Journal of Peptide Science
*J. Pept. Sci.* 2008; **14**: 401–406
Published online 12 November 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.939

JOURNAL OF PEPTIDE SCIENCE

Review

# Molecular interaction studies of peptides using steady-state fluorescence intensity. Static (de)quenching revisited‡

MARTA M. B. RIBEIRO,§ HENRI G. FRANQUELIM,§ MIGUEL A. R. B. CASTANHO and ANA SALOMÉ VEIGA*

Centro de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Ed C8, 1749-016 Lisboa, Portugal

*Received 29 May 2007; Revised 12 July 2007; Accepted 14 August 2007*

**Abstract:** Protein–protein interactions, as well as peptide–peptide and peptide–protein interactions are fields of study of growing importance as molecular-level detail is avidly pursued in drug design, metabolic regulation and molecular dynamics, among other classes of studies. In membranes, this issue is particularly relevant because lipid bilayers potentiate molecular interactions due to the high local concentration of peptides and other solutes.

However, experimental techniques and methodologies to detect and quantify such interactions are not abundant. A reliable, fast and inexpensive alternative methodology is revisited in this work.

Considering the interaction of two molecules, at least one of them being fluorescent, either intrinsically (e.g. Trp residues) or by grafting a specific probe, changes in their aggregation state may be reported, as long as the fluorophore is sensitive to local changes in polarity, conformation and/or exposure to the solvent. The interaction will probably lead to modifications in fluorescence intensity resulting in a decrease ('quenching') or enhancement ('dequenching'). Although the presented methodology is based on static quenching methodologies, the concept is extended from quenching to any kind of interference with the fluorophore.

Equations for data analysis are shown and their applications are illustrated by calculating the binding constant for several data-sets. 

**Keywords:** protein; peptide; fluorophore; interferent; (de)quenching; aggregation; association

## MOLECULAR INTERACTIONS OF PEPTIDES

Interactions between molecules, namely proteins and peptides, are fundamental to many biochemical processes, assuring the proper function of the cell [1]. Knowing how to quantify and analyze these interactions is of crucial importance. Targeting sites that modulate protein–protein interactions, for instance, represents an ongoing challenge for drug discovery [2].

The importance of studying related proteins and their eventual interaction with other molecules has increased [3], but few experimental techniques and methodologies have been developed to detect and quantify such interactions.

## FLUORESCENCE SPECTROSCOPY TO EVALUATE PEPTIDE AND PROTEIN INTERACTIONS

Some spectroscopy techniques are available to study these phenomena [4]. *Surface Plasmon Resonance* is a powerful technique, but needs dedicated expensive equipment [5], while *Circular Dichroism* (CD) can only report dimerization in case the association of molecules involves extensive conformational changes [6]. The possibility of false negative tests in CD is a serious drawback of the technique. Nevertheless, fluorescence spectroscopy can be used to study the association of peptides and proteins with other peptides and proteins, or even lipids or nucleic acids, thus becoming a powerful tool for monitoring biological events and elucidating the structure and function of biomolecules [1,7]. Such studies take advantage of the high sensitivity of fluorescence for measurements in dilute solutions, and an ubiquitous availability of the necessary equipment [8].

To evaluate these interactions, the fluorophore in the peptide/protein should display spectral features and/or quantum yield sensitive to local changes in polarity, intra-molecular interactions and/or exposure to the solvent, so that peptide–peptide or protein–protein associations lead to changes in a photophysical parameter [4]. This can be achieved by an intrinsic fluorescent amino acid residue, usually tryptophan, which is frequently present in proteins and peptides (typical in antimicrobial and cell-penetrating peptides, for instance [9]), or by grafting a specific probe [10].

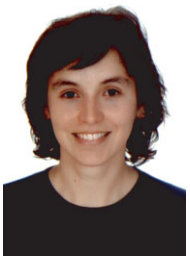
Four parameters have been used to quantify protein interactions *in vitro* and *in vivo*: polarization [11], lifetime, average energy (spectral shifts) and quantum yield [4].

* Correspondence to: Ana Salomé Veiga, Dep. Química e Bioquímica, Fac. Ciências da Univ. Lisboa, Campo Grande C8, P 1749-016 Lisboa, Portugal; e-mail: asveiga@fc.ul.pt

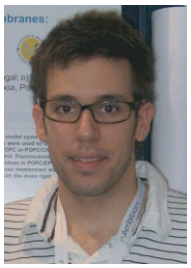
‡ This article is part of the Special Issue of the Journal of Peptide Science entitled "2nd workshop on biophysics of membrane-active peptides".

