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(54) Title: REAGENTLESS FLUORESCENT BIOSENSORS FROM NANOFITINS, RATIONAL DESIGN METHODS TO CREATE REAGENTLESS FLUORESCENT BIOSENSORS AND METHODS OF THEIR USE

(57) Abstract: The present invention relates to reagentless fluorescent biosensors from Nanofitins which comprise at least an OB-fold protein and a fluorophore and are specific for a target; the method for preparing such reagentless fluorescent biosensors comprises: (a) substituting each of the variable residues of said OB-fold protein, individually with a cysteine residue, thereby obtaining a set of cysteine mutants; (b) coupling said cysteine residue of each of the cysteine mutants of step (a) to a fluorophore, thereby obtaining biosensors; (c) purifying the biosensors of step (b); (d) measuring the equilibrium constant (KD) between each of said purified biosensors of step (c) and said target, or the dissociation (Koff) and association (kon) rate constants for each of said biosensors of step (c) and said target; and measuring the fluorescence variation of each of said biosensors of step (c) between a free and target bound state; and (f) determining the sensitivity (s) and/or relative sensitivity (sr) of each of said biosensors of step (c) from the measurements of step (d).



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Reagentless fluorescent biosensors from Nanofitins, rational design methods to create reagentless fluorescent biosensors and methods of their use

The present invention relates to reagentless fluorescent biosensors from Nanofitins which comprise at least an OB-fold protein and a fluorophore as well
5 as to methods to generate reagentless fluorescent (RF) biosensors, in particular wherein no structural data exists of the biosensor in combination with its molecular target.

A molecular biosensor transforms a specific molecular binding event into a detectable signal and comprises several modules: a recognition module, which
10 can also be called a receptor, can be of biological origin or biomimetic and which recognises at least one specific target such as an antigen, ligand or analyte during the binding event; a transduction module, which transforms the recognition event into a measurable signal; and a means of evaluating the measurable signal data.

The recognition and transduction modules should be integrated into
15 a compact device of molecular dimensions (Lowe, 1984) and a molecular biosensor can function without additional reagents and provide quantitative analytical information and follow the concentration of its target, continuously (Thevenot *et al.*, 2001).

The physical nature of the measurable signal can be very diverse (Morgan *et al.*, 1996). Fluorescence is an optical signal which allows one to detect
20 molecular interactions with great sensitivity. The transduction is based on a variation of the fluorescence properties of the biosensor when it interacts with its analyte (Altschuh *et al.*, 2006). The fluorescence of a protein biosensor can be intrinsic, e. g. provided by its component residues of tyrosine and tryptophan, or extrinsic, e. g. provided by the chemical coupling of fluorescent groups. The coupling of several
25 fluorophores to a unique molecule of biosensor can be beneficial but is usually difficult to implement (Smith *et al.*, 2005). Although intrinsic protein fluorescence can be used to study molecular interactions in purified experimental systems, extrinsic fluorescence is normally preferable to monitor specific interactions in complex media, without interference from other protein components (Foote and Winter, 1992).

30 The changes of fluorescence that occur upon recognition between a reagentless fluorescent biosensor and its target, result from different interactions between the fluorescent group and its environment in the free and bound forms of the

biosensor.

The binding of the target can occur in the neighborhood of the fluorescent group and directly modify its environment.

Alternatively, the binding of the target can induce a conformational
5 change in the biosensor and thus cause an interaction between the fluorescent group and the receptor indirectly.

Antibodies are perfectly suited to provide the recognition module of biosensors since they can be directed against almost any target with the potential exception of self-proteins. However, antibodies or their derivative fragments are often
10 difficult and expensive to produce because of their molecular complexity and/or their insufficient stability.

For these reasons, two new artificial families of target binding proteins, DARPins and Nanofitins (formerly known as Affitins) have recently been developed by engineering two different protein families that have a stable polypeptide
15 scaffold, are devoid of cysteine residues and disulfide bonds and consequently are well expressed in *Escherichia coli*. These new families of target binding proteins, DARPins and Nanofitins have been shown able to replace antibodies in many of their applications.

The family of the Designed Ankyrin Repeat Proteins (DARpins) is a
20 well characterized artificial family of protein-target binding proteins (Mosavi *et al.*, 2004; Li *et al.*, 2006).

The OB-fold protein family is a natural family of proteins which possess the oligonucleotide/oligosaccharide binding-fold (OB-fold), a five stranded β -barrel capped with an amphiphilic α -helix. Members of this family bind a wide range
25 of structurally different ligands : ssDNA, dsDNA, RNA, oligosaccharides, proteins, metallic ions and catalytic substrates (Agrawal and Kishan, 2003; Arcus, 2002; Theobald *et al.*, 2003).

Combinatorial libraries of DARPins and OB-fold proteins have been generated by randomization of residues that have a strong probability of contributing
30 to the target binding site, and for DARPins only, the subsequent assemblage of a random number of ankyrin modules between defined N- and C-terminal modules (Binz *et al.*, 2003). These libraries were used to select DARPins or Nanofitins that

bound specific targets, using ribosome or phage display. DARPins and Nanofitins with high affinities and specificities have been obtained against a wide variety of protein targets Binz *et al.*, 2004.; Stump *et al.*, 2008; WO 2008/068637; Mouratou *et al.*, 2007; Krehenbrink *et al.*, 2008; Cinier *et al.*, 2009).

5 The inventors and others have engineered reagentless fluorescent (RF) biosensors from antibodies and DARPins, first when the three-dimensional structure of the complex with their target is known and then in the absence of such a knowledge (Renard *et al.*, 2002; Renard *et al.*, 2003; Jespers *et al.*, 2004; Renard and Bedouelle 2004; Brient-Litzler *et al.*, 2010). This work also formed the basis of WO
10 2001/065258 and WO 2009/115919 which described such antibody and DARPin based biosensor molecules.

 The antibody is used in the form of a single-chain variable fragment or scFv. A residue of the single-chain variable fragment or the DARPin is identified which is in proximity to the target, when the target is in complex with the single-chain
15 variable fragment or the DARPin. The selected residue is changed into a cysteine by site-directed mutagenesis. A fluorophore is chemically coupled to the mutant cysteine. The binding of the target shields the fluorophore from the solvent and can therefore be detected by a change of fluorescence.

 The Inventors have now developed a new type of RF biosensors
20 which incorporate the advantages of Nanofitins relative to DARPins or antibodies. In particular, they combine, in a single molecule, the favorable properties of antibodies and DARPins, e.g., a wide diversity of ligands and a high stability, solubility and recombinant production yield without their respective disadvantages, e.g. the insufficient stability and inefficient recombinant production of the antibodies and a
25 binding limited to protein targets of the DARPins.

 In addition, Nanofitins can bind virtually any target. Therefore, the corresponding RF biosensors extend the range of molecules that can be detected with the existing RF biosensors based upon antibodies and DARPins. Also the inventors have unexpectedly found that RF biosensors with a high sensitivity are obtained more
30 often for the Nanofitins than for the DARPins.

 The inventors have also developed methods to design and produce such new RF biosensors when the three-dimensional structure of the complex with the

ligand is unknown.

Therefore, a first aspect of the present invention is a reagentless biosensor for a target, comprising at least:

an OB-fold protein;

5 a cysteine residue coupled to a fluorophore.

According to the present invention, "OB-fold protein" designates any polypeptide which comprises or consists of a domain having an OB-fold topology, as described by (Murzin, 1993) and (Arcus, 2002) and which binds said target specifically. The OB-fold is found in all kingdoms and was ranked as the 28th
10 most represented fold in a survey of 20 genomes (Qian *et al.*, 2001). This topology corresponds to an architecture which comprises a five-stranded β -barrel capped at one end by an amphiphilic α -helix. Referring to the CATH protein structure classification (Pearl *et al.*, 2003), the OB-fold topology corresponds to the 2.40.50 fold family (CATH database version 3.0.0: Released May 2006). The OB-fold presents a β -sheet
15 binding face that confers remarkable diversity in the types of compounds that can be recognized. In addition, the same positions of the binding face of OB-folds from different proteins are always involved in ligand recognition (Arcus, 2002; Mouratou *et al.*, 2007).

Such an OB-fold protein can be either a native protein (*i.e.*, an
20 isolated, purified or recombinant protein having the same sequence as a natural protein), or an engineered protein like, for example, a fragment of a native protein, a variant derived from a native OB-fold protein by mutations, for example in its binding site, a fusion protein comprising an OB-fold domain from a first protein, and another moiety from another protein.

25 The finding by the inventors that Nanofitins can be used to generate reagentless fluorescent biosensors and in particular that these Nanofitin based biosensors can compare and in some cases outperform, in terms of sensitivity, ease of production and other characteristics, the previous antibody and DARPin based biosensors they generated was unexpected.

30 The Nanofitins have a constant and small number of hypervariable positions. For example, Nanofitins with high affinity for various protein targets of interest were obtained by randomizing 14 residues of the Sac7d protein from

Sulfolobus acidocaldarius (WO 2008/068637; Mouratou *et al.*, 2007). The DARPins include a random number of ankyrin modules, exceptionally two and generally three or more (WO 2009/115919; Brient-Litzler *et al.*, 2010). Each ankyrin module has 7 hypervariable positions. Therefore a DARPin has exceptionally 14 and generally 21 or
5 more hypervariable positions. Antibodies have a much larger number of hypervariable positions with their six hypervariable loops. The low number of hypervariable positions in Nanofitins constituted an uncertainty but could be an advantage for the construction of RF biosensors. The inventors have unexpectedly found that despite the small number of hypervariable positions in Nanofitins, only part of them were impor-
10 tant for the binding of a specific target and it was possible to couple a fluorophore to the other ones without increasing K_d (*i. e.* decreasing affinity). Given this unexpected result, the small number of hypervariable positions in Nanofitins enables one to directly construct fluorophore conjugates in each of the hypervariable positions and determine their functionality and sensitivity (s_r) without having to characterize the
15 properties of changes into Ala of Cys, as for the previous antibody and DARPin based RF biosensors.

In addition, the inventors have found that it was possible to construct an operational RF biosensor by coupling a fluorophore to additional positions that have not been randomized even though they contact the ligand in some natural OB-
20 fold proteins. These positions, such as Lys39 in Sac7d based Nanofitins could represent universal positions for the construction of RF biosensors from Nanofitins.

Therefore several technical differences exist between antibodies, DARPins and Nanofitins. Importantly some of the technical differences between DARPins, antibodies and Nanofitins would probably have led the man skilled in the
25 art to consider Nanofitins inferior to antibodies and DARPins for the preparation of peptide biosensors according to the present invention.

Surprisingly the inventors have now proven that these inherent characteristics of Nanofitins do not affect the performance of Nanofitin based reagentless biosensors and also that such biosensors share the attractive properties of
30 Nanofitins, namely their solubility and stability, their ease of expression, purification and handling, and their wide diversity of ligands.

The inventors therefore provide a new class of RF biosensors which does not necessitate any manipulation, labelling or transformation of the sample. They have the advantages of a reagentless biosensor, namely a biosensor which can function without additional reagent. They can function in complex medium like
5 serum. Their response is immediate, specific, selective, sensitive, linear and quantitative. Thus they can provide quantitative analytical information and follow the concentration of its analyte continuously in complex mixtures, together with the robust bio/physico-chemical properties of Nanofitins and without any apparent loss of sensitivity or binding affinity. The inventors have validated this new class of RF biosensors
10 with the known Lys_H4 (also named H4), a Nanofitin which is directed against hen egg-white lysozyme (HEL; WO 2008/068637).

The inventors have shown that several RF biosensors based upon H4S (H4 mutant in which residue Cys29 has been changed into Ser29) work using the HEL protein as a model target.

15 Such reagentless fluorescent biosensors can be used in different formats: in solution, in microtiter-plates, in the form of protein chips, or at the tip of optical micro- or nano-fibers. They could be used for the continuous quantification of any target molecule of interest in complex mixtures, without any prior labelling of the target under analysis.

20 As mentioned above, the RF biosensors of the present invention which are based on Nanofitins have virtually no limitation concerning the target. For example, the target of interest can be a peptide, a protein, an oligosaccharide, a lipid, a lipopeptide, a carbohydrate (for example, a sugar), a single-stranded DNA, a double-stranded DNA, a RNA. Interestingly, the target of interest can be a natural or synthetic
25 small molecule, limited to a few atoms, or even to only one atom. Examples of such small molecules include any kind of haptens (*i.e.*, small molecules which can elicit an immune response only when attached to a large carrier), vitamins, or metallic ions.

In healthcare, they could be used for the bed side monitoring of patients, the controlled continuous delivery of drugs, the control of artificial organs,
30 some diagnostics, *in situ* measurements during surgical operations and the detection of doping drugs.

In industry, they could be used for the monitoring of reactions and processes, food control and pharmacokinetic studies.

In environmental protection/monitoring and civil or military defence, they could be used for the monitoring of pathogenic, toxic or polluting agents.

In fundamental research, they could be used in proteomics, for the profiling of cells, tissues or body fluids; in the biology of single cells, to continuously measure the concentration of an antigen within a single living cell; in neuro-chemistry and neuro-sciences, to measure the intra-cerebral concentration of neuro-peptides in response to external stimuli.

According to a particular embodiment of the invention, the biosensor comprises an OB-fold protein which is derived from a starting OB-fold protein by several substitutions at positions of the residues of the binding interface of said starting OB-fold protein with its native ligand, preferably by 5 to 32, more preferably by 8 to 20, even more preferably by 11 to 16 substitutions, possibly combined with the deletion of 1 to 4 residues and/or the insertion of 1 to 50 residues.

Such an OB-fold protein variant is named "Nanofitin" in the current Patent Application. The Nanofitins are derived from a starting OB-fold protein through the combinatorial mutation/selection process disclosed in WO 2008/068637 (page 2, line 16 to page 9, line 2; examples page 12 to page 34; figures 1 to 21 and sequence listing), the content of which is incorporated herein by reference. This combinatorial mutation/selection process consists in obtaining a combinatorial library corresponding to the randomization of a number of residues involved in the binding of a starting OB-fold protein with its native ligand, followed by a selection, in said library, of variants which bind specifically to a target of interest.

