A Peptide-Based, Ratiometric Biosensor Construct for Direct Fluorescence Detection of a Protein Analyte

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We present the design, synthesis, and functional evaluation of peptide-based fluorescent constructs for wavelength-ratiometric biosensing of a protein analyte. The concept was shown using the high-affinity model interaction between the 18 amino acid peptide pTMVP and a recombinant antibody fragment, Fab57P. pTMVP was functionalized in two different positions with 6-bromomethyl-2-(2-furanyl)-3-hydroxychromone, an environmentally sensitive fluorophore with a two-band emission. The equilibrium dissociation constant of the interaction between pTMVP and Fab57P was largely preserved upon labeling. The biosensor ability of the labeled peptide constructs was evaluated in terms of the relative intensity change of the emission bands from the normal (N*) and tautomer (T*) excited-state species of the fluorophore (I_{N*}/I_{T*}) upon binding of Fab57P. When the peptide was labeled in the C terminus, the I_{N*}/I_{T*} ratio changed by 40% upon analyte binding, while labeling close to the residues most important for binding resulted in a construct that completely lacked ratiometric biosensor ability. Integrated biosensor elements for reagentless detection, where peptides and ratiometric fluorophores are combined to ensure robustness in both recognition and signaling, are expected to become an important contribution to the design of future protein quantification assays in immobilized formats.

INTRODUCTION

The availability of sensitive, specific, and robust biomolecular quantification assays is crucial for fundamental characterization of biomolecular interactions as well as for disease diagnostics, environmental monitoring, and quality control in food production. In assay design, strategies for both biospecific recognition and detection have to be carefully considered. Proteins are attractive candidates as recognition elements, since they have evolved to specifically bind a wide variety of targets. In particular, antibodies and antibody fragments are preferred because of their target binding diversity, high affinity, and high specificity. For detection, fluorescence-based strategies are safe, inexpensive, and well-established, and offer a level of sensitivity still unrivalled by other methods. The accessibility of the technique is further expanded through a wide range of commercially available fluorophores that allow for labeling using a variety of coupling chemistries.

Today, many protein quantification systems rely on a traditional immunoassay-type of detection, involving either a multistep sandwich analysis or sample labeling. An interesting alternative is based on the design of so-called molecular (bio)sensors, where the recognition and signaling events have been molecularly integrated by the attachment of one or more fluorescent reporter probe(s) to the recognition element in a fashion that allows the probe(s) to respond to analyte binding (Figure 1) (*1*). The basis of detection is typically fluorescence resonance energy transfer (FRET) (*2, 3*), the formation of a nonfluorescent complex (*4*), fluorescence anisotropy (*5*), or, more relevant to the present work, the change of emission intensity of a single environmentally sensitive fluorophore in the presence of the analyte. Reagentless molecular sensing methods based on singly labeled proteins or peptides and capable of direct analyte detection have mainly been demonstrated for probing of ions and small molecules (*6–9*), but there are also examples where fluorophores have been conjugated to streptococcal protein G (*10*), antibodies (*11*), or synthetic peptides (*12*) for the detection of protein analytes.

The construction of protein-based molecular biosensors, which requires the large-scale production and purification of recombinant proteins and their derivatization with a fluorescent dye, represents a labor-intensive task with unpredictable success. Full-length proteins are susceptible to denaturation, a problem that is especially relevant if the biosensor concept should be applied in a microarray format where the immobilization of sensor molecules would be necessary. Moreover, the only current solution to the problem of site-specific fluorophore labeling of proteins in arbitrary positions is the production of single-cysteine mutants, which is difficult to implement on a general basis, since some proteins contain crucial cysteine residues in the wild-type sequence. Also, side reactions between thiol-reactive fluorophores and lysine side chains have been observed (*13, 14*). Synthetic binders such as peptide or nucleic acid aptamers (*15*) offer a promising alternative to recombinant proteins in molecular biosensor applications. In particular,

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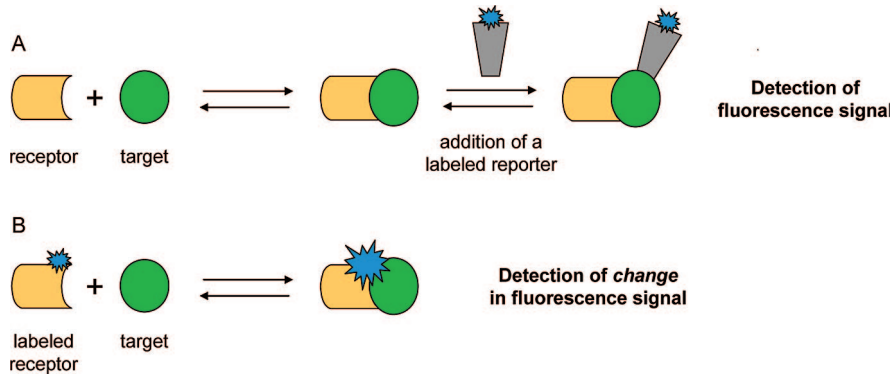

Figure 1. (a) A conventional sandwich assay for protein quantification relies on a labeled reporter molecule for detection. With this multistep approach, the target molecule must provide two distinct binding sites, and separation of free and bound molecules is required after each step. (b) If the recognition and reporting functions can be molecularly integrated, detection can be obtained in a homogeneous assay. This requires a fluorophore the emission properties of which are sensitive to changes in the local environment induced by binding to the target molecule.

synthetic peptides are ideal for molecular biosensor design since they can be straightforwardly produced at a low price, offer functional robustness superior to most proteins, and are well suited for long-term storage in dry, dissolved, and immobilized states (*16*). Also, site-specific functionalization is readily obtained.

