Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biosensors and Bioelectronics 20 (2005) 2061–2070
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Quantitative analysis of the response of an electrochemical biosensor for progesterone in milk

Y.F. Xu*, M. Velasco-Garcia, T.T. Mottram

Silsoe Research Institute, Bedfordshire MK45 4HS, UK

Received 24 May 2004; received in revised form 24 August 2004; accepted 14 September 2004
Available online 28 October 2004

Abstract

An electrochemical biosensor for progesterone in cow's milk was developed and used in a competitive immunoassay by Hart et al. (1977, Studies towards a disposable screenprinted amperometric biosensor for progesterone, Biosens. Bioelectron. 12, 1113–1121). The sensor was fabricated by depositing anti-progesterone monoclonal antibody (mAb) onto screen-printed carbon electrodes (SPCEs) which were coated with rabbit anti-sheep IgG (rIgG). This sensor was operated following the steps of competitive binding between sample and conjugate (alkaline-phosphatase-labelled progesterone) for the immobilised mAb sites and measurements of an amperometric signal in the presence of *p*-nitrophenylphosphate using either colorimetric assays or cyclic voltammetry. The hook effect of the progesterone biosensor was found in the concentration range of milk progesterone between 0 and 5 ng/ml when the sensor was fabricated using a loading of 25 ng rIgG per electrode of a diameter of 3 mm and a 1/50 dilution of mAb. A computer model has been developed in this study to simulate the operation of this progesterone biosensor with consideration of the fabrication processes. This paper presents the results of validating the computer model and the model has predicted the hook effect as observed in tests. The model thus reveals that the hook effect is determined by the total number of binding sites available and the rates of labelled and unlabelled progesterone diffusing towards the sensor surface and the binding rates.

Keywords: Hook effect; Computer model; Progesterone; Biosensor

1. Introduction

Biosensors have experienced continuous development since their introduction in 1960s. Among the many properties of a biosensor, the two most important are specificity and sensitivity (Sadana, 2002). Because of these properties, biosensors can be used to detect analytes at very low concentrations in complex biological media such as blood, serum, urine, foods with minimum sample treatment. It has been reported to measure analytes in the concentration ranges between 1 pM and 1 mM (Byfield and Abuknesha, 1994). A common feature of biosensor performance is the hook effect that can occur at either low or high concentrations of analytes.

Hook effect was first noted in a two-site immunometric assay by Miles et al. (1974) and it refers to the parabolic or flat shapes of the sensor response curves at either high or low concentrations of analytes. For the high-dose hook effect, low results are artificially obtained from samples having extraordinarily high concentrations of analytes and some examples were given by Selby (1999). High-dose hook effect could result in wrong treatments to patients in the clinical applications of immunoassay (Ismail et al., 2002). For the low-dose hook effect, samples containing very low analyte concentrations give ambiguous and high results. Using human growth hormone (hGH) in the tests, Fernando et al. (1992a) had studied the low-dose hook effect in a competitive homogeneous immunoassay and concluded that, in reagent-limited assays, the low-dose hook effect was caused by the cooperative interactions between antibodies. In the two-site, two-step sandwich assay also studied by Fernando et al. (1992b), the low-dose

* Corresponding author. Tel.: +44 1525 860000; fax: +44 1525 860156.
E-mail address: yongfu.xu@bbsrc.ac.uk (Y.F. Xu).

0956-5663/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2004.09.009

Nomenclature

A	area (m^2)
C	analyte concentration (mol m^{-3})
D	diffusivity (m^2 s^{-1})
h	constant
K	affinity constant (M^{-1})
K_B	Boltzmann's constant, 1.38054E −23 (JK^{-1})
k_a	association rate of antibody/antigen binding (M^{-1} s^{-1})
k_d	dissociation rate of antibody/antigen binding (s^{-1})
m	molecular weight (kg)
N_A	Avogadro number, 6.022E +23 molecules (mol^{-1})
n	total number of antigen types that are competing for binding sites ($n = 2$)
OD	optical density of colorimetric tests
$P(k)$	probability of one bound antigen blocking k binding sites, $k = 1, 2, 3, \ldots$
r'	hydrodynamic radius of an antibody (m)
T	temperature (K)
t	time (s)
U	velocity (m s^{-1})
V	partial specific volume of an antibody (m^3 kg^{-1})
y	Cartesian coordinate normal to the sensor surface

Greek letters

α	washing coefficient
Γ	surface density (mol m^{-2})
Γ_{ttl}	density of binding sites for the competitive binding (mol m^{-2})
Γ^{o}_{IgG}	maximum surface density of the adsorbed rIgG (mol m^{-2})
θ	surface coverage
μ	viscosity (kg m^{-1} s^{-1})
χ	sample concentration used to fabricate the progesterone sensor (mol m^{-3})

Subscripts

a	association
d	dissociation
i	ith antigen competing for binding sites, $i = 1, 2$ for this progesterone biosensor
IgG	properties of rabbit IgG
mAB	properties of sheep anti-progesterone monoclonal antibody

Superscripts

P	progesterone or labelled progesterone
s	surface of the biosensor

hook effect was attributed to the desorption of the bound analytes due to conformational changes after the reaction of the labelled antibody with several epitopes of the adsorbed analytes.

An electrochemical biosensor for cow's milk progesterone has been developed using a competitive immunoassay (Hart et al., 1997; Pemberton et al., 2001). This biosensor was fabricated by depositing sheep anti-progesterone monoclonal antibody (mAB) onto screen-printed carbon electrodes (SPCEs), which were coated with rabbit anti-sheep IgG (rIgG). This sensor was operated following the steps of competitive binding between sample and conjugate (alkaline-phosphatase-labelled progesterone) for the immobilised mAB sites and measurements of an amperometric signal in the presence of *p*-nitrophenylphosphate using either colorimetric assays or cyclic voltammetry. The hook effect of this progesterone biosensor was observed in the low concentration range of milk progesterone between 0 and 5 ng/ml when the sensor was fabricated using an rIgG loading of 25 ng per electrode and a mAB dilution rate of 1/50 (Pemberton et al., 2001).