Non-limitative examples of OB-fold proteins which can be used in the construction of a reagentless biosensor according to the current invention are: Sac7d from *Sulfolobus acidocaldarius* (PDB 1azp; UNIPROT or GenBank P13123 or Q4JC17; SEQ ID NO: 1) and the truncated form of Sac7d such as those described by McAfee *et al.*, 1995 (Sac7a (SEQ ID NO: 44) and Sac7b (SEQ ID NO: 45)), Sac7e from *Sulfolobus acidocaldarius* (UNIPROT or GenBank P13125 or Q4JBQ1; SEQ ID NO: 2), Sso7d from *Sulfolobus solfataricus* (PDB 1bf4; UNIPROT or GenBank

P39476 or P81550; SEQ ID NO: 3), DBP 7 from *Sulfolobus tokodaii* (UNIPROT or GenBank Q96X56; SEQ ID NO: 4), Ssh7b from *Sulfolobus shibatae* (UNIPROT or GenBank O59632; SEQ ID NO: 5), Ssh7a from *Sulfolobus shibatae* (UNIPROT or GenBank P61990, O59631, P80170, Q9UW18; SEQ ID NO: 6), p7ss from *Sulfolobus*
5 *solfataricus* (UNIPROT or GenBank P61991, O59631, P80170, Q9UW18; SEQ ID NO: 7), the N-terminal domain of SEB (Papageorgiou *et al.*, 1998), the chain A of the Shiga-like toxin IIe of *Escherichia coli* (PDB 2bosA or 1r4pB; SEQ ID NO: 8 and 9), the human Neutrophil Activating Peptide-2 (NAP-2, PDB 1tvxA; SEQ ID NO: 10), the Molybdenum Binding Protein (modg) of *Azotobacter vinelandii* (PDB 1h9j; SEQ
10 ID NO: 11), the N-terminal domain of SPE-C (Roussel *et al.*, 1997), the B₅ subunit of *E. coli* Shiga-like toxin (Kitov *et al.*, 2000), Cdc13 (Mitton-Fry *et al.*, 2002), the cold-shock DNA-binding domain of the human Y-box protein YB-1 (Kloks *et al.*, 2002), the *E. coli* inorganic pyrophosphatase EPPase (Samyгина *et al.*, 2001), or any of the proteins listed in Table 3 of the article by (Arcus, 2002) or in Table 3 of the present
15 Application, such as the Lysyl-tRNA synthetase LysS of *E.coli* (PDB 1krs ; UNIPROT or GenBank B7UHT9 ; SEQ ID NO : 12), the Asp-tRNA synthetase of *E.coli* (PDB 1c0aA; UNIPROT or GenBank P21889; SEQ ID NO: 13), the Asp-tRNA synthetase of *Thermococcus kodakaraensis* (PDB 1b8aA; UNIPROT or GenBank Q52428; SEQ ID NO: 14) , the Lysyl-tRNA synthetase LysU of *E.coli* (PDB 1lylA;
20 UNIPROT or GenBank P0A8N6; SEQ ID NO: 15), the human Replication protein A, 32kDa subunit (PDB 1quqA; UNIPROT or GenBank P15927; SEQ ID NO: 16), the human Replication protein A, 14kDa subunit (PDB 1quqB; UNIPROT or GenBank P35244; SEQ ID NO: 17), the human Replication protein A, 70kDa subunit (RPA70) fragment (PDB 1jmcA; UNIPROT or GenBank P27694; SEQ ID NO: 18), the
25 Telomere-end-binding protein of *Oxytricha nova* (PDB 1otcA and 1otcB: UNIPROT or GenBank P29549 and P16458; SEQ ID NO: 19 and 20), the human mitochondrial ssDNA-binding protein (PDB 3ulla; UNIPROT or GenBank Q567R6; SEQ ID NO: 21), the Pertussis toxin S5 subunit of *Bordetella pertussis* (PDB 1prtF; UNIPROT or GenBank P04981; SEQ ID NO: 22), the Pertussis toxin S5 subunit (ATP bound) of *B.*
30 *pertussis* (PDB 1bcpD; UNIPROT or GenBank P0A3R5; SEQ ID NO: 23), the Cholera Toxin of *Vibrio cholera* (PDB 3chbD; UNIPROT or GenBank D0OUTQ9; SEQ ID NO: 24), the Heat-labile toxin of *E. coli* (PDB 1tiiD; UNIPROT or GenBank

P43529; SEQ ID NO: 25), the Verotoxin-1/Shiga toxin, B-pentamer of *E. coli* (PDB 2bosA; UNIPROT or GenBank Q9MBZ7; SEQ ID NO: 26), the human TIMP-2 (PDB 1br9; UNIPROT or GenBank P16035; SEQ ID NO: 27), the Superantigen SPE-C of *Streptococcus pyogenes* (PDB 1an8 UNIPROT or GenBank Q8NKX2; SEQ ID NO: 28) , the Superantigen SPE of *Staphylococcus aureus* (PDB 3seb; UNIPROT or GenBank Q5MAA8; SEQ ID NO: 29), the Toxic shock syndrome toxin of *Staphylococcus aureus* (PDB 1aw7A; UNIPROT or GenBank A0FIN2; SEQ ID NO: 30) , the Major cold-shock protein of *E.coli* (PDB 1jmc; UNIPROT or GenBank P0A9Y1; SEQ ID NO: 31), the Initiation translation factor 5a of *Pyrobaculum aerophilum* (PDB 1bkb; UNIPROT or GenBank P56635; SEQ ID NO: 32), the S1 RNA-binding domain of PNPase of *E.coli* (PDB 1sro; UNIPROT or GenBank P05055; SEQ ID NO: 33), the human Initiation translation factor 1, eIF1a (PDB 1d7qA; UNIPROT or GenBank P47813; SEQ ID NO: 34), the Initiation translation factor 1, IF1 of *E.coli* (PDB 1ah9; UNIPROT or GenBank P69224; SEQ ID NO: 35), , the RNA guanylyltransferase of Chlorella virus, PBCV-1 (PDB 1ckmA; UNIPROT or GenBank Q84424; SEQ ID NO: 36), the ATP-dependent DNA ligase, of Bacteriophage T7 (PDB 1a0i; UNIPROT or GenBank P00969; SEQ ID NO: 37), the Staphylococcal nuclease, *Staphylococcus aureus* (PDB 1snc; UNIPROT or GenBank gi/224650; SEQ ID NO: 38), the DNA helicase RuvA subunit, N-terminal domain of *E.coli* (PDB 1hjp; UNIPROT or GenBank P0A811; SEQ ID NO: 39) , the Gene V protein of *Pseudomonas* bacteriophage pf3 (PDB 1pfsA; UNIPROT or GenBank P03672; SEQ ID NO: 40), the Gene V protein of Filamentous bacteriophage f1, M13 (PDB 1gvp; UNIPROT or GenBank D0U157; SEQ ID NO: 41), the Gene 32 protein (gp32) core of Bacteriophage T4 (PDB 1gpc; UNIPROT or GenBank B3IYU0; SEQ ID NO: 42), and the Inorganic pyrophosphatase of *Thermus thermophilus* (PDB 2prd; UNIPROT or GenBank Q72H95; SEQ ID NO: 43). It can be noted that OB-folds domains originating from toxins can be used as starting OB-fold protein even for purposes in which toxicity is to be avoided, since mutations in their binding site, and hence change in their binding specificity, can completely abolish their toxicity.

By superimposing several sequences and 3D-structures of OB-fold domains using the web sites WU-Blast2 (<http://www.ebi.ac.uk/blast2/index.html>) (Lopez *et al.*, 2003), T-COFFEE (<http://www.ch.embnet.org/software/TCoffee.html>)

(Notredame *et al.*, 2000), DALI lite (<http://www.ebi.ac.uk/DaliLite/>) (Holm and Park, 2000), and DALI (<http://www.ebi.ac.uk/dali/Interactive.html>) (Holm and Sander, 1998), the inventors have previously identified the positions of the residues involved in the binding of the OB-fold protein which could be substituted for obtaining
 5 Nanofitins (WO 2008/068637); these residues are often located in β -strands β 3, β 4 and β 5 and in loops 1, 3 and 4 of the OB-fold (figures 1b and 2 of WO 2008/068637).

Taking as a reference the sequence of Sac7d of SEQ ID NO: 1, the residues which can be substituted are V2, K3, K5, K7, Y8, K9, G10, E14, T17, K21, K22, W24, V26, G27, K28, M29, S31, T33, D36, N37, G38, K39, T40, R42, A44,
 10 S46, E47, K48, D49, A50 and P51. Still with Sac7d as a reference, the residues which can be deleted are: A59, R60, A61 and E64. Advantageously, insertions of 1 to 15 amino acid residues can be performed in loop 3 (as identified in figures 1b and 2 of WO 2008/068637), for example in the region of residues 25 to 30 of Sac7d, preferably between residues 27 and 28, insertions of 1 to 15 amino acid residues can be
 15 performed in loop 4 (as identified in figures 1b and 2 of WO 2008/068637), for example in the region of residues 35 to 40 of Sac7d, preferably between residues 37 and 38, and insertions of 1 to 20 amino acid residues can be performed in loop 1 (as identified in figures 1b and 2 of WO 2008/068637), for example in the region of residues 7 to 12 of Sac7d, preferably between residues 9 and 10.

20 The residues of any other OB-fold protein which are located at positions which are equivalent to those listed above can be easily identified by comparing the 3D-structure of said other OB-fold protein with that of Sac7d. For example, the online tool "DaliLite pairwise comparison of protein structures" (<http://www.ebi.ac.uk/Tools/dalilite/>) was used to superimpose the structures of the
 25 protein under consideration on the structure of Sac7d chain A by using their PDB coordinates (PDB code 1AZP for Sac7d chain A). A visual analysis of the superimposed structures with the Pymol software (Pymol; <http://www.pymol.org/>) was used to identify the correspondences between residues. The residues in positions 7, 8, 9, 21, 22, 24, 26, 28, 29, 31, 33, 39, 40, 42, 44, 46 on one side of Sac7d (SEQ ID
 30 NO: 1) OB-fold correspond respectively to the following residues on the corresponding side of the OB-fold of other proteins:

- residues in positions 7, 8, 9, 21, 22, 24, 26, 28, 29, 31, 33, 40, 41, 43, 45, 47 of Sso7d from *Sulfolobus solfataricus* (SEQ ID NO: 3),

- residues in positions 60, 59, 58, 9, 10, 12, 14, 17, 18, 20, 22, X, X, 27, 29, 31 of Shiga-like toxin IIe from *E.coli* (PDB 1r4pB ; SEQ ID NO: 9);

5 - residues in positions 25, 26, 27, 40, 41, 43, 45, 54, 55, 57, 59, 61, 63, 65, 67, 69 of the human Neutrophil Activating Peptide-2 (NAP-2, PDB 1tvxA ; SEQ ID NO: 10);

 - residues in positions 60, 61, 62, 86, 87, 89, 91, 93, 94, 96, 98, 105, 106, 108, 110, 112 of the Molybdenum Binding Protein (modg) from *Azotobacter*
10 *vinelandii* (PDB 1h9j; SEQ ID NO: 11)).

The same approach can be used to engineer a different binding site using the residues from another side of the OB-fold.

In a preferred embodiment of the invention, the biosensor comprises an OB-fold protein which is isolated from a combinatorial library obtained by the
15 randomization of 11, 12, 13, 14, 15, 16, 17 or 18 residues selected amongst those of said OB-fold protein sequence which correspond to K7, Y8, K9, K21, K22, W24, V26, K28, M29, S31, T33, K39, T40, R42, A44, S46, E47 and K48 of SEQ ID NO: 1, for example 11, 12, 13, 14, 15 or 16 residues selected amongst those of said OB-fold protein sequence which correspond to K7, Y8, K9, K21, K22, W24, V26, K28, M29,
20 S31, T33, K39, T40, R42, A44 and S46 of SEQ ID NO: 1.

In a more preferred biosensor of the above embodiment, the randomized residues comprise at least the residues of said OB-fold protein sequence which correspond to K7, Y8, K9, W24, V26, M29, S31, T33, R42, A44 and S46 of SEQ ID NO: 1. A specific library, "library 11" is obtained by the randomization of the
25 residues of said OB-fold protein sequence which correspond to K7, Y8, K9, W24, V26, M29, S31, T33, R42, A44 and S46 of SEQ ID NO: 1. In other combinatorial libraries, one, two or three additional residues selected amongst those of said OB-fold protein sequence which correspond to K21, K22 and T40 of SEQ ID NO: 1 are also randomized. Two other preferred libraries are "library 13", which is obtained by the
30 randomization of the residues of said OB-fold protein sequence which correspond to are K7, Y8, K9, K21, K22, W24, V26, M29, S31, T33, R42, A44 and S46 of SEQ ID NO: 1, and "library 14", which is obtained by the randomization of the residues said

OB-fold protein sequence which correspond to K7, Y8, K9, K21, K22, W24, V26, M29, S31, T33, T40, R42, A44 and S46 of SEQ ID NO: 1.

According to another particular embodiment of the invention, the Biosensor comprises an OB-fold protein which is selected from the group consisting of: Sac7d or Sac7e from *Sulfolobus acidocaldarius* (SEQ ID NO : 1 and 2), Sso7d from *Sulfolobus solfataricus* (SEQ ID NO : 3), DBP 7 from *Sulfolobus tokodaii* (SEQ ID NO : 4), Ssh7b from *Sulfolobus shibatae* (SEQ ID NO : 5), Ssh7a from *Sulfolobus shibatae* (SEQ ID NO : 6), p7ss from *Sulfolobus solfataricus* (SEQ ID NO : 7), Sac7a (SEQ ID NO: 44), Sac7b (SEQ ID NO: 45), and the Nanofitins derived from the preceding OB-fold proteins as defined in the current Patent Application..

A first more preferred Biosensor according to the invention comprises a Nanofitin which binds human immunoglobulins of class G (IgG) with high affinity ($K_d \leq 100 \mu\text{M}$), said Nanofitin having a sequence selected from:

SVKVKFX₁X₂X₃GEEKEVDTSKIRDVCRQGKNVKFLYNDNGK
 YGAGX₄VX₅EKDAPKELLDMLARAEREKK (SEQ ID NO: 46), wherein X₁ to X₅ are, independently from each other, any amino acids, and the variants derived from SEQ ID NO: 11 by 1 to 15, more particularly one, two, three, four, five or six mutations or deletions in positions selected in the group consisting of positions 1-6, 11-20, 23, 25, 27-28, 30, 32, 36-39, 41, 43, 45 and 47-66. Particular examples of such variants are those in which said one, two, three or four mutations or deletions are in positions selected in the group consisting of positions 13, 24, 36, 38, 47, 50 and 56 (mutations in positions 13, 36, 38, 47, 50 and 56 have been obtained by ribosome display, and position 24 was voluntarily mutated to avoid S-S bridging. Preferably, X₁X₂X₃ is selected in the group consisting of LLN, KYK, HSH, LCH, RYL and ARH. Simultaneously or independently, preferred immunoglobulin-binding proteins are so that X₄ is N or K, and/or X₅ is D, N or R.

