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(71) Applicant *(for all designated States except US)*: **NATIONAL RESEARCH COUNCIL OF CANADA** [CA/CA]; 1200 Montreal Road, Bldg. M-58, Room EG-12, Ottawa, Ontario K1A 0R6 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NI, Feng** [CA/CA]; 4507 Maplewood, Pierrefonds, Quebec H9A 1B2 (CA). **TOLKATCHEV, Dmitri** [CA/CA]; 110 Balmoral #103, La Prairie, Quebec J5R 4L5 (CA). **SU, Zhengding** [CA/CA]; 1445 Ouimet #45, St-Laurent, Quebec H4L 3R2 (CA).

(74) Agent: **McKAY, Margaret**; National Research Council of Canada, Intellectual Property Services Office, EG-12, Bldg. M-58, 1200 Montreal Road, Ottawa, Ontario K1A 0R6 (CA).

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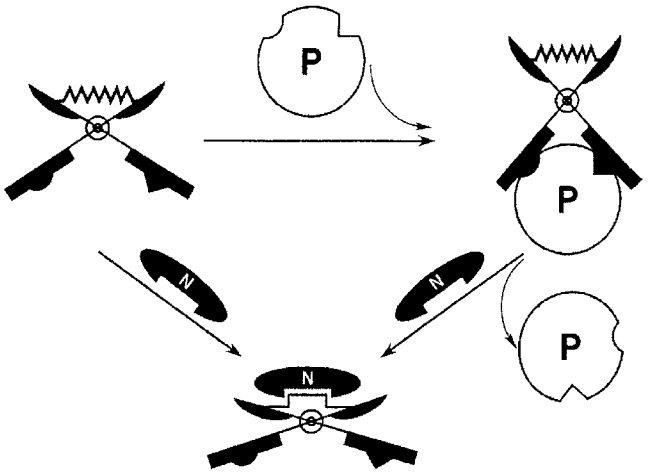
(54) Title: POLYPEPTIDE LIGANDS CONTAINING LINKERS



(57) Abstract: There is provided herein a multivalent binding molecule and uses thereof. The molecule is useful in binding a target under certain conditions and releasing it under other conditions. The molecule has the general formula (1) of BM1-L-$(BM2)_n$ (1) wherein, BM1 is a binding moiety 1 having an affinity for site 1 on the target, BM2 is a binding moiety 2 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 are selected such that in use each of the BM1 and BM2 existing separately has a lower binding affinity then the complex of BM1 and BM2 does when they are linked to form the molecule. BM2 may have a single binding region or multiple binding regions with affinity for the target. The binding affinity of BM1 or BM2 to the target alone is no more than $^1/_2$ the binding affinity of the molecule of formula (1). The molecule of formula (1) can be constructed using an oligomeric or polymeric linker, such as a polypeptide sequence. Such molecules can be useful in the delayed release of drugs, in screening assays, for stabilizing enzymes such as proteases, and for controlling reactions such as blood clotting.

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Title: Polypeptide Ligands Containing Linkers

Field of the Invention: The invention relates to the field of multivalent binding molecules containing polymeric linkers.

Background to the Invention:

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 moeity on a polymer backbone.

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

Summary of the Invention

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. Vise 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.

In an embodiment of the invention there is provided a multivalent binding molecule and uses thereof. The molecule is useful in binding a target under certain conditions and releasing it under other conditions. The molecule has the general formula (1) of

$$BM1\text{-}L\text{-}(BM2)_n \qquad (1)$$

wherein,

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

BM2 is a binding moiety 2 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 may 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 then the complex of BM1 and BM2 does when they are linked to form the molecule. BM1 and/or BM2 may each have a single binding region or multiple binding regions with affinity for the target. The binding affinity of BM1 or BM2 to the target alone is no more than ½ the binding affinity of the molecule of formula 1. The molecule of formula 1 can be constructed using an oligomeric or polymeric linker, such as a polypeptide sequence. Such molecules can be useful in the delayed release of drugs, in screening assays, for stabilizing enzymes such as proteases, and for controlling reactions such as blood clotting.

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.

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.

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.

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, or an amino acid sequence at least 90 % identical thereto.

In an embodiment of the invention there is provided an isolated or substantially isolated amino acid sequence of no more than 100 amino acids, said sequence comprising a series of contiguous amino acids having at least 80%, 90% or 95 % sequence identity to at least one of SEQ. ID. NO. 8, 12, 17, 24, 27, 28, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 128, 62, 63, 64, 65, 66, 67, 103, 104, 105, 106, 107, 108, 109, 110, 111, 118, 120, 123, 124, 125, 126, 127, 128, 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, 117, SEQ. 37, 38, 92, 93, 94, 95, 100, or 101. In an embodiment of the invention there is provided an isolated or substantially isolated nucleic acid sequence encoding one or more of the above amino acid sequences. In an embodiment of the invention there is provided a nucleic acid sequence substantially or completely complementary to at least one nucleic acid sequence described above. In

an embodiment of the invention there is provided vectors comprising one or more of the nucleic acid sequence described above.

In an embodiment of the invention there is provided a pharmaceutical composition comprising a molecule of formula 1 with a carrier. Also provided is a method of delivering a compound of interest for preferential release at a biological site of interest, comprising obtaining a molecule of formula 1 wherein BM1 and BM2 have binding affinities for the compound of interest and the linker is selected to undergo a conformational change in under conditions present or inducible at the biological site of interest so as to reduce multivalent binding f the compound by the molecule of formula 1 at the biological site of interest.

In an embodiment of the invention there is provided a method to screen a population of molecules to identify a ligand of interest. The method comprises: a) selecting a target of interest with a known affinity for a component; b) selecting a second molecule having a known affinity for the component of step (b), the second molecule binding a different region of the component from the region bound by the target; c) connecting the target and the second molecule with a suitable linker to create a bivalent structure; d) monitoring binding of the bivalent structure to the component in the presence of ligands; and, e) identifying a ligand which reduces the level of bivalent binding.

In an embodiment of the invention there is provided a method of assaying for the presence of a substance of concern in a sample. The method comprises: a) identifying a first target region known to be present on the substance of concern and less common within sample material which does not contain the substance; b) identifying a second target region known to be present on the substance of concern; c) obtaining a plurality of molecules comprising a first binding region having affinity for the first target region connected by way of a linker to a second binding region

having affinity for the second target region, wherein the linker undergoes an assayable conformational change during the transition between bivalent and monovalent binding; d) combining molecules from step (c) with the sample; and e) assaying for bivalent binding.

In an embodiment of the invention there is provided a method of identifying conditions affecting a structural characteristic of a polymeric molecule of interest. The method comprises: (a) obtaining a molecule according to claim 1 wherein the linker comprises the polymeric molecule of interest and binding moieties 1 and 2 are adapted to bind a known target when the linker has a first structural condition such that bivalent binding of binding moiety 1 and binding moiety 2 to the target causes a change in an assayable characteristic of the target; (b)permitting interaction between the molecule of step (a) and the target; (c)altering the conditions under which the interaction of step (b) occurs; and (d)assaying the effect of changes of conditions on the characteristic of the target.

In an embodiment of the invention there is provided method to enhance the stability of an enzyme. The method comprises reducing functional activity of the enzyme by binding to it a molecule of formula 1 having first and second binding moieties with affinity for the enzyme. Also provided is this method with a further step of releasing bivalent binding of the molecule of formula 1 by inducing a change in the structure and/or flexibility of the linker so as to allow an increase in functional activity of the enzyme. In some instances the enzyme is a protease.

In an embodiment of the invention there is provided a method of manufacture of a device capable of activation by an electromagnetic field. The method comprises: (a) obtaining a molecule of claim 1 wherein BM1 And BM2 bind to a target and L binds to an antidote which causes a change in the linker sufficient to reduce bivalent binding of the molecule to the target; (b)conjugating the antidote with a metal nanoparticle; (c) allowing interaction between the antidote and the target such that bivalent binding occurs. Also provided is a method of activating a device manufactured according to

the method above comprising releasing the ligand by heating the metal nanoparticle by means of electromagnetic radiation.

In an embodiment of the invention there is provided a method for the purification of a target. The method comprises: (a)immobilizing onto a solid support a molecule of claim 1 capable of binding to the target via BM1 and BM2; (b) allowing the target to bind the molecule of step (a); (c) eluting unbound materials; and, (d) eluting the target by inducing a condition which causes a change in structure and/or flexibility of the molecule of step (a) such that bivalent binding of the target by the molecule is reduced.

In an embodiment of the invention there is provided a method to obtain a molecule of formula 1 with high affinity to a protein target, said method comprising steps of: (a)obtaining at least two binding peptide moieties each having a binding affinity for a distinct binding site on the target based on already existing polypeptide ligands with high affinity; (b)establishing a weaker binding peptide moiety using NMR titration or NMR relaxation dispersion spectroscopy; (c)connecting the peptide moieties with a flexible linker; (d)increasing the bivalent affinity by sequence optimization of the weaker moiety by means of phage display.

In an embodiment of the invention there is provided a method to prolong the lifetime of a protease said method comprising the steps of: (a) Inhibiting the protease with a bivalent protease inhibitor containing a controllable linker; (b)Releasing and activating the protease with an appropriate linker-targeted antidote. In some instances the protease is thrombin. In some instances thrombin is a component of a fibrin sealant kit.

In an embodiment of the invention there is provided a method to detect an agent modifying the properties of the linker in a molecule of formula 1. The method comprises: (a) obtaining a multivalent ligand with the general structure of formula 1 having multivalent binding affinity for an enzyme catalyzing a detectable chemical reaction, for which the ligand is an inhibitor or an activator; (b) bringing the ligand and the enzyme in contact so as to form a complex with the ligand bound to the

enzyme; and (c) carrying out an enzymatic assay of the complex wherein the course of the detectable enzymatic reaction is compared in the presence and absence of conditions modifying the properties of the linker in the bivalent ligand. In some instances the linker includes at least two residues, selected from the group of tyrosine; serine; threonine; histidine; phosphotyrosine; phosphoserine; phoshothreonine; and, phosphohistidine.

Brief Description of the Figures

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-Pip)-$(GS)_{10}$-GDFEEIPEEYLQ.

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

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-$(Gly\text{-}Ser)_8$-Gly-DFEEIPEEYLQ.

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)-GSGSGSGSGKGSGSGSGSGS-GDFEEIPEEYLQ.

Figure 2k. Inhibition of fibrinogen clotting by the thrombin inhibitor Bbs-R-(D-Pip)-GSVVPRPQLHND-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.

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.

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-Val-Ser-Gly)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln.

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.

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.

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.

Figure 5. Inhibition of fibrinogen clotting assays for thrombin inhibitor of the formula Bbs-R-(D-Pip)-GEQKLISEEDLG-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:

Figure 6a. Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-NH_2 upon the addition of $CaCl_2$.

Figure 6b. Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Glu-Phe-Glu-NH_2 upon the addition of $CaCl_2$.

Figure 6c. Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH_2 upon the addition of $CaCl_2$.

Figure 6d. Changes in the proton NMR spectra of Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH_2 upon the addition of $CaCl_2$.

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 $CaCl_2$.

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 $CaCl_2$.

Figure 7. Effect of calcium on the inhibition of the amidolydic 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:

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.

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).

Figure 10. (A) Inhibition of the amidolydic activity of thrombin by CaM-DTI in the absence of Ca^{2+} (circle) and in the presence of 5 mM Ca^{2+} (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.

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

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.

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

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

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.

Detailed Description of the Preferred Embodiments

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 provided 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.

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.

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.

In an embodiment of the invention the controllable linker is a flexible peptide or peptide bound to another material. The linker will sometimes preferably also be a ligand to a well-structured macromolecular species, or an antidote. For example, many signaling proteins, or their subdomains are known to bind flexible peptides (Pawson and Linding, 2005, 1808-1814; Pawson and Nash, 2003, 445-452; Puntervoll and others, 2003, 3625-3630) conferring upon the latter a structure required for antidote effects. Once bound, such structured linkers will generally produce spatial orientation of binding moieties that will preclude simultaneous binding of the latter to the original target. 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.

Approaches taken in the Specific Examples

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*-butyl-benzenesulfonyl, 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 moeity, 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.

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; (△) 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; (□) 2nM; (△) 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; (□) 3nM; (△) 5 nM; (◇) 7 nM; and (●) 9 nM of a product of disulfide-bonded linkage between peptides Bbs-Arg-(D-Pip)-Gly-Cys and Cys-$(Gly\text{-}Ser)_8$-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; (△) 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; (△) 4 nM; (◇) 6 nM; (●) 8 nM; (■) 10 nM; (▲) 12 nM; and (◆) 15 nM; of the inhibitor with linker GSGSGSGSGKGSGSGSGSGS (SEQ. ID. NO. 58) at 25°C. Curves (k) represent OD_{420} time course in the presence of (○) 0 nM; (□) 0.5 nM; (△) 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; (△) 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; (△) 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; (△) 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 (△) 8 nM; of the inhibitor with linker GQSHNR (SEQ. ID. NO. 108) at 37°C.

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 interupt 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 ($ephrinB2_{301-309}$) 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).

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; (□) 2nM; (△) 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.

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.

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 OD_{420} time course after the addition of 0.6 nM thrombin in the presence of (a) (○) 2 nM P3161; (□) 2 nM P3161, 3 μM SH2; (△) 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, 3μM SH2; (△) 4 nM P3170; (◇) 4 nM P3170, 3μM SH2; (●) no inhibitor, no SH2; (■) no inhibitor, 3 μM SH2. Other experimental conditions were as used for assays described in Figure 3.

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 at 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).

Looking at the results of Figure 5, the curves represent OD_{420} 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.

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.

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-Ile-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.

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).

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-NH_2 (SEQ. ID. NO. 109) (P3230, panels **a,b**) and Ac-Asp-Lys-Asn-Ala-Asp-Gly-

Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH_2 (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-d_3 buffer, pH 5.5, containing 10% D_2O, of 1 µL (final $CaCl_2$ concentration ~0.22 mM), additional 2 µL (final $CaCl_2$ concentration ~0.66 mM), additional 10 µL (final $CaCl_2$ concentration ~2.8 mM) of 100 mM $CaCl_2$, and additional 10 µL (final $CaCl_2$ concentration ~23.9 mM) of 1 M $CaCl_2$. 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 $CaCl_2$ concentration ~0.22 mM), additional 2 µL (final $CaCl_2$ concentration ~0.66 mM) of 100 mM $CaCl_2$, and additional 10 µL (final $CaCl_2$ concentration ~22.2 mM) of 1 M $CaCl_2$.

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-Ile-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 OD_{405} 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 (○) no inhibitors; (□) 150 nM CaR ; (△) 150 nM CaR, 50 mM $CaCl_2$; (◇)150 nM CaR, 100 mM $CaCl_2$; (●) 2 nM P3150; (■) 2 nM P3150, 50 mM $CaCl_2$; and (▲) 2 nM P3150, 100 mM $CaCl_2$.

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.

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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-Asn-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 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 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 IC_{50} of 10±1 nM (Figure 8).

Looking at the results in Figure 8, curves (a) represent OD_{420} time course in the presence of (○) 0 nM; (□) 10 nM; (△) 30 nM; (◇) 50 nM; (●) 70 nM; and (■) 100 nM of mini-hirudin 1 at 37°C. Curves (b) represent OD_{420} time course in the presence of (○) 0 nM; (□) 4 nM; (△) 8 nM; (◇) 12 nM; (●) 20 nM; (■) 30 nM; (▲) 50 nM; and (◆) 100 nM of mini-hirudin 2 at 37°C. Curves (c) represent OD_{420} time course in the presence of (○) 0 nM; (□) 2.15 nM; (△) 4.3 nM; (◇) 8.6 nM; (●) 17.2 nM; and (■) 43 nM of mini-hirudin3 at 37°C.Other experimental conditions are as used for assays shown in Figure 2.

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 Ca^{2+}, an increase in apparent K_I from 480 nM (no calcium) to 2200 nM (Ca^{2+}) was observed (Figure 10).

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.

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.

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, *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 μM). An even weaker binding (K_d = 1160 μM) was observed between cCst20 and CaCdc42 with the current fluorescence titration strategy.

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 μM mCla4 (*filled*

circle) and cCst20 in the presence of 50 μM mCst20 (*filled triangle*). Solid lines represent fits of the data to a bimolecular association model.

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 μM to 207 μM (Table 2 and Figure 13c). Similarly, mCst20 affinity for CaCdc42 increased from 0.43 μM to 0.081μM 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.

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.

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 μM. Figure 14B shown that the apparent dissociation constant for the Cdc42-eCla4 complex is a function of the concentration of added SAP-SH2

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 OD_{420} time course after the addition of 0.6 nM thrombin in the presence of (C) (○) 0 nM P3291; (□) 10 nM P3291; (△) 15 nM P3291; (◇) 20 nM P3291; (●) 30 nM P3291; (■) 40 nM P3291; (▲) 50 nM P3291; (◆) 60 nM P3291; (D) (○) 0 nM P3291, 0 μM SAP-SH2; (□) 0 nM P3291, 5 μM SAP-SH2; (△) 25 nM P3291, 0 μM SAP-SH2; (◇) 25 nM P3291, 5 μM SAP-SH2; (E) (○) 0 nM P3291, 0 μM SAP-SH2; (□) 0 nM P3291, 10 μM SAP-SH2; (△) 25 nM P3291, 0 μM SAP-SH2; (◇) 25 nM P3291, 10 μM SAP-SH2. Other experimental conditions were as used in assays described in Figure 3.

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).

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-DTI and CaM-DTI2 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 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.

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 an NMR-based screening can also be applied to molecules that are either natural polypeptides or other chemical entities available only through chemical synthesis.

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.

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 oligonudeotides to peptides (Lin and others, 1995, 11044-11048) in the fabrication of biomolecular devices containing oligonucleotides as linkers (Figure 16C).

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 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).

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 ^{1}H-^{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 ^{1}H-^{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 H-^{15}N HSQC spectrum of the conjugated protein (see Figure 15B and 15C).

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 properties for bivalent ligand binding to its target.

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).

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.

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'-TAGCGATACTGCGTGGGTTGGGGCGGGTAGGGCCAGCAGTCTCGT-3' of Lin et al and Jayasena (Lin and others, 1995, 11044-11048) or 5'-GCGCCCTAAACTGGTGGT*GGAATGCGTCATGAGGGCGC-3' of Hamad-Schifferl 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).

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 μM, 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 CRIB 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).

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.

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).

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 stimuli-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.

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).

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.

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-Ile-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-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. 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 reconbinant 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.

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.; Nesset,A.; Dharmawardana,K.R.; and Bock,P.E., 2000, 16428-16434; Tolkatchev, 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.; Tolkatchev, 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).

In an embodiment of the invention, there is provided a method for the purification of a target protein, e.g. thrombin, prothrombin, Cdc42, or any other

protein for which a bivalent and retractable polypeptide ligand is designed. The bivalent polypeptide will be immobilized on a solid support for use as an affinity absorbent for the targeted protein. The absorbed protein can be eluted using molecular agents or temperature, which upon contact with the affinity matrix will inactivate the bivalent ligand and release the absorbed protein. In light of the disclosures provided here in, it will be apparent to ones skilled in the art what detailed procedures will need to be followed for the above-mentioned applications.

Specific Examples

Example 1. A tolerance of the bivalent mode of inhibition to the amino acid composition of the linker moeity on a series of bivalent inhibitors of thrombin with an active site binding moiety Bbs-Arg-(D-Pip)-Gly (H1, Bbs=4-*tert*-butyl-benzenesulfonyl, D-Pip=D-pipecolic acid, K_I in low μM 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 μM 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).

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-$(Gly\text{-}Ser)_8$-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-$(Gly\text{-}Ser)_8$-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).

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 (ephrinB2$_{301-309}$) to link the H1 and H2 moeities. 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 μM (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.

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 OD_{600}=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 $M^{-1}cm^{-1}$.

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 μM 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.

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 μM 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).

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-NH_2 (P3230) (SEQ. ID. NO. 109) and Ac-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu-NH_2 (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-d_3 buffer, pH 5.5, containing 10% D_2O. 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 $CaCl_2$ 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 μL of 1 μL (final $CaCl_2$ concentration ~0.22 mM), additional 2 μL (final $CaCl_2$ concentration ~0.66 mM), additional 10 μL (final $CaCl_2$ concentration ~2.8 mM) of 100 mM $CaCl_2$, and additional 10 μL (final $CaCl_2$ concentration ~23.9 mM) of 1 M $CaCl_2$. The changes in the spectra confirm binding of calcium with affinity in mM range.

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 μL of 1 μL (final $CaCl_2$ concentration ~0.22 mM), additional 2 μL (final $CaCl_2$ concentration ~0.66 mM) of 100 mM $CaCl_2$, and additional 10 μL (final $CaCl_2$ concentration ~22.2 mM) of 1 M $CaCl_2$. The changes in the spectra confirm binding of calcium and the peptide with affinity in mM range.

Samples tested for inhibition potency contained in the clotting buffer 0.6 nM thrombin, 50 μM 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.

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 minihirudin-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.

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.

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 $(\text{substrate concentration})^{-1}$ versus $(\text{initial velocity})^{-1}$ 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 $K_i = [I]/\{(SL_o/SL_1)-1\}$, where [I] is the inhibitor concentration, SL_o 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.

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.

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 (○) 0 nM; (△) 4.2 μM; and (□) 8.4 μM CaM-DTI2. Inhibition of the amidolytic reaction confirming the bivalent mode of binding as shown for CaM-DTI in Figure 10A.

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.

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.

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 pCaCdc42Δ13 (*Stevens & Ni, unpublished data*). A CaCdc42 expression vector encoding the R150K mutation was performed using the QuickChange Site-directed Mutegenesis Kit (Stratagene, La Jolla, CA). The sequences of the wild-type and R150K mutant CaCdc42 (Table 4) vectors were verified by DNA sequencing.

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 μg/ml aprotinin, leupeptin and pepstatin, and 10 μg/mL benzamidine 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.

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).

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 ε_{280nm} = 13,610 M^{-1} cm^{-1} (Gill, S. C. and von Hippel, P. H., 1989, 319-326), and using the absorbance of the sNBD moiety of ε_{463nm} = 22,000 M^{-1} 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.

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

$$P + L \underset{k_{off}}{\overset{k_{on}}{\leftrightarrow}} P \cdot L$$

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

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

where F_0 and F_t 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.

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 μM). An even weaker binding (K_d = 1160 μM) was observed between cCst20 and CaCdc42 with the current fluorescence titration strategy.

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 μM to 207 μM (Table 2 and Figure 13c). Similarly, mCst20 affinity for CaCdc42 increased from 0.43 μM to 0.081μM 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.

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).

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 μM, 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).

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±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 μM SAP-SH2 from the stock solution of 116 μM 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.

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 ^{1}H-^{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 ^{1}H-^{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.

Example 10. Molecular species and methods of 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 internal injury. The formulation that is commercially available (e.g. Tisseel VH Fibrin Sealant, Baxter) contains two major 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.

Thus, there is provided herein a new formulation, wherein the proteolytic activity of thrombin is inhibited by a specific bivalent 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 (P3170) (SEQ. ID. NO. 82) with a controllable polymeric linker binding to SH2 domain (Figure 4). Therefore in the modified formulation, thrombin and inhibitor can be premixed and will stay inactive (and stable) until exposed to SH2.

The middle auricular artery of eight rabbits was cut transversely with a scalpel. Two rabbits were left untreated to measure the bleeding time. A commercial source of 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. The fibrin glue containing inhibitor-stabilized and highly purified human α-thrombin (Haemotologics), replacing the "Thrombin 4" component from the Tisseel kit, was applied to the last pair of rabbits immediately after SH2 activation. The final table of applied formulations employed in the example was as follows:

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

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

In an embodiment of the invention there is provided a multivalent binding molecule and uses thereof. The molecule is useful in binding a target under certain conditions and releasing it under other conditions. The molecule has the general formula (1) of

$$BM1\text{-}L\text{-}(BM2)_n \qquad (1)$$

wherein,

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

BM2 is a binding moiety 2 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 may 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 then 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; phoshothreonine; phosphohistidine.

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

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

-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-;

-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-;

-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-;

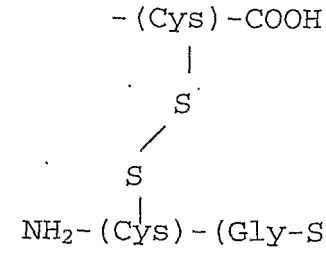
-Asn-Gly-Arg-Lys-Ile-Cys-Leu-Asp-Leu-Gln-Ala-Pro-Leu-Tyr-Lys-Lys-Ile-Ile-Lys-Lys-Leu-Leu-Glu-Ser-;

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

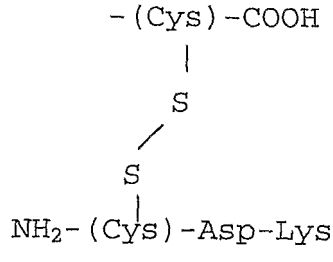
-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val-;

-Tyr-Met-Glu-Ser-Arg-Ala-Asp-Arg-;

-Gln-Ser-His-Asn-Arg-;



NH_2-(Cys)-$(Gly\text{-}Ser)_8$-Gly-;



NH_2-(Cys)-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-;

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

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

-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

$-(Gly\text{-}Ser)_2$-;

-(Gly-Ser)$_4$-;

-(Gly-Ser)$_6$-;

-(Gly-Ser)$_8$-;

-(Gly-Ser)$_{10}$-;

-(Gly-Ser)$_{12}$-;

-(Gly-Ser)$_{14}$-;

-Gly-Cys...Cys-(Gly-Ser)$_8$-;

-(Gly-Ser)$_4$-Gly-Lys-(Gly-Ser)$_5$-

-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-;

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

-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-;

wherein Tyr(P) is O-phosphotyrosine;

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

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

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

In some instances the molecule is a thrombin inhibitor;

In some instances the BM1 sequence is selected from the group consisting of:

Bbs-Arg-(D-Pip);

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

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

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;

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

Ile-Arg-Phe-Thr-Asp;

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

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;

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

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

Bbs-Arg-(D-Pip)-$(Gly-Ser)_2$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

Bbs-Arg-(D-Pip)-$(Gly-Ser)_4$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

Bbs-Arg-(D-Pip)-$(Gly-Ser)_6$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

Bbs-Arg-(D-Pip)-$(Gly-Ser)_8$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

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

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

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

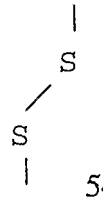
Bbs-Arg-(D-Pip)-Gly-Ser-Pro-His-Tyr-Glu-Lys-Val-Ser-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

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;

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;

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;

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



NH_2-(Cys)-$(Gly-Ser)_8$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

Bbs-Arg-(D-Pip)-$(Gly-Ser)_4$-Gly-Lys-$(Gly-Ser)_5$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;

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;

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;

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;

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;

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;

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;

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;

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;

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

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

NH_2-(Cys)-Asp-Lys-Asn-Ala-Asp-Gly-Trp-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-Leu-Gln;

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

In some instances the molecule is

WDPRPQRHADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAE LQDMINEVDADGNGTIDFPEFLTMMARKMKDTGGVKLIPSWTTVILVKSMLRKRS FGNPFGGDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIRE ADIDGDGQVNYEEFVQMMTAKDFEEIPEEYLQ;

In some instances the molecule is a ligand of Cdc42;

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

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

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

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

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

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

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;

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

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

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;

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;

In an embodiment of the invention there is provided a method to obtain the polypeptide molecule according to claim 3 with high affinity to a protein target, said method comprising steps of:

a) Identification of two binding peptide moieties to two different binding sites of the target based on already existing polypeptide ligands with high affinity;

b) Establishing a weaker binding peptide moiety using NMR titration or NMR relaxation dispersion spectroscopy;

c) Connecting the peptide moieties with a polymeric linker;

d) Increasing the bivalent affinity by sequence optimization of the weaker moiety by means of phage display;

In an embodiment of the invention there is provided a method to prolong the lifetime of reconstituted autocatalytic protease said method comprising the steps of

a) Inhibiting the protease with a bivalent protease inhibitor containing a controllable linker,

b) Releasing and activating the protease with an appropriate linker-targeted antidote;

In some instances the protease is thrombin.

