Chemical Synthesis of Triple-Labelled Three-Helix Bundle Binding Proteins for Specific Fluorescent Detection of Unlabelled Protein

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Site-specifically triple-labelled three-helix bundle affinity proteins (affibody molecules) have been produced by total chemical synthesis. The 58 aa affinity proteins were assembled on an automated peptide synthesizer, followed by manual on-resin incorporation of three different reporter groups. An orthogonal protection strategy was developed for the site-specific introduction of 5-(2-aminethylamino)-1-naphthalenesulfonic acid (EDANS) and 6-(7-nitrobenzofurazan-4-ylamino)-hexanoic acid (NBDX), constituting a donor/acceptor pair for fluorescence resonance energy transfer (FRET), and a biotin moiety, used for surface immobilization. Circular dichroism and biosensor studies of the synthetic proteins and their recombinant counterparts revealed that the synthetic proteins were folded and retained their binding specificities. The biotin-conjugated protein could be immobilized onto a streptavidin surface without loss of activity. The synthetic, doubly fluorescent-labelled affinity proteins were shown to function as fluorescent biosensors in an assay for the specific detection of unlabelled human IgG and IgA.

Introduction

The successful application of DNA microarrays to gene expression analysis has stimulated the development of similar array-based technologies for the global analysis of protein expression. Although still limited in terms of capacity and sensitivity, protein-capture microarrays have emerged as a promising tool for protein analysis in drug discovery, diagnostics and biological research in the last few years.[1] One of the main bottlenecks in the development of high-density protein microarrays is the large-scale generation of capture agents of high quality, with high affinity for their target and minimal cross-reactivity with other proteins. Although efforts are directed towards the production of antibodies to the human protein repertoire,[2,3] another strategy is to construct protein-capture arrays by using artificial affinity proteins selected from nonimmunoglobulin combinatorial protein libraries obtained by high-throughput in vitro methods, such as phage display.[4] One such class of artificial affinity proteins, which during the past ten years has been extensively studied and proven valuable in various biotechnological applications, comprises the "affibody" molecules. They are based on the 58 aa scaffold of the Z domain derived from staphylococcal protein A, and highly specific binders have been selected by phage display from a library generated by randomization of 13 amino acids in the protein.[5,6] As the resulting affibody molecules are attractive alternatives to antibodies in applications such as affinity chromatography, ELISA and Western blotting,[7–9] it is anticipated that affibody molecules will work equally well, or better, than antibodies in most assays based on affinity capture, including protein arrays.

A key issue in microarray-based methods, as well as in traditional immunoassays and biosensor applications, is the detection of the protein bound by the specific capture agent. As labelling of the protein sample prior to analysis often is associated with problems such as poor reproducibility, a label-free technology combining the binding event with the signaling in the form of a biosensor appears particularly attractive. A number of reagentless fluorescent biosensors,[10] in which the fluorescent properties of the binding molecule are altered in response to binding of the analyte, have been developed, and it has recently been suggested that this type of binding molecule could find applications in the development of protein microarrays. [11–13] Fluorescent biosensors can be composed of a binding molecule, such as an antibody or enzyme, derivatized with a single fluorescent probe, which is sensitive to changes in the local environment.[14-16] In other biosensors the binding molecule is labelled with two fluorophores.[17–19] In these molecules, the basis for signalling is that the interaction between the two fluorescent probes is altered upon ligand binding, either through changes in the conformation or in the local environment. A special case of using two fluorophores in a biosensor is to choose a pair suitable for fluorescence resonance energy transfer (FRET), which is a mechanism for nonradiative transfer of energy between an excited donor fluorophore and a proximal acceptor fluorophore.[20] As the efficiency of energy transfer is highly dependent on the distance between the two fluorescent probes, the mechanism of such a biosensor will in most cases be that binding of the analyte leads to a conformational change in the binding molecule that alters the distance

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between the probes. This can be monitored by a shift in the relative emission of the two fluorophores.[21,22] Since correct positioning of the fluorophores is crucial for the function of this type of biosensor, great emphasis has to be made on the design of the biosensor and on the site-specific labelling.

In order to introduce a fluorophore into a binding protein, a variety of fluorescent probes are available for covalent coupling to the functional groups of surface-exposed amino acid residues. However, it is generally difficult to predict which residues will be labelled, as the local environment affects the reactivity of the individual functional groups to a large extent. An alternative method for site-specific fluorescent labelling of proteins is their total chemical synthesis by using an orthogonal protection scheme. Peptides and small proteins are readily prepared by solid-phase peptide synthesis (SPPS), and several commonly used fluorophores are compatible with the conditions for on-resin incorporation and final cleavage (for molecular probes see: http://www.probes.com).[24] Chemical synthesis gives a product with a fluorophore incorporated at a defined position, but only peptides and smaller proteins can be synthesized by SPPS in reasonable yields. Although larger proteins can be prepared by ligation of peptide fragments,[25,26] this method requires considerably more synthetic effort and has not been demonstrated for proteins as large as antibodies.