Particular examples of immunoglobulin-binding proteins are:

SVKVKFLLNGEEKEVDTSKIRDVCRQGKNVKFLYNDNGKYG
 AGNVDEKDAPKELLDMLARAEREKK (SEQ ID NO: 47),
 SVKVKFLLNGEEKEVDTSKIRDVCRQGKNVKFLYNDNGKYG
 AGKVNEKDAPKELLDMLARAEREKK (SEQ ID NO: 48),

SVKVKFHHGEEKEVDTSKIRDVCRQGKNVKFLYNDNGKY
GAGNVDEKDAPKELLDMLARAEREKK (SEQ ID NO: 49),

SVKVKFLCHGEEKEVDTSKIRDVCRQGKNVKFLYNDNGKY
GAGNVDEKDAPKELLDMLARAEREKK (SEQ ID NO: 50),

5 SVKVKFRYLGEKEVDTSKIRDVCRQGKNVKFLYNDNGKY
GAGNVDEKDAPKELLDMLARAEREKK (SEQ ID NO: 51),

SVKVKFARHGEEKEVDTSKIRDVCRQGKNVKFLYNDNGKY
GAGNVREKDAPKELLDMLARAEREKK (SEQ ID NO: 52),

SVKVKFLLNGEEKEVDTSKIRDVCRQGKNVKFLYNDNGKYG
10 AGNVDEKDVPKELLDMLARAEREKK (SEQ ID NO: 53),

SVKVKFLLNGEEQEVDTSKIRDVCRQGKNVKFLYNDNGKYG
AGNVDEKDAPKELLDMLARAEREKK (SEQ ID NO: 54),

SVKVKFLLNGEEKEVDTSKIRDVCRQGKNVKFLYNDNGKYG
AGNVDKKDAPKELLDMLARAEREKK (SEQ ID NO: 55),

15 SVKVKFLLNGEEKEVDTSKIRDVCRQGKNVKFLYNDNDKYG
AGNVDEKDAPKELLDMLARAEREKK (SEQ ID NO: 56), and

SVKVKFLLNGEEKEVDTSKIRDVCRQGKNVKFLYNNNGKYG
AGNVDEKDAPKELLEMLARAEREKK (SEQ ID NO: 57).

Another more preferred Biosensor according to the invention
20 comprises a Nanofitin of: SEQ ID NO: 58 (H4S; Hen egg-white lysozyme binder).

According to another particular embodiment of the invention, the
Biosensor comprises a Nanofitin which is derived from a starting OB-fold protein
which is selected from the group consisting of the sequences SEQ ID NO: 8 to 42 and
the fragments of said sequences which include at least the OB-fold domain (the
25 positions of the OB-fold domain of these sequences is mentioned in Table 3 and in the
attached sequence listing).

According to another particular embodiment of the invention, the
biosensor according to the invention comprises an OB-fold protein which is coupled
to another protein moiety. The coupling between the two moieties can be either a
30 genetic fusion (the chimeric protein is then a fusion protein), or a chemical binding. In
the later case, the binding can be either covalent or non-covalent. For example, the
OB-fold protein is fused at its N- or C-terminal ends or both to another moiety

selected among: a binding moiety with different specificities than the OB-fold protein such as a peptide tag (His*6, FLAG, HA, myc, *etc.*), a tag for *in vivo* biotinylation (provided BirA is co-expressed) to enable oriented immobilization on a support (such as a protein chip), a poly-lysine tag (for example, Lys*6) for enabling oriented immo-
5 bilization on a support (such as an affinity chromatography resin); a targeting moiety such as a signal sequence (for example, to address the chimeric protein to a specific compartment like periplasm or nucleus). Advantageously, the fusion will encompass a peptide linker between the two moieties, which will be long enough to avoid inhibiting interactions between both moieties. The linker can optionally comprise a
10 recognition site for a protease, if easy separation of both moieties is wanted. For example, the linker can comprise the sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly, which is the optimum recognition site for TEV protease (a highly site-specific cysteine protease that is found in the Tobacco Etch Virus). This enzyme has a high specificity and a high activity rate and triggers cleavage between the Gln and Gly residues of the
15 above recognition site. It can hence be used, for example, for removing affinity tags from a purified chimeric protein according to the present invention.

In particular in the biosensor according to the present invention, the cysteine residue which is coupled to a fluorophore is either present in said OB-fold protein or is substituted for another suitable residue. In addition, nonessential cysteine
20 residues which are present in said OB-fold protein are substituted with small residues like serine or alanine residues by site-directed mutagenesis.

The site for the coupling of the fluorophore can be chosen using, either the rational approaches previously developed by the inventors for antibody and DARPin based RF biosensors, or a new approach that the inventors have developed
25 for Nanofitins according to a further aspect of the invention.

When structural data of the OB-fold protein in complex with its target are known the coupling site corresponds to a residue which is not in direct contact with the target but whose solvent accessible surface area (ASA) is modified when the OB-fold protein binds to the target. The methods previously described in
30 WO 2001/065258, WO 2009/115919, Renard *et al.*, 2002, Renard and Bedouelle 2004 and Brient-Litzler *et al.*, 2010 for producing antibody or DARPin based biosensors

using this design methodology can be used for producing OB-fold protein/Nanofitin based RF biosensors according to the current Patent Application.

However, structural data are rarely available and in the case of a newly generated Nanofitin with a selected specificity such structural data will not be available. The prior art methodology for choosing the coupling sites when no structural data are available relies on the identification of at least one residue which is adjacent (for example by reference to its sequence or to a canonical structure) to a residue which is functionally important for interaction with the target. Methods for producing antibody or DARPIn based biosensors using this design methodology are described in WO 2001/065258, WO 2009/115919 and Renard *et al.*, 2003. These methods comprise: (i) the identification of the residues (R_1) of the biosensor which make significant contribution to target binding by mutagenesis of all, or of a subset, of the residues of the biosensor and determining variations in at least one measurable chemical or physical parameter of interaction with said target (K_D , K_{off} , k_{on} , $\Delta\Delta G$, R_{eq}), (ii) the modification to cysteine of at least one of a second set of residues (R_2) which are located in sequence proximity or an in spatial proximity to one or more residues of the first set (R_1), and (iii) the coupling of the modified biosensor to a fluorophore at this cysteine.

The adjacent residues (R_2) are preferably the residues -1 and +1 along the peptide backbone relative to one or more of the residues (R_1) or the residues in Van-Der-Waals contact with one or more of the residues (R_1).

Such methods can be used for producing OB-fold protein/Nanofitin based RF biosensors according to the current Patent Application.

According to another particular embodiment of the invention, the biosensor has a fluorophore coupled to one residue of said OB-fold protein selected from the residues corresponding to V2, K3, K5, K7, Y8, K9, G10, E14, T17, K21, K22, W24, V26, G27, K28, M29, S31, T33, D36, N37, G38, K39, T40, R42, A44, S46, E47, K48, D49, A50, and P51 of SEQ ID NO: 1, the residue being changed to a cysteine residue if it is not already a cysteine residue.

In a first preferred embodiment of the invention, the fluorophore is coupled to one residue of said OB-fold protein selected from the residues corresponding to K7, Y8, K9, K21, K22, W24, V26, M29, S31, T33, T40, R42, A44

and S46 of SEQ ID NO: 1. Specific examples of said embodiment include the biosensors consisting of SEQ ID NO: 59, 60, 61 and 62 in which one of the residues W8, V21, K24 and A26 of H4S has been substituted with a cysteine residue and coupled to a fluorophore.

5 In a second preferred embodiment of the invention, the fluorophore is coupled to one residue of said OB-fold protein corresponding to K39 of SEQ ID NO: 1. This position is a universal position which can be used advantageously to engineer biosensors from OB-fold protein variants isolated from libraries which are randomized at other positions (library 11, 13 or 14 as described in the current Patent
10 Application). Non-limitative examples of OB-fold protein variants include variants of Sac7d and its homologs like Sso7d, Shiga-like toxin IIe, human Neutrophil Activating Peptide-2 and Molybdenum Binding Protein. A specific example of said embodiment is the biosensor consisting of SEQ ID NO: 63, in which the residue K39 of H4S has been substituted with a cysteine residue and coupled to a fluorophore.

15 A fluorophore is a component of a molecule which causes a molecule to be fluorescent. It is a functional group in a molecule which will absorb energy of a specific wavelength and re-emit energy at a different (but equally specific) wavelength. The amount and wavelength of the emitted energy depend on both the fluorophore and the chemical environment of the fluorophore.

20 In particular the fluorophore is selected from the group consisting of: 6-acryloyl-2-dimethylaminophthalene (acrylodan), 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (CNBD), 5-iodoacetamidofluorescein (5-IAF), (N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester), Cy3, Cy5 or a fluorophore having an aliphatic chain of 1 to 6 carbon atoms.

25 Another aspect of the present invention is a protein-based chip, characterized in that it consists of a solid support on which at least one biosensor as described in the current Patent Application is immobilized.

 Another aspect of the present invention is a solution comprising at least one biosensor as described in the current Patent Application.

30 Another aspect of the present invention is an optical fibre comprising at a first end thereof at least one biosensor as described in the current Patent Application and comprising at a second end thereof means to attach the optical

fibre to a device configured to receive and interpret the output of the at least one biosensor.

Another aspect of the invention is the use of a biosensor as described in the current Patent Application for screening libraries of molecules, for sorting
5 molecules, for sorting cells, for producing protein-based chips, for detecting, assaying or locating a target.

Another aspect of the invention is a reagent for detecting, assaying or locating targets, characterized in that it includes at least one biosensor as described in the current Patent Application.

10 Another aspect of the invention is a method for detecting, assaying or locating a ligand in a heterogeneous sample, characterized in that it comprises bringing said heterogeneous sample into contact with at least one reagent as described in the current Patent Application.

Another aspect of the invention is a kit for detecting, assaying or
15 locating a ligand in a heterogeneous sample, characterized in that it includes at least one reagent as described in the current Patent Application.

Another aspect of the invention is a method for producing reagentless biosensors which comprise at least an OB-fold protein and are specific for a target, characterized in that it comprises the following steps:

20 (a) substituting each of the variable residues of said OB-fold protein, individually with a cysteine residue, thereby obtaining a set of cysteine mutants,

(b) coupling said cysteine residue of each of the cysteine mutants in a) to a fluorophore, thereby obtaining biosensors,

25 (c) purifying the biosensors of step (b),

(d) measuring the equilibrium constant (K_D) between each of said purified biosensors and said target, or the dissociation (k_{off}) and association (k_{on}) rate constants for each of said biosensors and said target; and measuring the fluorescence variation of each of said biosensors between a free and target bound state; and

30 (e) determining the sensitivity (s) and/or relative sensitivity (s_r) of each of said biosensors from the measurements of step (d).

This method makes it possible to rank the biosensors according to their sensitivity and to determine the biosensors having the highest sensitivity, a parameter which is used to characterize any measuring instrument.

The variable residues in step (a) correspond to the residues of the binding interface of the starting OB-fold protein with its native ligand which are randomized in the combinatorial libraries of OB-fold protein variants which are used to isolate Nanofitins which bind to the target as described in the current Patent Application.

In particular prior to step (b), the cysteine mutants obtained in step (a) are subjected to a controlled chemical reduction.

In particular the biosensor may be purified in a soluble form.

In particular after step (c) or step (d) or step (e), the method comprises an additional step of immobilizing said biosensor on a solid support.

In particular after step (e), the method comprises an additional step of ranking the biosensors according to their sensitivity.

The OB-fold proteins which are used to generate new target specific Nanofitins comprise a canonical polypeptide backbone (OB-fold) in which some of the residues are fixed to provide the characteristic five stranded β -barrel capped with an amphiphilic α -helix and some of the residues of the β -sheet binding face that have a strong probability of contributing to target binding are varied, known as variable residues, in a random or semi random fashion so as to alter the binding properties of the Nanofitin.

The inventors have found that by focussing efforts upon these variable residues the method for producing biosensors works more efficiently. In addition, the inventors have found unexpectedly that this methods works with Nanofitins which have a small number of variable residues. For this reason, said method is particularly advantageous for making Nanofitin-based biosensors.

This method can be used also for producing reagentless biosensors from other natural or artificial families of target binding proteins which are similarly constructed like for examples antibody Fv fragments and DARPs.

For a better understanding of the invention and to show how the same may be carried into effect, there will now be shown by way of example only,

specific embodiments, methods and processes according to the present invention with reference to the accompanying drawings in which:

Figure 1. Shows the titration of H4S conjugates, monitored by fluorescence. The experiments were performed at 25 °C in buffer C. The total concentration of H4S conjugate, measured by A_{280} , was equal to 0.3 μM . The total concentration in homologous (HEL) or heterologous (BSA) antigen is given along the x axis. The continuous curves correspond to the fitting of Equation 5 to the experimental values of $\Delta F/F_0$ (see Materials and Methods for details). HEL as antigen: closed diamonds, fluorophore at position Trp8; closed triangles, Val21; closed circles, Lys24; open circles, Ala26; open triangles, Lys39. BSA as antigen: open diamonds, Lys24.

Figure 2. Shows the relative sensitivities s_r of the H4S conjugates at 25 °C in buffer C as a function of their concentration. This figure is a plot of Equation 8, using the parameters listed in Table 1. The s_r parameter relates the relative variation of fluorescence intensity $\Delta F/F_0$ and the relative concentration of antigen $[A]_0/[B]_0$ for the low values of $[A]_0$, where $[A]_0$ and $[B]_0$ are the total concentration of antigen and conjugate in the binding reaction, respectively (Equations 7 and 8). Closed diamonds, fluorophore at position Trp8; closed triangles, Val21; closed circles, Lys24; open circles, Ala26; open triangles, Lys39; open diamonds, Tyr42.

