

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
3 July 2008 (03.07.2008)

PCT

(10) International Publication Number  
**WO 2008/077406 A2**

(51) International Patent Classification:  
C07K 17/14 (2006.01)

[DK/DK]; Sjøllandsgade 92, 2. sal, DK-9000 Aalborg (DK). **STÅDE, Lars Wagner** [DK/DK]; Istedgade 14, 1. sal, DK-9000 Aalborg (DK). **ANDERSEN, Rune Wiik** [DK/DK]; Tornstedsgade 5, DK-9000 Aalborg (DK).

(21) International Application Number:  
PCT/DK2007/000569

(74) Agent: **ZACCO DENMARK A/S**; Hans Bekkevolds Allé 7, DK-2900 Hellerup (DK).

(22) International Filing Date:  
21 December 2007 (21.12.2007)

(25) Filing Language: English

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(26) Publication Language: English

(30) Priority Data:  
60/876,673 22 December 2006 (22.12.2006) US

(71) Applicant (for all designated States except US): **BIO-NANOPHOTONICS A/S** [DK/DK]; Niels Jernes Vej 10, DK-9220 Aalborg Øst (DK).

(72) Inventors; and

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(75) Inventors/Applicants (for US only): **PETERSEN, Maria Teresa** [DK/DK]; Kalmanparken 20, Frejlev, DK-9200 Aalborg (DK). **PETERSEN, Steffen Bjørn** [DK/DK]; Kalmanparken 20, Frejlev, DK-9200 Aalborg (DK). **DUROUX, Laurent** [FR/DK]; Kronosvej 127, DK-9210 Aalborg SØ (DK). **DUROUX, Meg** [GB/DK]; Kronosvej 127, DK-9210 Aalborg SØ (DK). **SKOVSEN, Esben** [DK/DK]; c/o Hanne & Bjarne Skovsen, Ørnevej 10, DK-6000 Kolding (DK). **STRANDDORF, Jon Weis** [DK/DK]; Borgmester Jørgensens Vej 5, 2.-131, DK-9000 Aalborg (DK). **JENSEN, Rasmus Lybech**

Published:

— without international search report and to be republished upon receipt of that report

(54) Title: COUPLING OF ELEMENTS

(57) Abstract: The present invention relates to a method of light-induced coupling two elements A and B via thiol binding. According to the method, molecules, proteins, peptides or peptide-containing elements can be coupled to a support or to a second molecule, protein, peptide or peptide-containing element, where the structural and functional properties of the coupled or immobilised elements are preserved, and the orientation of coupling can be controlled on a molecular scale. In particular, the method comprises generating an element A\* by providing an appropriate disulfide bridge or disulfide bridge-containing triad to said element A, irradiating element A\* to create an appropriate reactive thiol group from the disulfide bridge, and incubating the irradiated element A\* with said element B, thereby a coupling between element A and element B is obtained. Alternatively, element A\* can also be irradiated in the presence of B.

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## COUPLING OF ELEMENTS

### Technical field

The present invention relates to a method of coupling two elements. More  
5 particular, the invention concerns a method of coupling molecules, proteins,  
peptides or peptide-containing elements to a support or to a second mole-  
cule, protein, peptide or peptide-containing element, where the structural and  
functional properties of the coupled or immobilised elements are preserved,  
and the orientation of coupling can be controlled on a molecular scale.

10

### Background of the invention

Molecules can be immobilised on a support, carrier or solid surface either  
passively through hydrophobic or ionic interactions, or covalently by attach-  
ment to activated surface groups. In response to the enormous importance of  
15 immobilisation for solid phase chemistry and biological screening, the ana-  
lytical uses of the technology have been widely explored. The technology has  
found broad application in many different areas of biotechnology, e.g. diag-  
nostics, biosensors, affinity chromatography and immobilisation of molecules  
in immunoassays, such as ELISA. The value of immobilisation technology is  
20 demonstrated by the recent development of DNA microarrays, where multiple  
oligonucleotide or cDNA samples are immobilised on a solid surface in a spa-  
tially addressable manner. These arrays have revolutionised genetic studies  
by facilitating the global analysis of gene expression in living organisms.  
Similar approaches have been developed for protein analysis, where as little  
25 as one picogram of protein need be bound to each point on a microarray for  
subsequent analysis. The proteins bound to the microarrays, can then be as-  
sayed for functional or structural properties, facilitating screening on a scale,  
and with a speed, previously unknown. The biomolecules bound to the solid  
surface may additionally be used to capture other unbound molecules pre-  
30 sent in mixture.

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Development of this technology, with the goal of immobilising a biomolecule on a solid surface in a controlled manner, with minimal surface migration of the bound moiety and with full retention of its native structure and function, has been the subject of intensive investigation in recent years (Veilleux J  
5 (1996) *IVD Technology*, March p. 26-31). The simplest type of protein immobilisation exploits the high inherent binding affinity of surfaces to proteins in general. For example proteins will physically adsorb to hydrophobic substrates via numerous weak contacts, comprising van der Waals, and hydrogen bonding interactions. The advantage of this method is that it avoids  
10 modification of the protein to be bound. On the other hand, proteins bound in this manner may be distributed unevenly over the solid support and/or inactivated since, for example, their clustering may lead to steric hindrance of the active site/binding region in any subsequent functional assay.

15 Alternative methods of immobilisation rely on the use of a few strong covalent bonds to bind the protein to the solid surface (Wilson D.S., Nock S., 2001, *Current Opinion in Chemical Biology* 6:81-85). Examples include immobilisation of biotinylated proteins onto streptavidin-coated supports, and immobilisation of His-tagged proteins, containing a poly-histidine sequence, to Ni<sup>2+</sup>-  
20 chelating supports. Other functional groups on the surface of proteins which can be used for attachment to an appropriate surface include reacting an amine with an aldehyde via a Schiff-base, cross-linking amine groups to an amine surface with gluteraldehyde to form peptide bonds, cross-linking carboxylic acid groups present on the protein and support surface with carbodiimide, cross-linking based on disulfide bridge formation between two thiol  
25 groups and the formation of a thiol-Au bond between a thiol group and a gold surface.

Amine coupling is a widely used method of immobilisation chemistry. N-  
30 hydroxysuccinimide esters are formed from a fraction of the carboxyl groups of the carboxymethyl dextran matrix via reaction with N-hydroxysuccinimide

(NHS) and N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) in water, which then react spontaneously with amine groups on a protein to form covalent bonds (Johnsson B., *et. al.*, 1991, *Anal Biochem* 198:268-77). Following immobilisation, un-reacted N-hydroxysuccinimide esters on the support are deactivated with 1M ethanolamine hydrochloride to block areas devoid of bound proteins. The method is laborious since the reagents, used at each step of a chemical immobilization method, usually need to be removed prior to initiating the next step.

10 Methods for the immobilization of biomolecules via disulfide bridges are described by Veilleux J (1996) *supra*. Protein samples are treated with a mild reducing agent, such as dithiothreitol, 2-mercaptoethanol or tris(2-carboxyethyl)phosphine hydrochloride to reduce disulfide bonds between cysteine residues, which are then bound to a support surface coated with

15 maleimide. Alternatively primary amine groups on the protein can be modified with 2-iminothiolane hydrochloride (Traut's reagent) to introduce novel sulfhydryl groups, which are thereafter immobilized to the maleimide surface. Immobilization of proteins on a gold substrate via a disulfide bridge is shown for the cupredoxin protein plastocyanin from Poplar (Andolfi, L. *et al.* 2002,

20 *Arch. Biochem. Biophys.* 399: 81-88). Since this protein lacks a disulfide bridge, surface exposed residues Ile21 and Glu25 were both substituted with Cys. Disulfide bridge formation between the inserted cysteines was confirmed from the 3D crystal structure of the purified mutant plastocyanin. Mutant plastocyanin, expressed intracellularly in bacteria, is exposed to a reducing

25 environment in the cytoplasm, such that the inserted cysteines are reduced, and can thus mediate the direct adsorption of the isolated protein onto a gold substrate. The thiol group binding properties of the protein are thus dependent on *in vivo* or *in vitro* chemical reduction of the cysteine residues on the surface of the protein.

An alternative approach to engineering thiol-group binding properties into a protein has been described for ribonuclease (RnaseA), which has four essential cysteines (Sweeney, R.Y. et al. 2000 Anal Biochem. 286: 312-314). In this case a single cysteine residue was substituted for Ala19, located in a surface loop near the N-terminus of RNase A. The cysteine in the expressed RNase was protected as a mixed disulfide with 2-nitro-5-thiobenzoic acid. Following subsequent de-protection with an excess of dithiothreitol, the RNase was coupled to the iodoacetyl groups attached to a cross-linked agarose resin, without loss of enzymatic activity. Again, preparation of the protein for immobilisation requires its exposure to both protecting and de-protecting agents, which may negatively impact its native structure and/or function.

Methods are described in US 6,350,368 for the coupling of FAD-dependent enzymes onto electrodes, whereby an apoenzyme is complexed with a functionalised FAD that is covalently bonded to a binding moiety, capable of chemical association (e.g. through a thiol group) or attachment on the surface of an electrode (e.g. gold). The immobilised FAD-enzyme complex is further functionalised with an electron mediator group and can be used in electrochemical systems for detecting analytes or optical signals.

Light-induced immobilization techniques have also been explored, leading to the use of quinone compounds for photochemical linking to a carbon-containing support (EP0820483). Activation occurs following irradiation with non-ionising electromagnetic radiation in the range from UV to visible light. Masks can be used to activate certain areas of the support for subsequent attachment of biomolecules. Following illumination the photochemically active compound, anthraquinone, will react as a free radical and form a stable ether bond with a polymer surface. Since anthraquinone is not found in native biomolecules, appropriate ligands have to be introduced into the biomolecule. In the case of proteins, this additional sample preparation step may require thermochemical coupling to the quinone and may not be site specific.

A further development of light-induced immobilisation technology is disclosed in US 5,412,087 and US 6406844, which describe a method for preparing a linker bound to a substrate. The terminal end of the linker molecule is provided with a reactive functional group protected with a photo-removable protective group, e.g. a nitro-aromatic compound. Following exposure to light, the protective group is lost and the linker can react with a monomer, such as an amino acid at its amino or carboxy-terminus. The monomer, furthermore, may itself carry a similar photo-removable protective group, which can also be displaced by light during a subsequent reaction cycle. The method has particular application to solid phase synthesis, but does not facilitate orientated binding of proteins to a support.

Bifunctional agents possessing thermochemical and photochemical functional substituents for immobilising an enzyme are disclosed in US 3,959,078. Derivatives of arylazides are described which allow light mediated activation and covalent coupling of the azide group to an enzyme, and substituents which react thermochemically with a solid support. The orientation of the enzyme molecules immobilised by this procedure is not controlled.

A method for orientated, light-dependent, covalent immobilization of proteins on a solid support, using the heterobifunctional wetting-agent N-[m-[3-(trifluoromethyl)diazirin-3-yl]phenyl]-4-maleimidobutyramine, is described in WO 91/16425 and by Collioud A *et al.* (1993) in *Bioconjugate Chem.* 4: 528-536. The aryldiazirine function of this cross-linking reagent facilitates light-dependent, carbene-mediated, covalent binding to either inert supports or to biomolecules, such as proteins, carbohydrates and nucleic acids. The maleimide function of the cross-linker allows binding to a thiolated surface by thermochemical modification of cysteine thiols. Orientated binding of this cross-linking reagent to a protein can be attained by a thermochemical interaction between the maleimide function and an exposed thiol group on the

protein surface, however this treatment may modify the structure and activity of the target protein. Light-induced covalent coupling of the cross-linking reagent to a protein via the carbene function, however, has the disadvantage that it does not provide for controlled orientation of the target protein.

5

Common for most of the described immobilisation methods is their use of one or more thermochemical/chemical steps, sometimes with hazardous chemicals, some of which are likely to have a deleterious effect on the structure and/or function of the bound protein. The available methods are often invasive, whereby foreign groups are introduced into a protein to act as functional groups, which cause protein denaturation, as well as lower its biological activity and substrate specificity.

10

Disulphide bridges are known to be excellent quenchers of excited-state aromatic residues. Any aromatic residue, which is in close spatial proximity, can cause photo-induced disruption of a neighbouring disulphide bridge. Hence, the three aromatic amino acids, tryptophan, tyrosine and phenylalanine found in proteins, are all potential mediators of light-induced disulphide bridge disruption. While irradiation with light of a range of wavelengths extending from 240 nm to 300 nm will excite all aromatic residues, the individual aromatic residues have differing absorption maxima (Table 1; data obtained at neutral pH).

15

20

**Table 1**

In water	Absorption Max.	Emission Max.
Phe	254 nm	282 nm
Tyr	275 nm	303 nm
Trp	280 nm	350 nm

25

Since the excitation spectrum of the aromatic amino acid residues is only partially overlapping, protein irradiation at a single, narrow wavelength range

will excite the individual residues to different degrees. Irradiation at 295 nm can be used to selectively excite tryptophan residues in a protein. Irradiation at 280 nm will excite both tyrosine and tryptophan residues, which can both then cause photo-induced disulphide bridge disruption. Where irradiation is performed by multiple-photon excitation, for example when two-photon excitation is carried out, the sample is irradiated with photons (light) with half the energy (twice the wavelength) of the photons used in a single-photon experiment. For example, electronic excitation of tryptophan can both be achieved with ultraviolet light at 295 nm, or with two-photon excitation at a wavelength of approximately 690 nm. Furthermore, excited tyrosine residues can cause the excitation of neighbouring tryptophan residues by a mechanism called fluorescence resonance energy transfer, which in turn can cause disulphide bridge disruption.

15 When proteins are exposed to UV irradiation, some disulphide bridges are disrupted to form activated thiols. Although disulphide bridges are commonly found in the structural core and near/on the surface of folded proteins, those located in close proximity to aromatic amino acids are the most susceptible to UV-induced disruption. During UV exposure of proteins, energy absorbed by side chains of aromatic amino acid residues is transferred to spatial neighbouring disulphide bridges, which function as quenchers (Neves-Petersen MT., et al., 2002, *Protein Science* 11: 588-600). However, the flow of energy transferred to disulphide bridges and the likely formation of intermediate chemical species such as radicals/ions formed upon light excitation of the sample ultimately serves to trigger their disruption. Light may trigger ejection of electrons from the aromatic side chains. These electrons may become solvated, which can lead to the disruption of the disulfide the bridge (Bent and Hayon, *Journal of the American Chemical Society* 97 (10): 2612-2619). The presence of a disulphide bridge with tryptophan as a close spatial neighbour in a protein occurs frequently in nature, indicating that photo-induced disulphide bridge disruption is a widespread phenomenon (Petersen

MTN., et al., 1999, Protein Engineering 12: 535-548; Neves-Petersen MT et al., 2002, Protein Science 11: 588-600; Vanhooren A et al. 2002, Biochemistry 10; 41(36):11035-11043).

5 WO 04/065928 provides a method of coupling a protein to a support by inducing the formation of thiol groups in the protein with irradiation, and coupling the protein to the support. The formation of thiol group(s) in the protein is a consequence of the disruption of disulfide bridges following electronic  
10 to the disulfide bridge. The light-induced coupling method facilitates the orientated immobilization of a protein on a support. A prerequisite condition for obtaining the desired immobilisation is the existence of an essential triad, comprising an aromatic amino acid in close approximation to a disulfide  
15 bridge, in the protein. The contents of this application are included herein as reference.

Danish patent application PA 2005 00925 (unpublished) discloses a method of coupling a protein to a support by inducing the formation of thiol groups in the protein and in said support, where the support comprises disulfide bridge-  
20 containing triad-containing linkers on the surface of a support. The contents of this application are included herein as reference.

However, the majority of all molecules fail to comprise an appropriate disulfide bridge-containing triad, and most molecules are therefore excluded from  
25 coupling via thiol binding using irradiation for disruption of disulfide bridges. Also, the previously disclosed methods are only concerned with coupling of proteins to a support.

There is thus a need in the art for coupling and immobilisation by irradiation  
30 of other elements than protein with a natural content of the appropriate disulfide bridge-containing triad such as molecules, where the structural and func-

tional properties of the coupled or immobilised elements are preserved, and the orientation of coupling can be controlled on a molecular scale.

### Summary of the invention

5 The present invention satisfies the need in the art for coupling of two elements or immobilisation of molecules on a supports, where the structural and functional properties of the coupled or immobilised elements or molecules can be preserved if desired, and the orientation of coupling can be controlled on a molecular scale.

10

In WO 04/065928 the exploitation of appropriate naturally occurring triads for coupling of a molecule to a support or another molecule by thiol binding is disclosed. Such a triad is present when a disulfide bridge is located in sufficient proximity ( $< 10 \text{ \AA}$ ) to an aromatic amino acid residue in the folded protein, to allow for an absorption and quenching by disruption of the disulfide bridge of energy emission from light irradiated aromatic aminos. The thiol groups created in proteins in this way by light-induced disulfide bridge disruption may then used to immobilise the protein to a support in an orientation-dependent and controlled manner.

20

The present invention addresses the problem of coupling molecules and supports to other molecules or support, which molecules and/or support do not contain appropriate naturally occurring triad(s). In one case, the natural protein may not contain a triad or the triad(s) present is/are not accessible by light, or the created thiol(s) is/are not accessible for coupling because it is buried inside the protein or breakage of the disulfide bond will destroy the properties of the protein, such at binding capacity or enzymatic activity or the like. In such as case an appropriate disulfide bridge-containing triad advantageously may be added or providing to the protein. Similar for peptides and polypeptides to be coupled to another element.

25

30

The present invention also provides for coupling of two supports or two molecules both lacking appropriate thiol-binding properties, by utilizing the present principle of adding or providing appropriate disulfide bridge-containing triads. The principle can also be used to coupling of more than two elements. The principle may further be used to add the aromatic amino acid in a solution to the element(s) comprising an appropriate disulphide bridge.

Hence, the present invention relates to a method of providing an appropriate disulfide bridge-containing triad to an element A which lacks such appropriate triad for coupling element A to an element B via thiol binding. The invention also relates to a method of providing an appropriate disulfide bridge to element A and to methods of additional provision of appropriate disulfide bridge-containing triad or disulfide bridge to element B.

Thus, in a first aspect, the present invention relates to a method of coupling two elements A and B, by

1) generating an element A\* by providing an appropriate disulfide bridge or disulfide bridge-containing triad to element A, and if desired providing an appropriate disulfide bridge or disulfide bridge-containing triad to element B to generate element B\*

(2) a) irradiating said element A\* (and B\*) to create an appropriate reactive thiol group, and  
b) incubating said irradiated element A\* with said element B capable of binding a thiol group (or irradiated B\*), thereby obtaining a coupling between elements A and B;

or

a) incubating said element A\* with said element B capable of binding a thiol group or said element B\*, and  
b) irradiating said element A\* in the presence of said element B (or B\*) to create a reactive thiol group by disulfide bridge disruption in said

element A\* (and B\*), and thereby obtaining a coupling between element A and element B.

5 Elements according to the invention comprise molecules and supports, thus providing great flexibility, allowing for coupling combinations of molecule + molecule, support + support, molecule + support and/or support + molecule. In case of coupling support with support and molecule with molecule, different, similar or identical support(s) and/ molecule(s) can be coupled. In a specific embodiment, two or more elements placed on the same object may be  
10 coupled in order to create a desired conformation of the object, which may be a support or a molecule such as a protein or polypeptide. In an aspect of the invention, the molecule is a biomolecule, such as a peptide, a protein, a polynucleotide, a lipid, a sugar, a pharmaceutical, a cosme-  
tical, a pro-drug and the like.

15

In contrast to the state of the art, the invention is not limited to proteins, but allows virtually any molecule(s) and support to be coupled according to the invention, provided that an appropriate disulfide bridge or disulfide bridge-  
20 containing triad can be provided, for example by covalent attachment and/or by genetic engineering. This includes virtually any support(s), provided that they can be coupled according to the invention, i.e. that an appropriate linker can be coupled to the support and/or that the support capable of binding a thiol group. A support according to the invention comprises a soluble, semi-  
25 soluble or insoluble material to which an appropriate disulfide bridge or disulfide bridge-containing triad is capable of being attached. Such a support may also comprise a thiol reactive surface or a surface that can be made thiol re-  
active, e.g. a surface comprising gold or quartz. A support which is reactive for binding one or more molecule(s) is denoted "a carrier". A carrier may therefore be a support which by nature is reactive for binding one or more  
30 molecule(s) containing a thiol group or a support which is made reactive for binding one or more molecule(s) via thiol binding.

Another aspect of the invention relates to a linker molecule comprising an appropriate disulfide bridge or disulfide bridge-containing triad, which may be provided to an element lacking an appropriate disulfide bridge or disulfide  
5 bridge-containing triad. Such an appropriate linker molecule may comprise one or more copies of a peptide comprising any of the formulas (I)  $X1_m C X2_n C X3_o \emptyset X4_p$ , (II)  $X1_m C X2_n \emptyset X3_o C X4_p$  and/or (III):  $X1_m \emptyset X2_n C X3_o C X4_p$ , wherein  $X1_m$ ,  $X2_n$ ,  $X3_o$  and  $X4_p$  represent the same or different peptides, each peptide respectively consisting of m, n, o, and p amino acids, where m,  
10 n, o and p are mutually independent numbers between 0 and 1000 (or 100, 25, or 10), and  $m + n + o + p \leq 1000$  (or 100, 25, or 10), said amino acids being selected from all natural and synthetic amino acids, C is cysteine, and the two cysteines are covalently joined by a disulfide bridge, and  $\emptyset$  is an aromatic amino acid such as phenylalanine, tryptophane or tyrosine, or a peptide bond. The aromatic amino acid  $\emptyset$  may also be absent, or additional,  
15 similar or different aromatic amino acids  $\emptyset$  may be present.

In one aspect of the invention, the linker is provided to an element, support, molecule and/or protein, polypeptide or peptide through covalent binding of a  
20 linker molecule, e.g. using NHS (N-hydroxysuccinimide), EDC (N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride), activated ester, maleimide, disulfide formation, streptavidin/biotin, activated alcohol, vinylsulfone, Schiff base formation and/or "click" chemistry and the like.

25 In a another aspect of the invention, a linker providing an appropriate disulfide bridge and/or disulfide bridge-containing triad is provided through genetic engineering techniques, comprising: (i) N-terminal extension, (ii) C-terminal extension, (iii) internal extension, (iv) amino acid substitution, (v) amino acid insertion, (vi) amino acid deletion or (vii) any combination or combinations of  
30 said methods (i-vi). Furthermore, genetic engineering may also result in a

conformation change in a molecule, thereby bringing an aromatic amino acid and an appropriate disulphide bridge in the vicinity of each other.

5 In yet another aspect of the invention, coupling of two elements may be provided by adding a free aromatic amino acid (in solution), either alone or being part of a molecule, to the vicinity of one or more appropriate disulfide bridges.

10 The invention provides an irradiation step, comprising light of a wavelength that excites one or more aromatic amino acids. Such wavelength interval(s) comprises UV light in the wavelength interval of 250 to 305 nm (or 250 to 260nm, 270 to 280 nm and/or 290 to 300nm, or about 254, 275 or 295 nm), or with light having longer wavelengths that by means of non-linear processes and/or multiphoton excitation promotes the same electronic transitions as light in said wavelength interval of 250 to 305 nm.

15

An aspect of the invention provides a coupling between elements, resulting in an immobilization on a support or surface, which can be spatially controlled as disclosed in WO 04/065928. Such a support and/or surface may also be a derivatized support that is capable of binding a thiol group, such as a support and/or surface comprising a thiol group or a disulfide bridge. When appropriate, such a surface or support may comprise a spacer.

20

25 A further aspect of the present invention relates to a magnetic (nano)particle or a cantilever for atomic force microscopy, where the magnetic (nano)particles or a cantilever comprises a molecule coupled by irradiation of a disulfide bridge or disulfide containing triad.

25

Another aspect relates to dimers, such as homo- or heterodimers, consisting of two identical or different elements or subunits.

30

Yet another aspect relates to dendrimers, such as homo- or hetero dendrimers.

5 A further aspect relates to a vector or plasmid providing an appropriate disulfide bridge or triad fused to the N- or C-terminus of a protein to be expressed by said vector or plasmid in an appropriate host.

10 Another aspect relates to a vector or plasmid that provides an appropriate disulfide bridge or triad by fusion of a protein by C- or N-terminal fusion to a protein or part thereof or a natural or synthetic polypeptide or peptide. One example of a useful linker polypeptide is knottin (HpTX2) and/or mutant knottin (mutHpTX2).

15 Other aspects are disclosed in detail in a co-pending application by the same applicant filed on the same day.

#### **Brief description of the drawings**

20 **Figure 1** illustrates different embodiments of the invention concerning irradiation-mediated coupling of two elements (elements, supports and/or molecules A and B) where a linker is provided to element A or two linkers,  $L_A$  and  $L_B$ , are provided to elements A and B, respectively.

25 **Figure 2** is an embodiment according to the invention and shows an element A to which a cyclic peptide linker CXWXC is attached (C = cysteine; ■ = "body" of a cysteine, with S representing its sulphur atom; W = tryptophane; and X is any amino acid not comprising a reactive thiol group; Y is part of the linker and/or element A providing attachment of the linker to element A).

30 **Figure 3** illustrates further embodiments concerning linker molecules according to the invention (nomenclature = Figure 2).

**Figure 4** Cartoons of the B-FABP structure obtained by Balendiran et al. (2000) using X-ray diffraction with a resolution of 2.80 Å (PDB: 1FE3).

**Figure 5** W6/SS cross-linked B-FABP, labeled with 5(6)-carboxynaphthofluorescein, immobilized on thiol derivatized quartz slides using UV (280nm); A: 5 X 5 array; B: 9 X 1 array.

**Figure 6** 2 X 5 array of SS tagged B-FABP labeled with 5(6)-carboxynaphthofluorescein, immobilized on thiol derivatized quartz slides using UV (280nm).

**Figure 7** A schematic representation of the structural motif found in knottin peptides.

**Figure 8** shows different embodiments of the invention regarding homo- and heterodimers (A, B = elements and/or subunits;  $SS\emptyset_n$ : disulfide bridge-containing triad; n=1,2,3,4 indicating different subunits; SS appropriate disulfide bridge or disulfide bridge formed upon coupling;  $\emptyset$ : aromatic amino acid).

**Figure 9** shows different embodiments of the invention regarding dendrimer units before coupling ( $SS\emptyset_x$ ,  $SS\emptyset_y$ ,  $SS\emptyset_z$ : identical or different triads used for coupling;  $L_a$ ,  $L_b$ ,  $L_c$ : identical or different linkers):

**Figure 10** Ion exchange chromatogram of Human B-FABP after refolding.

**Figure 11** Agarose gel of PCR product of SS tagged B-FABP to determine the optimal annealing temperature.

**Figure 12** Agarose gel with the colony PCR products containing SS tagged B-FABP.

**Figure 13** SDS-PAGE gel with SS tagged B-FABP, W6/SS cross linked B-FABP, and native B-FABP.

**Figure 14** Emission measurements from 300 – 400 nm of native and W6/SS  
5 Cross linked B-FABP prior to heating.

**Figure 15** Emission measurements from 300 to 400 nm of native and W6/SS  
Cross linked BFABP after heating and subsequent cooling.

10 **Figure 16** Temperature scans of native and W6/SS Cross linked B-FABP.

**Figure 17** Emission scans of 5  $\mu\text{M}$  Native and 7  $\mu\text{M}$  SS Tagged B-FABP at  
pH 5.86 and a temperature of 11.5°C.

15 **Figure 18** Emission scans of 7  $\mu\text{M}$  SS tagged B-FABP before and after heat-  
ing.

**Figure 19** Thermal scans [11.5-82]°C of SS tagged B-FABP and native B-  
FABP.

20

**Figure 20** Static light scattering of W6/SS cross linked B-FABP prior to and  
after heating and subsequent cooling.

**Figure 21** Scattering scan of SS tagged B-FABP at 11.5°C.

25

**Figure 22** Structure of HpTX2 (left: Native HpTX2; middle: mutHpTX2; right:  
distances from W to each disulfide bridge).