In some instances thrombin is a component of a fibrin sealant kit.

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

Table 1. IC_{50} 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.
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
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
P3153, SEQ. ID. NO. 77	GSGSGSGSGSGSGSGSGSGS (SEQ. ID. NO. 54)	2.0±0.3	1.0±0.1	2e
P3160, SEQ. ID. NO. 78	GSGSGSGSGSGSGSGSGSGSGSGS (SEQ. ID. NO. 55)	4.6±0.8	2.8±0.1	2f
P3159, SEQ. ID. NO. 79	GSGSGSGSGSGSGSGSGSGSGSGSGSGS (SEQ. ID. NO. 56)	6.7±1.9	3.5±0.2	2g
P3172-P3165, SEQ. ID. NO. 83	Gly-Cys...Cys-GSGSGSGSGSGSGS (SEQ. ID. NO. 57)		1.1±0.2	2h
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
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
P3161, SEQ. ID. NO. 81	GSPHYEKVSGSPHYEKVS (SEQ. ID. NO.125)		0.7 (25°C)	3c

	Tandem of two peptide ligands to SH2 from Grb4, dephosphorylated			
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
P3174, SEQ. ID. NO. 84	GSGSGSGSGKGSGSGSGSGS (SEQ. ID. NO. 58) Lys in the middle of a long GS repeat linker		1.6±0.9 (25°C)	2j
P3181, SEQ. ID. NO. 85	GTLDLNTPVDKTSN (SEQ. ID. NO. 103) C5a receptor peptide		1.9±0.2	2i
P3182, SEQ. ID. NO. 13	GEQKLISEEDL (SEQ. ID. NO. 127) c-myc peptide	66±13	~25	
P3209, SEQ ID. NO. 32	GSVVPRPQLHND (SEQ. ID. NO. 105) Prothrombin-binding linker 1 (VV)		1.1	2k
P3210, SEQ. ID. NO. 33	GSHAPRPQIHND (SEQ. ID. NO. 104) Prothrombin-binding linker 2 (HA)		0.6	2l
P3234, SEQ. ID. NO. 88	GHHLGGAKQAGDV (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.	GQSHNR (SEQ. ID. NO. 108) Linkers conferring a RGDF		2±1	2o

ID. NO. 90	sequence, with potential binding to an integrin receptor			
3243-3255, SEQ. ID. NO. 91	Gly-Cys...Cys-DKNADGWIDN (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	

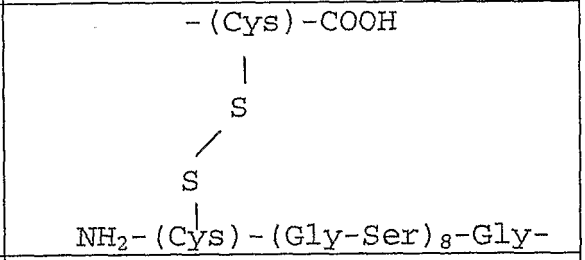
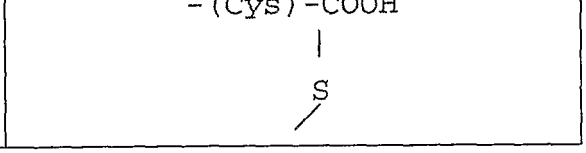
Table 2. Dissociation constants for binding of *Candida* CRIB fragments to CaCdc42 measured by fluorescence titration.

Peptide	mCla4	mCst20	cCla4	cCst20	eCla4	eCst20
K_d^m *(μM)*	4.2 ± 0.15	0.43 ± 0.03	275 ± 9	1160 ± 106	0.025 ± 0.002	0.046 ± 0.002
Peptide	mCla4 (+cCla4)	mCst20 (+cSt20)	cCla4 (+mCla4)	cCst20 (+mCst20)		
K_d^m *(μM)*	4.1 ± 0.13	0.081 ± 0.002	311 ±12	207 ± 10		
Peptide	mCla4-cCst20	mCst20-cCla4	mCst20-P-cCla4	eCla4-SG	eCla4-SLAM	
K_d^m *(μM)*	0.031 ± 0.002	2.64 ± 0.20	0.093 ± 0.01	0.067 ± 0.008	0.127 ± 0.07	

Table 3

SEQ. ID. NO.	SEQUENCE
SEQ. ID. NO. 1	Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 2	Bbs-Arg-(D-Pip)-linker-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 3	[-*linker*-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln]
SEQ. ID. NO. 4	Bbs-Arg-(D-Pip)-Gly-Cys
SEQ. ID. NO. 5	Cys-$(Gly-Ser)_8$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 6	Bbs-Arg-(D-Pip)-Gly-Cys
SEQ. ID. NO. 7	Cys-$(Gly-Ser)_8$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
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
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
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
SEQ. ID. NO. 15	Bbs-Arg-(D-Pip)-Gly-Cys...
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
SEQ. ID. NO. 17	Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-Gly-Asp-Phe-Glu
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
SEQ. ID. NO. 19	Trp-Asp-Pro-Arg-Pro-Gln-Arg-His
SEQ. ID. NO. 20	Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln

SEQ. ID. NO.	SEQUENCE
SEQ. ID. NO. 21	Trp-Asp-Pro-Arg-Pro-Gln-Arg-His-(CamCKK)-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
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)
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)
SEQ. ID. NO. 24	Ala-Lys-Val-Thr-Met-Thr-Cys-Ser-Ala-Ser-
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
SEQ. ID. NO. 26	*ISXPXXFXHXXHVGXD*
SEQ. ID. NO. 27	-Ser-Gly-Ser-Gly-
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-*tert*-butyl-benzenesulfonyl, D-Pip=D-pipecolic acid, K_I in low μM range

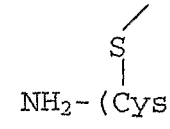
SEQ. ID. NO.	SEQUENCE
SEQ. ID. NO. 36	Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
SEQ. ID. NO. 37	-Gly-Gly-Asn-Ser-Gly-Val-Ser-Gly-Pro-Ile-Asn-Phe-Thr-His-Lys-Val-His-Val-Gly-Phe-Asp-Pro-Ala-Ser-Gly-Asn-Phe-Thr-Gly-Leu-Pro-Asp-Thr-Trp-Lys-Ser-Leu-Leu-Gln-His-Ser-Lys-Ile-Thr-
SEQ. ID. NO. 38	-Glu-Val-Asn-Ile-Lys-Ile-Ser-Thr-Pro-Phe-Asn-Ala-Lys-His-Leu-Ala-His-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-;
SEQ. ID. NO. 39	-Thr-Leu-Asp-Leu-Asn-Thr-Pro-Val-Asp-Lys-Thr-Ser-Asn-
SEQ. ID. NO. 40	-Ser-Val-Val-Pro-Arg-Pro-Gln-Leu-His-Asn-Asp-
SEQ. ID. NO. 41	-Ser-His-Ala-Pro-Arg-Pro-Gln-Ile-His-Asn-Asp-
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-
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-
SEQ. ID. NO. 44	-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val-
SEQ. ID. NO. 45	-Tyr-Met-Glu-Ser-Arg-Ala-Asp-Arg-
SEQ. ID. NO. 46	-Gln-Ser-His-Asn-Arg-
SEQ. ID. NO. 47	
SEQ. ID. NO. 48	

SEQ. ID. NO.	SEQUENCE
	S | NH_2-(Cys)-Asp-Lys-Asn-Ala-Asp-Gly-Trp-Ile-Asp-Asn-
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-

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

SEQ. ID. NO.	SEQUENCE
SEQ. ID. NO. 79	Bbs-Arg-(D-Pip)-$(Gly-Ser)_{14}$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln
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
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
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
SEQ. ID. NO. 83	Bbs-Arg-(D-Pip)-Gly-(Cys)-COOH | S / S | NH_2-(Cys)-$(Gly-Ser)_8$-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln;
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
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-

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
SEQ. ID. NO. 91	Bbs-Arg-(D-Pip)-Gly-(Cys)-COOH | S

SEQ. ID. NO.	SEQUENCE
	 NH2-(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. 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
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-

SEQ. ID. NO.	SEQUENCE
	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	Bbs-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-
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.	SEQUENCE
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
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-

SEQ. ID. NO.	SEQUENCE
	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-
SEQ. ID. NO. 130	GGNSGFPGPI NFTHKVHVGF DRKSLTIYAQ VQKNFTGLPD TWKSLLQHSK IT
SEQ. ID. NO. 131	YGRKKRRQRR RGGNSGFPGP INFTHKVHVG FDRKSLTIYA QVQKNFTGLP
SEQ. ID. NO. 132	GSSHHHHHHSSFNPRGSWYY GNVTRHQAECALNERGVEGDFLIRDSE SSPSDFSVSLKASGRNKHFKVQL VDSVYCIGQRRFHSMDELVEHYKKAP IFTSEHGEKLYLVRALQ
SEQ. ID. NO. 133	GSSHHHHHHSSFNPRGSD AVAVYHGKISRETGEKLLLATGLDG SYLLRDSESVPGVYCLCVLYHGYI

SEQ. ID. NO.	SEQUENCE
	YTYRVSQTETGSWSAETAPGVHKRYF RKIKNLISAFQKPDQGIVIPLQYPVEK
SEQ. ID. NO. 134	GSSHHHHHHSSGLVPRGSHMQTIKCVV VGDGAVGKTCLLISYTTSKFPA DYVPTVFDNYAVTVMIGDEPFTLGLF DTAGQEDYDRLRPLSYPSTDV FLVCFSVISPASFENVKEKWFPEVHHH CPGVPIIIVGTQTDLRNDDVI LQRLHRQKLSPITQEQGEKLAKELKA VKYVECSALTQRGLKTVFDEAIVAALE

Table 4. Full sequences of recombinant peptides and proteins

1. eCla4-SLAM

GGNSGFPGPI NFTHKVHVGF DRKSLTIYAQ VQKNFTGLPD TWKSLLQHSK IT
(SEQ. ID. NO. 130)

2. TAT-eCla4-SLAM:

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

3. Grb4-SH2

GSSHHHHHHSSFNPRGSWYYGNVTRHQAECALNERGVEGDFLIRDSESSPSDFSVSL
KASGRNKHFKVQLVDSVYCIGQRRFHSMDELVEHYKKAP IFTSEHGEKLYLVRALQ
(SEQ. ID. NO. 132)

4. SAP-SH2:

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

5. CaCdc42 (R150K)

GSSHHHHHHS SGLVPRGSH MQTIKCVV VGDGAVG KTCLLISY TTSKFPA
DYVPTVF DNYAVT VMIGDE PFTLGLF DTAGQED YDRLRPL SYPSTDV
FLVCFSV ISPASF ENVKEKW FPEVHHH CPGVPII IVGTQTD LRNDDVI
LQRLHRQ KLSPIT QEQGEKLA KEL**K**A VKYVEC SALTQRGLKT VFDEA
IVAALE
(SEQ. ID. NO. 134)

6. CaM-DTI:

WDPRPQRHADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQ
DMINEVDADGNGTIDFPEFLTMMARKMKDTGGVKLIPSWTTVILVKSMLRKRSFGNP
FGGDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGD
GQVNYEEFVQMMTAKDFEEIPEEYLQ (SEQ. ID. NO. 21)

7. CaM-DTI2:

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1. A molecule comprised of a structure useful in binding a target, said molecule having the general formula (1) of

$BM1\text{-}L\text{-}(BM2)_n$ (1)

wherein,

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

BM2 is a binding moiety 2 having an affinity for a site other than site 1 on the target, 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,

n is 1 or greater;

wherein BM1 and BM2 may be the same or different, and where n>1, different BM2 moieties may have affinities for different binding sites on the target; BM1 and BM 2 being selected such that in use each of the BM1 and BM2 existing separately has a lower binding affinity then the complex of BM1 and BM2 does when they are linked to form the molecule.

2. The molecule of claim 1 wherein the binding affinity of BM1 or BM2 alone is no more than ½ the binding affinity of the molecule.

3. The molecule of claim 1 wherein the linker is an oligomeric or polymeric linker.

4. The molecule of claim 1 wherein the linker comprises an amino acid sequence.

5. The molecule of claim 4 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.

6. The molecule of claim 1 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.

7. The molecule of claim 1 wherein BM2 comprises an amino acid sequence selected from SEQ. ID. NO. 1, 20, 36, 96-99.

8. The molecule of claim 1 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, 130, 131, or 117.

9. An isolated amino acid sequence of no more than 100 amino acids, said sequence comprising a series of contiguous amino acids having at least 90% sequence identity to at least one of SEQ. ID. NO. 8, 12, 17, 24, 27, 28, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 128, 62, 63, 64, 65, 66, 67, 103, 104, 105, 106, 107, 108, 109, 110, 111, 118, 120, 123, 124, 125, 126, 127, or 128.

10. A nucleic acid sequence encoding the amino acid sequence of claim 9.

11. An amino acid sequence having at least 80% sequence identity to at least one 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 or 117.

12. A nucleic acid sequence encoding the amino acid sequence of claim 11.

13. A pharmaceutical composition comprising a molecule of claim 1 with a carrier.

14. A method of manufacture of a medicament useful in the localized modulation of biological function, said method comprising combining the molecule of claim 1 with a carrier.

15. A method to screen a population of molecules to identify a ligand of interest, said method comprising:

a) selecting a target of interest with a known affinity for a component;

b) selecting a second molecule having a known affinity for the component of step (b); said second molecule binding a different region of the component from the region bound by the target;

c) connecting the target and the second molecule with a suitable linker to create a bivalent structure;

d) monitoring binding of the bivalent structure to the component in the presence of ligands;

e) identifying a ligand which reduces the level of bivalent binding.

16. The method of claim 15 wherein the linker selected is one which undergoes an assayable conformation change during the transition between bivalent and monovalent binding, such that this conformation change can be readily assayed.

17. The method of claim 16 wherein the linker is selected to undergo a structural change assayable by nuclear magnetic resonance ("NMR"), surface-plasmon resonance ("SPR"), and/or H/D exchange.

18. The method of claim 16 wherein the linker is selected to undergo a structural change assayable by a change in fluorescence, optical chiral properties or absorbance properties.

19. A method of assaying for the presence of a substance of concern in a sample, said method comprising:

a) identifying a first target region known to be present on the substance of concern and less common within sample material which does not contain the substance;

b) identifying a second target region known to be present on the substance of concern;

c) obtaining a plurality of molecules comprising a first binding region having affinity for the first target region connected by way of a linker to a second binding region having affinity for the second target region, wherein the linker undergoes an assayable conformational change during the transition between bivalent and monovalent binding;

d) combining molecules from step (c) with the sample; and

e) assaying for bivalent binding.

20. The method of claim 19 wherein the linker is selected to undergo a structural change assayable by nuclear magnetic resonance ("NMR"), surface-plasmon resonance ("SPR"), and/or H/D exchange.

21. The method of claim 19 wherein the linker is selected to undergo a structural change assayable by a change in fluorescence, optical chiral properties or absorbance properties.

22. The molecule of claim 1 wherein the linker is a controllable flexible linker.

23. The molecule of claim 22 wherein the linker contains a specific binding site for a binding molecule.

24. The molecule of claim 23 wherein the linker contains at least one amino acid which is a substrate for a kinase or phosphatase.

25. The molecule of claim 24 wherein the linker conformation is modulated by SH2 binding.

26. The molecule of claim 22 wherein the linker contains a specific binding site for an antibody.

27. The molecule of claim 26 wherein the antibody is a monoclonal antibody.

28. The molecule of claim 27 wherein the specific binding site is SEQ. ID. NO. 12 and the antibody is 9E10.

29. The molecule of claim 22 wherein the linker contains residues capable of forming disulfide bonds with each other.

30. The molecule of claim 22 wherein the linker has an affinity for a metal ion.

31. The molecule of claim 30 wherein the linker comprises SEQ. ID. NO. 17 and the metal ion is a calcium ion.

32. The molecule of claim 30 wherein the linker comprises SEQ. ID. NO. 21 and the metal ion is calcium.

33. The molecule of claim 22 wherein the linker undergoes a folding transition in response to a change in pH.

34. The molecule of claim 22 wherein the linker undergoes a folding transition in response to a change in ionic strength.

35. The molecule of claim 22 wherein the linker undergoes a folding transition in response to a change in temperature.

36. A peptide comprising SEQ. ID. NO. 25.

37. A nucleic acid sequence encoding the peptide of claim 36.

38. Use of the peptide of claim 38 in modulating thrombin activity.

39. A polypeptide sequence comprising at least one of SEQ. 37, 38, 92, 93, 94, 95, 100, or 101.

40. The polypeptide sequence of claim 39 further comprising at least one of SEQ. ID. NO. 96, 97, 98 or 99.

41. The molecule of claim 1 wherein the linker has a metal and/or magnetic nanoparticle conjugated to it.

42. Use of the molecule of claim 1 with a binding molecule having a magnetic and/or metal nanoparticle conjugated to it.

43. The molecule of claim 1 wherein bivalent binding of the molecule causes the linker to undergo a change in fluorescent, chromogenic, or chiral properties.

44. Use of the molecule of claim 1 in screening for the presence of the binding protein, kinase or phosphatase.

45. Use of a molecule of claim 24 as a carrier to deliver a bound molecule to a location where release of the molecule is effected by modulation of linkers flexibility and/or conformation.

46. A polypeptide comprising SEQ. ID. NO. 29.

47. A kit comprising the polypeptide of claim 46 together with SH2 and thrombin.

48. Use of the kit of claim 47 in modulating blood flow.

49. The use of claim 49 wherein the bound molecule is thrombin.

50. The use of claim 49 wherein the bound molecule is at least one of: a chemotherapeutic, an antifungal agent, an anticoagulant, and antibiotic, an antioxidant, a neurotransmitter or mimetic thereof, an antiviral agent, a degratory enzyme, a kinase, a phosphatase, an anaesthetic, an analgesic, a trans-acting 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

51. The molecule of claim 1 wherein the linker has an affinity for prothrombin.

52. The molecule of claim 1 comprising at least one of SEQ. ID. NO. 32 and 33.

53. A kit comprising:

(a) a controllable polymeric linker having a first end and a second end; and

(b) instructions for the conjugation of a binding moiety to the first and second end of the linker.

54. The kit of claim 53 wherein the linker is designed to undergo a structural change in response to a change in physical environment .

55. A method of identifying conditions affecting a structural characteristic of a polymeric molecule of interest, said method comprising:

a) obtaining a molecule according to claim 1 wherein the linker comprises the polymeric molecule of interest and binding moieties 1 and 2 are adapted to bind a known target when the linker has a first structural condition such that

bivalent binding of binding moiety 1 and binding moiety 2 to the target causes a change in an assayable characteristic of the target;

b) permitting interaction between the molecule of step (a) and the target;

c) altering the conditions under which the interaction of step (b) occurs; and

d) assaying the effect of changes of conditions on the characteristic of the target.

56. The method of claim 53 wherein the assayable chacteristic of the target is its ability to catalyze a chemical reaction.

57. A method to enhance the stability of an enzyme, said method comprising:

a) reducing functional activity of the enzyme by binding to it a molecule of claim 2 having first and second binding moieties with affinity for the enzyme.

58. The method of claim 57 further including a further step of releasing bivalent binding of the molecule of claim 2 by inducing a change in the structure and/or flexibility of the linker so as to allow an increase in functional activity of the enzyme.

59. The method of claim 57 or 58 wherein the enzyme is a protease.

60. A method of manufacture of a device capable of activation by an electromagnetic field, said method comprising:

a) obtaining a molecule of claim 1 wherein BM1 And BM2 bind to a target and L binds to an antidote which causes a change in the linker sufficient to reduce bivalent binding of the molecule to the target;

b) conjugating the antidote with a metal nanoparticle;

c) allowing interaction between the antidote and the target such that bivalent binding occurs.

61. A method of activating a device manufactured according to the method of claim 60, said method comprising releasing the ligand by heating the metal nanoparticle by means of electromagnetic radiation.

62. A method for the purification of a target, said method comprising:

a) immobilizing onto a solid support a molecule of claim 1 capable of binding to the target via BM1 and BM2;

b) allowing the target to bind the molecule of step (a)

c) eluting unbound materials;

d) eluting the target by inducing a condition which causes a change in structure and/or flexibility of the molecule of step (a) such that bivalent binding of the target by the molecule is reduced.

63. The molecule of claim 1 covalently attached to the target at a point in addition to BM1 or BM2.

64. The molecule of claim 64 wherein the molecule is covalently attached to its target.

65. The molecule of claim 65 wherein the molecule is attached to its target by means of recombinant conjugation.

66. The method of claim 15 or 55, wherein a change in linker structure or flexibility is assayable by nuclear magnetic resonance, surface plasmon resonance, and/or H/D exchange.

5

67. The method of claim 15 or 55, wherein the linker is selected to undergo a structural change assayable by a change in fluorescence, optical chiral properties or absorbance properties.

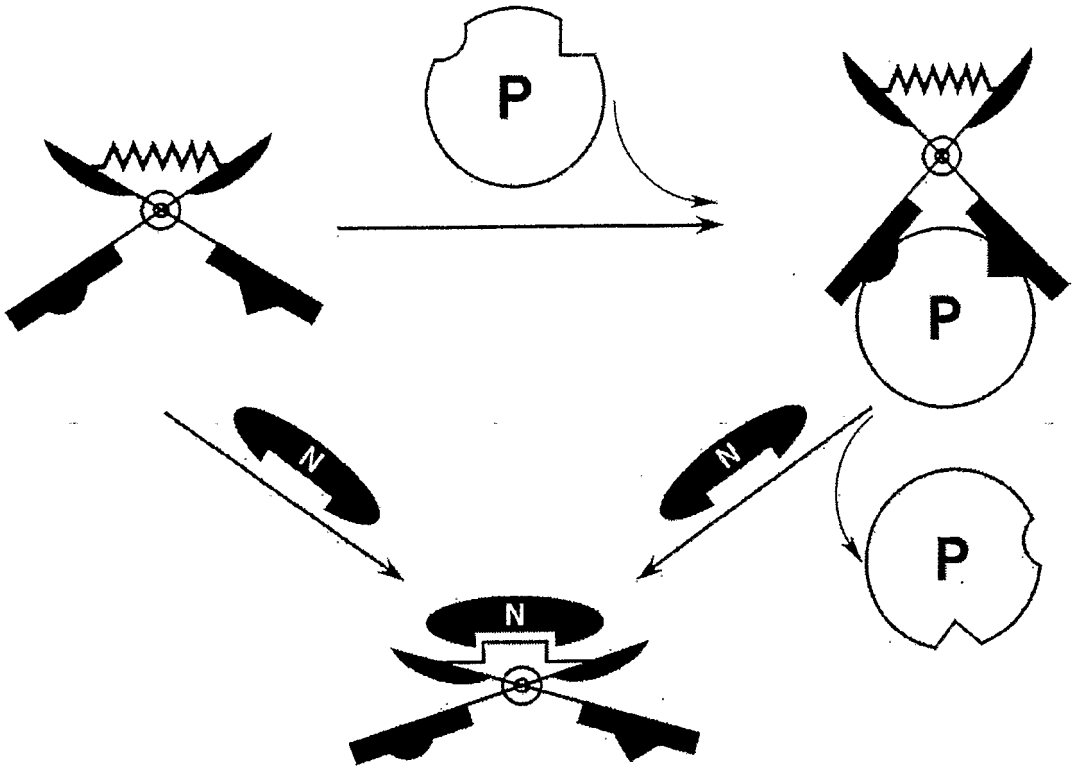

Figure 1A.

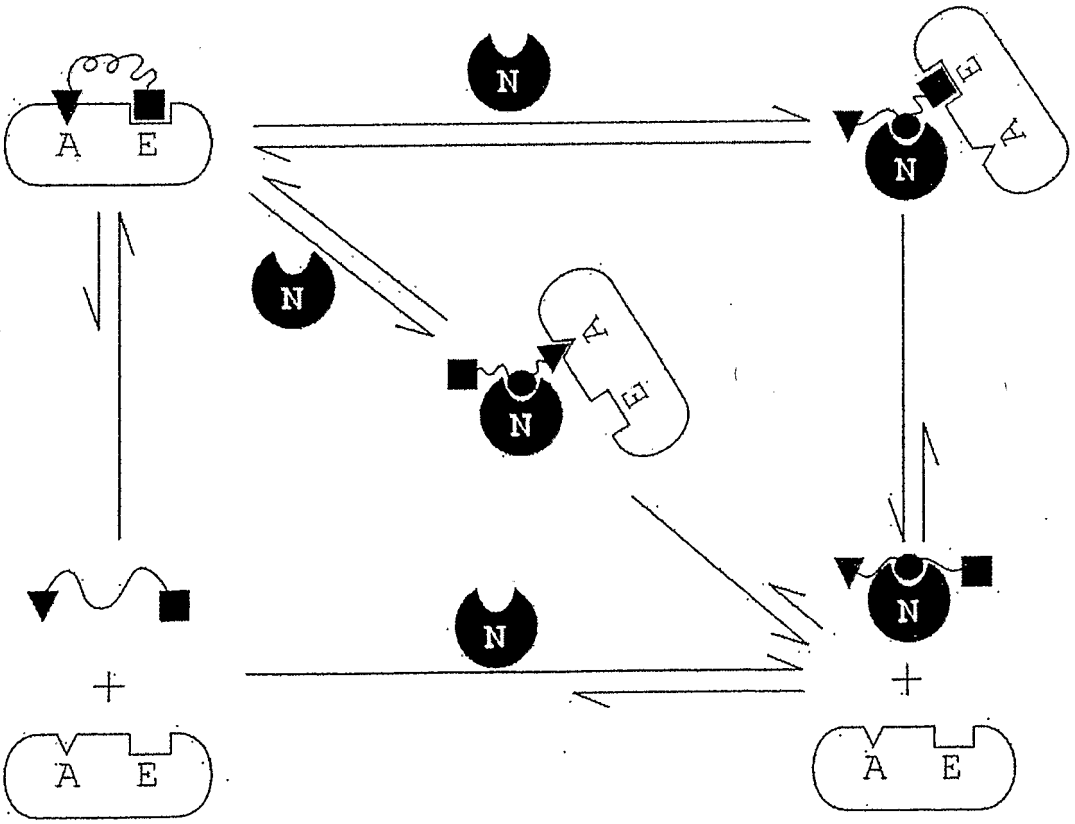

Figure 1B.