Figure 3. Shows the effect of the concentration in serum on the fluorescence signals for conjugates between IANBD and either H4S(K24C) or 2-mercaptoethanol. The experiments were performed in a mixture (v:1-v) of serum and buffer C. The total concentration of conjugate was equal to 0.3 μM . The total concentration of HEL was equal to 10 μM and thus saturating. Open squares, 2-mercaptoethanol-ANBD (the results were identical in the presence or absence of HEL); open circles, H4S(K24ANBD) without HEL; closed circles, H4S(K24ANBD) with HEL. AU, arbitrary units of fluorescence. The continuous curves were drawn only for clarity.

Figure 4. Shows the positions of the hypervariable positions in a structural model of H4S. The model was created with the Swiss Model program in the alignment mode and the crystal structure of the Sac7d protein at a resolution of 1.6 Å (PDB: 1azp) as a template (Arnold *et al.*, 2006; Robinson *et al.*, 1998). Dark grey,

positions where the coupling of the fluorophore strongly decreased the free energy of interaction between H4S and HEL ($\Delta\Delta G \geq 1.5$ kcal mol⁻¹); medium grey, positions where the coupling mildly decreased the energy of interaction ($0.5 \leq \Delta\Delta G \leq 1.2$ kcal mol⁻¹) and resulted in the most sensitive conjugates; light grey, positions where the coupling did not affect the interaction and resulted in little sensitive or insensitive conjugates.

Figure 5. Shows quenching of the H4S(K24ANBD) fluorescence by KI. F and F^0 , fluorescence of the conjugate with and without quencher respectively. The concentration of conjugate was equal to 0.3 μ M and the experiments were performed at 25 °C in buffer C. The continuous curves were obtained by fitting Equation 11 to the experimental data. Closed circles, conjugate in the absence of HEL; open circle, conjugate in the presence of a saturating concentration of HEL (10 μ M). The corresponding Stern-Volmer constants K_{SV} were equal to 6.7 ± 0.1 M⁻¹ and 2.5 ± 0.1 M⁻¹ respectively (value $\pm$ SE in the fitting).

15 **EXAMPLE 1: MATERIALS AND METHODS**

1.1 Materials and genetic constructions

Phosphate buffered saline (PBS), bovine serum albumin (BSA), hen egg-white lysozyme (HEL), calf serum, dithiothreitol (DTT) and Tween 20 were purchased from Sigma; N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester), LB broth and 2-YT broth from Invitrogen; the *Escherichia coli* strain NEB-Express-I^q from New England Biolabs; the plasmid vector pQE30 and the fast-flow NiNTA resin from Qiagen. A stock solution of the IANBD ester was made at a concentration of 10 mg/mL in dimethylformamide. Ampicillin was used at a concentration of 100 μ g/mL and chloramphenicol at 10 μ g/mL. Buffer A was 500 mM NaCl, 20 mM imidazole, 50 mM Tris-HCl, pH 8.0; buffer B, 500 mM NaCl, 200 mM imidazole, 50 mM Tris-HCl, pH 8.0; buffer C, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4; buffer D, 0.005% (v/v) Tween 20, 0.1 mg/mL BSA in buffer C; buffer E, 5 mM DTT in buffer D.

The H4S protein is a recombinant derivative of the Sac7d protein from *Sulfolobus acidocaldarius* that has been evolved by ribosome display to bind hen egg-white lysozyme HEL (WO 2008/068637). Its sequence is identical to

that previously published, except that residue Cys29 has been changed into Ser29:

NH₂-MRGSHHHHHHGSVKVKFFWNGEEKEVDTSKIVWVKRAGKSVLFIYDD
NGKNGYGDVTEKDAPKELLDMLARAEREKKLN-COOH (SEQ ID NO: 58).

Note that the numbering does not take the first 11 residues into account. Plasmid
5 pH4S (SEQ ID NO: 64), which codes for the H4S protein, is a derivative of pQE30
(Qiagen). Changes of residues were introduced into H4S at the genetic level, by
mutagenesis of pH4S with the Quickchange II site-directed mutagenesis kit
(Stratagene).

1.2 Protein production, purification and characterization

10 The parental protein H4S(wt) and its mutant derivatives were
produced in the cytoplasm of the recombinant strain NEB-Express-I^q(pH4S) and its
mutant derivatives as follows. The producing strains were grown at 30 °C. They were
streaked on plates of LB agar, supplemented with ampicillin and chloramphenicol. A
pre-culture in 2-YT broth, supplemented with the same two antibiotics, was inoculated
15 with an isolated colony and grown overnight. A larger culture (650 mL),
supplemented with ampicillin alone, was inoculated with an aliquot of the preculture
(30 mL), grown until $A_{600nm} = 0.7$, induced with 1 mM IPTG, and then grown further
for 24 hours. The following purification steps were performed at 4 °C. The culture
was chilled on ice, centrifuged at 8000 g for 20 min and the resulting pellet was frozen
20 and kept at -20 °C. The pellet was thawed, resuspended in 30 mL of Buffer A, and the
cells were disintegrated by sonication. The lysate was centrifuged at 8600 g for 30
min and the supernatant filtered through a 0.22 µm Millex filter (Millipore). H4S and
its derivatives were purified through their hexahistidine tag by affinity
chromatography on a column of Ni-NTA resin. The proteins were eluted from the
25 resin with Buffer B. The purification fractions were analyzed by SDS-PAGE, in the
presence or absence of 2.5 % (v/v, 0.4 M) 2-mercaptoethanol, and the protein bands
were quantitated with the Un-scan-it software (Silk Scientific) as described (Brient-
Litzler et al., 2010). The pure fractions (> 98 % homogeneous in reducing conditions),
were pooled and kept at -80 °C. The protein concentrations were measured by
30 absorbance spectrometry. The coefficients of molar extinction were calculated from
the amino acid sequences with the ExPASy ProtParam tool (Gasteiger et al., 2003). All
the binding experiments were performed at 25 °C.

1.3 Fluorophore coupling

The conjugates between the cysteine mutants of H4S (Nanofitin) and the IANBD ester were prepared as follows. The H4S mutants were reduced with 5 mM DTT for 30 min at 30°C with gentle shaking and then the buffer was exchanged to PBS by size exclusion chromatography with a PD10 column (GE Healthcare). From this point on, all the experiments were done in the dark. The thiol-reactive fluorophore IANBD ester was added in a 10:1 molar excess over the Nanofitin and the coupling reaction was carried out for 2.5 hours at 30 °C with gentle shaking. The denatured proteins were removed by centrifugation for 30 min at 13200 g, 4 °C. The conjugate was separated from the unreacted fluorophore by chromatography on a Ni-NTA column (0.5 mL of resin) and eluted with Buffer B. The conjugate between 2-mercaptoethanol and the IANBD ester was prepared by mixing the two molecules in stoichiometric amounts and then incubating the mixture for 30 min at 25 °C. The coupling yield y_c , i. e. the average number of fluorophore molecule coupled to each Nanofitin molecule, was calculated as described below, with $\epsilon_{280}(\text{ANBD}) = 2100 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{500}(\text{ANBD}) = 31800 \text{ M}^{-1} \text{ cm}^{-1}$, both measured with a conjugate between IANBD and 2-mercaptoethanol (Renard *et al.*, 2002).

Let P be a protein; B, a monoconjugate between P and IANBD; Φ , the conjugated form of IANBD; A_{280} and A_{500} , the absorbances of the mixture of P and B that results from the coupling reaction and elimination of the unconjugated fluorophore. By definition,

$$y_c = [B]/([B] + [P]) \quad (1)$$

where [B] and [P] are concentrations. Then, the reciprocal of the coupling yield is given by the following equation where ϵ is a molar absorbance (Brient-Litzler *et al.*, 2010):

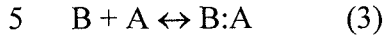
$$y_c^{-1} = (A_{280}/\epsilon_{280}(P))(A_{500}/\epsilon_{500}(\Phi))^{-1} - \epsilon_{280}(\Phi)/\epsilon_{280}(P) \quad (2)$$

1.4 Fluorescence measurements and antigen binding

The binding and fluorescence experiments were performed at equilibrium as if the preparations of conjugates were homogeneous, i. e. as if $y_c = 1$. The binding reactions were conducted by incubating 0.3 μM of conjugate with variable concentrations of the HEL or BSA antigens in a volume of 1 mL, for 30 min

in the dark with gentle shaking. The reactions were carried out in Buffer C or in a mixture v:(1-v) of calf serum and Buffer C as indicated in example 2.

The conjugate (or biosensor) B and antigen A form a 1:1 complex B:A according to the reaction:



At equilibrium, the concentration [B:A] of the complex is given by the equation:

$$[B:A] = 0.5 \{ [B]_0 + [A]_0 + K_d - (([B]_0 + [A]_0 + K_d)^2 - 4 [B]_0 [A]_0)^{1/2} \} \quad (4)$$

where K_d is the dissociation constant, and $[A]_0$ and $[B]_0$ are the total concentrations of A and B, respectively (Renard *et al.*, 2003).

10 The fluorescence of the IANBD conjugates was excited at 485 nm (5 nm slit width) and its intensity measured between 520 and 550 nm (20 nm slit width) with a FP-6300 spectrofluorometer (Jasco). The signal of HEL alone or BSA alone was measured in an independent experiment and subtracted from the global signal of the binding mixture to give the specific fluorescence intensity F of each conjugate.

15 The intensity F at a given value of $[A]_0$ satisfies the following equation:

$$(F - F_0)/F_0 = \Delta F/F_0 = (\Delta F_\infty/F_0)([B:A]/[B]_0) \quad (5)$$

where F_0 and F_∞ are the values of F at zero and saturating concentrations of A (Renard *et al.*, 2003). The values of $\Delta F_\infty/F_0$, K_d , and $[B]_0$ could be determined by fitting Equation 5, in which [B:A] is given by Equation 4, to the experimental values
20 of $\Delta F/F_0$, measured in a titration experiment. The fittings were performed with the Kaleidagraph software (Synergy Software).

The sensitivity s and relative sensitivity s_r of a conjugate can be defined by the following equations for the low values of $[A]_0$, i. e. in the initial part of the titration curve:

$$25 \quad \Delta F = s[A]_0 \quad (6)$$

$$\Delta F/F_0 = s_r[A]_0/[B]_0 \quad (7)$$

s and s_r can be expressed as functions of characteristic parameters of the conjugate:

$$s_r = (\Delta F_\infty/F_0)([B]_0/(K_d + [B]_0)) \quad (8)$$

$$s = f_b s_r \quad (9)$$

30 where $f_b = F_0/[B]_0$ is the molar fluorescence of the free conjugate (Renard and Bedouelle, 2004). Equation 8 implies that s_r is maximal when the biosensor B is

present at a concentration $[B]_0 \gg K_d$, as every newly titrated molecule A then forms a complex. Equation 6 implies that the lower limit of detection $\delta[A]_0$ of the conjugate is linked to the lower limit of measurement of the spectrofluorometer δF by the following equations:

$$\delta[A]_0 = s^{-1} \delta F = s_r^{-1} [B]_0 (\delta F / F_0) \quad (10)$$

1.5 Quenching by potassium iodide

The experiments of fluorescence quenching by KI were performed at 25°C in Buffer C, essentially as described above. The Stern-Volmer Equation 11 was fitted to the experimental data, where F and F^0 are the intensities of fluorescence for the H4S conjugate in the presence or absence of quencher, respectively. The Stern-Volmer constant K_{SV} was used as a fitting parameter.

$$F^0/F = 1 + K_{SV} [KI] \quad (11)$$

1.6 Affinity in solution as determined by competition Biacore

The affinity in solution between H4S and HEL was determined with the following modifications of a published procedure (Brient-Litzler *et al.*, 2010). The binding reactions (250 μ L) were conducted by incubating 20 nM of H4S with variable concentrations of HEL for 30 min in buffer D. The concentration of free H4S was then measured by surface plasmon resonance with a Biacore 2000 instrument (Biacore). A high density of HEL (1700 Resonance Units, RU) was immobilized on the surface of a CM5 sensorchip (GE Healthcare) and each reaction mixture was injected in the sensor chip at a flow rate of 30 μ L min⁻¹. The chip surface was regenerated between the runs by injecting 10 μ L of a 0.05 % SDS solution.

1.7 Kinetic measurements by Biacore

The kinetics were measured in buffer D at a flow rate of 30 μ L min⁻¹ with CM5 sensor chips. A first cell of the sensor chip was used as a reference, i.e. no ligand was immobilized on the corresponding surface. A second cell was loaded with 500-1000 resonance units (RU) of HEL. Solutions (200 μ L) of the H4S derivatives at 15 different concentrations (1 nM to 6 μ M) were injected to monitor association and then buffer alone (150 μ L) for dissociation. The chip surface was regenerated between the runs by injecting 10 μ L of a 0.05% SDS solution. The signal of the buffer alone was subtracted from the raw signals to obtain the protein signals, and then the protein

signal on cell 1 was subtracted from the protein signal on cell 2 to obtain the specific signal of interaction. The kinetic data were cleaned up with the Scrubber program (Biologic Software) and then the kinetic parameters were calculated by applying a simple kinetic model of Langmuir binding and a procedure of global fitting, as implemented in the Bia-evaluation 4.1 software (Biacore).

EXAMPLE 2: BIOSENSORS FROM NANOFITINS-RESULTS USING BIOSENSORS DERIVED FROM H4S

2.1 Production and oligomeric state of cysteine mutants

The artificial family of Nanofitins is derived from Sac7d, an OB-fold protein, and comprises 14 randomized positions. Two additional positions, 28 and 39, have not been randomized although the residues at the corresponding positions form contacts between some OB-fold proteins and their cognate partners (Mouratou et al., 2007). The residues at the 14 fully randomized positions of the H4S protein, directed against HEL, and at its positions Lys28 and Lys39 were changed individually into cysteine by site-directed mutagenesis of the coding gene. The parental protein, H4S(wt), and its mutant derivatives were produced in the cytoplasm of *E. coli* and purified through their hexahistidine tag. The yields of purified soluble protein varied between 4 mg/L and 46 mg/L of culture in flask, with an average of 18 ± 3 mg/L (mean $\pm$ standard error). The yields for the mutant proteins were not very different from the yield for the parental protein, i. e. 40 mg/L.