One of the main problems of using this biosensor in agricultural and veterinary applications for health diagnosis and quality control has been in the practicalities of biosensor deployment, issues such as stability, instrumentation design and the fabrication of low cost sensors (Velasco-Garcia and Mottram, 2003). A computer model has been developed to simulate the operation of this progesterone biosensor with consideration of the fabrication processes with the aim to understand and optimise its performance. The model includes estimation of the immobilised mAB binding sites and computational solutions of the competitive binding between progesterone and labelled progesterone. This model was validated against experimental data and was used to quantitatively determine the factors that have contributed to the hook effect observed. This model can be used to optimise design and operation of biosensors for higher accuracy and wider detecting ranges in shorter detection times.

2. Fabrication and operation of the progesterone sensor

Disposable screen-printed carbon electrodes (SPCEs) were printed using D14 ink to a thickness of 20 μm onto PVC sheets with a thickness of 0.5 mm. Fabrication of this biosensor include deposition onto a circular working area of the electrode (3 mm diameter) a layer of rabbit anti-sheep IgG (to capture and orientate the sheep anti-progesterone monoclonal antibodies), which was formed by loading a 5 μl solution onto an electrode and incubating overnight at 4 °C in a highly humidified chamber and washing away the remaining solution on the electrode after the incubation. A second layer of anti-progesterone monoclonal antibody (mAB) was formed over the rIgG layer by loading a 5 μl solution of mAB onto the surface and keeping at room temperature for 2 h in a highly humidified chamber and then washing away the

remaining mAB solution on the electrode after the incubation. Full details of the chemicals and reagents used to fabricate the progesterone sensor were given by Hart et al. (1997) and Pemberton et al. (2001).

This biosensor employs a competitive immunoassay format. It relies upon the reduction in binding of alkaline-phosphatase-labelled progesterone in the presence of endogenous milk progesterone within 30 min allowed for competitive binding. To test its performance using a colorimetric assay, the sensor was rinsed, dried, cut and placed into individual wells of a 96-well immunoassay plate for 60 min. Enzyme substrate used was *p*-nitrophenylphosphate and the *p*-nitrophenol produced in the enzymatic reaction was detected by measuring the absorbance at 405 nm. The absorbance is inversely proportional to the concentration of progesterone in milk.

3. The computer model

3.1. Immobilisation of rabbit IgG (rIgG)

The binding sites on the sensor surfaces for competitive binding were formed by immobilised sheep anti-progesterone monoclonal antibody (mAB) on the SPCE surface coated with rabbit anti-sheep IgG, as shown in Fig. 1. The total number of binding sites formed on the surfaces depends on the quality and quantities of the rIgG and mAB layers and the washing conducted during the fabrication.

After studying adsorption of IgG onto surfaces using AFM, You and Lowe (1996) confirmed that IgG adsorbed onto surfaces in the form of a flat monolayer with a thickness of 5 nm in non-specific binding and in the form of closely packed monolayer with a thickness of 11 nm in specific binding. After washing the monolayer with SDS, AFM studies found that surface coverage by the strand-like structure is typically less than 40% of the flat monolayer, whereas the granular structure has a surface coverage of typically more than 80% of the closely packed monolayer. Similar effect on IgG adsorption by washing was also found in the study by Malmsten et al. (1996) using Ellipsometry, total internal reflection fluorescence spectroscopy (TIRF) and enzyme immunoassay (EIA).

The adsorption of rIgG onto the carbon electrode is non-specific and so rIgG is adsorbed on the surface in the form of a flat monolayer with a thickness of 5 nm as observed by You and Lowe (1996). When the adsorption reaches equilibrium overnight, the surface coverage of rabbit IgG can be estimated using Langmuir adsorption isotherm as:

$$\theta_{\mathrm{IgG}} = \frac{K_{\mathrm{IgG}}\chi_{\mathrm{IgG}}}{1 + K_{\mathrm{IgG}}\chi_{\mathrm{IgG}}} \tag{1}$$

Therefore the density of rIgG on the sensor working area is $\Gamma^{\mathrm{o}}_{\mathrm{IgG}}\,\theta_{\mathrm{IgG}}$ when the concentration of rIgG loading is x_{IgG}. This density reaches its maximum when the surface is fully covered.

Even though Langmuir isotherm was found too simple for the non-specific binding of proteins onto continuous sites (Hall, 2001), because it was used here in conjunction with experimental data as illustrated later in this paper, the inaccuracy caused by the simplicity of Langmuir isotherm should have been taken into account through the experimental data to a large extent.

Assuming the adsorbed rIgG can form a closely packed monolayer as the plan view shown in Fig. 2 and the area covered by each rabbit IgG is A_{IgG}, the maximum density of adsorbed rabbit IgG is:

$$\Gamma^{\mathrm{o}}_{\mathrm{IgG}} = \frac{1}{N_{\mathrm{A}} A_{\mathrm{IgG}}} \tag{2}$$

The SPCE surfaces coated with rIgG was then washed using a solution containing 0.05% Tween 20 (a detergent) before mAB was loaded over it and a large amount of loosely bound rIgG will be lost during the washing. The rIgG density available for immobilising mAB is estimated as:

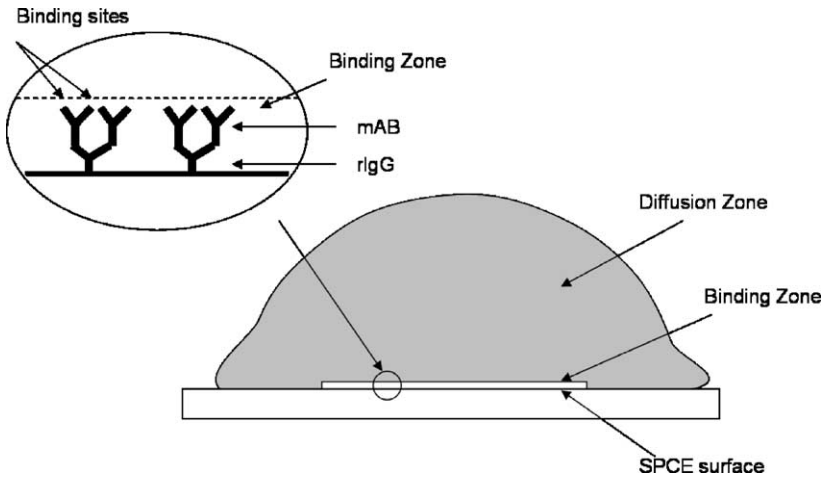

Fig. 1. Models for surface fabrication and competitive binding of the progesterone biosensor.