**Figure 23** The two figures show a possible conformation of HpTX2 after mu-  
30 tation of Tyr20  $\rightarrow$  Trp20 and Trp25  $\rightarrow$  Phe25.

**Figure 24** Another two possible conformations of HpTX2 after mutation of Tyr20 → Trp20 and Trp25 → Phe25.

**Figure 25** Verification of a mutHpTX2 Insert in pET-11a by\_colony PCR.

5

**Figure 26** Schematically illustration of a method and apparatus for material deposition by light induced molecular immobilization. Shown is a light source (102) in the form of a laser.

## 10 Definitions

The term "element" as used herein is set out to encompass all kinds of molecules, particles and solid supports which are the two or more objects to be coupling by thiol binding.

15 As used herein, the term "support" comprises a soluble, semi-soluble or insoluble material to which an appropriate disulfide bridge or disulfide bridge-containing triad is capable of being attached. For insoluble and solid supports may also be used the term "surface". Examples of an insoluble support is  
20 electronic chips, slides, wafers, particles, resins, wells, tubes or membranes which include but are not limited to any material comprising polymers such as Topaz, polystyrene, polyethylene, polyester, polyethermides, polypropylene, polycarbonate, polysulfone, polymethylmethacrylate [PMMA], poly(vinylidene flouride) [PVDF], siliciumoxidecontaining materials such as silicon; diamond; glass e.g. quartz and silica; silicon e.g. silicon wafers; metals such as gold,  
25 silver and aluminium; membranes e.g. nylon membranes, nitrocellulose filters; porous materials such as gels, agarose or cellulose; ceramics etc. which furthermore include all forms of derivatisation of the support which facilitate attachment or binding of the peptide linker to the support. Examples of a  
soluble support are a soluble compound or polymer such as hydrocarbons or  
30 another biomolecule, for example collagen or a polypeptide, or a magnetic

particle. A support can also be a polypeptide or another biomolecule such as DNA or synthetic biomolecules such as aptamers.

5 As used herein, the term "carrier" refers to a support which is or is made re-active for binding one or more molecules.

As used herein, the term "appropriate disulfide bridge" refers to a disulfide bridge susceptible of being broken by irradiation when in vicinity of an aromatic amino acid and placed in a desired part or region of the element. In  
10 peptides, polypeptides or proteins, disulfide bridges may be provided via two cysteines. Disulfide bridges may be intra molecular (part of the same molecule) or inter molecular (covalently linking to peptides, polypeptides or proteins) via a disulfide bridge.

15 As used herein, the terms "appropriate triad" and "appropriate disulfide bridge-containing triad" refers to a spatial configuration of a disulfide bridge and an aromatic amino acid, which are in sufficient vicinity to each other to allow irradiation-mediated breakage of the disulfide bond and placed or situated in a desired part or region of the element. In other words, disulfide  
20 bridge and aromatic amino acid are "spatial neighbours". However, it is not enough that the element, e.g. the protein, comprises a disulfide bridge-containing triad or disulfide bridge, if after breaking the bridge, the thiol group is buried in the interior of the protein, ruins the property, e.g. binding capacity or enzymatic activity, of the protein or cannot be used to secure a desired  
25 uniform spatial configuration on a support.

"Spatial neighbours" relates to the physical distance between two chemical groups within a compound or composition, such that groups lying in three-dimensional close proximity are considered to be spatial neighbours. A disul-  
30 phide bridge in e.g. a protein which is a spatial neighbour to an aromatic residue may function as a quencher for excitation energy absorbed by the

aromatic amino acid following irradiation. The physical distance between half cystines of a disulphide bridge, which are spatial neighbours to one or more aromatic residues such as tryptophan residues and may act as quenchers, can be, but is not limited, to a range of 1 to 15 Å, more particular 1-12 Å or 1-10 Å.

As used herein, "reactive thiol group" relates to a thiol group which is capable of covalent coupling to another thiol group creating a disulphide bond.

The linker used in the method according to the invention is designed so it is activated by irradiation e.g. UV-beam illumination and thereby making spatially controlled immobilisation possible. This is an advantage of the present method compared to known methods for spot-size immobilisation using UV-beam immobilisation where the support, carrier or surface is capable of binding without activation. When the polypeptide to be immobilised is in a solution, known spot-size immobilisation methods may allow polypeptide that were activated above the surface to diffuse to an area outside the UV-beam and be immobilised. Using the method according to the present invention, this problem is solved since immobilisation now requires activation of the support and/or carrier. Activated polypeptide that diffuses outside the immobilisation area will not immobilise because the surface outside the UV-beam area is not activated using the present method.

As used herein, the term "linker" relates to a molecule to be provided to an element, e.g. to a molecule or a support, in order to provide to said element a disulfide bridge or disulfide bridge-containing triad capable of being activated by irradiation to contain reactive thiol group(s) (-SH group(s)). When activated, the thiol group should preferably be available for coupling according to the invention. A linker comprising an appropriate disulfide bridge and/or disulfide bridge-containing triad may include, but is not limited to a linker comprised solely or partly by amino acids. Thus a linker may include other mole-

5 cules than amino acids and may be comprised by one or more peptide groups and one or more groups of organic or non-organic materials, e.g. containing a peptide group and one or more carbohydrate groups, including small sugar molecules, oligosaccharides, large carbohydrate-based polymers. Inorganic part(s) of the linker may include e.g. metallic groups based on gold, silver, aluminium, silicon, and/or non-metallic groups based e.g. on ceramic.

10 The term "amino acid" comprises both natural amino acids such as Ala (alanine), Cys (cysteine), Asp (aspartic acid), Glu (glutamic acid), Phe (phenylalanine), Gly (glycine), His (histidine), Ile (isoleucine), Lys (lysine), Leu (leucine), Met (methionine), Asn (asparagines), Pro (praline), Gln (glutamine), Arg (arginine), Ser (serine), Thr (threonine), Val (valine), Trp (tryptophan), Tyr (tyrosine) and synthetic or modified amino acids such as  
15 Aad (2-aminoadipic acid), bAad (3-Aminoadipic acid), bAla (beta-alanine, beta-aminopropionic acid), Abu (2-aminobutyric acid), 4Abu (4-aminobutyric acid, piperidinic acid), Acp (6-aminocaproic acid), Ahe (2-aminoheptanoic acid), Aib (2-aminoisobutyric acid), bAib (3-aminoisobutyric acid), Apm (2-aminopimelic acid), Dbu (2,4 diaminobutyric acid), Des (desmosine), Dpm  
20 (2,2'-diaminopimelic acid), Dpr (2,3-diaminopropionic acid), EtGly (N-ethylglycine), EtAsn (N-ethylasparagine), Hyl (hydroxylysine), aHyl (allo-hydroxylysine), 3Hyp (3-hydroxyproline), 4Hyp (4-hydroxyproline), Ide (isodesmosine), alle (allo-isoleucine), MeGly (N-methylglycine, sarcosine), Melle (N-Methylisoleucine), MeLys (6-N-methyllysine), MeVal (N-  
25 methylvaline), Nva (norvaline), Nle (norleucine) and Orn (ornithine). The amino acids are preferably natural amino acids. In the formulas, the IUPAC amino acid letter code may be used.

30 The aromatic amino acids according to the invention comprise phenylalanine, tryptophan and/or tyrosin and/or their derivatives. These derivatives do not significantly alter the excitation/emission behaviour.

The terms "peptide" and "polypeptide" are in the present context intended to mean molecules comprising amino acids covalently linked via peptide bonds, and the term encompasses both short peptides of from 2 to 10 amino acid  
5 residues, oligopeptides of from 10 to 20 amino acid residues, and polypeptides of more than 20 amino acid residues, for example 25, 30, 50, 100, 200 or even 1000 amino acid residues. The peptides may be natural occurring or synthesized. Furthermore, the term is also intended to include proteins, i.e. functional biomolecules comprising at least one polypeptide; when  
10 comprising at least two polypeptides, these may form complexes, be covalently linked, or may be non-covalently linked. The polypeptide(s) in a protein can be glycosylated and/or lipidated and/or comprise prosthetic groups. The term "polypeptides" includes enzymes, antibodies, antigens, transcription factors, immunoglobulin, binding proteins e.g. DNA binding proteins, or protein  
15 domains or fragments of proteins or any other amino acid based material. The term "polyamino acid" denotes a molecule constituted by at least 3 covalently linked amino acid residues. The terms may also relate to functional parts or fragments of proteins. Such function may be as providing an appropriate disulfide bridge or disulfide bridge-containing triad or binding, enzymatic or other biological activity.  
20

As used herein, the terms "UV light" or "irradiation" or UV illumination" or "UV irradiation" are a range of wavelengths or a single wavelength of UV or visible light. Furthermore, these terms comprise any wavelength that by means  
25 of non-linear effects and/or multiphoton excitation will excite aromatic amino acid residues electronically. These aromatic amino acids residues comprise phenylalanine, tryptophan and tyrosine.

As used in the context of the present invention, the term "click" chemistry refers to methods of linking different molecules as described in "Diverse  
30 Chemical Function from a Few Good Reactions" by Hartmuth C. Kolb, M. G.

Finn, and K. Barry Sharpless (Angewandte Chemie International Edition Volume 40, 2001, p. 2004-2021).

As used herein, the term "biosensor" comprises an analytical device incorporating biological or biologically-derived sensing elements, such as an amino acid (e.g., cysteine), protein, antibody, nucleic acid, microorganism or cell. The sensing element is either integrated within or intimately associated with a physicochemical transducer. The general aim of a biosensor is to produce either discrete or continuous signals that are proportional to a single analyte or a related group of analytes such as e.g. digital electronic signals or light signals.

As used herein, the term "pharmaceutical drug" comprises articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and articles (other than food) intended to affect the structure or any function of the body of man or other animals and articles intended for use as a component of any article specified above.

As used herein, the term "nanoparticle" refers to (i) "a particle having one or more dimensions of the order of 100nm or less", (ii) "an aggregate of anywhere from a few hundred to tens of thousands of atoms that combine into a crystalline form with a diameter ranging from 3 -25 nanometers", and/or (iii) "a microscopic, nanoscale particle whose size is measured in nanometers". Nanoparticles are often used in medical applications acting as drug carriers or imaging agents.

As used herein, the term "dimer" refers to a structure consisting of two monomers, subunits or elements. These may be coupled by a thiol group or disulfide bridge according to the invention. In case of identical subunits, this dimer is also called "homo-dimer", in contrast to a "hetero-dimer", which consists of

two different subunits. These subunits are not limited to molecules, but can also comprise any elements according to the invention.

As used herein, the term "dendrimer" relates to a tree-like structure, consisting of elements or subunits. The subunits or elements are not limited to molecules, but can also comprise one or more elements according to the invention. The dendrimer may comprise similar or different elements and/or subunits. These subunits may be coupled by one or more thiol groups or disulfide bridge(s) according to the invention. The building block/units of the dendrimer may contain several branching points. These may also contain one or more coupling units and/or branching points that are different from those provided by appropriate disulfide bridges and/or disulfide bridge-containing triads according to the invention.

#### 15 **Detailed description of the invention**

The present invention relates to a method of providing an appropriate disulfide bridge-containing triad to an element A which lacks such appropriate triad for coupling element A to an element B via thiol binding. The invention also relates to a method of providing an appropriate disulfide bridge to element A and to methods of additional provision of appropriate disulfide bridge-containing triad or disulfide bridge to element B. Coupling is achieved while preserving the native structural and functional properties of the coupled elements.

25 Thus, in a first aspect, the present invention relates to the a method of coupling two element A and B, by

- 2) generating an element A\* by providing an appropriate disulfide bridge or disulfide bridge-containing triad to element A, and optionally to element B to generate element B\*
- 30 (2) a) irradiating said element A\* (and B\*) to create a reactive thiol group, and

- b) incubating said irradiated element A\* with said element B capable of binding a thiol group (or B\*), thereby obtaining a coupling between elements A and B;
- or
- 5 a) incubating said element A\* with said element B capable of binding a thiol group or B\*, and
- b) irradiating said element A\* in the presence of said element B (or B\*) to create a reactive thiol group by disulfide bridge disruption in said element A\* (and B\*), and thereby obtaining a coupling between element A and element B.
- 10

Elements according to the invention are molecules and supports, either different or identical, allowing for all permutations, such as A and B are molecules, A is a molecule and B is a support, A and B are supports, and A is a support and B is a molecule; in case of molecule–molecule or support–support couplings, these can be either identical or different.

15

Elements according to the invention comprise molecules and supports, thus providing great flexibility, allowing for coupling combinations of molecule + molecule, support + support, molecule + support and/or support + molecule. In case of coupling support + support and/or molecule + molecule, different, similar or identical support(s) and/or molecule(s) can be coupled. In one aspect of the invention, the molecule is a biomolecule, such as a peptide, a protein, a polynucleotide, a lipid, a sugar, a pharmaceutical, a cosme-

20

25

drug and the like.

Furthermore, in contrast to the state of the art, the invention is not limited to proteins, but allows virtually any molecule(s) and support to be coupled according to the invention, provided that an appropriate disulfide bridge or disulfide bridge-containing triad can be provided, for example by covalent attachment and/or by genetic engineering. This includes virtually any support(s),

30

provided that they can be coupled according to the invention, i.e. that an appropriate linker can be coupled to the support and/or that the support capable of binding a thiol group. A support according to the invention comprises a soluble, semi-soluble or insoluble material to which an appropriate disulfide  
5 bridge or disulfide bridge-containing triad is capable of being attached. Such a support may also comprise a thiol reactive surface or a surface that can be made thiol reactive, e.g. a surface comprising gold. A support which is made reactive for binding one or more molecule(s) is denoted "a carrier".

10 This contrasts with traditional coupling methods for molecule- or protein immobilisation, which typically involve several chemical and/or thermal reactions, which can be costly, time-consuming as well as deleterious to the structure/function of the bound protein. Furthermore, the orientation of the protein, coupled according to the method of the present invention, can be  
15 controlled, such that the functional properties, (e.g. enzymatic activities and/or substrate specificities) are preserved. In comparison, the majority of known protein coupling methods lead to a random orientation of the proteins immobilised on a support, with the significant risk of lower biological activity and thereby e.g. raised detection limits. Furthermore, all functional/structural  
20 assays performed on molecules, such as polypeptides, which are immobilised in a uniform orientation according to the methods of the present invention, will generate data derived from a uniform population of proteins. The structural and functional uniformity of the immobilised proteins, and retention of their native state, is of primary importance for screening or assaying pro-  
25 teins for catalytic, binding, or any other biological properties and provides one of the many valuable advantages of the present invention. In the case of proteins having anti-microbial properties, it is useful to be able to immobilise said proteins on a surface (e.g. food, skin, packaging) in order to prevent microbial growth and infection.

The present invention describes a method, where an element is modified in such a way that it can react with another element to form a covalent coupling upon irradiation. The method according to the invention involves attachment of a linker providing an appropriate disulphide bridge or disulfide bridge-  
5 containing triad, which is convertible by irradiation to contain fully reactive thiol(s) capable of coupling a desired element (support or molecule), said element containing at least one reactive thiol group or at least one disulphide bridge capable of being activated by irradiation to reactive thiol groups. This makes it possible to selectively irradiate pre-determined regions in order to  
10 convert the disulphide-containing linker to contain reactive thiol(s) and thereby obtain coupling of the linker containing element on the activated regions of a support or another molecule. This spatially addressability is a major advantage afforded by the method of the invention.

15 A particular advantage of the present invention is its avoidance of the several disadvantages associated with the chemical generation of free thiols in a protein. Some proteins, e.g., cutinase, are inactivated by the reducing agents (DTT or beta-mercaptoethanol) used to generate free thiols. If a reducing agent comprising a thiol group, is used to chemically generate free thiols in a  
20 protein, it must be removed before immobilisation by can be performed, during which step the disulfide bonds can reform. Alternative reducing agents, such as 2-carboxyethyl)phosphine, lacking a thiol group, have the disadvantage that they are reactive to other groups. The use of reducing agents to disrupt disulfide bonds has the additional disadvantage that they are pH dependent, both with regards their chemical stability and their reducing activity.  
25

In a further aspect of the present invention, the orientation of the immobilised element, such as a polypeptide can be controlled in a uniform and reproducible manner.

30 It is, however, also possible according to the invention to immobilise elements already containing at least one reactive thiol group. According to this

aspect of the invention, only activation of the linker is necessary. The element may contain such reactive thiol group(s) in their native form or they may have been formed after chemical treatment.

- 5 In one aspect of the invention, elements A or B or elements A and B comprise a linker molecule, containing an appropriate disulphide bridge or disulphide bridge-containing triad, and the elements are activated by irradiation to create reactive thiol groups in the same step or sequentially. The element, molecule or support may comprise one or more disulphide bridges or reactive
- 10 thiols and the disulphide linker may after irradiation thus be coupled to the molecule or support with either one or more disulphide bonds.

In one aspect, the molecule or support comprises one disulphide bridge, and said molecule or support are irradiated simultaneously with the linker containing

15 molecule to create reactive thiol groups.

In another aspect of the invention, the free thiols in said molecule or support are formed after chemical treatment e.g. using a disulphide reducing agent such as DTT.

20

#### Linker

Another aspect of the invention relates to a linker molecule comprising an appropriate disulfide bridge or disulfide bridge-containing triad, which may be provided to an element lacking an appropriate disulfide bridge or disulfide

25 bridge-containing triad.

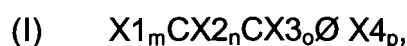
The invention allows for a variety of different combinations concerning couplings between elements. Non-exclusively, Figure 1 illustrates a variety of different embodiments of the invention concerning irradiation-mediated coupling

30 of two elements (elements, supports and/or molecules), where a linker  $L_A$  is provided to an element A, or a linker  $L_A$  and a linker  $L_B$  are provided to both

elements. Non-exclusively, these embodiments comprise: (a) A linker L is provided to element A, thus generating element A\*, which is now capable of being coupled to element B; (b) A linker L<sub>A</sub> is provided to element A, and a linker L<sub>B</sub> is provided to element B, thus generating elements A\* and B\*, which are now capable of being coupled; (c) Element A comprising a linker L is coupled to a support; (d) Element A comprising a linker L<sub>A</sub> is coupled to a support B via a linker L<sub>B</sub> provided to support B; (e) Support A comprising a linker L is coupled to a support B; (f) Support A comprising a linker L<sub>A</sub> is coupled to a support B via a linker L<sub>B</sub> provided to support B; (g) A linker L is provided to element A, which is now capable of being coupled to element B; elements A and B are part of the same structure; (h) A linker L<sub>A</sub> is provided to element A, and a linker L<sub>B</sub> is provided to element B, thus generating elements A\* and B\*, which are now capable of being coupled; elements A and B are part of the same structure; (i) A linker L is provided to support at position A, which is now capable of being coupled to another position (B) of said support; by such "internal" coupling, the structure may be bent or twisted the desired way. Alternatively, coupling may also occur via a linker L<sub>B</sub> provided to B (not shown). Another embodiment of the invention comprises examples as above, where more than one linker is provided to element A or elements A and B (not shown).

As exemplified in Figure 1 (g-i), elements A and B can be part of a common structure, such as part of a support, (e.g. carrier, surface and the like), and/or part of a molecule). In order to achieve such a coupling, or as a result of a coupling or both, the structure may be bent or twisted. In a further embodiment of the invention, the support comprises a foil-like structure, which can be bent or folded, and remains bent or folded after coupling.

In an alternative embodiment of the invention, a linker peptide comprises one or more copies of a peptide comprising any of the formulas:



(II)  $X1_mCX2_n\emptyset X3_oCX4_p$ ,

(III)  $X1_m\emptyset X2_nCX3_oCX4_p$ ;

wherein  $X1_m$ ,  $X2_n$ ,  $X3_o$  and  $X4_p$  represent the same or different (poly) peptides, each peptide respectively consisting of  $m$ ,  $n$ ,  $o$ , or  $p$  amino acids, where  $m$ ,  $n$ ,  $o$ ,  $p$  are mutually independent numbers between 0 and 1000 (or 100, 25, 10) and  $m + n + o + p \leq 1000$  (or 100, 25, 10), said amino acids being selected from all natural and synthetic amino acids, C is cysteine, both the two cysteines are covalently joined by a disulfide bridge, and  $\emptyset$  is an aromatic amino acid such as phenylalanine, tryptophane or tyrosine, or their derivatives; or  $\emptyset$  represents a peptide bond.

In a further embodiment, the different polypeptides  $X1_m$ ,  $X2_n$ ,  $X3_o$  and  $X4_p$  do not comprise an additional reactive thiol group.

In yet further embodiment, one or more aromatic amino acids are present in the different polypeptides  $X1_m$ ,  $X2_n$ ,  $X3_o$  and  $X4_p$ .

When the linker is activated by irradiation to contain reactive thiol groups, part of the linker may be set free (a leaving group) as a by-product. The by-product (or leaving group) will usually be washed away from the surface if they interfere with subsequent reactions. The free thiol group still part of the linker can participate in the formation of a new disulphide bond to a free thiol in the polypeptide. In one embodiment of the invention, the linker is designed so as to not leave any by-product. In another embodiment of the invention, the linker is designed so to contain a leaving group.

In a further alternative embodiment of the invention, the peptide linker comprises one or more of the formula

(IV) LD;

wherein L is attached to the support and comprises at least one amino acid which does not contain a reactive thiol and which is different from an amino

acid which is capable of being activated by irradiation to contain at least one reactive thiol group and D is a non-cyclic sequence of amino acids or a cyclic sequence of amino acids, which non-cyclic or cyclic sequence comprises at least two cysteines (C) covalently joined by a disulphide bridge and wherein  
5 one of the cysteines (C) is bound to L. In one embodiment of the invention the two cysteines (C) are covalently joined by a disulphide bridge (-C-S-S-C-). In another embodiment, they are covalently joined by a disulphide bridge and also by a peptide bond (-C-C-). In a further embodiment, the peptide linker comprises at least one aromatic amino acid which may be  
10 part of either D or L. In an alternative embodiment, neither D nor L contains an aromatic amino acid.

According to one embodiment of the invention, L comprises 1-30 amino acids, 3-20 amino acids or 5-10 amino acids. According to a further aspect of  
15 the invention, D comprises 2-30 amino acids, 3-20 amino acids or 5-10 amino acids.

According to still another embodiment of the invention, L comprises one or more aromatic amino acids, such as phenylalanine, tryptophane, tyrosine or  
20 their derivatives.

In a further aspect of the invention, L comprises an aromatic amino acid separated from the cysteine (C) in D bound to L by at least one amino acid.

25 In a further embodiment of the invention, D is a cyclic sequence of amino acids.

According to one embodiment of the invention, D has the following sequence  $C(X)_n C$ , wherein X independently is any amino acid which does not comprise  
30 a reactive thiol group, n is from 1 to 10, 2 to 8 or 3 to 6, and the two cysteines (C) are covalently joined by a disulphide bridge. In another aspect of the in-

- vention, D has the following sequence  $CC(X_1)_{n1}$ , wherein  $X_1$  independently is any amino acid which does not comprise a reactive thiol group,  $n1$  is from 0 to 10, 2 to 8 or 3 to 6, and the two cysteines (C) are covalently joined by a disulphide bridge and a peptide bond. In another aspect of the invention D
- 5 has the following sequence  $C-S-S-C(X_1)_{n1}$ , wherein  $X_1$  independently is any amino acid which does not comprise a reactive thiol group,  $n1$  is from 0 to 10, 2 to 8 or 3 to 6, and the two cysteines (C) are covalently joined by a disulphide bridge.
- 10 In a further embodiment of the invention, L has the following sequence  $(X_3)_{n3}W(X_4)_{n4}$ , wherein  $X_3$  and  $X_4$  independently are any amino acid which does not comprise a reactive thiol group, W is tryptophan and  $n3$  and  $n4$  each independently are from 1 to 5.
- 15 In yet a further embodiment of the invention, LD has the following sequence  $K(X_5)_{n5}WX_6CGGGC$ , wherein  $X_5$  and  $X_6$  independently are any amino acid which does not comprise a reactive thiol group, W is tryptophan, K is lysine,  $n5$  is 3, G is glycine and the two cysteine molecules (C) are covalently joined by a disulphide bridge, for example the following sequence KAM-
- 20 HAWGCGGGC-NH<sub>2</sub>, wherein CGGGC is cyclic and the two cysteine molecules (C) are covalently joined by a disulphide bridge, K is lysine, A is alanine, M is methionine, H is histidine, W is tryptophan, and G is glycine. In another embodiment of the invention, LD has the following sequence  $KAMHAWGC-S-S-CX_7X_8-NH_2$ , wherein  $X_7$  and  $X_8$  independently are any
- 25 amino acid which does not comprise a reactive thiol group, the two cysteine molecules (C) are covalently joined by a disulphide bridge, K is lysine, A is alanine, M is methionine, H is histidine, W is tryptophan, and G is glycine preferably the following sequence  $KAMHAWGC-S-S-CGG-NH_2$ , wherein the two cysteine molecules are covalently joined by a disulphide bridge, K is ly-
- 30 sine, A is alanine, M is methionine, H is histidine, W is tryptophan, and G is glycine.

In any of the above sequences, any of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ ,  $X_6$ ,  $X_7$ , and  $X_8$  are independently any amino acid except cysteine. In one aspect of the invention, the amino acid is selected from the group consisting of basic amino acids such as Lys, Arg or His, acidic and amidic amino acids such as Asp, Glu, Asn or Gln, amino acids with non-charged side chains such as Gly, Ala, Val, Leu, Ile, Pro, Ser, Thr, Met, Phe, Tyr or Trp. In a further aspect of the invention, the amino acid is selected from the group consisting of Gly, Ala, Val, Leu or Ile. In another aspect of the invention, the amino acid is selected from the group consisting of Gly, Ala, Val or Leu, and in a further aspect the amino acid is selected from the group consisting of Gly, Ala or Val and in still a further aspect the amino acid is selected from the group consisting of Gly or Ala such as Gly.

15 In an alternative embodiment, L, D or LD comprises phenylalanine, tyrosine or derivatives of tryptophane, phenylalanine and tyrosine, instead of tryptophane (W).

20 In one embodiment according to the invention, D is as described above and is directly attached to the support, and D comprises one or more aromatic amino acids.

25 In another embodiment, L, D or LD does not comprise an aromatic amino acid. Such a linker comprises an appropriate disulfide bridge, if said disulfide bridge can be activated/broken by an aromatic amino not part of the linker, such as an amino acid provided by the element A the linker  $L_A$  (L, D or LD) is coupled to. In an alternative embodiment, the amino acid is provided by the other element B or by the linker  $L_B$  (L, D or LD), being part of element B. In an alternative embodiment, the amino acid is provided as free amino acid.

Figure 2 illustrates an embodiment of the invention and shows an element A, to which a cyclic peptide linker CXWXC is attached, the cysteine joined via a disulphide bridge, which disulphide bridge is disrupted when irradiated to create reactive thiol groups, W is tryptophane (or another aromatic amino acid and X is any amino acid which does not comprise a reactive thiol group, and Y (not to be confused with the single letter code for tyrosine) is part of the linker and/or element A providing attachment of the linker to element A. In this embodiment element B contains a disulphide bridge which is activated to contain reactive thiol groups when element A\* (i.e. element A + linker) and element B are irradiated. Further non-exclusive embodiments are depicted in Figure 3: (a) cyclic peptide linker KXXXXWXCXXXC, wherein C is cysteine joined via a disulphide bridge. Upon irradiation, one or two or both cysteines may be involved in coupling to the other element; (b) non-cyclic peptide linker KXXXXWXC-S-S-CXX, wherein two cysteines are joined via a disulphide bridge and via a peptide bond; (c) cyclic peptide linker CXWXC, where a part of the linker is a leaving group as both cysteines are only joined via a disulphide bridge; (d) non-cyclic peptide linker C-S-S-CXW, wherein C are cysteines joined via a disulphide bridge and via a peptide bond. Upon irradiation, one or two or both cysteines may be involved in coupling to the element; (e) non-cyclic peptide linker KXXXXWXCCX, where a part of the linker is a leaving group as both cysteines are only joined via a disulphide bridge W is tryptophan, K is lysine, X is any amino acid which does not comprise a reactive thiol group, and Y (not to be confused with the single letter code for tyrosine) is part of the linker providing attachment of the element to which the linker is coupled.