In a previous study, the B domain of the immunoglobulin binding staphylococcal protein A, was labelled with two different fluorescent probes for intramolecular FRET to monitor the binding of the Fc region of immunoglobulin G.[27] In the presence of this specific ligand, the donor emission increased and the acceptor emission decreased, whereas the presence of a control protein did not affect the emission ratio; this shows that the system can be used for specific detection of unlabelled protein. As affibody molecules are based on the Z domain, which is a synthetic version of the B domain, it was suggested that this was a valid model of the interaction between an affibody and its protein ligand. In this study, a single cysteine residue was introduced into the B domain by site-directed mutagenesis. The recombinant protein was then labelled at this cysteine residue with a donor fluorophore and at the N terminus with an acceptor fluorophore, thus positioning the fluorophores on opposite sides of the binding surface. To achieve site-specific labelling of the N terminus, the reaction was carried out at low pH, which favours labelling of the N-terminal α-amino group instead of the side-chain ε-amino groups, but as absolute selectivity was not possible, the correct product had to be separated from side products by RP-HPLC. In a follow-up of this study, the concept of dual fluorescent labelling was further evaluated as a general strategy for protein detection by using affibody molecules that bind other protein ligands. However, it was found that when the same labelling protocol was used for incorporating the fluorophores in an affibody, pH control of the reaction did not provide sufficient selectivity for labelling the N terminus of the protein and it was difficult to purify the doubly labelled protein to homogeneity.

In this study, chemical synthesis was investigated as an alternative method for the production of site-specifically fluorescent-labelled affibody molecules. An orthogonal protection scheme was devised that allows for selective incorporation of a donor fluorophore, an acceptor fluorophore and an affinity handle, which is used for the immobilization of the protein on the surface of a sensor chip. The study includes an investigation of the folded structure of the synthetic proteins and an evaluation of the performance of the fluorescent-labelled proteins in a fluorimetric binding assay for the detection of unlabelled protein.

Results

Chemical synthesis of fluorescent-labelled affibody molecules

To facilitate the preparation of site-specifically doubly fluorescent-labelled affibody molecules, we investigated whether a protocol for solid-phase synthesis on an automated peptide synthesizer followed by manual on-resin incorporation of the fluorescent probes could be developed. In a first attempt, the 58 aa Z domain was synthesized by an Fmoc/*t*Bu (Fmoc = 9-fluorenylmethyloxycarbonyl) strategy under standard conditions. MS analysis of the product revealed the presence of a side product with a mass difference of −18 Da compared to the theoretical value; this suggested that a dehydration side reaction had occurred. In order to localize the site of the side reaction, the peptide was digested with trypsin, and the fragments were analyzed by MS/MS. The side reaction was traced to a tryptic fragment corresponding to residues 1–7, which contains an Asp-Asn dipeptide sequence known to be susceptible to aspartimide formation.[28] When Asp2 was substituted with Glu in subsequent syntheses (Table 1), no dehydration side product was detected; this further corroborated that the side reaction in the original peptide was aspartimide formation.

By using the same strategy, the affibody Z_{IgA} that had been previously selected by phage display as a specific binder of human IgA was synthesized.[29] In the first trial synthesis, the presence of several truncated peptides decreased the yield of the correct product. The critical positions were identified by MS analysis of the truncated peptides, and it was found that incomplete acylation was primarily a problem in the coupling of bulky, trityl-protected amino acid derivatives towards the end of the synthesis. To minimize this problem, selected amino acids were doubly coupled in subsequent syntheses (Table 1).

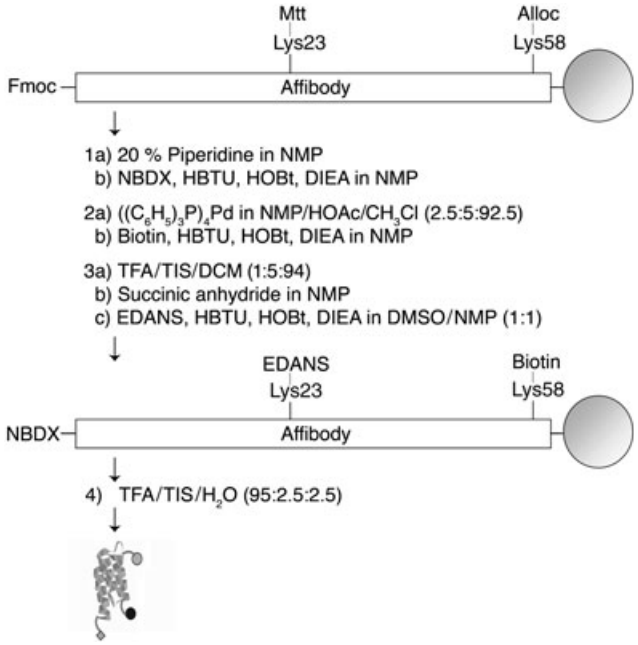
Table 1. Sequences of the synthetic peptides.

Z(D^2E, $N^{23}K$)	VE**N**K**FN**KE**QQN**AF**Y**EI**LH**LPNLK[a]EE**QRN**AFI**Q**SL**K**DDPSQSANLLAEAKKLNDAQAP**K**[b]-resin
Z_{IgA}(D^2E, $N^{23}K$)[c]	VENKF**N**KE**TIQ**A**SQ**EI**RL**LPNLK[a]**GRQKL**AFI**H**SL**L**DDPSQSANLLAEAKKLNDAQAPK[b]-resin

[a] Mtt-protected. [b] Alloc-protected. [c] Originally denoted Z_{IgA1} in ref. [29]. Bold residues were double coupled. Underlined residues correspond to randomized residues in the original affibody library described in ref. [6].

Furthermore, free amino groups remaining after acylation were capped with acetic anhydride to prevent the formation of deletion peptides.