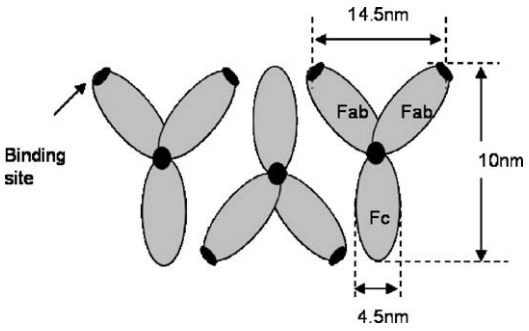

Fig. 2. Plan view of the closely packed rabbit IgG with a flat orientation on a surface.

$$\Gamma_{\mathrm{IgG}} = \alpha_{\mathrm{IgG}}\theta_{\mathrm{IgG}}\Gamma^{\mathrm{o}}_{\mathrm{IgG}} \tag{3}$$

α_{IgG} is the surface coverage of an IgG monolayer after washing. You and Lowe (1996) found that the surface coverage of the strand-like structures is typically less than 40% after washing a surface fully covered by IgG using SDS.

3.2. Immobilisation of mAB

When mAB is loaded onto the rIgG layer, anti-progesterone monoclonal antibodies bind specifically on the Fabs of the IgG and form the binding sites for the competitive binding between the progesterone in milk and labelled progesterone. The total number of binding sites produced is determined by a number of factors, including the density of rIgG Fabs, the concentration of the mAB buffer, incubation time, reaction between mAB and the IgG monolayer and the washing. The incubation is normally long enough for the adsorption of mAB to rIgG to reach equilibrium, the coverage of mAB onto the IgG is also estimated using Langmuir adsorption isotherm as:

$$\theta_{\mathrm{mAB}} = \frac{K_{\mathrm{mAB}}\chi_{\mathrm{mAB}}}{1 + K_{\mathrm{mAB}}\chi_{\mathrm{mAB}}} \tag{4}$$

The density of mAB bound onto the sensor surface is

$$\Gamma_{\mathrm{mAB}} = \alpha_{\mathrm{mAB}}\theta_{\mathrm{mAB}}\Gamma_{\mathrm{IgG}} \tag{5}$$

Combining Eqs. (3) and (5) and because each rIgG has two identical Fabs for immobilising mAB and each immobilised mAB has two identical binding sites, the density of sites for the competitive binding is:

$$\Gamma_{\mathrm{ttl}} = 4\alpha_{\mathrm{mAB}}\alpha_{\mathrm{IgG}}\theta_{\mathrm{mAB}}\theta_{\mathrm{IgG}}\Gamma^{\mathrm{o}}_{\mathrm{IgG}} \tag{6}$$

3.3. The competitive binding

A mixture of milk and label progesterone was then loaded on the sensor surface with immobilised mAB binding sites. The fluid domain formed over the biosensor surface is divided into two zones, i.e. the diffusion zone and the binding zone as shown in Fig. 1. The movement of analytes inside the diffusion zone is governed by Navier–Stokes equation as:

$$\frac{\partial C_i}{\partial t} + U\nabla C_i - D_i\nabla^2 C_i = 0 \tag{7}$$

U is the flow velocity inside the diffusion zone, which is zero if fluid is loaded on the surface through a micro-dispenser and will be very slow in the case of flow-cells.

The binding zone contains all the immobilised mAB and it has a depth equivalent to the height of the mAB on the sensor working area, which is about 15 nm. Flow movements and analyte diffusion are ignored inside the binding zone, so only competitive binding occurs inside it. Antigen binding onto immobilised antibodies is simulated using the one-order reaction model as:

$$\mathrm{Ab} + \mathrm{Ag} \underset{k_{\mathrm{d}}}{\overset{k_{\mathrm{a}}}{\Leftrightarrow}} \mathrm{Ab}\cdot\mathrm{Ag} \tag{8}$$

$$\frac{\partial \Gamma_i}{\partial t} = k_{\mathrm{a},i}C_i^{\mathrm{s}}\left(\Gamma_{\mathrm{ttl}} - \sum_{j=1}^{n}\Gamma_j\right) - k_{\mathrm{d},i}\Gamma_i \tag{9}$$

The flux of antigen through the interface between the two zones is

$$D\frac{\partial C_i}{\partial y}|_{y=0} = \frac{\partial \Gamma_i}{\partial t} \tag{10}$$

The quantities of progesterone and labelled progesterone bound onto the sensor are obtained by solving Eqs. (8)–(10) using the density of binding sites estimated from Eq. (6) as the boundary condition.

4. Input parameters for the model

4.1. Diffusivities of the progesterone and labelled progesterone

The diffusion of progesterone and labelled progesterone in solution is the result of continuous collision between macromolecules. The diffusivity can be expressed by the Stokes–Einstein relation as (Berg and van Hippel, 1985; Cantor and Chemmel, 1980):

$$D = \frac{K_{\mathrm{B}}T}{6\pi\eta r'} \tag{11}$$

The hydrodynamic radius r' can be calculated using

$$r' = \left(\frac{3mV}{4\pi N_{\mathrm{A}}}\right)^{1/3} \tag{12}$$

Progesterone has a molecular weight of 314 Da and a hydrodynamic radius of 0.5 nm (Nghiem et al., 2004), the estimated diffusivity of progesterone in the mixture of milk and buffer (a ratio of 3:5) using Eq. (11) is $3.13 \times 10^{-10}\ \mathrm{m^2\ s^{-1}}$.

The molecular weight of alkaline-phosphatase-labelled progesterone is 140 kDa, which is much heavier than that of progesterone, so its physical properties are likely to be

largely determined by alkaline-phosphatase. At near room temperature, the partial specific volume, i.e. V in Eq. (12), of all globular proteins studied to date are between 0.69 and 0.76 $cm^3\,g^{-1}$ (Chalikian, 2003). A partial specific volume of 0.725 cm^3/g was used here to estimate the diffusivity of alkaline-phosphatase-labelled progesterone. The diffusivity of labelled progesterone in the mixture of milk and buffer (a ratio of 3:5) estimated using Eqs. (11) and (12) is $5.0 \times 10^{-11}\,m^2/s$.