The introduction of a cysteine residue could lead to inter-molecular disulfide bonds. To characterize the oligomeric state of the H4S mutants, their purified preparations were analyzed by SDS-PAGE after denaturation in the presence or absence of a reducing agent. Under reducing conditions, a single protein species was observed with an apparent molecular mass that was consistent with the theoretical mass of an H4S(wt) monomer, 9146.3. Under non-reducing conditions, a second species was observed with an apparent molecular mass that was consistent with the theoretical mass of a dimer. The proportion of protomers in a dimeric state was calculated from the intensities of the protein bands. It was ≤ 20 % for the five mutants that we analyzed.

2.2 Conjugation and its yield

The purified preparations of the H4S mutants was submitted to a reaction of reduction before coupling with the thiol reactive fluorophore N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester), to break open the potential intermolecular disulfide bonds and ensure that the mutant cysteine residue would be in a reactive state. The products of the coupling reaction were separated from the unreacted fluorophore by chromatography on a nickel ion column. The coupling yield y_c , defined as the number of fluorophore groups per H4S molecule, was calculated from the absorbance spectra of the purified reaction product (Materials and Methods; Table 1). It varied from 43 to 90 % (72 ± 3 , mean $\pm$ SE). Such position dependent variations have already been observed for other Antigen Binding Proteins (AgBPs; Brient-Litzler *et al.*, 2010; Renard *et al.*, 2003; Renard *et al.*, 2002). The synthesis yield y_s of the coupling procedure, i. e. the proportion of protein molecules that remained at the end of the procedure, was similar for all the H4S mutants, 65 ± 3 % (mean $\pm$ SE).

Table 1.

Table 1 shows the properties of H4S conjugates, as derived from fluorescence experiments. Column 1, residue with which the fluorophore was coupled. y_c , number of molecules of fluorophore per molecule of H4S in a purified preparation of the conjugate (coupling yield). $\lambda_{\max}$, wavelength of the maximal value of F_0 . f_b , molar fluorescence of the free conjugate at $\lambda_{\max}$. The total concentration of conjugate was equal to $0.3y_c \mu\text{M}$. $\Delta F_\infty/F_0$, maximal variation of F at $\lambda_{\max}$. The entries for $\Delta F_\infty/F_0$ and K_d give the value and associated SE from the fitting of Equation 5 to the data points in the titration experiments. s_r , relative sensitivity of the conjugate at a fixed concentration of $0.3 \mu\text{M}$. nm, not measurable. The fluorescence experiments were performed in buffer C. The experiments for Trp8, Val21, Lys24, Ala26, Ser29, Leu31 and Lys39 were performed in duplicate, with nearly identical results. The Pearson parameter in the fittings was $R > 0.992$ except for Phe7 ($R = 0.98$) and Trp22 ($R = 0.72$). The K_d value for H4S(wt) was equal to $40.3 \pm 1.6 \text{ nM}$, as measured by competition Biacore in buffer D (see Materials and Methods for details).

Residue	γ_c	$\lambda_{\max}$ (nm)	f_b (FU μM^{-1})	$\Delta F_{\infty}/F_0$	K_d (nM)	s_r
Phe7	0.86	539.0	1760	0.26 ± 0.01	42 ± 13	0.22
Trp8	0.72	536.5	427	1.47 ± 0.04	189 ± 50	0.90
Asn9	0.43	539.0	232	0.52 ± 0.01	7 ± 2	0.50
Val21	0.87	537.0	531	6.30 ± 0.04	296 ± 19	3.17
Trp22	0.77	529.5	4375	0.3 ± 0.1	1226 ± 1141	0.06
Lys24	0.68	536.0	433	8.8 ± 0.1	98 ± 17	6.61
Ala26	0.54	539.0	664	7.9 ± 0.1	206 ± 27	4.66
Lys28	0.75	537.5	1888	0.26 ± 0.01	28 ± 8	0.24
Ser29	0.73	538.5	1428	0.55 ± 0.01	33 ± 6	0.50
Leu31	0.80	538.0	193	4.9 ± 0.4	8356 ± 2754	0.17
Ile33	0.90	537.5	191	nm	nm	nm
Lys39	0.68	538.5	764	1.65 ± 0.02	126 ± 14	1.16
Asn40	0.65	537.5	458	0.57 ± 0.05	2004 ± 894	0.07
Tyr42	0.69	537.5	286	6.6 ± 0.2	2479 ± 487	0.72
Asp44	0.70	537.0	689	1.50 ± 0.04	623 ± 106	0.49
Thr46	0.85	538.5	1922	0.36 ± 0.01	100 ± 32	0.27

2.3 Fluorescence properties of the conjugates

The free conjugates were excited at 485 nm and their emission spectra were recorded. The maximum of fluorescence intensity had a wavelength $\lambda_{\max}$ that varied slightly between conjugates, from 529.5 to 540 nm (Table 1). The following experiments of spectrofluorometry were performed at the $\lambda_{\max}$ value thus determined for each conjugate.

The responsiveness of the H4S conjugates to the binding of their antigen was tested by measuring the relative variation $\Delta F/F_0 = (F - F_0)/F_0$ in their fluorescence intensity F between their HEL-bound and free states. The total concentration of conjugate $[B]_0$ was chosen to fulfill two requirements. (i) The fluorescence intensity F_0 of the free conjugate had to be within the dynamic interval of the spectrofluorometer. (ii) The dynamic interval of F had to cover more than one order of magnitude in antigen concentration. In these experiments, a concentration that was

identical for all the conjugates and equal to 0.3 μM was chosen. The values of F_0 for the various conjugates were comprised between 37 and 1921 FU (fluorescence units) at this concentration and corresponded to molar fluorescences f_b comprised between 191 and 4375 FU μM^{-1} (Table 1).

5 The theoretical Equation 5, linking $\Delta F/F_0$ and the total concentration of antigen $[A]_0$, was fitted to the experimental data with the maximal variation $\Delta F_\infty/F_0$, the dissociation constant K_d and the total concentration of conjugate $[B]_0$ as floating parameters (Materials and Methods). The titrations of the conjugates were performed with ≥ 17 concentrations of their antigen, HEL (Figure 1). The
10 corresponding values of $\Delta F_\infty/F_0$ and K_d are given in Table 1. The values of K_d varied widely between conjugates, between 7 nM and 8 μM , with five values being close to the value for H4S(wt) (see legend to Table 1). The value of $\Delta F_\infty/F_0$ was > 0.5 for eleven of the conjugates, i. e. their fluorescence intensity F increased by > 1.5 fold on HEL binding.

15 2.4 Classification of the conjugates

 The conjugates of H4S gave a wide range of values for $\Delta F_\infty/F_0$ and K_d . They were classified according to their sensitivity, a parameter which is used to characterize any measuring instrument. This sensitivity can take two forms for a RF biosensor, a relative sensitivity s_r and an absolute sensitivity s . The relative sensitivity
20 s_r relates the relative variation $\Delta F/F_0$ of the fluorescence signal to the relative concentration $[A]_0/[B]_0$ of antigen for the low values of the latter, where $[A]_0$ and $[B]_0$ are the total concentrations of antigen and conjugate, respectively, in the titration reaction (Equation 7 in Materials and Methods; $[A]_0/[B]_0$ can be viewed as the concentration of antigen, normalized to the concentration of conjugate). s_r is an intrinsic dimensionless
25 parameter. Its value does not depend on the spectrofluorometer or its set up, and should remain constant between experiments, instruments and laboratories. The value of s_r depends on the values of $[B]_0$ and K_d according to a saturation law and its maximal value is equal to $\Delta F_\infty/F_0$ (Equation 8). The absolute sensitivity s relates ΔF and $[A]_0$ for the low values and is equal to $f_b s_r$, where f_b is the molar fluorescence of
30 the free conjugate (Equations 6 and 9). The s^{-1} parameter relates the lower limit of

detection $\delta[A]_0$ for the conjugate to the lower limit of measurement δF for the spectrofluorometer.

The variations of s_r (Figure 2) and s^{-1} were calculated for each conjugate as functions of $[B]_0$ in buffer C from Equations 8 and 9. These variations showed that the classification of the conjugates according to their values of s_r could vary as a function of $[B]_0$. For s_r and with $[B]_0 = 0.3 \mu\text{M}$, i. e. the concentration at which the experiments were performed, the coupling positions ranked in the following decreasing order, i. e. starting with the highest relative sensitivity: Lys24 > Ala26 > Val21 > Lys39 > Trp8 > Tyr42 (Table 1). For s^{-1} and $[B]_0 = 0.3 \mu\text{M}$, the coupling positions ranked in the following increasing order, starting with the lowest limit of detection: Ala26 < Lys24 < Val21 < Lys39 < Ser29 < Thr46. The conjugate at position Lys24, H4S(Lys24ANBD), had a value $s_r = 6.6$ when used at a concentration $[B]_0 = 0.3 \mu\text{M}$, and therefore a lower limit of detection $\delta[A]_0 = 0.35 \text{ nM}$ since the Jasco FP-6300 spectrofluorometer which was used could detect a relative variation of fluorescence $\delta F/F_0 = 1.5 \%$ in these experimental conditions (Equation 10). The ranking of the conjugates according to s^{-1} was valid because all our measurements were done with the same spectrofluorometer and set-up of the instrument.

2.5 Specificity and selectivity

To test the specificity of the recognition between the H4S conjugates and HEL, H4S(K24ANBD) and H4S(A26ANBD) were titrated with bovine serum albumin (BSA) in buffer C. Both conjugates bound BSA but with values of K_d much higher and values of $\Delta F_\infty/F_0$ much lower than for HEL: $K_d = 1.0 \pm 0.3 \mu\text{M}$ and $\Delta F_\infty/F_0 = 0.26 \pm 0.01$ for H4S(K24ANBD); $K_d = 1.4 \pm 0.3 \mu\text{M}$ and $\Delta F_\infty/F_0 = 0.73 \pm 0.04$ for H4S(A26ANBD) (Figure 1). As a result, the relative sensitivities s_r of the two conjugates were 106 and 36 fold lower for BSA than for HEL, respectively, at the concentration of conjugate $[B]_0 = 0.3 \mu\text{M}$ used for these experiments. Therefore, the variation of the $\Delta F/F_0$ signal was indeed specific for HEL, the cognate antigen.

The selectivity of a biosensor refers to the extent to which it can recognize a particular analyte in a complex mixture without interference from other components (Vessman *et al.*, 2001). The selectivity of the H4S(K24ANBD) conjugate was characterized by comparing its fluorescence properties in serum and in buffer C.

More specifically, the variations of the F_0 and $F_{10\mu\text{M}}$ parameters were measured as functions of the concentration in serum (Figure 3). Given that $K_d = 0.1 \mu\text{M}$ for H4S(K24ANBD), this conjugate was fully saturated with HEL at $10 \mu\text{M}$. We observed that the value of $F_{10\mu\text{M}}$ for H4S(K24ANBD) decreased linearly with the
 5 concentration in serum. As expected, the absorbance of the serum alone increased linearly with its concentration, in agreement with the Beer-Lambert law, at both 485 nm and 536 nm, which were the wavelengths of fluorescence excitation and emission in our experiments. Therefore, the absorption of the excitation and emission lights by serum could account for the variation of $F_{10\mu\text{M}}$. Surprisingly, F_0 increased with the
 10 concentration in serum, up to 40 % (v/v) of serum, and then decreased. The same increase and decrease was observed when measuring the fluorescence of a conjugate between IANBD and 2-mercaptoethanol. Thus, the increase resulted from the interaction between the fluorescent group and molecules of the serum, and the decrease from the absorbance of the serum, as observed for $F_{10\mu\text{M}}$. The variation of
 15 $(F_{10\mu\text{M}} - F_0)/F_0$ with the concentration of serum led to the conclusion that H4S(K24ANBD) could still operate in ≤ 50 % serum $((F_{10\mu\text{M}} - F_0)/F_0 \geq 0.25)$.

2.6 Binding parameters

The dissociation constants K_d in solution between the H4S conjugates and HEL were determined by fluorescence in titration experiments (Table
 20 1). This method was not applicable to the parental Nanofitin H4S(wt). Therefore, the value of its K_d was determined in solution by experiments of competition Biacore. It was equal to $40.3 \pm 1.6 \text{ nM}$ (legend to Table 1). Comparison of the K_d values for the conjugates and H4S(wt), all of them determined in solution, showed that conjugation of the fluorophore at positions Trp22, Leu31, Ile33, Asn40, Tyr42 and Asp44 strongly
 25 decreased the free energy of interaction between H4S and HEL ($\Delta\Delta G \geq 1.5 \text{ kcal mol}^{-1}$, Set 1). Conjugation at positions Trp8, Val21, Lys24, Ala26 and Lys39 affected it more mildly ($0.5 \leq \Delta\Delta G \leq 1.2 \text{ kcal mol}^{-1}$, Set 2). Conjugation at positions Phe7, Asn9, Lys28, Ser29 and Thr46 did not affect adversely the interaction ($\Delta\Delta G \leq 0.5 \text{ kcal mol}^{-1}$, Set 3). Note that the conjugation at position Asn9 improved the interaction.
 30 These experiments suggested that the residues of Set 1, which form a continuous patch at the surface of H4S, belong to the binding site for HEL; that the residues of Set 2 are

at the periphery of the binding site; and that those of Set 3 are located outside of it (Figure 4). The positions that gave the most sensitive conjugates belonged to Set 2 (Table 1).

To determine whether the variations of affinity were due to the mutation of the parental side chain into Cys or to the grafting of the fluorescent group, the kinetic parameters of interaction were measured between H4S(wt) and five of its cysteine mutants on the one hand, and HEL on the other hand by Biacore (Materials and Methods). The kinetics were measured in reducing conditions to prevent the dimerization of the cysteine mutants. The kinetic data were analyzed with a 1:1 model, the corresponding dissociation constant were calculated from the rate constants, i. e. $K_d' = k_{off}/k_{on}$, and the dissociation constant at steady state K_d'' were also derived from these kinetic experiments (Table 2). The values of K_d , K_d' and K_d'' were found close for H4S(wt), 40 ± 2 nM, 31 nM and 18 nM respectively. The value determined in solution was slightly higher than the values determined at the interface between a solid and a liquid phase. Comparison of the K_d , K_d' and K_d'' values between H4S(wt) and either the Cys mutants or the conjugates indicated that the lower affinity of the conjugates relative to H4S(wt) was mainly due to the mutation into Cys at positions Val21 and Lys24, whereas it was mainly due to the grafting of the fluorophore at positions Trp8, Ala26 and Lys39.