In this work, we present the design and functional evaluation of synthetic, singly labeled peptide constructs as a promising alternative to recombinant protein-based conjugates for biosensing of a protein target. The concept was shown with an antibody fragment serving as the analyte. We further provide the first demonstration of the applicability of 3-hydroxychromone (3-HC) derivatives for wavelength-ratiometric biosensing of proteins, as an alternative to conventional dyes, which typically offer changes in absolute steady-state fluorescence intensities upon analyte binding. When relying on relative fluorescence signals for quantification, interpretation problems related to instrument parameters, photobleaching, and concentration variations of the fluorophore between samples can be avoided (*17*).

EXPERIMENTAL SECTION

Peptide Synthesis and Modification. Fluorophores 2-(2-furanyl)-3-hydroxychromone (FC) (*18*) and 6-bromomethyl-2-(2-furanyl)-3-hydroxychromone (BMFC) (*19*) were synthesized as described. The peptides pTMVP, corresponding to amino acids 134−151 of the tobacco mosaic virus coat protein (sequence: H_2N-RGTGSYNRSSFESSSGLV-$CONH_2$), C-pTMVP (H_2N-CRGTGSYNRSSFESSSGLV-$CONH_2$), R134K (H_2N-KGTGSYNRSSFESSSGLV-$CONH_2$), S146C (CH_3CO-KRGTGSYNRSSFECSSGLV-$CONH_2$), and V151C (CH_3CO-KRGTGSYNRSSFESSSGLC-$CONH_2$) were synthesized on a Pioneer automated peptide synthesizer (Applied Biosystems) using standard fluorenylmethoxycarbonyl (Fmoc) chemistry. The synthesis of each peptide was performed on a 0.1 mmol scale using an Fmoc-PAL-PEG-PS polymer (Applied Biosystems). Before each coupling, the Fmoc group was removed by treatment with 20% piperidine in *N,N*-dimethylformamide (DMF). Each amino acid, used at 4-fold excess, was activated with a mixture of *O*-(7-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 0.5 M in DMF; Alexis Biochemicals) and diisopropylethylamine (DIPEA, 1 M in DMF). Coupling times were 60−120 min. In the synthesis of pTMVP, S146C, and V151C, a pseudoproline dipeptide, Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH (Calbiochem-Novabiochem AG), was used in two positions instead of the amino acid form of Ser in order to improve yields.

To allow for site-specific labeling of S146C and V151C, the side chain of Cys was protected by 4-methoxytrityl, which can be selectively removed by treatment with 1% trifluoroacetic acid (TFA). The final N termini of the peptides were capped with 0.3 M acetic anhydride in DMF. Cys was orthogonally deprotected by treatment with TFA, triisopropylsilane (TIS), and dichloromethane (DCM) (1:2:97 v/v), and the process was visually monitored by the yellow color of the detached trityl ion. For fluorophore coupling, the resins were mixed with 1.2 equiv of BMFC and 6 equiv of DIPEA in DMF (6 mL per gram of polymer) and were left at room temperature in the dark with careful swirling for 48−72 h to yield S146C-FC and V151C-FC.

Resins were rinsed in DCM and dried. Peptides were cleaved from the solid phase by treatment with 95% TFA for 2 h at room temperature (TMVP, R134K: TFA/TIS/H_2O 95:2.5:2.5 v/v; C-pTMVP, S146C, V151C: TFA/TIS/H_2O/ethanedithiol 94:2.5:2.5:1 v/v). After filtration, TFA was evaporated and peptides were precipitated by the addition of cold diethyl ether, washed, and lyophilized. They were then purified by reversed-phase HPLC on a semipreparative C-8 column with aqueous acetonitrile (ACN) containing 0.1% TFA as the mobile phase and at a flow rate of 8−10 mL/min. Elution conditions were 20−40% ACN; 40 min gradient (pTMVP), 18% ACN (C-pTMVP and R134K), and 10−50% ACN; 30 min gradient (S146C and V151C). Purified peptides were identified by MALDI-TOF mass spectrometry.

Expression and Purification of Fab57P. Cloning and expression of recombinant Fab57P in *Escherichia coli* was performed as described (*20*) with some modifications. Cells were grown in 30 × 500 mL of LB medium containing 0.3 mM ampicillin. After induction at OD_{600nm} ~0.9 with 1 mM isopropyl thiogalactoside (IPTG; final concentration), Fab57P was expressed at 30 °C for 15 h. Cells were harvested by centrifugation and treated with 0.6 M sucrose in 30 mM Tris-HCl pH 8.0 containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.15 mg/mL lysozyme for 3.5 h on ice. The supernatant (600 mL) was dialyzed twice against HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4) containing 0.2 mM phenylmethanesulfonyl fluoride. Dialyzed samples were filtered (0.22 μm) and stored at 4 °C. Presence of Fab57P was confirmed by Western blotting using a rat anti(mouse κ light chain) antibody conjugated to alkaline phosphatase for revelation.

Fab57P was affinity purified using CNBr-activated Sepharose 4B (Amersham Biosciences) and R134K as the specific capture molecule. R134K (22 mg, 12 μmoles) dissolved in 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (5 mL) was added to 4 mL washed gel and coupling was allowed for 2 h at room temperature. Blocking of unreacted CNBr groups was performed in 0.1 M Tris-HCl pH 8.0 (15 mL) for 2 h at room temperature.

The gel was then washed according to the manufacturer's instructions. The peptide coupling reaction was monitored by dot blotting of coupling and washing solutions, followed by incubation with mAb174P (*21*), which binds to TMVP and pTMVP. For revelation, a secondary goat antimouse antibody conjugated to alkaline phosphatase was used.