To permit site-specific deprotection and coupling of a reporter group, a substitution was introduced at position 23, in which the original asparagine residue was replaced by lysine (Table 1). An orthogonal protection scheme was devised (Scheme 1), based on the base-labile Fmoc group in combination with the 4-methyltrityl (Mtt) group,[30] which is removed with dilute acid, and the allyloxycarbonyl (Alloc) group,[31] which is removed by Pd^0-catalyzed allyl transfer. Thus, by selective removal of the semipermanent, orthogonal protecting groups Mtt at Lys23, Alloc at Lys58 and Fmoc at the N terminus, three amino groups could sequentially be liberated for site-specific modification. The peptides were functionalized by manual coupling of NBDX to the N^{α}-amino group of Val1 and coupling of EDANS through a succinyl linker to the ε-amino group of Lys23. Lys58(Alloc) was either left intact or the protecting group was cleaved and replaced with a biotin moiety.



Scheme 1. Synthetic scheme for the on-resin incorporation of reporter groups and the final deprotection of the modified peptide. NBDX = 6-(7-nitrobenzofurazan-4-ylamino)-hexanoic acid, HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorphosphate, HOBt = 1-hydroxybenzotriazole, NMP = *N*-methylpyrrolidone, TFA = trifluoroacetic acid, TIS = triisopropylsilane, EDANS = 5-(2-aminethylamino)-1-naphthalenesulfonic acid; DIEA = diisopropylethylamine.

By using this modified synthetic protocol, doubly fluorescent-labelled affibody scaffold Z and affibody Z_{IgA} could be successfully synthesized on solid phase. The experimentally determined molecular mass of the purified products correlated well with the theoretical mass (Table 2). The yield of unlabelled full-length product was estimated from analytical RP-HPLC. A representative chromatogram of sZ_{IgA} is shown in Figure 1. Depending on the character of the peptide, the overall yield ranges between 20–30%, which corresponds to an average yield of 97–98% for each coupling step, well within the expected yield range for a 58 aa peptide. The overall yield of the doubly labelled full-length products varies between 12–17%, which corresponds to a combined yield of approximately 60% for the deprotection of the orthogonal protecting groups and the introduction of the two fluorophores.

Table 2. Characterization of synthetic and recombinant proteins.

Protein	Target protein	$M_{r\,theor.}$	$M_{r\,exp.}$	Helical content [%][a]	K_D [M][b]
$sZ^{(NBDX,EDANS)}$	IgG	7375.6	7375.0	62.3	1.1×10^{-8}
$rZHis_6$	IgG	8389.0	8389.0	52.4	1.8×10^{-8}
$sZ_{IgA}^{(NBDX,EDANS)}$	IgA	7191.6	7191.0	59.5	1.7×10^{-7}
$Z_{IgA}His_6$	IgA	8206.0	8206.0	44.2	2.2×10^{-7}

M_r indicates relative molecular mass. [a] Helical content was determined by circular dichroism. [b] The dissociation constants were determined by SPR biospecific interaction analysis.

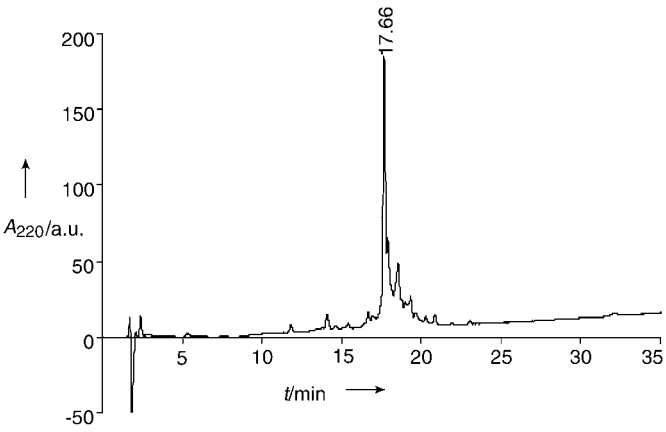


Figure 1. Chromatogram of analytical RP-HPLC (20–60% B in 5–35 min) of crude synthetic Z_{IgA}. The correct product elutes at 17.66 min.

Analysis of the secondary structure by CD spectroscopy

Circular dichroism was used to compare the secondary structure content of the synthetic, fluorescent-labelled proteins with recombinant reference proteins. Recombinant Z and Z_{IgA} were produced as fusion proteins with a C-terminal His_6 tag, to allow for immobilized metal affinity chromatography (IMAC) purification. The purity and correct identity of $rZ\text{-}His_6$ and $rZ_{IgA}\text{-}His_6$ were verified by SDS-PAGE (results not shown) and MS analysis (Table 2). The four proteins $sZ^{NBDX,EDANS}$, $sZ_{IgA}^{NBDX,EDANS}$, $rZ\text{-}His_6$ and $rZ_{IgA}\text{-}His_6$ generated very similar CD spectra (Figure 2) with double minima at 208 and 222 nm, and a maximum between 194 and 196 nm, characteristic of an overall α-helical secondary structure. This correlates well with the known three-helix-bundle structure of the Z domain and a previously studied affibody variant.[32–35] Further analysis of the CD spectra showed that the synthetic, labelled proteins had higher α-helical content than their recombinant counterparts, and that Z was slightly more helical than Z_{IgA} (Table 2).

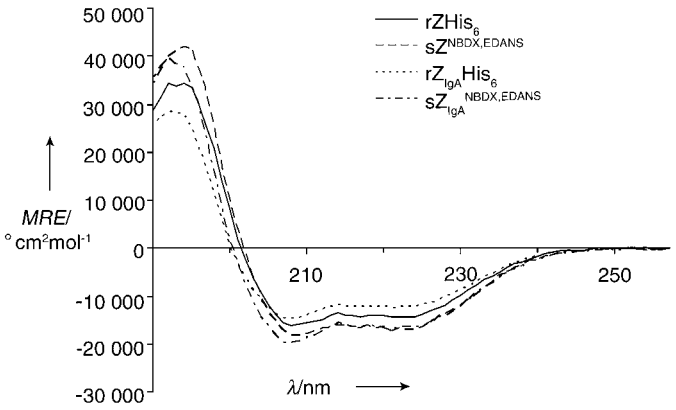


Figure 2. CD spectra showing the mean residue ellipticity (MRE) of the synthetic, fluorescent-labelled proteins $sZ^{NBDX,EDANS}$ and $sZ_{IgA}^{NBDX,EDANS}$ and the corresponding recombinant reference proteins rZ-His_6 and rZ_{IgA}-His_6.