Mutation	Monomer (%)	k_{on} ($10^5 \text{ M}^{-1} \text{ s}^{-1}$)	k_{off} (10^{-2} s^{-1})	K_d' (nM)	K_d'' (nM)
WT	100	4.9	1.5	31	18
W8C	86	4.5	3.0	68	66
V21C	88	0.4	1.1	332	389
K24C	100	1.6	2.3	117	104
A26C	80	3.8	2.3	60	54
K39C	85	3.1	1.2	38	24

Table 2.

Table 2 shows binding parameters of H4S derivatives, as determined by Biacore experiments. The percentage of monomers was quantified with the Un-scan-it software, as described in Materials and Methods. HEL was immobilized on a CM5 sensorchip. The association and dissociation rate constants, k_{on} and k_{off} , were

determined at 25 °C in buffer E and used to calculate $K_d' = k_{\text{off}}/k_{\text{on}}$ (Materials and Methods). K_d'' is the dissociation constant at steady state. A simple kinetic model of Langmuir binding was applied.

2.7 Mechanism of fluorescence variation

5 Eleven of the conjugates were sensitive to the binding of HEL, with $\Delta F_{\infty}/F_0$ between 0.5 and 8.9. Potassium iodide (KI) was used to explore the physico-chemical mechanism by which the fluorescence intensity of the conjugates varied on antigen binding. KI, up to 250 mM, does not affect the interaction between proteins (Brient-Litzler *et al.*, 2010). The fluorescence of the H4S(K24ANBD) conjugate was
10 quenched by KI, both in its free and HEL-bound states. The quenching varied linearly with the concentration of KI (Figure 5). This law of variation indicated that the molecules of fluorophore were identically exposed to KI and constituted a homogeneous population in either case (Lakowicz, 1999). It confirmed that the fluorescent group was specifically coupled to the mutant cysteine. The Stern-Volmer
15 constant was higher for the free conjugate than for its complex with the target antigen: $K_{SV} = 6.7 \pm 0.1 \text{ M}^{-1}$ versus $2.5 \pm 0.1 \text{ M}^{-1}$ (Figure 5). These values indicated a lower accessibility of the fluorophore to KI in the bound state of the conjugate than in its free state. They showed that the fluorescence increase was due to a shielding of the fluorescent group from the solvent by the binding of the antigen, as previously
20 observed for other conjugates with IANBD (Brient-Litzler *et al.*, 2010; Renard *et al.*, 2003).

2.8 Conclusion

The inventors have constructed reagentless fluorescent biosensors from Nanofitins in the absence of specific structural data, by using their peculiar
25 method of construction. The method comprises: (i) individually changing the residues of the hypervariable positions into cysteine at the genetic level, (ii) chemically coupling a solvatochromic fluorophore with the mutant cysteine, and (iii) ordering the resulting conjugates through their relative sensitivity s_r , that involves both their affinity for the antigen and their relative variation of fluorescence signal. This method
30 could be applied to any antigen binding protein in the same conditions. The inventors have validated the method by constructing sixteen conjugates between the IANBD

fluorophore and H4S, a Nanofitin which is directed against hen egg-white lysozyme (HEL) and for which no specific structural data is available.

The residues at the 14 fully randomized positions and at positions Lys28 and Lys39 of H4S were changed individually into cysteine by site-directed mutagenesis of the coding gene. The sixteen cysteine mutants were then conjugated to the thiol reactive fluorophore IANBD ester, and the fluorescence and binding properties of the conjugates were analyzed.

Five conjugates had values of K_d close to that of H4S(wt). The value of $\Delta F_\infty/F_0$ was > 0.5 for eleven of the conjugates, i. e. their fluorescence intensity F increased by > 1.5 fold on HEL binding. The inventors ranked the conjugates according to their relative sensitivity s_r and their lower limit of detection (proportional to s_r^{-1}). The positions that gave the most sensitive conjugates were at the periphery of the binding site ($0.5 \leq \Delta\Delta G \leq 1.2$ kcal mol $^{-1}$). Comparison of the K_d , K_d' and K_d'' values between H4S(wt) and either the Cys mutants or the conjugates indicated that the lower affinity of the conjugates relative to H4S(wt) was mainly due to the mutation into Cys at positions Val21 and Lys24, whereas it was mainly due to the grafting of the fluorophore at positions Trp8, Ala26 and Lys39. One of the conjugate, H4S(Lys24ANBD), had a value $s_r = 6.6$ when used at a concentration $[B]_0 = 0.3$ μ M, and therefore a lower limit of detection $\delta[A]_0 = 0.35$ nM. This biosensor had an affinity only two-fold lower than the affinity of the parental protein. H4S(Lys24ANBD) bound its cognate antigen specifically and selectively and could still operate in ≤ 50 % serum. Its fluorescence signal increased linearly and specifically with the concentration of antigen. Titration with potassium iodide indicated that the fluorescence variation was due to a shielding of the fluorescent group from the solvent by the antigen.

These new results on Nanofitins and previous results on antibodies and DARPins show that it is possible to construct reagentless fluorescence biosensors from any natural or artificial family of antigen binding protein by targeting in priority the hypervariable positions that are not important for the interaction with the antigen.

The sensitivities of the Nanofitin conjugates were much higher than those for scFv fragments of antibodies and generally higher than those for DARPins.

Therefore, the Nanofitins which have a higher stability than antibodies and may have an extended range of antigens compared to DARPins constitute a promising family of target binding proteins, for the construction and the multiple applications of reagentless fluorescent biosensors directed to various targets.

SEQ ID NO:	Protein Data Bank (PDB) accession number	Protein name Organism	UniProt/GenBank accession number	SEQUENCE
12	1krs	Lysyl-tRNA synthetase LysS (<i>Escherichia coli</i>)	B7UHT9	MSEQHAQCADAVVDLNNELKTRREKLANLEQGIAPFNDFFRDHTSDQLHAEFDGKENEELALNIEVAVAG RMTTRIMGRASFVTLQDVGGRIQLYVARDDLPEGVYNEQFKWDLGLGAKGLFKTKTGELS IHCTELRL LLTKALRPLPKFHLGQDQARYRQRYLDLISNDESRNFKVRSQILSGIRQFVNRGFMVEVETPMQVPIPG GAAARPFITHNALDMLYRIAPELYLKLVLVGGPERVFEINRFRNEGISVRHNPEFTMMELYMAYADYK DLIELTESLFRITLAQDILGTEVTYGDVTLDGKPEKLTNRKAIKKYRSETDMA DLNDFDSAKAIAESIGI HVEKSWGLGRIVTEIFEVAAEHLIQPTFITETEPAEVSPPLARRNDVNPEITDRFEFFIGGREIGNGSELNDA AEDQAQRFLDQVAAKADAGDDEAMFYDEYVTALEHGLPPTAGLGIGIDRMVMLFTNSHTTIRDVILFFPAMRPV K
13	1c0aa	Asp-tRNA synthetase (<i>Escherichia coli</i>)	P21889	MRTEYCGQLRSLSHVGQOVTLCGWVNRRLDGLSLIFIDMRDREGIVGVFFDDPRADALKASELRNEFCIQVT GTVRARDEKNINRDMATGEIEVLASSLTIINRADVLPLDSNHVNTPEARLKYRLDLRRPEMAQRKTRAKI TSLVRRFMDHGFLDIETPMLTKATPEGARDYLPSRVHKGKFFALPQSPQLFKQLMMSGFDRYYQIVKCF RDEDLRADRQPEFTQIDVETSFMTAPQVREYMEALVRHLWLEVKVGLDGFVMTFAEAERRYGSKDPDLRN PMELTDVADLLKSVEFAVAGPANDPKGRVAALRVPGGASLTRKQIDEGNFVKIYGAKGLAYIKVNERAKG LEGINSVPVAKFLNAEIIEDILDRTAQDGMIFFGADNKKIVADAMGALRLKVGKDIGLTDSEKWAPLWVID PFMFEDDGEGLTAMHHPTSPKDMTAAELKAAAPENAVANAYDMVINGEYVGGGSRVHNHNGDMQQTVFGILG INEEEQREKFGFLLDALKYGTPTPPHAGLAFGLDRLTLLTGTDNIRDVIAFPKTTAAACLMTTEAPSFANPTAL AELSIQVVK
14	1b8aa	Asp-tRNA synthetase (<i>Thermococcus kodakaraensis</i>)	Q52428	MYRTHYSSEITEELNGQKVKVAGWVWEVKDLGGIKFIWIRDDGIVQITAPKKKVDPELFLIKPILKRSDEVV AVEGVVNFTPKAKLGFELLPEKIVVLNRAETPLDPTGKVKAELETRLDNRFMDLRRPEVMAIFKIRSSVF KAVRDFPHENGFEIHTPKIATATGEGTELPMPKYFEEDAFQAQSPQLYQIMMASGLDRVYEIAPIFRAE EHNTTRHLINEAWSIDSEMAFIEDEEVMSFLERLVAHAINYVREHNAKELDIINFELEEKLPFPFRVSYDKA LEILGDSLGKEIPWGEDIDTEGRLLGKYMENENAPLYFLYQYPSAKPFYIMKYDNKPEICRAFDELYRGV EISSGGQREHRHDILVEQIKEKGLNPESFEFYIKAFRYGMPHGGFGLGAERLIKQMLDLNIREVILFPDR RRRLTP
15	1lyla	Lysyl-tRNA synthetase LysU (<i>Escherichia coli</i>)	POA8N6	SEQETRGANEALDFNDELNRREKLAALRQQGVAFPNDFRDRDHTSDQLHEEFDAKDNQELSLNIEVSVAGR MMTRRINGKASFVTLQDVGGRIQLYVARDSLPEGVYNDQFKWDLGLDIGARTLFTKTGTGELS IHCTELRL ITKALRPLPKFHLGQDQEVRYRQRYLDLISNDESRNFKVRSQILSGIRQFVNRGFMVEVETPMQVPIPGG ASARPFITHNALDMLYRIAPELYLKLVLVGGPERVFEINRFRNEGISVRHNPEFTMMELYMAYADYHD LIELTESLFRITLAQDILGTEVTYGDVTLDGKPEKLTNRKAIKKYRSETDMA DLNDFDSAKAIAESIGI VEKSWGLGRIVTEIFEVAAEHLIQPTFITETEPAEVSPPLARRNDVNPEITDRFEFFIGGREIGNGSELNDA EDQAERTQEQVNAKAGDDEAMFYDEYVTALEHGLPPTAGLGIGIDRMVMLFTNSHTTIRDVILFFPAMRPQK

SEQ ID NO:	Protein Data Bank (PDB) accession number	Protein name Organism	UniProt/GenBank accession number	SEQUENCE
16	1quqA	Replication protein A, 32 kDa subunit (<i>Homo sapiens</i>)	P15927	MWNSGFESYSSGGAGGYTQSPGGFSPAPSAEKKSPARAGHIVPTISQLLSATLVDEVEFRIGNVEIS QVTVIGIIRHAEKAPTNIYKIDMTAAPMDVROMVDTDDTSSNTVVPETPYVKVAGHLRSFQNKKSIVAF KIMPLEDMNEFTTHILEVINAHVLSKANSQPSAGRAPISNPGMSEAGNFGGNSFMPANGLITVAQNQVNLNI KACPRGEGINFQDLKNQKHMSVSSIKQAVDFLSNEGHIYSTVDDHFKRSTDAE
17	1quqB	Replication protein A, 14 kDa subunit (<i>Homo sapiens</i>)	P35244	MVDMMDLPRSRINAGMLAQFIDKPVCFVGRLEKIHPTGKMFILSDGEGKNGTILMEPLDEEISGIVEVVG VTAKATILCTSYVQFKEDGSHPFDLGLYNEAVKIIHDFPQFYPLGIVQHD
18	1jmcA	Replication protein A, 70 kDa subunit (RPA70) fragment (<i>Homo sapiens</i>)	P27694	MVGQLSEGAIAAIMOKGDTNIKPILOVINIRPIITGNSPPRYRLIMSDGLNTLSFMLATQNLPLVEEQLS SNVCQIHRFIVNTLKDGRVVIIMELEVLKSAEAVGVLGNPVPYNEGLQGPQVAPPAAPAAASSRPQP QNGSSGMGSTVSKAYGASKTGKAAGPSLSHTSGCTQSKVVPVPIASLTPTYOSKWTICARVTNKQIRTWNSR GEGKLFSELVDESGEIRAFANEOVDKFEPLIEVNKYVYFSKGLKIANKQFTAVKNDYEMTNNETSVMP CEDDHLPTVQFDFTGIDDLLENKSKDSLVDIIGICKSYEDATKITVRSNNREVAKRNIYLMDTSGKVVTATL WGEDADKFDGSRQPVLAIKGARVSDFGGRSLSVLSSTTIANPDIEAYKLRGWFDAEQALDGVSI SLDKS GGVGSNTNWKTLVEYKSENILGQGDKPDYFSSVATVVYLKNCWYQACPTQDCNKKVIDQONGLYRCEKCD TEFPNFKYRMILSVNIADFQENQWVTCFQBSAEAILQNAAYLGELKDKNEQAEEVFQANFRSFI FRVRV KVETYNDESRIKATVMDVKPVDYREYGRRLVMSIRRSALM
19	1otcA	Telomere-end-binding protein (<i>Oxytricha nova</i>)	P29549	MSTAAKQNRSTSRVSKKKTAAAPKEGAACKSKDKGHKYYEVELAKASILTSAQPOHFYAVVIDATFPYKTNQERY ICSLKIVDPTLYLKQKQAGDASDYATLVLYAKRFEDLP IHRAGDIIRVHRATRLRYNGQRFQFNANVFYSS SWALFSTDKRSVTQEIINNQDAVSDTTPFSSSKHATIEKNEISILQNLKRWANOVFSSYVSSDMYTALNK AQAAQGDGDFVVAKILQVHELDEYTNELKLDASGQVYTYLLSLKLPHPHVTGEVVRIRSATYDETSTQKKVL ILSHYSNIIITFIQSSKLAKELRAKIQDDHSEVAVSLKKNVSLNAVVLTEVDKHAALPSTSLQDLFHHADS KELQADTFRFTQFYVTKIEPSDVKEWVKGYDRKTKKSSSLKSGASGKGDNI FQVQFLVKDASTQLNNTYRVL LYTQDGLGANFNVKADNLHKNADARKKLEDSAEILLTKFNSYVDAVVERNGFYLIKDKTKLIY
21	3ulla	Mitochondrial ssDNA-binding protein (<i>Homo sapiens</i>)	Q567R6	MFRPVLQVLRQFVRHESETTSLVLESLNRVHLGRVGQDPVLRQVEGKNPVTIFSLATNEMWRSQDSEV YQLGDVYSQKTTWHRISVFRPGLRDVAYQYVKKRSRIYLEGKIDYGEYMDKNNVRROATTIADNIIIFLSDQT KEKE
22	1prtF	Pertussis toxin S5 subunit (<i>Bordetella pertussis</i>)	P04981	MOROAGLPLKANPMHTIASILLVLGYSPADVAGLPTHLKNTFTVQELAKLKGKNEQFCGLTAFMSGRSLV RACLSAGHEHDTWFDTMLGFAISAYALKSRALTVEDSPFGTPTGDLLELOICPLNGYCE
23	1bcpD	Pertussis toxin S5 subunit (ATP bound; <i>Bordetella pertussis</i>)	P0A3R5	MLRRFTRTTAPQGGARRSRVRALAWLLASGAMTHLSPALADVPYVLYKTNMVTSVAM KPEYVFTRMVLCGIAAKLGAASSPDHVPFCFGKDLKRGSSSPMEVMLRAVFMQQRPL RMFLGPKQLTTEGKPALELIIRNVECSGKQDCP