4.2. Surface density of the immobilised binding sites Γ_{ttl}

As shown in Eq. (6), the coverage of rIgG and mAB, i.e. θ_{IgG} and θ_{mAB}, are needed for the estimation of the density of total available binding sites. However, it is difficult to obtain them directly because of the difficulties to measure the affinity constants K_{IgG} and K_{mAB}, and the uncertainty in quantifying sample concentrations used to produce the immobilised binding sites, i.e. x_{IgG} and x_{mAB}.

In order to estimate the density of total available binding sites of the progesterone biosensor, it is assumed here that the absorbance at 405 nm of the colorimetric tests is linearly related to the density of binding sites. Taking into account Eq. (6), the absorbance, i.e. optical density (OD), is expressed as:

$$\mathrm{OD} = h\alpha_{mAB}\alpha_{IgG}\theta_{mAB}\theta_{IgG}\Gamma^{o}_{IgG} + \mathrm{OD}_0 \quad (13)$$

where h is a constant and OD_0 is the offset.

For the colorimetric results of the progesterone biosensor shown in Fig. 3 (Hart et al., 1997), only one of the parameters in Eq. (13), either x_{IgG} or x_{mAB}, was changed each time. Using these colorimetric results and Eq. (13), the calculations of θ_{IgG} and θ_{mAB} are given in Tables 1 and 2.

Table 1
Estimating the coverage of rIgG, i.e θ_{IgG}

	IgG concentration ($mol\,m^{-3}$)	OD
Input data		
IgG loading 0 ng per electrode	0	0.02
IgG loading 10 ng per electrode	x_{IgG}	0.51
IgG loading 100 ng per electrode	$10x_{IgG}$	0.67
Estimated		
h	0.675	
$K_{IgG}\,x_{IgG}$ in Eq. (1)	2.8	
θ_{IgG} at the loading 25 ng per electrode	0.875	

Table 2
Estimating the coverage of mAB, i.e. θ_{mAB}

	mAB concentration ($mol\,m^{-3}$)	OD
Input data		
Nil mAB	0	0.18
mAB dilution 1:100	x_{mAB}	0.48
mAB dilution 1:10	$10x_{mAB}$	0.86
Estimated data		
h	0.722	
$K_{mAB}\,x_{mAB}$ in Eq. (4)	0.711	
θ_{mAB} at dilution rate 1:200	0.262	
θ_{mAB} at dilution rate 1:100	0.416	
θ_{mAB} at dilution rate 1:50	0.587	

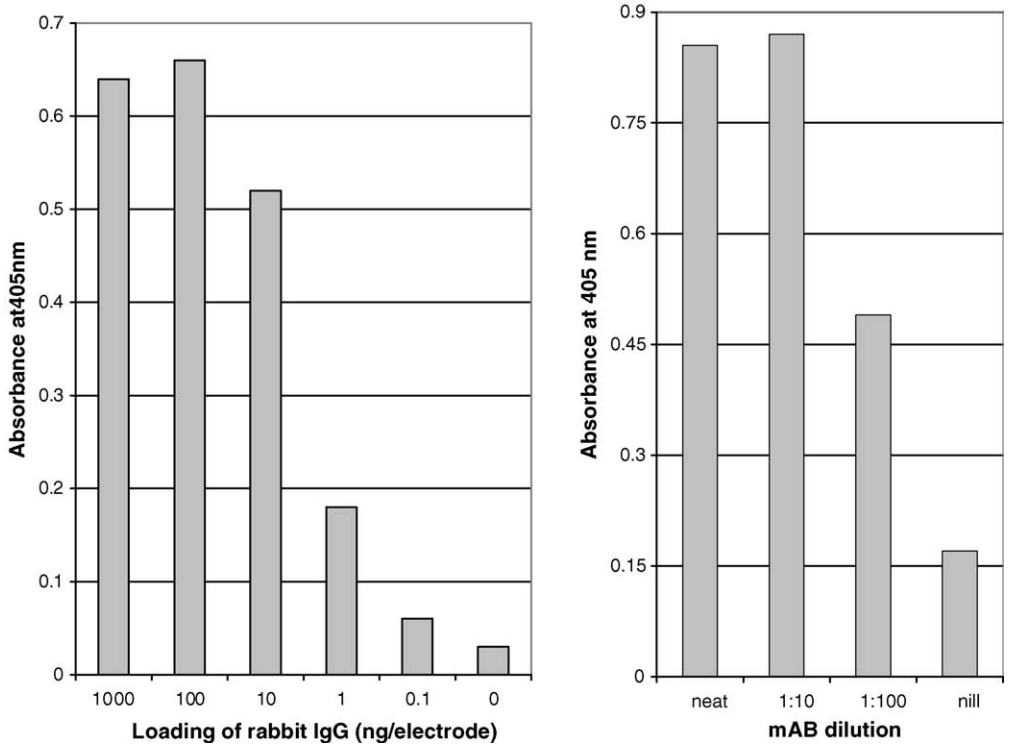

Fig. 3. Optimisation of the rIgG loading and mAB dilution rates for fabricating the progesterone biosensor (Hart et al., 1997).

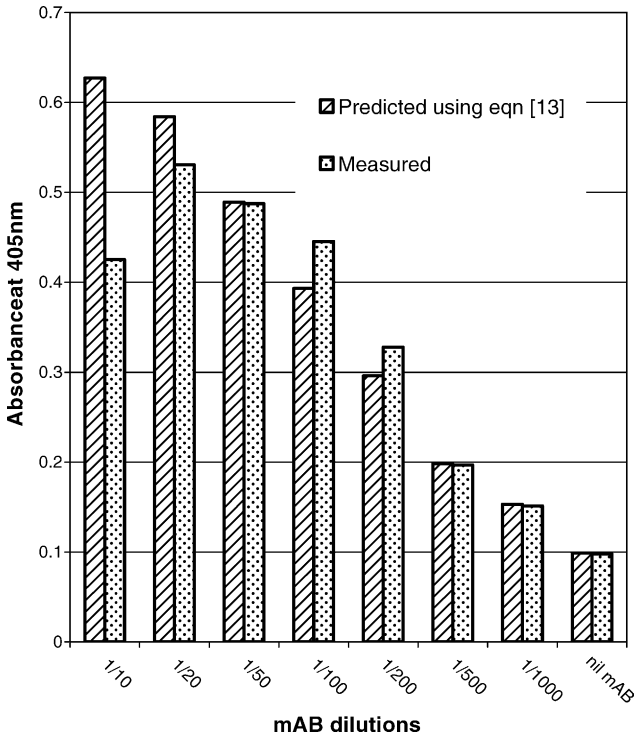

Fig. 4. Comparing measurements with the predictions using Eq. (13).