In another embodiment, the aromatic amino acid  $\emptyset$  is absent; such a linker comprises an appropriate disulfide bridge, if it can be activated/broken by an aromatic amino not part of the linker, such as an amino acid provided by the element A the linker  $L_A$  is coupled to. In an alternative embodiment, the amino acid is provided by the other element B or by the linker  $L_B$ , being part

of element B. In a further alternative embodiment, the amino acid is provided as free amino acid.

5 In yet another embodiment, the linker comprises an aromatic amino and not a disulfide bridge. Thereby, the linker provides an appropriate disulfide containing triad, by bringing an aromatic amino acid in the vicinity of an appropriate disulfide bridge, said disulfide bridge being part of either element A, element B or elements A and B. In a further embodiment, the linker comprises only one amino acid, said amino acid being an aromatic amino acid, or its derivative. In an embodiment, s

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In still a further alternative embodiment, the linker provides a cysteine, capable of forming a disulfide bridge with element A or B, thus providing an appropriate disulfide bridge according to the invention. Such a linker may also provide the aromatic amino acid necessary for forming a triad. Alternatively, the aromatic amino acid is provided by either element A, element B or both, or as free amino acid.

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In yet another alternative embodiment, a triad is formed via interaction of to linker.

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#### Leaving group

As illustrated in Figure 3 (c) and (e), part of the linker may be set free (a leaving group) as a by-product upon disruption of the linker's disulphide bridge.

25 When the disulphide-containing linker is activated by irradiation to obtain reactive thiol groups, part of the linker may be set free (a leaving group) as a by-product. The by-product (or leaving group) will usually be washed away from the surface if they interfere with subsequent reactions. The free thiol group still part of the linker can participate in the formation of a new disulphide bond to a free thiol in the polypeptide. In one aspect, the disulphide-containing linker is designed so as to not leave any by-product.

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### Linker – covalent binding

In one aspect of the invention, a linker as described above is provided to an element through covalent binding of a linker molecule. Many methods are available for the skilled person for making the disulphide-containing linker used in the present invention such as e.g. described in Kimmerlin T. Seebach D. (2004). '100 years of peptide synthesis': ligation methods for peptide and protein synthesis with applications to b-peptide assemblies', J Pept Res, Vol 65, Issue 2, pp. 229-260, McKay, F.C. & Albertson, N.F. (1957) New amine-masking groups for peptide synthesis. J. Am. Chem. Soc. 79, 4686–4690 and Carpino, L.A. (1957) Oxidative reactions of hydrazines. IV. Elimination of nitrogen from 1,1-disubstituted-2-arenesulfonhydrazides. J. Am. Chem. Soc. 79, 4427–4430, or "click" chemistry ("Diverse Chemical Function from a Few Good Reactions" by Hartmuth C. Kolb, M. G. Finn, and K. Barry Sharpless (Angewandte Chemie International Edition Volume 40, 2001, p. 2004-2021) are incorporated herein by reference.

Methods for attaching the linker according to the invention to a molecule will be apparent to those skilled in the art and comprise e.g. binding an amine in the peptide linker with an aldehyde via a Schiff-base, cross-linking amine groups in the peptide linker to an amine surface with gluteraldehyde to form peptide bonds, cross-linking carboxylic acid groups present in the peptide linker and molecule to be coupled with carbodiimide, cross-linking based on disulphide bridge formation between two thiol groups and the formation of a thiol-Au bond between a thiol group and a gold surface, preferably the support is aldehyde derivatised silicon or quartz and is attached via a Shiffs bond to the lysine amine or the N-terminal amine of lysine. Other linker-support attachment methods include covalent coupling via e.g. ester bonds, amide bonds, as well as non-covalent coupling such as ionic bonding and hydrophobic interactions.

In one embodiment of the invention, the linker is provided to an element, support, molecule and/or protein, polypeptide or peptide through covalent binding of a linker molecule, e.g. using NHS (N-hydroxysuccinimide), EDC (N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride), activated ester, maleimide, disulfide formation, streptavidin/biotin, activated alcohol, vinylsulfone, Schiff base formation and/or "click" chemistry and the like.

In another embodiment of the invention, the linker is provided via chemical coupling to a protein by NHS, EDC or NHS and EDC chemistry.

In yet another embodiment of the invention, the reaction products will be fractionated and/or purified, in order to remove unwanted reaction products. These can comprise molecules, where more than one linker has been attached to, or where one or more linkers are bound inappropriately with respect to the desired coupling reaction according to the invention, or where attachment of the linker impeded desired function and/or activity of the molecule.

#### B-FABP

Human Brain Fatty Acid Binding Protein (B-FABP) was selected as model protein, as a representative for proteins containing neither an appropriate disulfide bridge nor a disulfide bridge-containing triad for irradiation induced coupling according to the invention. It belongs to a family known as the intracellular 14-15 kDa lipid binding proteins. This group of proteins is known for their ability to bind amphiphils like bile acids, retinoids, eicosanoids, and fatty acids [Balendiran et al., 2000]. Human B-FABP consists of 131 residues and has a molecular weight of 14777.8 Da [www.pdb.org, accessed 05-12-2006]. Its primary structure is given in Table 2 (see Example1), and the dimensions are, using DeepView 3.7, found to be approximately 40 Å x 35 Å x 35 Å. The tertiary structure of the protein constitutes a β-barrel. This β barrel consists of 10 antiparallel β strands making up two almost perpendicular β sheets.

These  $\beta$  sheets surround a hydrophobic cavity in which the substrate is bound. The cavity is filled with water molecules of which some are released upon substrate binding, resulting in a change in protein intrinsic fluorescence [Reese and Banaszak, 2004]. One end of the  $\beta$  barrel is in some conforma-  
5 tions blocked by a helix-turn-helix structure. This structure constitutes a lid which acts as a binding site cavity entrance and exit for the substrate. Opposite to this binding site entrance is both the N- and C-terminus of the protein, and they are both solvent accessible [Chmurzynska, 2006], Figure 4 (Left: Side view. Right: View rotated 90° to the right). There are several solvent ex-  
10 posed Glu, Asp, Lys; none of the Trp residues are solvent exposed; one Cys residues is solvent exposed. The structure is obtained from [www.pdb.org, accessed 04-02-06] and the images are generated using PyMOL 0.98.

B-FABP is in its native form not expected to be suitable for light induced im-  
15 mobilization, as no appropriate disulfide bridge and thus no disulfide bridge-containing triad exists in this protein. It does however contain two unlinked cysteine residues at positions 5 and position 80. One of the two residues is surface exposed and considered reactive towards e.g. thiol activated sur-  
20 faces. This residue is positioned close to the N- and C-terminus. The protein contains two Trp at position 8 and 97. Trp8 is fully buried while Trp97 is partially buried.

With respect to coupling of a linker according to the invention using EDC/NHS chemistry using the primary amines, B-FABP contains 12 Lys  
25 residues and the N-terminus. Also of interest for EDC/NHS chemistry are carboxyl groups. The protein contains 10 Asp residues, 10 Glu residues and the C-terminus. Investigating these residues and termini in terms of solvent accessibility using the Sting Millennium Suite program [mirrors.rscb.org, accessed 05-25-2006] and the PDB file 1FE3 it can be seen that the protein  
30 contain approximately 13 solvent accessible primary amines and 18 solvent accessible carboxyl groups (Figure 4).

The successful UV-induced coupling of Human Brain Fatty Acid Binding Protein (B-FABP) to a quartz surface by chemical cross-linking of a peptide linker has been demonstrated in Examples 1, 2, 4-9. In short, B-FABP, devoid of an appropriate disulfide bridge or disulfide bridge-containing triad, was chemically coupled with a linker peptide containing a disulfide bridge-containing triad, and coupled to thiol activated quartz surface according to the invention. The examples contain the protocols used for the chemical coupling of W6/SS peptide (Table 2), comprising an appropriate disulfide bridge-containing triad with the amino acid sequence KAMHAWGCGGGC to B-FABP and the UV-irradiation-mediated coupling of the W6/SS-B-FABP to a support (thiol-activated quartz surface) via thiol bonds. This includes expression of recombinant B-FABP in *E. coli*, folding and purification, buffer exchange, EDC/NHS chemistry for chemically coupling a linker molecule, fluorophore-labeling of cross linked protein and UV-light induced coupling to quartz surface (Figure 5).

#### Genetic engineering

A different aspect of the invention relates to the use of techniques involving molecular biology and/or recombinant DNA techniques in order to provide an appropriate disulfide bridge or disulfide bridge-containing triad to a protein. By the use of these molecular techniques, novel, artificial DNA sequence can be created, or existing sequences can be altered or combined. Although processes such as crossing over technically produce recombinant DNA, the term is generally reserved for DNA produced by joining molecules derived from different biological sources. Recombinant DNA techniques can be used to introduce subtle changes in a protein, such as exchange of individual amino acids, point mutations or more substantial changes, such as insertion, deletion or replacement of a string of amino acids, or even fusion of two different polypeptides by providing the appropriate changes on the DNA level. Alternatively, completely new DNA and polypeptide sequences can be cre-

ated. By exploiting the universal genetic code, these molecular techniques can be used to express virtually any desired protein in a wide range of host organisms. The appropriate host or expression organisms and expression systems are known to the artisan. Often, the genes and/or cDNAs in ques-  
5 tions are cloned in vectors such as plasmids, and a wide range of commercial vectors are available, including expression vectors for a wide range of organisms. Alternatively to using vectors and plasmids, the DNA sequences of interest can be inserted into the genome of the desired host organism.

### 10 Triad

The features of the amino acid composition around a spatial disulfide bridge-containing triad (SSØ) in a protein, which may be used to predict whether it will be susceptible to UV-induced disruption, are as follows: 1) an aromatic amino acid residue is located within 15, 12 or 10 Å of the disulfide bond; 2)  
15 those SSØ in which the angle between the plane of the emitting dipole of the aromatic side chain is orthogonal to the plane of the absorbing dipole of the disulfide bridge are not expected to exchange excitation energy between excited state of the aromatic amino acid and a disulfide bridge. Furthermore, a specific subset of amino acids residues are found within an 8Å radius of a  
20 UV-disruptable Trp S-S triad of a protein has been demonstrated for cutinase, lysosyme, lipase, plasminogen, chymosin and α-lactalbumin, and immunoglobulin which are distinct from the average frequency of occurrence of amino acids in a protein in general. In this specific subset of amino acids, the amidic amino acid residues (Asn, Gln) are over-represented, and in the ma-  
25 jority of cases the occurrence of short aliphatic residues (Gly, Ala, Val) and/or long aliphatic residues (Leu, Ile) are also over-represented.

Furthermore, the location of appropriate disulphide bridges, forming free thiol groups upon irradiation, can be predicted from the protein's structure, where  
30 it is known from three-dimensional models, nuclear magnetic resonance (NMR) or X-ray diffraction crystallography analysis. In those cases where only a single thiol group is induced by irradiation of tryptophan residues in a

protein, as is the case for cutinase, then immobilisation of the protein on a support will occur exclusively via this thiol group. In contrast to alternative methods of disulphide bridge disruption, the light-induced method of the present invention leads to targeted disruption of disulphide bridges forming one  
5 or only a few accessible thiol groups, whose position can be precisely predicted. The subsequently immobilised proteins will thus have a single or very limited number of orientations.

The artisan will be able to locate and or design appropriate disulfide bridges  
10 and/or disulfide bridge containing triads by the use of protein modelling. Furthermore, the formation of new thiol groups formed in proteins following light-induced disulphide bond disruption can be analyzed by a 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB] based assay (Elleman's assay, see Example 6).

15 Thus, one embodiment of the invention relates to methods of recombinant DNA technology, which are utilized to introduce amino acid substitutions, insertions or deletions into a protein sequence, to create an appropriate disulfide bridge and/or disulfide bridge-containing triad, by e.g. introducing an aromatic amino acid in the vicinity of a disulphide or cysteines capable of  
20 forming a disulphide bridge may be introduced in close spatial proximity to an endogenous aromatic residue. Alternatively, an appropriate disulfide bridge and/or disulfide bridge-containing triad may be introduced.

Another embodiment of the invention relates to creating a change in the con-  
25 formation of a protein through molecular techniques, thereby bringing an aromatic amino acid and an appropriate disulphide bridge in the vicinity of each other. A further embodiment of the invention relates to providing to provide an appropriate disulfide bridge or disulfide bridge-containing triad through amino acid exchanges, insertions, deletions or any combination, re-  
30 sulting in an appropriate disulfide bridge or disulfide bridge-containing triad.

Providing a linker by genetic engineering

In another major aspect of the invention, recombinant DNA technology is used to provide a linker comprising an appropriate disulfide bridge and/or disulfide bridge-containing triad. These techniques may comprise the use DNA sequences coding for N-terminal extension, C-terminal extension, or internal extension (insertion), amino acid substitution, amino acid insertion, amino acid deletion or any combination or combinations of said methods. Such a linker may comprise any of the sequences (formulas I-IV) as discussed above.

10

The disulphide-containing linker is in one aspect of the invention a peptide linker comprising at least one amino acid. In a further aspect, the peptide linker comprises at least one aromatic amino acid.

15

The successful UV-induced coupling of a recombinant, SS-fusion tagged Human Brain Fatty Acid Binding Protein (B-FABP) to a quartz surface has been demonstrated in Examples 3-9. In short, B-FABP was fused with a disulphide containing peptide (SS peptide) to the C-terminus of B-FABP by conventional molecular biology /recombinant DNA techniques, and the fusion protein was coupled onto a thiol activated quartz surface using UV (Figure 6). This represents an example of providing a linker to a molecule, said linker providing an appropriate disulfide bridge and the molecule is providing the aromatic amino sufficiently close to the disulfide bridge to forming the appropriate triad.

20

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Figure 6 shows fluorescence emission at 670 nm from a slide onto which SS tagged and labeled B-FABP has been deposited. It is seen that the fluorescence is more intense from small spots of the 5x5 array, which corresponds to the areas illuminated with the 280 nm laser. The fact that emission intensity is highest for the areas subjected to UV light indicates that the immobilization is through disulphide bond formation between a Cys residue from a

30

broken disulphide bridge and the thiol activated glass surface. Had the immobilization been through non-covalent interactions (e.g. adsorption), it would have resulted in a uniform immobilization and therefore, a uniform emission from the surface. Such a result would also be expected if the coupling had been between a solvent exposed Cys residue and the thiol activated surface, as this reaction would be independent of UV-illumination. Even though immobilization was achieved through disruption of the disulphide bridge, the SS tagging peptide without Trp is not a universal peptide for immobilization. The peptide is only expected to enable immobilization of proteins with amino acid arrangements similar to that of B-FABP. Specifically, B-FABP contains one Trp residue (8) and one Tyr residue (128) near the C-terminus, where the SS-tagging peptide is coupled.

Thus, for proteins where no aromatic residue is found in the proximity of the C-terminus, the position of an existing disulphide bridges must be investigated. If a disulphide bridge is close to the surface, introduction of a Trp or a Tyr residue within 15, 12 or 10 Å may enable light induced immobilization of the protein and *vice versa*. Therefore, cloning strategies C- or N-terminal or internal protein manipulation for enabling light induced immobilization may also comprise: (i) providing a peptide with a disulfide bridge-containing triad; (ii) providing a linker with an appropriate disulfide bridge disulphide bridge, when an aromatic amino acid is provided by the target protein or (iii) providing an aromatic amino acid in the target protein, thereby forming the triad with the appropriate disulfide bridge of the linker.

25

#### Fusion with protein

A different embodiment of the invention relates to generating a fusion, comprising a portion of a protein providing the appropriate disulfide bridge or disulfide bridge-containing triad fusion with the target protein to be coupled, which is lacking such an appropriate disulfide bridge or disulfide bridge-containing triad.

30

With respect to the requirements for a suitable linker, the peptide or polypeptide must have a free C- or N-terminus, preferably with sufficient spacing to the first cystine residue. Additionally the three dimensional structure of the peptide must be solved to allow three dimensional modeling which may help to determine if the above stated requirements can be achieved.

In the quest for a suitable protein providing an appropriate disulfide-containing triad via fusion to proteins lacking such a photo-active triad, the available literature was searched using following criteria in the mentioned order:

1. Solved three-dimensional structure.
2. Length of the amino acid sequence, preferably below 30.
3. Presence of Trp residue.
4. Position of Trp residue, must be within only a few Å of at least one disulphide bridge, preferably more than one.
5. If these requirements are not met, the following must be considered:
  - a. Presence of mutable amino acid in good position with respect to the disulphide bonds. Preferably Tyr residues because of the resemblance in size with Trp.
  - b. Other needed mutations and the ease by which these can be performed. For example the removal of unwanted Trp residues.

### Knottin

As illustrative of the present invention, a family of peptides was selected which was suitable for this approach, namely the knottin peptides. An online database exists which contains the known knottin peptides. The database is found at <http://knottin.com> and is described in Gelly et al. (2004). The family of knottin peptides is a collection of relatively small peptides. This family is characterized by a structural knot facilitated by disulphide bonds. This structure is a special structural motif found in a growing number of peptides. In

fact the motif is so widespread that peptides are classified according to the motif although the conservation of the amino acid sequence between them is very low [Craik et al., 2001]. Knottins are both found in plants, insects, and animals and are among others utilized as toxins [Craik et al., 2001], [Gelly et al., 2004]. To make a knot in a peptide there needs to be three disulphide bonds arranged as a ring made by two disulphide bonds with a third disulphide bond penetrating the ring. In Figure 7 it can be seen that Cys(I-IV) and Cys(II-V) form a ring which is penetrated by Cys(III-VI). The cystines are numbered from the N-terminus to the C-terminus. The image is adapted from [www.knottin.com, accessed 05-12-2006]. Right image: In order to clarify the structure of a knottin peptide HpTX2 (PDB: 1EMX) is shown as cartoon with disulphide bonds as lines; the cystines are numbered as in the left image. By comparing the right image with the left schematic, it is seen that HpTX2 indeed adopts a knotted structure. Above the right image the primary sequence is seen with disulphide bonds indicated and cystines highlighted. The image has been generated using PyMOL 0.98. The structure of HpTX2 is found at [www.pdb.org, accessed 05-12-2006].

A general characteristic of the knottin peptides is their very high stability which is believed to originate from the three disulphide bonds and the knotted topology. In addition the knot is normally very densely packed leading to reduced mobility of the structure hence also increasing the stability [Craik et al., 2001], [Gelly et al., 2004]. The knottin peptides show different possibilities for several therapeutic treatments primarily owing to their toxic nature. Some knottin peptides block sodium channels which make them applicable for pain therapy while others are potential antibacterials because of antimicrobial properties. Most important in this context is their potential for use as scaffolds in protein engineering. This utilizes the great stability of knottin peptides because substantial changes can be introduced in the loop regions of the peptide giving it new properties without destroying the stability [Craik et al., 2001].

Vector providing a linker

Examples 10-14 illustrate the development of a mutant knottin peptide (HpTX2) for recombinant fusion to proteins lacking an appropriate disulfide  
5 bridge or disulfide bridge-containing triad. A universal expression vector was designed and produced for fusion of an engineered, cysteine-rich (S-S rich) peptide, based on the knottin fold HpTX2, to the C terminus of virtually any protein devoid of an appropriate disulfide bridge or disulfide bridge, like B-FABP.

10

A further aspect relates to a vector or plasmid providing an appropriate disulfide bridge or catalytic triad fused to the N- or C-terminus of a protein to be expressed by said vector or plasmid in an appropriate host.

15 Another aspect relates to a vector or plasmid that provides an appropriate disulfide bridge or catalytic triad by fusion of a protein by C- or N-terminal fusion to a protein or part thereof or a natural or synthetic polypeptide or peptide. One example of a useful linker peptide is knottin (HpTX2).

20 Experimental evidence of two suitable vectors, based on a fusion construct with a mutated knottin providing a disulfide-bridge containing triad is given by the inventors. Examples 13-14 demonstrate the feasibility of a universal expression vector, such that a linker peptide, which contains the UV reactive triad in form of a disulfide bridge-containing triad and any protein are trans-  
25 lated during protein synthesis as one fusion protein.

Free aromatic amino acid

In yet another aspect of the invention, coupling of two elements may be provided by adding a free aromatic amino acid, either alone or being part of a  
30 molecule, to the vicinity of one or more appropriate disulfide bridges-

In another embodiment, free amino acid is provided in solution. The invention is not limited to a single, free aromatic amino acid but may also comprise a mixture of two or more different aromatic amino acids.

- 5 In a further embodiment, the free aromatic amino acid is part of a molecule.

In yet another embodiment, more than one aromatic amino acid or their analogues or derivatives with comparable excitation and emission profiles are provided being part of one or more molecules, not attached to either element A  
10 or B.

#### Irradiation

The irradiation step according to the invention comprises light of a wavelength that excites one or more aromatic amino acids. This may be achieved  
15 using UV light, such as UV light in the wavelength interval of 250 to 305 nm, or with light having longer wavelengths that by means of non-linear processes and/or multiphoton excitation promotes the same electronic transitions as light in said wavelength interval of 250 to 305 nm.

20 In one embodiment, the wavelength interval is preferably 250 to 260nm, 270 to 280 nm or 290 to 300nm, and more preferably about 254, 275 or 295 nm. Alternatively, a combination of different wavelengths may be applied, which may comprise the above mentioned intervals.

25 In another embodiment of the invention, the irradiation is preferably performed with a light source of a wavelength of between about 250 nm and about 320 nm, more preferred between about 275 nm and about 300 nm, or IR/visible light for multi-photon excitation. In yet another embodiment of the invention the irradiation step comprises light of a wavelength that specifically  
30 excites one or more aromatic amino acids, or other molecular system that may mimic aromatic amino acids, preferably light of a wavelength that excites

one specific aromatic amino acid such as e.g. the wavelength of approximately e.g. 295 nm, 275 nm or 254 nm that excites respectively tryptophan, tyrosine or phenylalanine, most preferably the wavelength about 295 nm that excites tryptophan or the wavelength about 275 nm that excites tyrosin, or  
5 multi-photon excitation, for example 2-photon excitation of between e.g. 500 nm and 640 nm or 3-photon excitation of between 750 nm and 960 nm. In one aspect of the invention, the polypeptide is irradiated in the presence of a free aromatic amino acid, such as Trp, Tyr and Phe.

10 It will be apparent to those skilled in the art that the disruption of disulphide bonds in a given protein at a selected wavelength can be predicted from the location and amino acid neighbours of each disulphide bridge in the 3D structure of the protein. Disulphide bridges placed in the spatial vicinity of aromatic amino acid residues are likely to be the most labile to UV light. The 3D structures of a subset of proteins containing the spatial triad Trp Cys-Cys, in close  
15 spatial proximity, have been examined in order to identify which amino acids are located in immediate vicinity of the tryptophan residues of the triad (WO04/065928). This analysis has identified those proteins having a similar amino acid neighbourhood composition around the triad to that of cutinase,  
20 which can be used to predict which proteins will have the disulphide bond of the triad broken upon UV illumination.

Irradiation with 295 nm light permits the selective excitation of tryptophan residues in a protein, which in turn may lead to the disruption of a single or a  
25 limited number of disulphide bonds. A variety of light sources suitable for the irradiation of proteins at a range of wavelengths, for the photo-induction of disulphide bond disruption include, but are not limited to, a 75-W Xenon arc lamp from a research grade spectrometer such as a RTC PTI spectrometer, a deuterium lamp, a high pressure mercury lamp. Irradiation at a single  
30 wavelength can be obtained by coupling the light source to a monochroma-

tor. A source of single and multiple photon excitation includes a high peak-power pulsed or CW laser.

5 Prolonged selective excitation of tryptophan residues in a polypeptide such as a protein will only lead to disruption of those disulphide bridges to which excitation energy is transferred.

10 It has been shown that a 5 sec illumination is sufficient for successful immobilization of W6/SS-cross linked B-FABP or SS-tagged B-FABP (Figures 5 and 6). In another embodiment, illumination time is around 1 msec, 10 msec, 100 msec, 1 sec, 5 sec, 10 sec, 30 sec, 1 min, 5 min or more.

#### Support/surface

15 An aspect of the invention provides a coupling between elements, resulting in an immobilization on a support or surface, which can be spatially controlled. Such a support and/or surface may also be a derivatized support that is capable of binding a thiol group, such as a support and/or surface comprising a thiol group or a disulfide bridge. When appropriate, such a surface or support may comprise a spacer.

20

This method of light induced thiol coupling can also be used to immobilise a protein on a support. The most common types of bonds that are formed during coupling to a support are disulfide bonds and sulfur-metal bonds (primarily sulfur-gold) where a self-assembled layer is formed. As both types of  
25 bonds are stable, extensive washing after immobilization will not displace the protein.

30 In one embodiment of the invention, the support comprises a spacer. In Figures 2 and 3, such a spacer may be represented in the form of "Y". In an alternative embodiment, the spacer is covalently bound to the support. In another embodiment, the spacer is a part of the linker molecule to be coupled

according to the invention. In yet another embodiment, the spacer improves the coupling reaction according to the invention.

5 The support to which a molecule is coupled according to the invention may be any biological, non-biological, organic, inorganic or a combination of any of these materials existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. It can have any convenient size and shape.

10 In one embodiment of the invention, the support is insoluble.

In a further embodiment of the invention, the support comprises a material selected from the group consisting of polymer such as polystyrene, polyethylene, polyester, polyetherimide, polypropylene, polycarbonate, polysulfone,  
15 polymethylmethacrylate, or poly(vinylidene fluoride), and silicon or quartz.

In still a further embodiment, the support is selected from the group consisting of an electronic chip, slide, wafer, resin, well, tube, micro array and membrane. In another aspect the support comprises a material selected from the  
20 group consisting of polystyrene, polyethylene, polyester, polyetherimide, polypropylene, polycarbonate, polysulfone, polymethylmethacrylate, poly(vinylidene fluoride), and silicon.

In yet another embodiment of the invention, the insoluble support may be  
25 coated with a layer of the disulphide-containing linker.

In yet a further embodiment of the invention, the polypeptide coupled to the support is selected from the group consisting of an enzyme such as selected from the group consisting of cutinase, chymosin, glucose oxidase, lipase, lysozyme, alkaline phosphatase and plasminogen, transcription factor, protein  
30

domain, binding protein, antigen and immunoglobulin, such as a F(ab) fragment.

5 In another embodiment, the invention relates to an insoluble coupled support comprising one or more polypeptide(s) coupled by the method according to the invention or another method. The coupled polypeptide may suitably be specifically oriented. The coupled support according to the invention comprises a material, such as one selected from the group consisting of an electronic chip, slide, wafer, resin, well, tube, micro array and membrane and the  
10 material of the support may be selected from but is not limited to the above group consisting of polystyrene, polyethylene, polyester, polyetherimide, polypropylene, polycarbonate, polysulfone, polymethylmethacrylate, poly(vinylidene fluoride); and silicon. The polypeptide coupled to the support is preferably spatially controlled.

15 In one embodiment of the invention, the coupled support is used in a bio-functional reaction such as a bio-sensor, chromatography, immunodetection, enzyme assay, nucleotide binding detection, protein-protein interaction, protein modification, support targeting and protein targeting.

20 In another embodiment of the invention, the coupled support may also be used in a diagnostic or biosensor kit.

25 In another aspect of the invention, the use of the method according to the invention for the production of polypeptide-based surface coating is provided, in particular for use in the production of polypeptide-based biosensors, polypeptide-based micro arrays, and food packing materials with polypeptide-based surface coatings e.g. for the production of anti-microbial food packing materials.

30

The method according to the invention of light induced thiol coupling can also be used to immobilise a protein on a support. The disulphide bond between the linker and the protein is stable, and extensive washing after immobilisation will not displace the protein. The density of proteins on a support can be controlled by varying the protein concentration, or the intensity and duration of UV-irradiation, and subsequently blocking the remaining activated thiol groups on the surface with reagents such as L-cysteine, (2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) or with a thiol-lipid bilayer (Hong Q., et. al., 2001, Biochemical Society Transactions 29(4):587-582).