SEQ ID NO:	Protein Data Bank (PDB) accession number	Protein name Organism	UniProt/GenBank accession number	SEQUENCE
24	3chbD	Cholera Toxin (<i>Vibrio cholerae</i>)	D0UTQ9	MTPQNTDLCAYHNTQIHTLNDKIFSYTESLAGKREMAITTFKNGATFQVEVPGSQHIDSOKKAIERMKDT LRIAYLITE
25	1tiid	Heat-labile toxin (<i>Escherichia coli</i>)	P43529	MSFKKIIKAFVIMAAVSVQAHAGASQFFKDCNCRNTASLVEGVELTKYISDINNNTDGM YVVSSTGGVWRISRAKDYDPDVMFTAEMRKIAMAAVLSCMRVNMCPASSPNVIWAIELE AE
26	2bosA	Verotoxin/Shiga toxin, B-pentamer (<i>Escherichia coli</i>)	Q9MBZ7	MKKMFIATLVALFALVSNAMAADCAKGKIEFSKYNEEDNTFTVKVSGREYWTNRWNLPQLLSAQLTGMTVTIIS NTCSSGSGFQVKFN
27	1br9	TIMP-2 (<i>Homo sapiens</i>)	P16035	MGAARTLRALGLLLATLLRPADACSCSPVHPQOAFCNADVIRAKAYSEKEVDSGNDIYGNFIKRIQYE IKQIRMFKGPEKDIIEFIYTAPSSAVCGVSLDVGGRKEYLIAGKAEQDGMHITLQDFIVPDWTLSSTQKKS NHRVQMGCECKITRCPMIPCYISSPDECLMMDWVTEKNINGHQARFFACIKRSDSCAWYRGAAFPKQEFLLD IEDP
28	1an8	Superantigen SPE-C (<i>Streptococcus pyogenes</i>)	Q8NKX2	MKKINIIVFIITVILISTISPIIKSDSKDI SNVKSDDLAYITPYDYKDCRVNFSTHTLIDTQKYR GKDYISSEMSYEASQKFKRDDHVDVFGGLFYLNSHTGEYIGGTPAQNKNVNHKLLGNLFISGESQOQNLN NKIILEKDI VTFQEI DFKIRKYLMDNYKIYDATSPYVSGRIEIGTKDGKHEQIDLPDSPNEGTRSDIFAKYK DNRIINMKNFSHFYILEK
29	3seb	Superantigen SPE (<i>Staphylococcus aureus</i>)	Q5MAA8	ILVISTPNVLAESQDPKDELHKSKFTGLMENMKVLYDDNHVSAINVKSIDQELFYFDLIYSIKDTKLGNY DNVRVEFKNKDLADKYDKYVDVFGANYCYCYFSKKTNDINSHQDKRKTCTMYGCVTEHNGNQLDKYRSIT VRVFEQGNLLSFDVQTNKKKVTQAQLDYLTPRHLYLVKNKLLYEFNNSPYETGYIKFIENENSFWYDMMPPAG DKFDQSKYLMYNDNMVDSKDVKIEVYLITTKK
30	1aw7A	Toxic shock syndrome toxin (<i>Staphylococcus aureus</i>)	A0FIN2	MNKKLMIFFTVSPLLLATIATDFTVPVPLLSNQIIKTAKASTNDNIDKLLDWYSSGSDFTTNSEVLNLSGS MRIKNTDGSISLIIFFSPYYPSPAFTKGEKVDLNTKTKKSQHTSEGYIHFQISGVNTTEKLPTELPKLV KVHGKDSPLKYWPKFKKQLAISTLDLFEIRHQLTQIHGLYRSDTKGYWKITMNDGSTYQSDLSKKFEYNT EKPPINIDEIKTIEAEIN
31	1mjc	Major cold-shock protein, (<i>Escherichia coli</i>)	P0A9Y1	MSGKMTGIVKWFNADKGFGITPPDDGSKDVVFHFSALQNDGYKSLDEGQKYSFTIESGAKGPAAGNVTSL
32	1bkb	Initiation translation factor a (<i>Pyrobaculum aerophilum</i>)	P56635	MVLKWMSTKYVEAGELKEGSYVVIDGEPCRVVEIEKSKTGKHSKAKARIYAVGVFDGGKRTLSLPVDAQVE VPDIEKFTAQILSVSGDVIQLMDMRDYKTIEVPMKVEEAKGRUAPGAEEVWQILDYKRIIRVK

SEQ ID NO:	Protein Data Bank (PDB) accession number	Protein name Organism	UniProt/GenBank accession number	SEQUENCE
33	1sro	S1 RNA-binding domain of PNPase (<i>Escherichia coli</i>)	P05055	MLNPVIRKFPYQGHVTVLTETGMARQATAAAMVSMDDTAVFTVTVGQKAKPGQDFPFLTVNYQERTYAAAGR IPGSFFRREGRPSEGETLIARLIDRPIRPLFPEGVNEQVIATVSVNPNQNPDIAMIGASAAALSLGIP FNGPIGAARVGYINDQVNLPTQDELKESKLDLVAGTEAAVLMVESQAQLLQMLGAVFGEHQQQVVI QINELVKEAGKPEWDQPEPVNEALNARVAALAEARLSDAYRITDKQERYAQVDVVKSETIATLLAEDETL DENLGEILHAIENKVVSRVLAGEPRIDGREKDMIRGLDVRTGLVPRTHGSALFTRGETQALVTATLTGTAR DAQVLDELGERTDTFLFHYNFPFYSVGETGMVSGPKRREIGHRLAKRGVLAVMPDMDFKFFTVRVVSEIT ESNGSSMASVCGASLALMDAGVPIKAAVAGIAMGLVREGDNYVVLSDILGDEHLGDMDFKVGASRDGISA LQMDIKIEGITKEIMQVALNOAKARHLILGVMEQAINAPRGDISEFAPRIHTIKINPKIKDVIKGGSVI RALTEETGTTIEIEDDGTVKIAATDGEKAKHARRIEETAEIEVGRVTGKTRIVDFGAFVAIGGKEGL VHISQIADRRVEKVTDYLOMGQEVFVKVLEVDROGRIRLSIKEATEQSQPAAPEAPAAEQGE
34	1d7qa	Initiation translation factor eIF4 (<i>Homo sapiens</i>)	P47813	MPKNGKGGKNNRRRKNENESEKRELVEKEDGQYQAQVTKMLNGRLEAMCFDGVKRLCHIRGLRKKVWIN TSDIILVGLRDYQDNKADVILKYNADARSCLKAYGELPEHAKINETDTFGPDDDEIQFDDIGDDEDDDDI
35	1ah9	Initiation translation factor IF (<i>Escherichia coli</i>)	P69224	MAKEDNIEMQGTVLETLPTMTFRVELENGHVVTAAHISGMRKNYIRILTGDKVTVELTPYDLSKGRIVFRSR
36	1ckmA	RNA guanylyltransferase (Chlorella virus, PBCV-1)	Q84424	MVPTINTGKNIITERRAVLTNLGLQIKLHKVVGESRDDIVAKMKDLAMDHDKFPRLPGPNPVSIERKDFEKL KQNKVVSEKTDGIRFMMFTTRVFGKVCITIIDRAMTVLLPFKNIPRVLFQGSIFDGLCVDIVKKEKFAFV LFDVAVVSGVTVSQMDLASRFFAMKRSLEKFNVPEDPADILRYKEWIPLEHPTIKDHLKKAIAIYHTDGLI IMSDPEPVIYGRNENFLFKLPGTHHTIDFTIMSEDGTIGIFDPLNLRKNVPVGLDGYYNKGSIVECGFADGT WKYIOGRSDKNQANDRLTYEKTLLNIEENITIDELDLFEWE
37	1a0i	ATP-dependent DNA ligase, (Bacteriophage T7)	P00969	MMNKTNPFKAVSFVESAIKKALDNAGYLIAEIKYDGVRCNIVDNTANSYWLRSVSKTIPALEHLNGFDVR WKRINDDDRCFYKDGFMLDGELMVKGVDFTGTGGLLRTKWTDTNQEFHEELFVEPIRKKDKVPFKLHTGHL HIKLYAILPLHIVESGEDCDVMTLLMQEHVKNMPLLLQYFPEIWEQAESYEVYDMVLOQLYEQKRAEGH EGLIVKDPMCIIYKRGKKSGWKKMPENEAAGLIQGLVWGFKGLANEGKVGIGFVLLLESGLVFNATNISRALM DEFTETVKEATLSQWGFSPYIGIGNDACTINPYDGWACQISYMEETPDGSLRHPFVMEFRGTEDNPNQEKM
38	1snc	Staphylococcal nuclease, (<i>Staphylococcus aureus</i>)	gi1224650	RLILKLTKKREVLNMTYLLSAGICMAIVSILLIGMAISNVSKGQYAKRFFFTFATSLVTLVVVSSLS SANASQTDNGVNRSGSEDPVYSATSTKKLHKPATLIIKALDGDVTVKMYKGQPMTEFRLLLVDTPETKHP KKGVEKYGPASAFTKMKMVENAKIEVEFDKQRTDKYGRGLAYIYADGRMVNEALVRQGLAKVAYVYKP NNTHEQHRLRSEAQAQKKEKLNWSEDNADSGQ
39	1hjp	DNA helicase RuvA subunit N- terminal domain (<i>Escherichia coli</i>)	P0A811	MIGRLRGIIITEKOPPLIVLIEVGGVGYEVHMPMTCTFYPELAGQEAIVFTHFVVREDQAQLLYGENNKQERTLF KELIITNGVGFKLALAILSGMSAQQFVNAVEREEVGAALVKLPKGKKTARLIVEMKDRFKGLHGLDFTFAA DLVLTSPASPATDDAEQAEVAALVAIKYKQEAASRMVSKTARPDASSETTLIREALRAAL

SEQ ID No:	Protein Data Bank (PDB) accession number	Protein name Organism	UniProt/GenBank accession number	SEQUENCE
20	1otcB	Telomere-end binding protein (<i>Oxytricha nova</i>)	P16458	MSKGASAPQOQSAFKQLYTELFNNEGDFSKVSSNLKPKCYKESYPHELVTGCGFFVAPYFTKEAVNEFH AKFPNNIVDLTDKIVIVINWSLELRVNSAEVFTSYANLEARLIVHSFENLQERLNPTRYPVNLFRDDEF KTTIOHFRHTALQAAINKTVKGDNLVDISKVADAAKGGKGVDAIVKASASKGDFSDFSKEGNTATLKIA DIFVQKGGKDALNKAADHTDCAKVKGGAKGKGAATAKAGKKL
40	1pfsA	Gene V protein (<i>Pseudomonas bacteriophage pf3</i>)	P03672	<u>MNIQITFTDTSVRQGTSAKGNPYTFQEGFLHLEDKPEPLQCFVVESVIPAGSYQVYPYRINNVNNGRPELAFDE</u> <u>KAMKRA</u>
41	1gvp	Gene V protein (Filamentous bacteriophage f1, M13)	D0U157	MIKVEIKPSQAQFTTRSGVSRQGPYSLNEQLCYVDLGNYPVLVKITLDEGQPAYAPGLYTVHLSSFKIGQ EGSLMIDRLRLVPAK
42	1gpc	Gene protein gp32 core (Bacteriophage T4)	B31YU0	MFKRKSTAEALAAQMAKLNKNGKGFSSDKGEWKLKLDNAGNGQAVIRFLPSKNDEQAPFALLVNHGFKKNGKW YIETCSSTHGDIYDSCPCQYISKNLDLYNTDNKEYSLVKRRTSYWANILVVKDDPAAPENEGVKFYRFGKKIW DKINAMIAVDVEMGETPVDVTCPEWEGANFVLKVKQVSGFSNYDESKFLNQSAIPNIDDESQKELFEQWVDL SEMTSKDKFKSFEELNTKFGQVMGTAVMGAAATAAKKADKVADDLDLAFNVDDFNKTEDDFMSSSSGSSSS ADDT
43	2prd	Inorganic pyrophosphatase (<i>Thermus thermophilus</i>)	Q72H95	MANLKSLPVGDKAPEVVHVMVIEVPRGSGNKYEYDPLGAIKLDRLVLPGAQFYPGDYGFIPSTLAEDGDPDLDG LVLTSTYPLLPGVVVEVRVVGILLMEDEKGGDAKVIGVVAEDQRLDHIQIDIGDVPEGVKQEIQHFFETYYKALE <u>AKKGKWKVTGWRDRKAALAEVVRACIARYKG</u>

Table 3.

Table 3 shows OB-fold protein sequences. The OB-fold domain sequence is underlined. Sequence differences between PDB and UniProt/GenBank data-bases are indicated in bold.