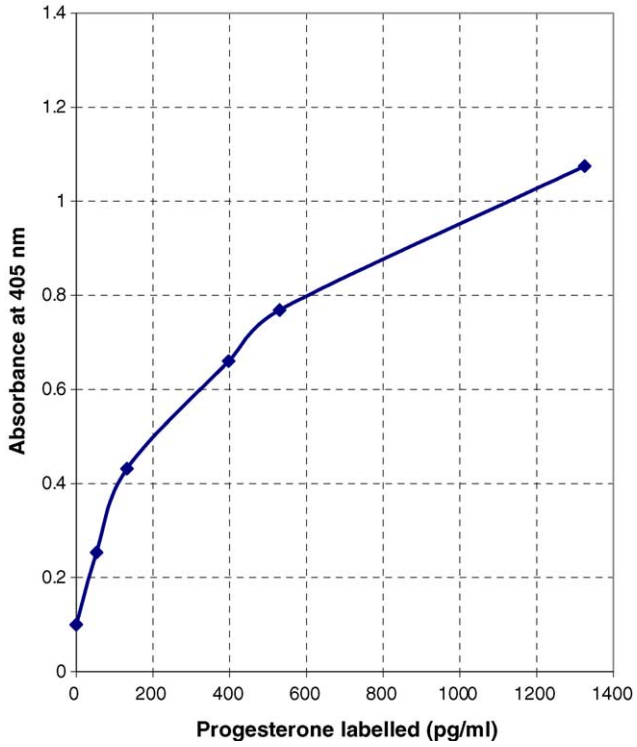

Fig. 5. Experimental results relating absorbance at 405 nm to the concentration of labelled progesterone inside the wells of an immunoassay plate.

Constant h is 0.722 from the estimation of mAB coverage and it is 0.675 from the rIgG coverage estimation. The difference between them is only 6.6% and this indirectly indicates that this simplified approach of estimating surface coverage is producing reasonable results within the concentration ranges of the tests shown in Fig. 3.

To prove the capability of this simplified approach on estimating surface coverage, new tests were carried out following the same procedures as described by Hart et al. (1997). The new tests were done using progesterone in buffer alone and the sensors used have a circular working area of a diameter of 3 mm, instead of a square working area of 3 mm × 3 mm as used by Hart et al. The comparison between measured absorbance and the predicted absorbance using Eq. (13) is given in Fig. 4. Eq. (13) has produced satisfactory predictions in the range of mAB dilution rates from nil to 1/20. However, Eq. (13) cannot be used to optimise the sensor performance.

4.3. Steric hindrance

Depending on distribution of the immobilised binding sites, one antigen, e.g. progesterone or labelled progesterone, could block more than one binding sites, i.e. steric hindrance. The proportion of an antigen blocking more than one binding sites can be calculated using stochastic theory. When the binomial distribution was approximated with a Poisson distribution as done by Muller et al. (1998) for multivalent binding to fixed ligands, and assuming the binding sites are distributed randomly and the bindings are specific, the probability of one progesterone blocking k binding sites is:

$$P(k) = \frac{(\Gamma_{\mathrm{ttl}} A^{\mathrm{P}})^k}{k!} \frac{\mathrm{e}^{-\Gamma_{\mathrm{ttl}} A^{\mathrm{P}}}}{(1 - \mathrm{e}^{-\Gamma_{\mathrm{ttl}} A^{\mathrm{P}}})} \tag{14}$$

where in Eq. (14) $k = 1, 2, 3, \ldots$.

4.4 Colorimetric output

Tests were carried out to relate the predicted surface binding of labelled progesterone to the absorbance at 405 nm. Samples with fixed amount of labelled progesterone were put into individual wells of an immunoassay plate and 1 mg/ml *p*-nitrophenylphosphate was added into each well to a total volume of 100 μl, i.e. the volume used by Hart et al. (1997). After 60 min of incubation, the formation of *p*-nitrophenol was recorded by measuring the absorbance at 405 nm. The test results were given in Fig. 5.

Assuming all the labelled progesterone bound onto the sensor working area reacts with *p*-nitrophenylphosphate within 60 min, the binding predicted by model was converted to the concentrations of labelled progesterone in the wells of an immunoassay plate and the corresponding absorbance at 405 nm was decided using Fig. 5.

5. Results and discussions

5.1 The association rate and coefficient α_{mAB}

Apart from the parameters shown in Table 3, the association rate k_a in Eq. (9) and coefficient α_{mAB} in Eq. (6) are still required by the model, but no published data were found on them and no simple methods were found to measure them directly. To make the model useful for the progesterone biosensor, these two parameters were determined using the computer model by fine tuning the predicted results against experimental data at selected test cases. Then predictions with these determined k_a and α_{mAB} were compared with more test data to validate the model and the validated model was consequently used to predict the sensor performance.

Table 4 shows the measured kinetic constants of antigen–antibody reactions found in literatures. Much higher affinity constants, up to 10^{12} and 10^{13} M^{-1}, were reported by Voss (1993).

The affinity constant of progesterone and sheep anti-progesterone monoclonal antibody was $2.82 \pm 0.64 \times 10^{10}$ l mol^{-1} (O'Connor and Gosling, 1997) which is within the range reported in Table 4. To decide the association rate between them, it was varied between 10^3 and 10^8 in the model and Fig. 6 shows the predicted results for the sensors fabricated using mAB dilution rates of 1/200 and 1/50 and a rIgG loading of 25 ng per electrode. It is clearly shown that the sensor is not sensitive to the association rate as long as it is higher than 10^4, which is below the lowest association rate found between antibody and antigen as shown in Table 4. Therefore it is assumed here that the performance of this progesterone immunosensor is not sensitive to the association rate and it was given a value of 10^4 in the model. The dissociation rate was estimated using the affinity constant and the association rate of 10^4.