§ Both authors contributed equally to the work.

## BIOGRAPHY



**Ana Salomé Veiga** is a biochemist finishing her doctoral studies in the University of Lisbon, Portugal. Her research focuses on the study of peptide (and protein) interactions with model membrane systems and peptide–peptide interactions using fluorescence spectroscopy and, to a less extent, IR and circular dichroism spectroscopies, and surface plasmon resonance.



**Henri Franquelim** was born in Esch-Alzette (Luxembourg) in 1984. He obtained his degree in Biochemistry in 2007 at the Faculty of Sciences, University of Lisbon, Portugal. His research interests comprise the applications of spectroscopic techniques (steady-state and time-resolved fluorescence spectroscopy and light-scattering spectroscopy) and atomic force microscopy in the resolution of problems related to structural biochemistry and biomembranes. His present research is focused on membrane-active peptides, particularly viral fusogenic peptides and fusion inhibitors.

**Miguel Castanho** graduated in Biochemistry (University of Lisbon, Portugal, 1990), has a Ph.D. degree in Molecular Biophysics (Technical University of Lisbon, Portugal, 1993) and habilitation in Physical Biochemistry (University of Lisbon, Portugal, 1999). He became a group leader in the Faculty of Sciences (University of Lisbon, Portugal), where he started working on the mechanism of action of membrane-active peptides at the molecular level. His work includes the development of methodologies aiming at specific functional and structural information, related to cell-penetrating, antimicrobial, viral fusion inhibitor and neuropeptides. Both *in-vitro* and *in-vivo* work is carried out, using mainly optical spectroscopic techniques. Miguel Castanho has recently been appointed full professor (Biochemistry) at the Faculty of Medicine (University of Lisbon, Portugal).

These photophysical parameters can be measured using steady-state or time-resolved techniques. Although time-resolved analysis may provide a more complete information, data analysis is more demanding, and in some cases, the complexities of the decays may render the methodologies extremely complicated. Therefore, we will focus on steady-state data.

## BIOGRAPHY

**Marta Ribeiro** was born in Lisbon, Portugal, in 1985. In 2007, she graduated in Biochemistry at the Faculty of Sciences, University of Lisbon, Portugal. Her research interests comprise the application of spectroscopic techniques in structural biochemistry of biomembranes and in the evaluation of in vivo nociception, mainly focusing on neuropeptides with analgesic properties.

## (DE)QUENCHING

Considering two molecules, at least one of them having a fluorophore group, if the contact interaction between both leads to a change in the fluorescence intensity being measured, either decreasing – 'quenching' – or enhancing – 'dequenching' – then, the change can be used to quantify and monitor the interaction.

The methodology reviewed in this paper considers a fluorescent peptide, $F$, and the associating (de)quencher molecule, $Y$, which can be a peptide/protein, for instance. However, the methodology can be generalized to any molecule that interferes with the fluorophore's fluorescence property being detected.

Both static and dynamic quenching require molecular contact of the quencher with the fluorophore [12]. We will focus on common static-quenching methodologies, which consider the association between the molecules prior to the excitation of the fluorophores [13]. Nevertheless, the concept is extended from quenching to any kind of interference with the fluorophore that may result in an alteration of the quantum yield due to association with other molecules, the interference species.

Herein, we report a method for studying molecular peptide association interactions using adapted known quenching equations.

## METHODOLOGY

Static quenching is a frequent complicating factor in the analysis of quenching data, but can also be a valuable source of information about binding between the fluorescent molecule and the quencher [8]. If the fluorophore, $F$, and the interference specie, $Y$, associate to form a complex, the system is described by the association constant, $K_b$, which in the case of 1 : 1 stoichiometry is [13]:

$$F + Y \rightleftharpoons FY$$

$$K_b = \frac{[FY]}{[F][Y]} \quad (1)$$




DOI: 10.1002/psc

Some authors generalize this equation to the possibility of different stoichiometric proportions [14,15]. For the sake of simplicity, we will restrict our analysis to the 1 : 1 stoichiometry.