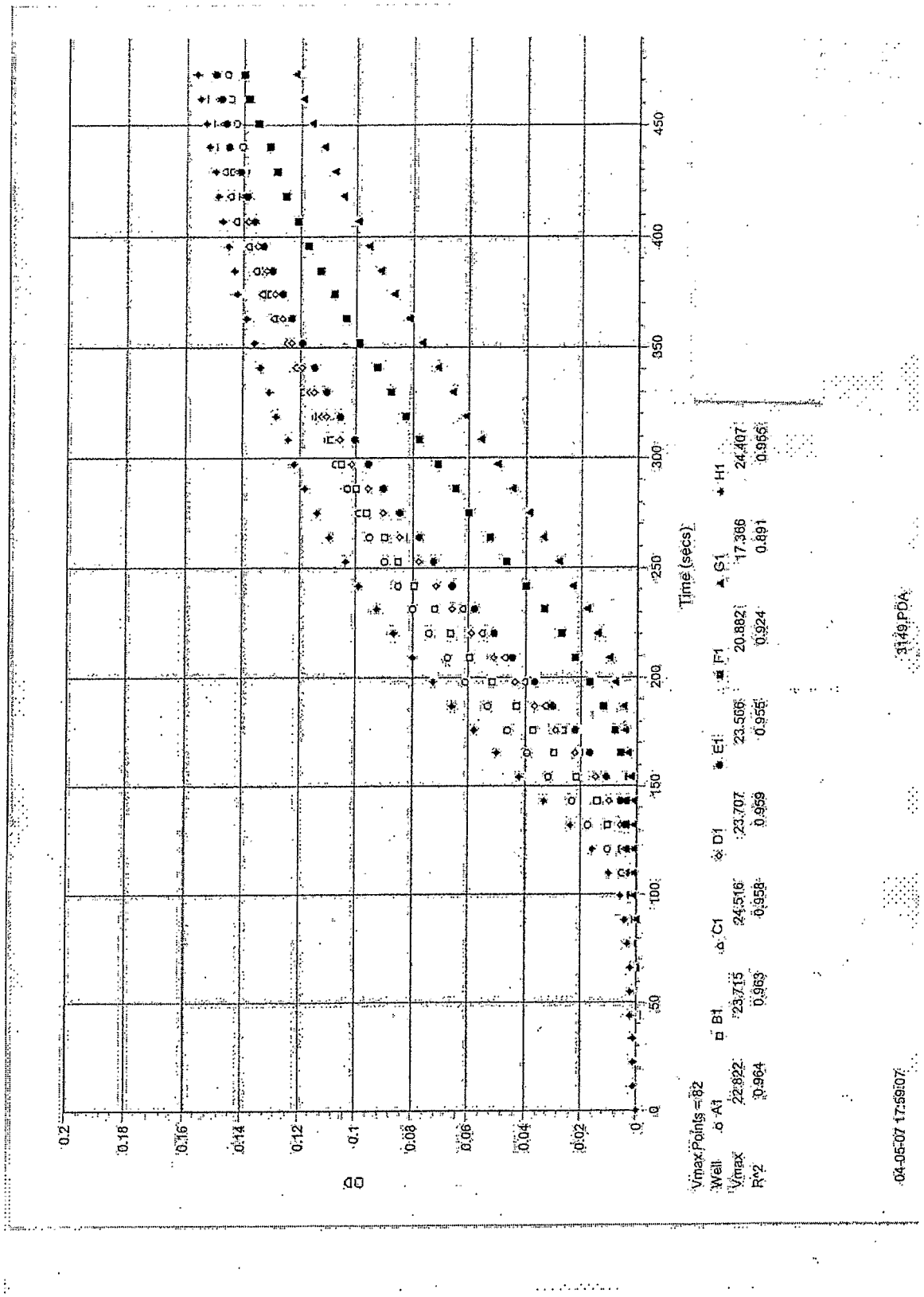
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Figure 2a.

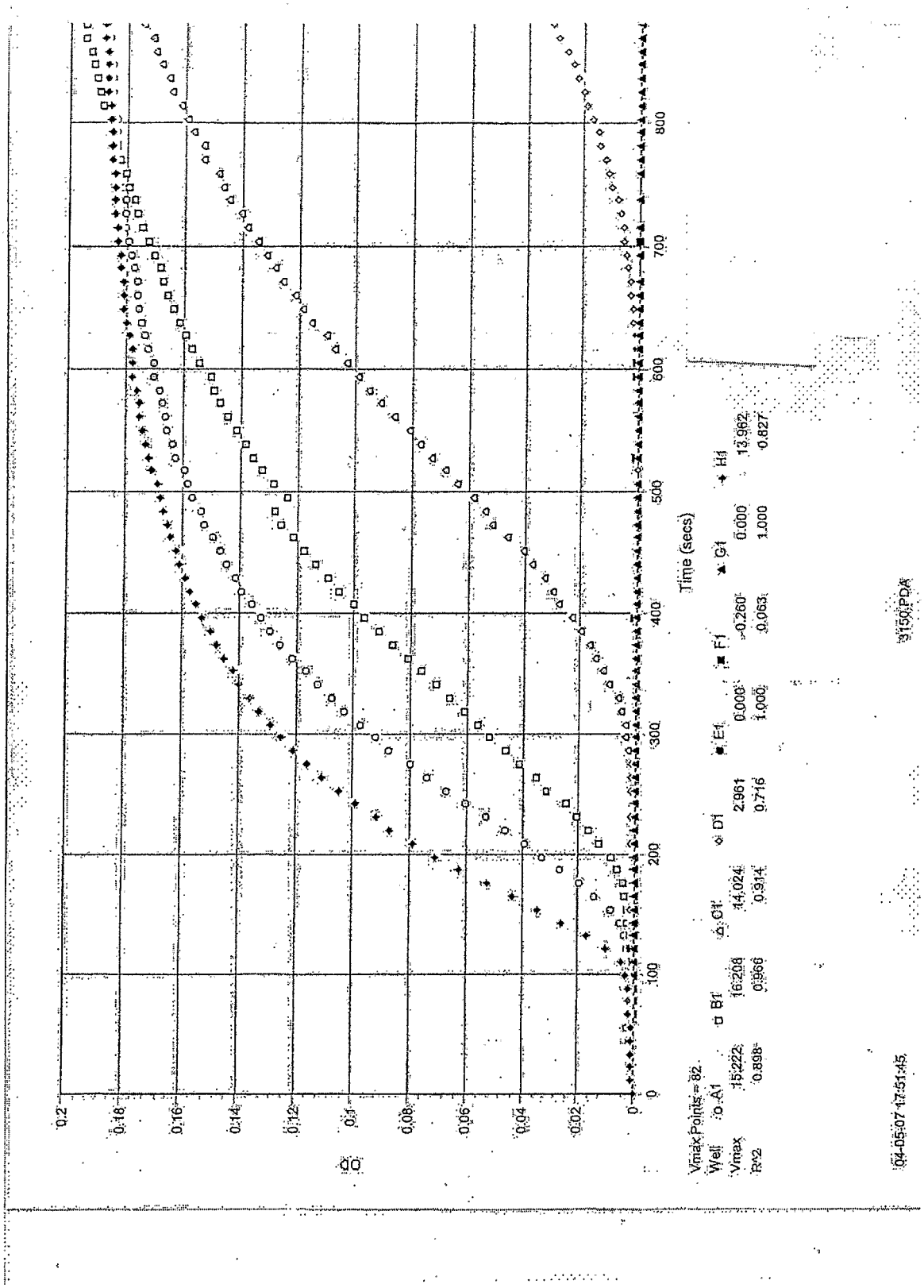
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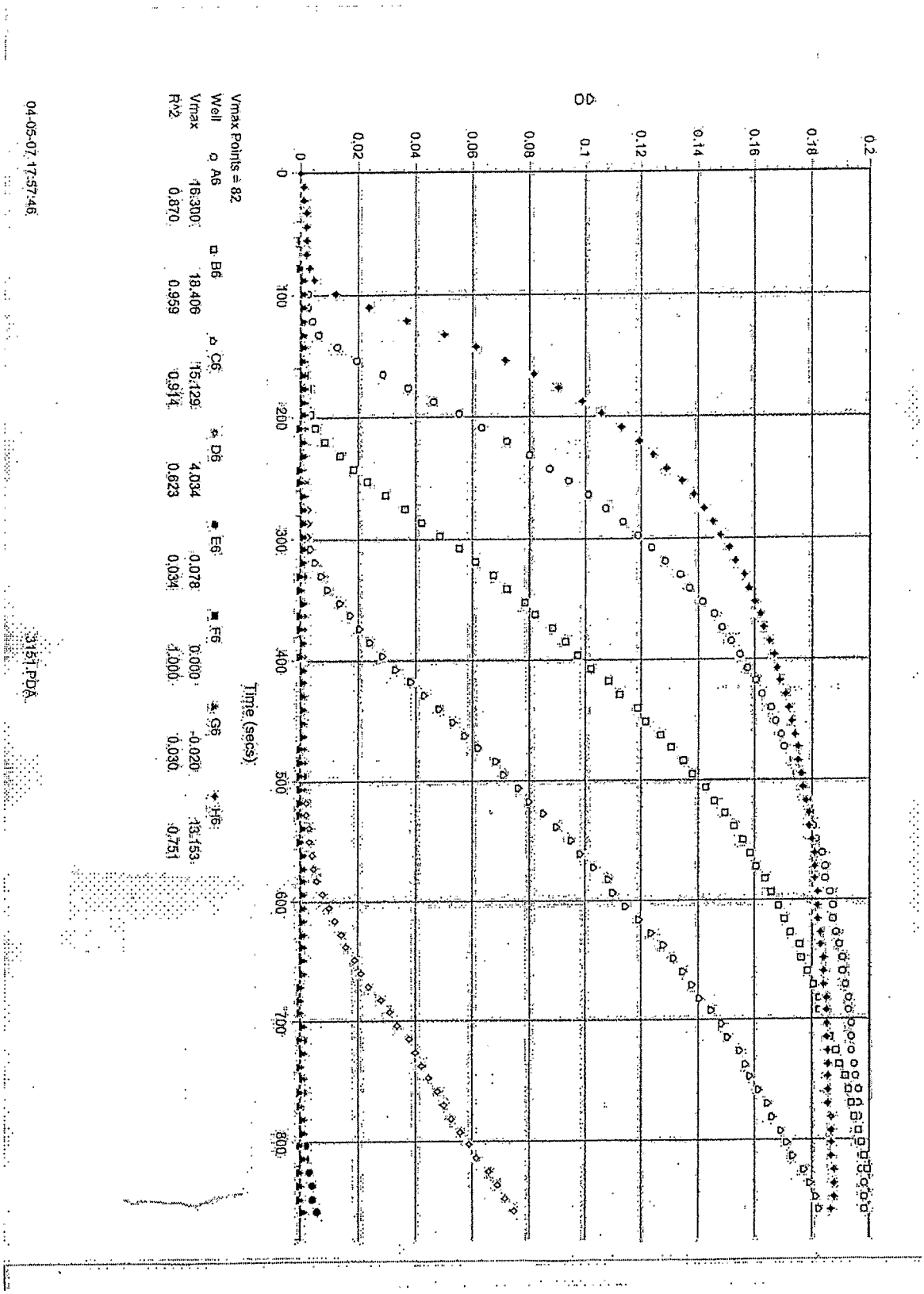
Figure 2b.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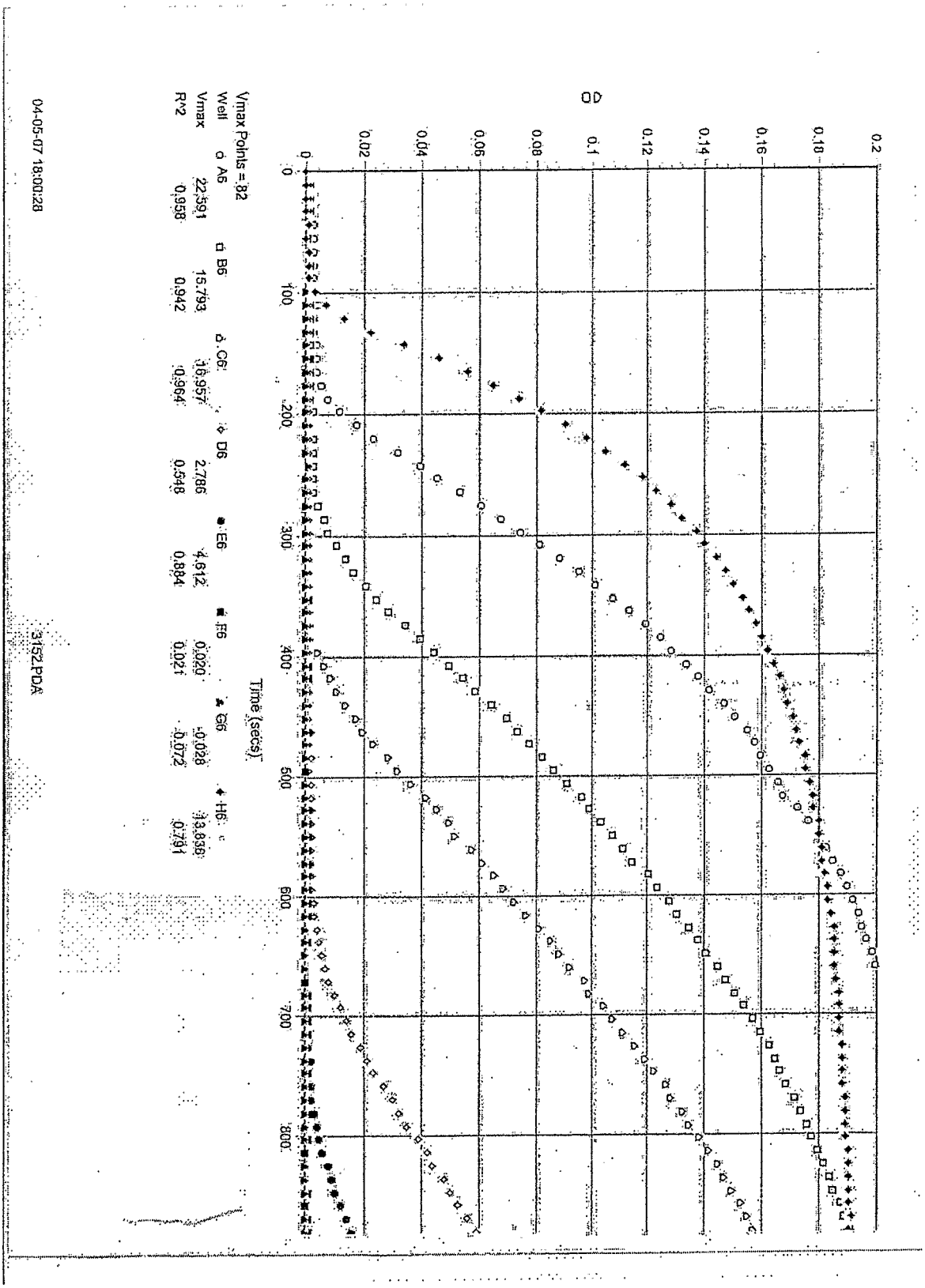
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Figure 2d.

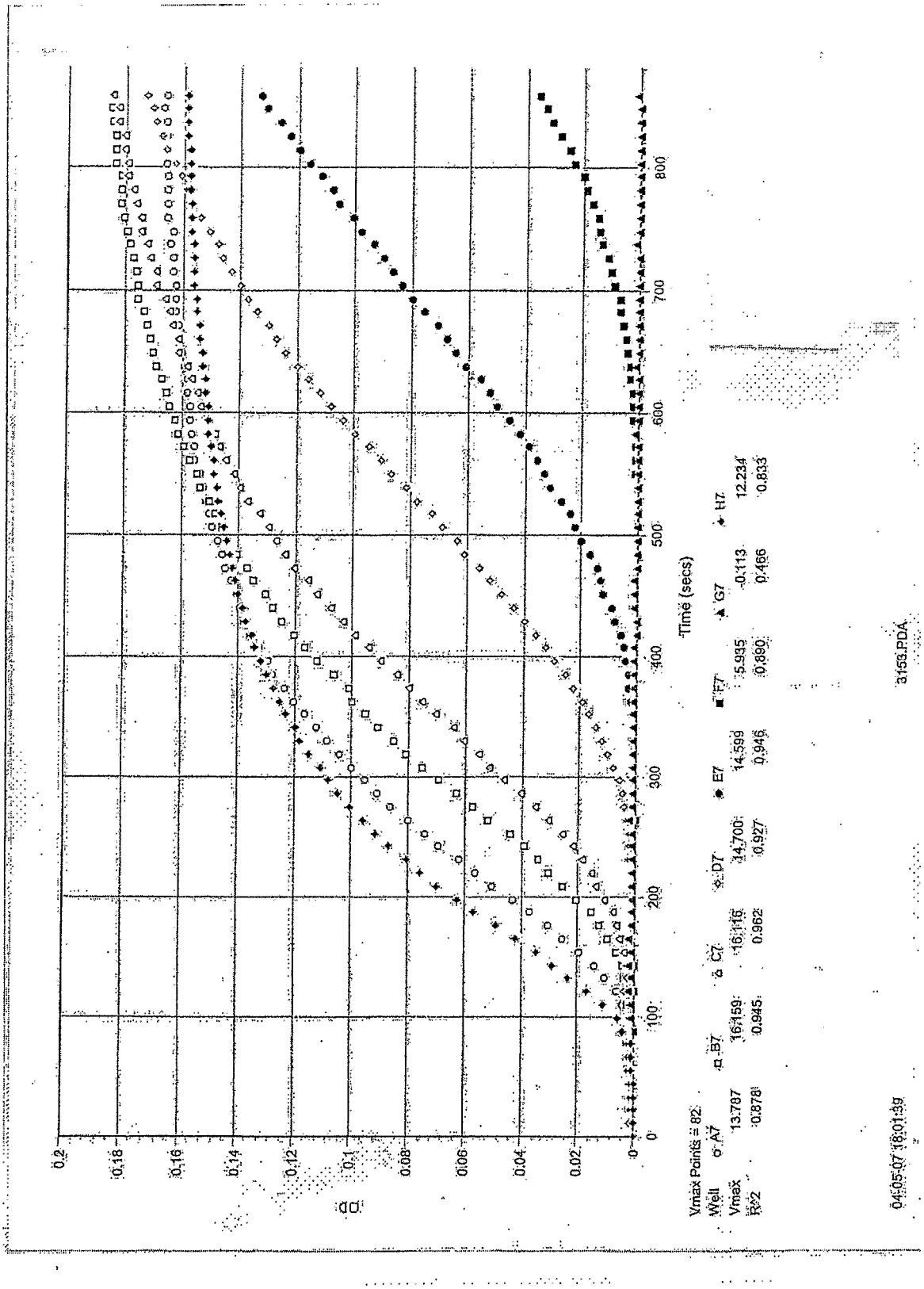
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Figure 2e.

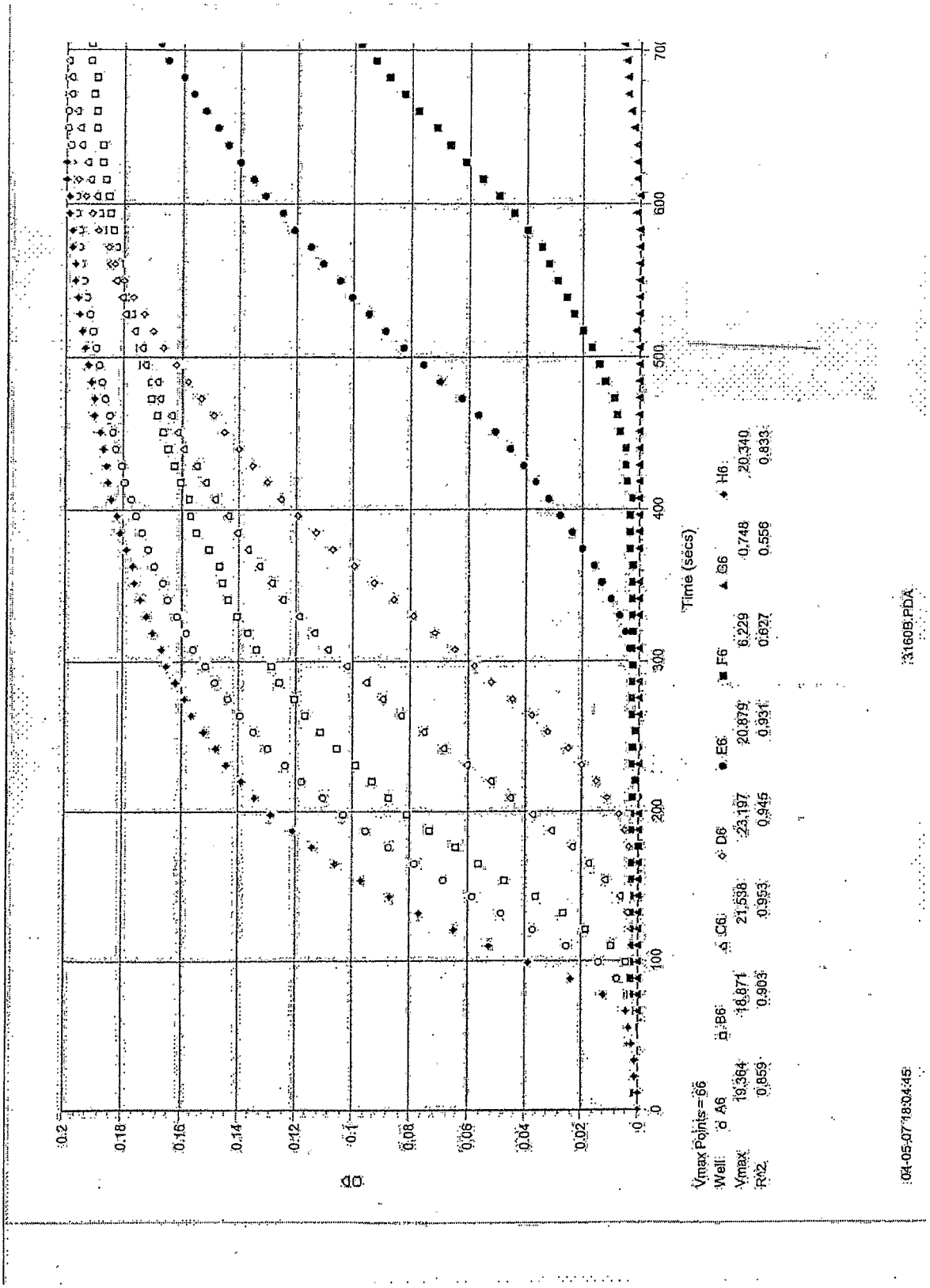
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Figure 2f.

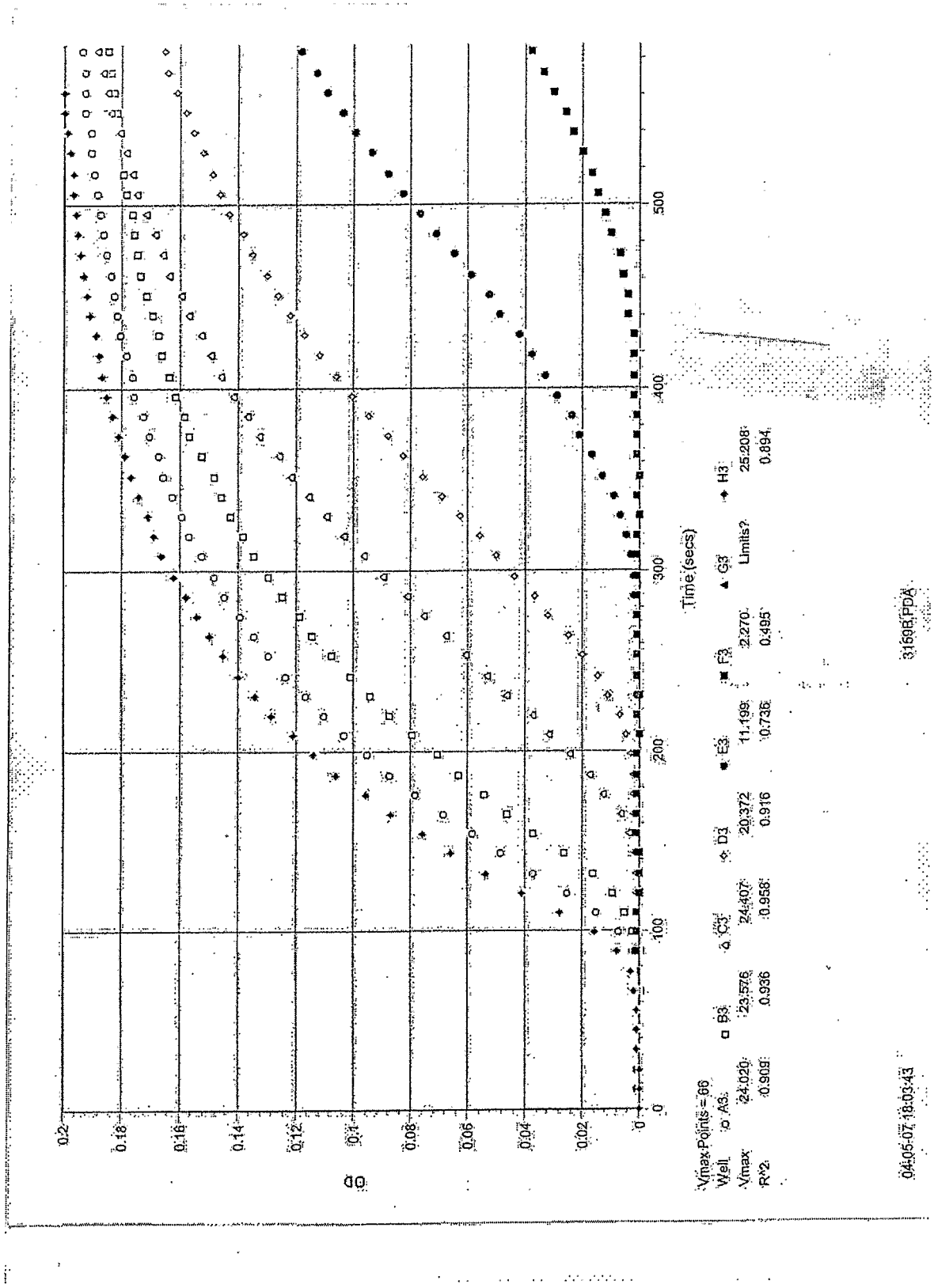
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Figure 2g.