Analysis of the binding kinetics by biospecific interaction analysis

Biosensor studies with the Biacore system based on surface plasmon resonance (SPR) were used to investigate whether the binding affinities of the synthetic, fluorescent-labelled proteins were different from those of the recombinant proteins. It was shown that the dissociation constants (K_d) were similar for the interaction of $sZ^{NBDX,EDANS}$ and rZ-His_6 with IgG, and for the interaction of $sZ_{IgA}^{NBDX,EDANS}$ and rZ_{IgA}-His_6 with IgA (Table 2). The dissociation constants correlate well with those previously reported.[29,36]

Fluorescence spectroscopy of the doubly labelled synthetic proteins

Fluorescence measurements were carried out in order to study the potential of the synthetic, fluorescent-labelled proteins as biosensor reagents for the specific detection of protein. The doubly labelled synthetic proteins were excited at 336 nm and emission from the donor EDANS (480 nm) and the acceptor NBDX (520 nm) was detected by collecting spectra from 400 to 600 nm (Figure 3). Emission spectra were recorded in the presence of increasing concentrations of IgG or IgA, and the ratio of acceptor/donor emission was calculated and normalized with the emission ratio of the labelled protein in the absence of target protein. It was shown that titration with increasing concentrations of target protein generated a shift in the relative emission of the two fluorophores, whereas the presence of the control protein did not affect the emission ratio over the same concentration range (Figure 4). According to the titration curves, the limit of detection (calculated as the mean value of the negative control signals plus the mean value of the standard deviations of the negative control signals, multiplied by 2) was lower than 100 nM for both $sZ^{NBDX,EDANS}$ binding to IgG and for $sZ_{IgA}^{NBDX,EDANS}$ binding to IgA.

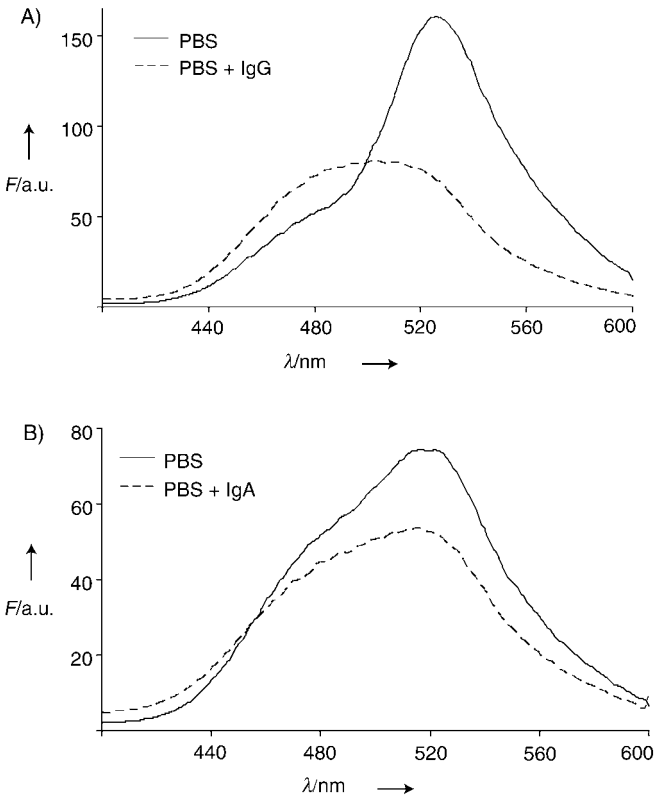


Figure 3. Fluorescence emission spectra showing the relative emission fluorescence (F) in arbitrary units (a.u.) of doubly fluorescent-labelled synthetic protein in the absence and presence of target protein. A) Emission spectra of $sZ^{NBDX,EDANS}$ (4 μM) in PBS, pH 7.4, +/− IgG (1 μM). B) Emission spectra of $sZ_{IgA}^{NBDX,EDANS}$ (4 μM) in PBS, pH 7.4, +/− IgA (1 μM).

Directed immobilization on a streptavidin sensor chip

To verify that a third label could be introduced and be used for immobilization of the protein onto a chip surface without disrupting the binding function, a biotin moiety was coupled to the side chain of Lys58 in Z. By using the high-affinity interaction between streptavidin and biotin, $sZ^{NBDX,EDANS,biotin}$ was immobilized onto a streptavidin-coated sensor chip. Biospecific interaction analysis showed that immobilization had been successful and that specific binding of IgG was retained in the immobilized, biotinylated, doubly labelled, synthetic Z domain (Figure 5).

