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A FLOW-THROUGH ENZYME-LINKED IMMUNOASSAY FOR PROGESTERONE

A thesis

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Abstract

Bovine reproductive performance is one of the most important factors influencing dairy farm profitability. Present-day techniques for oestrus- and pregnancy-detection are unreliable and labour-intensive. Although measuring milk-progesterone at regular intervals allows the fertility status of a cow to be determined reliably, the labour cost of collecting and analysing samples is prohibitive.

This project aimed to develop a progesterone sensing system that could be automated and integrated with the milking unit, thus minimising labour costs. The proposed system involved mixing the milk sample with an enzyme-antibody conjugate and then passing the sample through a column containing immobilised progesterone. Any progesterone in the milk would inhibit conjugate binding to the column. An enzyme substrate would then flow through the column and bound conjugate would be detected as a colour change at the column's outlet.

Periodate-coupling was used to attach horseradish peroxidase enzyme to anti-progesterone antibody, and progesterone-3-carboxymethyloxime was immobilised on the polystyrene bead surface using amine-coupling. Both techniques are widely used. Initial experiments attempted to verify the success of these two reactions simultaneously, whereas later experiments focused on the bead-coating. Beads were suspended in a specially-constructed syringe and the antibody activity of the eluted solution was measured by SPR. However, a combination of non-specific binding and antibody stability and activity issues meant neither reaction was conclusively verified. Many trials were done to investigate how to overcome the problems encountered but a suitable, workable procedure was not developed.

Despite poor progress, the problems encountered did not undermine the project's potential. There remains optimism of developing an on-line method if research were to continue.

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Glossary

Abbreviation	Description
5AS	5-aminosalicylic acid
Ab	Antibody
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AMS	Automatic Milking System
bPAG	Pregnancy Associated Glycoprotein
BSA	Bovine Serum Albumin
DMF	Dimethylformamide
ECF	Early Conception Factor
EDA	Ethylene diamine
EDC	N-ethyl-N'(3-ethylaminopropyl) carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay
EIA	Enzyme Immunoassay
EID	Electronic Cow Identification
LED	Light Emitting Diode
HDPE	High Density Polyethylene
HMS	Herd Management System
HRP	Horseradish Peroxidase
mAb	Monoclonal Antibody
mAb1	Monoclonal Antibody CL425 from UC Davis received on 16 Jan 2004
mAb2	Monoclonal Antibody CL425 from UC Davis received on 1 May 2006
mAb3	Monoclonal Antibody CL425 from UC Davis received on 10 July 2006
NHS	N-hydroxysuccinimide
NSB	Non-Specific Binding
OPD	o-Phenylenediamine
P4	Progesterone (4-pregnene-3,20-dione)
P4-3CMO	Progesterone-3-carboxymethyloxime
pAb	Polyclonal Antibody
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PSA	Prostate Specific Protein
PSPB	Pregnancy Specific Protein B
RIA	Radioimmunoassay
RU	Response Units
SDS	Sodium Dodecyl Sulphate
SEC	Size Exclusion Chromatography
SPR	Surface Plasmon Resonance
TIRF	Total Internal Reflection Fluorescence
TMB	Tetramethylbenzidine
UHT	Ultra High Temperature (pasteurisation)

Chapter 1 Background

The dairy industry is New Zealand's primary exporter, producing over 40% of our total export earnings. Nearly all dairy farms operate under a pasture-fed regime, with supplementation from hay or silage when grass growth is low. Milk is collected at a central milking parlour, normally twice daily (although some farms collect only once daily). Towards the end of lactation, collection may decrease to once daily.

In New Zealand, cows normally calve in July, August or September and commence lactation at this time. This calving time is optimal so that maximum milk production will coincide with the spring peak in grass growth. After this peak, the cows' milk production gradually decreases until they are dried-off in April or May, depending on grass growth. The annual milking cycle is not unique to New Zealand. Dairy systems worldwide are normally based on an annual reproductive cycle and therefore depend on successful mating at a specific time of the year - spring in New Zealand.

The New Zealand industry uses a combination of artificial insemination and natural mating to breed cows. Since 1909, herd testing, measuring the yield (litres) and solids content of milk from every cow in the herd, has been used to identify high performing cows, whose progeny are likely to be valuable. Artificial insemination allows farmers to obtain the genes of the highest quality sires without owning the animal. However, for successful conception, it is crucial that insemination occurs at the correct time in the oestrous cycle. Pregnancy then needs to be confirmed so that re-insemination can occur if required.