Recalling that the total concentration of fluorophore and interferent is given by:

$$[F] + [FY] = [F]_t \text{ and } [F] + [FY] = [F]_t \tag{2}$$

and considering $X_i$ the molar fraction due to total concentration of $i$ in solution:

$$X_F = \frac{[F]}{[F] + [FY]} \quad (X_{FY} + X_F = 1) \tag{3}$$

Equation (1) can be rewritten as:

$$X_F = \frac{1}{1 + [Y]K_b} \tag{4}$$

In the classical static-quenching equation the fluorescence quantum yield of the complex, $\phi_{FY}$ is nil, $\phi_{FY} = 0$, so, $I_f \propto X_F$, $I_f$ being the measured fluorescence emission intensity in the presence of interferent. Therefore: $I_f \propto \frac{1}{1+[Y]K_b} \Leftrightarrow \frac{I_0}{I_f} = 1 + [Y]K_b$, where $I_0$ is the fluorescence intensity in the absence of quencher. In conditions where $[Y] \approx [Y]_t$ (i.e. $K_b$ is small enough), a linear Stern–Volmer relationship ($I_0/I_f$ vs. $[Y]_t$) is obtained.

From Eqns (2) and (3):

$$\begin{aligned} &[F] - [Y] = [F]_t - [Y]_t \Leftrightarrow \\ &[Y] = [Y]_t - [F]_t X_{FY} \end{aligned} \tag{5}$$

Replacing $[Y]$ in Eqn (4):

$$X_{FY}^2([F]_t K_b) - X_{FY}(1 + K_b[Y]_t + K_b[F]_t) + K_b[Y]_t = 0 \tag{6}$$

For the sake of clarity, Eqn (6) can be simplified to the notation of a quadratic equation of:

$$ax^2 + bx + c = 0 \tag{7}$$

where $a = [F]_t K_b$, $b = 1 + K_b[Y]_t + K_b[F]_t$ and $c = K_b[Y]_t$

Therefore:

$$X_{FY} = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \tag{8}$$

Considering that the complexation of $Y$ in $FY$ may change the fluorescence intensity of $F$, either decreasing (quenching) or enhancing (dequenching), the measured fluorescence emission intensity in the presence of interferent is: $I_f = I_{f,F} + I_{f,FY} \propto (X_F I_{t,F} + X_{FY} I_{t,FY})$, where $I_{t,F}$ and $I_{t,FY}$ are the intensities which would be measured if every fluorescent molecule were free or bound, respectively. So,

$$I_f \propto \left(X_F + X_{FY}\frac{I_{t,FY}}{I_{t,Y}}\right) = 1 + \left(\frac{\phi_{FY}}{\phi_F} - 1\right) X_{FY} \tag{9}$$

Adopting a Stern–Volmer formulation:

$$\frac{I_0}{I_f} = \frac{I_{t,F}}{I_f} = \frac{1}{1 + \left(\frac{\phi_{FY}}{\phi_F} - 1\right) X_{FY}} \tag{10}$$

Other authors have used equivalent equations to report interactions between molecules [16–18].

The case of static-quenching is obtained by setting $\phi_{FY} = 0$:

$$\frac{I_0}{I_f} = \frac{1}{1 - X_{FY}} = \frac{1}{1 + \frac{b \pm \sqrt{b^2 - 4ac}}{2a}} \tag{11}$$

i.e., $I_0/I_f$ depends on $[Y]_t$, $[F]_t$ and $K_b$.

However, a linear relationship is not obtained at variance with classical Stern–Volmer plots, so there is no reason to work with the reciprocal of $I_f$. Data sets for illustrative results from the literature were fitted with the reciprocal of Eqn (10), having $[F]_t$ and $[Y]_t$ as known values (Figure 1 and Table 1).

A more general situation would be the one involving two fluorescent molecules. The derivation procedure is similar:

$$I_f - I_Y = I_F - \frac{g \pm \sqrt{g^2 - 4fh}}{2f}(I_{F,FY} - I_F + I_{Y,FY} - I_Y) \tag{12}$$