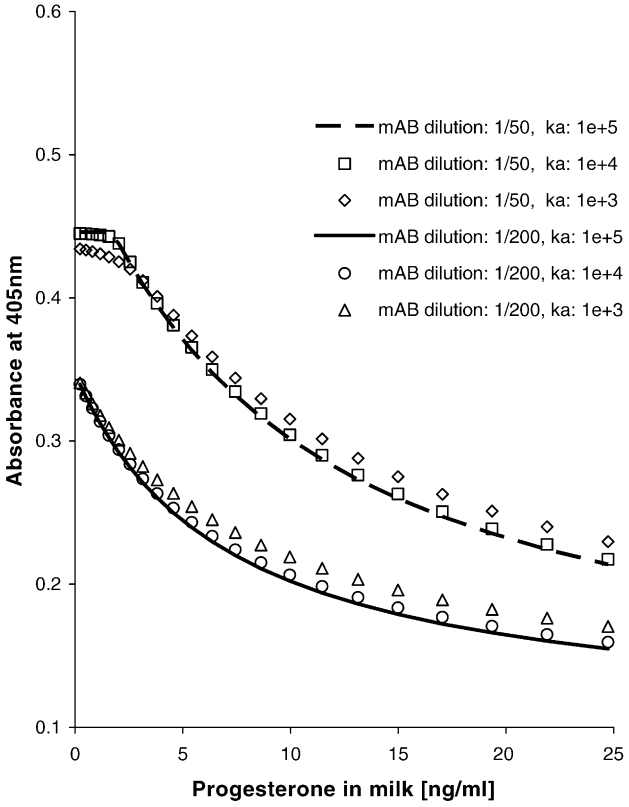

Fig. 6. Determining the association rate between mAB and progesterone or labelled progesterone.

Table 3
Other parameters used in the model

Constant	Value
Milk viscosity (kg m^{-1} s^{-1})	0.002
Conjugate viscosity (kg m^{-1} s^{-1})	0.001
Conjugate and milk ratio	5:3
Conjugate concentration (ng progesterone /ml)	6.25
Temperature (K)	293
A_{IgG}	95 nm^2 (estimated using the dimension shown in Fig. 2)
A^p for progesterone (m^2)	Using a diameter of 1.0 nm (Nghiem et al., 2004)
A^p for labelled progesterone (m^2)	Using a diameter of 6.9 nm estimated using Eq. (12)
α_{IgG}	0.4 [as observed by You and Lowe (1996)]

Table 4
Kinetic constants of immunochemical reactions

Constant	Steward (1977) [quoted by Bongrand (1999)]	Mason and Williams (1986) and Hoylaerts et al. (1990)
Affinity constant K (M^{-1})	10^4–10^{10}	10^7–10^{11}
Association rate k_a (M^{-1} s^{-1})	8×10^6–1.8×10^8	10^4–10^7
Dissociation rate k_d (s^{-1})	3.4×10^{-4}–6×10^3	10^{-5}–10^{-3}

The coefficient α_{mAB} was determined by fine tuning the predictions against a measured sensor performance curve when the sensor was fabricated using a mAB dilution rate of 1/200 and a rIgG loading of 25 ng per electrode, as reported by Pemberton et al. (2001). At this high dilution rate, the effect of steric hindrance on the surface should be weak and no hook effect was observed in the sensor's performance. Fig. 7 shows the comparison of the test results and the prediction when coefficient α_{mAB} was fixed at 0.48. The factors that could have contributed to α_{mAB} include: surface washing after mAB immobilisation, non-specific binding of progesterone and labelled progesterone onto the sensor working area and the binding of impurity, such as fat and milk proteins as reviewed by Nakanishi et al. (2001).

The test results shown in Fig. 4 were used to validate the model with the determined k_a and α_{mAB} and the results are given in Fig. 8. The maximum differences between the measured and predicted absorbance at 405 nm is 23% at the mAB dilution rate of 1/10, which is 48% when Eq. (13) is used as shown in Fig. 4. The maximum difference within the mAB

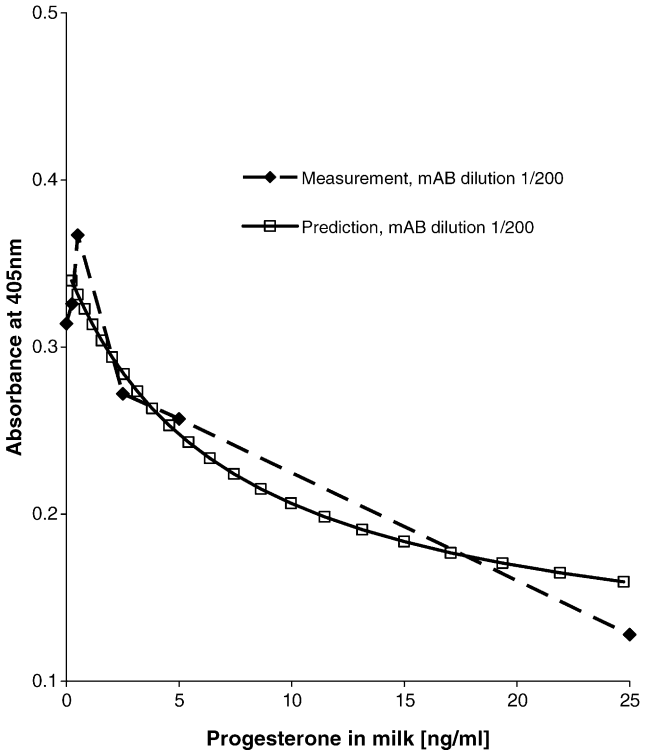

Fig. 7. Determining the washing coefficient α_{mAB}.

