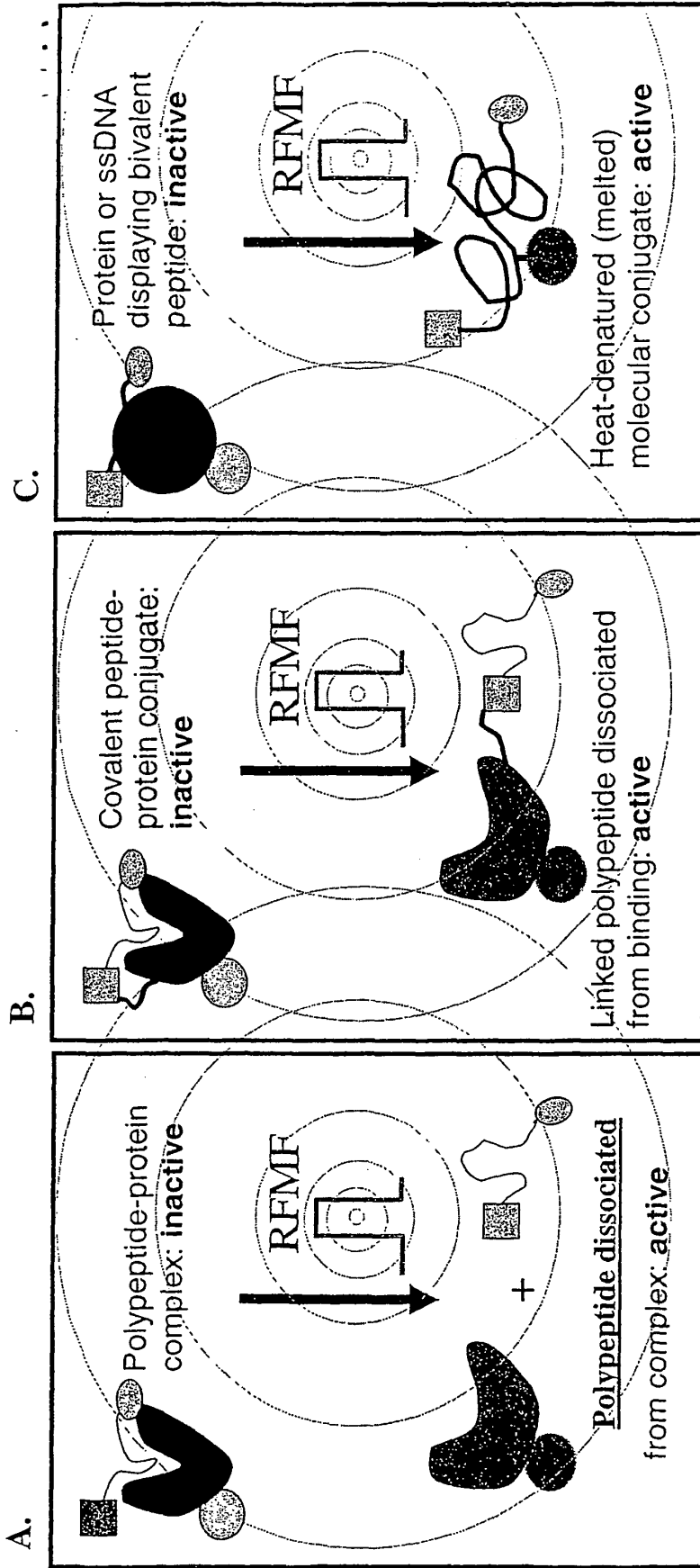


Figure 16

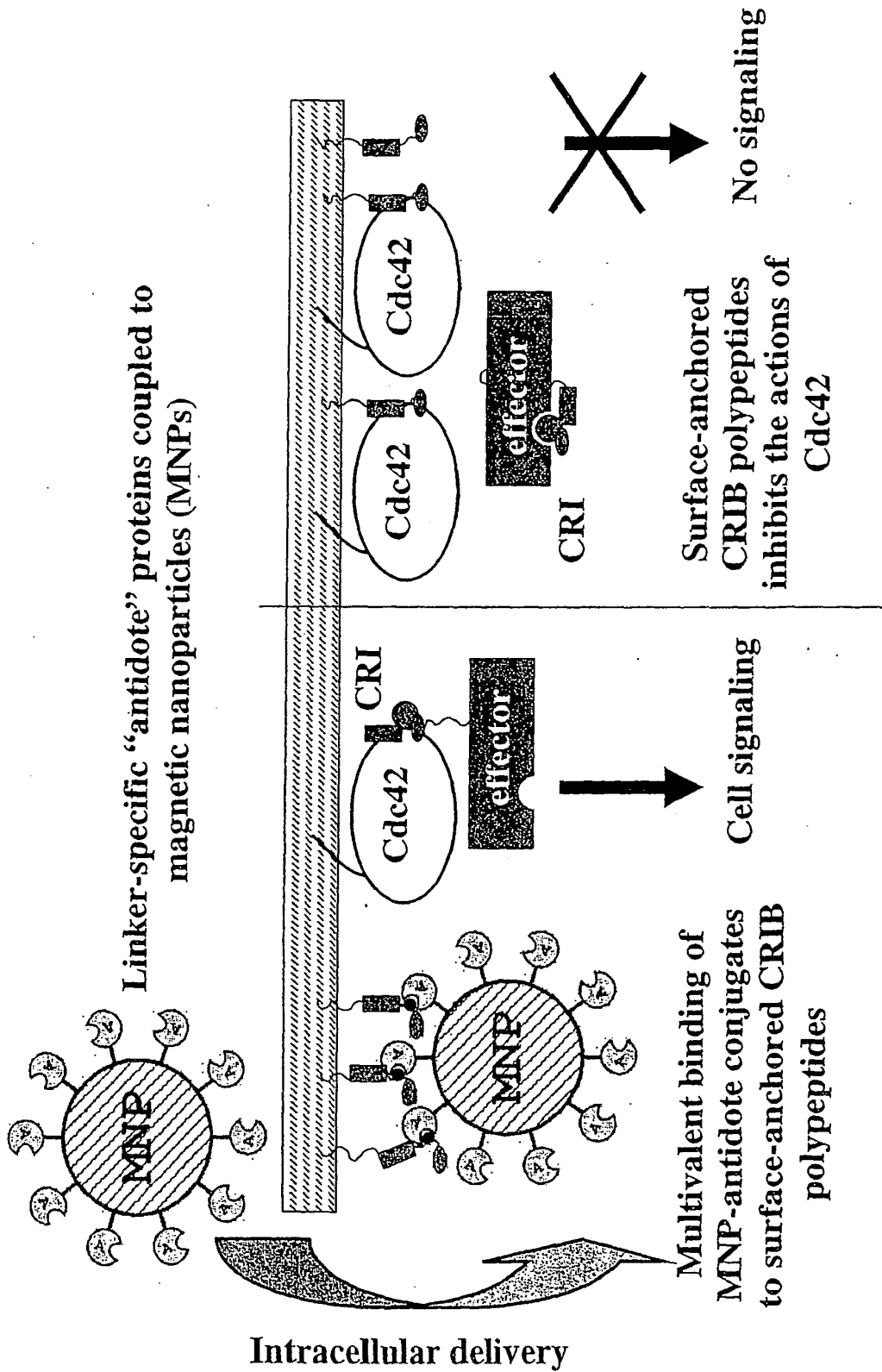
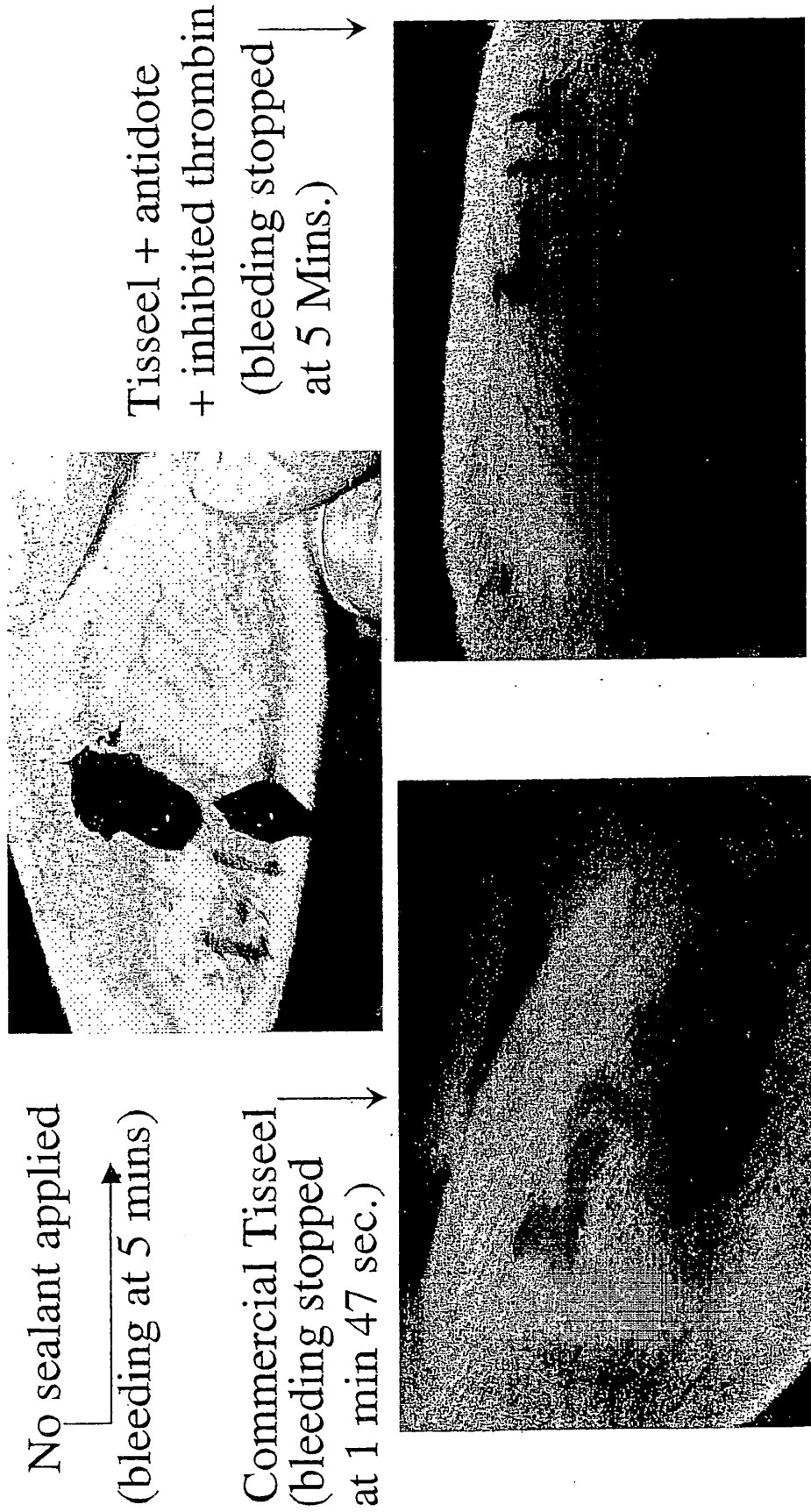


Figure 17

Figure 18



REFERENCES CITED IN THE DESCRIPTION

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摘要(译)

本文提供了一种多价结合分子及其用途。该分子可用于在某些条件下结合靶标并在其他条件下释放靶标。该分子具有BM1-L-(BM2)n的通式(1)(1)其中, BM1是对靶标上的位点1具有亲和力的结合部分1, BM2是对靶点1具有亲和力的结合部分2。在靶标上除位点1以外的位点, n为1或更大, L为连接BM1和BM2的接头, 所述接头适于通过构象和/或柔性的变化来响应其环境的变化, 其中BM1和BM2可以相同或不同, 并且被选择为使得在使用中分开存在的BM1和BM2中的每一个都具有比它们连接形成分子时的BM1和BM2的复合物低的结合亲和力。BM2可以具有对靶标具有亲和力的单个结合区或多个结合区。单独的BM1或BM2与靶标的结合亲和力不超过式(1)的分子的结合亲和力的1/2。式(1)的分子可以使用寡聚或聚合接头, 例如多肽序列来构建。此类分子可用于药物的延迟释放, 筛选试验, 稳定酶(如蛋白酶)和控制反应(如凝血)。