**Table 1** Affinity of several interference species with intrinsic or extrinsic fluorescent peptides. Binding constant, $K_b$, and $\Phi_{FY}/\Phi_F$ were obtained by fitting Eqn (10) to the experimental data presented in Figure 1

| Peptide (Fluophore) | Interference species | Ref. | $K_b(\mu M^{-1})$ | $\Phi_{FY}/\Phi_F$ |
|---|---|---|---|---|
| USP7 (Trp) | $p53_{355-393}$ (monomer) | [17] | $0.079 \pm 0,002$ | $1.657 \pm 0.006$ |
| | $p53_{311-393}$ (tetramer) | | $0.113 \pm 0,002$ | $1.636 \pm 0.004$ |
| TnC (1,5-IAEDANS) | $TnI_{96-115}$ | [16] | $2.010 \pm 0,286$ | $2.132 \pm 0.038$ |
| | $TnI_{104-115}$ | | $0.351 \pm 0.151$ | $1.916 \pm 0.185$ |
| HCII (TNS) | $Heparin_{(10-mer)}$ | [18] | $0.399 \pm 0.040$ | $1.401 \pm 0.015$ |
| | $Heparin_{(26-mer)}$ | | $1.168 \pm 0.104$ | $0.723 \pm 0.006$ |



DOI: 10.1002/psc

The parameters $f$, $g$ and $h$ being:

$$f = K_b$$
$$g = -[K_b([Y]_t + [F]) + 1]$$
$$h = K_b[F]_t[Y]_t$$

where $I_f$ is the total fluorescence intensity and $I_F$ and $I_Y$ are the intensities in the absence of the interference specie, $Y$, and fluorophore, $F$, respectively.

## DATA ANALYSIS

Several experimental results were found which report molecules' interaction [14–25]. To illustrate how Eqn (10) can be used to fit interactions between any two molecules, three datasets have been chosen. Two of them focus on peptide–peptide interaction, having as a fluorophore the amino acid residue tryptophan [17] or a probe [16]. The other [18] refers to a peptide-interference species interaction (Figure 1).

Equation (10) can be rewritten as:

$$\frac{I_f}{I_0} = 1 + \left(\frac{\phi_{FY}}{\phi_F} - 1\right) X_{FY} \Leftrightarrow \frac{\Delta I_f}{I_0} = \frac{\Delta I_{\max}}{I_0} X_{FY} \qquad (13)$$

where $\Delta I_f$ is the fluorescence intensity change upon addition of $Y$ and $\Delta I_{\max}$ the maximal fluorescence intensity change. This equation, however, disregards the useful information obtained from $\phi_{FY}/\phi_F$.

Ubiquitin-specific protease 7 (USP7) or herpes-associated ubiquitin-specific protease (HAUSP) was first identified by virtue of its interaction with herpes simplex virus type 1 immediate early protein (ICP0) [26,27]. It is also known as the target of another herpes virus protein, namely, the EBNA1 protein of Eppstein-Barr [28]. Although this protein is most known for its interaction with herpes virus proteins, the main cellular function of USP7 is the regulation of the turnover of p53 [29].

Peptide-binding titrations of p53 fragments, containing USP7-binding sequence (355–393) alone or both USP7-binding sequence and tetramerization domain (311–393), with USP7 were analyzed by Holowaty *et al.* [17]. (Figure 1(a)). Because the p53 peptides do not contain any tryptophan residue in its sequences, the intrinsic fluorescence properties of USP7 were used and the changes in fluorescence signal enabled the measurement of the degree of binding between p53 and USP7. Very similar $\Phi_{FY}/\Phi_F$ and $K_b$ were obtained for the interaction of USP7 with p53 monomer and tetramer peptides (Table 1), indicating no significant differences in the mode of binding of these two fragments with USP7.

Troponin (Tn) is a $Ca^{2+}$-dependent protein complex that is involved in the regulation of the vertebrate skeletal muscle contraction. Tn consists of three