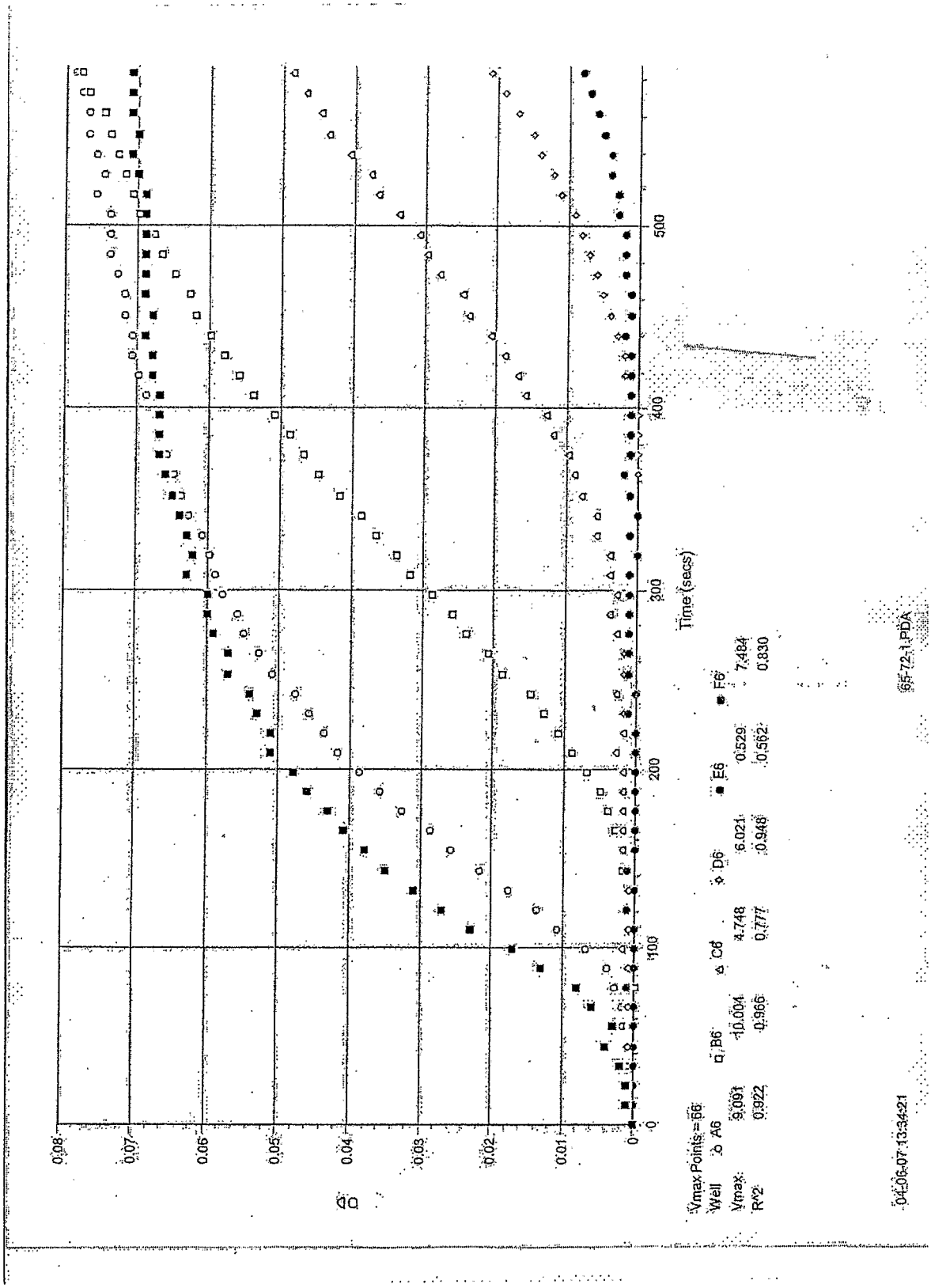
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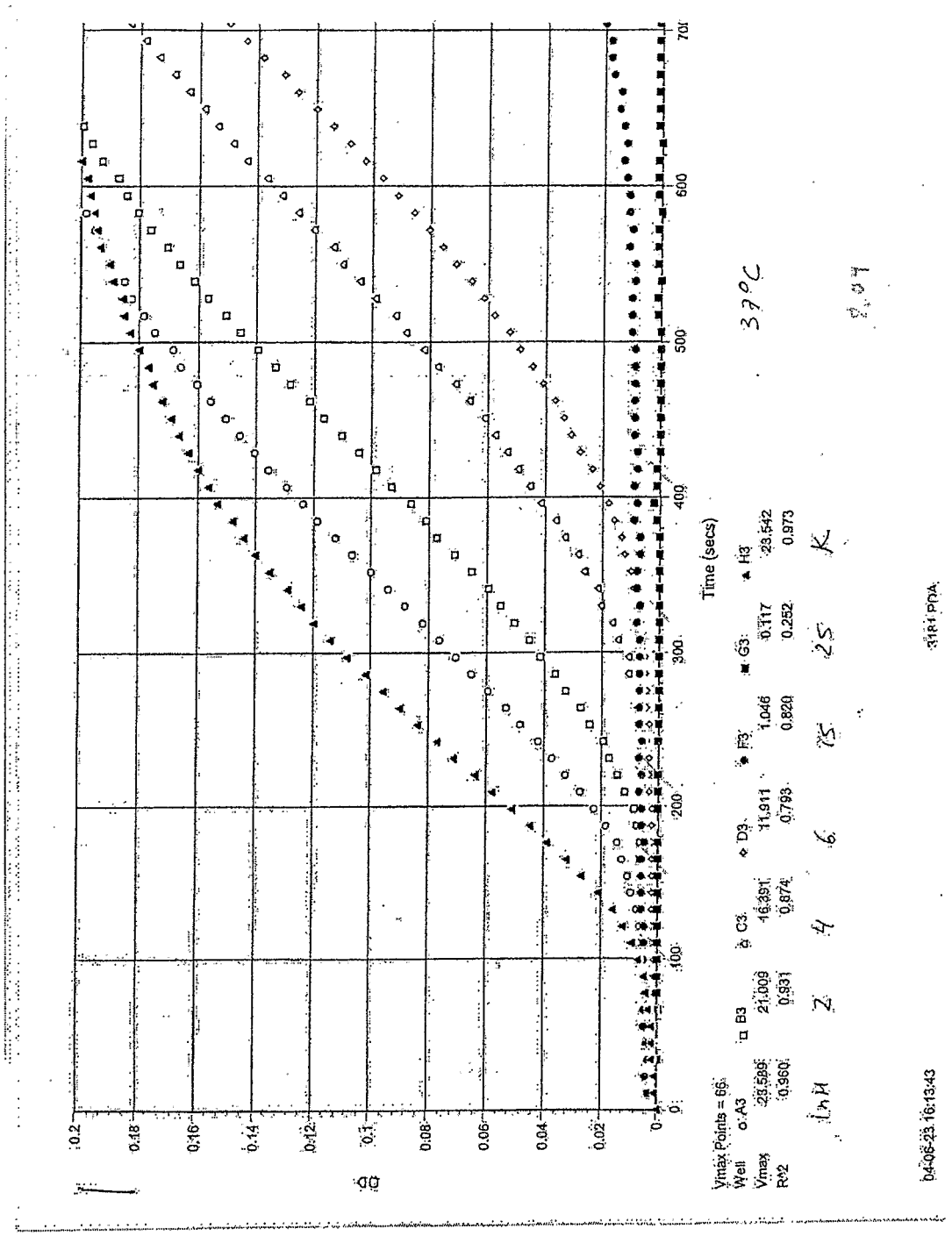
Figure 2h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04-06-23 16:13:43
37°C

Figure 2i

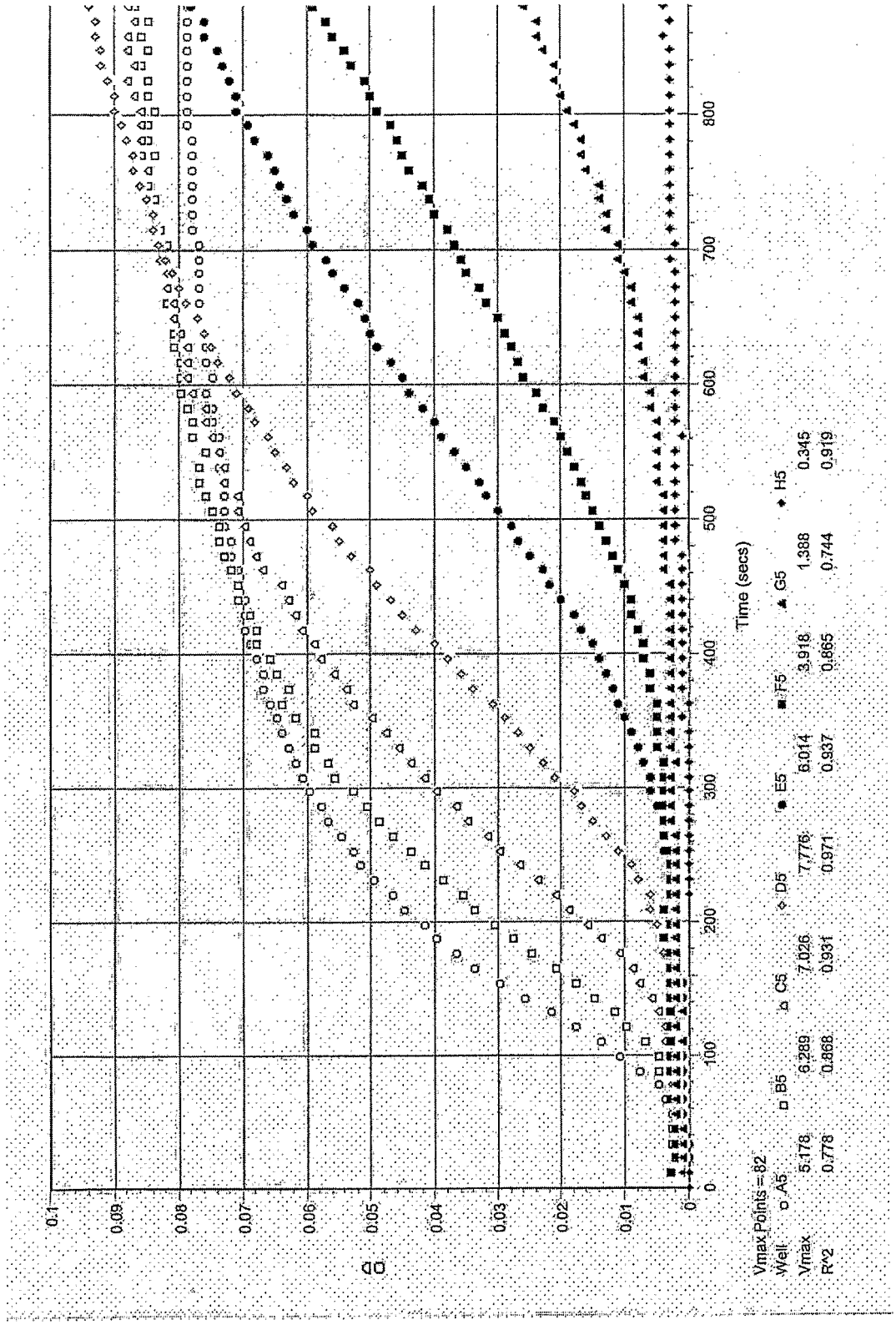

Time (secs)
Vmax Points = 82
Well A5 B5 C5 D5 E5 F5 G5 H5
Vmax 5.178 6.289 7.026 7.776 6.014 3.918 1.388 0.345
R^2 0.778 0.868 0.931 0.971 0.937 0.865 0.744 0.919

Figure 2j

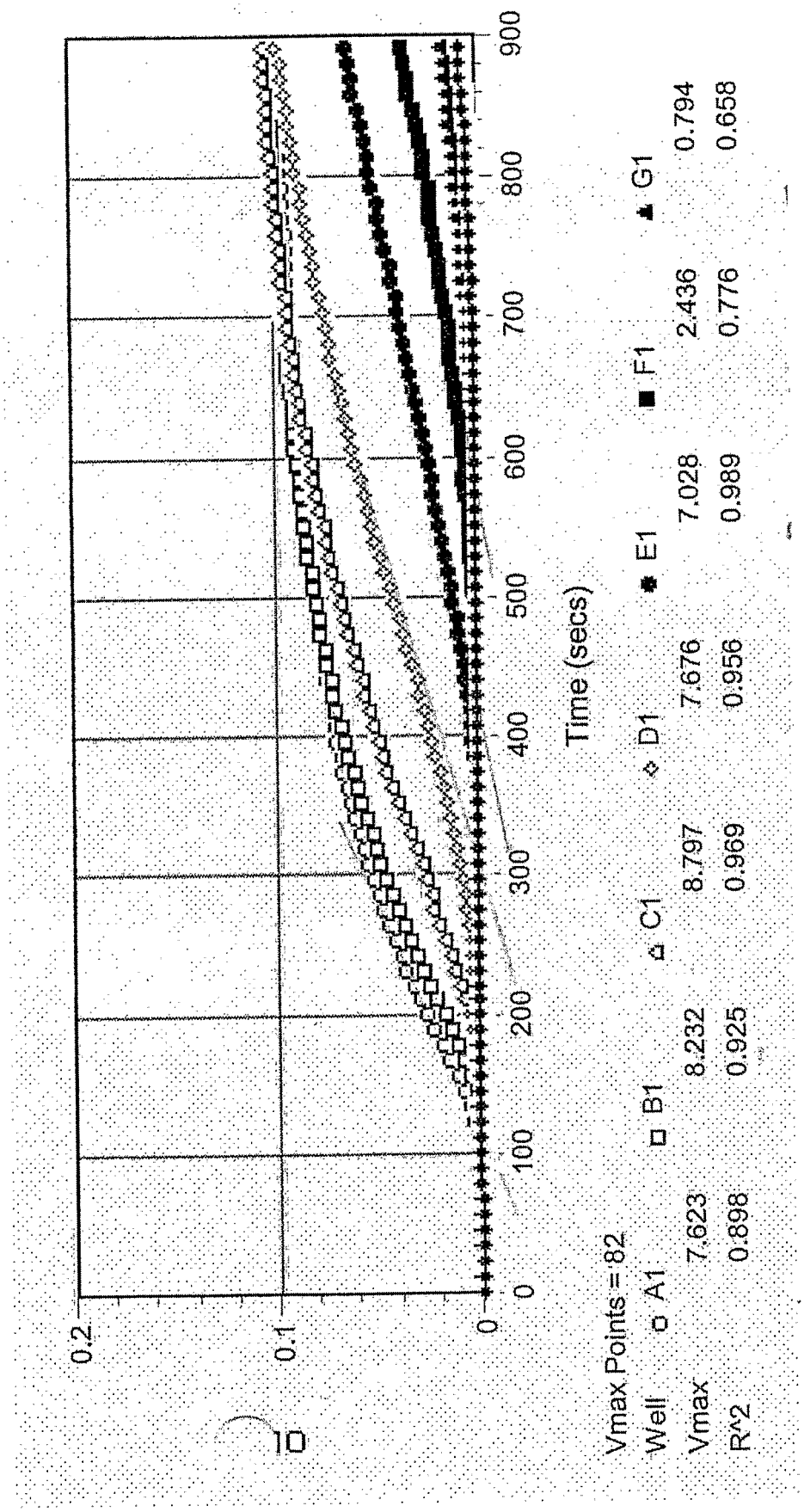

0.2
0.1
0
0
100
200
300
400
500
600
700
800
900
Time (secs)
Vmax Points = 82
Well
A1
B1
C1
D1
E1
F1
G1
Vmax
7.623
8.232
8.797
7.676
7.028
2.436
0.794
R^2
0.898
0.925
0.969
0.956
0.989
0.776
0.658

Figure 2k

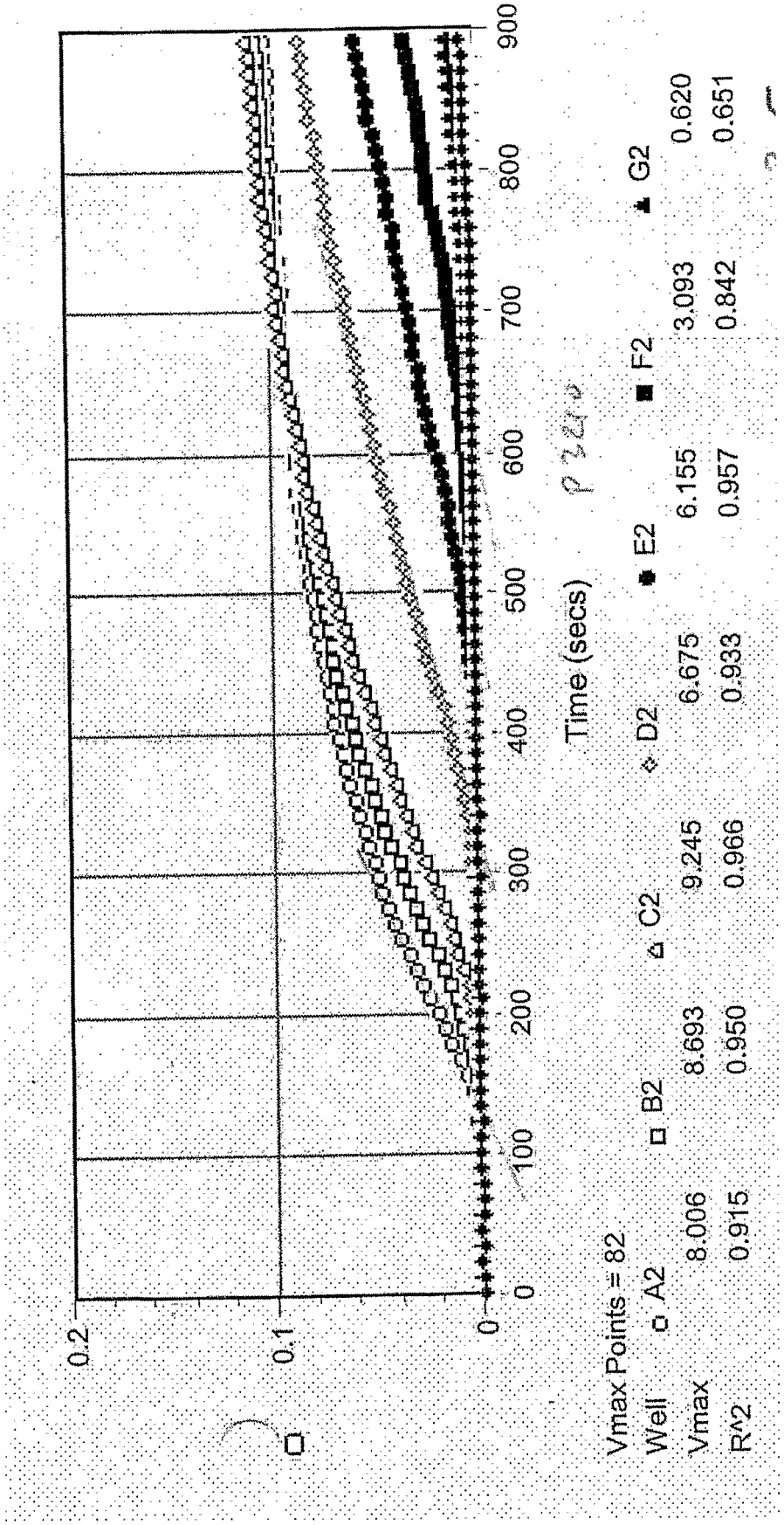

0.2
0.1
0
0
100
200
300
400
500
600
700
800
900
Time (secs)
Vmax Points = 82
Well
A2
B2
C2
D2
E2
F2
G2
Vmax
8.006
8.693
9.245
6.675
6.155
3.093
0.620
R^2
0.915
0.950
0.966
0.933
0.957
0.842
0.651

Figure 2l

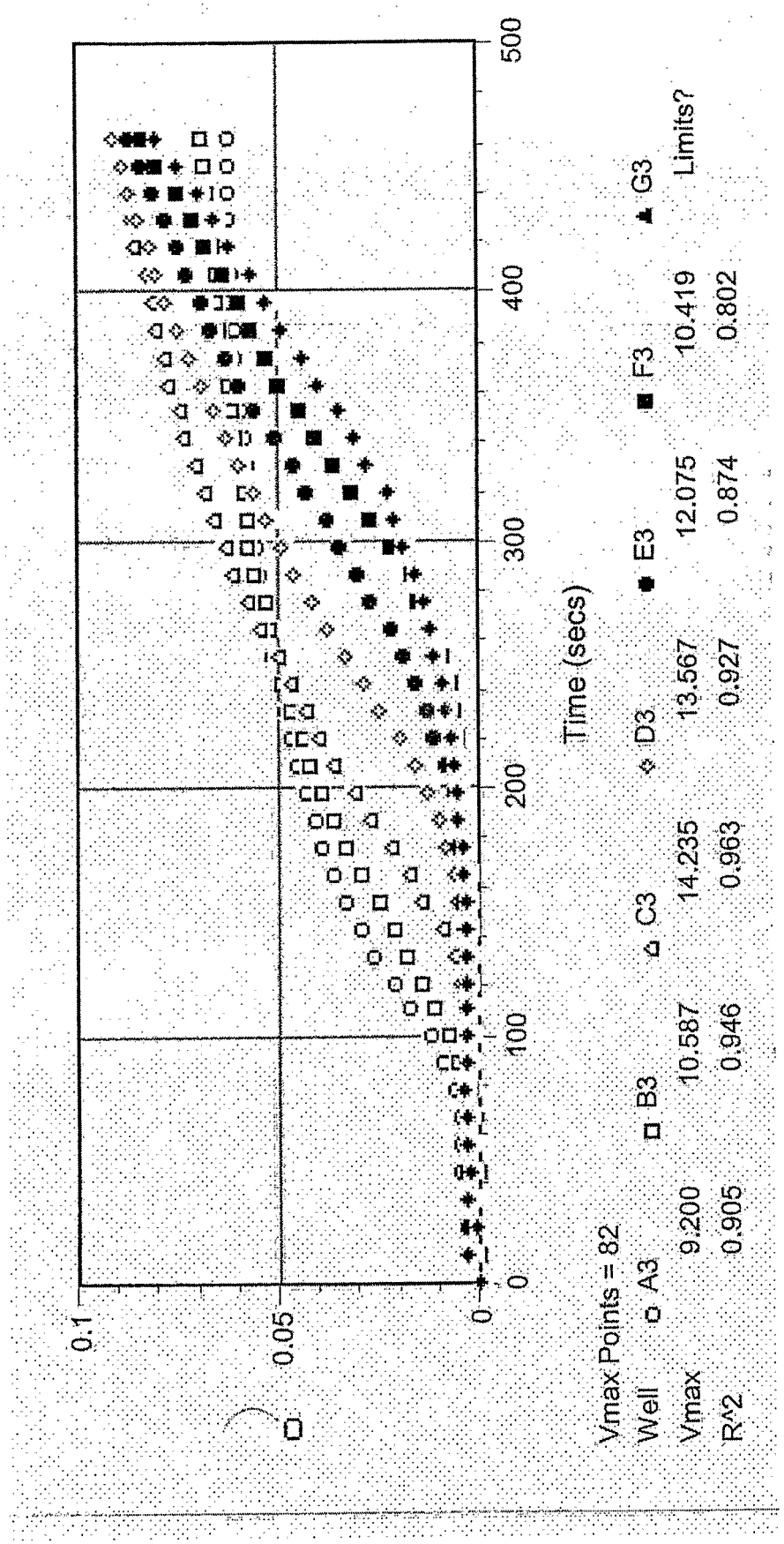
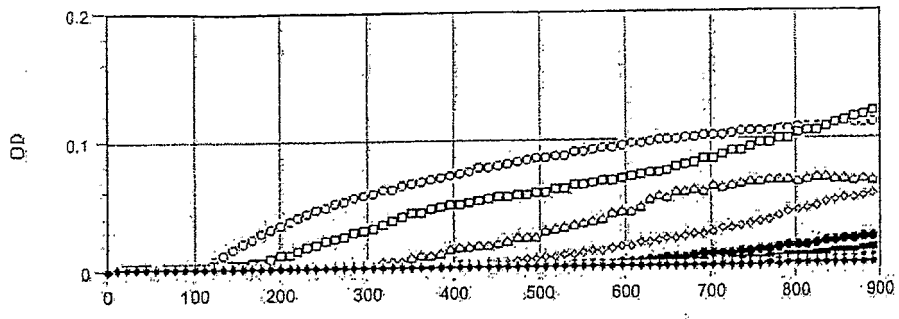
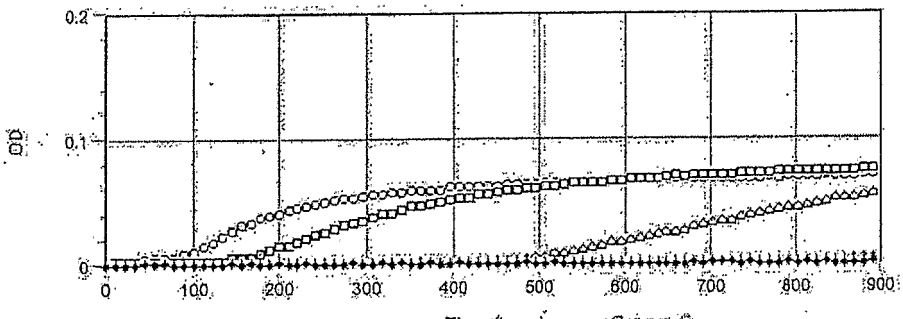

Time (secs)
0
100
200
300
400
500
0
0.05
0.1
Vmax Points = 82
Well A3 B3 C3 D3 E3 F3 G3 Limits?
Vmax 9.200 10.587 14.235 13.567 12.075 10.419
R^2 0.905 0.946 0.963 0.927 0.874 0.802

Figure 2m



Vmax Points = 82							
Well	A2	B2	C2	D2	E2	F2	G2
Vmax	8.290	8.363	5.940	3.517	1.144	0.567	0.203
R^2	0.932	0.987	0.921	0.794	0.654	0.567	0.440

Figure 2n



Vmax Points = 82							
Well	A4	B4	C4	D4	E4	F4	G4
Vmax	4.320	5.965	3.893	0.165	-0.545	0.006	-0.005
R^2	0.755	0.916	0.809	0.496	0.125	0.003	0.003

Figure 2o

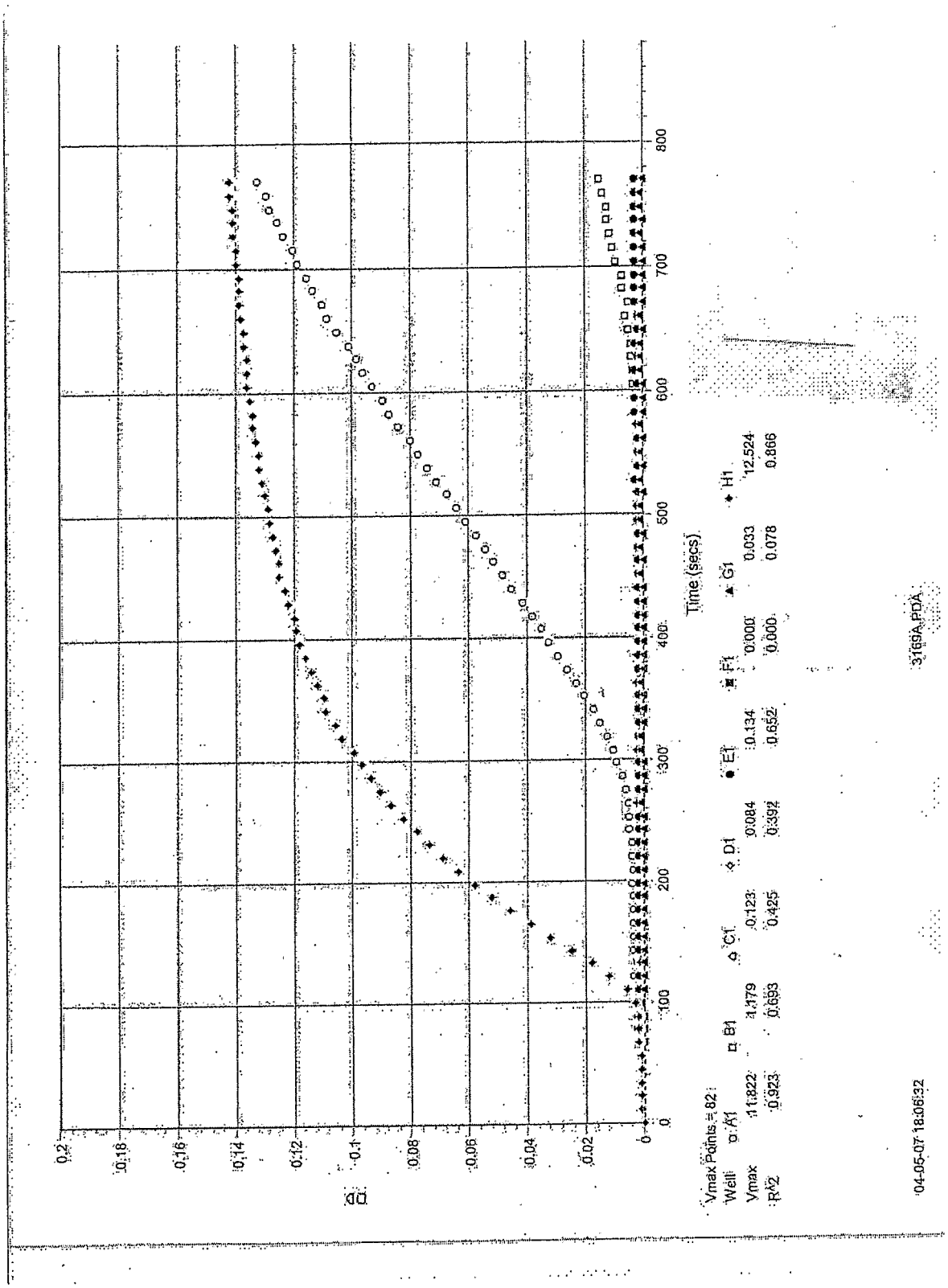

0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
100
200
300
400
500
600
700
800
Time (secs)
Vmax Points = 62
Well A1 B1 C1 D1 E1 F1 G1 H1
Vmax 1.1822 1.179 0.123 0.084 0.134 0.000 0.033 12.524
R^2 0.923 0.693 0.425 0.392 0.652 0.000 0.078 0.866
04-05-07 18:06:32
3169A.PDA