Filtered cell extracts containing Fab57P were applied to the gel column following equilibration at pH 7.4. The protein was eluted with 10 mM glycine pH 2.2 in 2 mL fractions. A small amount of 1 M Tris-HCl pH 8.0 was added promptly to the eluate to give a final pH of 7.2−7.5. With this protocol, 90−95% of the protein eluted within the first 8 mL. The purified protein was stored in aliquots at −20 °C until use.

Analysis of Binding Kinetics. Kinetic analysis was performed with a Biacore 2000 instrument (Biacore AB) at 25 °C using using HEPES-buffered saline (HBS-EP: 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4) as the running buffer. S146C-FC and V151C-FC were immobilized in separate flow channels of a CM5 sensor chip at low (90 or 120 RU) or medium (190 or 340 RU) levels. pTMVP was immobilized to yield 10 and 20 RU. Immobilization levels were defined as the baseline difference before and promptly after the injection of the peptide. A reference flow cell without immobilized peptide was included for analysis. A detailed coupling protocol can be found in Supporting Information.

The experimental conditions were checked for mass transport limitations through variation of the analyte flow rate (30, 50, and 70 μL/min) at a constant Fab57P concentration (25 nM). For kinetic analysis, sensorgrams were collected for six analyte concentrations between 1.6 nM and 50 nM, at a flow rate of 50 μL/min. Samples were diluted in HBS-EP. One of the samples was run in duplicate to check the stability of the sensor surface. Regeneration was efficiently achieved with 50 mM HCl (30 s) after each protein injection. Reference subtraction and kinetic evaluation of the sensorgrams were performed with the *BIAevaluation 4.1* software, fitting data from the entire injection phase and the first 100 s of the postinjection (dissociation) phase in each concentration series using the Langmuir bimolecular binding model in a global fit algorithm.

Fluorescence Biosensor Experiments. Fluorescence biosensor experiments were performed in a Safire2 microplate detection system (Tecan Trading AG) using 96-well plates (Corning) at 23 °C. The sample volume in each well was 75 μL. The excitation wavelength was 365 nm and emission was monitored in the range 400−700 nm. Excitation and emission slits were 10 nm. All measurements were done in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4). For each peptide, three samples were measured in duplicate: 5 μM peptide, 5 μM peptide + 5 μM Fab57P, and 5 μM peptide + 2.5 μM goat antirabbit F(ab′)$_2$ (Sigma-Aldrich; product number R9130). F(ab′)$_2$ was used as a negative control. As it contains two Fab units, it was added at half the concentration relative to Fab57P. With V151C-FC, a measurement series of 10 μM peptide, 10 μM peptide + 10 μM Fab57P, and 10 μM peptide + 5 μM F(ab′)$_2$ was performed as well. At the excitation wavelength used, Fab57P and F(ab′)$_2$ did not give rise to any background fluorescence. The ratio of the maximum intensity values of the N* band (at 432 nm) and the T* band (at 520 nm), I_{N*}/I_{T*}, was calculated for each sample and the fractional signal change upon introduction of Fab57P or F(ab′)$_2$ was obtained from

$$C = \frac{|(I_{N*}/I_{T*})_{-\mathrm{Fab}} - (I_{N*}/I_{T*})_{+\mathrm{Fab}}|}{(I_{N*}/I_{T*})_{-\mathrm{Fab}}} \cdot 100\%$$

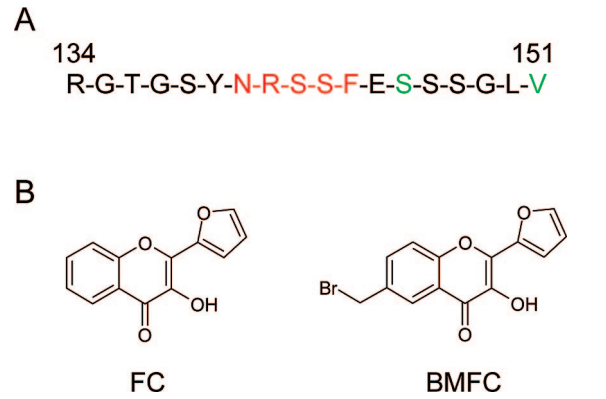

Figure 2. (a) Sequence of pTMVP, corresponding to amino acids 134−151 in TMVP and comprising the residues most important for binding of Fab57P (red). Positions targeted for fluorophore labeling are shown in green. (b) Structure of the ratiometric fluorophore 2-(2-furanyl)-3-hydroxychromone (FC) and its thiol-reactive derivative, 6-bromomethyl-2-(2-furanyl)-3-hydroxychromone (BMFC).

where the subscripts +Fab and −Fab refer to the samples with and without added Fab, respectively.