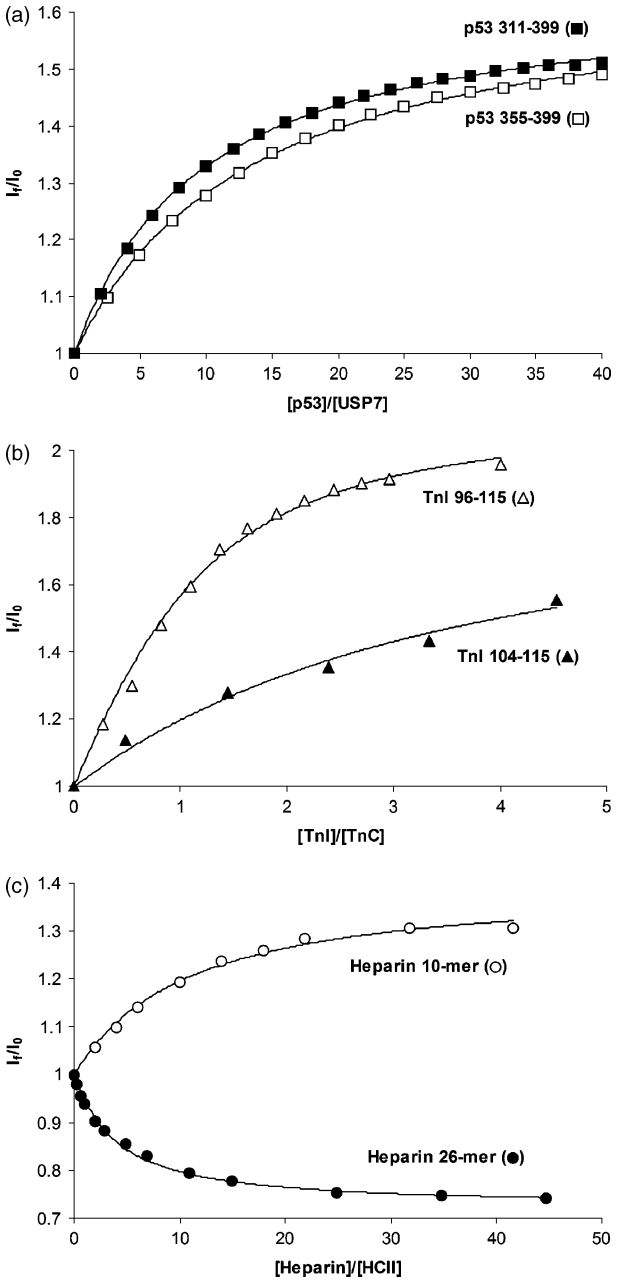



**Figure 1** Interaction of several interference species with intrinsic or extrinsic fluorescent peptides. Changes in fluorescence emission intensities at fixed peptides' concentrations were obtained with increasing concentrations of the interference species. Fitting Eqn (10) to the experimental data gave the binding curves shown. (a) Titration of p53 peptides (containing amino acid residues 311–399 or amino acid residues 355–399) into a solution of 1.0 μM USP7, followed by tryptophan fluorescence at 350 nm, using an excitation wavelength of 295 nm [17]. (b) Titration of TnI peptides ($TnI_{104-115}$ or $TnI_{96-115}$) into a solution of 1.0 μM Cys98 IEDANS-labeled TnC in the presence of $Ca^{2+}$. Excitation was at 340 nm and the emission spectra were recorded from 340 to 650 nm [16]. (c) Heparin (26-mer or 10-mer) titrations into a solution of 0, 25 μM HCII and 10 μM TNS. Excitation was at 330 nm and fluorescence emission spectra were collected from 360 to 500 nm [18]. (b) and (c) Each datum point represents the relative emission area.



DOI: 10.1002/psc

subunits: TnI, TnC and TnT. While TnT anchors the troponin complex to the thin filament; TnI (inhibitory protein) binds actin and inhibits the actomyosin $Mg^{2+}$-ATPase and TnC ($Ca^{2+}$-binding protein) releases the inhibitory effect of TnI [30–33].

After binding of $Ca^{2+}$, localized conformational changes in both C- and N-domains of TnC occur, resulting in the exposure of hydrophobic residues, thus permitting the interaction with TnI [34–36].

Mapping studies have identified the inhibitory region of TnI in the position 96–115 of its sequence [37] and the binding site for the inhibitory region on the C-domain of TnC [38–40].

Tripet's work consisted in the interaction of IEDANS labeled TnC (on the C-domain) with TnI fragment peptides (Figure 1(b)) [16]. As shown in Table 1, TnI fragment 96–115 (which corresponds to the full inhibitory region) has a higher binding affinity to TnC relative to the 104–115 fragment of TnI (which corresponds to a partial segment of the inhibitory region). $\Phi_{FY}/\Phi_F$ is similar for both peptides, suggesting a similar mode of action, i.e. the peptides interact with the labeled C-domain of TnC with similar docking.