Discussion

Proteins modified with fluorescent dyes, enzymes and other reporter groups are valuable tools with widespread use in immunology and biochemical research. The preparation of protein conjugates is typically carried out by labelling the protein in solution by using coupling reagents that react with the different functional groups present in proteins, that is, amino, thiol, hydroxyl and carboxyl groups. Although a certain degree of chemical selectivity can be achieved by a careful consideration of the reaction conditions, site-specific labelling of proteins in solution is usually difficult to perform, in particular for the in-

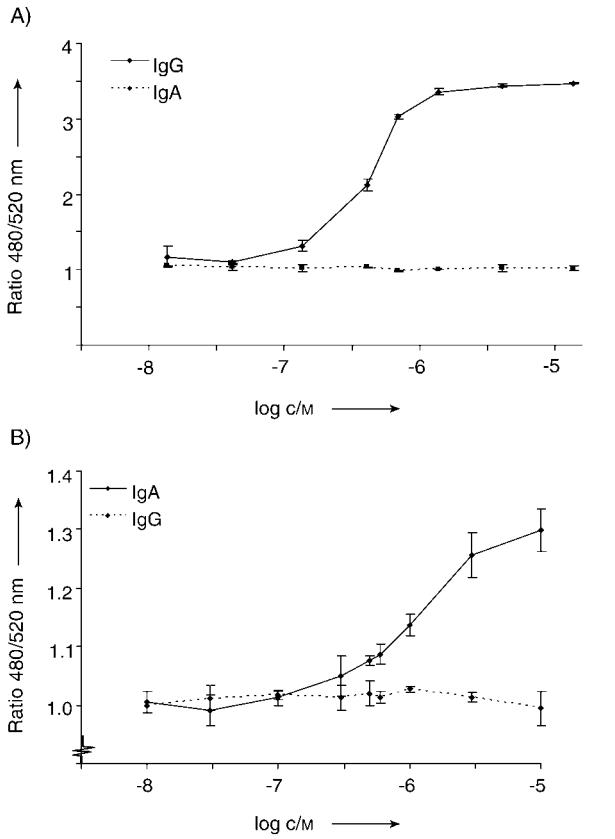


Figure 4. Relative fluorescence emission ratio 480/520 nm for titration of doubly fluorescent-labelled synthetic protein with increasing concentrations of target and control protein. The mean of three independent experiments and the calculated standard deviation are plotted for each concentration. A) Titration of $sZ^{NBDX,EDANS}$ (1 μM) with increasing concentrations of IgG and IgA. B) Titration of $sZ_{IgA}^{NBDX,EDANS}$ (1 μM) with increasing concentrations of IgA and IgG.

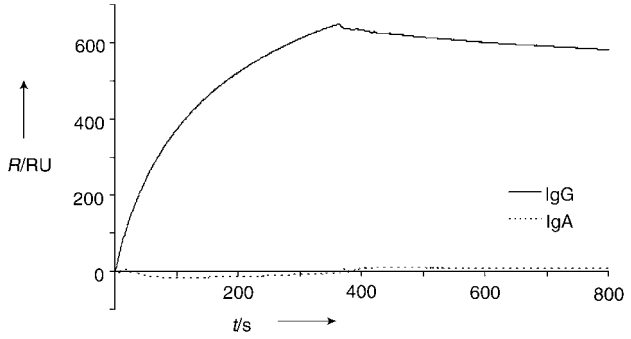


Figure 5. Overlay of sensorgrams showing relative response (R) in response units (RU) obtained from injections with 50 nM IgG and 50 nM IgA solutions over $sZ^{NBDX,EDANS,biotin}$ immobilized on a streptavidin SA sensor chip.

troduction of more than one probe. Here we present a simplified strategy for the preparation of site-specifically labelled protein based on solid-phase peptide synthesis in combination with an orthogonal protection scheme for on-resin introduction of three different reporter groups.

Solid-phase peptide synthesis has over the past 40 years matured to a highly efficient technology and small- to medium-sized peptides (< 60 aa) can now be synthesized in high yield with routine methods.[37] Problems that limit the synthesis of longer peptides are incomplete acylation and deprotection caused by interchain aggregation of the resin-bound peptides.[38] The resulting deletion peptides are also often difficult to separate from the correct product. In the present study, the two 58-residue proteins, Z and Z_{IgA}, were synthesized on solid phase in 20–30% overall yield, with an average yield of 97–98% for each coupling step. Ten residues in Z and nine in Z_{IgA} that were difficult to couple were identified, and by subjecting only these positions to double couplings (Table 1), the synthetic yield could be increased while still keeping both cost and time required for the synthesis at a minimum.

In the synthesis of these proteins, aspartimide formation in Asp2–Asn3 was identified as a major side reaction. As aspartimide formation is base-catalyzed and occurs during the piperidine-mediated deprotection of the Fmoc group, a feasible way to suppress this side reaction would be to synthesize the protein by Boc/benzyl strategy (Boc = *tert*-butyloxycarbonyl). However, for the development of a general procedure for the synthesis of labelled affibody molecules, it was desirable to avoid the harsh conditions of the final HF cleavage used in Boc chemistry, which might be harmful to more sensitive reporter groups. To avoid aspartimide formation while synthesizing the proteins by Fmoc/*t*Bu strategy, Asp2 was substituted with glutamic acid. According to NMR studies of the Z domain,[33] the four N-terminal residues are unstructured, and it was therefore anticipated that any structural changes introduced by the substitution would not influence the overall folded structure of the protein. Also, by choosing glutamic acid, the net charge was kept constant.

One of the main advantages of using solid-phase synthesis instead of recombinant methods for the production of peptides and proteins is that unnatural amino acids and different reporter groups can easily be introduced. In this study, an orthogonal protection scheme was used to enable incorporation of a fluorescent probe at the N terminus and a second fluorophore at position 23. Position 23 is located in the loop connecting helices 1 and 2, outside the randomized binding surface in the affibody library (Table 1).[5,6] As this position is constant in the library, it can be substituted without affecting the binding specificity of an affibody, and modifications at this position should cause little interference with the ligand binding. Furthermore, by using the N terminus and the side chain of amino acid 23 for labelling, the two fluorophores were placed on opposite sides of the binding surface, at a distance of approximately 30 Å, which is suitable for FRET. The fluorophores NBDX and EDANS are compatible with the reaction conditions, as shown by the correct molecular mass of the labelled proteins (Table 2). The protocol is general; this makes it easy to test other fluorescent dyes or to vary the positions for modification.