5

10 The support, with evenly distributed immobilised proteins, is therefore blocked to prevent non-specific binding. According to the present invention, the method of immobilisation does not involve any chemical steps, since the thiol-activated proteins formed by UV radiation, spontaneously self-assemble on the support. The described thiol and disulphide exchange reactions are an effective and rapid way to bind molecules to supports.

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Immobilisation of a polypeptide on a support can also be spatially controlled. Present day laser technology allow for focal spots with dimensions of 1 micrometer or less. If a specific polypeptide or target molecule, containing SS bridge(s), is incubated with the disulphide-containing support the light-induced thiol group formation and coupling could be limited to the focal points of illumination. This approach would allow for an extremely dense packing of identifiable and different molecules on a support surface. Thus, the method of the present invention could be used for charging micro arrays with molecules.

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#### Oligomers and polymers

One different aspect of the invention relates to generation of dimmers, oligomers, polymers and/or dendrimers. This method of coupling of two elements according to invention can be used to construct various types of disulphide-linked oligomers or polymers. Light-induced thiol groups in a given protein or peptide or other biomolecules can be coupled to a support. Provided

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that the concentration of protein and support molecules in the coupling reaction is sufficiently high, SS based cross-linking between neighbouring molecules will take place. While the light-induced protein or molecule should contain an SS bridge, the possession of an aromatic residue as close spatial neighbour is not essential, since the aromatic contribution to the reaction may be supplied by aromatics amino acids or their derivatives, added to the coupling reaction.

### *Dimers*

Another aspect relates to dimers, such as homo- or heterodimers, consisting of two identical or different elements or subunits. Upon coupling of two elements according to the invention, a dimer is formed. Figure 8 shows examples of different embodiments of the invention regarding homo- and heterodimers (A, B = elements and/or subunits;  $SS\emptyset_n$ : disulfide bridge-containing triad;  $n=1,2,3,4$  indicating different subunits; SS appropriate disulfide bridge or disulfide bridge formed upon coupling;  $\emptyset$ : aromatic amino acid):

(a) Homo-dimer ( $A = B$ ) without additional triad;  $SS\emptyset_1$  triad used for coupling); (b) Homo-dimer ( $A = B$ );  $SS\emptyset_1$  triad used for coupling; units contain an additional triad  $SS\emptyset_2$ ; (c) Hetero-dimer ( $A \neq B$ ); both units contain different triads  $SS\emptyset_1$  and  $SS\emptyset_2$  for coupling; (d) Hetero-dimer ( $A \neq B$ );  $SS\emptyset_1$  triad used for coupling; A contains an additional triad  $SS\emptyset_2$  not used for coupling; (e) Hetero-dimer ( $A \neq B$ );  $SS\emptyset_1$  triad used for coupling; A + B contain different, additional triads  $SS\emptyset_2$  and  $SS\emptyset_3$  not used for coupling; (f) Hetero-dimer ( $A \neq B$ );  $SS\emptyset_1$  and  $SS\emptyset_3$  are different triads used for coupling; A + B contain different, additional triads ( $SS\emptyset_2$ ,  $SS\emptyset_4$ ) not used for coupling. (g) Homo-dimer ( $A = B$ ); the units do not comprise a triad, but coupling is mediated via an aromatic amino acid in solution; (h) Homo-dimer ( $A = B$ ); the units comprise a triad  $SS\emptyset_1$  not used for coupling; coupling is mediated via an aromatic amino acid in solution; (i) Hetero-dimer ( $A \neq B$ ); unit B contains a triad not used for coupling reaction ( $SS\emptyset_1$ ); both linkers lack a triad at the coupling site in the absence of an aromatic amino acid, but coupling is mediated via

an aromatic amino acid in solution; (j) Hetero-dimer ( $A \neq B$ ); units A and B contain different triads ( $SS\emptyset_1$ ,  $SS\emptyset_2$ ) not used for coupling reaction; both linkers lack a triad at the coupling site in the absence of an aromatic amino acid, but coupling is mediated via an aromatic compound (amino acid) in solution.

Another embodiment relates to dimers, homo or hetero, where one or more appropriate disulfide bridges and/or one or more additional triads can be activated by different irradiation wavelengths than the first appropriate disulfide bridge or triad.

A further embodiment concerns the formation of heterodimers, where the formation of (undesired) homodimers is reduced or avoided. This can be achieved by designing the elements/units in such a way, that e.g. one unit provides one (or more) aromatic amino acids, and the other one the appropriated disulfide bridge, thus preventing the formation of homodimers.

#### *Dendrimers*

Yet another aspect relates to dendrimers, such as homo- or hetero dendrimers. These tree-like, or generational structures can be synthesized using the coupling according to the invention, using identical or different units, elements or building blocks according to the invention. Dendrimers can be used in drug delivery (Sezen Gurdag et al., 2006). There are two defined methods of dendrimer synthesis, divergent synthesis and convergent synthesis. The former assembles the molecule from the core to the periphery and the latter from the outside to termination at the core. Both methods are possible using the coupling method according to the present invention.

Figure 9 shows examples of different embodiments of the invention regarding dendrimer units/elements before coupling ( $SS\emptyset_x$ ,  $SS\emptyset_y$ ,  $SS\emptyset_z$ : identical or different triads used for coupling;  $L_a$ ,  $L_b$ ,  $L_c$ : identical or different linkers): (a)

Dendrimer unit with three identical or different triads ( $SS\emptyset_x$ ,  $SS\emptyset_y$ ,  $SS\emptyset_z$ ) located on one or more identical or different internal linkers; additional, internal triads, not intended for coupling ( $SS\emptyset_i$ ) may be present; (b) Dendrimer unit before coupling comprising an internal triad  $SS\emptyset_x$  intended for coupling;  $SS\emptyset_y$  and  $SS\emptyset_z$  are located on identical or different linkers; additional, identical or different internal triads not intended for coupling may be present (not shown)); (c) Dendrimer unit comprising two internal triads ( $SS\emptyset_x$  and  $SS\emptyset_y$ ) intended for coupling;  $SS\emptyset_z$ : triad on linker used for coupling; additional, identical or different internal triads not intended for coupling may be present (not shown)); (d) Dendrimer unit comprising three internal triads ( $SS\emptyset_x$ ,  $SS\emptyset_y$ , and  $SS\emptyset_z$ ) intended for coupling; additional internal triads not intended for coupling may be present (not shown)).

#### Biomolecules

The coupled support according to the invention may suitably be used for drug delivery. The polypeptide is in one aspect of the invention selected from the group consisting of an enzyme such as selected from the group consisting of cutinase, chymosin, glucose oxidase, lipase, lysozyme, alkaline phosphatase and plasminogen, a transcription factor, a protein domain, a binding protein, an antigen and an immunoglobulin, such as a F(ab) fragment.

In one embodiment of the invention, a method of delivering a drug or prodrug to a patient is provided, comprising: (i) providing a support coupled to one or more polypeptides, (ii) administering the support-coupled polypeptide to a patient, (iii) irradiating the support-coupled polypeptide to create a thiol group in the molecule by disulphide bridge disruption, and thereby releasing the polypeptide from the support. In the above method of the invention, the support in itself may be a pharmaceutical drug and in another aspect of the invention, the coupled polypeptide is a drug or a prodrug.

This method of light-induced coupling to a support can be usefully applied to other types of support molecule, such as pharmaceutical drugs, in order to facilitate their effective delivery. For example, light-induced thiol coupling of a water-soluble molecule containing a disulphide bridge (including but not limited to a peptide or protein) to a drug can help the solubilisation and delivery of water-insoluble, poorly soluble or hydrophobic drugs. Furthermore, the molecule coupled to the drug may serve to protect the drug from its physiological environment, and hence improve its stability *in vivo*. This particular feature makes this technology attractive for the delivery of labile drugs such as proteins. Localized delivery of the molecule-coupled drug, by implantation at the site of treatment, would reduce systemic exposure of the patient to the drug. Support-linked prodrugs are generally defined as prodrugs that contain a temporary linkage of a given active substance to a transient support group that produces improved physicochemical or pharmacokinetic properties and that can be easily removed *in vivo*, usually by a hydrolytic cleavage. In the present invention, light-mediated disruption of the disulphide bond linking a drug to a molecule can be used to achieve a controlled release of the active drug from the molecule-coupled form, implanted in the patient. This would minimise the frequency of drug delivery to the patient, and provide for light-controlled dosing. The process of drug delivery may be optimised, by only illuminating those regions of the body where drug release is necessary. These features would improve patient compliance, especially for drugs used for chronic indications, requiring frequent injections (such as for deficiency of certain proteins or metabolites). Controlled drug release could be induced by infra-red light (via two-photon excitation) in the case of transdermal drug delivery, within the penetration range of infra-red light, while the greater penetration of UV light (or infra-red light via three-photon excitation) would facilitate drug release deeper within the patient. Also, the use of optical fibers allows the delivery of light at various depths in the body, as used in PDT (photodynamic therapy), as for example in the treatment of cancer/tumor patients. Since a solvent exposed disulphide bridge will be broken in a reducing

environment, the drug could also be released when the support coupled with the drug has entered a reducing environment such as the cytoplasmic space of a cell. In an alternative embodiment, the support comprises a (nano)particle or a magnetic (nano)particle (see below for further embodiments regarding magnetic (nano)particles).

### Sensor

Another aspect of the invention relates to sensor method or device, comprising elements coupled according to the invention. In embodiment, the sensor comprises fiber optics, to which molecules, such as biomolecules are coupled to the area or surface of point of entry or exit of light, either directly or via a spacer or another support. This method and/or device may be used to detect changes of light based on interaction of the sensor with its environment, comprising luminescence, light scattering, absorbance, reflection, transflection, fluorescence and/or light quenching reactions, or any combination of the above.

Another embodiment of the invention relates to a method or device comprising a sensor for monitoring interactions between molecules, said device comprising elements coupled according to the invention. A further embodiment, relates to cantilever for atomic force microscopy. It is known in the field that the correct orientation of the molecules coupled to the tip of such a cantilever is crucial in order to obtain more accurate and precise measurements, and/or an improved signal to noise ratio. This desired, correct orientation of the molecules can be provided by the invention.

A further aspect of the present invention relates to a cantilever for atomic force microscopy, where the cantilever comprises a molecule coupled by irradiation of a disulfide bridge or disulfide containing triad.

### Release

In a further aspect of the present invention, the bond immobilising the protein to the support may be disrupted, releasing the protein into solution. Disulphide bridges between a protein and the support can be disrupted e.g. with UV irradiation, in the same way as disrupting a disulphide bond on a protein, where an aromatic amino acid is a spatial neighbour. The aromatic amino acid can either be located on the immobilised protein itself, or be supplied in the form of a solution of an aromatic amino acid, such as tryptophan, applied to the support surface. Disulphide bonds (SS) themselves are known to be disrupted by approximately 254 nm light. Alternatively, disulphide bridges between a protein and a support can be disrupted with (dithiothreitol) DTT, or other reducing agents known to persons skilled in the art. Following disruption of the immobilisation bond, the released protein can be purified, if necessary, and used in further experiments.

15 A further aspect of the present invention is regenerating a gold surface by removing proteins that are immobilised through a thiol-Au bond with O<sub>2</sub>-plasma treatment or Piranha, thereby removing the top layer of the gold surface including the proteins.

20 In one embodiment of the invention, a coupled molecule or polypeptide may be released from the support by irradiating the molecule or polypeptide to create a thiol group in the polypeptide by disulphide bridge disruption. In another embodiment of the invention, the wavelengths used for immobilization and release are different. In yet another embodiment, the release is provided by another disulfide bridge-containing triad. In a further embodiment, the release is provided by triggered by providing a free amino acid to the disulfide bridge formed during coupling.

#### Magnetic (nano)particles

30 Magnetic particles can be coupled with elements, such as molecules, polypeptides, drugs, prodrugs, biomolecules or the like, according to the inven-

tion. The particles se can vary in size and shape, and may even be as small as nanoparticles. In one embodiment, magnetic (nano)particles are used in the concentration, extraction, removal or purification of substances, such as recovering enzymes, toxic-, fluorescent or radioactive compounds.

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A further embodiment relates to a magnetic (nano)particle, where the magnetic (nano)particles comprises a (bio)molecule coupled by irradiation of a disulfide bridge or disulfide containing triad.

10 In another embodiment, magnetic (nano)particles are used in the treatment of a patient, human or animal, where one or more biologically active substances are concentrated in the area of treatment, by providing an appropriate magnetic field. Thereby the activity is increased at the area of activity and/or other parts of the organism are protected against it.

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In yet another embodiment, the magnetic (nano)particles comprising biomolecules coupled according to the invention are used in cancer treatment, such as chemotherapy or radiation treatment.

20 In a further embodiment, an active portion of the biologically active substance is released upon irradiation according to the invention.

In yet a further embodiment, the magnetic (nano)particles comprise dendrimers coupled according to the invention.

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In an alternative embodiment, one or more biologically active substances are removed from the patient by the use of magnetic nanoparticles and providing an appropriate magnetic field.

30 Alternatively, the magnetic (nano)particles according to the invention are used in combination with a fluorescent label or radioactive compound. In an-

other alternative embodiment, the magnetic (nano)particles according to the invention are used in combination with a substance, molecule, biomolecule, polypeptide and the like, that can be recycled, i.e. that retains a significant or considerable portion of its activity after use, and thus maintains its capability of being reused, either in its form after use or after reactivation or modification. Such radioactive magnetic (nano)particles can also be used to remove (radioactive) markers from a tube system, such as water pipes in a heating system, in combination or after search for leaks.

10 In still another embodiment, the total dosage of molecules, polypeptides or biomolecules can be reduced by the use of magnetic (nano)particles.

#### Device for bioarrays

Figure 27 schematically illustrates a more detailed specific aspect of material deposition by light induced molecular immobilization. Shown is a light source (102) in the form of a laser. Such a method and/or device may be useful for providing bioarrays. Examples of one embodiment of a bioarray are given in Figures 5 and 6: In an alternative embodiment, the bioarray is coupled with molecules and/or biomolecules according to the invention, such as protein, peptide, polypeptide, DNA, RNA and the like, either in natural, derivatized, modified, engineered or synthetic form, or in combination. Such molecules may also comprise a combination of different elements, such as molecules or supports, which may also be coupled according to the invention.

25 In one embodiment, the laser is a solid state diode-pumped mode-locked titanium-Sapphire (Ti-Sapphire) laser delivering e.g. 0.9 W of 840 nm near-infrared and approximately 80 femtosecond long laser pulses at a repetition rate of 80 MHz. One example of such a laser is e.g. the Tsunami 3960 laser by Spectra Physics, Mountain View, CA. The laser may e.g. be pumped by a high power (5W at 532 nm) solid state laser such as Millennia V by Spectra Physics.

In this exemplary embodiment, the laser pulses are passed through a pulse picker (110) reducing the repetition rate to 8 MHz before entering a doubler/tripler unit (111) (e.g. GWU from Spectra Physics) where the reduced laser pulses are used to generate the third harmonic of 840 nm to yield approximately 1mW of approximately 200 femtosecond long 280nm UV pulses at 8 MHz. The pulse picker (110) is not necessary for immobilization but gives a simple way of controlling the power of the light.

The resulting UV pulse train is then passed to (e.g. using mirrors, optical fibres and/or components, and/or the like (113)) a light exposure controlling system (104) comprising in this particular embodiment a shutter (112), being controlled by the controller (101), receiving the UV pulse train and passing the UV pulse train through (when the shutter is controlled to be open) to a beam expander (114) (or an optical lens) expanding the beam, an iris diaphragm (115) and a finally a focusing lens (116) focusing the beam into a spot (or an area) of an appropriate size (e.g. about 25 micrometers but via an appropriate light source (102) and/or light exposure controlling system (104) even down to 1 micrometer and below in the nanometer range).

Please note, that the shutter is not needed for embodiments using a mask, a template, a micro-lens array and/or by the use of a so-called digital micro-mirror device (DMD), etc. where the object or a larger portion (than a spot) of the object is illuminated by the light source.

The object (103) comprising molecules to be immobilized is, in this particular embodiment, mounted on a 2D or 3D translation stage (117) (controlled by controller (101)) with the surface of the object comprising molecules to be immobilized in substantially the focal plane (at least when light is to immobilize the molecules on the object (103)) of the UV-light).

The translation stage may also be used to bring the surface out of focus, i.e. bring the object out of the focal plane, e.g. if an immobilization pattern is to

be produced due utilizing diffraction. Additional aspects are given in the co-pending application by the same applicant filed on the same day.

## Examples

The following, non-limiting Examples illustrate a variety of aspects of the invention.

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### Example 1

#### **Production of human brain fatty acid binding protein (B-FABP)**

##### Cloning of B-FABP for recombinant expression in *Escherichia coli*.

A plasmid bearing the coding sequence for human Brain Fatty Acid Binding Protein (B-FABP) was constructed for expression in *Escherichia coli*. The coding sequence for B-FABP was PCR amplified from a cDNA from the I.M.A.G.E. collection (GeneBank accession: BC012299) with the following forward and reverse primers, LD007fw and LD009rv (Table 2), respectively. The PCR amplicon was cloned into the NdeI and BamHI restriction sites of the pET11a plasmid (Novagen).

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##### Expression and purification of B-FABP:

Wild-type B-FABP was expressed in *Escherichia coli* (*E.coli*) BL21(DE3). Tagged B-FABP with S-S containing peptides was expressed in *Escherichia coli* (*E.coli*) Origami(DE3). In any case, B-FABP was expressed in inclusion bodies using following protocol: A preculture of 3 ml LB medium with a selected colony is grown overnight with an appropriate antibiotic, Ampicillin<sup>100</sup> (100 µg/ml) or Carbenicillin<sup>50</sup> (50 µg/µl). 500 ml of enriched medium e.g. SOB medium with Amp<sup>100</sup> or Carb<sup>50</sup> is prepared, and the preculture was added.

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The cell culture is grown to an OD of 0.6, after which 200µM of IPTG is added. The cell culture is allowed to induce over night at 30°C under continuous stirring. The induced cell culture is then centrifuged at 5000 rcf for 30 minutes at 4°C, and the supernatant is discarded. The cell pellet is then re-suspended in 100 ml TES buffer and 10 ml of lysis buffer (lysozyme 1mg/ml in TES buffer) is added. The mix is kept on ice for 30 minutes to allow for cell lysis, and frozen before further processing. After thawing, 20 ml of detergent

buffer is added to the lysate, which is then centrifuged at 5000 rcf for 10 minutes. The pellet is washed 3 times with 20 ml 1% Triton X100 and 3 times with 20 ml of Milli-Q H<sub>2</sub>O during each wash the sample is centrifuged at 5000 rcf for 10 minutes, and finally resuspended in 100 ml of Milli-Q H<sub>2</sub>O. In order to ensure proper resuspension of the pellet, and shearing of mRNA, the lysate is pulse sonicated with 500,000 J for 5 minutes (30 sec pulse, 30 sec wait), and centrifuged at 5000 rcf for 10 minutes at 4°C. The sedimented pellet was recovered and frozen for further use.

10 Materials: Isopropyl β-D-thiogalactopyranoside (IPTG) (C<sub>9</sub>H<sub>18</sub>O<sub>5</sub>S), Cas: 367-93-1, Nova Biochem; SOB medium with Amp<sup>100</sup>; TES buffer; Lysozyme (hen egg white), Cas: 12650-88-3, Fluka; Urea (CH<sub>4</sub>N<sub>2</sub>O), Cas: 57-13-6, BDH; Triton X100, Cas: 9002-93-1, Sigma; Milli-Q H<sub>2</sub>O; Sonics Vibra Cell VCX500 500W ultrasonic processor; Sorvall RC 5C Plus centrifuge.

15

Refolding:

The pellet containing B-FABP inclusion bodies was dissolved in a 10 ml pH 7.5 solution containing 8 M urea, 1 mM DTT, and 10 mM Tris. This solution was stirred using a magnetic stir bar for 15 minutes. Following 1 μl was taken out for subsequent concentration determination through OD measurements. The remaining solution was then diluted to 1.5 M urea using a pH 7.5 solution consisting of 1 M arginine, 1 mM DTT, and 10 mM Tris reaching a B-FABP concentration of approximately 0.1 mg/ml. After diluting the solution was stirred intensively for 10 minutes at 4°C. Subsequently the solution was dialysed in 10 mM Tris pH 7.5 for 24 hours with gentle stirring. After dialysis the pH was adjusted to pH 7.5 and the sample was filtered through a 220 nm pore size filter. Previous results in the laboratory have shown that the protein refolds totally in these conditions and that the protein is essentially pure, based on SDS-PAGE analysis (data not shown).

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Materials: Urea (CH<sub>4</sub>N<sub>2</sub>O), Cas: 57-13-6, BDH; DTT (diluted from 1M stock);

Tris[hydroxymethyl]aminomethane (Tris), Cas: 77-86-1, Sigma; B-FABP inclusion bodies; L-Arginine (C<sub>6</sub>H<sub>14</sub>N<sub>4</sub>O<sub>2</sub>), Cas: 74-79-3, Aldrich; Spectrum Spectra/Por Dialysis bags, MWCO: 6-8000, vol/length: 1.7ml/cm.

5 **Table 2** List of DNA and amino acid sequences

Primer	Sequence (DNA or protein)
LD007fw	5' GGG AATTCCATATGGTGGAGGCTTTCTGTGCT
LD009rv	5' CGCCACTATGAGAAGGCATAATAAGGATCCCG
LD035fw	5' AACCCGGGGATCCGGTGATTGCGGCAAAC TGT TTAGCGGCTGCGATA CCAATGCGGATTGCTGCGAAGGCTGGGTGTGC
LD036rv	5' CGGCTGCGATACCAATGCGGATTGCTGCGAAGGCTGGGTGTGCCGTC TGTTTTGCAAAC TGGATTAATAAAGATCTATT
LD037rv	5' ATCTTTATTAATCCAGTTTGC
LD039rv	5' AATGGATCCTTATTAGCAACCGCCACCACAGCCCGGGCCTTCTCAT AGTGGCGAACAGCAACCACATCACC
T7fw	5' CGAAATTAATACGACTCACTATAG
T7rv	5' GCTAGTTATTGCTCAGCGGTGGCA
B-FABP	5' ATGGTGGAGGCTTTCTGTGCTACCTGGAAGCTGACCAACAGTCAGAA CTTTGATGAGTACATGAAGGCTCTAGGCGTGGGCTTTGCCACTAGGCAG GTGGGAAATGTGACCAAACCAACGGTAATTATCAGTCAAGAAGGAGACA AAGTGGTCATCAGGACTCTCAGCACATTC AAGAACACGGAGATTAGTTT CCAGCTGGGAGAAGAGTTTGATGAAACCACTGCAGATGATAGAACTGT AAGTCTGTTGTTAGCCTGGATGGAGACAACTTGTT CACATACAGAAAT GGGATGGCAAAGAAACAAATTTTGTAAGAGAAATTAAGGATGGCAAAT GGTTATGACCCTTACTTTTGGTGATGTGGTTGCTGTT CGCCACTATGA- GAAGGCATAA  MVEAFCATWKL TNSQNFDEYMKALGVGFATRQVGNVTKPTV I I SQEGDK VVIRTLSTFKNTE I SFQLGEEFDETTADDRNCKSVVSLDGDKLVHIQKW DGKETNFVREIKDGKVMVMTLTFGDVVAVRHYEKA
mutHpTX2	5' AACCCGGGGATCCGGTGATTGCGGCAAAC TGT TTAGCGGCTGCGATA CCAATGCGGATTGCTGCGAAGGCTGGGTGTGCCGCTGTTTTGCAAAC T GGATTAATAAAGATCTATT  GDCGKLFSGCDTNADCCEGWVCR L FCKLD
SS peptide	PGCGGGC

TABLE 2 LIST OF SEQUENCES (CONTINUED)

<b>W6/SS peptide</b>	KAMHAWGCGGGC - amidated
<b>B-FABP/ mutHpTX2 Fusion</b>	<p>5' ATGGTGGAGGCTTTCTGTGCTACCTGGAAGCTGACCAACAGTCAGAACTTTGATGAGTACATGAAGGCTCTAGGCGTGGGCTTTGCCACTAGGCAGGTGGGAAATGTGACCAAACCAACGGTAATTATCAGTCAAGAAGGAGACA AAGTGGTCATCAGGACTCTCAGCACATTCAAGAACACGGAGATTAGTTTCCAGCTGGGAGAAGAGTTTGTATGAAACCACTGCAGATGATAGAACTGT AAGTCTGTTGTTAGCCTGGATGGAGACAACTTGTTACATACAGAAATGGGATGGCAAAGAAACAAATTTTGTAAAGAGAAATTAAGGATGGCAAATGGTTATGACCCTTACTTTTGGTGATGTGGTTGCTGTTCCGCCACTATGAG AAGGCAGGATCCGGTGATTGCGGCAAACCTGTTTAGCGGCTGCGATACCAATGCGGATTGCTGCGAAGGCTGGGTGTGCCGTCTGTTTTGCAAACCTGGA TTAATAAAGATCTATT</p> <p>MVEAFCATWKLNSQNFDEYMKALGVGFATRQVGNVTKPTV IISQEGDKVVIRTLSTFKNTEISFQLGEEFDETTADDRNCKSVVSLDGDKLVHIQKW DGKETNFVREIKDGKVMMLTFGDVVAVRHYEKAGDCGKLFSGCDT - NADCCGWWCRLFCKLD</p>
<b>B-FABP / W3-SS fusion</b>	<p>5' ATGGTGGAGGCTTTCTGTGCTACCTGGAAGCTGACCAACAGTCAGAACTTTGATGAGTACATGAAGGCTCTAGGCGTGGGCTTTGCCACTAGGCAGGTGGGAAATGTGACCAAACCAACGGTAATTATCAGTCAAGAAGGAGACA AAGTGGTCATCAGGACTCTCAGCACATTCAAGAACACGGAGATTAGTTTCCAGCTGGGAGAAGAGTTTGTATGAAACCACTGCAGATGATAGAACTGT AAGTCTGTTGTTAGCCTGGATGGAGACAACTTGTTACATACAGAAATGGGATGGCAAAGAAACAAATTTTGTAAAGAGAAATTAAGGATGGCAAATGGTTATGACCCTTACTTTTGGTGATGTGGTTGCTGTTCCGCCACTATGAG AAGGCCCCGGGCTGTGGTGGCGGTTGCTAATAAGGATCCATT</p> <p>MVEAFCATWKLNSQNFDEYMKALGVGFATRQVGNVTKPTV IISQEGDKVVIRTLSTFKNTEISFQLGEEFDETTADDRNCKSVVSLDGDKLVHIQKW DGKETNFVREIKDGKVMMLTFGDVVAVRHYEKAPGCGGGC</p>

**Anion exchange chromatography on QEA-sepharose**

After dialysis and equilibration in 10 mM Tris buffer pH 7.5, the protein was

5 further purified by anion-exchange FPLC, using a gradient system consisting of two buffers: buffer A (Tris-Cl 10mM, pH7.5) and buffer B (Tris-Cl 10mM, pH7.5 + NaCl 1M). In Figure 10 a chromatogram of the conducted ion exchange (anion, QEA-sepharose) experiment is shown. The primary y-axis is milli Absorbance Units ( $A = \log(T_0/T)$ ) and the x-axis is the elution volume in

10 ml. The salt concentration affecting the ionic strength / conductivity and thereby the binding affinity of the protein to the column matrix are given. The

secondary y-axis is the proportion of buffer B in the eluent (in %, v/v). From 0ml to 110ml the column is flushed with buffer A. From 110ml to 140ml buffer B is raised to 10%. At 130ml of elution, the protein is released from the column, as seen by a peak of absorbance at 133ml. The sample fraction from 130ml to 136ml is expected to hold the highest concentration of protein and is collected (indicated by red triangles). From 140ml the salt concentration is rapidly raised to 100% releasing the remaining contaminant proteins from the column. From 150ml to 170ml smaller peaks in the blue and purple curves show that some excess protein is released.

10

Materials: Tris(hydroxymethyl)aminomethane (Tris) (C<sub>4</sub>H<sub>11</sub>NO<sub>3</sub>), Cas: 77-86-1, AppliChem; Sodium chloride (NaCl), Cas: 7647-14-5, J.T. Baker; Folded B-FABP sample; Amersham Biosciences Äkta Purifier controlled by Unicorn 5.01 software; HiTrap Q HP anion column.