Heparin cofactor II (HCII) is a GAG-activated inhibitor of thrombin that circulates in the blood at very high concentration [41–45]. Because HCII lacks an intrinsic fluorescence signal, *O'Keeffe et al.* used an extrinsic fluorescence probe, TNS (environment-sensitive probe that binds weakly with HCII) to determine the binding constant of HCII to heparin [18]. Heparin binding to HCII-TNS causes a change in the environment of HCII-bound TNS [18,21]. TNS fluorescence changes depended on the length of heparin (Figure 1(c)). In chains smaller than 13 monosaccharide units (minimal heparin size to fully occupy the heparin-binding site on HCII) a fluorescence enhancement – 'dequenching' – was observed, which is the case of 10-mer heparin, $\Phi_{FY}/\Phi_F > 1$. At variance, for larger heparin units, a fluorescence quenching ($\Phi_{FY}/\Phi_F < 1$) was reported, being 26-mer heparin, the example presented in Table 1.

The apparent increase in the binding affinity for the 26-mer heparin chain can be understood as a statistical effect of the number of overlapping HCII sites available, due to the nonspecificity of the binding of heparin to HCII [18]. Considering the enormous change in TNS fluorescence, visualized in the value of $\Phi_{FY}/\Phi_F$, it is probable that in the case of the larger heparin not only the affinity but also the binding mode/docking are affected.

## DISCUSSION

In the present work, a frequently overlooked but valuable tool, with a broad utility to analyze interactions between two molecules having as the only requirement one of them being fluorescent, is revisited. The methodology is of general use and can be applied directly to the quenching or dequenching data, with no assumptions regarding the associated peptides' quantum yield. The binding constant, $K_b$, and $\Phi_{FY}/\Phi_F$ of some illustrative data were revisited using the proposed methodology. 'Affinity' ($K_b$) effects can be distinguished from conformational effects (likely to affect the fluorescence quantum yield). Therefore, a resourceful methodology that can be used in most cases was present.

The equations can be used in membranes to a certain extent as long as local concentrations in the lipid media are used, i.e. accounting for partition of the peptides [46,47].

## Acknowledgement

A. S. Veiga acknowledges grant SFRH/BD/14336/2003 from FCT (Portugal).

## REFERENCES

1. Cusick ME, Klitgord N, Vidal M, Hill DE. Interactome: gateway into systems biology. *Hum. Mol. Genet.* 2005; **14**: R171–R181.
2. Saldanha SA, Kaler G, Cottam HB, Abagyan R, Taylor SS. Assay principle for modulators of protein-protein interactions and its application to Non-ATP-competitive ligands targeting protein kinase A. *Anal. Chem.* 2006; **78**: 8265–8272.
3. Remaut H, Waksman G. Protein-protein interaction through $\beta$-strand addition. *Trends Biochem. Sci.* 2006; **31**: 436–444.
4. Yan Y, Marriott G. Analysis of protein interactions using fluorescence technologies. *Curr. Opin. Chem. Biol.* 2003; **7**: 635–640.
5. Goodrich TT, Wark AW, Corn RM, Lee HJ. Surface plasmon resonance imaging measurements of protein interactions with biopolymer microarrays. In *New and Emerging Proteomic Techniques*, chap. 7, Nedelkov D, Nelson RW (ed.). Human Press: Totowa, 2006.
6. Zsila F, Bikadi Z, Fitos I, Simonyi M. Probing protein binding sites by circular dichroism spectroscopy. *Curr. Drug Discov. Technol.* 2004; **1**: 133–153.
7. Sculimbrene BR, Imperiali B. Lanthanide-binding tags as luminescent probes for studying protein interactions. *J. Am. Chem. Soc.* 2006; **128**: 7346–7352.
8. Lakowicz JR. *Principles of Fluorescence Spectroscopy*. 2nd edn, chap. 8 and 16. Kluwer Academic/Plenum Press: New York, 1999.
9. Henriques ST, Melo MN, Castanho MARB. Cell-penetrating peptides and antimicrobial peptides: how different are they? *Biochem. J.* 2006; **399**: 1–7.
10. Park S, Raines RT. Green fluorescent protein as a signal for protein-protein interactions. *Protein Sci.* 1997; **6**: 2344–2349.
11. Monteiro RQ, Bock PE, Bianconi ML, Zingali RB. Characterization of bothrojaracin interaction with human prothrombin. *Protein Sci.* 2001; **10**: 1897–1904.
12. Santos N, Castanho M. Fluorescence spectroscopy methodologies on the study of proteins and peptides. On the 150th anniversary of protein fluorescence. *Trends Appl. Spectrosc.* 2002; **4**: 113–125.
13. Castanho MARB, Prieto MJE. Fluorescence quenching data interpretation in biological systems: the use of microscopic models for data analysis and interpretation of complex systems. *Trends Appl. Spectrosc.* 1998; **1373**: 1–16.
14. Pimenta DC, Nantes IL, De Souza ES, Le Bonniec B, Ito AS, Tersariol ILS, Oliveira V, Juliano MA, Juliano L. Interaction of