RESULTS

Design of Molecular Biosensor Constructs. A biomolecular model system with a mapped binding site and well-characterized interaction kinetics was selected to demonstrate the design of a robust molecular biosensor for ratiometric detection of a protein analyte. The 18 kDa four-helix bundle tobacco mosaic virus coat protein (TMVP) is recognized by Fab57P, a recombinant antibody fragment (*20*), and essential residues of the epitope have been mapped to positions 140−144 (*22*). In earlier studies, Fab57P has been found to bind TMVP and a synthetic peptide derivative, pTMVP, with similar affinities (*23*). pTMVP consists of eighteen amino acids, corresponding to TMVP residues 134−151, thus comprising the epitope (Figure 2a). The fact that the affinity of the protein toward Fab57P is retained in the peptide makes pTMVP an ideal recognition element for the further design into a robust molecular biosensor. In order to obtain peptide constructs for ratiometric sensing, the recently developed thiol-reactive dye 6-bromomethyl-2-(2-furanyl)-3-hydroxychromone (BMFC; Figure 2b), which has been successfully used for protein functionalization earlier (*19*), was chosen for labeling of pTMVP. BMFC is a derivative of the 3-HC fluorophore 2-(2-furanyl)-3-hydroxychromone (FC; Figure 2b) (*18, 24*). These dyes undergo excited-state intramolecular proton transfer (ESIPT) between the normal (N*) and a phototautomer (T*) excited-state species, which are both highly emissive and give rise to a two-band fluorescence spectrum. The intensity ratio of the high energy band from the N* state and the low energy band from the T* state, I_{N*}/I_{T*}, is a very sensitive indicator of solvent polarity and hydrogen bonding ability (*25–27*). In aprotic solvents such as acetonitrile, FC exhibits emission predominantly from the T* state, which gives a low value of the I_{N*}/I_{T*} ratio (Figure 3). In protic media, when increasing solvent polarity in the line octanol−ethanol−methanol−water, FC exhibits a gradual increase in the I_{N*}/I_{T*} ratio until almost complete disappearance of the T* emission (Figure 3). The I_{N*}/I_{T*} ratio of an FC derivative attached to the peptide recognition element was expected to be useful as a signal for analyte binding in the molecular biosensor context explored here, where the local probe environment was expected to change upon interaction.

In a design where the biosensor response should be based on spectral changes induced by direct contact of the probe with the analyte upon binding, the position of labeling has to be

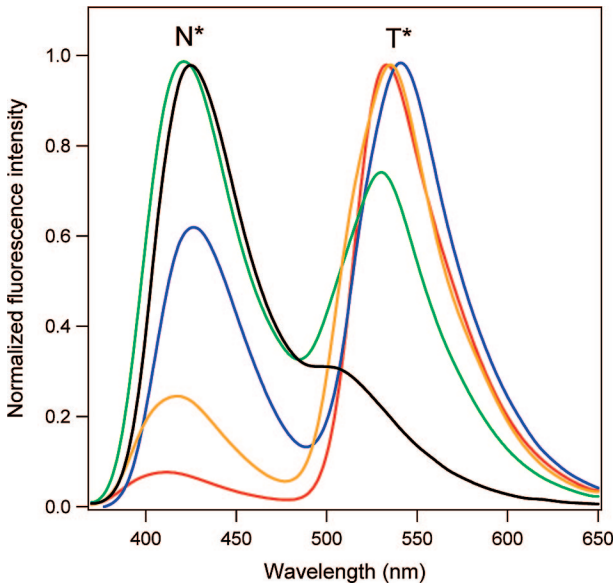

Figure 3. Normalized fluorescence spectra of FC in acetonitrile (red), octanol (orange), ethanol (blue), methanol (green), and water (black). The excitation wavelength was 350 nm. Positions of N* and T* bands are indicated.

chosen close to the binding site. However, it is important that the probe does not interfere with the interaction, thus reducing affinity. Although structural data are helpful in the selection of suitable sites, it is very difficult to rationally predict the optimal position for labeling. Here, in the absence of structural data, two positions were targeted: Ser146 in the immediate vicinity of positions mapped as essential for binding, and Val151 in the peptide C terminus. The numbering of the amino acid positions from TMVP was used also for pTMVP. Labeling in position 146 was assumed not to dramatically reduce the high affinity between Fab57P and pTMVP, as the interaction is tolerant to amino acid substitutions in this position (*22*). Both sites were assumed to allow for spectral changes of the attached probe upon peptide−protein interaction, but the local peptide flexibility was thought to be more pronounced in position 151 than in position 146.

Peptide Synthesis and Functionalization. Two variants of pTMVP were produced, where either Ser146 or Val151 was changed into a cysteine to allow for probe coupling. The peptides S146C and V151C were synthesized on the solid phase. To avoid labeling of the N terminal amine group, the peptide N termini were acetylated. BMFC was introduced after selective deprotection of the thiol at the side chain of Cys146 or Cys151 to form S146C-FC and V151C-FC. The peptides were cleaved from the resin by 95% trifluoroacetic acid to yield a C terminal amide, purified by reversed phase HPLC, and identified from their MALDI-TOF mass spectra. Yields of labeled peptides were very low, possibly due to poor accessibility of the coupling sites caused by the peptide forming secondary structure on the polymer.

Expression, Purification, and Concentration Determination of Fab57P. Fab57P was expressed in *E. coli* and identified by Western blotting. Affinity purification was performed with a lysine-containing derivative of pTMVP, R134K, as the capture molecule. The concentration of native Fab57P in cell extracts and after purification was determined in a surface plasmon resonance (SPR) assay. A pTMVP-derived peptide with an additional N terminal cysteine, C-pTMVP, was synthesized and immobilized to a Biacore CM5 sensor chip at high concentration, and Fab57P samples were introduced in solution. From the slopes of the binding curves, it was found that the production protocol resulted in approximately 0.3 mg native Fab57P per liter of culture. The purification yield was 74%. Experimental details for the concentration determination assay can be found in Supporting Information.

Kinetic Analysis. The association rate constant k_a and the dissociation rate constant k_d describing the interaction between C-pTMVP and nonpurified Fab57P have been determined by SPR (*22*). It was found that $k_a = 7.8 \times 10^5$ M^{-1} s^{-1} and $k_d = 1.4 \times 10^{-3}$ s^{-1}, resulting in a equilibrium dissociation constant $K_d = 1.8$ nM. Here, the kinetic analysis was repeated using immobilized pTMVP and purified Fab57P. The average kinetic parameters were $k_a = 5.8 \times 10^5$ M^{-1} s^{-1} and $k_d = 0.93 \times 10^{-3}$ s^{-1}, resulting in $K_d = 1.6$ nM (Table 1).