Herd reproductive performance is one of the most important factors influencing dairy farm profitability. Cows that conceive late, calve late, and therefore begin to milk late, and may spend less time in milk. Every day a cow remains empty costs the farmer money. Although a cow may have high milk-producing capability, she may be culled if she fails to conceive.

Despite the importance of reproductive performance to dairy farming profitability, there are relatively few tools available and in wide use for the farmer to manage fertility. Oestrus detection typically involves observing animal behaviour. In some herds oestrus is synchronised across the entire herd by the use of hormone-impregnated controlled-release vaginal implants. Pregnancy is typically detected by rectal palpation by a trained technician.

Milk progesterone measurement has long been thought to have potential as a fertility management tool. Progesterone concentration is very low at oestrus and slightly above normal levels throughout pregnancy, so it can be used for oestrus detection and cautiously for pregnancy confirmation. Although several different technologies have been investigated, no on-line progesterone biosensor is available. Therefore, samples need to be collected from all cows every three or four days and analysed quickly before the insemination-window passes. This system is not very practical so attempts to implement lab-based milk progesterone schemes have failed.

The aim of this thesis was to develop an on-line biosensing platform for progesterone, based on a flow-through enzyme-linked immunosorbent assay using a solid phase column of beads coated with immobilised progesterone. The milk sample would be mixed with an enzyme-antibody conjugate and then flow through the column. Any progesterone in the milk would inhibit conjugate from binding to the immobilised progesterone on the beads. An enzyme substrate would then flow through the column and the bound conjugate would be detected as a colour change at the outlet of the column. A large colour change indicates a large amount of bound conjugate and therefore a low milk progesterone concentration. The key requirements are that the biosensor is simple and robust, and has low running costs and relatively low capital costs. The milking environment is very different from the lab environment and the sensor needs to be designed accordingly.

Chapter Two discusses the current state-of-the-art methods in dairy fertility management, and describes how milk progesterone measurement can be used in this area. It also discusses possible technologies for development into an online progesterone biosensor, before outlining the specific aims of this research. The materials and methods are described in Chapter Three. Chapter Four presents the methodology and results of each trial, and Chapter Five gives an overall discussion of the findings. The conclusions and recommendations from the research are presented in Chapter Six.

Chapter 2 Dairy Cow Fertility Management

This chapter presents the literature on the current state-of-the-art methods in dairy fertility management, and describes how milk progesterone measurement can be used in this area. It then goes on to discuss possible technologies for development into an online progesterone biosensor. Finally, it outlines the specific aims of this research.

2.1 Financial Implications

Fertility management is a critical aspect of dairy farming. It has a direct effect on milk produced per cow per day, number of replacements produced, and rates of voluntary and involuntary culling (Britt, 1985). These all have a direct effect on profitability. For an average yielding cow, the net cost of one day's delay in conception is estimated to be £2.41 in the period 85-100 days post-calving and £5.02 in the period 146-175 days post-calving (Esslemont *et al.*, 2001).

In recent years, dairy cow fertility has been declining at a rate of approximately 1% per annum. The decline has been attributed to factors such as: genetic selection for high yield cows; changes in nutrition targeting high yields; increasing herd sizes; and more crowded environments (Butler, 2000; Royal *et al.*, 2000; Silvia, 2003). Improved reproductive management is required to compensate for this decline.

2.2 Oestrus Detection

One of the most important and difficult aspects of dairy reproductive management is detecting oestrus, which indicates when to inseminate. The most common technique used historically (and currently) is observing cow mounting behaviour. A cow that is ovulating will typically stand still and allow herself to be mounted by another cow, whereas a cow that is not ovulating will normally not stand still when mounted. The major drawback to

this approach is that it is labour-intensive, requiring constant monitoring of cows, and therefore not particularly reliable.

Several systems that record evidence of standing mount behaviour have been developed to automate oestrus identification. These include tail chalk/paint or pressure-sensitive tags placed on the tailhead, which will be rubbed off or activated during mounting. KaMaR tags are pressure-sensitive and change colour when pressure is applied (Williamson *et al.*, 1972) whilst the HeatWatch system records time and duration of mounting events on a computer (At-Taras and Spahr, 2001). These systems reduce the labour requirement, but there is a significant chance of false detection.