The B domain is known to be a fast-folding protein that folds without the assistance of chaperones or other cofactors.[39] In a previous study of an affibody produced by chemical synthesis, the synthetic protein was shown to have a helical structure and the same binding specificity as its recombinant

counterpart.[40] However, since the introduction of amino acid substitutions and fluorescent probes might adversely affect the folding of a protein, the structure and function of the synthetic, doubly labelled proteins prepared in this study were studied by CD spectroscopy and biosensor analyses and compared with that of recombinant, unmodified proteins. It was found that the CD spectra were very similar for all proteins analyzed (Figure 2); this suggests that the synthetic affibody molecules fold in the same manner as the recombinant proteins and that the structure is not affected by the amino acid substitutions and the presence of the fluorescent probes. CD spectroscopy showed that the helical content was somewhat higher in the synthetic proteins than in the recombinant proteins; this can be explained by the lack of the C-terminal His_6 purification tag and the N-terminal E′ leader sequence in the synthetic protein. Biosensor studies gave further evidence that the synthetic, doubly labelled proteins were correctly folded and functional, as the affinity for the target protein was essentially the same for the synthetic as for the recombinant proteins (Table 2).

One purpose of the study was to develop fluorescent biosensors for the detection of unlabelled proteins. Fluorescence spectroscopy was therefore used to study the spectral properties of the synthetic proteins $sZ^{NBDX,EDANS}$ and $sZ_{IgA}{}^{NBDX,EDANS}$ and to evaluate their performance in a fluorescence-based binding assay (Figure 3). The emission spectra showed that both fluorophores were functional in the proteins, as demonstrated by the fluorescence emission at 480 (EDANS) and 520 nm (NBDX). In $sZ^{NBDX,EDANS}$, the NBDX emission peak was more dominant than the EDANS emission peak; this suggests that FRET was efficient in the doubly labelled protein. In $sZ_{IgA}{}^{NBDX,EDANS}$, the emission of EDANS was more pronounced than the NBDX emission, which could be due to less efficient FRET in this protein, possibly caused by differences in the local environment of the fluorophores, or structural differences, as suggested by the lower helical content in Z_{IgA} according to the CD spectrum (Table 2).

When fluorescence spectra were recorded in the presence of increasing concentrations of IgG or IgA, it was shown that the relative emission of the two fluorophores was shifted in the presence of the specific ligand, but not by the presence of the control protein. The emission ratio could be plotted to yield a binding curve (Figure 4); this showed that the assay could be used for the detection of unlabelled target protein. The dose-dependent shift in fluorescence emission can be explained by a decrease in the efficiency of FRET between the donor and the acceptor groups, which could be mediated by a conformational change in the labelled protein upon binding to its target, or a change in the local environment of the two fluorophores. When compared to the previous study, in which NBDX/EDANS-labelled recombinant B domain was titrated with increasing concentrations of Fc fragments, $sZ^{NBDX,EDANS}$ titrated with IgG gave a similar titration curve with a slightly higher relative response.[27] This clearly shows that the synthetic strategy gives results comparable to or better than those obtained with recombinant methods. Both the system based on $sZ^{NBDX,EDANS}$ and the affibody $sZ_{IgA}{}^{NBDX,EDANS}$ gave a specific fluorescent signal upon binding to their target proteins, but the limit of detection was higher for the IgA system, probably due to the difference in affinity for the respective ligands (Table 2). In addition, the relative response was lower for the IgA system; this is largely due to the difference in the relative emission of the two fluorophores in the absence of added target protein (Figure 3).

For high-throughput protein analysis, chip-based formats are attractive, and for that reason it was important to ensure that the fluorescent-labelled protein could be successfully site-specifically immobilized on a surface without disruption of activity. In addition to the two fluorophores, a biotin moiety was introduced as an affinity handle, to allow for immobilization onto the streptavidin surface of a sensor chip. Biosensor studies of immobilized $sZ^{NBDX,EDANS,biotin}$ showed that the protein was functional and retained its binding specificity (Figure 5).

Conclusion

In conclusion, an efficient strategy for chemical synthesis of site-specifically labelled affibody molecules has been developed. By using solid-phase peptide synthesis in combination with orthogonal protection chemistry, double fluorescent-labelled Z domain, binding IgG, and the affibody Z_{IgA}, binding IgA, were synthesized. The synthetic proteins were shown to be correctly folded and to bind their specific ligands. Titration of the fluorescent-labelled protein with increasing concentration of protein showed that the relative emission of the fluorescent probes was shifted in the presence of the specific target protein; this suggests that the assay can be used for the detection of unlabelled protein. In addition to the two fluorophores, a biotin moiety could be site-specifically incorporated in the protein and allow for immobilization of the protein onto a streptavidin-coated surface with retention of binding specificity. As the fluorophores and the affinity handle were introduced at nonrandomized positions in the affibody sequence, the concept can easily be extended to include affibody molecules with other binding specificities. Other functional groups or affinity handles can be introduced in the same manner; this makes the synthetic affibody a valuable tool in future applications based on molecular recognition, such as protein microarrays.