The kinetics of the interaction between Fab57P and the functionalized pTMVP derivatives S146C-FC and V151C-FC were analyzed in order to evaluate the effects on the interaction from labeling. The values of the kinetic parameters obtained at two different levels of peptide immobilization are summarized in Table 1. Affinities were only slightly decreased compared to that of the unlabeled peptide. In the case of S146C-FC, labeling in the immediate vicinity of residues important for binding resulted in a 2-fold decrease in the association rate and a 2-fold increase in the dissociation rate (average $K_d = 6.3$ nM). The goodness of fit was assessed by inspection of the residual plots and by relating the average squared residuals, χ^2, to the experimental value of the maximum SPR response ($R_{max,\ exp}$). The fitted curves ran closely to the experimental data and statistical parameters were good with a $\chi^2/R_{max,\ exp}$ ratio lower than 0.05.

In order to show that the equilibrium dissociation constants obtained for the labeled peptides were reasonable and that the peptide molecules were available for binding Fab57P in solution, an SPR experiment was performed where the ability of S146C-FC and V151C-FC to inhibit the interaction between Fab57P and immobilized C-pTMVP was analyzed. Fab57P was mixed with peptides at a range of concentrations and introduced to a sensor surface where C-pTMVP was immobilized at high density. The concentration of unbound Fab57P in the mixture was calculated under the assumption of K_d values obtained from the kinetic analysis and the expected slope of a binding curve from Fab57P at this concentration was compared to the experimental data. Experimental details can be found in Supporting Information. It was found that the inhibition ability of both pTMVP and the labeled peptides corresponded reasonably well with calculated values (Table S-1, Supporting Information). Consequently, the inhibition experiment showed that the kinetic data of the labeled peptides are reliable, and thus justified the conclusion that labeling did not dramatically reduce the affinity of the functionalized peptides toward Fab57P compared to that of pTMVP.

Fluorescence Biosensor Analysis. Fluorescence quantum yields Φ of 3-HC dyes are rather low in water and other polar solvents (*27*). Using quinine sulfate as a reference, it was found that Φ of S146C-FC and V151C-FC in HEPES-buffered saline pH 7.4 was 0.061 and 0.046, respectively (average of two measurements; experimental details can be found in Supporting Information). These values are in very good agreement with what could be expected, considering that Φ of FC in water is 0.05 (*27*). Moreover, they are sufficiently large to record useful fluorescence signals from the peptide constructs in a biosensor analysis.

Incorporating BMFC in a peptide or a protein can change its emission profile substantially. The I_{N*}/I_{T*} ratio of FC in water is >3.2 (Figure 3) but when attached to the αA subunit of bovine α-crystallin the ratio drops below 0.3 (*19*). Such differences could reflect a decrease in the local polarity and hydrogen-bonding ability experienced by the fluorophore due to the

Table 1. Kinetic Parameters Obtained for the Interactions Between Fab57P and TMVP-Derived Peptides[a]

peptide	level of immob.[b] (RU)	$R_{max,\ exp}$[c] (RU)	$R_{max,\ theor}$[d] (RU)	k_a (10^5 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})	K_d (10^{-9} M)	χ^2 (RU^2)	$\chi^2/R_{max,\ exp}$ (RU)
pTMVP	20	323	529	5.94	8.65	1.46	2.93	0.0091
	10	106	265	5.57	9.87	1.77	0.343	0.0032
				5.76	**9.26**	**1.61**		
S146C-FC	190	190	4107	3.72	21.7	5.84	0.640	0.0034
	190	180	4107	3.70	21.6	5.84	0.775	0.0043
	90	15	1945	2.82	19.3	6.83	0.274	0.018
				3.27[e]	**20.5**[e]	**6.27**[e]		
V151C-FC	340	130	7388	4.87	9.08	1.86	3.47	0.027
	340	134	7388	5.01	9.68	1.93	4.95	0.037
	120	45	2608	3.97	11.9	2.99	0.539	0.012
				4.42[e]	**10.49**[e]	**2.37**[e]		

[a] Numbers in bold refer to average values. [b] The level of immobilization was defined as the baseline difference before and promptly after the injection of the peptide. [c] Experimental R_{max} obtained from the global fit. [d] Theoretical R_{max} obtained from the immobilization level and the molecular weight of Fab57P (~50 kDa). [e] Average of the first measurement at the higher immobilization level and the single measurement at the lower immobilization level.

Table 2. Ratiometric Response from Biosensor Constructs upon Binding of Analyte[a]

		I_{N*}/I_{T*}			change in I_{N*}/I_{T*} (%)	
peptide	measurement	peptide	peptide + Fab57P	peptide + $F(ab')_2$	Fab57P	$F(ab')_2$
S146C-FC[b]	1	0.70	0.69	0.69	1.4	1.4
	2	0.73	0.70	0.74	4.1	1.4
		0.72	**0.70**	**0.71**	**2.8**	**1.4**
V151C-FC[b]	1	0.94	0.60	0.92	36	2.1
	2	0.95	0.56	0.86	41	9.5
		0.95	**0.58**	**0.89**	**39**	**5.8**
V151C-FC[c]	1	0.91	0.52	0.91	43	0

[a] Numbers in bold refer to average values. [b] Measurements at 5 μM of peptide. [c] Measurements at 10 μM of peptide.

