TOWARDS A NEW HOMOGENEOUS IMMUNOASSAY FOR GONADOTROPIN-RELEASING HORMONE BASED ON TIME-RESOLVED FLUORESCENCE ANISOTROPY

Peter D. Dowd
Department of Bioengineering, University of Strathclyde, Wolfson Centre, 106 Rottenrow, Glasgow, U.K.

Jan Karolin, Carol Trager-Cowan, David J. S. Birch
Department of Physics, Centre for Molecular Nanometrology, University of Strathclyde, SUPA John Anderson Building, 107 Rottenrow, Glasgow, G4 0NG, U.K.

William H. Stimson
Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde Royal College, 204 George Street, Glasgow, U.K.

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Abstract: A new method for detecting gonadotropin-releasing hormone type 1 (GnRH-1) is proposed. The method is based on a fluorescence polarisation immunoassay where the GnRH-1 vies for the binding site on the anti-GnRH (antibody 7B10.1D10) with a labelled peptide consisting of a fragment of GnRH labelled with fluorescein. Time-resolved anisotropy decays indicate increased correlation times for the labelled fragment in the presence of 7B10.1D10 compared with the correlation times for the labelled peptide fragment alone. When GnRH-1 is added to the solution of the labelled peptide in the presence of 7B10.1D10 it has the effect of reducing the correlation times. The results demonstrate the possibility of introducing the labelled fragment as a competitor to the antigen in the fluorescence polarisation immunoassay for GnRH-1 and, more generally, in the case where there is a disadvantage in labelling the antigen, such as GnRH-1, with a fluorescent probe.

1 INTRODUCTION

In this report we describe a potential method for detecting gonadotropin-releasing hormone type-1 (GnRH-1) using a fluorescence polarisation immunoassay (FPI). Conventionally in FPI's, an antigen labelled with a fluorescent probe is displaced from a specific antibody binding site by an unlabelled antigen. In this method we have replaced the labelled antigen with a labelled 'fragment' of the antigen thus eliminating the problematic issues surrounding GnRH-1.

GnRH-1 (the mammalian form of GnRH) is a hypothalamic hormone and it plays a fundamental role in the events leading to the synthesis and release of leuteinising hormone (LH) and follicule stimulating hormone (FSH) into the circulatory system (Conn & Crowley, 1991). GnRH is essential in reproductive function and expression of GnRH has even been reported in tissues such as the ovary, breast and prostate (Chen et al, 2002). Although GnRH influences conditions such as GnRH-dependent precocious puberty, the expression of GnRH in tissue areas outside the hypothalamic region and in the immune system is not fully understood. Nevertheless, due to the suggestion that GnRH expression maybe a possible marker for certain tumours and the success of GnRH agonists used in a variety of disorders, monitoring the levels and effects of GnRH are of significant clinical interest (Tanriverdi et al, 2004).

FPI'S are appealling for medical diagnostic and point of care testing (POC) applications because they can be performed in a homogeneous format and

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with relatively few preparation steps (Price, 2001). Using antigens labelled with fluorescent markers, FPI's rely on the difference in anisotropy (or polarisation) measurements of a labelled antigen compared with the anisotropy of the labelled antigen in association with an antibody (Terpetsching et al, 1994). More specifically, in FPI's the emission from the free labelled antigen must be depolarised. Thus the rotational correlation time for the antigen must be much shorter than that of the probe. Therefore, when this method is applied to antigens with a low molecular weight, fluorescein with a lifetime near 4ns is typically chosen as the fluorescent probe (Spencer et al, 1973). In comparison with the free labelled antigen the polarisation will increase when the labelled antigen binds to the antibody.