Figure 3a

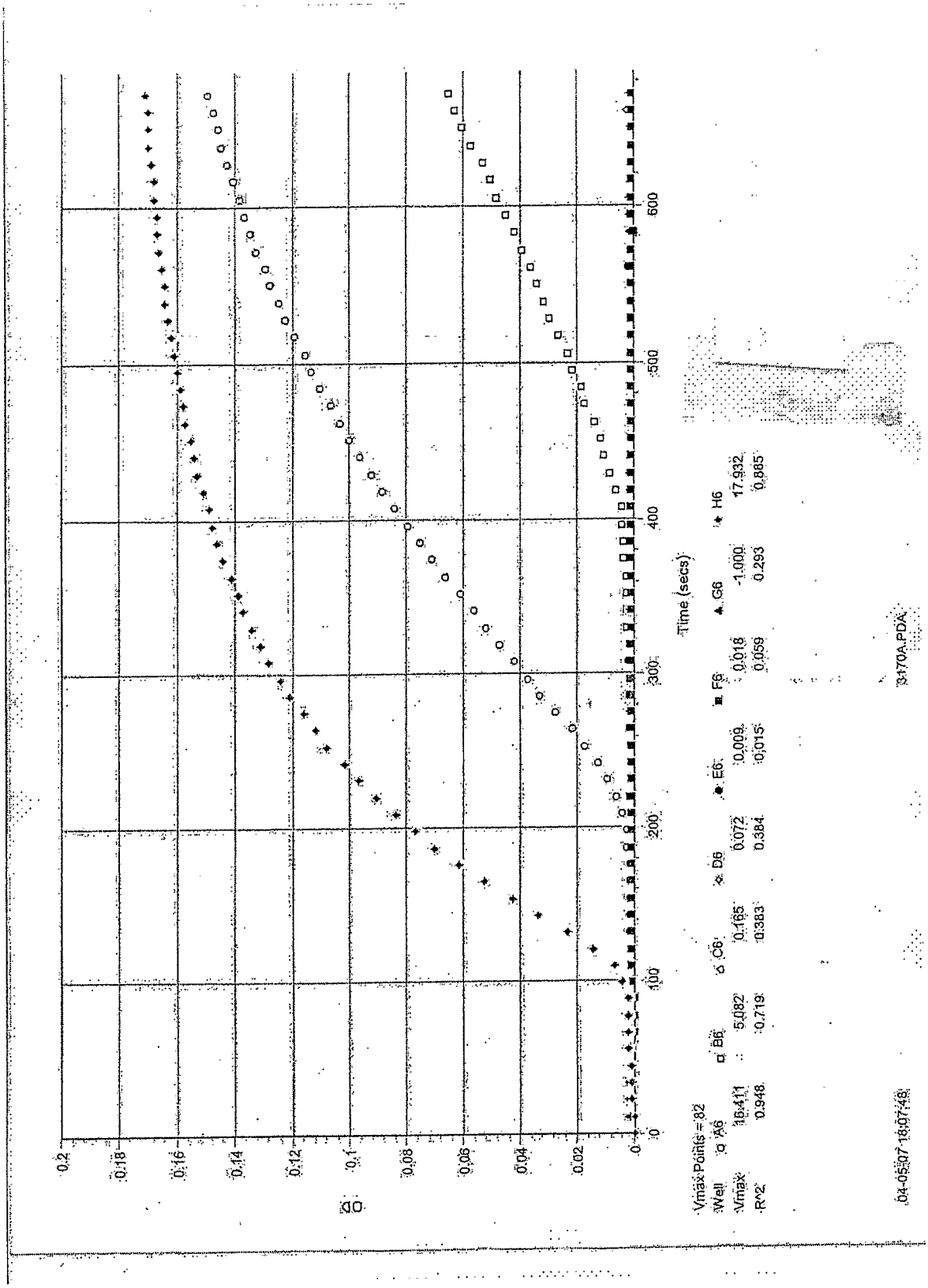

OD
Time (secs)
Vmax Points = 82
Well A6 B6 C6 D6 E6 F6 G6 H6
Vmax 16.411 5.082 0.166 0.072 0.009 0.018 -1.000 17.932
R^2 0.948 0.719 0.383 0.384 0.015 0.059 0.293 0.885
04-05-07 18:07:48
3-170A.PDA

Figure 3b

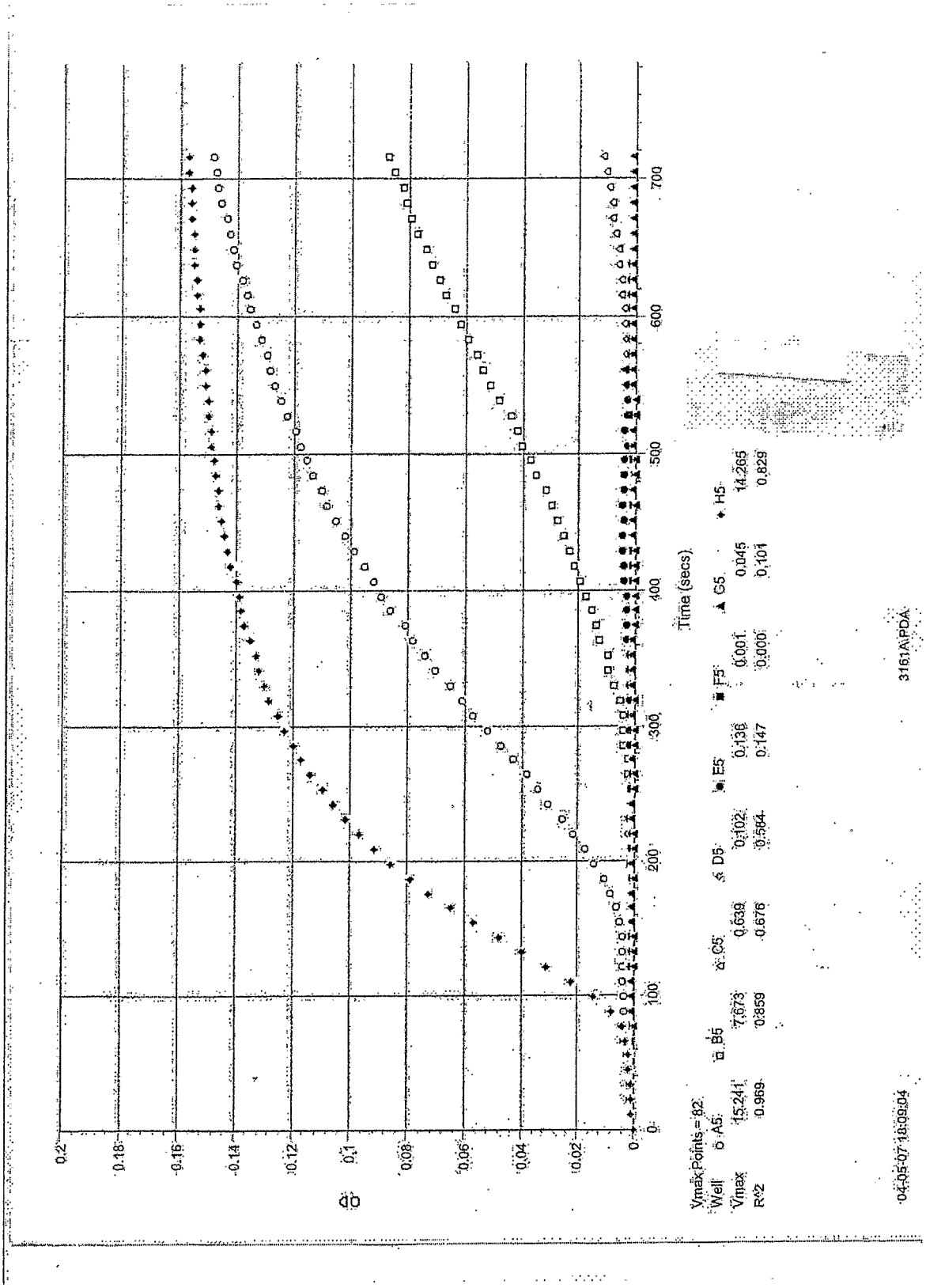

OD
Time (secs)
Ymax Points = 82
Well A5 B5 C5 D5 E5 F5 G5 H5
Vmax 15.241 7.673 0.639 0.102 0.138 0.001 0.045 14.265
R^2 0.969 0.859 0.676 0.564 0.147 0.000 0.101 0.829
04-05-07 18:09:04
3161A:PDA

Figure 3c

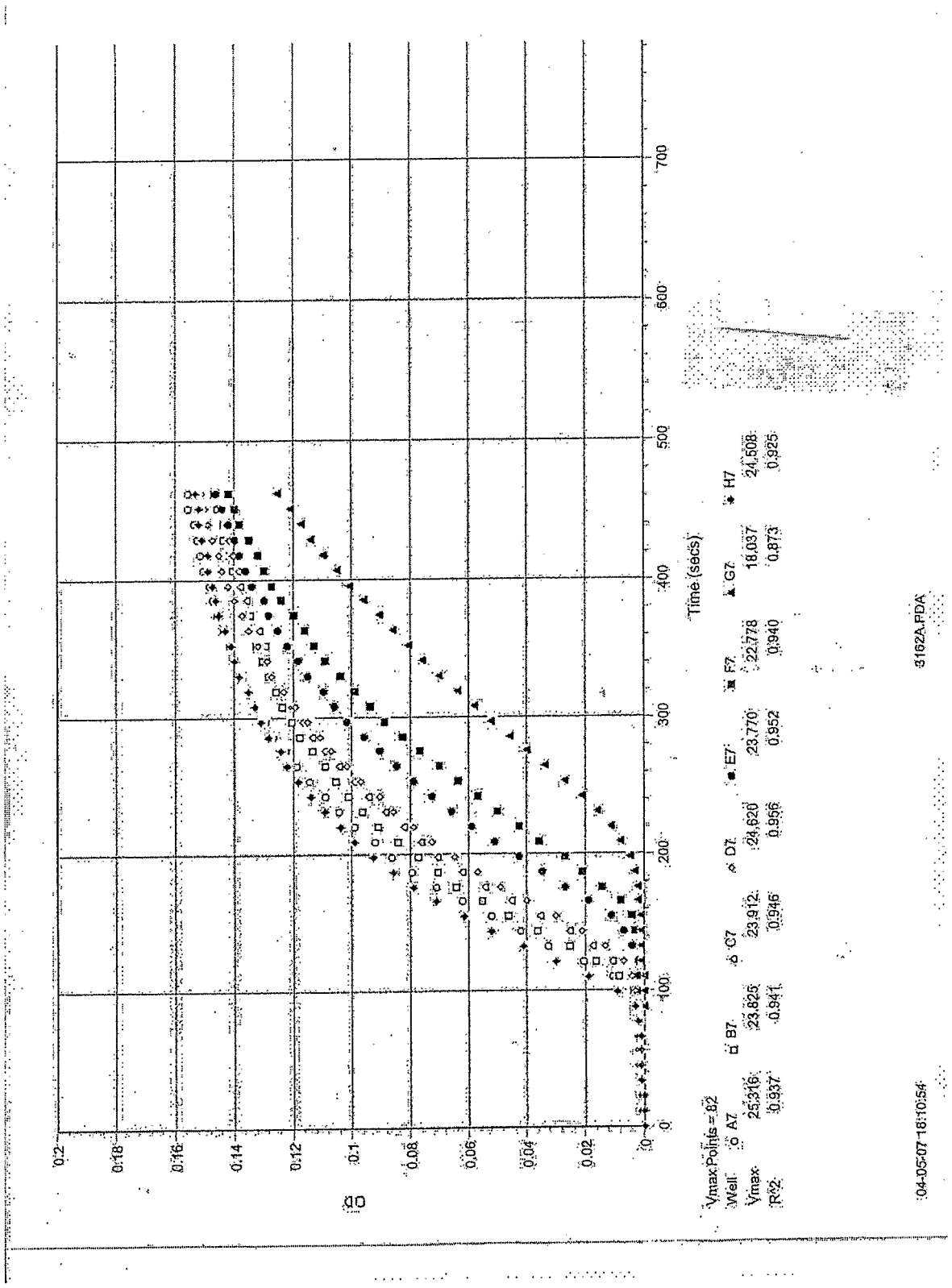

OD
Time (secs)
Vmax Points = 82
Well A7 B7 C7 D7 E7 F7 G7 H7
Vmax 25.316 23.825 23.912 24.620 23.770 22.778 18.037 24.508
R^2 0.937 0.941 0.946 0.956 0.952 0.940 0.873 0.925
04-05-07 18:10:54
3162A.PDA

Figure 3d

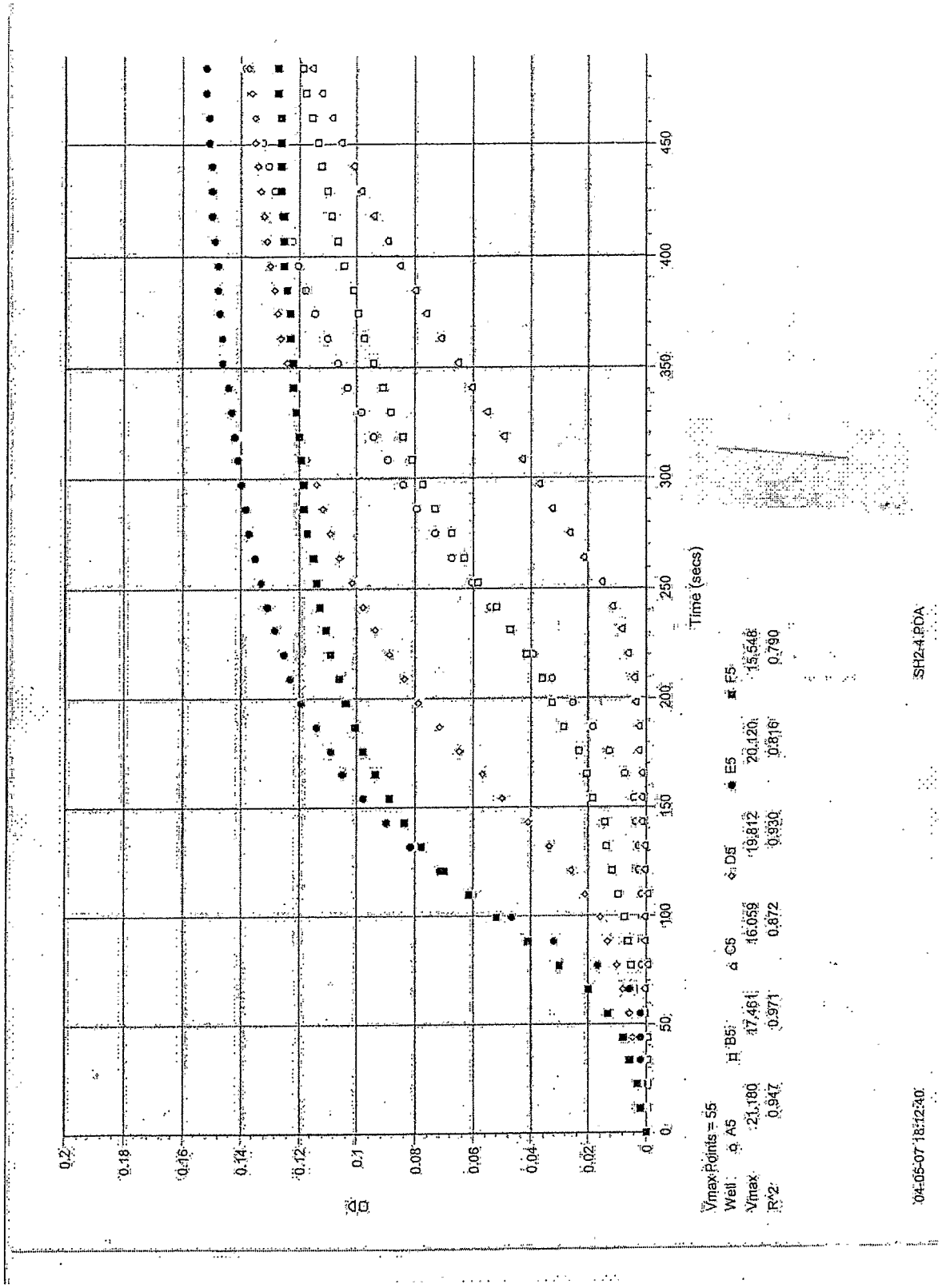

dO
Time (secs)
Vmax Points = 55
Well A5 B5 C5 D5 E5 F5
Vmax 21.180 17.461 16.059 19.812 20.120 15.548
R^2 0.947 0.971 0.872 0.930 0.816 0.790
04-05-07 18:12:40
SH24.PDA

Figure 4a

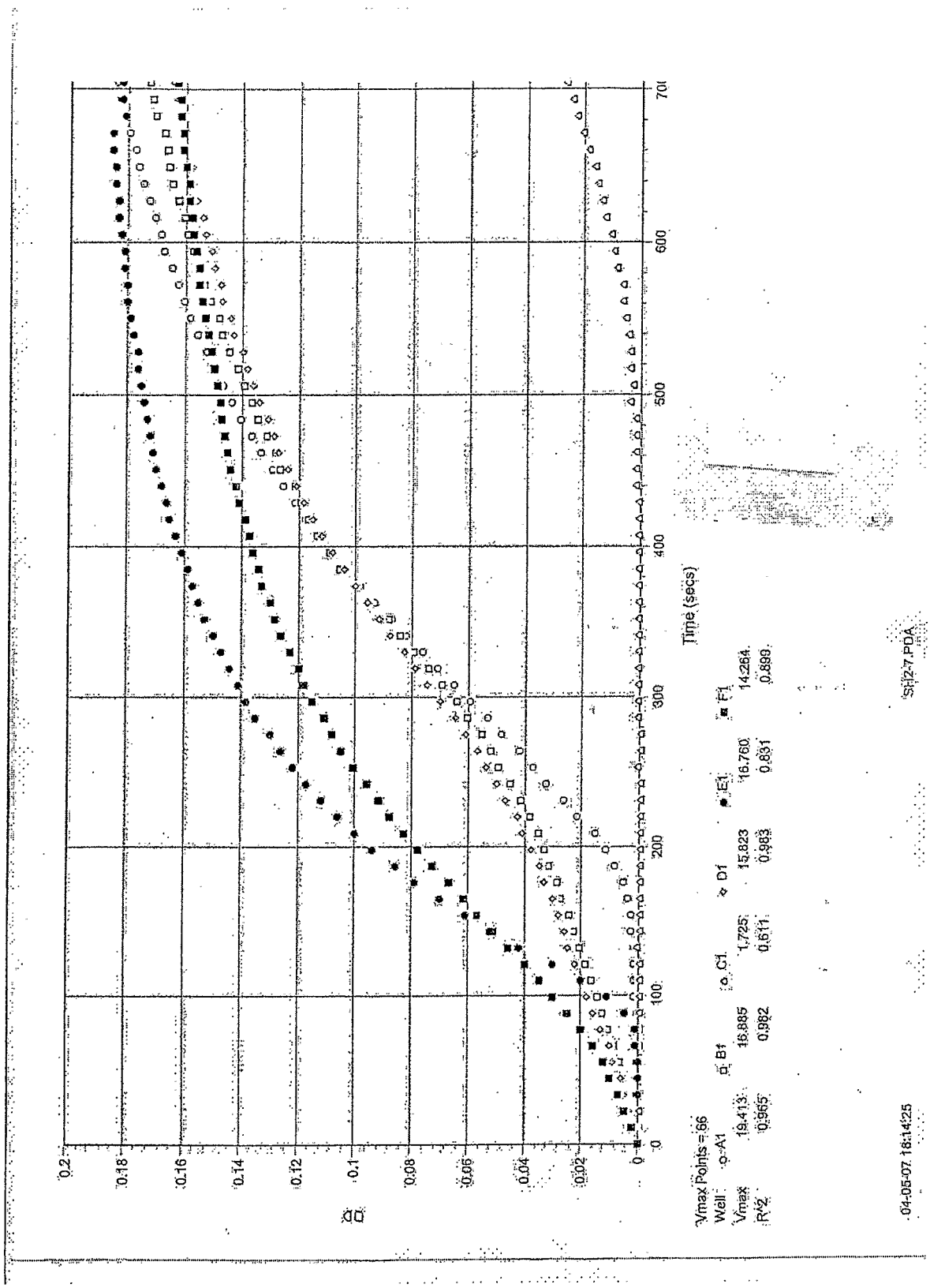

ΔD
Time (secs)
Vmax Points = 66
Well A1 B1 C1 D1 E1 F1
Vmax 19.413 16.885 1.725 15.823 16.760 14.264
R^2 0.965 0.982 0.611 0.983 0.831 0.899
04-05-07 18:14:25
SH2-7.PDA

Figure 4b

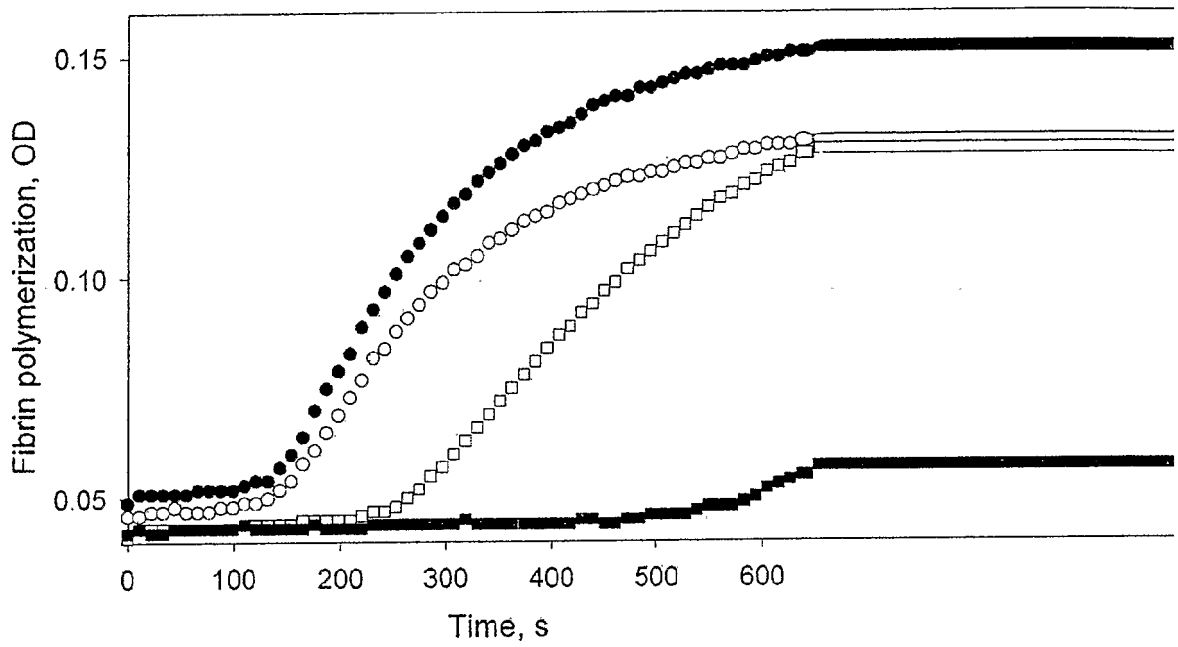

0.15
0.10
0.05
Fibrin polymerization, OD
0
100
200
300
400
500
600
Time, s

Figure 5

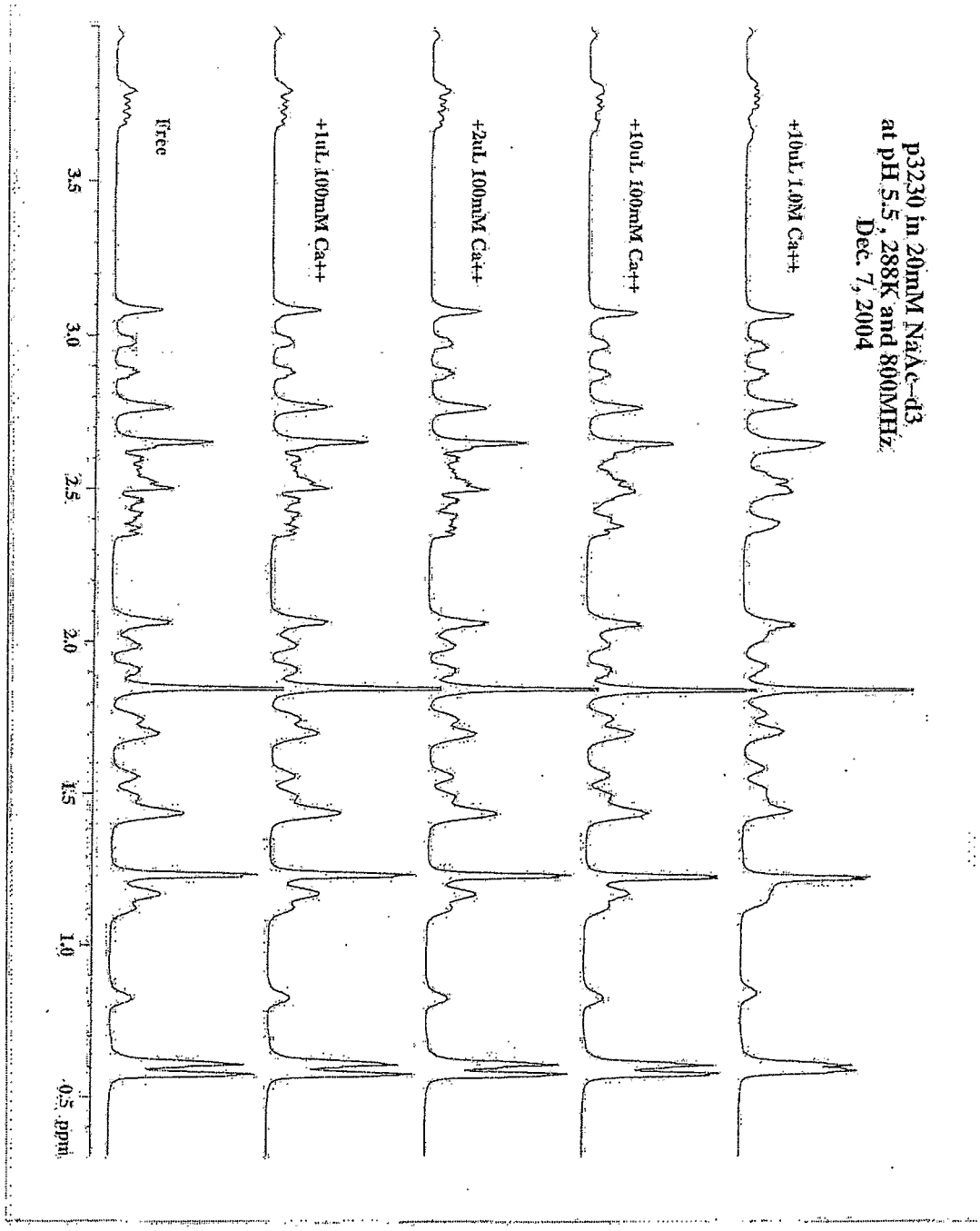

p3230 in 20mM NaAc-d3
at pH 5.5 , 288K and 800MHz
Dec. 7, 2004
+10uL 1.0M Ca++
+10uL 100mM Ca++
+2uL 100mM Ca++
+1uL 100mM Ca++
Free
3.5
3.0
2.5
2.0
1.5
1.0
0.5 ppm