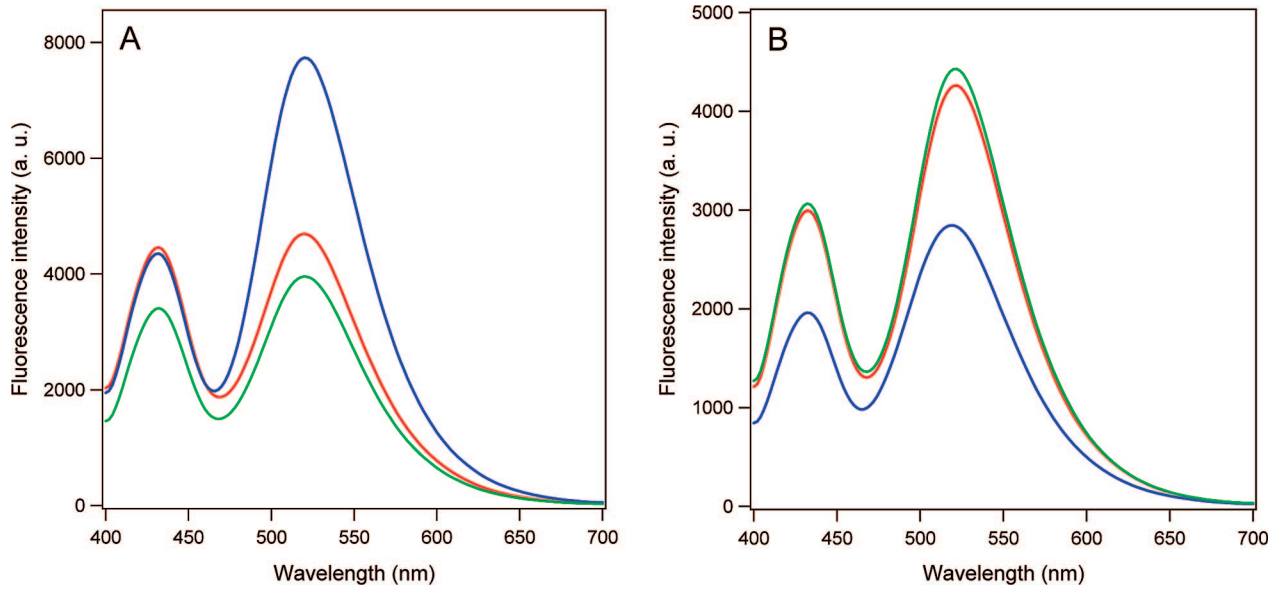

Figure 4. Fluorescence spectra of (a) V151C-FC and (b) S146C-FC in the absence (red) and presence (blue) of saturating amounts of Fab57P. A $F(ab')_2$ fragment with unrelated specificity was used as a negative control (green).

adjacent peptide chain screening it from bulk water. However, attaching BMFC to a polypeptide chain *per se* should not change the emission properties of the fluorophore, since the conjunction is realized through the formation of a thiomethylene group, which should not allow the electronic structure of FC to be modified to any significant extent.

Like in the case of labeling of α-crystallin, the I_{N*}/I_{T*} ratios of S146C-FC (I_{N*}/I_{T*} ~0.7) and V151C-FC (I_{N*}/I_{T*} ~0.9) are dramatically reduced compared to the corresponding value of FC in water (Table 2, Figure 3, Figure 4). The difference in the I_{N*}/I_{T*} value between the two constructs indicates that the choice of labeling site is important, since it can substantially influence the original signal of the molecular biosensor and thus probably the fractional change that can be obtained upon binding of the analyte. The observed spectral difference could reflect the hydrogen-bonding ability of the fluorophore being different in the two positions, as a result of screening from bulk water being less efficient in the case where the probe is attached in the peptide C terminus. However, it is also possible that specific intramolecular interactions between the probe moiety and amino acid residues are responsible for this effect. As confirmed by circular dichroism spectroscopy (data not shown), the peptide constructs do not form any well-defined secondary structure that can aid in determining which of these phenomena are primarily responsible for the spectral differences of S146C-FC and V151C-FC.

For the biosensor evaluation, the fluorescence signal from each peptide construct alone was compared to that where a fraction of >96% of the peptide molecules were bound to Fab57P, according to affinities obtained from the SPR study.

Equimolar amounts of peptide and Fab57P (5 μM or 10 μM) were used. Addition of the analyte to V151C-FC caused a pronounced change in the I_{N*}/I_{T*} ratio, from >0.9 to <0.6 (~40% decrease; Table 2, Figure 4a), without affecting the positions of the N* and T* bands (at 432 and 520 nm, respectively). The most immediate interpretation of this observation is that binding of Fab57P results in a decrease of hydrogen-bonding ability of the probe due to exclusion of water molecules from the interaction site. In contrast, the introduction of a negative control antirabbit $F(ab')_2$ did not have any significant impact on the ratiometric emission profile. V151C-FC thus demonstrates the ratiometric biosensor concept, where the two emission bands are differently affected by the binding of a protein analyte.

In contrast to V151C-FC, S146C-FC did not show any significant change in the I_{N*}/I_{T*} ratio when binding the analyte (Table 2, Figure 4b). This result was rather unexpected taking into account that the fluorophore in S146C-FC is situated very close to the residues most important for binding and should not be less shielded from buffer upon introduction of Fab57P than when attached in the C terminus. It can be speculated that if the spectral differences of the peptide constructs observed in the absence of the analyte can be attributed to specific intramolecular interactions between the peptide and the probe, upon binding of the analyte, these interactions in S146C-FC might be preserved or substituted for similar interactions with residues of Fab57P. However, without any structural data of the binding complex, it is difficult to deduce the detailed biosensor mechanism of V151C-FC and the reason for the insensitivity of S146C-FC to Fab57P binding.

DISCUSSION

Current assays for biomolecular quantification are based on a vast number of different strategies, many of them typically involving a multistep procedure where color reagents or labeled secondary antibodies are used. In contrast, we have focused on the concept of reagentless biosensing, where a single molecular construct integrates recognition specificity and reporting ability (*1*).