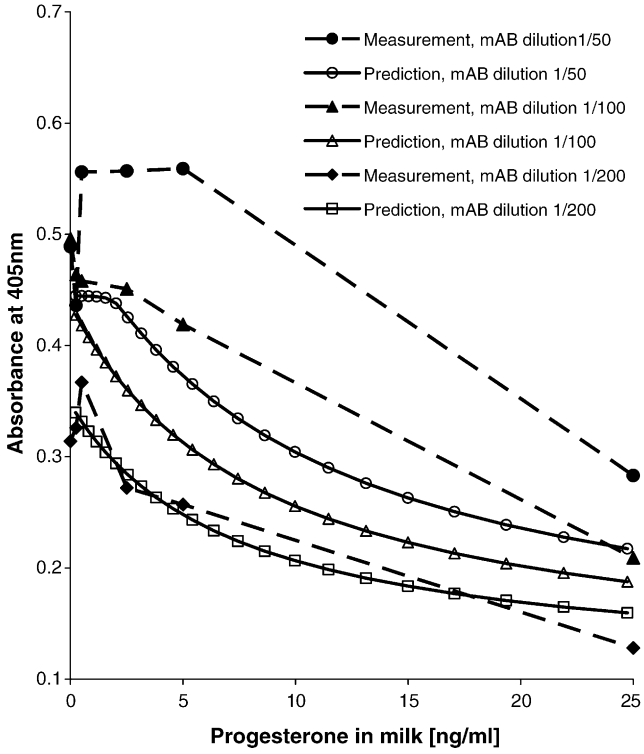

Fig. 9. Comparing the predicted absorbance at 405 nm of the progesterone biosensor using the computer model with measurements reported by Pemberton et al. (2001).

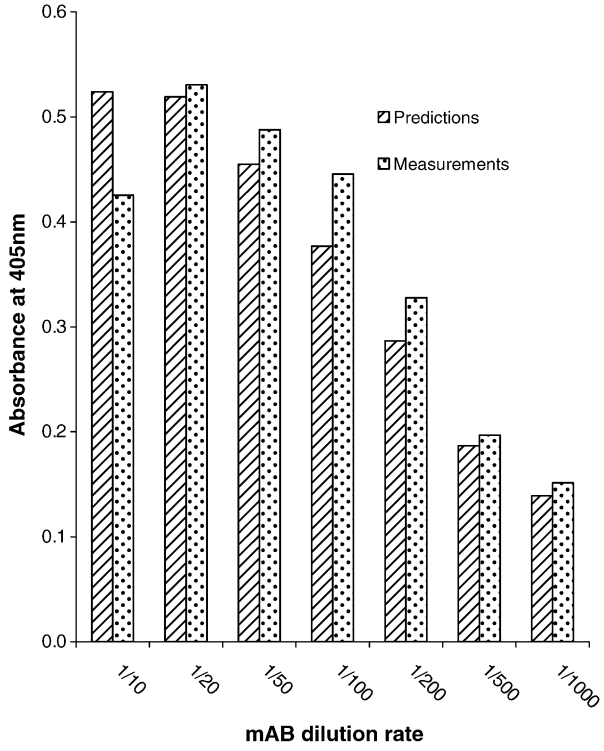

Fig. 8. Comparison of the predicted absorbance using the computer model with the measured absorbance at 405 nm for progesterone in buffer.

dilution rates between 1/1000 and 1/20 is 14% using the computer model and is 12% when Eq. (13) is used. So the computer model developed here is producing satisfactory results within the mAB dilution rates between 1/1000 and 1/20. The complexity of the computer model allows the functioning of the progesterone sensor to be explored in details and this should lead to optimised sensor performance.

5.2 Hook effect

Hook effect was reported in the sensor performance when it was fabricated using 25 ng rIgG per electrode and a mAB dilution rate of 1/50 by Pemberton et al. (2001) and no hook effect was observed when the mAB dilution rate used was 1/100 or 1/200. Fig. 9 shows the comparison of the measured and predicted absorbance at 405 nm. As observed in the tests, the predicted absorbance decreases noticeably with the increase of progesterone concentrations in milk and no hook effect is predicted for the sensors when the mAB dilution rate used is either 1/100 or 1/200. However when the mAB dilution rate is 1/50, the decrease of the predicted absorbance with the increase of milk progesterone concentrations in the range between 0 and 2 ng/ml is very slight and this flat section of the response curve indicates the low-dose hook effect.

The model has shown that the hook effect is mainly determined by the total number of binding sites available to the competitive binding and the rates at which progesterone and labelled progesterone diffuse towards the sensor surface

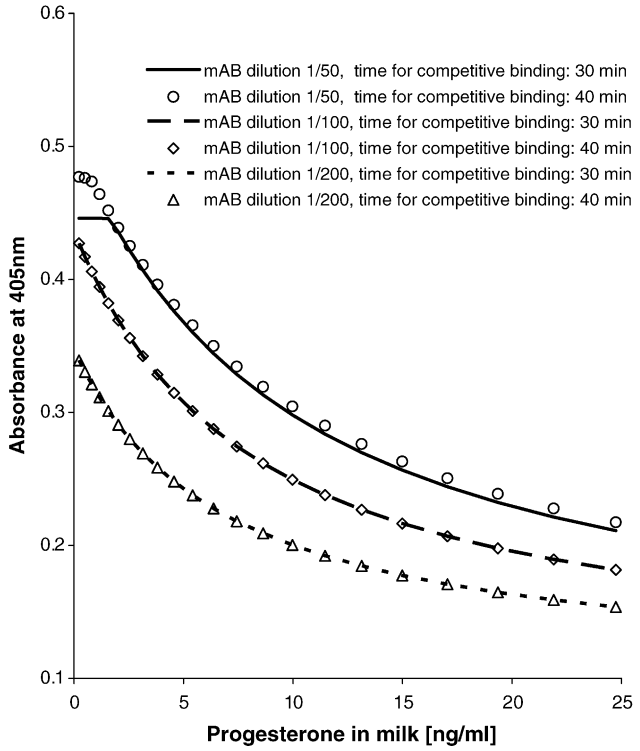

Fig. 10. The change of sensor performance when the time for competitive binding is increased from 30 to 40 min.

and the binding rates. Naturally, there is an upper limit on the amount of progesterone and labelled progesterone that can be diffused into the binding zone within a fixed duration, such as the 30 min allowed for the competitive binding in this progesterone biosensor. If there are not enough binding sites for all the progesterone and labelled progesterone diffused into the binding zone to be bound within the 30 min, they will compete for the limited binding sites and no hook effect would occur. However, if there are enough binding sites for all the progesterone and labelled progesterone diffused into the binding zone, no competitive binding happens and consequently hook effect occurs.

The computer model has generally under-predicted the absorbance at 405 nm as shown in Fig. 9. The likely causes of the under-prediction include the uncertainty in quantifying the conjugate concentration, conformational changes of progesterone and labelled progesterone during the tests and the simplification of Eq. (7) to one-dimensional for the competitive binding carried out in batch.