## 15 **Example 2**

### **Chemical coupling of W6/SS peptide and B-FABP**

As the EDC/NHS reaction is preferably not conducted in a Tris buffer, this buffer was exchanged with MES buffer before chemical coupling between the Tag peptide and B-FABP. The B-FABP solution was transferred to dialysis bags. These were placed in a 50 mM MES buffer pH 5.8 at room temperature under stirring. After 3 hours the MES buffer was replaced by a fresh solution and the dialysis was continued over night. The concentration of B-FABP after dialysis was determined by OD measurements at 280nm, assuming a molar extinction coefficient of 13940 M<sup>-1</sup> cm<sup>-1</sup>.

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The cross linking of W6/SS peptide and B-FABP was performed using EDC/NHS chemistry, to produce W6/SS Cross linked B-FABP. Thereto, a solution of the following was made and was left to react for 2 hours at room temperature: 1 ml 43 µM B-FABP, 4 µl 43 µM peptide, 5 µl 5 mM EDC, 5 µl 5

mM NHS. After reacting 0.2 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> pH 9.0 was added to the solution. The solution was dialysed against 200 ml 10 mM Na<sub>2</sub>CO<sub>3</sub> buffer pH 9.0.

Materials: 2-(N-Morpholino)ethanesulfonic acid (MES) (C<sub>6</sub>H<sub>13</sub>NO<sub>4</sub>S), Cas: 4432-31-9, AppliChem; Spectrum Spectra/Por dialysis bags, MWCO: 6-8,000, vol/length: 1.7 ml/cm; N-hydroxysuccinimide (NHS) (C<sub>4</sub>H<sub>5</sub>NO<sub>3</sub>), Cas: 6066-82-6, Aldrich; 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) (C<sub>8</sub>H<sub>17</sub>N<sub>3</sub>), Cas: 25952-53-8, Sigma-Aldrich; Sodium carbonate-10-hydrate (Na<sub>2</sub>CO<sub>3</sub>·10H<sub>2</sub>O), Cas: 6132-02-1, Riedel-de Haën; W6/SS Peptide; Folded and purified B-FABP.

### Example 3

#### Cloning, expression and purification of SS Tagged B-FABP into pET-11a

The coding sequence for human B-FABP was PCR-amplified with the following forward and reverse primers, LD007fw and LD039rv (Table 2), respectively. The reverse primer was designed to create an in-frame C-terminal fusion of the peptide sequence PGCGGGC to the coding sequence of B-FABP. The PCR amplicon was cloned into the *Nde*I and *Bam*HI restriction sites of the pET11a plasmid (Novagen). Optimization of the annealing temperature for the hybridisation and extension PCR using the primers LD007fw and LD039rv coding for the SS tag and the B-FABP template strand can be seen in Figure 11. The wells were loaded with increasing temperature from left to right (45°C to 65°C), the agarose gel was 1.5%, and the ladder step size was 100 bp. For all samples a clear band is seen at 450 bp as expected. The intensity of the bands was found to increase with increasing annealing temperature. To confirm the PCR amplification of SS tagged B-FABP, the DNA samples were analyzed on agarose gels. DNA was then cleaned using a PCR cleaning prep kit and the concentration was determined by OD measurements. A double digest was conducted with 40 µl SS tagged B-FABP DNA (100 ng/ul), 0.5 µl *Nde*I, 1 µl *Bam*HI, 20 µl *Y*/Tango buffer and 39 µl of

sterile Milli-Q H<sub>2</sub>O overnight. The digestion product was cleaned using the phenol-chloroform extraction and the end product verified on a 1.5 % agarose gel and concentration determined by OD measurements. For the ligation 7 µl of SS tagged B-FABP (100 ng/µl) insert was mixed with 2 µl of pET-11a  
5 (600 ng/µl), 2 µl of ligation buffer, 2 µl of T4 ligase, and 6 µl of Milli-Q H<sub>2</sub>O. The ligated plasmid was transformed into competent *E. coli* DH5α cells, and plated onto LB-Amp<sup>100</sup> plates. After incubation over night at 37°C, 20 colonies were picked and replated onto a new LB-Amp<sup>100</sup> plate and left at 37°C over night. The replated colonies were subjected to a screening PCR with  
10 primers T7fw and T7rv and analyzed on 1.5% agarose gels (see Figure 12 ; lanes 1, 2, 3, 5 and 6 holds the colonies 14, 15, 16, 17, and 18, respectively, while a 100 bp ladder is in well 4). Bands were seen at 550 bp for the colonies 14, 15, 16 and 18 as expected, while a 950 bp band was seen for colony 17. The ladder size is 100bp and the gel is a 1.5% agarose gel. Based on  
15 visual confirmation from the gel analysis, colonies were picked and transferred into 3 ml of LB medium with Amp<sup>100</sup>, and left over night. In order to ensure that the picked colonies still contained the desired pET11a with SS tagged B-FABP insert after culturing, a screening PCR was conducted with T7fw primer and T7rv primer. After incubation 1 ml of preculture was ex-  
20 tracted and 100 µl of glycerol was added to cryo preserve the sample before it was frozen at -85°C. The additional 2 ml of culture were purified using a Quiagen plasmid mini prep kit. The purified plasmid was suspended in 100 µl of elution buffer. The plasmid containing the cloned SS tagged B-FABP was then transferred into *E. coli* Origami (DE3) cells for expression (see Example  
25 1 for details regarding expression and purification). 6 colonies were picked onto a new Amp<sup>100</sup> plate. A test expression was conducted by washing the Amp<sup>100</sup> plate with 4 ml of LB medium. 3 ml of washed bacteria culture was extracted to an inoculation tube along with 6 µl of Amp<sup>100</sup> and 12 µl/ml IPTG. The tube was left at 37°C for 3 hours, and then placed at 4°C. To express the  
30 modified B-FABP with linker, 500ml SOB medium with Amp<sup>100</sup> was grown with the selected colony to an OD of 0.6, after which 12 µl/ml of IPTG was

induced, and the cell culture was allowed to induce over night at 30°C under continuous stirring. Upon purification, the weight of the inclusion body pellet was 56 mg. 14 mg of the pellet was extracted and resuspended in 0.5ml Urea 8M. The 14 mg protein was purified and refolded according to the re-  
5 folding protocol (Example 1).

Materials: PCR primers: LD007fw; LD039rv; T7fw and T7rv, TAGCopenhagen (see Table 2); *NdeI* restriction enzyme, 20u/μl, New England BioLabs; *BamHI* restriction enzyme, 10u/μl, Fermentas; Buffer Y/Tango with BSA 10x,  
10 MBI-Fermentas; pET-11a vector DNA, Novagen; T4 DNA ligase, Roche Diagnostics GmbH; Ligation buffer 10x, Roche Diagnostics GmbH; LB medium with Amp<sup>100</sup>; Glycerol 99% (C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>), Cas: 56-81-5, Sigma; *E. coli* Origami (DE3) cells; Isopropyl β-D-thiogalactopyranoside (IPTG) (C<sub>9</sub>H<sub>18</sub>O<sub>5</sub>S), Cas: 367-93-1, Nova Biochem; SOB medium; Sterile Milli-Q H<sub>2</sub>O; Quagen plasmid mini prep kit, Sonics Vibra Cell VCX500 500W ultrasonic processor.  
15

#### Characterization of SS-tagged B-FABP by SDS-PAGE

The construct corresponding to clone 15 was transferred to Origami(DE3) cells. The recombinant protein was expressed as inclusion bodies and allowed to refold in Tris buffer pH 7.5. Figure 13 shows 15%a SDS-PAGE gel  
20 holding SS tagged B-FABP in lane 2 with W6/SS Cross linked BFABP in lane 3 and native B-FABP in lane 4. The marker in lanes 1 and 5 is MBI marker SM0671. The band height for SS tagged B-FABP and W6/SS Cross linked B-FABP appears slightly higher than for native B-FABP. All three bands are  
25 found below the 15 kDa marker, which is in good agreement with the fact that the tagged protein bears additional amino acids.

#### **Example 4**

##### **Steady State Fluorescence Spectroscopy (SSFS)**

30 For steady state fluorescence spectroscopy, 600 μl of 5 μM protein in 10 mM NaH<sub>2</sub>PO<sub>4</sub> were prepared. The pH was adjusted to 5.86 with 1% H<sub>3</sub>PO<sub>4</sub>, the

sample was placed in a 600  $\mu$ l quartz cuvette, and placed in the steady state spectrofluorometer. The protein samples analyzed were native B-FABP, W6/SS Cross linked B-FABP, and SS tagged B-FABP. For each sample emission spectra were recorded from 300 nm to 400 nm at 11.5°C before and after heating with excitation at 292 nm. Static scatter spectras were recorded from 450 nm to 550 nm at 11.5°C with excitation at 500 nm. In both emission specters and static scatter specters 5 accumulations were acquired and the integration was 0.1-second per point. Thermal scans were recorded at 350nm from 11.5°C to 82°C and again from 82°C to 11.5°C with excitation at 292 nm and a temperature step size of 1.5°C per minute and 1 point per second. All SSFS measurements were averaged by a smoothing function in FeliX32.

Materials: Sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), Cas: 7558-80-7, Sigma; Phosphoric acid  $\geq 85\%$  ( $\text{H}_3\text{PO}_4$ ), Cas: 7664-38-2, Sigma; Fluorescence system QM-6 Steady State Spectrofluorometer (PTI); Light source: 75W Xenon bulb, USHIO UXL-75XE; FeliX32 software.

Figure 14 shows measurements of 300 – 400 nm emission from native and W6/SS Cross linked B-FABP prior to heating using (SSFS). Excitation was done at 292 nm. The pH was 5.86 and the temperature was 11.5°C for both samples. The ratio of 330/350 nm emission is respectively seen to be 1.47 and 1.37. Finally it is noticed that the emission intensity of the W6/SS Cross linked B-FABP is higher than that of the native B-FABP. The curves were generated using a smooth function in FeliX32 software.

Figure 15 shows measurements of 300 to 400 nm emission from W6/SS Cross linked B-FABP before heating and after heating to 82°C and subsequent cooling to 11.5°C using SSFS. Excitation was at done 292 nm. The pH was 5.86 and the temperature was 11.5°C for both samples. It is seen that the ratio of 330/350 nm emission decreases from 1.37 to 1.22. This indicates

that the Trp residues become exposed to a more polar environment.

Figure 16 displays the result of a thermo scan conducted on native and one  
conducted on W6/SS Cross linked B-FABP. Excitation was done at 292 nm  
5 whereas emission was collected at 350 nm. The pH was 5.86 for both sam-  
ples. It is seen that the curvature of the two curves are similar. The transition  
mid point for both samples is approximately 55°C, indicating that the two pro-  
teins bear the same conformation. As with the emission scans it is noticed  
that the emission intensity of the W6/SS Cross linked B-FABP is higher than  
10 that of native B-FABP.

Emission scans of SS tagged B-FABP and native B-FABP were conducted in  
order to compare the tryptophan distribution, see Figure 17. The emission  
scans were recorded from 300-400 nm upon 292 nm excitation. The curves  
15 were processed using a smooth function in FeliX32 software. It is seen that  
emission at 350 nm is higher for SS tagged B-FABP than for native B-FABP,  
while the two have equal amounts of 330 nm emission. The ratio of 330/350  
nm for SS tagged B-FABP before heating is 1.243, compared to 1.469 for na-  
tive B-FABP. This indicate that tryptophans are in a more polar environment  
20 in SS tagged B-FABP than in native B-FABP.

To investigate whether or not the conformation of SS tagged B-FABP is re-  
tained after heating to 82°C and subsequent cooling to 11.5°C, emission  
scans were conducted. Figure 18 shows that the emission spectra for SS  
25 tagged B-FABP do not change after heating and subsequent cooling. The pH  
was 5.86 and the temperature was 11.5°C for both samples. Both emission  
scans were recorded from 300-400 nm upon 292 nm excitation, as an aver-  
age over 5 accumulations. The curves were processed using a smooth func-  
tion in FeliX32 software. The ratio of 330/350 nm emission before heating is  
30 1.243 and 1.223 after heating. This shows that no conformational change oc-  
curred to the protein after heating and cooling.

The thermal stability of SS tagged B-FABP was compared to native B-FABP by heating both proteins from 11.5°C to 82°C with a step rate of 1.5°C pr. minute at pH 5.86. Figure 19 shows no increase in 350 nm emission at 51°C for SS tagged B-FABP like the native B-FABP. The thermal scans subsequently smoothed using Felix32 software. No transition mid point can be seen for SS tagged B-FABP, as can be seen for native B-FABP around 56°C. Apart from a small kink around 20°C SS tagged B-FABP decreases nearly linearly from around 21°C, confirming a lack of structure for SS tagged B-FABP.

### Example 5

#### Total Internal Reflection Fluorescence (TIRF)

In order to investigate the ligand binding properties of W6/SS Cross linked B-FABP and SS tagged B-FABP, total internal reflection fluorescence (TIRF) measurements were conducted. Thereto, thiol activated slides were flushed with Milli-Q H<sub>2</sub>O, 35.5 µM B-FABP with peptide, Milli-Q H<sub>2</sub>O, 5 µM DHA, 10 mM phosphate buffer at pH 7.0 with 0.1% Tween 20, and Milli-Q H<sub>2</sub>O in the named order. The times at which the different substances were added can be seen in Table 3. The TIRF apparatus settings were: Gain: 4.7 V, Signal: 3.9 V, Excitation wavelength: 285 nm, Emission wavelength: 340 nm, Pump speed: 4.16 µl/s.

Materials: Docosahexaenoic acid, 22:6 omega-3 (DHA) (C<sub>22</sub>H<sub>32</sub>O<sub>2</sub>), Cas: 6217-54-5, Sigma; W6/SS Cross linked B-FABP; Sodium phosphate (NaH<sub>2</sub>PO<sub>4</sub>), Cas: 7558-80-7, Sigma; di-Sodium hydrogen phosphate dihydrate (Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O), Cas: 10028-24-7, Merck KGaA; Polyethylene glycol sorbitan monolaurate (Tween 20) (C<sub>58</sub>H<sub>114</sub>O<sub>26</sub>), Cas: 9005-64-5, Sigma-Aldrich; Glycerol (C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>), Cas: 56-81-5, Sigma; Milli-Q H<sub>2</sub>O; TIRF system: BioElectroSpec Inc. with a Photon Technology Int. PTI QM-2000 fluorescence spectrophotometer and a 75W Xenon arc source.

sample was placed in a 600  $\mu$ l quartz cuvette, and placed in the steady state spectrofluorometer. The protein samples analyzed were native B-FABP, W6/SS Cross linked B-FABP, and SS tagged B-FABP. For each sample emission spectra were recorded from 300 nm to 400 nm at 11.5°C before and after heating with excitation at 292 nm. Static scatter spectras were recorded from 450 nm to 550 nm at 11.5°C with excitation at 500 nm. In both emission specters and static scatter specters 5 accumulations were acquired and the integration was 0.1-second per point. Thermal scans were recorded at 350nm from 11.5°C to 82°C and again from 82°C to 11.5°C with excitation at 292 nm and a temperature step size of 1.5°C per minute and 1 point per second. All SSFS measurements were averaged by a smoothing function in FeliX32.

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Materials: Docosahexaenoic acid, 22:6 omega-3 (DHA) (C<sub>22</sub>H<sub>32</sub>O<sub>2</sub>), Cas: 6217-54-5, Sigma; W6/SS Cross linked B-FABP; Sodium phosphate (NaH<sub>2</sub>PO<sub>4</sub>), Cas: 7558-80-7, Sigma; di-Sodium hydrogen phosphate dihydrate (Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O), Cas: 10028-24-7, Merck KGaA; Polyethylene glycol sorbitan monolaurate (Tween 20) (C<sub>58</sub>H<sub>114</sub>O<sub>26</sub>), Cas: 9005-64-5, Sigma-Aldrich; Glycerol (C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>), Cas: 56-81-5, Sigma; Milli-Q H<sub>2</sub>O; TIRF system: BioElectroSpec Inc. with a Photon Technology Int. PTI QM-2000 fluorescence spectrophotometer and a 75W Xenon arc source.

Figure 20 displays static light scattering measurements of W6/SS Cross linked B-FABP before and after heating using total internal reflection fluorescence (TIRF) analysis. The pH was 5.86 and the temperature was 115°C for both samples. It is seen that heating and subsequent cooling of the sample leads to a nearly 9 fold increase in static light scattering by W6/SS Cross linked BFABP. Also it can be noticed that the emission peak before heating compared to the wavelength of excitation, is red shifted 1 nm. After heating the emission peak is red shifted 0.5 nm compared to wavelength of excitation.

To establish if there is formation of aggregates upon heating from 11.5°C to 82°C, static scatter measurements were conducted on native and SS tagged B-FABP. Figure 21 shows an increase in scattering intensity upon heating and subsequent cooling of SS tagged B-FABP. The pH was 5.86 and the temperature was 11.5°C for both samples. The scatter scans were recorded between 450 nm and 550 nm. The intensity for SS tagged B-FABP was approximately 6 times greater than native B-FABP. Furthermore both spectra are red shifted from the excitation wavelength at 500 nm by approximately 1 nm.

Table 3: The time of injection and time of flushing for the substances used during TIRF experiment.

Injected Substance	Time of Injection (s)	Duration of flushing (s)
Milli-Q H2O	245	275
35.5 $\mu$ M B-FABP with peptide	520	864
Milli-Q H2O	1384	580
5 $\mu$ M DHA	1964	728
10 mM phosphate buffer at pH 7.0	2692	644
With 0.1% Tween 20		
Milli-Q H2O	3336	2605

### Example 6

#### 5 Ellman's assay

To investigate whether or not the disulphide bond in the peptide was formed and to get an indication as to whether or not the protein was folded correctly, Ellman's assays were performed. The experiment were performed on both the SS tagged B-FABP and the W6/SS Cross linked B-FABP and both on folded protein and protein denatured in urea. Thereto, the solutions were mixed according to Table 4 and 4  $\mu$ l 10 mM Ellman's reagent was added to the solutions in complete darkness and left to react for 15 minutes likewise in darkness. Absorbance was measured at 412nm.

15 Materials: W3/SS tagged B-FABP; W6/SS Cross linked B-FABP; Phosphate buffered saline (PBS); Urea (CH<sub>4</sub>N<sub>2</sub>O), Cas: 57-13-6, BDH; 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (C<sub>14</sub>H<sub>8</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>), Cas: 69-78-3, Sigma.

Table 4 Solutions mixed for Ellman's assay.

Chemical	W3/SS Tagged B-FABP	Denatured W3/SS Tagged B-FABP	W6/SS Cross linked B-FABP	Denatured W6/SS Cross linked BFABP
Protein	20 $\mu$ l 50 $\mu$ M	20 $\mu$ l 50 $\mu$ M	28.5 $\mu$ l 35 $\mu$ M	28.5 $\mu$ l 35 $\mu$ M
10x PBS	20 $\mu$ l pH >8	20 $\mu$ l pH >8	20 $\mu$ l pH >8	20 $\mu$ l pH >8
Urea	0 $\mu$ l	100 $\mu$ l 8 M	0 $\mu$ l	100 $\mu$ l 8 M
Milli-Q H2O	156 $\mu$ l	76 $\mu$ l	147.5 $\mu$ l	47.5 $\mu$ l

- The principle of Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) (DTNB), is that it reacts with free and solvent exposed thiol groups to form mixed disulfides, *i.e.* it leads to the release of a free chromogenic 5-thio-2-nitrobenzoic acid (TNB) moiety, whereas the other half of DTNB reacts with one free thiol (R-SH) to form a non chromogenic mixed disulfide. The quantification of free thiol groups is done by measuring absorbance on the sample. Specifically measurements are conducted on TNB which has an extinction coefficient of 14150 M<sup>-1</sup> cm<sup>-1</sup> at 412 nm. Because of the low extinction coefficients of DTNB (211 M<sup>-1</sup> cm<sup>-1</sup>) and the TNB which have reacted with the free -SH group (210 M<sup>-1</sup> cm<sup>-1</sup>) absorbance at 412 nm can be used to calculate the concentration of reacted protein sulfhydryls [Riener et al., 2002].
- Table 5 displays the measured absorbance values of W6/SS cross linked B-FABP when refolded and subsequently denatured in 8 M urea. The ratio( $C_{EII}/C_{B-FABP}$ ) indicate that the refolded proteins on average contain 2.20 free solvent exposed cysteines, and in denatured conditions 2.15. In denatured conditions, a total of two out of four free cysteines were expected. These results indicate that one disulphide bridge exists in the protein.

Table 5 The result of Ellman's assay on folded and denatured W6/SS cross linked B-FABP. It is seen that the proteins on average contain 2.20 and 2.15 free solvent exposed cysteines.

	W6/SS cross linked B-FABP	W6/SS cross linked B-FABP denatured in 8 M urea
Abs <sub>EII</sub> :	0.156	0.152
C <sub>EII</sub> :	11.02 $\mu$ M	10.74 $\mu$ M
C <sub>B-FABP</sub> :	5 $\mu$ M	5 $\mu$ M
Ratio(C <sub>EII</sub> /C <sub>B-FABP</sub> ):	2.20	2.15

5

The Absorbance of 5-thio-2-nitrobenzoic acid (TNB<sup>2-</sup>) absorbance was measured at 412 nm and the concentration was calculated using Lambert-Beers law and a molar extinction coefficient of 14150 M<sup>-1</sup> cm<sup>-1</sup>. The calculated concentration was compared with the known concentration of B-FABP to find an estimate of the number of solvent exposed and free cysteine residues per B-FABP molecule.

Table 6 displays the measured absorbance and the amount of disulphide bridges calculated from the absorbance for the SS tagged B-FABP. It is seen that the proteins on average contain 0.41 free solvent exposed cysteines. This means that there is at least 1 disulphide bridge formed, maybe 2.

Table 6: The result of Ellman's assay on folded and denatured SS tagged B-FABP. It is seen that the proteins on average contain 0.41 and 0.44 free solvent exposed cysteines respectively.

	SS tagged B-FABP	SS tagged B-FABP denatured in 8 M urea
Abs <sub>EII</sub> :	0.041	0.044
C <sub>EII</sub> :	2.89 $\mu$ M	3.11 $\mu$ M
C <sub>B-FABP</sub> :	7 $\mu$ M	7 $\mu$ M
Ratio(C <sub>EII</sub> /C <sub>B-FABP</sub> ):	0.41	0.44

20

### Example 7

#### Labeling of the W6/SS coupled B-FABP with fluorescent probe

To determine whether the immobilization using light induced immobilization was successful, it was necessary to label the W6/SS Cross linked B-FABP and SS tagged B-FABP with a fluorescent probe according to following protocol: The dye, 5(6)-carboxynaphthofluorescein N-succinimidyl ester, was added (0.5  $\mu$ l) to the solution in a molar excess of two compared to the protein or peptide (200  $\mu$ l). Following the reaction was incubated under agitation and at room temperature for 4 hours. After incubation 20  $\mu$ l of 1M Tris and 1M glycine buffer pH 8.1 was added to the sample. To remove excess dye BioRad 2 ml spin-column cartridges was first added 1 ml Sephadex G-25 Superfine (Amersham Biosciences) and then centrifuged at 3000 rcf for 5 minutes. The protein/peptide solution was added and the cartridge was again centrifuged at 3000 rcf for 5 minutes. The protein/peptide was contained in the flow through. The protein/peptide could subsequently be washed for salt and small molecules by 20 ml Milli-Q H<sub>2</sub>O. Additionally, native B-FABP and the W6/SS peptide were labeled for control experiments. All proteins and peptides were in 10mM Na<sub>2</sub>CO<sub>3</sub> buffer at pH 9.0. The procedure for SS tagged B-FABP differed in the way that the incubation was done overnight.

20

Materials: Protein or Peptide in 10 mM Na<sub>2</sub>CO<sub>3</sub> buffer at pH 9.0, Tris[hydroxymethyl]aminomethane (Tris) (C<sub>4</sub>H<sub>11</sub>NO<sub>3</sub>), Cas: 77-86-1, Sigma; Glycine (NH<sub>2</sub>CH<sub>2</sub>COOH) Cas: 56-40-6, AppliChem; 5(6)-carboxynaphthofluorescein Nsuccinimidyl ester (C<sub>33</sub>H<sub>19</sub>NO<sub>9</sub>), Cas: 150347-58-3, Invitrogen.

25

### Example 8

#### Thiol Activation of Quartz Surfaces

In order to immobilize the B-FABP on glass slides using light induced immobilization it was necessary to thiol activate the quartz surfaces. Optically flat slides from ArrayIt Microarray Technology were used (average flatness of 2.0

30

nm) according to the following protocol: In order to clean the quartz slides they were immersed in 75°C chromosulphuric acid for 1 hour. Afterwards the slides were washed with deionised water. Then, for hydroxylation of the surfaces, they were immersed in 99 - 100°C 5 w/v% K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> for 1 hour. Following the hydroxylation the slides were washed with deionised water and then dried using N<sub>2</sub>. After the cleaning and hydroxylation the slides were ready for thiol activation. This was done by incubating slides covered by 0.3% 3-mercaptopropyl-trimethoxysilane for 30 minutes at room temperature. If the slides were to be used on the day of activation they were incubated for 1 hour at 100°C prior to use.

**Materials:** Chromosulphuric acid (H<sub>2</sub>SO<sub>4</sub> ≥92%, CrO<sub>3</sub> ≥1.3%), Cas: 1333-82-0 and 7664-93-9, Merck KGaA; Potassium persulphate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>), Cas: 7727-21-1, Aldrich; 3-mercaptopropyl-trimethoxysilane (C<sub>6</sub>H<sub>16</sub>O<sub>3</sub>SSi), ≥97%, Cas: 4420-74-0, Fluka; 1,3-dimethylbenzen (m-xylene) (C<sub>8</sub>H<sub>10</sub>), Cas: 108-38-3, Bie & Berntsen; 70% EtOH, Milli-Q H<sub>2</sub>O, Grant GD120 1.5kW water bath.

### **Example 9**

#### **Light induced protein immobilization**

W6/SS coupled B-FABP and SS tagged B-FABP were coupled onto an activated quartz surface using a laser system for UV irradiation. The protocols used thereto included surface activation, labeling by fluorescent probe (see previous examples), and light induced protein immobilization by laser. The solution of protein to be immobilized was dispensed onto a thiol activated optically flat glass slide. The laser system provided pulses with a duration of 50-60 fs and a wavelength of 280 nm. The laser focal spot was focused on the surface of the glass slide. The duration of illumination of each spot was 5 seconds and the spot were separated by roughly 500 μm. After immobilization the slide was washed with 4 ml of 1x PBS solution with 0.1% Tween 20

and thereafter rinsed with Milli-Q H<sub>2</sub>O. Finally the slide was scanned with a green (532 nm) laser with 6 μm resolution.

Materials: Protein/peptide to be immobilized; Thiol activated optically flat  
5 glass slide; 1x Phosphate buffered saline (PBS); Polyethylene glycol sorbitan monolaurate (Tween 20) (C<sub>58</sub>H<sub>114</sub>O<sub>26</sub>), Cas: 9005-64-5, Sigma-Aldrich; Milli-Q H<sub>2</sub>O; Spectra Physics Tsunami 3960 titanium-sapphire laser; Spectra Physics Millennia V solid state laser; Tecan LS 200 Scanner.

Figure 5 shows two examples of W6/SS cross-linked B-FABP labeled with  
10 5(6)-carboxynaphthofluorescein, immobilized on thiol derivatized quartz slides using UV; (A) 5 X 5 array; (B) 9 X 1 array. Distance between spots: 250 μm, average spot size: 50μm, time of immobilization: 5 seconds/spot. Array visualized with Tecan LS200 Array Scanner. Excitation: 633 nm (red laser). Emission filter: 670 nm. Gain 180. The images were processed in Im-  
15 ageJ 1.36b. Figure 6 shows SS cross-linked B-FABP labeled with 5(6)-carboxynaphthofluorescein, immobilized on thiol derivatized quartz slides using UV on a 2 X 5 array; Distance between spots: 250 μm, average spot size: 50μm, time of immobilization: 5 seconds/spot. Array visualized with Tecan LS200 Array Scanner. Excitation: 633 nm (red laser). Emission filter: 670 nm.  
20 Gain 180. The images were processed in ImageJ 1.36b.