There is a relationship between increased cow physical activity and oestrus, and pedometers have been used to measure the activity and predict oestrus (Koelsch *et al.*, 1994a; At-Taras and Spahr, 2001; Cross *et al.*, 2004). Semi-permanent electronic intravaginal probes have been developed to measure the chemical and physical changes in the vagina during oestrus (Heckman *et al.*, 1979; Lane and Wathes, 1988; Kyle *et al.*, 1998). Morais and coworkers (2006) developed a device to measure temperature and electrical conductivity. The intention was to implant the device in the vulvar tissue to detect these changes and identify oestrus. All of these technologies suffer because the variables measured can easily change for reasons other than oestrus.

Milk temperature immediately from the udder has also been used to predict oestrus (Gil *et al.*, 1997) although this can be affected by variables such as mastitic infection (Hillerton, 2000) and ambient temperature.

The oestrous cycle of an entire herd can be synchronised using hormone-impregnated implants. Examples of this technology include CIDR (Controlled Intravaginal Drug Release) and PRID (Progesterone Releasing Intravaginal Device) (Rathbone *et al.*, 1998), which are implanted in the vagina and release hormones to prevent ovulation. A cow will ovulate shortly after the device is removed, so removing the devices simultaneously from the herd will

synchronise ovulation. This is undoubtedly a useful technology, but another method must be used to actually detect oestrus itself. It also involves putting hormones in the animal, thus raising potential ethical and food safety concerns.

2.3 Pregnancy Testing

Once a cow is inseminated, it is critical to identify successful conception. Early knowledge of a failed insemination allows re-insemination at the next ovulation, thus maximising reproductive efficiency. However, early detection of pregnancy can be confounded by embryonic loss at a later date (Rajamahendran *et al.*, 1994) so further confirmation of the pregnancy later is advisable. Knowledge of the pregnancy-status of a cow is useful for herd-management decisions such as culling.

The most common technique for diagnosing pregnancy is palpating the uterus through the rectum. This approach has many drawbacks: it is labour intensive; the animal must be isolated and restrained and then inspected physically; a skilled practitioner (most commonly a veterinarian) is required for reliable results; the procedure can induce embryonic loss when not performed correctly; and the earliest useful diagnosis can be made at about 35 days post-insemination. A non-pregnant cow will have ovulated again at about day 22 (Romano *et al.*, 2007). In practice, oestrus detection may well infer a non-pregnant cow at day 22 so re-insemination can occur. Only cows that aren't detected as ovulating would be palpated. However, detecting oestrus, for example by observing standing mount behaviour, is not reliable. Six percent of cows may exhibit standing mount behaviour during normal pregnancy (Sheldon and Noakes, 2002).

Ultrasonography, also performed per rectum, can detect pregnancy earlier than palpation and there is no risk of damage to the foetus. However, the cows need to be isolated and restrained and a skilled practitioner is required (Ribadu

and Nakao, 1999). Pregnancy can be detected as early as day 21 under controlled experimental conditions, but day 25 is a more practical estimate. Thus, as for palpation, ultrasonography cannot detect non-pregnancy before the next ovulation. The cost of ultrasonography equipment ($8000-16000) is relatively small compared with the cost of poor reproductive management, especially if the cost is spread across many farms (Fricke and Lamb, 2005).

Two pregnancy-specific trophoblastic proteins, Pregnancy Specific Protein B (PSPB) and Pregnancy Associated Glycoprotein (bPAG), have been identified in the maternal blood. These can be detected in a laboratory by immunoassay and are reliable indicators of pregnancy (Humblot, 2001). Pregnancy can be detected at day 25 and confirmed at any later stage in the pregnancy (Sheldon *et al.*, 2006). The major drawback of this approach is that a blood sample is required. There appears to be no literature on these proteins in milk, which is far more accessible than blood.

Another pregnancy-associated glycoprotein, Early Conception Factor (ECF), can be detected in the milk within 3 days of conception. Commercially available qualitative test kits are available (EDP Biotech Corp.), which claim "reliable detection of ECF to determine whether or not conception has occurred as early as 7 days after breeding". However the literature suggests that the test is not yet reliable (Cordoba *et al.*, 2001; Sheldon and Noakes, 2002).