Hook effect produces false sensor outputs and reduces its accuracy. It could be removed or delayed using the following methods:

- Prolonging the period for competitive binding: Fig. 10 shows the predicted sensor output if the time allowed for the competitive binding is increased from 30 to 40 min. The increase in incubation time has little effect on the sensors fabricated using mAB dilution rates of 1/100 and 1/200, because all the binding sites are occupied within 30 min. However, the hook effect is almost eliminated for the sensors fabricated using a mAB dilution rate of 1/50. The drawback of this approach is that it requires a longer detection time.
- Increasing the concentration of labelled progesterone: this should result in more labelled progesterone to be diffused onto the sensor surface for the competitive binding. More labelled progesterone bound onto the sensor should also improve the quality of its output amperometric singles, but the signals may be less sensitive to small changes of progesterone concentrations in milk.
- Reducing the density of immobilised binding sites in the sensor working area. However, this would cause a reduction of sensor output signals.
- Engineering increased antigen diffusion towards the working area or an increased sensor working area.

Each of these approaches suggested here to remove or delay hook effect of the progesterone biosensor has its own drawbacks in sensor applications and the sensor may need to be optimised using a combination of the approaches suggested.

6. Conclusions

A computer model has been developed to predict the performance of an electrochemical biosensor for measuring progesterone in milk. This model estimates the density of the immobilised binding sites created by the fabrication process and predicts the competitive binding between progesterone and labelled progesterone. The predicted sensor performance compares well with the measurements, even though the model has under-predicted the absorbance at 405 nm of colorimetric tests. The model has predicted the hook effect as observed in tests and revealed that the hook effect is mainly determined by the density of binding sites available to the competitive binding and the rates at which progesterone and labelled progesterone diffuse towards the sensor surface and the binding rates.

Acknowledgements

The authors would like to thank Professor J. Hart and Dr. R. Pemberton at University of West England for discussions and data on the progesterone biosensor.

References

Berg, O.G., van Hippel, P.H., 1985. Diffusion-controlled macromolecular interactions. Ann. Rev. Biophys. Biophys. Chem. 14, 131–160.

Bongrand, P., 1999. Ligand–receptor interaction. Report Progress Phys. 62 (6), 921–968.

Byfield, M.P., Abuknesha, R.A., 1994. Biochemical aspects of biosensors. Biosens. Bioelectron. 9, 373–400.

Cantor, C., Chemmel, P., 1980. Biophysical Chemistry. Part II. Techniques for the Study of Biological Structure and Function. Freeman, New York.

Chalikian, T.V., 2003. Volumetric properties of proteins. Ann. Rev. Biophys. Biomol. Struct. 32, 207–236.

Fernando, S.A., Sportsman, J.R., Wilson, G.S., 1992a. Studies of the low dose hook effect in a competitive homogeneous immunoassay. J. Immunol. Methods 151, 27–46.

Fernando, S.A., Sportsman, J.R., Wilson, G.S., 1992b. Multiple epitope interactions in the two-step sandwich immunoassay. J. Immunol. Methods 151, 67–86.

Hall, D., 2001. Use of optical biosensors for the study of mechanistically concerted surface adsorption processes. Anal. Biochem. 288, 109–125.

Hart, J.P., Pemberton, R., Luxton, R., Wedge, R., 1997. Studies towards a disposable screenprinted amperometric biosensor for progesterone. Biosens. Bioelectron. 12, 1113–1121.

Hoylaerts, M.F., Bollen, A., De Broe, M.E., 1990. The application of enzyme kinetics to the determination of dissociation constants for antigen–antibody interaction in solution. J. Immunol. Methods 126, 253–261.

Ismail, A.A., Walker, P.L., Barth, J.H., Lewandowski, K.C., Jones, R., Burr, W.A., 2002. Wrong biochemistry results, two case reports and observational study in 5310 patients on potentially misleading Thyroid-stimulating hormone and gonadotropin immunoassay results. Clin. Chem. 48, 2023–2029.

Malmsten, M., Lassen, B., Holmberg, K., Thomas, V., Quash, G., 1996. Effect of hydrophilisation and immobilisation on the interfacial behaviour of immunoglobulins. J. Colloid Inter. Sci. 177, 70–78.

Mason, D.W., Williams, R.F., 1986. Kinetics of antibody reactions and the analysis of cell surface antigen. In: Weir, D.W. (Ed.), Handbook of Experimental Immunology. Blackwell, Oxford.

Miles, L.E., Lipschitz, D.A., Beiber, C.P., Cook, J.D., 1974. Measurement of serum ferritin by a two-site immunometric assay. Anal. Chem. 61, 209–224.

Muller, K., Arndt, K., Plukthun, A., 1998. Model and simulation of multivalent binding to fixed ligands. Anal. Biochem. 261, 149–159.

Nakanishi, K., Sakaharu, T., Imamura, K., 2001. On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. J. Biosci. Bioeng. 91, 233–244.

Nghiem, L.D., Schafer, A.I., Elimelech, M., 2004. Removal of natural hormones by nanofitration membranes: measurement, modelling and mechanisms. Environ. Sci. Technol. 38, 1888–1896.

O'Connor, T., Gosling, J.P., 1997. The dependence of radioimmunoassay detection limits on antibody affinity. J. Immunol. Methods 208, 181–189.

Pemberton, R.M., Hart, J.P., Mottram, T.T., 2001. An electrochemical immunosensor for milk progesterone using a continuous flow system. Biosens. Bioelectron. 16, 715–723.

Sadana, A., 2002. Engineering Biosensors: Kinetics and Design Applications. Academic Press, San Diego.

Selby, C., 1999. Interference in immunoassay. Ann. Clin. Biochem. 36, 704–721.

Velasco-Garcia, M., Mottram, T.T., 2003. Biosensor technology addressing agricultural problems: a review. Biosyst. Eng. 84 (1), 1–12.

Voss, E.W., 1993. Kinetic measurements of molecular interactions by spectrofluorometry. J. Mol. Recognit. 6 (2), 51–58.

You, H.X., Lowe, C.R., 1996. AFM studies of protein adsorption: characterization of immunoglobulin G adsorption by detergent washing. J. Colloid Interface Sci. 182, 586–601.