In the case of GnRH-1, labelling demands either the addition or removal of an amino acid. The addition of an amino acid can lead to an alteration in conformation due to the increase in size and change in charge. This is less likely to occur if an amino acid is removed. Therefore, in an attempt to preserve the antibody binding site which is in the centre of the peptide (Khan et al, 2003), we describe a method that incorporates the 9-amino acid fragment of GnRH-1 labelled with fluorescein to act as a replacement for the conventional labelled antigen in the assay. Furthermore, we use time-resolved anisotropy techniques in an effort to reveal any underlying issues that may arise from using the labelled fragment in place of the labelled antigen. Fluorescence spectroscopy is a technique that offers sensitivity down to a single-molecule level, with a signal-to-niose ratio that improves with measurement duration, and is therefore well placed for the development of ultrasensitive biosensors such as fluorescence-based immunoassays.

2 THEORY

The timed-resolved fluorescence anisotropy, $r(t)$, is an angular correlation function that conveniently is defined as (Lakowicz, 2006)

$$r(t) = \frac{I_{vv}(t) - I_{vh}(t)}{I_{vv}(t) + 2I_{vh}(t)} \quad (1)$$

where $I_{vv}(t)$ and $I_{vh}(t)$ are the recorded fluorescence intensities at time t following an excitation event at $t = 0$, and where the first and second subscript refers to the orientation of the excitation and emission polariser respectively. In the simplest case, with a fluorophore rigidly attached to a macromolecule that undergoes free isotropic rotational diffusion, the anisotropy is described by a mono-exponential decay function

$$r(t) = r_0 \exp\left(-\frac{t}{\phi_{global}}\right) \quad (2)$$

The time-zero ansiotrpoy r_0 can take the maximum value 0.4 for one-photon excitation (assuming the transition dipoles for absorption and emision are parallel). The correlation time ϕ_{global} is related to the hydrodynamic volume, V, of the macromoleule and can be calculated form the Stokes-Einstein Debye relationship

$$\phi_{global} = \frac{\eta V}{k_b T} \quad (3)$$

wher η is the viscosity of the solvent, k_b Boltzmann constant and T the absolute temperature.

It is often observed when analysing the fluorescence anisotropy recorded from fluorescently labelled macromolecules, for example proteins, that the anisotropy expression is given by a bi-exponential decay law

$$r(t) = b_1 \exp\left(-\frac{t}{\phi_{local}}\right) + b_2 \exp\left(-\frac{t}{\phi_{global}}\right) \quad (4)$$

The correlation times are then often interpreted to reflect on a local wobbling motion of the fluorophore in its binding site, ϕ_{local}, and an overall rotational diffusion of the macromolecule, ϕ_{global}. If $\phi_{\text{global}} >> \tau$, it will not be resolved in a time correlated single-photon counting experiment, and will only be evident by a plateau value of value r_∞. If there is also a fraction of free fluorophore that is not bound to the macromolecule, as in our case, there might be a fraction of the antigen that is not bound to the antibody and then the anisotropy expression reads

$$r(t) = b_1 \exp\left(-\frac{t}{\phi_{free}}\right) + b_2 \exp\left(-\frac{t}{\phi_{local}}\right) + r_\infty \quad (5)$$

In the present work anisotropy data was analysed according to equation (5).

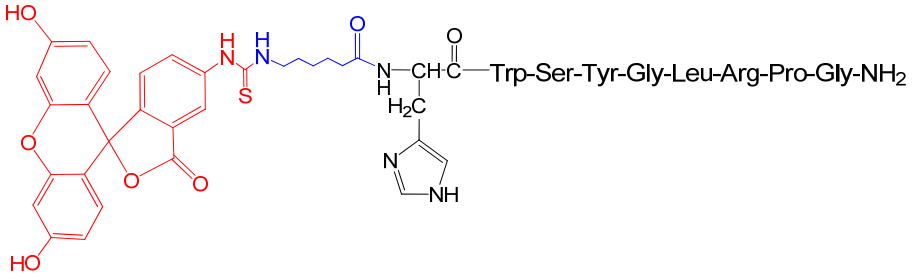


Figure 1: The pyroGlu on GnRH-1 is replaced by a fluorescein that is attached to the peptide backbone of the peptide fragment by a caproic acid linker.