Figures 5 and 6 demonstrate the successful immobilizations B-FABP to a thiol activated quartz slide via thiol bonds, either chemically coupled or C-terminally extended with a linker, comprising a disulfide bridge-containing  
25 triad or an appropriate disulfide bridge, respectively. No immobilization is seen in the areas not illuminated by the laser. Controls were made using native B-FABP and SS tag peptide. Native B-FABP showed no immobilization (not shown).

**Example 10****Selection of a Knottin peptide (HpTX2) as template for photoactive multiple disulfide tagging and design of mutations in HpTX2**

A screening of all 3D structure files corresponding to knottin peptides was done, in order to find a suitable template for tagging recombinant proteins lacking a photoactive triad (S-S/Trp). Heteropodatoxin-2 (HpTX2), which is a toxin from the venom of the spider *Heteropoda venatoria* (PDB: 1EMX) was selected. HpTX2 consists of 30 amino acids with one Tyr residue at position 20, and two Trp residues at positions 25 and 30. There are three disulphide bridges between residues 3 and 17, 10 and 22, 16 and 26. Figure 22 [www.pdb.org, accessed 05-01-06] shows the structure of HpTX2 (PDB: 1EMX, solved by NMR); Native HpTX2. Backbone: green; Disulphide bridges: yellow sticks; Tyr: red; Trp: blue. Middle: Mutated HpTX2 (mutHpTX2), Tyr20 is mutated to Trp (blue), Trp25 is mutated to Phe (orange), and Trp30 is deleted. Right: The distances from Trp to each disulphide bridge in mutHpTX2 is seen. Images are generated using PyMOL 0.98. HpTX2 was chosen as the length is only 30 amino acids and because it has a Tyr residue buried between the disulphide bridges only 5.71Å, 7.53Å, and 9.90Å from these, see Figure 22. Tyr20 would be mutated to Trp20 because of the higher extinction coefficient of Trp residues [Creighton, 1993]. Furthermore, Trp25 would be substituted by a Phe residue and Trp30 would be removed altogether to ensure that the transmitted energy stems from Trp20 exclusively for the purpose of simplifying the characterization of the peptide upon photo-activation.

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**Modeling on native HpTX2 using DeepView 3.7**

Prior to selecting HpTX2 as a scaffold for a recombinant immobilization method, molecular modeling on native HpTX2 was done using DeepView 3.7. This was done in order to see if the desired mutations were feasible. From the selected knottin gene, HpTX2, mutations were introduced at position 25 (Tyr→Trp) and 25 (Trp→Phe). Additionally Trp30 was removed.

30

HpTX2 was first examined in DeepView 3.7, to investigate the impact of mutations on the structure. The basic criteria for examining the mutations was to look at energy minimized conformations to check whether conformers not forming atomic clashes were predicted. Table 7 shows different  $\zeta$  values calculated for possible mutant conformations calculated by DeepView 3.7. The results show that a stable conformation for the HpTX2 Y20W mutant can be expected.

Table 7:  $\zeta$  for different conformations calculated using DeepView. The conformations relate to the mutations Tyr20→Trp20, and Trp25→Phe25. The value of  $\zeta$  corresponds to intramolecular clashes arising from the mutations, for each described conformation.

Conformation	$\zeta$ (Tyr20 → Trp20)	Conformation	$\zeta$ (Trp25 → Phe25)
1	182	1	134
2	226	2	0
3	82	3	60
4	46	4	0
5	16	5	134
6	180	6	18
7	2	7	0
8	214	8	28
9	22	9	136
10	48	-	-
11	244	-	-
12	196	-	-
13	44	-	-
14	0	-	-
15	60	-	-

The values of  $\zeta$  relate to intramolecular clashes, and should be as close to zero as possible. From the table it is evident, that conformations can be found where both the Tyr20 → Trp20 and the Trp25 → Phe25 mutations show  $\zeta$  values of zero, and it is reasonable to believe for these low energy conformers, that the conformation of mutated HpTX2 does not differ significantly from the native conformation of HpTX2. Figures 23 and 24 show two

possible conformations of HpTX2 after mutation of Tyr20 → Trp20 and Trp25 → Phe25, and removal of Trp30 (Figures 23). The values of the energy minimization calculations done in DeepView 3.7 was for this conformation 0 and 0 corresponding to the conformations 14 and 2 for Trp and Phe) shows the conformation with the smallest  $\zeta$  value. The values for the energy minimization done in DeepView 3.7 was for this conformation 60 and 136 corresponding to the conformations 15 and 9 for Trp and Phe) displays a conformation with higher  $\zeta$  values, where intramolecular atomic clashes are clearly seen. The  $\zeta$  values are taken as a measure of intramolecular clashes and must be as close to zero as possible for a conformation to be favorable.

### **Example 11**

#### **Design and construction of a vector for “universal” fusion of mutHpTX2 knottin to recombinant proteins**

The strategy with the HpTX2 peptide was to mutate it into the desired sequence and then implement the corresponding DNA sequence into the pET-11a vector thereby creating a new expression system. To test the concept, the gene coding for B-FABP should be inserted into this vector such that it is fused at the C-terminus to the N-terminus of the mutHpTX2 during expression.

#### Design of Oligonucleotide Sequences for the mutHpTX2 adapter

As described in the introduction the HpTX2 peptide must be mutated with regards to three of the residues; Tyr20 should be changed to a Trp and the two intrinsic Trp knocked out by replacing Trp25 with a Phe and by removing Trp30. The translated peptide sequence should be extended with restriction sites to be used for the incorporation into the plasmids along with a stop codon to terminate the translation. The amino acid sequence, and the nucleotide sequence for the mutated peptide, and the nucleotide sequence for the adapter peptide are illustrated in Table 2, which also included the added restriction sites and the stop codons. The nucleotide sequence has been op-

timized for *E. coli* usage.

The DNA adapter coding mutHpTX2 can be generated by annealing of self-complementary adapter primers LD035fw (Table 2) and filled-in with *Pfu* polymerase. Restriction sites for *Bam*HI at the 5'-end and *Bg*II at the 3'-end are added to the adapter for ligation into pET11 vector and restoration of a *Bam*HI site in the new vector, for subsequent ligations. An additional *Sma*I site is added in 5' for additional flexibility in ligations and a double stop codon is added at the 3'-end to stop translation.

10

Cloning strategy in pET-11a to generate a new vector pET-mutHpTX2 for C-terminal tagging of recombinant proteins

The construct with pET-11a utilizes the restriction sites for *Bam*HI and *Bg*II. *Bam*HI and *Bg*II overhangs are compatible, however if the overhangs are ligated the resulting annealing sequence is non-recognizable, i.e. not re-cleavable by *Bam*HI. A ligation mix of the mutant HpTX2-DNA digested with both *Bam*HI and *Bg*II and pET-11a digested with *Bam*HI results in a modified plasmid. The mutant HpTX2-DNA has become an intrinsic part of pET-11a, due to the non-recognizable annealing sequence and a new restriction site for *Bam*HI is created. But due to the compatibility of the two overhangs the insert may be oriented in two directions. It is therefore necessary to ensure that the orientation is correct such that the sequence of the translated DNA reads from N-terminus to C-terminus, thereby having the restriction site for *Bam*HI situated immediately upstream the insert and not vice versa. The result is a construct that allows for the in-frame C-terminal fusion of a protein coding DNA sequence to the mutated HpTX2 by digesting the plasmid and the target gene (insert) with *Bam*HI and *Nde*I.

20  
25

Results: Cloning of mutHpTX2 into pET-11a

Double stranded DNA coding the mutHpTX2 gene was generated by annealing to complementary oligonucleotides (LD035fw / LD036rv) and filling the 3'

30

ends with Pfu Polymerase. The resulting DNA fragments were digested with *Bam*HI and *Bgl*II and cloned into pET-11a. After transformation into DH5 $\alpha$ , positive transformants were screened by colony-PCR with vector specific T7fw and T7rv. A number of positive clones were found which were sub-

5 screened for correct orientation of the insert with T7fw and LD037rv primers (Table 2). Finally three clones were found with the correct insertion (data not shown). Clone 1 shows a size close to 220 bp, as expected, whereas clones 8 and 17 presents a size of approximately 300 bp, which is longer than expected.

10

Materials: PCR primers LD035fw, LD036rv, LD037rv, T7fw, TAGCopenhagen; *Bam*HI restriction enzyme, 10 u/ $\mu$ l, Fermentas; *Bgl*II restriction enzyme, 10 u/ $\mu$ l, New England BioLabs; Buffer Y<sup>+</sup>/Tango with BSA 10x, MBI-Fermentas; pET-11a vector DNA, Novagen; Calf intestine alkaline phosphatase (CIAP), MBI-Fermentas; 10x reaction buffer (0.1 M Tris-HCl pH 7.5, 0.1 M MgCl<sub>2</sub>), MBI-Fermentas; T4 DNA ligase, Roche Diagnostics GmbH; Ligation buffer 10x (660 mM Tris-HCl, 50 mM MgCl<sub>2</sub>, 10 mM Dithioerythritol, 10 mM ATP, pH 7.5), Roche Diagnostics GmbH; Sterile Milli-Q H<sub>2</sub>O.

20 Methods: To amplify the coding sequence for mutHpTX2, the two primers LD035fw and LD036rv were hybridized and extended by running a primer hybridisation and extension PCR. The DNA concentration was determined by OD measurements. The DNA was then cleaned using a phenol-chloroform extraction, and the remaining pellet was resuspended in 100  $\mu$ l of sterile Milli-

25 Q H<sub>2</sub>O, and digested using a double digest of *Bam*HI and *Bgl*II. Since a 10 times over digest was required, 50  $\mu$ l of mutated HpTX2 DNA (280 ng/ $\mu$ l), 20  $\mu$ l of Y<sup>+</sup>/Tango buffer, 27  $\mu$ l of sterile Milli-Q H<sub>2</sub>O, 1  $\mu$ l of *Bam*HI, and 2  $\mu$ l of *Bgl*II were mixed and left at 37°C for 10 hours. Upon digestion, the sample was cleaned and the DNA was precipitated using phenol-chloroform extrac-

30 tion, and following resuspended in 20  $\mu$ l of Milli-Q H<sub>2</sub>O. 1  $\mu$ l was extracted and used for concentration determination by OD measurements. To prepare

the pET-11a plasmid for insertion, 2  $\mu$ l of pET-11a (1300 ng/ $\mu$ l) was digested with 1  $\mu$ l BamHI in 20  $\mu$ l Y<sup>+</sup>/Tango buffer and 77  $\mu$ l sterile Milli-Q H<sub>2</sub>O for 5 hours at 37°C. The plasmid was cleaned and precipitated by use of phenol-chloroform extraction. The cleaned plasmid was resuspended in 20  $\mu$ l of sterile Milli-Q H<sub>2</sub>O. 1  $\mu$ l was extracted and used for concentration determination by OD measurements. The resuspended digested plasmid pET-11a was dephosphorylated using calf intestine alkaline phosphatase (CIAP) in order to avoid self ligation 18  $\mu$ l of plasmid was mixed with 5  $\mu$ l of 10x reaction buffer, 26  $\mu$ l of sterile Milli-Q H<sub>2</sub>O and 1  $\mu$ l of CIAP. The mix was incubated at 37°C for 30 minutes. The ligation of the digested mutHpTX2 with the digested dephosphorylated pET-11a was done by mixing 4  $\mu$ l of pET-11a (405 ng/ $\mu$ l) with 2  $\mu$ l of mutHpTX2 (67.5 ng/ $\mu$ l) and 2  $\mu$ l ligation buffer, 2  $\mu$ l T4 ligase and 10  $\mu$ l sterile Milli-Q H<sub>2</sub>O. The mix was incubated at 12°C for 72 hours and following inactivated at 65°C for 10 minutes. The ligated plasmid was transformed into competent E. coli DH5 $\alpha$  cells and plated onto LB-Amp<sup>100</sup> plates and incubated at 37°C overnight. After incubation 20 colonies were picked and replated onto a new LB-Amp<sup>100</sup> plate and left at 37°C over night. The replated colonies were subjected to a screening PCR and analyzed on 1.5% agarose gels using a 100bp marker. The PCR was redone with the T7fw and LD037rv primers on selected colonies to screen for those having the insert in the correct orientation. The plasmid DNA for the selected samples was expressed and purified.

### Example 13

#### 25 pET-43.1a:

The *Sma*I restriction site, in the mutant HpTX2-DNA sequence was designed to be used with the pET-43.1a plasmid along with a *Bgl*II site. This plasmid is designed for high-level expression of recombinant proteins, by fusing them with the highly expressed, highly cyto-soluble Nus protein (495 AA), to generate a so-called Nus•Taq. Digesting pET-43.1a and the adapter peptide with *Sma*I and *Bgl*II fuses mutHpTX2 DNA to Nus. This construct allows thereby

for the high-level production of the mutated HpTX2 peptide for a thorough characterization.

**Example 14**

**5 Cloning of B-FABP into mutHpTX2 Vector**

10  $\mu$ l of the purified plasmid DNA from the three positive colonies (1 (680 ng/ $\mu$ l), 8 (1910 ng/ $\mu$ l) and 17 (840 ng/ $\mu$ l)) were digested with 0.5  $\mu$ l NdeI and 1  $\mu$ l BamHI in 10  $\mu$ l Y<sup>+</sup>/Tango 10x and 28.5  $\mu$ l Milli-Q H<sub>2</sub>O overnight. To amplify the B-FABP for insert into the mutHpTX2 vectors, a PCR was conducted with the primers LD007fw and LD041rv and a template strand coding for B-FABP. The PCR product was cleaned using a DNA cleaning kit and the concentration was determined by OD measurements. To make the DNA compatible with the vectors 40  $\mu$ l of the cleaned product was digested with 0.5  $\mu$ l NdeI and 1  $\mu$ l BamHI in 20  $\mu$ l Y<sup>+</sup>/Tango 10x and 39  $\mu$ l Milli-Q H<sub>2</sub>O. Both digestion products were cleaned and precipitated according to the phenol-chloroform protocol and for each of the three samples of mutHpTX2 vector a ligation mix with B-FABP was prepared according to Table 8 for three colonies 1, 8 and 17, and left for 4°C for three days.

20 Table 8: Ligation solutions for three mutHpTX2 vectors

<b>mutHpTX2 vector</b>	<b>1 (325 ng/<math>\mu</math>l)</b>	<b>8 (875 ng/<math>\mu</math>l)</b>	<b>17 (780 ng/<math>\mu</math>l)</b>
B-FABP (320 Ng/ $\mu$ l)	3 $\mu$ l	3 $\mu$ l	3 $\mu$ l
mutHpTX2 vector	4 $\mu$ l	3 $\mu$ l	3 $\mu$ l
Ligase Buffer	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l
T4 ligase	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l
Milli-Q H <sub>2</sub> O	9 $\mu$ l	10 $\mu$ l	10 $\mu$ l

After ligation the samples were inactivated by heating to 65°C for 10 minutes. The ligated constructs were transformed into competent *E. coli* DH5 $\alpha$  cells, and following plated onto LB-Amp<sup>100</sup> plates and incubated at 37°C overnight.

25 After incubation 20 colonies were picked and replated onto a new LB Amp<sup>100</sup> plate and left at 37°C over night. The replated colonies were subjected to a

screening PCR and analyzed on 1.5% agarose gels using a 100 bp marker.

Materials: BamHI restriction enzyme, 10 u/μl, Fermentas; NdeIII restriction enzyme, 20 u/μl, New England BioLabs; Buffer Y<sup>+</sup>/Tango with BSA 10x, MBI-  
5 Fermentas; T4 DNA ligase, Roche Diagnostics GmbH; Ligation buffer 10x (660 mM Tris-HCl, 50 mM MgCl<sub>2</sub>, 10 mM Dithioerythritol, 10 mM ATP, pH 7.5), Roche Diagnostics GmbH; Sterile Milli-Q H<sub>2</sub>O.

Verification of mutHpTX2 Insert in pET-11a

10 Figure 26 shows a 1.5% agarose gel with DNA from a colony PCR using primers T7fw and LD037rv on pET-11a ligated with mutHpTX2. Lane 1 and 14 holds a 100 bp ladder, while lane 6, 10, and 12 corresponds to colony 8, 1 and 17 respectively. Bands corresponding to products of ~300 bp length are found for colonies 8 and 17, while a band corresponding to a ~220 bp prod-  
15 uct is found for colony 1. A PCR using these two primers and pET11-a with mutHpTX2 should yield a 226 bp product. It is expected that colony 1 has the correct insert, however as none of the clones were sequenced it was decided to keep all three colonies at this point. Conclusive evidence requires se-  
quencing of the clones.

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**Claims**

1. A method of coupling an element A to an element B via thiol binding, said element A lacking an appropriate disulfide bridge or disulfide bridge-containing triad and said element B being capable of binding a thiol group, comprising:
- 5
- (1) generating an element A\* by providing an appropriate disulfide bridge or disulfide bridge-containing triad to said element A, and
- (2) a) irradiating said element A\* to create an appropriate reactive thiol group from said disulfide bridge, and
- 10
- b) incubating said irradiated element A\* with said element B, thereby obtaining a coupling between element A and element B via thiol binding;
- or
- 15
- a) incubating said element A\* with said element B, and
- b) irradiating said element A\* in the presence of said element B to create an appropriate reactive thiol group by disulfide bridge disruption in said element A\*, thereby obtaining a coupling between element A and element B via thiol binding.
- 20
2. A method of coupling an element A to an element B via thiol binding, said element A and said element B each lacking an appropriate disulfide bridge or disulfide bridge-containing triad, comprising:
- (1) a) generating an element A\* by providing an appropriate disulfide bridge or disulfide bridge-containing triad to said element A,
- 25
- b) generating an element B\* by providing an appropriate disulfide bridge or disulfide bridge-containing triad to said element B, and
- (2) c) irradiating said element A\* and said element B\* to create an appropriate reactive thiol group from said disulfide bridge in each element,
- 30
- and

- d) incubating said irradiated elements A\* and B\*, thereby obtaining a coupling between element A and element B via thiol binding;
- or
- c) incubating said element A\* with said element B\*, and
- 5 d) irradiating said elements A\* and B\* to create an appropriate reactive thiol group by disulfide bridge disruption in each element, thereby obtaining a coupling between element A and element B via thiol binding.
- 10 3. A method according to claims 1 or 2, wherein element A and element B are parts of a common structure.
4. A method according to any of the claims 1-3, wherein element A and element B independently are a support or a molecule.
- 15 5. A method according to claim 1-4, wherein the elements A and B are selected from the combinations: A and B are molecules, A is a molecule and B is a support, A and B are supports, and A is a support and B is a molecule,.
- 20 6. A method according to any of claims 3-5, wherein said molecule comprises a peptide, polypeptide or protein.
7. A method according to any of claims 3-6, wherein said molecule is a peptide, polypeptide or protein.
- 25 8. A method according to any of the claims 1 to 7, wherein the appropriate disulfide bridge or disulfide bridge-containing triad is provided to said element(s) by a linker molecule.
- 30 9. A method according to claim 8, wherein said linker molecule is a peptide, polypeptide, or protein, or a natural or synthetic derivate or analogue thereof.

10. A method according to claim 9, wherein said peptide, polypeptide or protein are natural, synthetic or mutated natural molecules.

5 11. A method according to claim 10, wherein said linker comprises one or more copies of any of the formulas

I:  $X1_m C X2_n C X3_o \emptyset X4_p$ ,

II:  $X1_m C X2_n \emptyset X3_o C X4_p$ ,

10 III:  $X1_m \emptyset X2_n C X3_o C X4_p$ ;

wherein  $X1_m$ ,  $X2_n$ ,  $X3_o$  and  $X4_p$  represent the same or different peptides, each peptide consisting of m, n, o, and p amino acids respectively, where

m, n, o and p are mutually independent numbers between 0 and 1000,

15 and

$m + n + o + p \leq 1000$ ,

said amino acids being selected from all natural and synthetic amino acids;

C is cysteine, and the two cysteines are covalently joined by a disulfide bridge, and

20  $\emptyset$  is an aromatic amino acid such as phenylalanine, tryptophane or tyrosine, or a peptide bond.

12. A method according to claim 11, wherein

m, n, o and p are mutually independent numbers between 0 and 100,

25 and

$m + n + o + p \leq 100$ .

13. A method according to claim 11, wherein

m, n, o and p are independently and individually any number between

30 0 and 25, and

$m + n + o + p \leq 25$ .

14. A method according to claim 11, wherein  
m, n, o and p are mutually independent numbers between 0 and 10,  
and  
5  $m + n + o + p \leq 10$ .
15. A method according to any of the claims 8 to 14, wherein said appropriate disulfide bridge or disulfide bridge-containing triad is provided to said elements(s) through covalent binding of said linker molecule.  
10
16. A method according to claim 15, where said linker molecule is covalently bound by using NHS (N-hydroxysuccinimide), EDC (N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride), activated ester, maleimide, disulfide formation, streptavidin/biotin, activated alcohol, vinylsulfone,  
15 Schiff base formation and/or "click" chemistry.
17. A method according to claim 16, comprising a fractionation, selection and/or purification step.
- 20 18. A method according to any of the claims 1 to 14, wherein said linker or said appropriate disulfide bridge and/or disulfide bridge-containing triad is provided through genetic engineering techniques.
- 25 19. A method according to claim 18, wherein genetic engineering techniques comprise: (i) N-terminal extension, (ii) C-terminal extension, (iii) internal extension, (iv) amino acid substitution, (v) amino acid insertion, (vi) amino acid deletion or (vii) any combination or combinations of said methods (i-vi).
- 30 20. A method according to claim 19, wherein the genetic engineering results in a conformation change in a molecule, thereby bringing an aromatic amino acid and an appropriate disulphide bridge in the vicinity of each other.

21. A method according to any of the preceding claims, wherein coupling of said elements A and B is provided by adding a free aromatic amino acid (in solution) to the vicinity of one or more appropriate disulfide bridges.
- 5
22. A method according to claim 21, where said free aromatic amino acid is present in a molecule.
23. A method according to any of the preceding claims, wherein said irradiation step comprises light of a wavelength that excites one or more aromatic amino acids present in the triad or as free amino acid.
- 10
24. A method according to claim 23, wherein said irradiation step comprises UV light in the wavelength interval of 250 to 305 nm, or with light having longer wavelengths that by means of non-linear processes and/or multi-photon excitation promotes the same electronic transitions as light in said wavelength interval of 250 to 305 nm.
- 15
25. A method according to claim 24, wherein said wavelength interval comprises 250 to 260nm, 270 to 280 nm and/or 290 to 300nm, preferably about 254, 275 or 295 nm.
- 20
26. A method according to any of the preceding claims, wherein said element A comprises a biomolecule, such as a peptide, a protein, a polynucleotide, a lipid, a sugar, a pharmaceutical, a cosmetical, a pro-drug, and the like.
- 25
27. A method according to any of the claims 4-26, wherein said support comprises a thiol reactive surface or a surface that can be made thiol reactive.
28. A method according to claim 27, wherein said surface comprises gold.
- 30

29. A method according to claim 27 or 28, wherein said coupling is an immobilization on said support.
30. A method according to claim 29, wherein said immobilization is spatially  
5 controlled.
31. A method according to any of the claims 27-30, wherein said support is a derivatized support that is capable of binding a thiol group.
- 10 32. A method according to claim 31, wherein said support comprises a thiol group or a disulfide bridge.
33. A method according to any of the claims 4-32, wherein the support comprises a spacer.  
15
34. A method according to any of the claims 4-33, wherein the support is a carrier for the molecule and may be a biomolecule such as a binding molecule, a peptide, polypeptide, protein, nucleic acid, lipid, carbohydrate, etc, or a (nano)particle such as a collagen particle, liposome, magnetic particle, etc,  
20 or the support is a label, such as a dye, a chromophore, a binding partner such as a receptor, a radioisotope, etc.
35. A method according to any of the claims 4-33, wherein the support is in the form of a surface for immobilising reagents in a bioassay, biosensor, or  
25 for trapping and/or separating molecules, or for immobilising molecule(s) for measuring interactions between molecules, etc.
36. A carrier, magnetic particle or cantilever comprising one or more molecule coupled by irradiation of a disulfide bridge or disulfide bridge-containing  
30 triads provided to said molecule and/or to said carrier, magnetic particle or cantilever.

37. A dimer, homo-dimer, hetero-dimer or dendrimer coupled by irradiation of a disulfide bridge or disulfide bridge-containing triads provided to said dimer, homo-dimer, hetero-dimer or dendrimer.

5

38. A vector or plasmid comprising a nucleic acid sequence concoding a peptide comprising a disulfide bridge or disulfide bridge-containing triad to be fused to the N- or C-terminus of a protein or peptide to be expressed by said vector or plasmid.

10

39. A vector/plasmid according to claim 38 wherein said peptide comprising an appropriate disulfide bridge-containing triad is knottin (HpTX2) or a functional fraction thereof.

15

40. A vector/plasmid according to claim 38 wherein said peptide comprising an appropriate disulfide bridge or disulfide bridge-containing triad is a linker as defined in claims 11-14.

20

41. An element comprising an appropriate disulfide bridge or disulfide bridge-containing triad provided according to the present invention for use in thiol coupling via irradiation of an aromatic amino acid in close proximity of said disulfide bridge.