The key criterion for a useful fluorescent biosensor construct — attaching the fluorophore in a position where it experiences large environmental differences upon analyte binding but does not interfere with the interaction — was met in a design where the peptide recognition element was labeled seven amino acids away from the residues most important for binding. Interestingly, labeling in the immediate vicinity of the epitope core was not a sufficient condition for the realization of a ratiometric biosensor. The significance of the choice of labeling position when designing a molecular biosensor has also been thoroughly demonstrated on a larger scale by others (*28, 11*). De Lorimier et al. attached eight environmentally sensitive fluorophores in different amino acid positions of eleven bacterial periplasmic binding proteins. The choice of positions for labeling was based on structural considerations and both allosteric and peristeric sites were addressed. A minority of the resulting 320 constructs were judged as being useful for sensing purposes and in only five protein mutants, there was a biosensor signal above the criterion level from all the analyzed fluorophores (*28*). This shows that rational prediction of a labeling site from where a probe will emit a useful signal is very difficult also when structural data is available, and that the choice of fluorophore may critically influence the biosensor performance. The studies referred to above were focused on the labeling of full-length proteins. Our results indicate that finding a suitable labeling position may require some effort also when targeting peptides. However, the number of candidate positions is more limited, which in combination with straightforward peptide synthesis procedures facilitates screening for the optimal site.

Limited reproducibility in biosensor results sometimes observed for protein constructs labeled with conventional single-band fluorophores (*11, 29*) may very well be due to batch-to-batch variations in the degree of labeling and in the extent of photobleaching of the fluorophore. Due to the ratiometric nature of detection, the influence of such factors is minimized in the molecular biosensor design reported here. Also, ratiometric signaling will be highly interesting in future microarray applications with immobilized biosensor constructs, since the surface density of immobilized molecules is generally more difficult to control than the concentration in corresponding solution assays.

A challenging issue for the implementation of synthetic molecular biosensors on a more general level is related to binding specificity. Antibodies are highly attractive providers of specific binding, since their recognition ability is extremely versatile with respect to the nature of the analyte. In order to constitute a competitive alternative, synthetic binders displaying high-affinity, specific interactions toward virtually any target are needed. Examples of such classes of molecules are nucleic acid aptamers selected from large combinatorial libraries by selective evolution of ligands by exponential enrichment (SELEX) (*30, 31*) and affibodies based on a robust, 58 amino acid scaffold protein derived from staphylococcal protein A (*32–34*). The combination of such libraries with ratiometric fluorophores attached at carefully selected positions should eventually surpass conventionally labeled protein-based biosensor molecules as components in, e.g., immobilized protein detection platforms, where robustness of the biosensor element with respect to both recognition and signaling is imperative for reliable results.

In conclusion, we have expanded the repertoire of fluorescence strategies available for protein detection assays by demonstrating the concept of wavelength-ratiometric biosensing in solution based on straightforwardly obtained steady-state emission spectra from a two-band fluorophore. Optimization of the labeling position of the recognition element will require some work in future constructs, especially in cases where the recognition site might not be as well-characterized. However, the prospect of reagentless, robust sensing should be a strong motive for investing this effort.

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Supporting Information Available: Additional information on experimental procedures; results from SPR inhibition study. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Altschuh, D., Oncul, S., and Demchenko, A. P. (2006) Fluorescence sensing of intermolecular interactions and development of direct molecular biosensors. *J. Mol. Recognit. 19*, 459–477.

(2) Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature 388*, 882–887.

(3) Karlström, A., and Nygren, P. Å. (2001) Dual labeling of a binding protein allows for specific fluorescence detection of native protein. *Anal. Biochem. 295*, 22–30.

(4) Wei, A. P., Blumenthal, D. K., and Herron, J. N. (1994) Antibody-mediated fluorescence enhancement based on shifting the intramolecular dimer-monomer equilibrium of fluorescent dyes. *Anal. Chem. 66*, 1500–1506.

(5) Jiskoot, W., Hoogerhout, P., Beuvery, E. C., Herron, J. N., and Crommelin, D. J. A. (1991) Preparation and application of a fluorescein-labeled peptide for determining the affinity constant of a monoclonal antibody hapten complex by fluorescence polarization. *Anal. Biochem. 196*, 421–426.

(6) Walkup, G. K., and Imperiali, B. (1996) Design and evaluation of a peptidyl fluorescent chemosensor for divalent zinc. *J. Am. Chem. Soc. 118*, 3053–3054.

(7) Hamachi, I., Nagase, T., and Shinkai, S. (2000) A general semisynthetic method for fluorescent saccharide-biosensors based on a lectin. *J. Am. Chem. Soc. 122*, 12065–12066.

(8) Morii, T., Sugimoto, K., Makino, K., Otsuka, M., Imoto, K., and Mori, Y. (2002) A new fluorescent biosensor for inositol trisphosphate. *J. Am. Chem. Soc. 124*, 1138–1139.

(9) Zheng, Y. J., Gattas-Asfura, K. M., Konka, V., and Leblanc, R. M. (2002) A dansylated peptide for the selective detection of copper ions. *Chem. Commun.* 2350–2351.

(10) Sloan, D. J., and Hellinga, H. W. (1998) Structure-based engineering of environmentally sensitive fluorophores for monitoring protein-protein interactions. *Protein Eng. 11*, 819–823.

(11) Renard, M., Belkadi, L., Hugo, N., England, P., Altschuh, D., and Bedouelle, H. (2002) Knowledge-based design of reagentless fluorescent biosensors from recombinant antibodies. *J. Mol. Biol. 318*, 429–442.