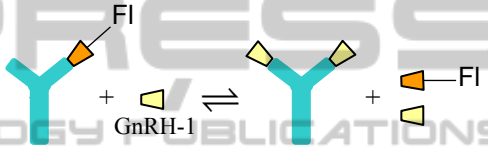
3 MATERIALS AND METHODS

GnRH-1 is a decapeptide with the amino acid sequence pyroGlu[1]-His[2]-Trp-[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2. GnRH-1 (cat. no. L7134) and [des-pGlu[1]]-RH-LH (cat. no. L8762) were purchased from Sigma-Aldrich, Inc. The labelled fragment ([des-pGlu[1]]-RH-LH-Acp-FITC) was purchased from GL Biochem (Shanghai) Ltd (Figure 1).

Antibody specific to GnRH-1 was produced from the monoclonal antibody secreting cell line, 7B10.1D10, by using standard cell culture techniques (Khan et al, 2003). From experiment, the antibody was found to have an IgG_1 isotype and was shown to bind to both GnRH-1 and [des-pGlu[1]]-RH-LH by ELISA. The antibody was also shown to bind to Protein L agarose resin purchased from Pierce (cat. no. 20510) thus implying that the antibody has kappa light chains and, more specifically, Vκl light chains. The Protein L agarose resin was used in the antibody purification procedure following the Pierce protocol. All solutions for fluorescence measurements were prepared using a 20 mM sodium phosphate buffer, pH 7.4.

Time-resolved fluorescence and anisotropy decay time measurements were performed using the time-correlated single photon counting technique (TCSPC) on a FluoroCube (Horiba Jobin Yvon IBH Ltd, Glasgow), (Apperson et al, 2009). The excitation source was a 482 nm PicoBrite running at 10 MHz repetition rate and with an instrumental full width half maximum of 50 ps. The emitted fluorescence was observed on a TBX-04 detector and data was analysed in IBH DAS6 software suite.

In the first instance the purpose of the experiment was to compare the time-resolved fluorescence measurements of the labelled fragment with those from the labelled fragment in the presence of the antibody 7B10.1D10 to confirm that the altered GnRH structure could be captured by the antibody. Secondly, it was to assess that the effect could be reversed by adding GnRH-1 to the mixture (Figure 2).



Labelled fragment bound to antibody

Figure 2: Schematic illustrating the initial bulk solution condition where it is most likely that one labelled fragment is bound to the antibody and the end bulk solution condition where the antibody binding sites are most likely to be occupied by GnRH-1.

The time-resolved fluorescence, recorded in the magic angle condition, and anisotropy decays of six different mixtures (Table 1) were measured. Solution A contained the labelled fragment and solution B contained the labelled fragment in the presence of antibody where the number of potential antibody binding sites is much greater than the number of labelled fragment molecules in the solution. GnRH-1 is added to the mixture giving solutions C-F.

Table 1: The concentrations of antibody, labelled fragment and GnRH-1 for each mixture.

sample	[7B10.1D10] μM	[labelled fragment] nM	[GnRH-1] M
A	-	4.94	-
B	0.33	1.97	-
C	0.33	1.10	0.56×10^{-6}
D	0.11	0.66	1.13×10^{-3}
E	0.11	0.66	1.69×10^{-3}
F	0.11	0.65	5.61×10^{-3}

Table 2: Lifetimes resolved into two components τ_1 and τ_2 and also anisotropies at r_0, r_∞ and correlation times ϕ_1, ϕ_2 for all solutions.

sample	τ_1 / ns		τ_2 / ns		χ^2	r_0	ϕ_1 / ns		ϕ_2 / ns		r_∞	χ^2
A	0.76	(8.94%)	2.88	(91.06%)	1.03	0.34	0.22	(29.7%)	0.59	(70.3%)	0.00	1.00
B	1.17	(4.82%)	4.09	(95.18%)	1.03	0.49	1.70	(78.1%)	0.16	(21.9%)	0.20	1.06
C	0.93	(5.46%)	3.99	(94.54%)	1.17	0.37	0.96	(67.7%)	0.24	(32.3%)	0.21	1.10
D	0.61	(10.19%)	3.25	(89.71%)	1.43	0.31	0.00	(0.0%)	0.41	(100.0%)	0.13	1.18
E	0.68	(10.55%)	3.26	(89.45%)	1.43	0.36	0.14	(9.4%)	0.43	(90.6%)	0.10	0.94
F	0.90	(13.98%)	3.21	(86.02%)	1.21	0.37	0.12	(10.8%)	0.47	(89.2%)	0.07	1.03

4 RESULTS

On examining the time-resolved intensity decay times of each mixture it is evident that the decay time must be resolved into two or more components. Analysis for two components is shown in Table 2. The trend is more clearly shown qualitatively in the decay curves.