25

42. Two or more elements coupled together according to the method of claims 1-35.

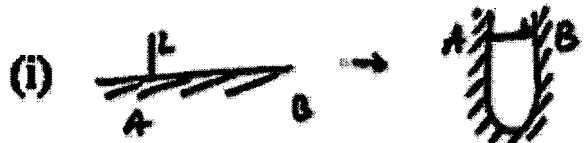
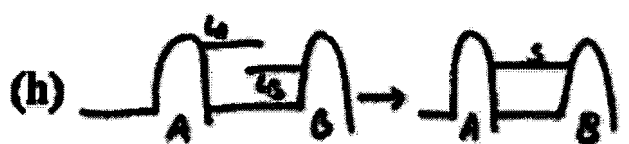
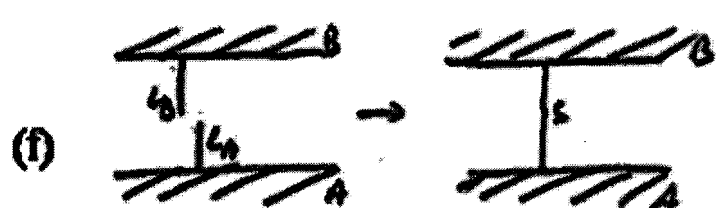
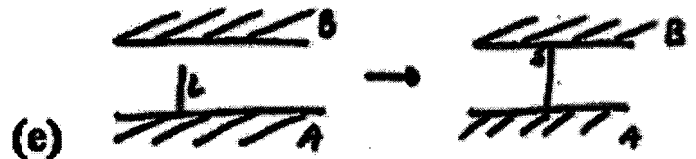
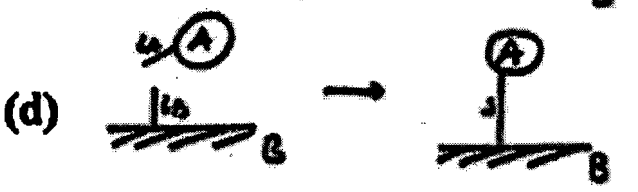
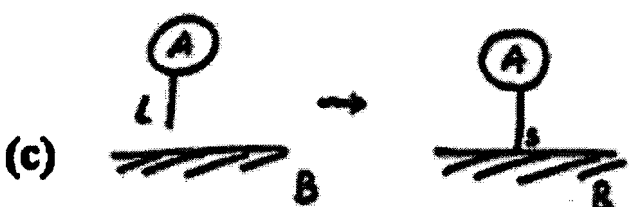
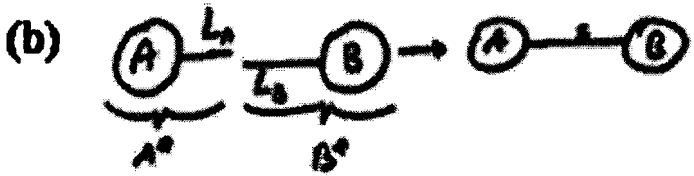
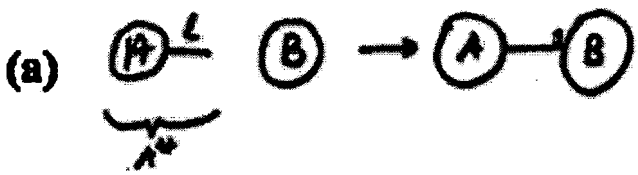


Figure 1

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Cyclic peptide linker (without leaving group)

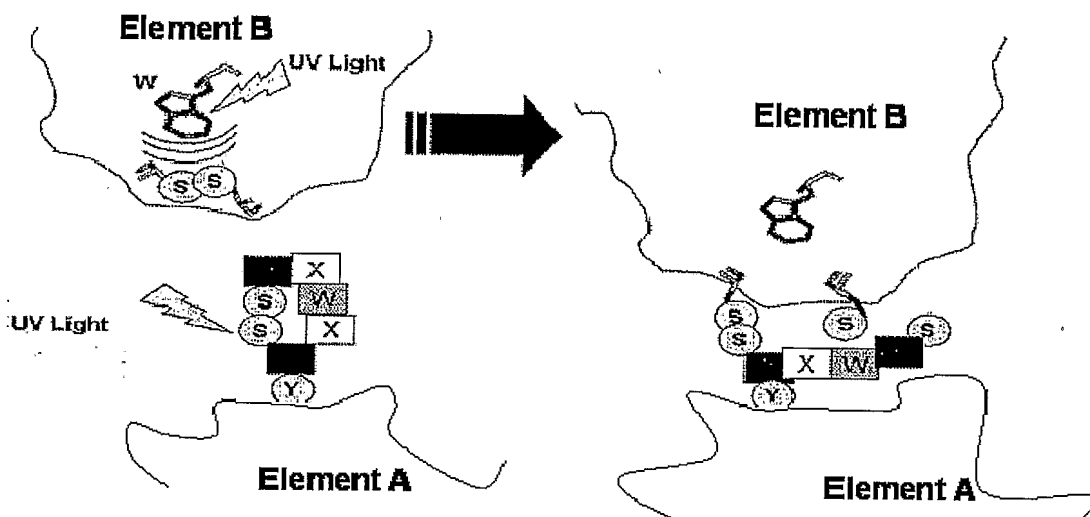


Figure 2

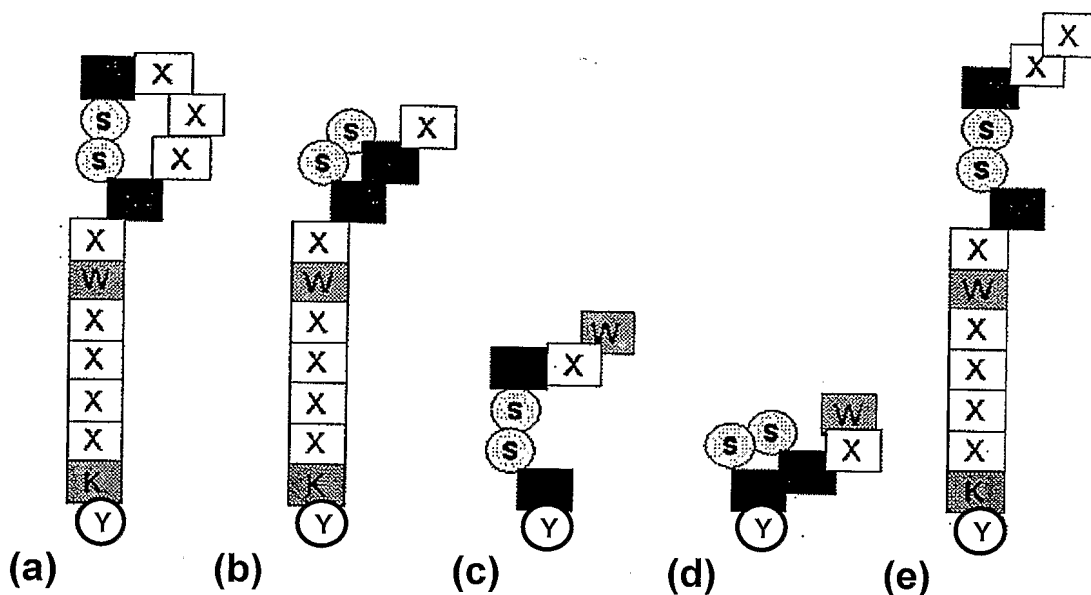


Figure 3

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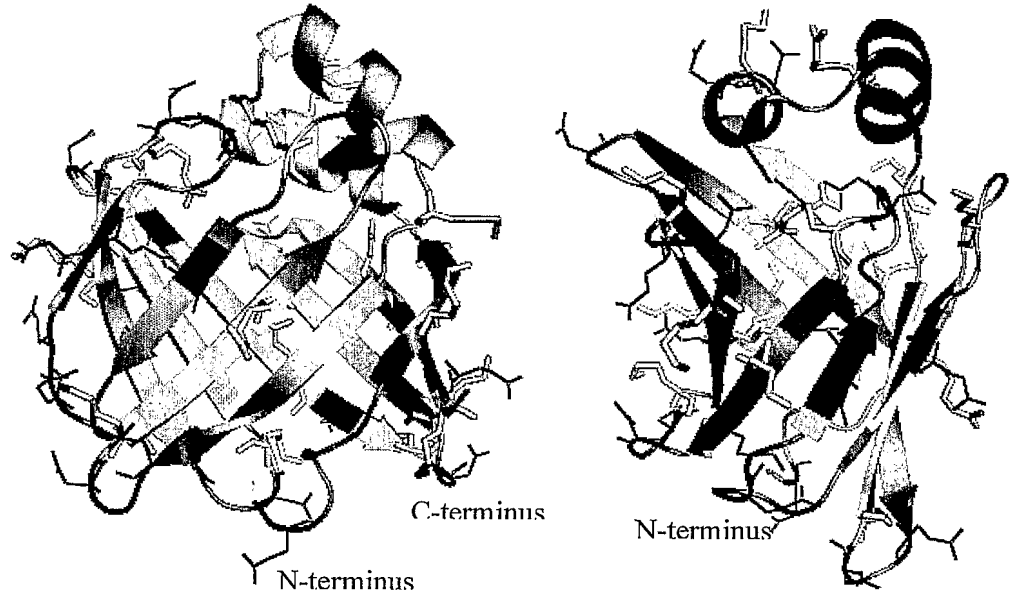


Figure 4

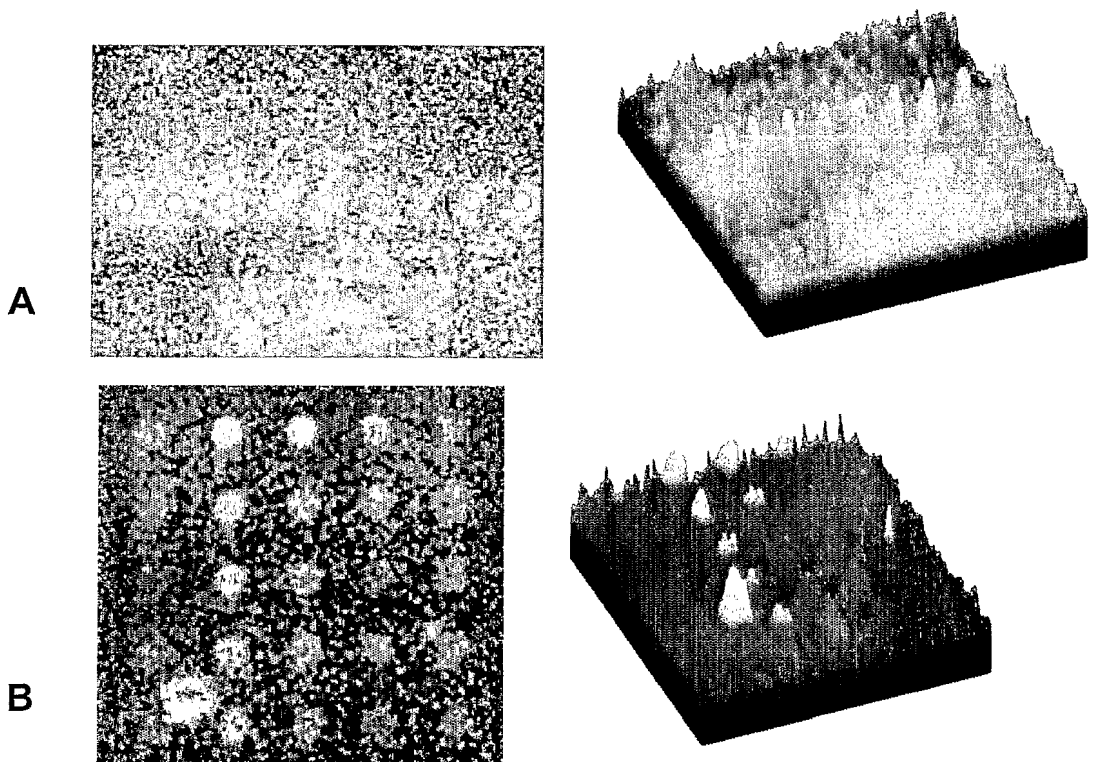


Figure 5

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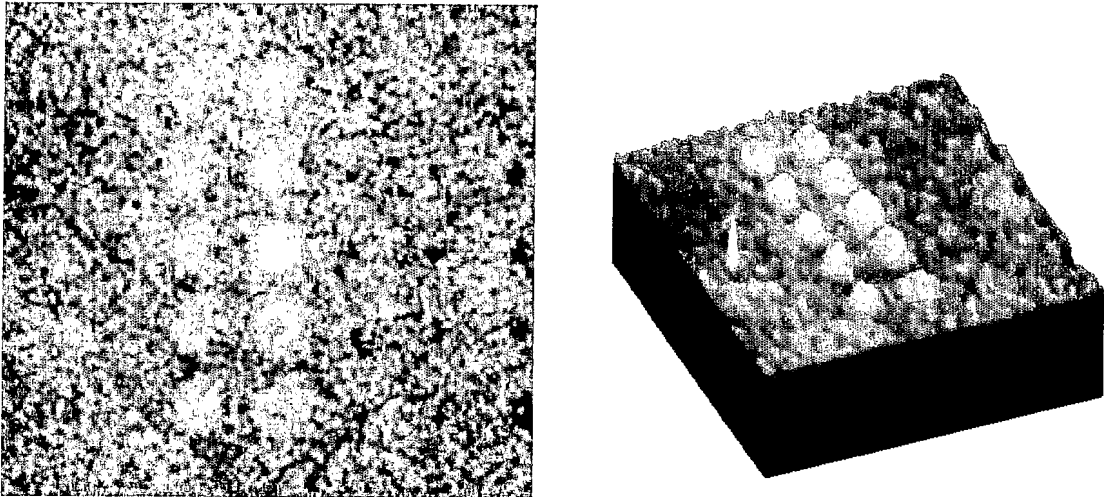


Figure 6

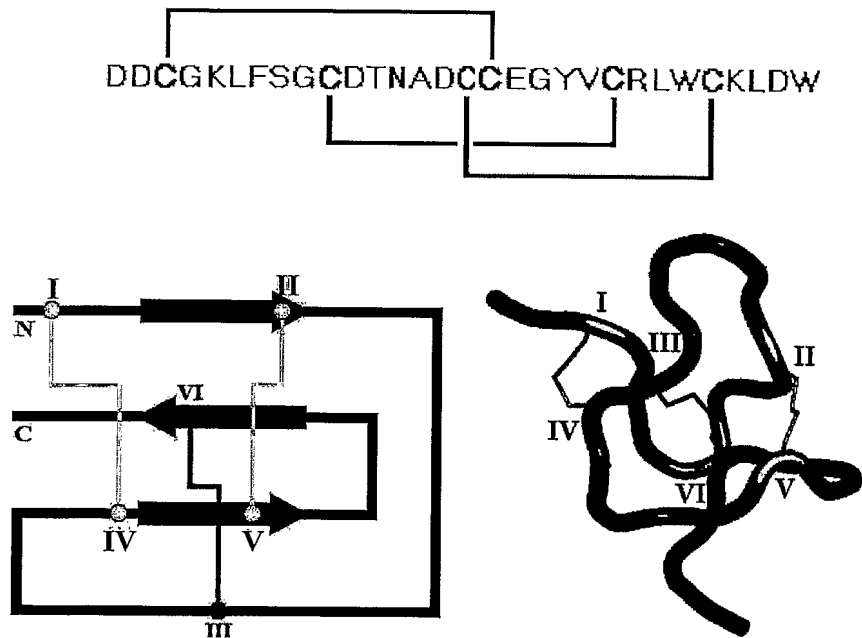


Figure 7

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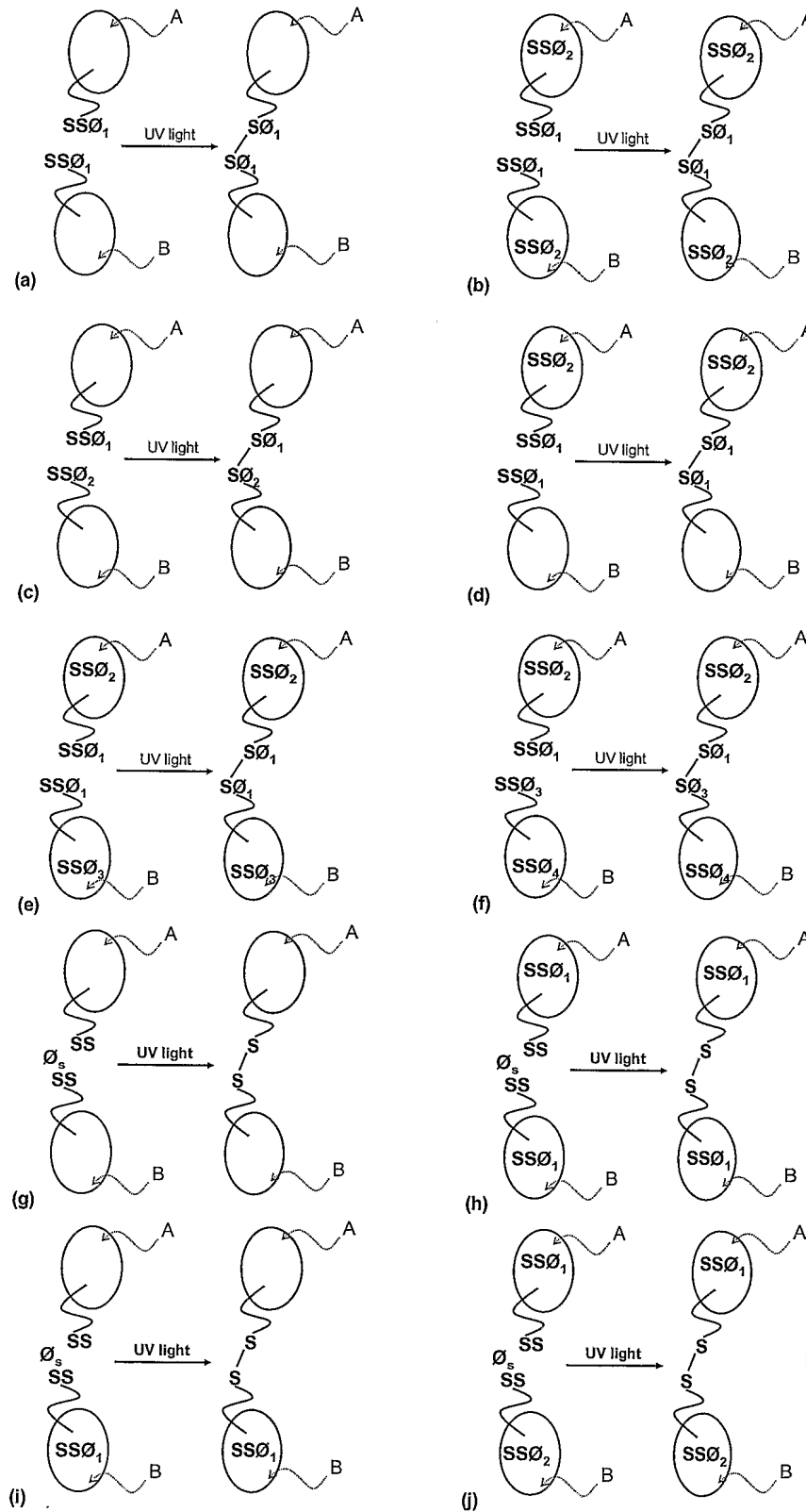


Figure 8

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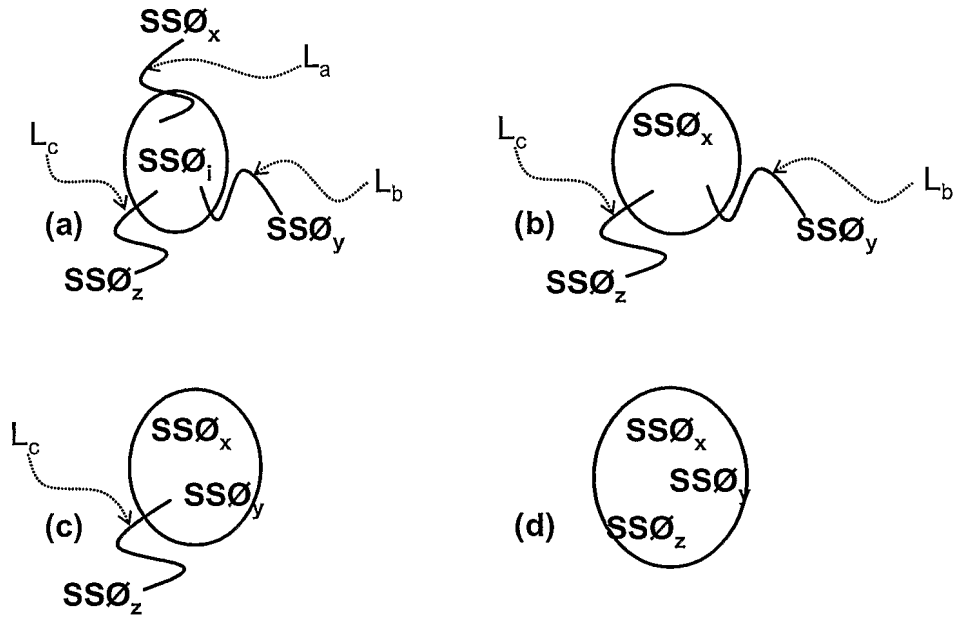


Figure 9

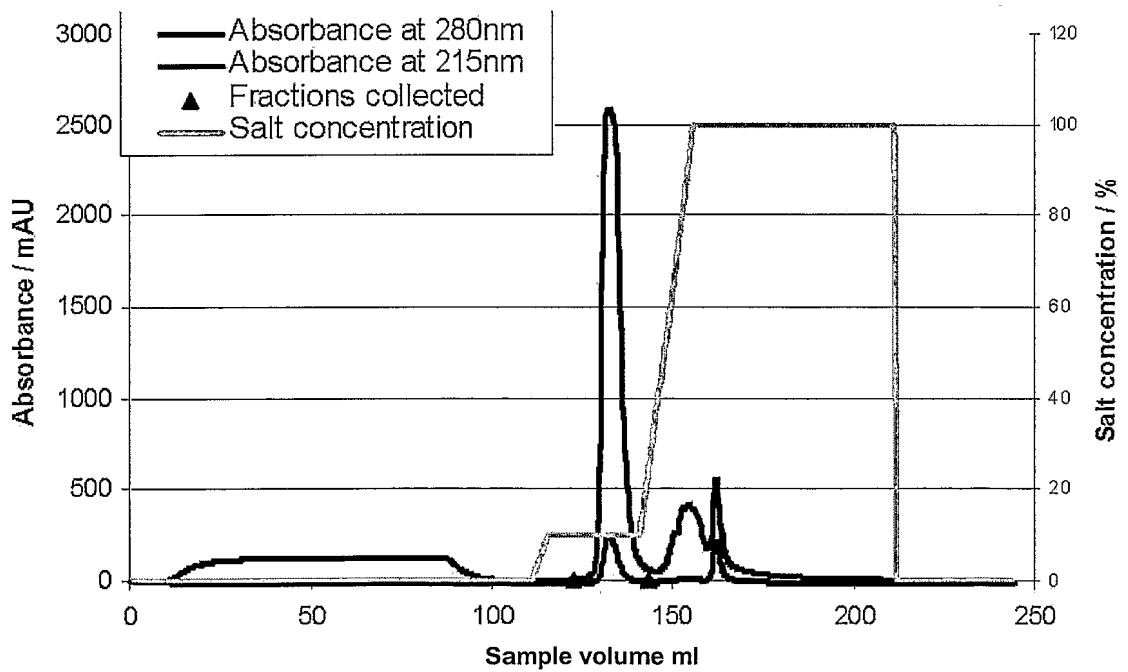


Figure 10

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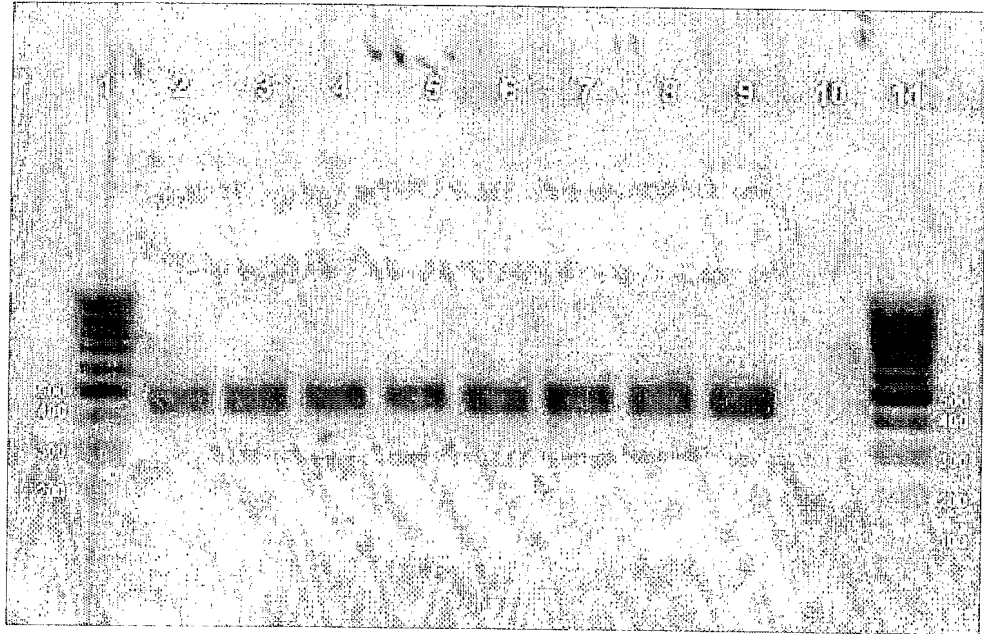


Figure 11

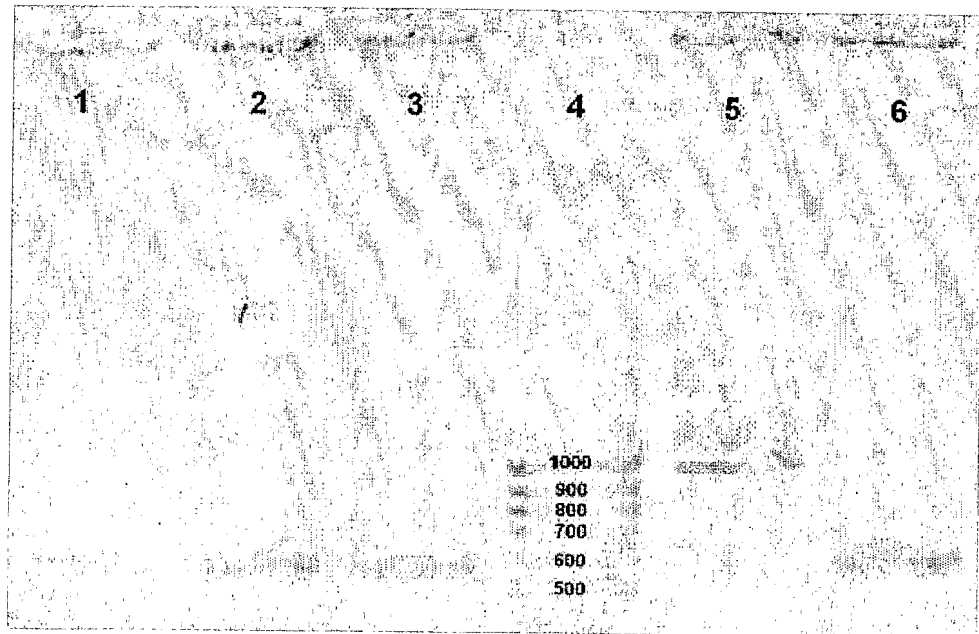


Figure 12

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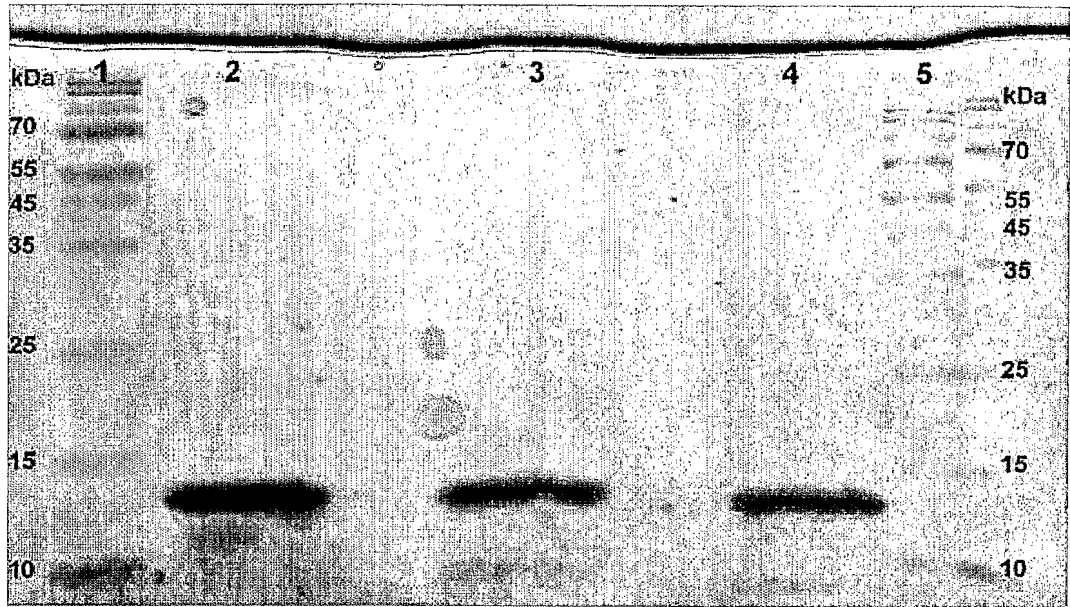