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CLAIMS

1. A reagentless biosensor for a target, comprising at least:
an OB-fold protein;
a cysteine residue coupled to a fluorophore.
2. The biosensor of claim 1, wherein the OB-fold protein is derived from a starting OB-fold protein by 5 to 32, preferably 8 to 20, more preferably 11 to 16 substitutions at positions of the residues of the binding interface of said starting OB-fold protein with its native ligand, possibly combined with the deletion of 1 to 4 residues and/or the insertion of 1 to 50 residues.
3. The biosensor of claim 2, wherein the residues which are substituted are selected amongst those of said OB-fold protein sequence which correspond to V2, K3, K5, K7, Y8, K9, G10, E14, T17, K21, K22, W24, V26, G27, K28, M29, S31, T33, D36, N37, G38, K39, T40, R42, A44, S46, E47, K48, D49, A50 and P51 of SEQ ID NO: 1.
4. The biosensor of claim 3, which comprises an OB-fold protein which is isolated from a combinatorial library obtained by the randomization of 11, 12, 13, 14, 15 or 16 residues selected amongst those of said OB-fold protein sequence which correspond to K7, Y8, K9, K21, K22, W24, V26, K28, M29, S31, T33, K39, T40, R42, A44 and S46 of SEQ ID NO: 1.
5. The biosensor of claim 4, wherein the randomized residues comprise at least those of said OB-fold protein sequence which correspond to K7, Y8, K9, W24, V26, M29, S31, T33, R42, A44 and S46 of SEQ ID NO: 1.
6. The biosensor of claim 5, wherein one, two or three additional residues selected amongst those of said OB-fold protein sequence which correspond to K21, K22 and T40 of SEQ ID NO: 1 are also randomized.
7. The biosensor of any one of claims 2 to 6, wherein said OB-fold protein comprises the insertion of 1 to 15 random amino acid residues in the region which corresponds to the residues in positions 25 to 30 of SEQ ID NO : 1, preferably between the residues corresponding to G27 and K28 of SEQ ID NO : 1, and/or the insertion of 1 to 15 random amino acid residues in the region corresponding to the residues in positions 35 to 40 of SEQ ID NO : 1, preferably between the residues corresponding to N37 and G38 of SEQ ID NO : 1, and/or the insertion of 1 to 20

random amino acid residues in the region corresponding to the residues in positions 7 to 12 of SEQ ID NO : 1, preferably between the residues corresponding to K9 and G10 of SEQ ID NO : 1.

8. The biosensor of any one of claims 2 to 7, wherein said OB-fold
5 protein comprises the insertion of 1 to 4 amino acid residues selected amongst those which correspond to A59, R60, A61 and E64 of SEQ ID NO : 1.

9. The biosensor of claim 1, wherein said OB-fold protein comprises a sequence selected from the group consisting of SEQ ID NO: 1 to 45.

10. The biosensor of any one of claims 2 to 8, wherein the OB-fold
10 protein is derived from a starting OB-fold protein comprising a sequence selected from the group consisting of SEQ ID NO: 1 to 45.

11. The biosensor of claim 10, wherein said OB-fold protein is selected from the group consisting of the sequence :
SVKVKFX₁X₂X₃GEEKEVDTSKIRDVCRQGKNVKFLYNDNGKYGAGX₄VX₅EK
15 DAPKELLDMLARAEREKK (SEQ ID NO: 46), wherein X₁ to X₅ are, independently from each other, any amino acids, and the variants derived from SEQ ID NO: 46 by 1 to 15, more particularly one, two, three, four, five or six mutations or deletions in positions selected in the group consisting of positions 1-6, 11-20, 23, 25, 27-28, 30, 32, 36-39, 41, 43, 45 and 47-66; and wherein said biosensor is specific for human IgG.

20 12. The biosensor of claim 10 or claim 11, wherein said OB-fold protein comprises any of SEQ ID NO: 47 to 63.

13. The biosensor of any one of claims 1 to 12, wherein said OB-fold protein is coupled to a second protein moiety.

14. The biosensor of any one of claims 1 to 13, wherein said
25 cysteine is either present in the OB-fold protein or substituted for another suitable residue.

15. The biosensor of claim 14, wherein said cysteine or substituted residue have a solvent accessible surface area (ASA) which is modified when said OB-fold protein binds to the target but are not in direct contact with said target.

30 16. The biosensor of claim 14, wherein said cysteine or substituted residue are located in proximity or are in spatial proximity with a residue which is functionally important for interaction of said OB-fold protein with said target.

17. The biosensor of any one of claims 1 to 16, wherein said fluorophore is coupled to one residue of said OB-fold protein which is selected from the residues which correspond to V2, K3, K5, K7, Y8, K9, G10, E14, T17, K21, K22, W24, V26, G27, K28, M29, S31, T33, D36, N37, G38, K39, T40, R42, A44, S46, E47, K48, D49, A50 and P51 of SEQ ID NO: 1, the residue being changed to cysteine residue if it is not already a cysteine residue.

18. The biosensor of claim 17, wherein said fluorophore is coupled to one residue of said OB-fold protein which is selected from the residues which correspond to K7, Y8, K9, K21, K22, W24, V26, M29, S31, T33, T40, R42, A44 and S46 of SEQ ID NO: 1.

19. The biosensor of claim 17, wherein said fluorophore is coupled to one residue of said OB-fold protein which corresponds to K39 of SEQ ID NO: 1.

20. The biosensor of any one of claims 1 to 19, wherein said fluorophore is selected from the group consisting of: 6-acryloyl-2-dimethylaminophthalene, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole, 5-iodoacetamidofluorescein, (N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole or a fluorophore having an aliphatic chain of 1 to 6 carbon atoms.

21. The biosensor of any one of claims 1 to 20, wherein said target is selected from the group consisting of: a peptide, a protein, an oligosaccharide, a lipid, a lipopeptide, a carbohydrate, a single-stranded DNA, a double-stranded DNA, a RNA, an hapten, a vitamin or a metallic ion.

22. A protein-based chip, characterized in that it consists of a solid support on which at least one biosensor as claimed in any one of claims 1 to 21 is immobilized.

23. An optical fibre comprising at a first end thereof at least one biosensor as claimed in any one of claims 1 to 21 and comprising at a second end thereof means to attach the optical fibre to a device configured to receive and interpret the output of the at least one biosensor.

24. The use of a biosensor as claimed in any one of claims 1 to 23 for screening libraries of molecules, sorting molecules, sorting cells, producing protein-based chips, or detecting, assaying or locating a target.

25. A method for producing reagentless biosensors which comprise at least an OB-fold protein and are specific for a target, as claimed in any one of claims 1 to 21, characterized in that it comprises the following steps:

- (a) substituting each of the variable residues of said OB-fold protein,
5 individually with a cysteine residue, thereby obtaining a set of cysteine mutants,
- (b) coupling said cysteine residue of each of the cysteine mutants of step (a) to a fluorophore, thereby obtaining biosensors,
- (c) purifying the biosensors of step (b), and
- (d) measuring the equilibrium constant (K_D) between each of said
10 purified biosensors of step (c) and said target, or the dissociation (K_{off}) and association (k_{on}) rate constants for each of said biosensors of step (c) and said target; and measuring the fluorescence variation of each of said biosensors of step (c) between a free and target bound state; and
- (f) determining the sensitivity (s) and/or relative sensitivity (s_r) of
15 each of said biosensors of step (c) from the measurements of step (d).

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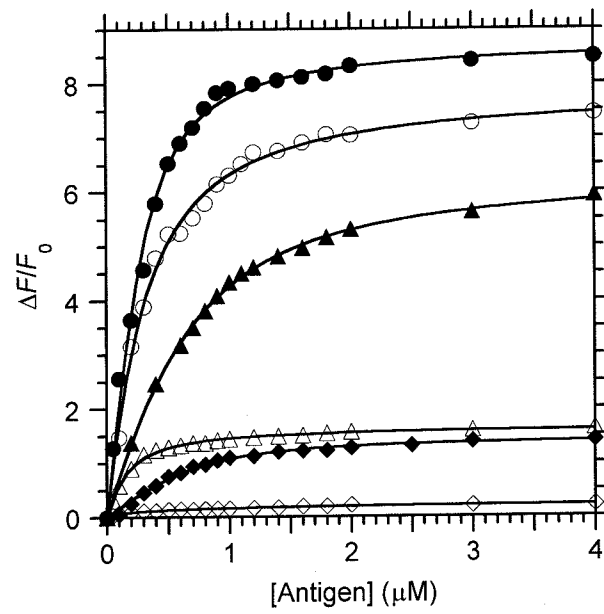


FIGURE 1

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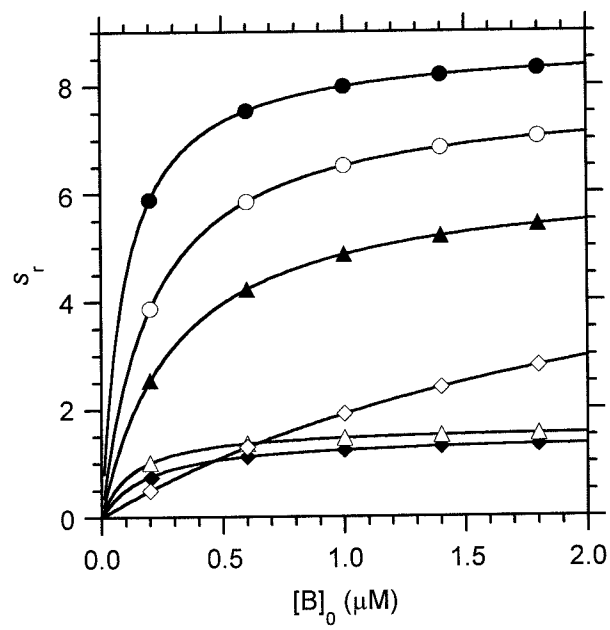


FIGURE 2

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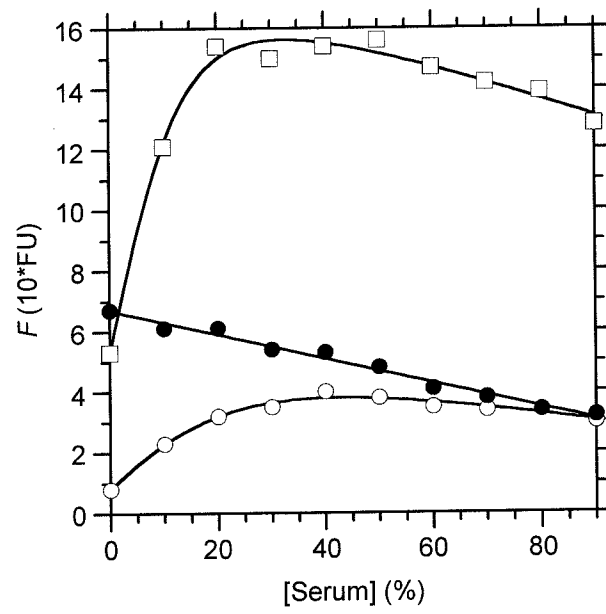


FIGURE 3

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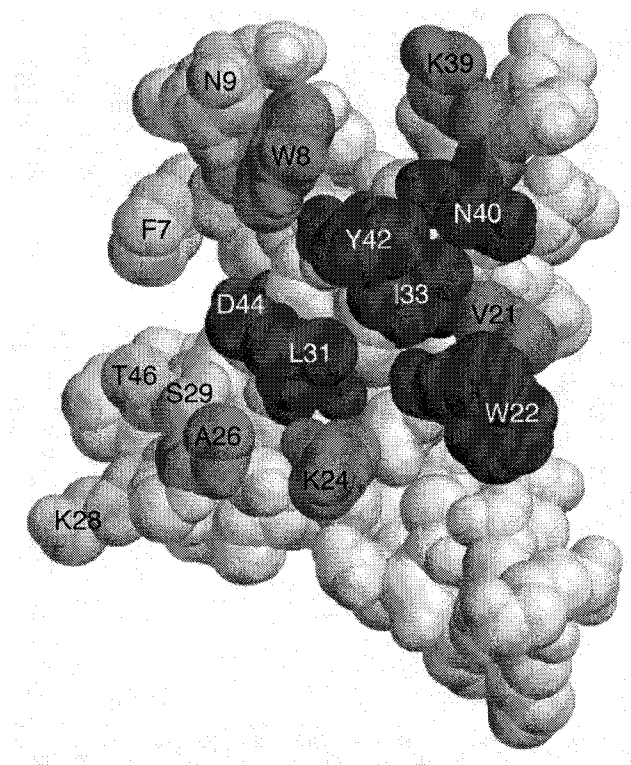


FIGURE 4

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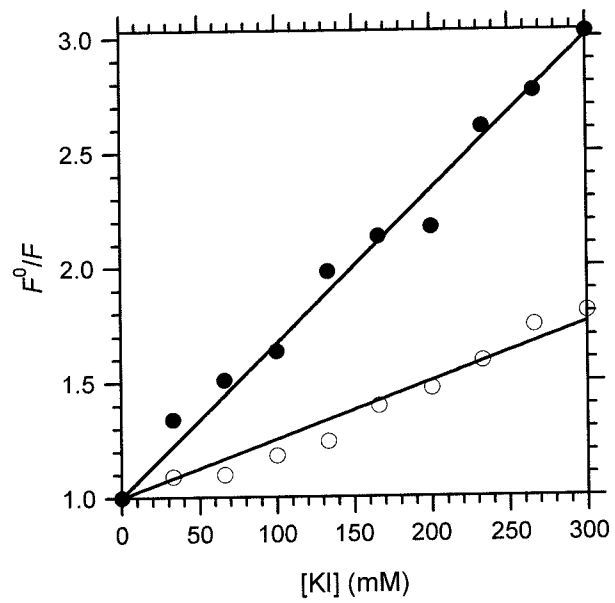


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/055853

A. CLASSIFICATION OF SUBJECT MATTER INV. G01N33/533 G01N33/542 C07K14/195 C12N15/10 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) G01N C07K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BEILSTEIN Data, BIOSIS, CHEM ABS Data, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 01/65258 A1 (PASTEUR INSTITUT [FR]; CENTRE NAT RECH SCIENT [FR]; RENARD MARTIAL [FR]) 7 September 2001 (2001-09-07) cited in the application the whole document	1-25
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-/--		
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
17 April 2012	08/05/2012	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Cervigni, S	

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X	----- B. MOURATOU ET AL: "Remodeling a DNA-binding protein as a specific in vivo inhibitor of bacterial secretin PulD", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 104, no. 46, 13 November 2007 (2007-11-13), pages 17983-17988, XP055000032, ISSN: 0027-8424, DOI: 10.1073/pnas.0702963104 cited in the application the whole document -----	1-25

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