(12) Enander, K., Dolphin, G. T., Andersson, L. K., Liedberg, B., Lundström, I., and Baltzer, L. (2002) Designed, folded polypeptide scaffolds that combine key biosensing events of recognition and reporting. *J. Org. Chem. 67*, 3120–3123.

(13) Yem, A. W., Epps, D. E., Mathews, W. R., Guido, D. M., Richard, K. A., Staite, N. D., and Deibel, M. R. (1992) Site-specific chemical modification of interleukin-1-beta by acrylodan at cysteine-8 and lysine-103. *J. Biol. Chem. 267*, 3122–3128.

(14) Mims, M. P., Sturgis, C. B., Sparrow, J. T., and Morrisett, J. D. (1993) Acrylodan can label amino as well as sulfhydryl-groups - results with low-density lipoprotein, lipoprotein a, and lipid-free proteins. *Biochemistry 32*, 9215–9220.

(15) Cho, E. J., Collett, J. R., Szafranska, A. E., and Ellington, A. D. (2006) Optimization of aptamer microarray technology for multiple protein targets. *Anal. Chim. Acta 564*, 82–90.

(16) Kodadek, T. (2002) Development of protein-detecting microarrays and related devices. *Trends Biochem. Sci. 27*, 295–300.

(17) Demchenko, A. P. (2005) The problem of self-calibration of fluorescence signal in microscale sensor systems. *Lab Chip 5*, 1210–1223.

(18) Klymchenko, A. S., Pivovarenko, V. G., and Demchenko, A. P. (2003) Perturbation of planarity as the possible mechanism of solvent-dependent variations of fluorescence quantum yield in 2-aryl-3-hydroxychromones. *Spectrochim. Acta, Part A 59*, 787–792.

(19) Avilov, S. V., Bode, C., Tolgyesi, F. G., Klymchenko, A. S., Fidy, J., and Demchenko, A. P. (2005) Temperature effects on alpha-crystallin structure probed by 6-bromomethyl-2-(2-furanyl)-3-hydroxychromone, an environmentally sensitive two-wavelength fluorescent dye covalently attached to the single Cys residue. *Int. J. Biol. Macromol. 36*, 290–298.

(20) Chatellier, J., Rauffer-Bruyère, N., van Regenmortel, M. H. V., Altschuh, D., and Weiss, E. (1996) Comparative interaction kinetics of two recombinant Fabs and of the corresponding antibodies directed to the coat protein of tobacco mosaic virus. *J. Mol. Recognit. 9*, 39–51.

(21) Al Moudallal, Z., Briand, J. P., and van Regenmortel, M. H. V. (1985) A major part of the polypeptide chain of tobacco mosaic virus protein is antigenic. *EMBO J. 4*, 1231–1235.

(22) Choulier, L., Laune, D., Orfanoudakis, G., Wlad, H., Janson, J. C., Granier, C., and Altschuh, D. (2001) Delineation of a linear epitope by multiple peptide synthesis and phage display. *J. Immunol. Methods 249*, 253–264.

(23) Choulier, L., Rauffer-Bruyère, N., Ben Khalifa, M., Martin, F., Vernet, T., and Altschuh, D. (1999) Kinetic analysis of the effect on Fab binding of identical substitutions in a peptide and its parent protein. *Biochemistry 38*, 3530–3537.

(24) Klymchenko, A. S., Kenfack, C., Duportail, G., and Mély, Y. (2007) Effects of polar protic solvents on dual emissions of 3-hydroxychromones. *J. Chem. Sci. 119*, 83–89.

(25) Ercelen, S., Klymchenko, A. S., and Demchenko, A. P. (2002) Ultrasensitive fluorescent probe for the hydrophobic range of solvent polarities. *Anal. Chim. Acta 464*, 273–287.

(26) Klymchenko, A. S., and Demchenko, A. P. (2003) Multiparametric probing of intermolecular interactions with fluorescent dye exhibiting excited state intramolecular proton transfer. *Phys. Chem. Chem. Phys. 5*, 461–468.

(27) Klymchenko, A. S., and Demchenko, A. P. (2004) 3-Hydroxychromone dyes exhibiting excited-state intramolecular proton transfer in water with efficient two-band fluorescence. *New J. Chem. 28*, 687–692.

(28) De Lorimier, R. M., Smith, J. J., Dwyer, M. A., Looger, L. L., Sali, K. M., Paavola, C. D., Rizk, S. S., Sadigov, S., Conrad, D. W., Loew, L., and Hellinga, H. W. (2002) Construction of a fluorescent biosensor family. *Protein Sci. 11*, 2655–2675.

(29) Renard, M., and Bedouelle, H. (2004) Improving the sensitivity and dynamic range of reagentless fluorescent immunosensors by knowledge-based design. *Biochemistry 43*, 15453–15462.

(30) Ellington, A. D., and Szostak, J. W. (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature 346*, 818–822.

(31) Tuerk, C., and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment - RNA ligands to bacteriophage T4 DNA-polymerase. *Science 249*, 505–510.

(32) Nilsson, B., Moks, T., Jansson, B., Abrahmsen, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T. A., and Uhlén, M. (1987) A synthetic IgG-binding domain based on staphylococcal protein A. *Protein Eng. 1*, 107–113.

(33) Nord, K., Nilsson, J., Nilsson, B., Uhlén, M., and Nygren, P.Å. (1995) A combinatorial library of an α-helical bacterial receptor domain. *Protein Eng. 8*, 601–608.

(34) Nord, K., Gunneriusson, E., Ringdahl, J., Ståhl, S., Uhlén, M., and Nygren, P. Å. (1997) Binding proteins selected from combinatorial libraries of an α-helical bacterial receptor domain. *Nat. Biotechnol. 15*, 772–777.

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