heparin with internally quenched fluorogenic peptides derived from heparin-binding consensus sequences, kallistatin and antithrombin III. *Biochem. J.* 2002; **366**: 435–446.
15. Kolakowski J, Wrzosek A, Da Browska R. Fesselin is a target protein for calmodulin in a calcium-dependent manner. *Biochem. Biophys. Res. Commun.* 2004; **323**: 1251–1256.
16. Tripet B, De Crescenzo G, Grothe S, O'Connor-McCourt M, Hodges RS. Kinetic analysis of the interactions between troponin C and the C-terminal troponin I regulatory region and validation of a new peptide delivery/capture system used for surface plasmon resonance. *J. Mol. Biol.* 2002; **323**: 345–362.
17. Holowaty MN, Sheng Y, Nguyen T, Arrowsmith C, Frappier L. Protein interaction domains of the ubiquitin-specific protease, USP7/HAUSP. *J. Biol. Chem.* 2003; **278**: 47753–47761.
18. O'Keeffe D, Olson ST, Gasiunas N, Gallagher J, Baglin TP, Huntington JA. The heparin binding properties of heparin cofactor II suggest an antithrombin-like activation mechanism. *J. Biol. Chem.* 2004; **279**: 50267–50273.
19. Cox JA, Comte N, Fittonsll JE, DeGradoll WF. The interaction of calmodulin with amphiphilic peptides. *J. Biol. Chem.* 1985; **260**: 2527–2534.
20. Orosz F, Veartessy BG, Salerno C, Crifo C, Capuozzo E, Ovadi J. The interaction of a new anti-tumour drug, KAR-2 with calmodulin. *Br. J. Pharmacol.* 1997; **121**: 955–962.
21. Meagher JL, Olson ST, Gettins GW. Critical role of the linker region between Helix D and strand 2A in heparin activation of antithrombin. *J. Biol. Chem.* 2000; **275**: 2698–2704.
22. Raja SM, Chhablani N, Swanson R, Thompson E, Laffan M, Lane DA, Olson ST. Deletion of P1 arginine in a novel antithrombin variant (antithrombin london) abolishes inhibitory activity but enhances heparin affinity and is associated with early onset thrombosis. *J. Biol. Chem.* 2003; **278**: 13688–13695.
23. Garzón MT, Lidón-Moya MC, Barrera FN, Prieto A, Gómez J, Mateu MG, Neira JL. The dimerization domain of the HIV-1 capsid protein binds a capsid protein-derived peptide: a biophysical characterization. *Protein Sci.* 2004; **13**: 1512–1523.
24. Halden Y, Rek A, Atzenhofer W, Szilak L, Wabnig A, Kungl AJ. Interleukin-8 binds to syndecan-2 on human endothelial cells. *Biochem. J.* 2004; **377**: 533–538.
25. Ronga L, Langella E, Palladino P, Marasco D, Tizzano B, Saviano M, Pedone C, Improta R, Ruvo M. Does tetracycline bind helix 2 of prion? An integrated spectroscopical and computational study of the interaction between the antibiotic and a helix 2 human prion protein fragments. *Proteins: Struct., Funct., Bioinformat.* 2007; **66**: 707–715.
26. Meredith M, Orr A, Everett R. Herpes simplex virus type 1 immediate-early protein Vmw110 binds strongly and specifically to a 135-kDa cellular protein. *Virology* 1994; **200**: 457–469.
27. Everett R, Meredith M, Orr A, Cross A, Kathoria M, Parkinson J. A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. *EMBO J.* 1997; **16**: 1519–1530.
28. Holowaty MN, Zeghouf M, Wu H, Tellam J, Athanasopoulos V, Greenblatt J, Frappier L. Protein profiling with epstein-barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7. *J. Biol. Chem.* 2003; **278**: 29987–29994.
29. Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* 2002; **416**: 648–653.
30. Farah CS, Reinach FC. The troponin complex and regulation of muscle contraction. *FASEB J.* 1995; **9**: 755–767.