Figure 6a

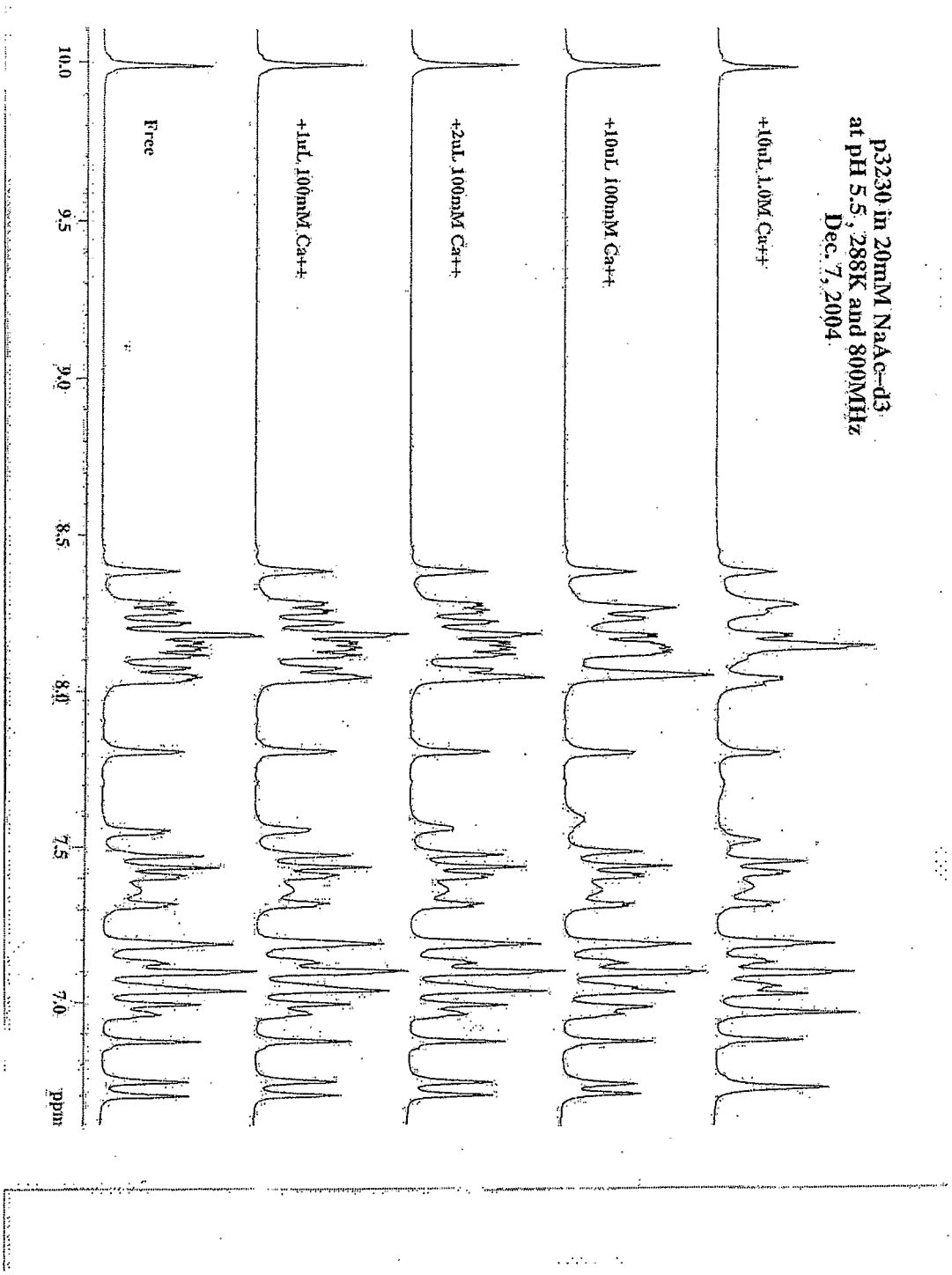

p3230 in 20mM NaAc-d3
at pH 5.5, 288K and 800MHz
Dec. 7, 2004
+10uL 1.0M Ca++
+10uL 100mM Ca++
+2uL 100mM Ca++
+1uL 100mM Ca++
Free
10.0
9.5
9.0
8.5
8.0
7.5
7.0
ppm

Figure 6b

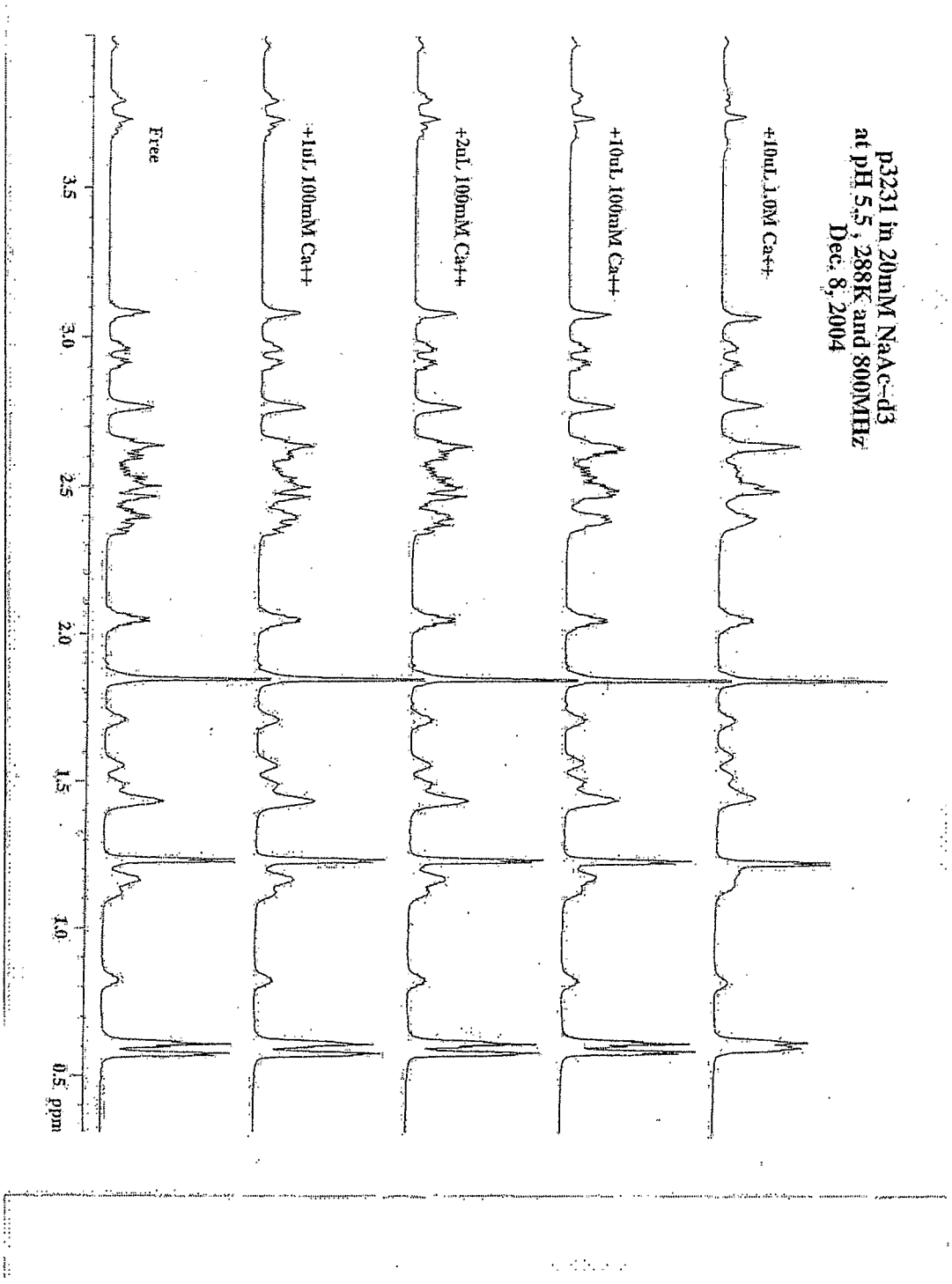

p3231 in 20mM NaAc-d3
at pH 5.5, 288K and 800MHz
Dec. 8, 2004
+10uL 1.0M Ca++
+10uL 100mM Ca++
+2uL 100mM Ca++
+1uL 100mM Ca++
Free
3.5
3.0
2.5
2.0
1.5
1.0
0.5 ppm

Figure 6c

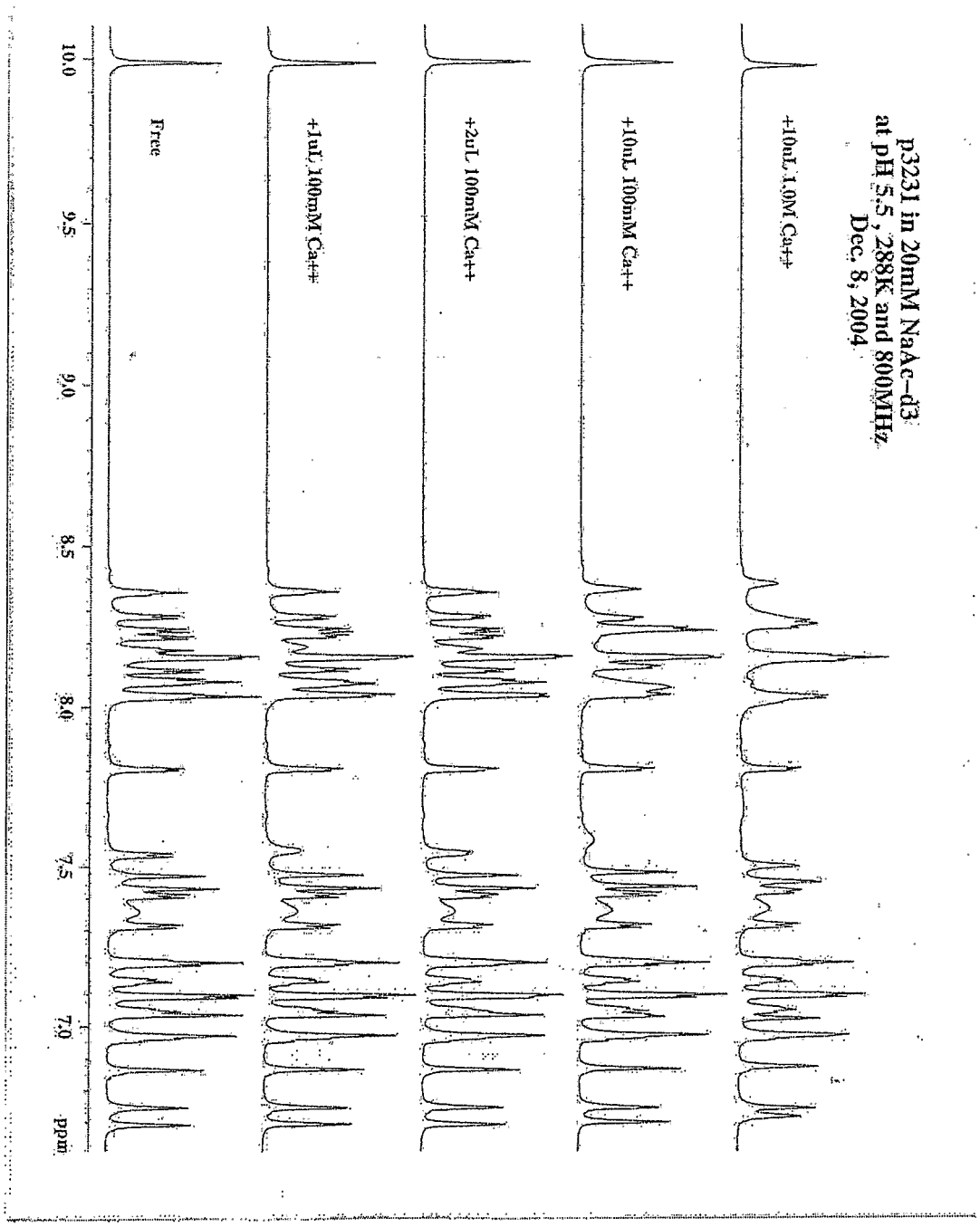

p3231 in 20mM NaAc-d3
at pH 5.5, 288K and 800MHz
Dec. 8, 2004
+10uL 1.0M Ca++
+10uL 100mM Ca++
+2uL 100mM Ca++
+1uL 100mM Ca++
Free
10.0
9.5
9.0
8.5
8.0
7.5
7.0
ppm

Figure 6d

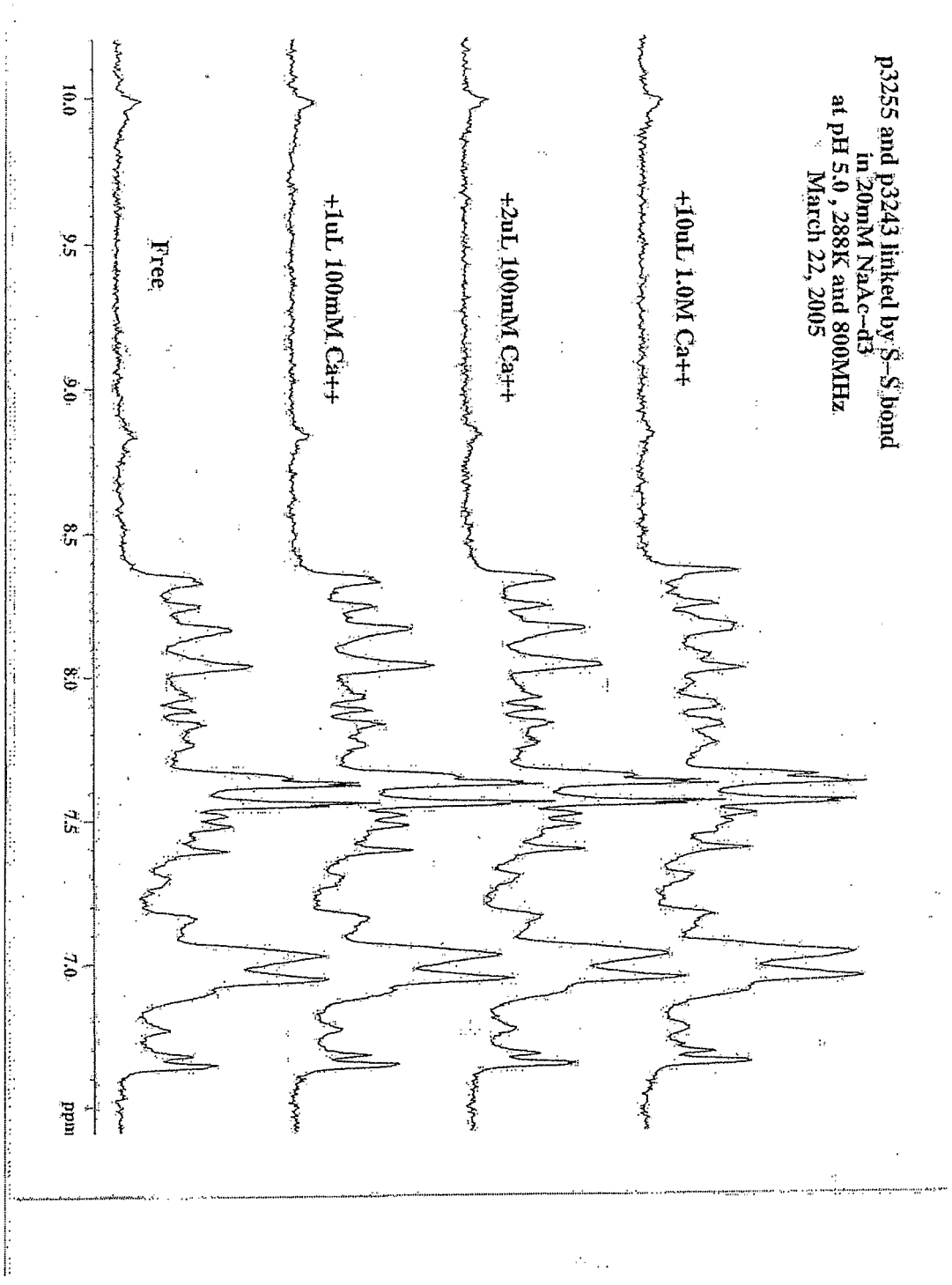

p3255 and p3243 linked by S–S bond
in 20mM NaAc–d3
at pH 5.0 , 288K and 800MHz
March 22, 2005
+10uL 1.0M Ca++
+2uL 100mM Ca++
+1uL 100mM Ca++
Free
10.0
9.5
9.0
8.5
8.0
7.5
7.0
ppm

Figure 6e

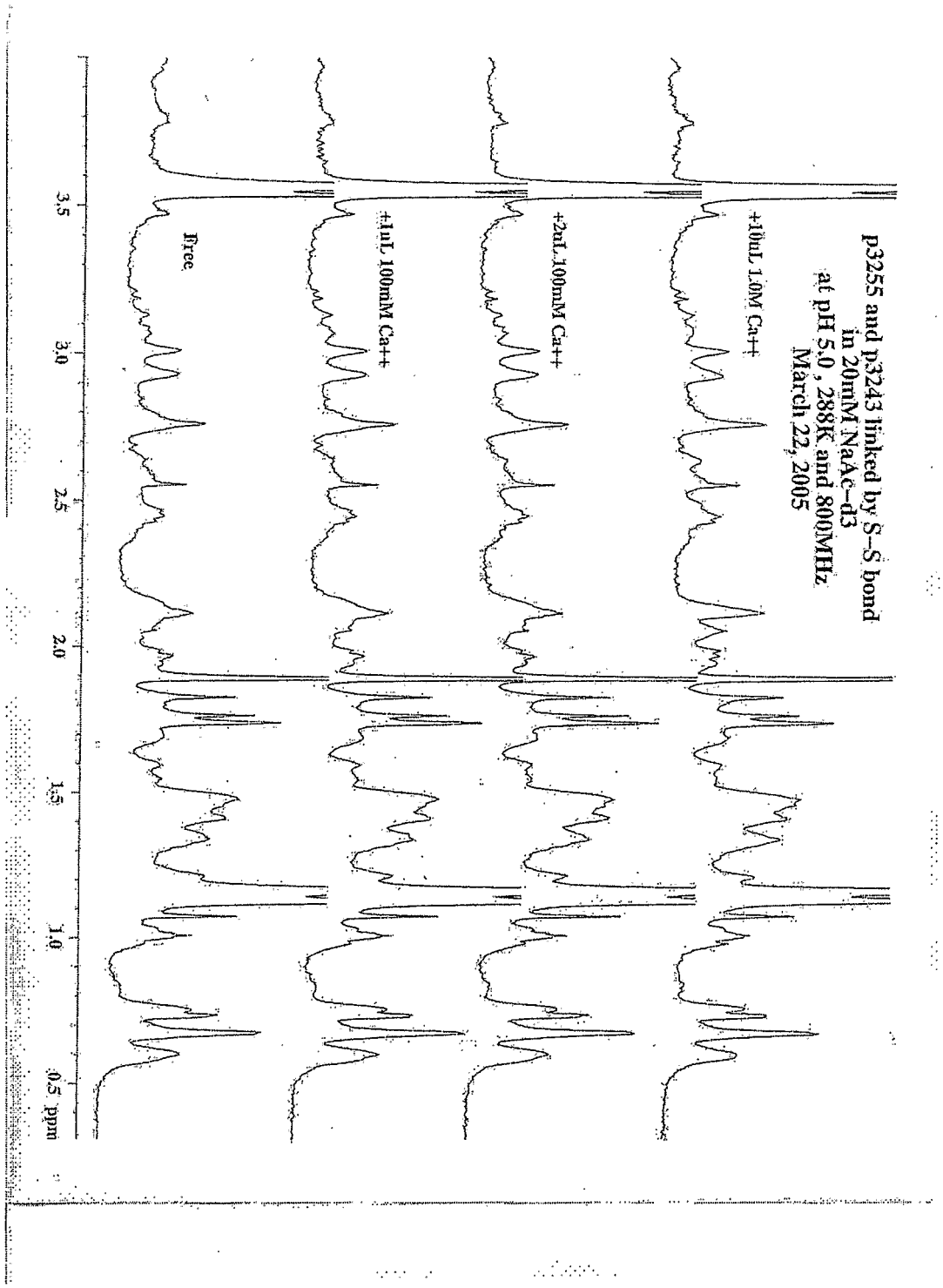

p3255 and p3243 linked by S–S bond
in 20mM NaAc–d3
at pH 5.0 , 288K and 800MHz
March 22, 2005
+10uL 1.0M Ca++
+2uL 100mM Ca++
+1uL 100mM Ca++
free
3.5
3.0
2.5
2.0
1.5
1.0
0.5 ppm

Figure 6f

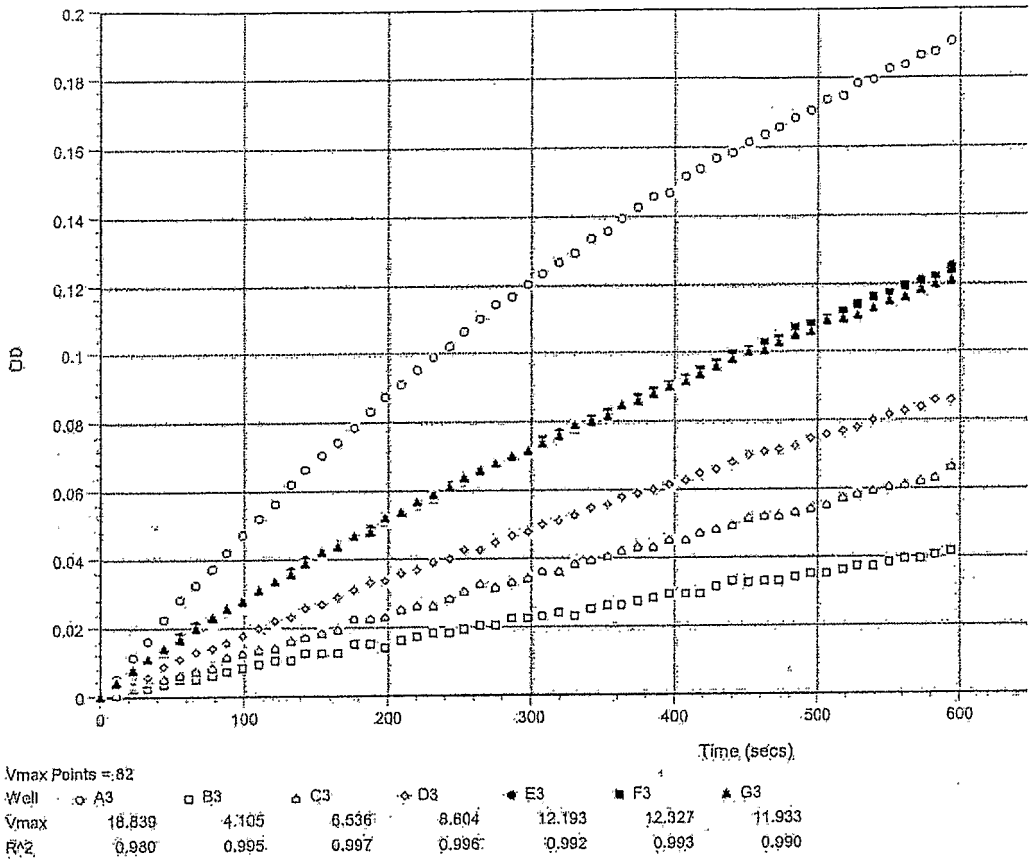

0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD
0
100
200
300
400
500
600
Time (secs)
Vmax Points =:82
Well A3 B3 C3 D3 E3 F3 G3
Vmax 18.839 4.105 6.536 8.604 12.193 12.327 11.933
R^2 0.980 0.995 0.997 0.996 0.992 0.993 0.990

Figure 7.

Figure 8a.

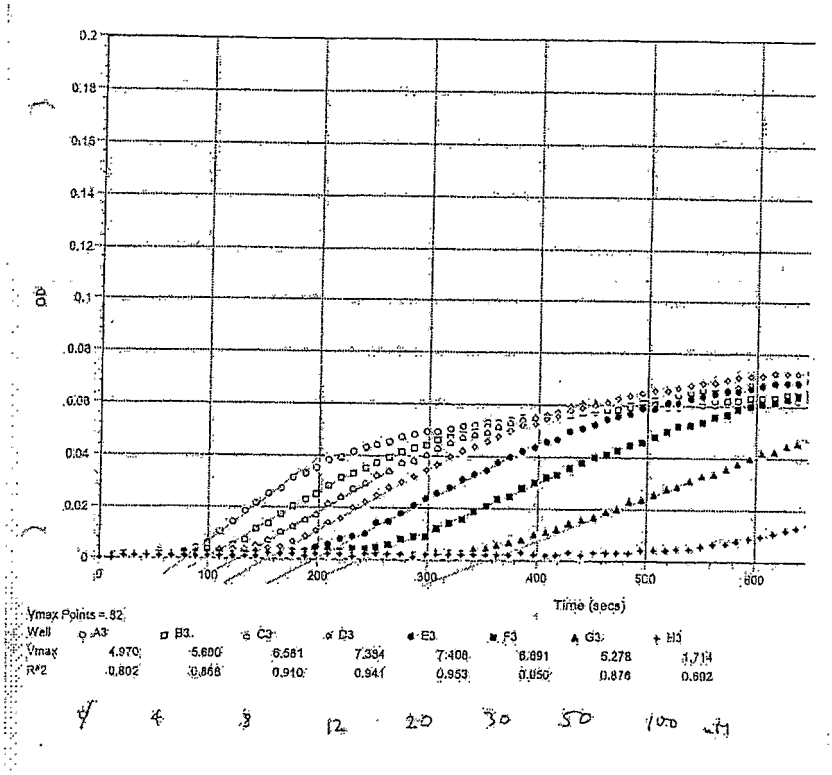
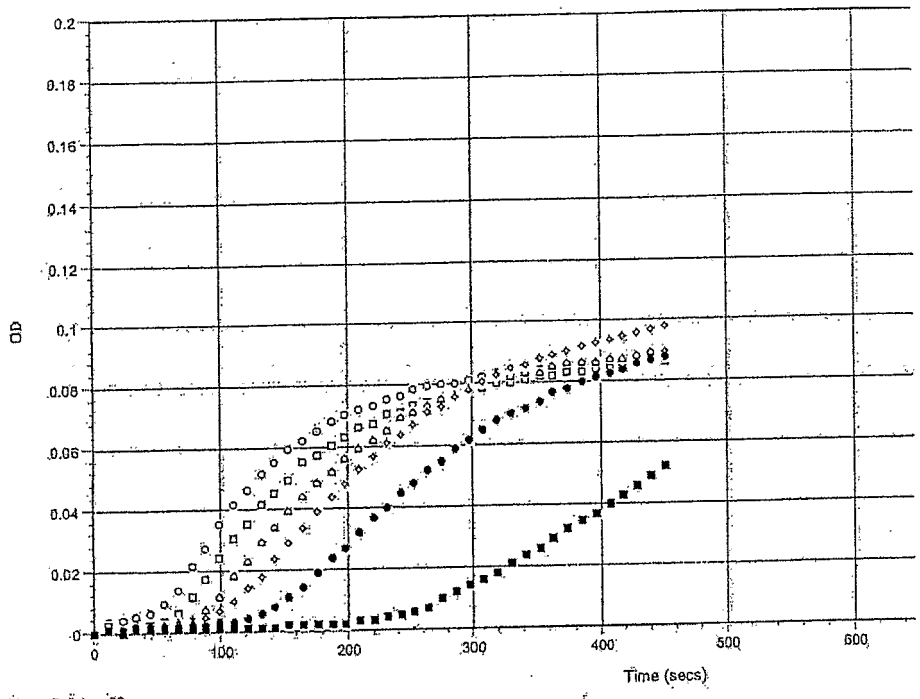

0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD
0
100
200
300
400
500
600
Time (secs)
Ymax Points = 82
Well A3 B3 C3 D3 E3 F3 G3 H3
Ymax 4.970 5.600 6.581 7.394 7.408 6.891 6.278 1.714
R^2 0.802 0.868 0.910 0.941 0.953 0.950 0.876 0.602

Figure 8b.



Vmax Points = 82.