Pregnancy-specific hormones such as oestrone sulphate are found in the milk and can also be measured by immunoassay and used to detect pregnancy. A test kit is commercially available (Confirm®, ICPBio). The disadvantage is that oestrone sulphate in milk cannot reliably indicate pregnancy until day 120 (Henderson *et al.*, 1994).

2.4 Progesterone

Progesterone is a steroid hormone (Figure 1) produced in female mammals. It is secreted from the corpus luteum in the ovary, beginning shortly after ovulation, and its function is to prepare the uterus for pregnancy. If the egg is successfully fertilised, the corpus luteum will persist throughout gestation and the progesterone concentration will remain high (slightly elevated). If the egg is not fertilised, it will decay and the progesterone concentration will fall (Frobenius, 1999).

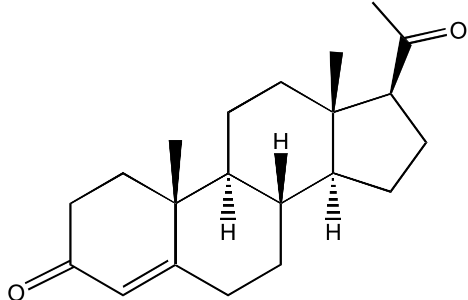


Figure 1: Molecular structure of progesterone (4-pregnene-3,20-dione).

2.5 Dairy Cow Fertility Management Using Progesterone

Regular measurement of a cow's milk or plasma progesterone concentration can be used to follow the oestrous cycle and applied as a general purpose reproductive management tool. Many studies have been completed examining the various uses of milk progesterone measurement to manage bovine fertility (for example: Laing and Heap, 1971; Dobson and Fitzpatrick, 1976; Laing, 1976; Lamming and Bulman, 1976; Foote *et al.*, 1979; Gunzler *et al.*, 1979; McLeod *et al.*, 1991; Petersson *et al.*, 2006).

Progesterone measurements are required every 3-4 days, and possibly more often near ovulation, to establish a progesterone profile for each cow to identify oestrus. Some example milk-progesterone profiles are shown in Figure 2. When the progesterone concentration falls to very low concentrations in a non-pregnant cow, ovulation will occur soon afterward, so it is a good time to inseminate.

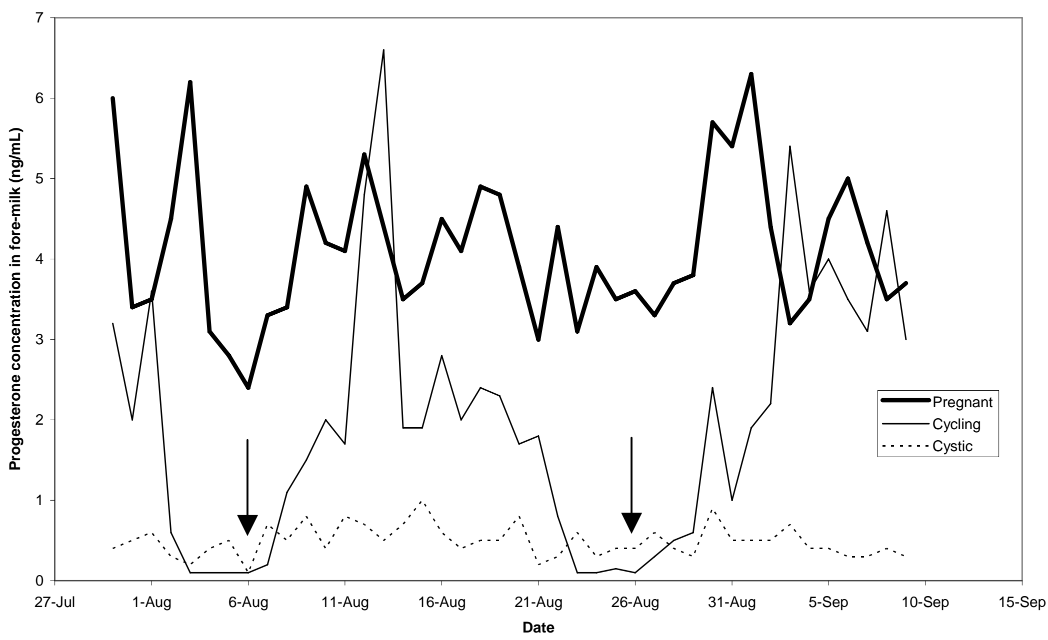


Figure 2: Examples of progesterone profiles for different fertility conditions. The arrows indicate the approximate day of ovulation for the cycling cow. (Source: Claycomb, 1996)