Figure 13

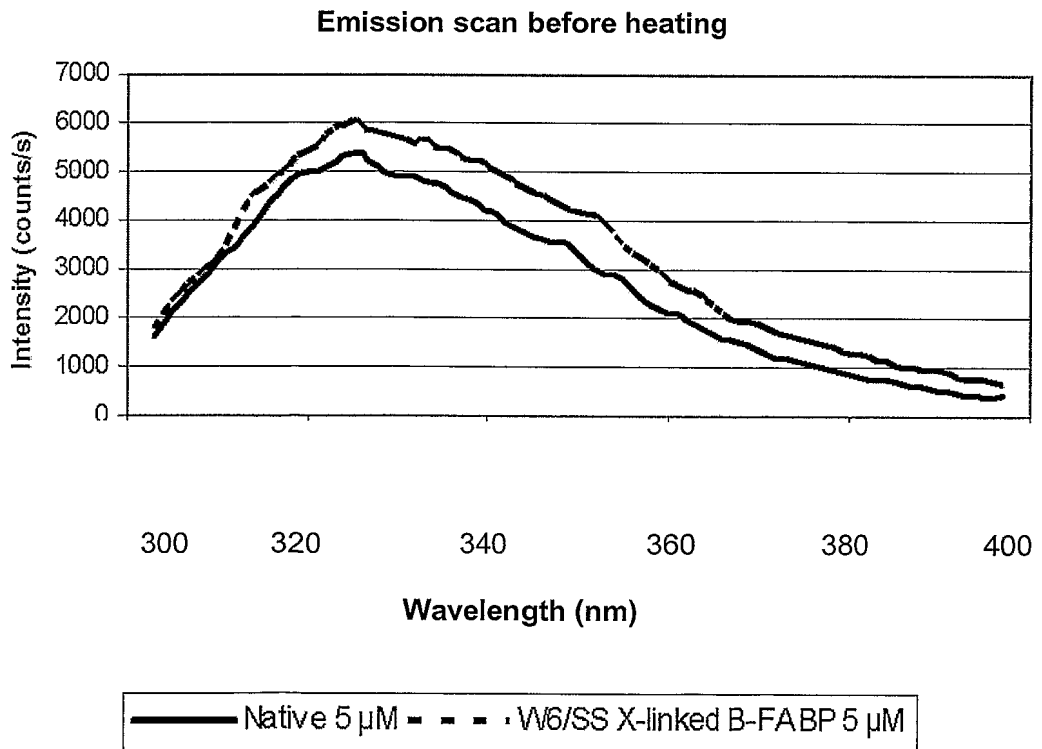


Figure 14

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Emission scan W6/SS X-linked B-FABP

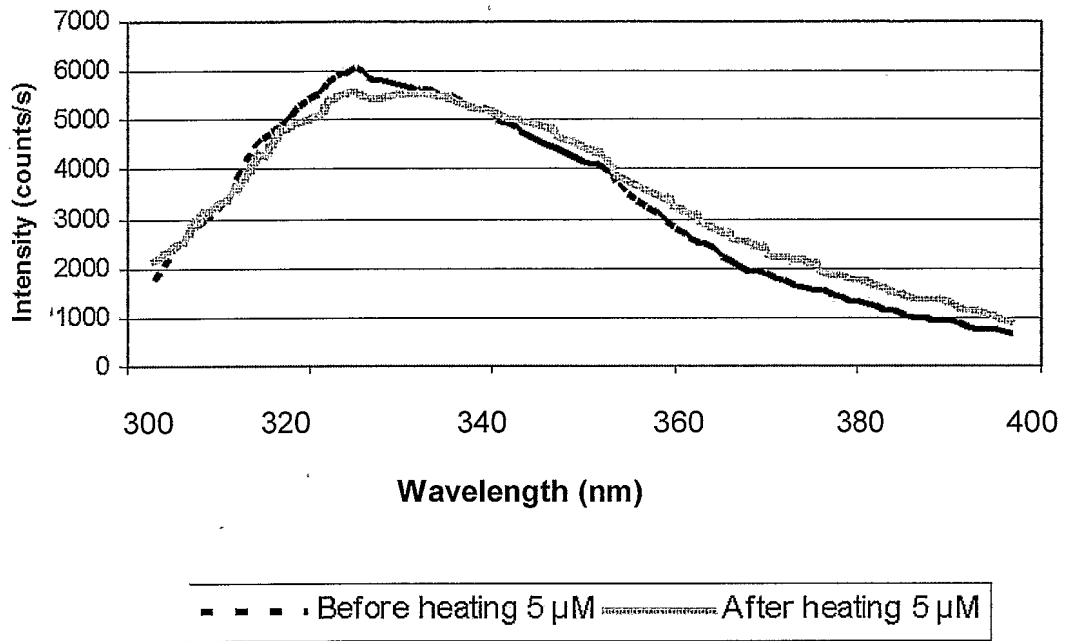


Figure 15

Thermal scan

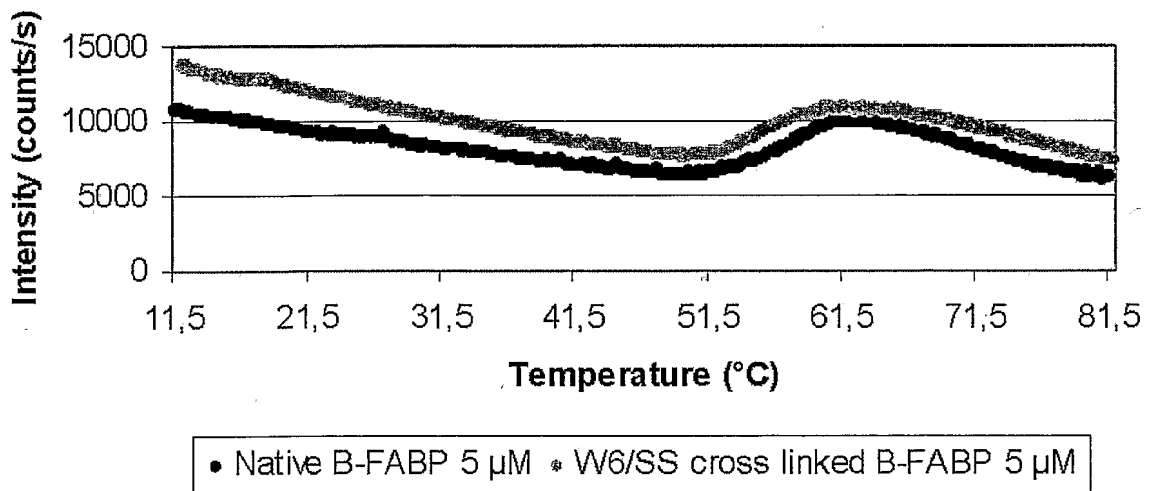


Figure 16

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Scattering scan of W6/SS cross linked B-FABP 5  $\mu$ M

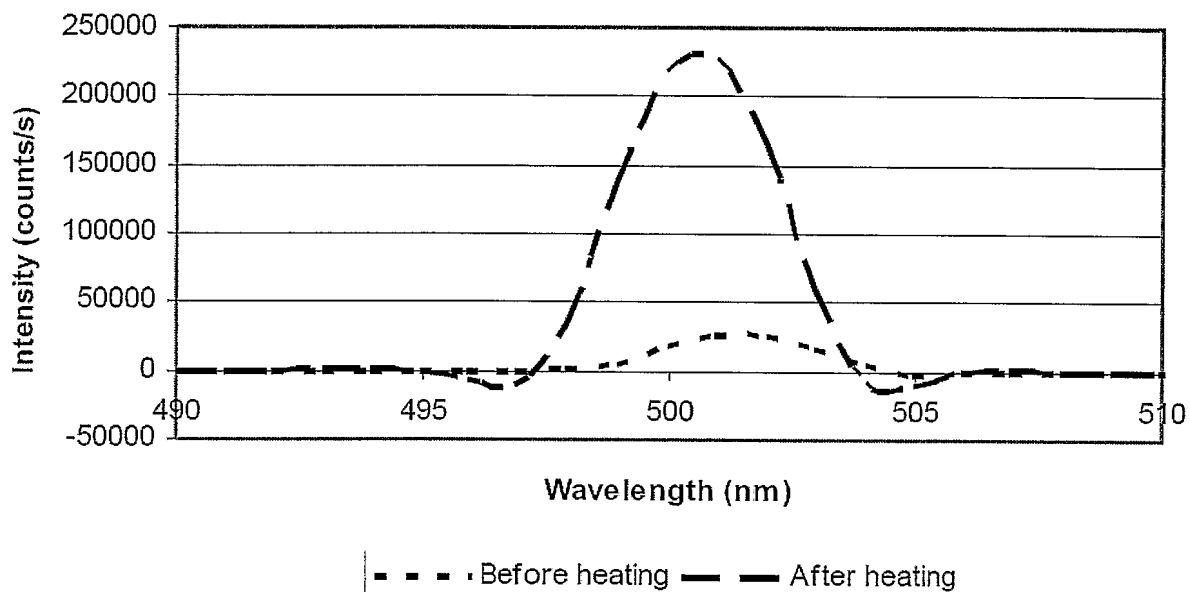


Figure 17

Emission scan before heating

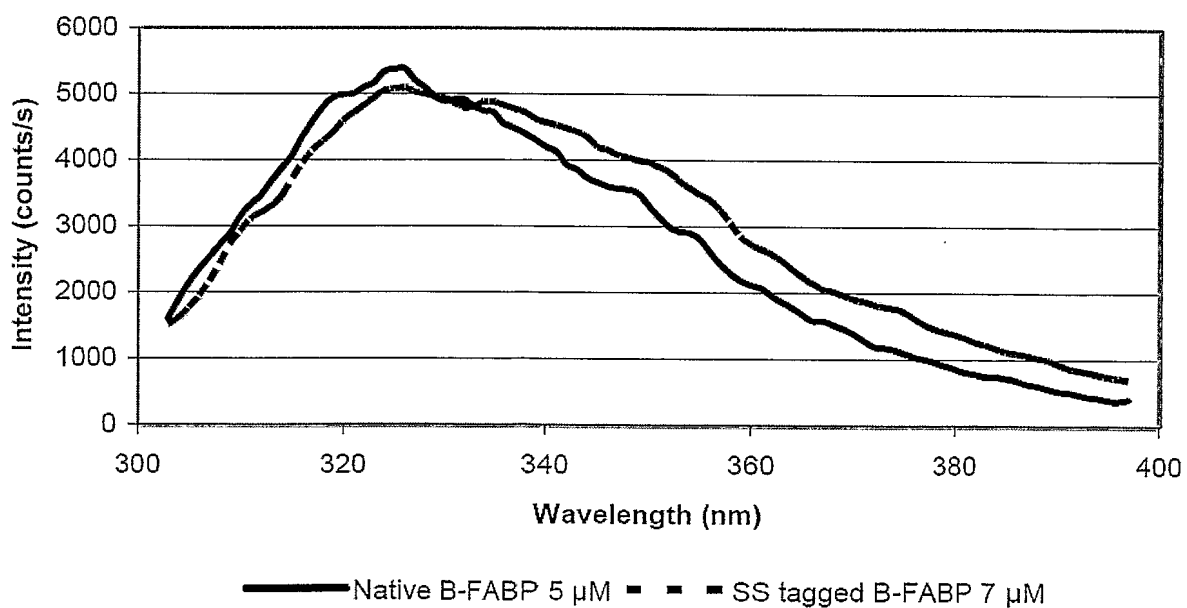


Figure 18

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Emission scan for SS tagged B-FABP

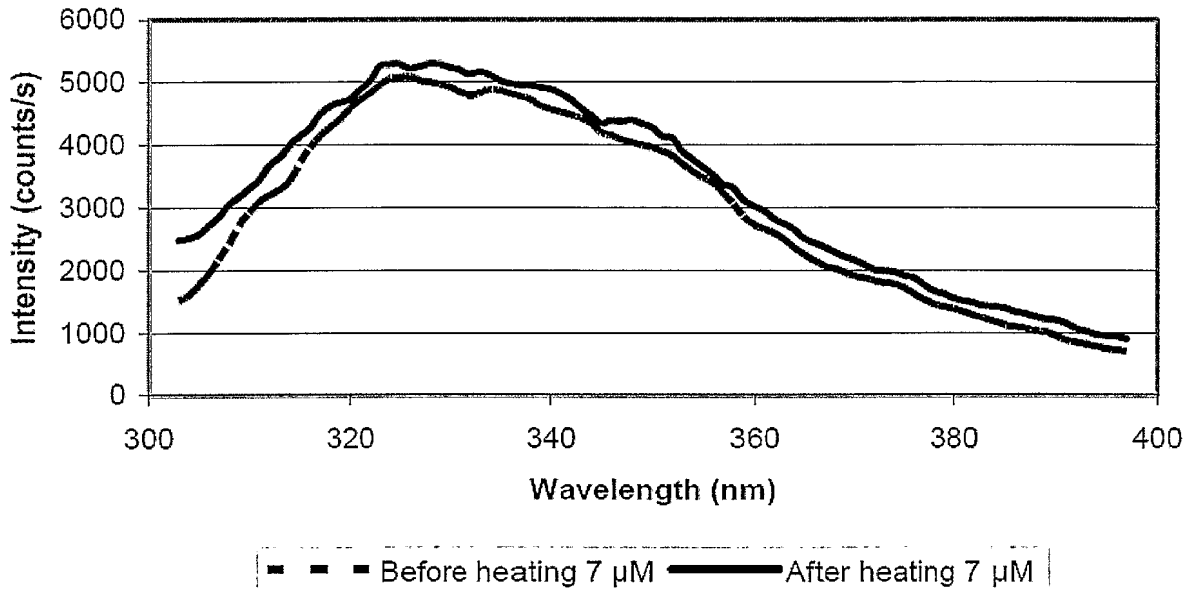


Figure 19

Thermal scan

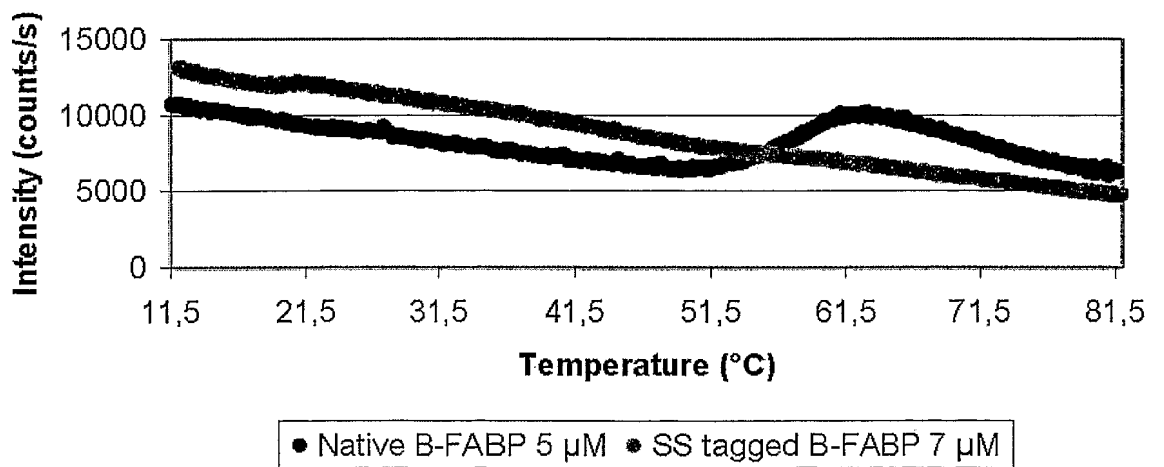


Figure 20

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Scatter scan of SS tagged B-FABP

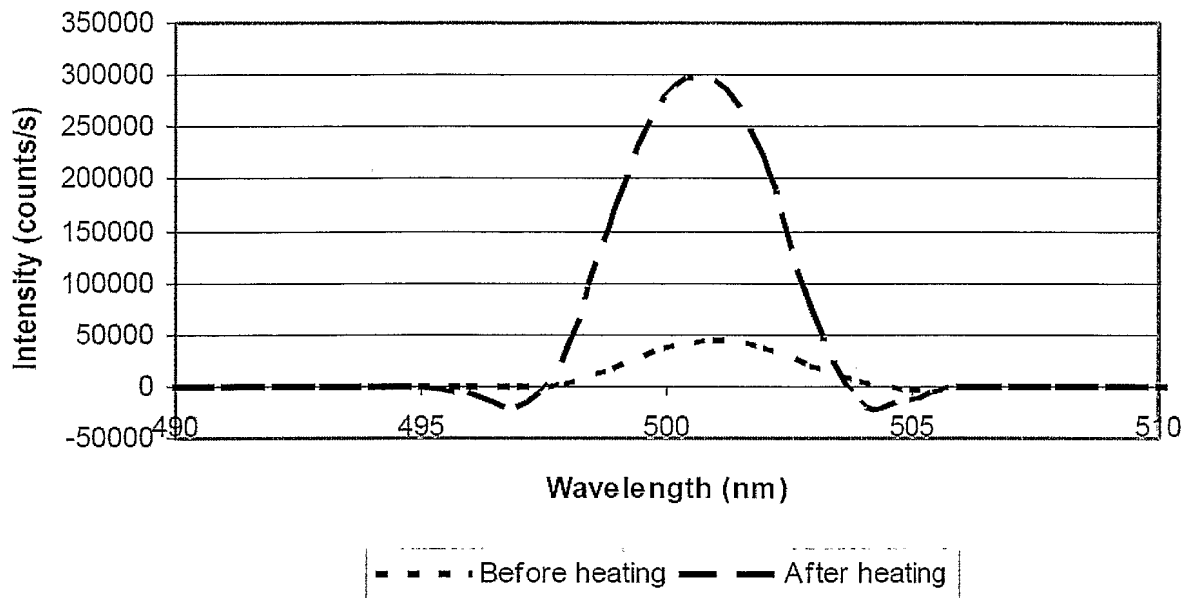


Figure 21

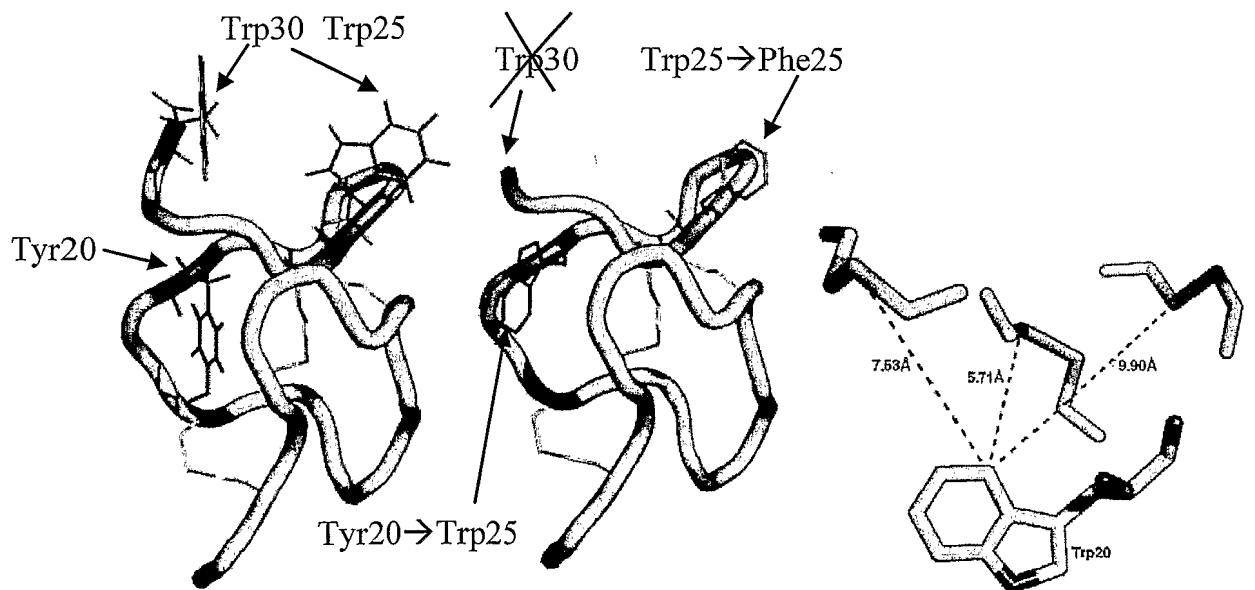


Figure 22

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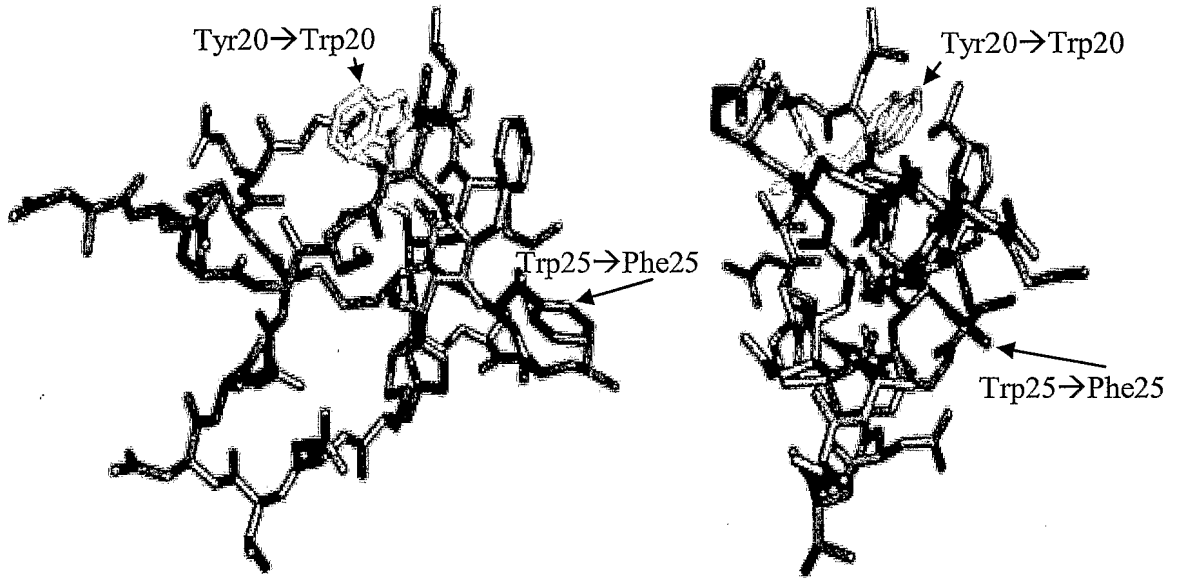


Figure 23

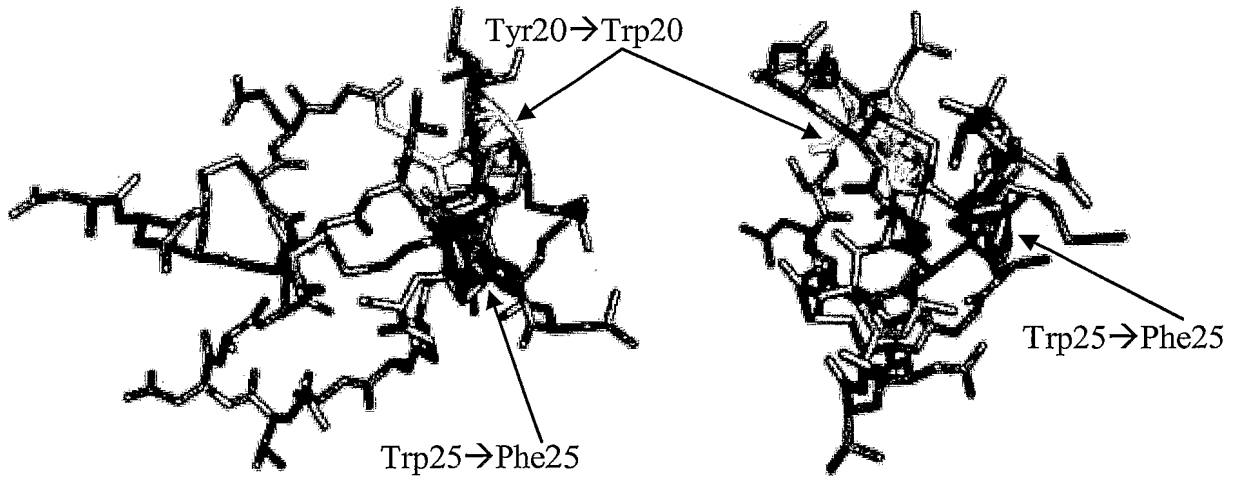


Figure 24

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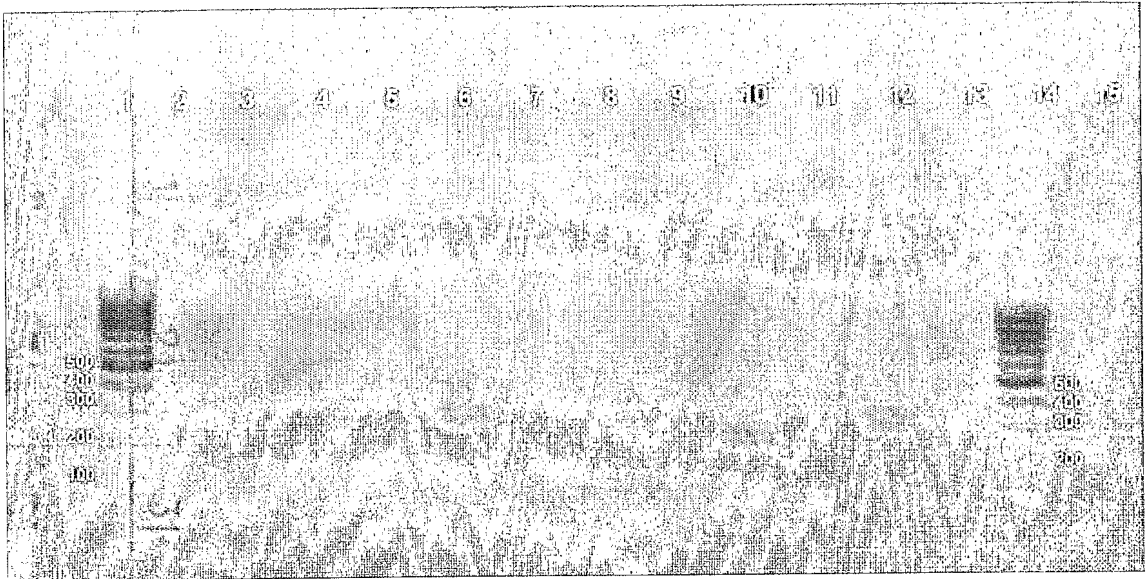


Figure 25

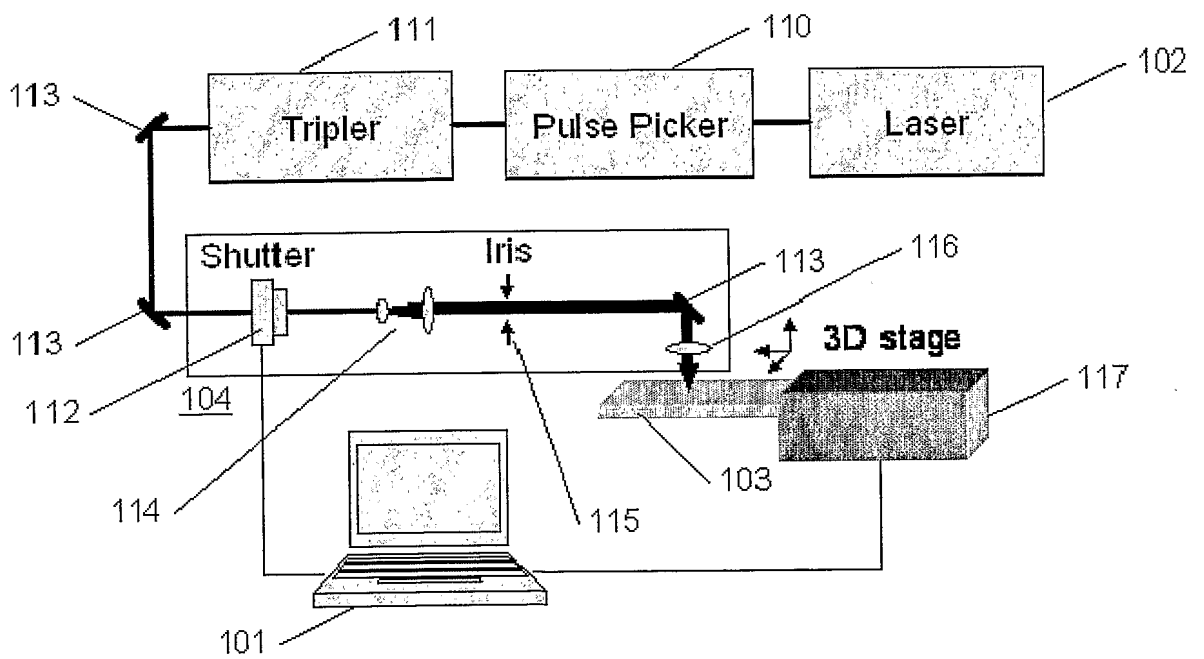


Figure 26