Well	A7	B7	C7	D7	E7	G7
Vmax	12.255	12.967	14.395	15.873	14.315	6.388
R^2	0.847	0.888	0.929	0.962	0.958	0.790
K	2.15nM	4.3nM	8.6nM	17.2nM	43 nM	

p.327, fraction 50-52

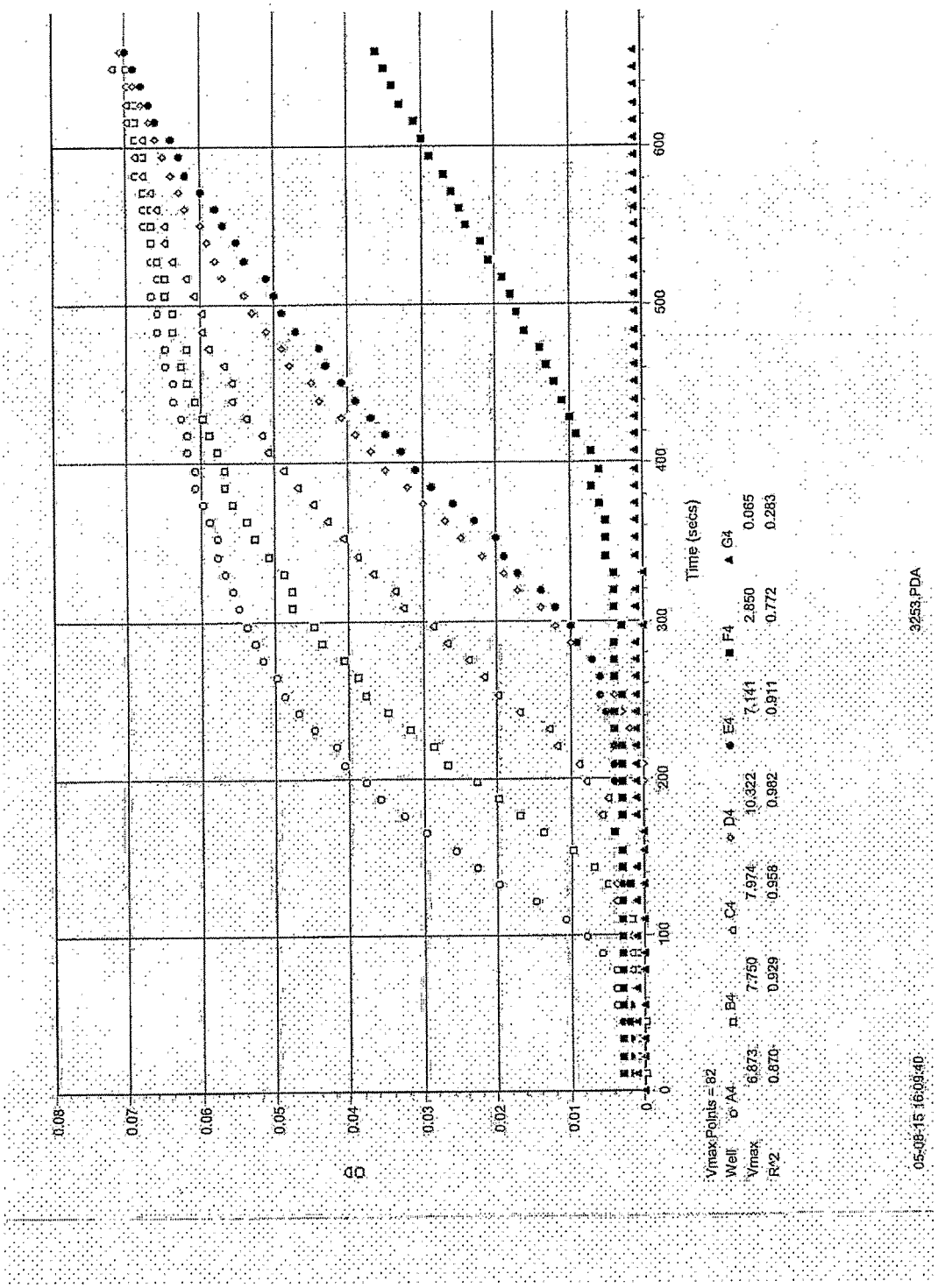

OD
Time (secs)
Vmax Points = 82
Well A4 B4 C4 D4 E4 F4 G4
Vmax 6.873 7.750 7.974 10.322 7.141 2.850 0.065
R^2 0.870 0.929 0.958 0.982 0.911 0.772 0.283
05-08-15 16:09:40
3253.PDA

Figure 8c.

CaM-DTI:

*WDPRPQRH*ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQN
PTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTGGVKLIPSWTTVI
LVKSMLRKRSFGNPFGGDSEEEIREAFRVFDKDGNGYISAAELRHVMTNL
GEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK*DFEEIPEEYLQ*

CaM-DTI2:

*IRFTDGEG*ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNP
TEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDNGGVKLIPSWTTVIL
VKSMLRKRSFGNPFGGDSEEEIREAFRVFDKDGNGYIRAAELRHVMTNLG
EKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK*DFEEIPEEYLQ*

Figure 9.

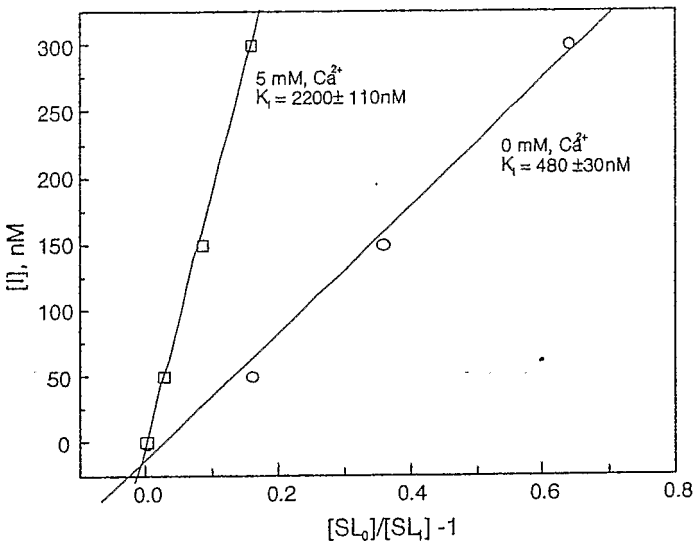

300
250
200
150
100
50
0
[I], nM
5 mM, Ca2+
Ki = 2200± 110nM
0 mM, Ca2+
Ki = 480 ±30nM
0.0
0.2
0.4
0.6
0.8
[SL0]/[SLi] -1

Figure 10A.

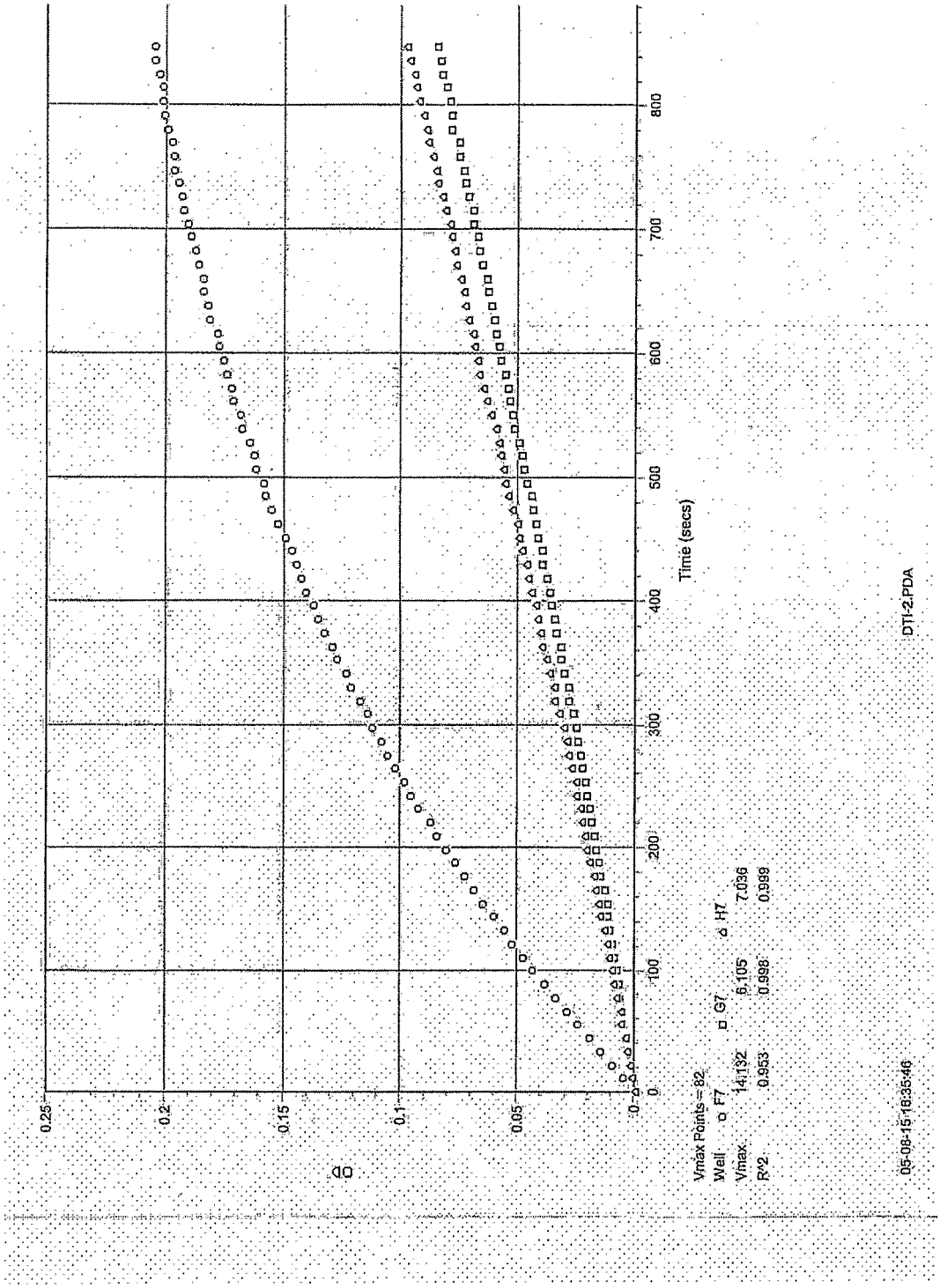

0.25
0.2
0.15
0.1
0.05
0
OD
0
100
200
300
400
500
600
700
800
Time (secs)
Vmax Points = 82
Well F7 G7 H7
Vmax 14.132 6.105 7.036
R^2 0.953 0.998 0.999
05-08-15 16:35:46
DTI-2.PDA

Figure 10B

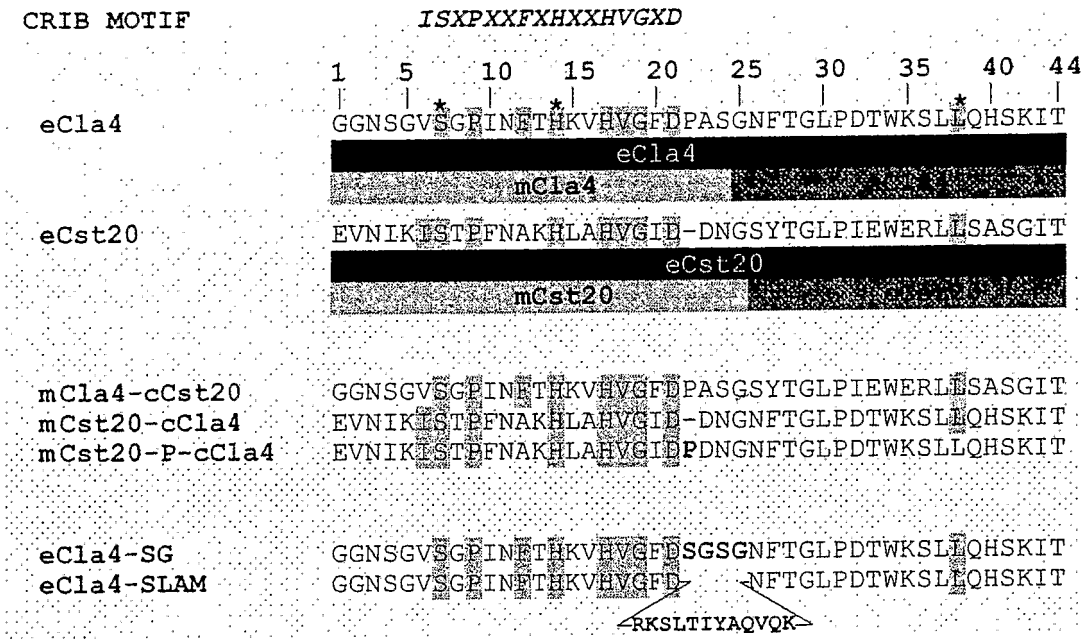

CRIB MOTIF
ISXPXXFXHXXHVGXD
1 5 10 15 20 25 30 35 40 44
eCla4
GGNSGVSGPINETHKVHVGFDPASGNFTGLPDTWKSLLQHSKIT
eCla4
mCla4
eCst20
EVNIKISTPFNAKHLAHVGIDDNGSYTGLPIEWERLLSASGIT
eCst20
mCst20
mCla4-cCst20 GGNSGVSGPINETHKVHVGFDPASGSYTGLPIEWERLLSASGIT
mCst20-cCla4 EVNIKISTPFNAKHLAHVGIDDNGNFTGLPDTWKSLLQHSKIT
mCst20-P-cCla4 EVNIKISTPFNAKHLAHVGIDPDNGNFTGLPDTWKSLLQHSKIT
eCla4-SG GGNSGVSGPINETHKVHVGFDSGSGNFTGLPDTWKSLLQHSKIT
eCla4-SLAM GGNSGVSGPINETHKVHVGFD NFTGLPDTWKSLLQHSKIT
RKSLTIYAQVQK

Figure 11

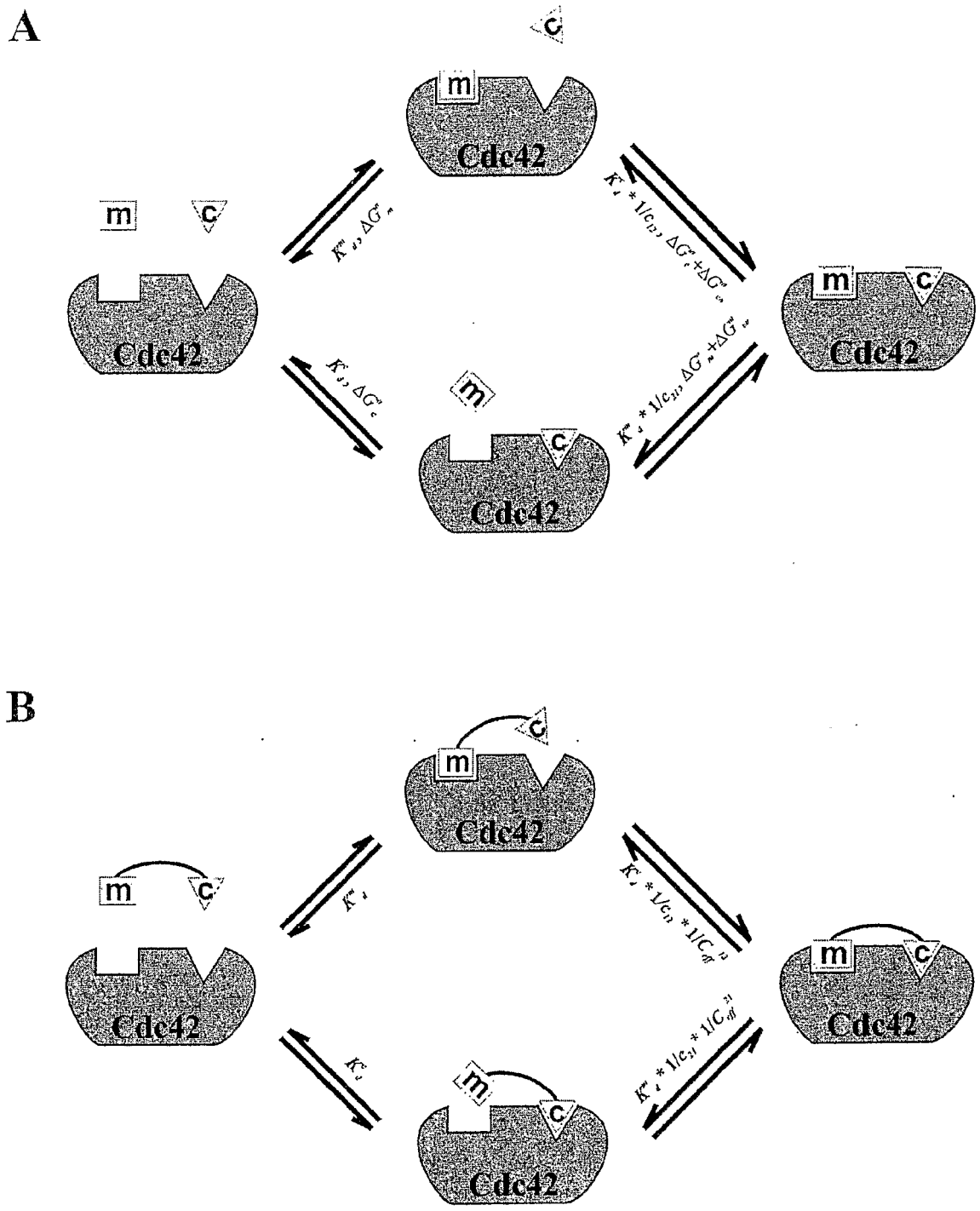


Figure 12.

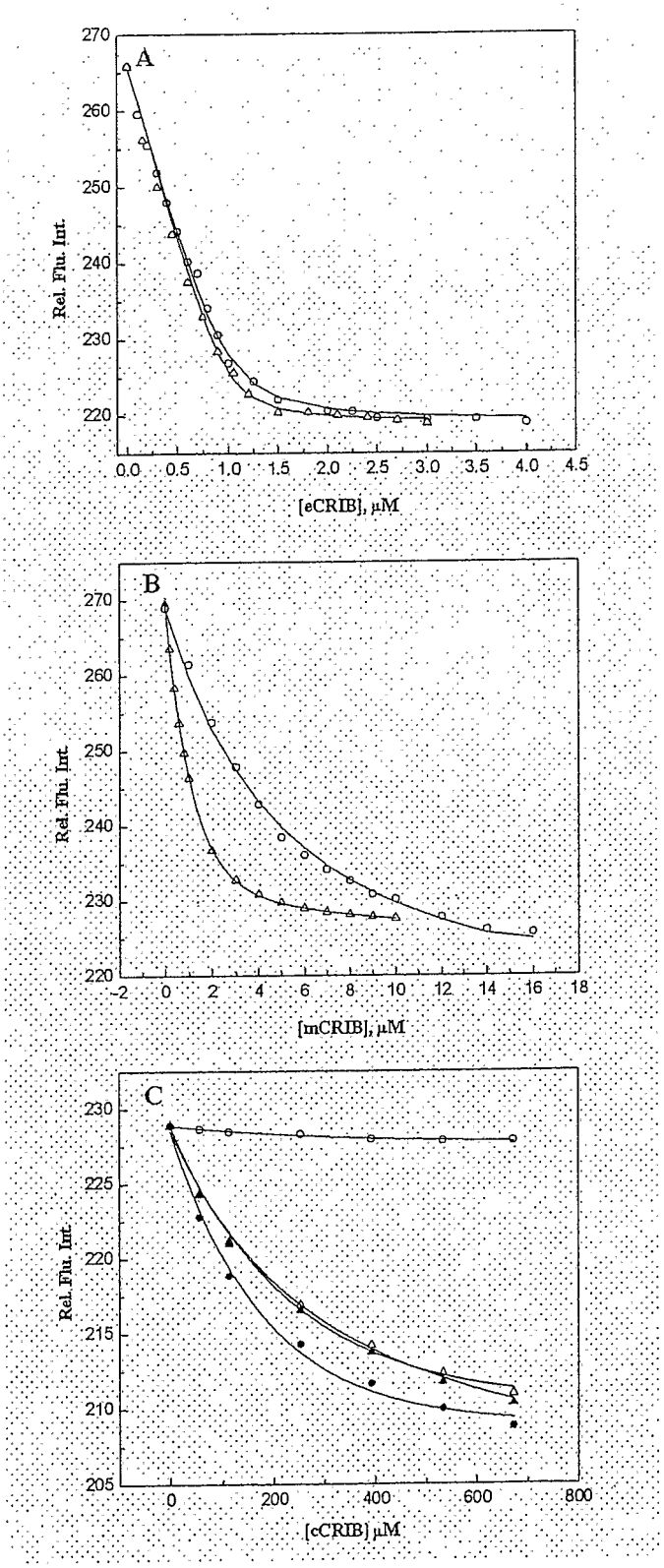

A
Rel. Flu. Int.
270
260
250
240
230
220
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
[eCRIB], μM
B
Rel. Flu. Int.
270
260
250
240
230
220
-2 0 2 4 6 8 10 12 14 16 18
[mCRIB], μM
C
Rel. Flu. Int.
230
225
220
215
210
205
0 200 400 600 800
[cCRIB] μM

Figure 13

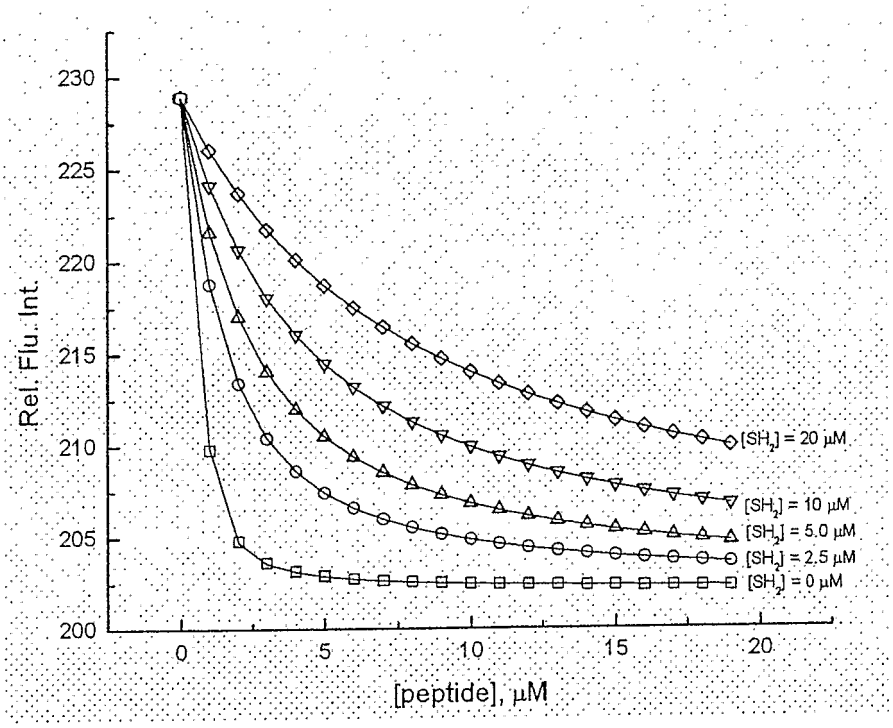


Figure 14A

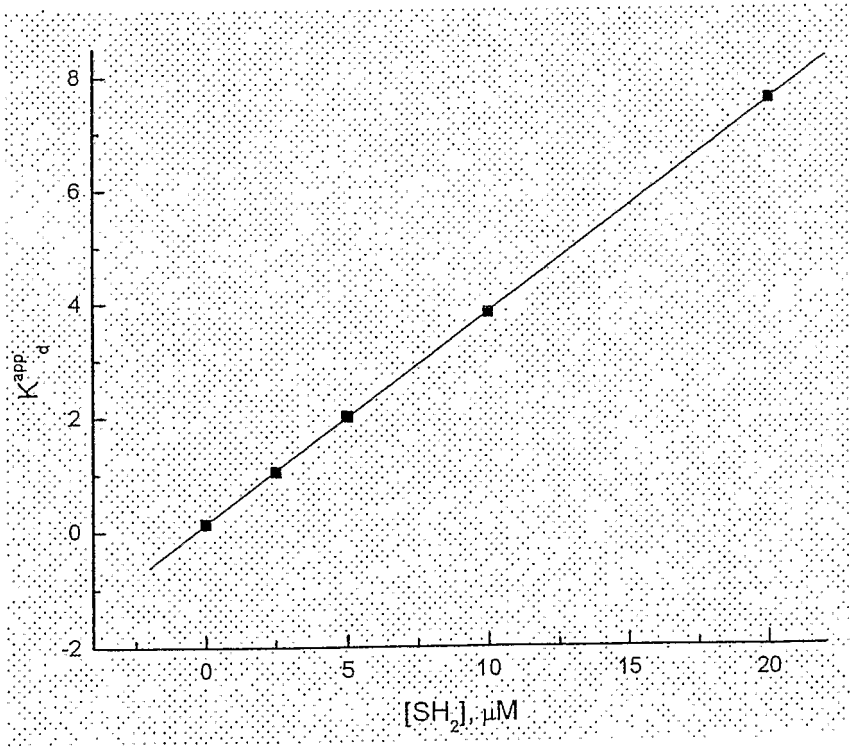
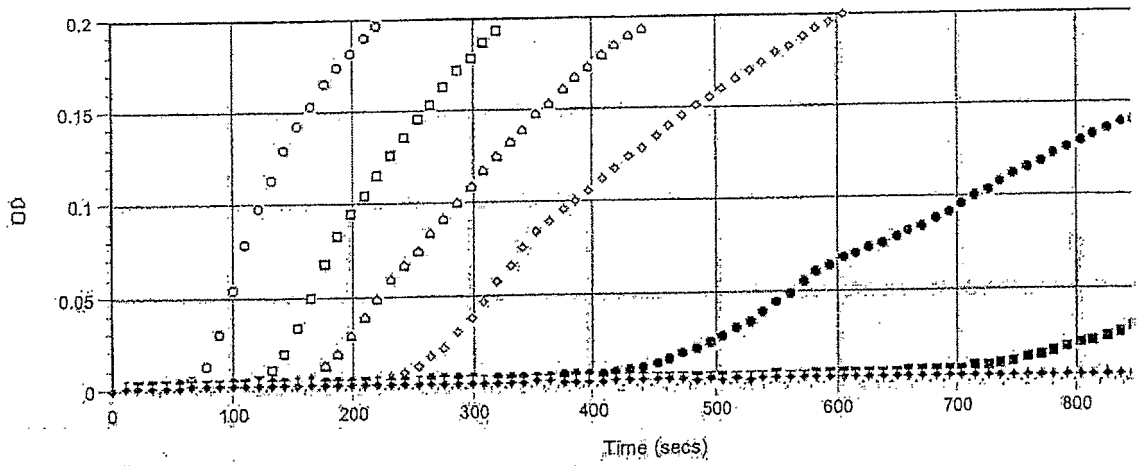


Figure 14B



Vmax Points = 82								
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

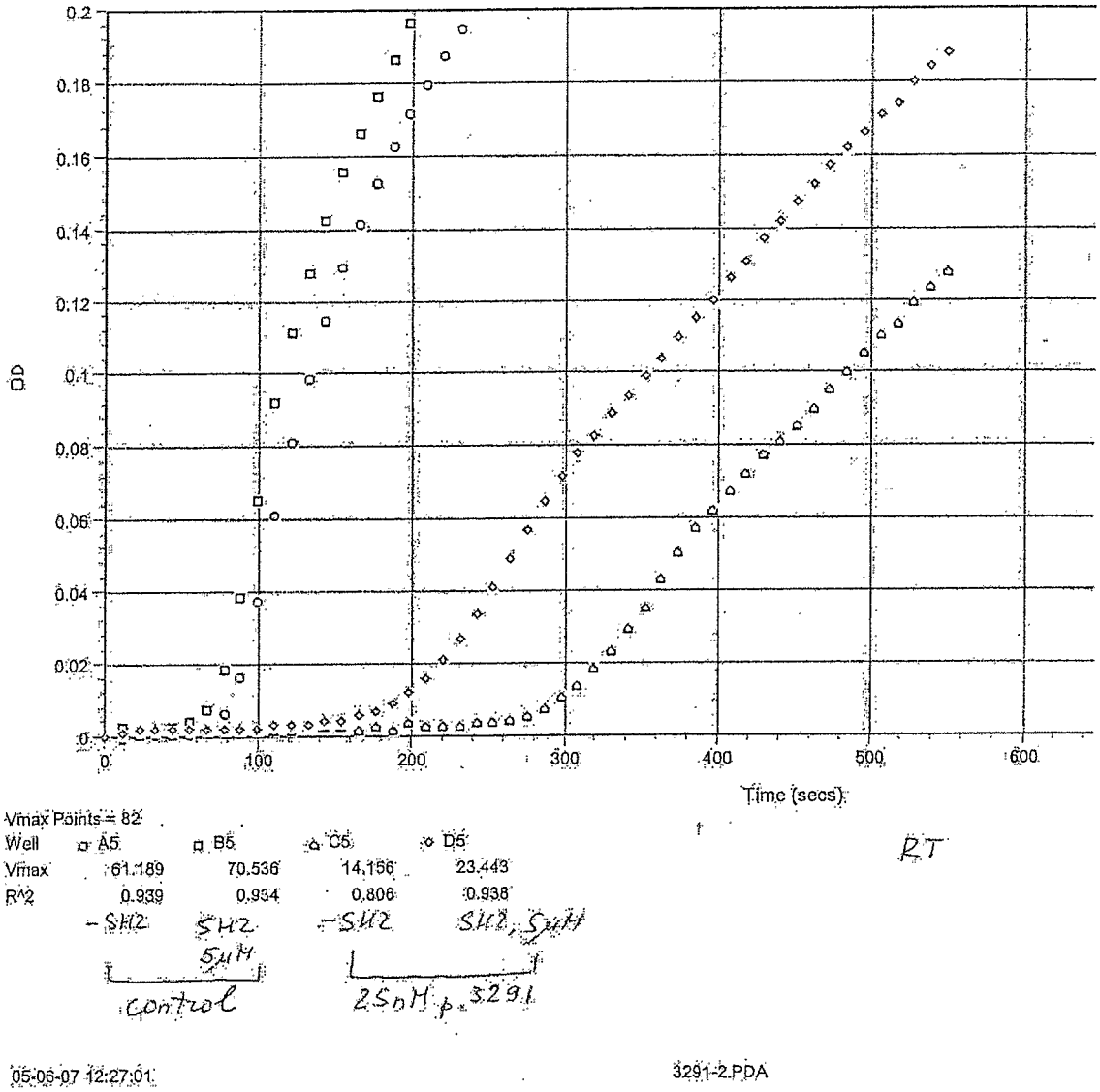

0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD
0
100
200
300
400
500
600
Time (secs)
Vmax Points = 82
Well A5 B5 C5 D5
Vmax 61.189 70.536 14.156 23.443
R^2 0.939 0.934 0.806 0.938
- SH2
SH2 5μM
control
- SH2
SH2, 5μM
250M p. 3291
RT
05-06-07 12:27:01
3291-2.PDA