31. Houdusse A, Love ML, Dominguez R, Grabarek Z, Cohen C. Structures of four $Ca^{2+}$-bound troponin C at 2.0A° resolution: further insights into the Ca2+-switch in the calmodulin superfamily. *Structure* 1997; **5**: 1695–1711.
32. Perry SV. Troponin I: inhibitor or facilitator. *Mol. Cell. Biochem.* 1999; **190**: 9–32.
33. Tobacman LS. Thin filament-mediated regulation of cardiac contraction. *Annu. Rev. Physiol.* 1996; **58**: 447–481.
34. Gagne SM, Tsuda S, Li MX, Smillie LB, Sykes BD. Structures of the troponin C regulatory domains in the apo and calcium-saturated states. *Nature Struct. Biol.* 1995; **2**: 784–789.
35. Herzberg O, James MN. Structure of the calcium regulatory muscle protein troponin-C at 2.8 A° resolution. *Nature* 1985; **313**: 653–659.
36. Strynadka NC, Cherney M, Sielecki AR, Li MX, Smillie LB, James MN. Structural details of a calcium-induced molecular switch: X-ray crystallographic analysis of the calcium-saturated N-terminal domain of troponin C at 1.75 A° resolution. *J. Mol. Biol.* 1997; **273**: 238–255.
37. Tripet B, Van Eyk JE, Hodges RS. Mapping of a second actin–tropomyosin and a second troponin C binding site within the C terminus of troponin I, and their importance in the $Ca^{2+}$-dependent regulation of muscle contraction. *J. Mol. Biol.* 1997; **271**: 728–750.
38. Dalgarno D, Grand R, Levine B, Moir A, Scott G, Perry S. Interaction between troponin I and troponin C. *FEBS Lett.* 1982; **150**: 54–58.
39. Drabikowski W, Dalgarno DC, Levine BA, Gergely J, Grabarek Z, Leavis PC. Solution conformation of the C-terminal domain of skeletal troponin C. Cation, trifluoperazine and troponin I binding effects. *Eur. J. Biochem.* 1985; **151**: 17–28.
40. Kobayashi T, Leavis PC, Collins JH. Interaction of a troponin I inhibitory peptide with both domains of troponin C. *Biochim. Biophys. Acta* 1996; **1294**: 25–30.
41. Huntington JA, Baglin TP. Targeting thrombin–rational drug design from natural mechanisms. *Trends Pharmacol. Sci.* 2003; **24**: 589–595.
42. Tollefsen DM. Heparin cofactor II deficiency. *Arch. Pathol. Lab Med.* 2002; **126**: 1394–1400.
43. Aihara K, Azuma H, Takamori N, Kanagawa Y, Akaike M, Fujimura M, Yoshida T, Hashizume S, Kato M, Yamaguchi H, Kato S, Ikeda Y, Arase T, Kondo A, Matsumoto T. Heparin cofactor II is a novel protective factor against carotid atherosclerosis in elderly individuals. *Circulation* 2004; **109**: 2761–2765.
44. He L, Vicente CP, Westrick RJ, Eitzman DT, Tollefsen DM. Heparin cofactor II inhibits arterial thrombosis after endothelial injury. *J. Clin. Invest.* 2002; **109**: 213–219.
45. Takamori N, Azuma H, Kato M, Hashizume S, Aihara K, Akaike M, Tamura K, Matsumoto T. High plasma heparin cofactor II activity is associated with reduced incidence of in-stent restenosis after percutaneous coronary intervention. *Circulation* 2004; **109**: 481–486.
46. Veiga S, Yuan Y, Li X, Santos N, Liu G, Castanho M. Why are HIV-1 fusion inhibitors not effective against SARS-CoV? Biophysical evaluation of molecular interactions. *Biophys. Biochem. Acta – Gen. Subj.* 2006; **1760**: 55–61.
47. Castanho MA, Fernandes MX. Lipid membrane-induced optimization for ligand-receptor docking: recent tools and insights for the "membrane catalysis" model. *Eur. Biophys. J.* 2006; **2**: 92–103.