Experimental Section

Materials: The Fmoc-protected amino acids were purchased from Applied Biosystems (Warrington, UK) with the side-chain protecting groups: *tert*-butyl (*t*Bu) for Asp, Glu, Ser, Thr and Tyr, *tert*-butyloxycarbonyl (Boc) for Lys and Trp, trityl (Trt) for Asn, Gln and His, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. Fmoc-lysine protected with 4-methyltrityl (Mtt) and allyloxycarbonyl (Alloc) was obtained from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland) and Applied Biosystems, respectively. D-Biotin, DMSO, 6-(7-nitrobenzofurazan-4-ylamino)-hexanoic acid (NBDX), 5-(2-aminethylamino)-1-naphthalenesulfonic acid (EDANS), chloroform, formic acid and tetrakis(triphenylphosphine) palladium(0) $[((C_6H_5)_3P)_4Pd]$ were purchased from Sigma–Aldrich. Triisoproylsilane (TIS), trifluoroacetic acid (TFA) and *tert*-butyl methyl ether were purchased from Merck. Acetic acid was from VWR Interna-

tional. All other chemicals for peptide synthesis were from Applied Biosystems.

General procedure for peptide synthesis: Z(D^2E, $N^{23}K$) and Z_{IgA}(D^2E, $N^{23}K$; Table 1) were synthesized by stepwise solid-phase peptide synthesis using an Fmoc/*t*Bu protection strategy. The syntheses were carried out on a 433 A Peptide Synthesizer (Applied Biosystems, Foster City, CA) with a synthetic protocol based on the *FastMoc*0.1 ΩMonPrevPk chemistry file of the SynthAssist 2.0 software package (Applied Biosystems). The peptides were synthesized on a 0.1 mmole scale using an acid-labile Fmoc amide resin (Applied Biosystems; loading 0.67 mmol g^{-1}). Deprotection of the Fmoc group was carried out with piperidine/NMP (NMP = *N*-methylpyrrolidone; 20%) for 10 min. A tenfold molar excess of amino acid in NMP activated with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorphosphate (HBTU; 0.45 M in NMP), 1-hydroxybenzotriazole (HOBt; 0.45 M in DMF) and diisopropylethylamine (DIEA; 1.6 M in NMP) was used for all couplings. A single coupling of 10 min was used, except for selected residues (Table 1), which were coupled twice. The coupling was followed by capping with acetic anhydride (0.5 M), DIEA (0.125 M) and HOBt (0.015 M) in NMP for 5 min. Standard side-chain protection was used in all positions except in position 23, in which Fmoc-Lys(Mtt)-OH was used, and in position 58, in which Fmoc-Lys(Alloc)-OH was used.

Introduction of chemical modifications: The resin-bound peptides were manually modified by incorporation of fluorescent probes at position 1 and 23. The N-terminal Fmoc group of the full-length peptide was initially cleaved with piperidine/NMP (20%) for 1 min. Deprotection byproducts were then removed, fresh reagent was added and the reaction allowed to proceed for a further 30 min. NBDX (2 equiv), HBTU (2 equiv), HOBt (2 equiv) and DIEA (4 equiv) were dissolved in a small volume of NMP and transferred to the resin for coupling for 45 min. The ε-amino protecting Mtt group at Lys23 was removed by repeated treatment (8×2 min) with TFA/TIS/DCM (1:5:94). Succinic anhydride (10 equiv) in NMP was added to the resin and coupled for 2×30 min. EDANS (4 equiv) was dissolved in a small volume of DMSO with DIEA (4 equiv) and added to the resin, followed by the addition of an equal volume of NMP with HBTU (2 equiv) and HOBt (2 equiv) and allowed to react for 30 min. The reactions were monitored by Kaiser tests.[41] The chemical modifications resulted in doubly labelled synthetic peptides denoted $sZ^{NBDX,EDANS}$ and $sZ_{IgA}{}^{NBDX,EDANS}$, respectively.

In addition to the doubly labelled constructs, a peptide with three different reporter groups was prepared by the incorporation of a biotin moiety in position 58 of sZ(D^2E, N^{23} K). NBDX was attached to the N terminus as described above. The Alloc protection group on Lys58 was removed by treating the resin-bound peptide with $[((C_6H_5)_3P)_4Pd]$ (6 equiv) in NMP/HOAc/$CHCl_3$ (2.5:5:92.5) for 2 h. The reaction was performed under nitrogen on an Applied Biosystems 433 A Peptide Synthesizer. D-Biotin (3 equiv), HBTU (3 equiv), HOBt (3 equiv), and DIEA (6 equiv) were dissolved in a small volume NMP, added manually to the resin-bound peptide and allowed to react for 30 min. Removal of the Mtt group at Lys23 and coupling of succinic anhydride and EDANS was performed as described above to give $sZ^{NBDX,EDANS,biotin}$.

Cleavage and purification of synthetic peptides: The side-chain protecting groups were removed and the peptides were cleaved off the resin in a single step by treatment with TFA/TIS/H_2O (95:2.5:2.5) at room temperature for 2 h. The peptides were extracted in H_2O/*tert*-butyl methyl ether (1:1), followed by filtration and lyophilization of the aqueous phase. The crude peptides were analyzed by analytical RP-HPLC by using a 4.6×150 mm polystyrene/divenylbenzene matrix column with a particle size of 5 μm (Amersham Biosciences), a 30 min gradient of 20–60% B (A: TFA/H_2O (0.1%); B: TFA/CH_3CN (0.1%)) and a flow rate of 1 mL min^{-1}. Eluted fractions were lyophilized and analyzed by MS (see below). The peptides were purified by RP-HPLC using the same column and a 20 min gradient of 30–40% B, followed by verification of the correct product by MS. Stock solutions were prepared by dissolving the peptides in NH_4OAc (5 mM; pH 5.5) and the final concentrations were determined by amino acid analysis (Aminosyraanalyscentralen, Uppsala, Sweden).