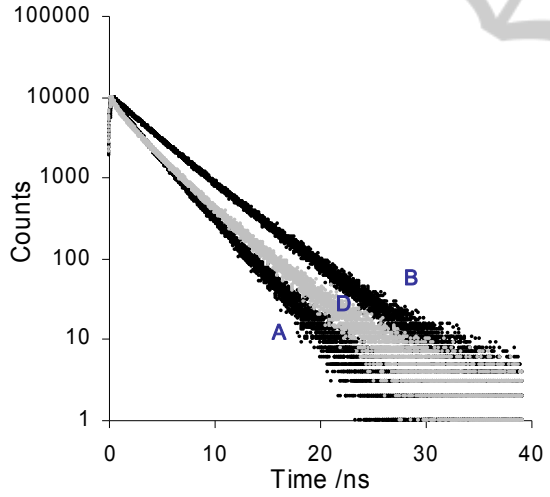


Figure 3: Time-resolved decays showing qualitatively the difference between decay times of the labelled fragment (A), the labelled fragment in the presence of antibody (B) and when an excess of GnRH-1 is added to the solution (D).

The most dramatic changes are shown in Figure 3 where the greatest difference in the decay time is seen between the labelled fragment (A) and the labelled fragment in the presence of antibody (B).When an excess of GnRH-1 is added to the solution (D) the mean decay time is reduced and the photophysics is also altered.

A similar pattern is repeated in the time-resolved anisotropy measurements and the greatest change in anisotropy occurs between the labelled fragment (A) and the labelled fragment in the presence of antibody (B) (Figure 4).

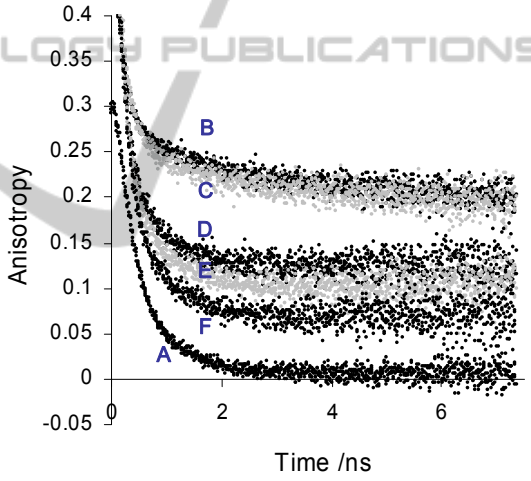


Figure 4: Time resolved anisotropy decays for all solutions. The anisotropy reduces as more GnRH-1 is added to the solution (C-F).

As more GnRH-1 is added to the solution the anisotropy falls suggesting that more of the labelled fragment is spending more time free in solution than bound to the antibody thus revealing the competition between the labelled fragment and the GnRH-1 for the 7B10.1D10 binding sites. Time-resolved anisotropy decay measurements reveal information about the rotational motion of the labelled peptide molecules. Again, in almost all cases there are two significant contributions to the rotational motion but no attempt has been made as yet to explain the results quantitatively.

5 CONCLUSIONS

As a first step in designing an FPI for GnRH-1 we used time-resolved fluorescence techniques (where the antigen GnRH-1 competes with a synthetic labelled fragment for the binding sites on the specific GnRH-1 antibody). We demonstrated qualitatively that the labelled fragment can bind to the antibody and that competition between the labelled fragment and the GnRH-1 for the binding sites on the antibody 7B10.1D10 does occur.

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