Figure 14D

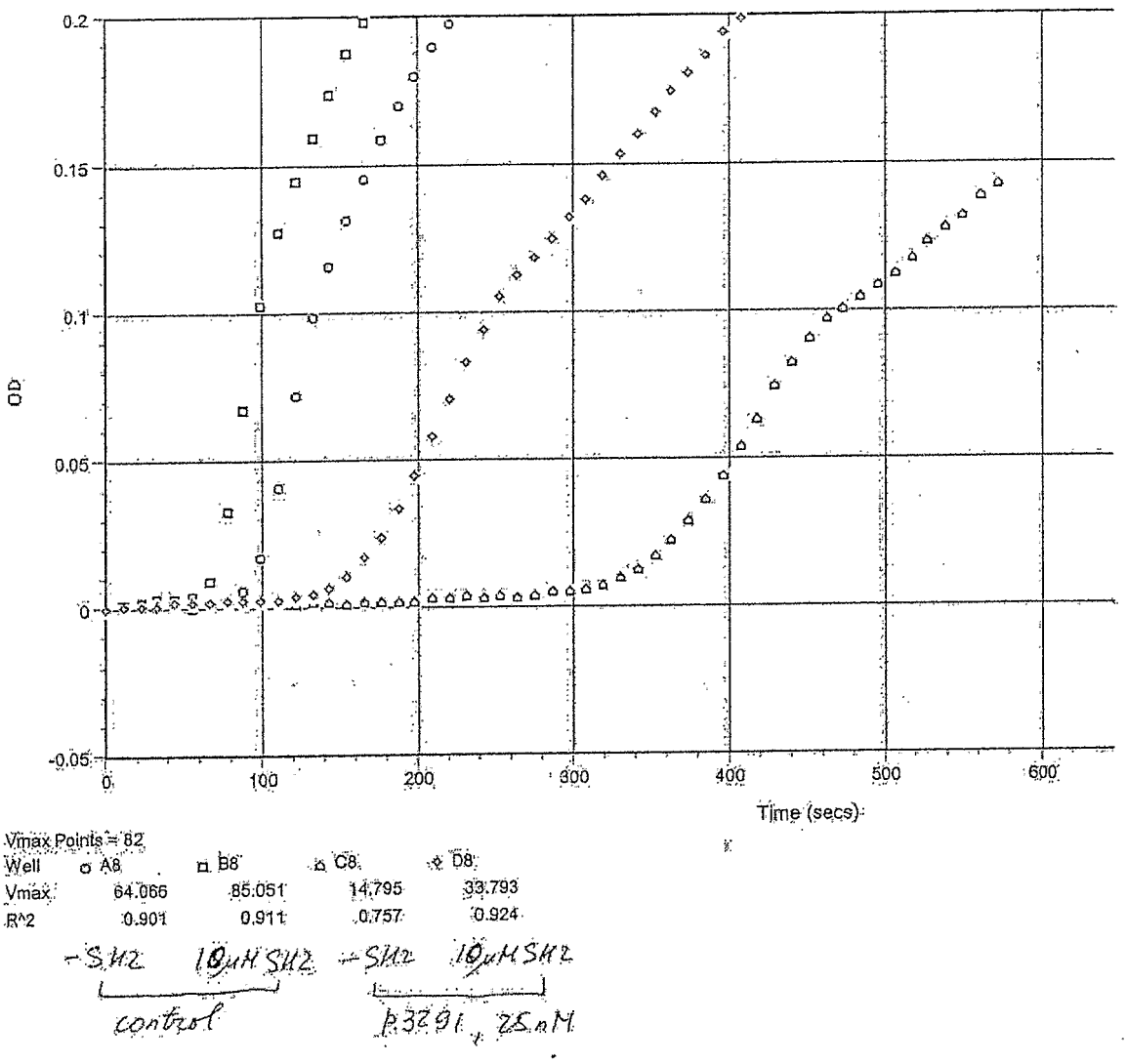

0.2
0.15
0.1
0.05
0
-0.05
OD
0
100
200
300
400
500
600
Time (secs)
Vmax Points = 82
Well A8 B8 C8 D8
Vmax 64.066 85.051 14.795 33.793
R^2 0.901 0.911 0.757 0.924
-SH2 10μM SH2 -SH2 10μM SH2
control
p3291, 25nM

Figure 14E

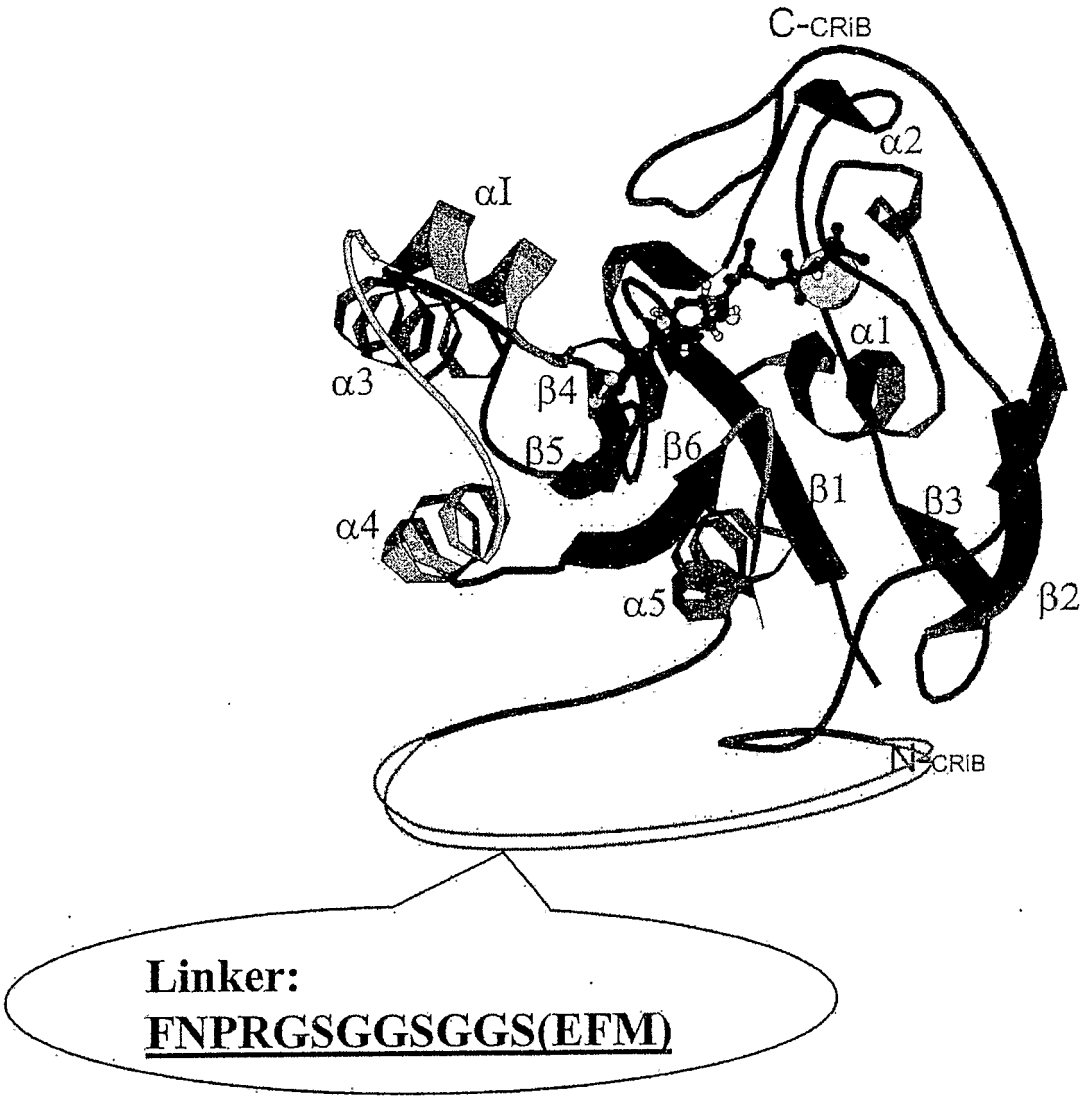

C-CRIB
α2
αI
α1
α3
β4
β5
β6
β1
β3
α4
α5
β2
N-CRIB
Linker:
FNPRGSGGSGGS(EFM)

Figure 15a

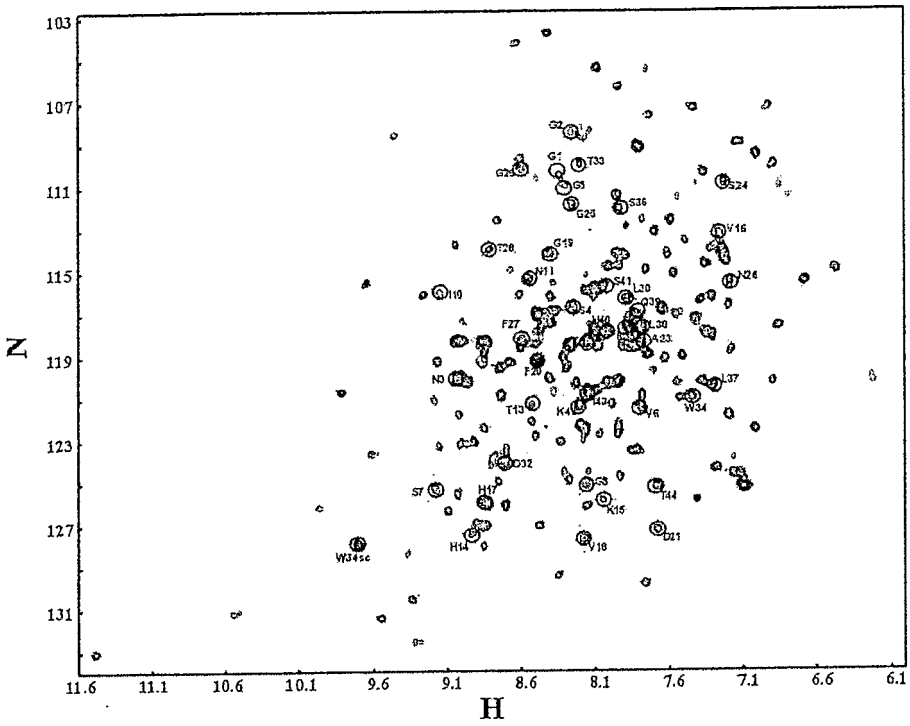


Figure 15b

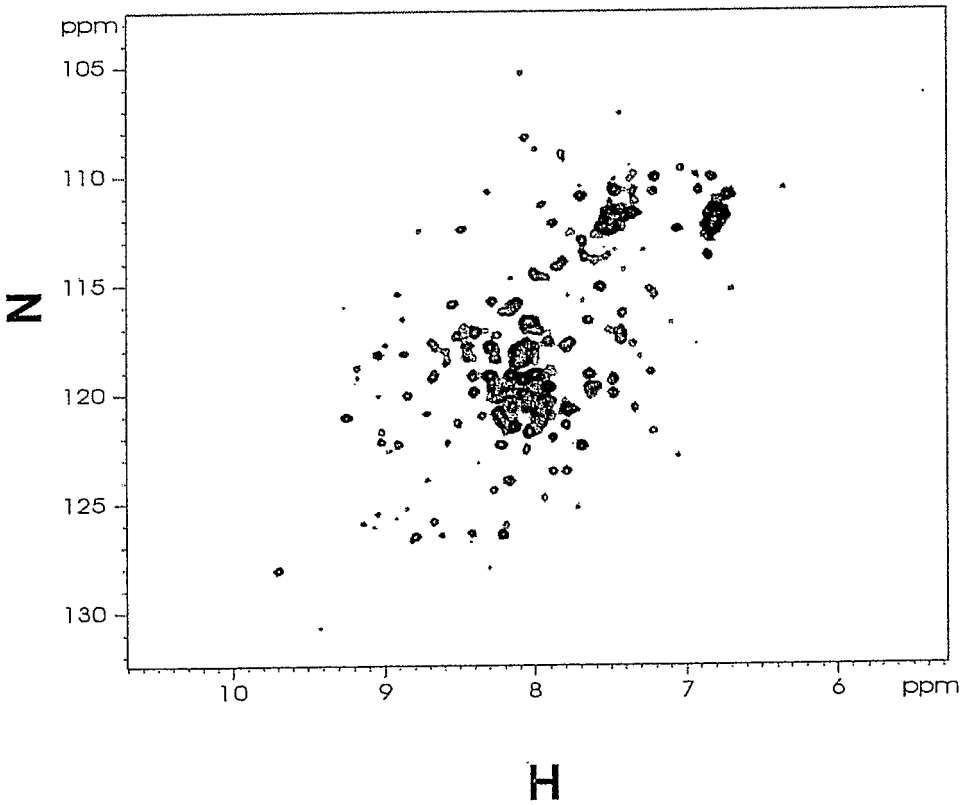
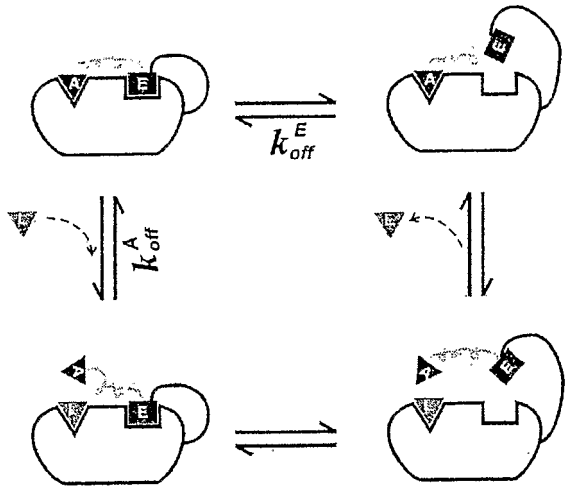


Figure 15c



If [L] is finite and $K_d^L \to 0$,

(when L is a stronger and specific binder than A)

then

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

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

Figure 15d.

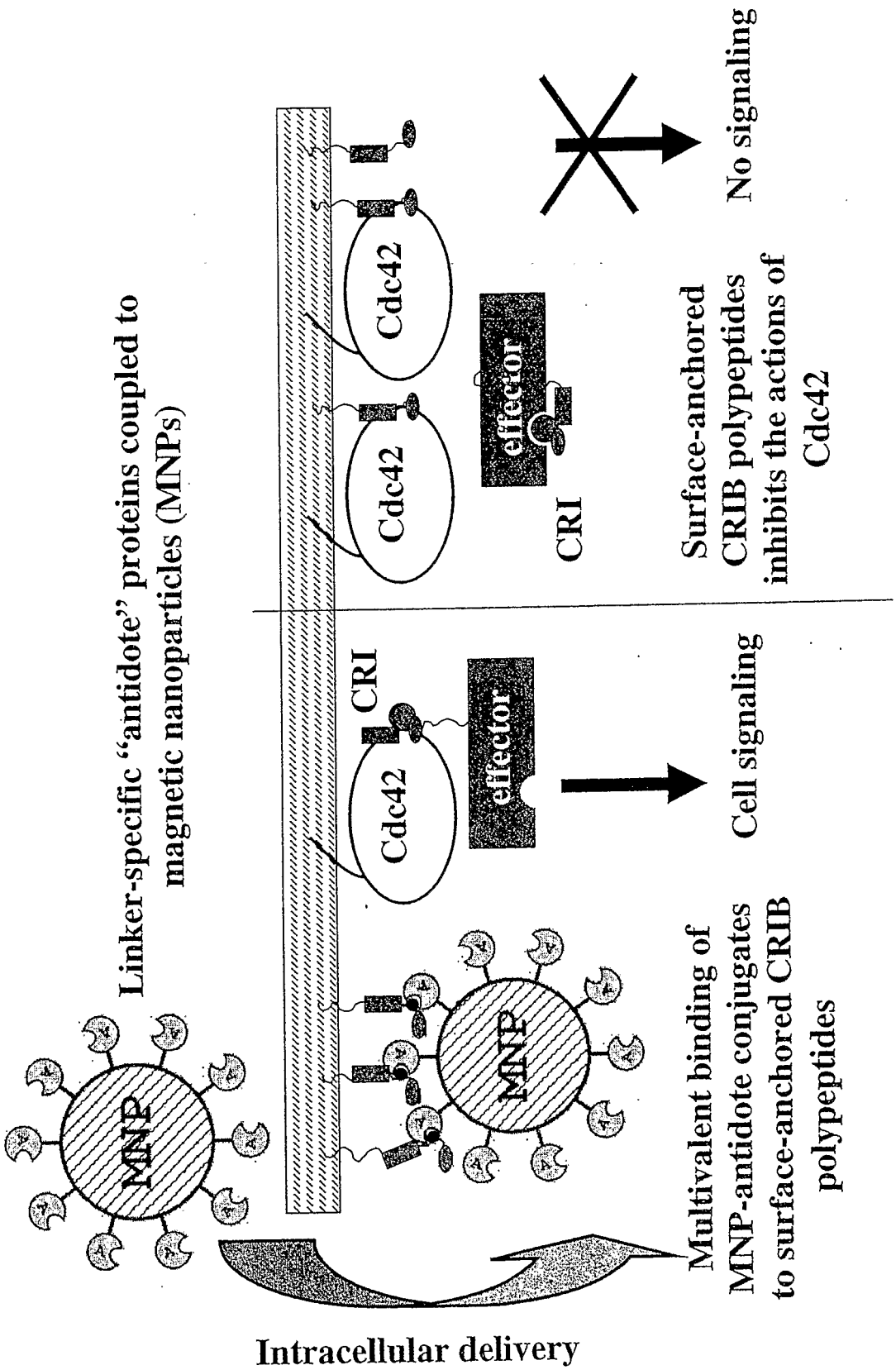

Linker-specific "antidote" proteins coupled to magnetic nanoparticles (MNPs)
MNP
Multivalent binding of MNP-antidote conjugates to surface-anchored CRIB polypeptides
Cell signaling
Cdc42
CRI
effector
Surface-anchored CRIB polypeptides inhibits the actions of Cdc42
No signaling
Intracellular delivery

Figure 17

A.
Polypeptide-protein complex: inactive
RFMF
+
Polypeptide dissociated from complex: active
B.
Covalent peptide-protein conjugate: inactive
RFMF
Linked polypeptide dissociated from binding: active
C.
Protein or ssDNA displaying bivalent peptide: inactive
RFMF
Heat-denatured (melted) molecular conjugate: active

Figure 16

Figure 18

No sealant applied
(bleeding at 5 mins)
Commercial Tisseel
(bleeding stopped
at 1 min 47 sec.)
Tisseel + antidote
+ inhibited thrombin
(bleeding stopped
at 5 Mins.)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/000951

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7): C12N 15/11, C07K 14/00, C07K 7/00, A61K 38/04, A61K 38/16, G01N 33/53, C12N 9/96, C12Q 1/56, C07K 1/14

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(7): C12N 15/11, C07K 14/00, C07K 7/00, A61K 38/04, A61K 38/16, G01N 33/53, C12N 9/96, C12Q 1/56, C07K 1/14

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
WEST; Delphion; STN (Bioeng, Biosis, Caplus, Medline); GenomeQuest; Canadian Patent Database; Keywords: ligand(s), spacer(s), (bi-, oligo-, multi-, poly-)valent, (flexible) linker(s), (modulat-, regulat-, cleav-, switch-, adjust-)able

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US6225100 B1 (DCV, INC. (US)) 1 May 2001 - Table 5, SEQ ID NO:10	11, 12
A		1-8, 13, 14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)
X	US5674838 A (HOECHST AKTIENGESELLSCHAFT (DE)) 7 October 1997	11, 12
A	- Example 4, SEQ ID NO:5	1-8, 13, 14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)
X	EP372670 A2 (TRUSTEES of BOSTON UNIVERSITY (US)) 13 June 1990	11, 12
A	- claim 2	1-8, 13, 14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 October 2005 (17-10-2005)	26 October 2005 (26-10-2005)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001(819)953-2476	Michael W. De Vouge (819) 997-2952

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/000951

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

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

1. [ ] Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. [ ] Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

- as indicated on Extra Sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

1-8, 11-14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO94/25491 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE (US)) 10 November 1994 - hirutonin 2 - page 39, claim 7	11, 12
A		1-8, 13, 14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)
X	WO03/057258 A2 (NATIONAL RESEARCH COUNCIL OF CANADA (CA)) 17 July 2003 - Example 5, figure 14 (peptide CaCla4)	11, 12
A		1-8, 13, 14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)
X	US6533819 B1 (BIOELASTICS RESEARCH LTD (US)) 18 March 2003 - SEQ ID NO: 14	11, 12
A		1-8, 13, 14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)
P,X	WO2004/076484 A1(NATIONAL RESEARCH COUNCIL OF CANADA (CA)) 10 September 2004 -table 1, SEQ ID NO: 28	11, 12
P,A		1-8, 13, 14, 19-38, 39 (partial), 40 (partial), 41-52, 55-65, 66 (partial), 67 (partial)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/000951

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US6225100 B1	01-05-2001	AU4326397 A	10-02-1998
		WO9803636 A1	29-01-1998
US5674838 A	07-10-1997	AU697362 B2	01-10-1998
		AU1163795 A	17-08-1995
		CA2142173 A1	11-08-1995
		DE4404168 A1	17-08-1995
		EP0667355 A1	16-08-1995
		JP7252299 A	03-10-1995
EP0372670 A2	13-06-1990	AU623882 B2	28-05-1992
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		AU3098289 A	07-09-1989
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		DK210690 A	03-06-1991
		EP0333356 A2	20-09-1989
		FI904329D D0	03-09-1990
		FI904330D D0	03-09-1990
		HU55799 A2	28-06-1991
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		JP4500802T T	13-02-1992
		JP4503660T T	02-07-1992
		NO903833 A	02-11-1990
		NO903834 A	02-11-1990
		US5256559 A	26-10-1993
		WO9003391 A1	05-04-1990
		WO9006128 A1	14-06-1990
WO9425491 A1	10-11-1994	CA2161772 A1	10-11-1994
		EP0713496 A1	29-05-1996
		JP8509618T T	15-10-1996
		US5443827 A	22-08-1995
WO03057258 A2	17-07-2003	AU2003201554 A1	24-07-2003
		CA2472193 A1	17-07-2003
		EP1463952 A2	06-10-2004
US6533819 B1	18-03-2003	AU2798599 A	15-09-1999
		CA2319558 A1	02-09-1999
		EP1056413 A1	06-12-2000
		JP2002507437T T	12-03-2002
		US6699294 B2	02-03-2004
		US2002116069 A1	22-08-2002
		WO9943271 A1	02-09-1999
WO2004076484 A1	10-09-2004	NONE	

...continued from Box III:

The International Search Authority found multiple groups of inventions in this international application, as follows:

I. Claims 1-8, 11-14, 19-38, 39 & 40 (partial), 41-52, 55-65, 66 & 67 (partial):
Target binding molecule of the general formula BM1-L-$(BM2)_n$ wherein BM1 and BM2 are binding moieties having affinities for different sites on a target, and L is a linker adapted to respond to changes in environment; specific embodiments of such molecules; polynucleotides sequences encoding these; methods of use thereof; kit comprising SEQ ID NO: 29 and its use in modulating blood flow; use of peptide comprising SEQ ID NO: 25 in modulating thrombin activity; peptides comprising SEQ ID NOs: 100, 101.

II-XXXIX. Claims 9, 10 (partial):
Amino acid sequences of no more than 100 amino acids and nucleotide sequences encoding these, wherein the sequences have at least 90% identity to SEQ ID NOs: 8; 12; 17; 24; 27; 28; 39; 40; 41; 42 & 43 (considered as one invention); 44; 45; 46; 47; 48; 49; 50-56 (considered as one invention); 57; 58; 59 & 60 (considered as one invention); 62; 63; 64 & 66 (considered as one invention); 65; 67; 103; 104; 105; 106; 107; 108; 109 & 110 (considered as one invention); 111; 118 & 120, 123 & 124, 125 & 126 (each pair considered as one invention); 127; 128.

XL-XLI. Claims 9, 10 (partial), 39, 40 (partial):
Polypeptides and nucleotide sequences encoding these, wherein the polypeptides have at least 90% sequence identity to SEQ ID NOs: 37; 38.

XLII. Claims 15-18, 66 and 67 (partial):
Methods of screening a population of molecules to identify a ligand of interest, using a bivalent structure comprising a target and a second molecule both having affinity for a component, and linked by a linker.

XLIII-XLVI. Claims 39, 40 (partial):
Polypeptide sequences comprising SEQ ID NOs: 92; 93; 94; 95.

XLVII. Claims 53, 54:
Kit comprising a controllable polymeric linker.

The special technical feature of Invention I is considered to be the structure of the molecule of claim 1. Inventions XLII and XLVII are considered to be distinct inventions in that molecules recited in these methods do not fully share the structural features of the molecule of Invention I. The International Search Authority further considers that the polypeptide linkers of Inventions II-XLI and XLIII-XLVI are classes of peptides that fail to exhibit a significant structural similarity. Their common functional feature as a linker is not held to be a special technical feature, as linkers are well-known in the art and any polypeptide may potentially be capable of serving as a linker. Thus, the linker embodiments are considered to be distinct inventions.

专利名称(译)	含有接头的多肽配体		
公开(公告)号	EP1766011A1	公开(公告)日	2007-03-28
申请号	EP2005759242	申请日	2005-06-20
[标]申请(专利权)人(译)	加拿大国家研究委员会		
申请(专利权)人(译)	加拿大国家研究委员会的		
当前申请(专利权)人(译)	加拿大国家研究委员会的		
[标]发明人	NI FENG TOLKATCHEV DMITRI SU ZHENGDING		
发明人	NI, FENG TOLKATCHEV, DMITRI SU, ZHENGDING		
IPC分类号	C12N15/11 C07K14/00 C07K7/00 A61K38/04 A61K38/16 G01N33/53 C12N9/96 C12Q1/56 C07K1/14		
CPC分类号	G01N33/542 A61K38/00 A61K47/66 B82Y5/00 C07K14/40 G01N33/53 G01N2500/02		
优先权	60/581703 2004-06-23 US		
其他公开文献	EP1766011A4 EP1766011B1		
外部链接	Espacenet		

摘要(译)

本文提供了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并在其他条件下释放它。该分子具有BM1-L-（BM2）<sub的通式（1）