Tryptic digestion: Synthetic Z (8 μg) was digested for 19 h at 37 °C with sequencing-grade modified trypsin (0.4 μg; Promega) in NH_4OAc (50 mM; pH 5.5). Tryptic peptides were desalted for MS analysis by washing samples bound to a C-18 ZipTip unit (Millipore) according to the manufacturer's instructions prior to elution with H_2O/CH_3CN (1:1) supplemented with HCOOH (0.1%).

ESI-MS analysis: Mass spectrometric analysis was carried out on a Q-Tof II mass spectrometer fitted with a nano Z spray source (Waters Corporation, Micromass MS Technologies, Manchester, U.K.). External mass calibration was obtained over the *m/z* range of 50–2000 by using a solution of NaI (2 mg mL^{-1}) in propan-2-ol/H_2O (1:1). Intact proteins and tryptic digests were introduced into the mass spectrometer from a solution in H_2O/CH_3CN (1:1) supplemented with HCOOH (0.1%) by using a syringe pump (1–5 pmol μL^{-1}; 300–500 nL min^{-1}). The quadrupole ion filter was operated at a resolution of about $m/z = 1$ during MS/MS experiments, and collision energy was varied depending upon precursor ion resilience. The Maximum Entropy 3 (MaxEnt3) algorithm was used to deconvolute isotopic and charge state information in raw combined MS/MS spectra to aid data interpretation.

Production of recombinant protein: Recombinant Z and Z_{IgA} were expressed from a modified version of the pKN1 vector,[5,29] with N-terminal E′ tag AQHDEA and C-terminal His_6 purification tag SSTHHHHHH. rZ-His_6 was produced in *E. coli* RV308 and rZ_{IgA}-His_6 was produced in *E. coli* RR1ΔM15.[42,43] Protein expression was induced by isopropylthiogalctoside (IPTG; Sigma–Aldrich), and release of the perpiplasmic protein fraction by repeated freeze–thaw cycles was carried out as described previously.[27] The proteins were purified by IMAC by using TALON (Co^{2+}) resin (BD Biosciences Clontech, Palo Alto CA) as described previously.[29] The purity of the proteins was analyzed on a Coomassie Brilliant Blue stained SDS-PAGE gel (20%) by using the Phast system (Amersham Biosciences, Uppsala Sweden). MS was used to verify the correct mass of each protein (see above). The samples were dissolved in NH_4OAc (5 mM; pH 5.5), and the final protein concentrations were determined by amino acid analysis (Aminosyraanalyscentralen, Uppsala, Sweden).

CD spectroscopy: For CD measurements the synthetic and the recombinant proteins (15 μM) were dissolved in K_2HPO_4 (10 mM; pH 7.4). CD spectra were collected from a 0.1 cm quartz cell by using a J-720 spectropolarimeter (JASCO, Tokyo, Japan). Each spectrum represents the mean of three accumulated scans from 260–190 nm.

Biosensor analysis: The binding kinetics of the fluorescent-labelled synthetic proteins and the recombinant proteins were analyzed by real-time biospecific interaction analysis (BIA) based on SPR using a BIAcore 2000 instrument (Biacore, Uppsala, Sweden). Human IgG and IgA were diluted in NaOAc (10 mM; pH 3.8) and immobilized onto a CM5 sensor chip (Biacore AB, Sweden) by using EDC/NHS coupling chemistry (EDC = 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride, NHS = *N*-hydroxysuccinimide) generating a

response of approximately 4000 response units (RU). The binding analyses were carried out at 25 °C with a flow rate of 30 μL min^{-1} and a sample volume of 150 μL. HBS (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P20 (Bicacore AB), pH 7.4) was used as running buffer. Each protein sample was prepared at ten different concentrations (Z_{wt} ranging from 2 nM to 15 μM and Z_{IgA} ranging from 125 nM to 64 μM) by dilution in HBS. Injections were made in duplicate at random order and followed by regeneration with HCl (10 mM). The dissociation constant of the binding interaction was determined by using the BIAevaluation 3.2 software (Biacore AB) and a 1:1 Langmuir model. In a different experiment, biotinylated, synthetic $sZ^{NBDX,EDANS,Biotin}$ was immobilized on a streptavidin-coated SA sensor chip (Biacore AB). IgG (30 μL; 50 nM) was injected at a flow rate of 5 μL min^{-1}. The surface was regenerated with HCl (5 μL, 10 mM) and the procedure was repeated using human IgA as a negative control.

Fluorescence spectroscopy: Spectra from synthetic, doubly fluorescent-labelled $sZ^{NBDX,EDANS}$ and $sZ_{IgA}^{NBDX,EDANS}$ were recorded by using a Perkin-Elmer LS 50B fluorimeter. The proteins were dissolved in phosphate buffered saline (PBS, pH 7.4) at a concentration of 4 μM, and emission spectra were recorded in the presence and absence of target protein (1 μM). Titration curves were obtained by recording emission spectra of the proteins at a concentration of 1 μM with increasing concentrations of target protein. The target proteins used were human IgG (Kabi Pharmacia, Stockholm, Sweden) and human IgA (Sigma–Aldrich), and the concentration was titrated from 10 nM to 14 μM. The excitation wavelength was 337 nm and emission scans were recorded from 400 to 600 nm. The slit width for both excitation and emission was 10 nm.

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