After ovulation, the progesterone concentration will increase. If insemination is successful, the progesterone concentration will remain high throughout pregnancy. Failed insemination can be detected by progesterone measurement as early as day 21 when the next ovulation occurs. This is earlier than all other pregnancy detection methods described in Section 2.3 other than ECF. Any loss of the foetus is quickly followed by a fall in progesterone concentration.

Because progesterone concentration directly reflects ovarian activity, an atypical progesterone profile can be used to detect possible fertility disorders associated with the ovary, although such disorders can also cause persistent high progesterone concentrations that may give false positives for pregnancy.

Milk is a convenient fluid to sample for progesterone analysis and fertility management. The typical dairy cow will be lactating during the stages of the bovine reproductive cycle when it is most important to know the cow's fertility status: start of the oestrous cycle after calving; insemination; and the early stages of pregnancy. Thus, daily milk samples can be obtained. Although the milk-matrix complicates the analysis and can introduce measurement

errors, the determination is still possible because progesterone's affinity to milk fat makes the concentration in milk much higher than in blood plasma (Erb *et al.*, 1977).

2.6 Progesterone Measurement

Chemical techniques for measuring progesterone were developed in the 1940s and 50s. They were typically based on solvent extraction, chromatography and/or spectrophotometric quantification (Edgar, 1953; Short, 1958). A radio-assay for steroids developed in the 1960s (Murphy *et al.*, 1963; Murphy, 1967) led to progesterone radioassays (Bassett *et al.*, 1969; Demetriou and Austin, 1971) and radio-immunoassays (Heap *et al.*, 1976; Holdsworth *et al.*, 1979).

The development of enzyme-linked immunosorbent assays (ELISAs) (Engvall *et al.*, 1971; van Weemen and Schuurs, 1971) led to many ELISAs for progesterone (for example: Dray *et al.*, 1975; Arnstadt and Cleere, 1981; Sauer *et al.*, 1981; Chang and Estergreen, 1983; Munro and Stabenfeldt, 1984). These ELISA formats allowed widespread use of progesterone analysis. A chemiluminescence immunoassay for progesterone was also developed (Miller *et al.*, 1988).

More recently, other immunoassay technologies have been used for laboratory progesterone analysis. Gillis and coworkers and Wu and coworkers (2002) have independently presented surface plasmon resonance assays for progesterone which were further refined (Mitchell *et al.*, 2005; Gillis *et al.*, 2006). Ehrentreich-Förster and coworkers have developed an assay for progesterone in whole blood using an integrated optical grating coupler. In 2004, Tschmelak and coworkers developed a fluorescence-based progesterone assay using a RIANA optical biosensor (Tschmelak *et al.*, 2004; Tschmelak *et al.*, 2005; Käppel *et al.*, 2007). The latter two technologies have extraordinarily wide dynamic ranges compared to typical ELISAs even when dealing with difficult sample matrices such as whole blood and milk. Of the methods

developed for progesterone, only Gillis and Tschmelak measured progesterone in milk with a view to using their technology for dairy reproductive management. However, any of these new technologies may have the potential to be developed into on-line milk progesterone sensors.

Many cow-side progesterone tests, using samples directly from the cow, have been developed and many attempts have been made to use these and lab-based tests to manage dairy reproduction (Nebel *et al.*, 1987; 1989). Most cow-side tests are simplified enzyme immunoassays, with several in a lateral-flow format (Laitinen and Vuento, 1996; Sananikone *et al.*, 2004). Recently, two automated (off-line) progesterone EIA analysers were released . Off-line progesterone testing schemes have the main disadvantage of a high labour requirement for taking and coordinating samples and running the tests. The major breakthrough will occur if an economically-feasible fully-automated on-line progesterone sensor is developed.

Some technologies have been used to develop on-line milk-progesterone sensors. Koelsch and coworkers (1994) developed a sensor based on measuring surface mass change using the resultant frequency change of a 10-MHz quartz crystal. Claycomb (1996) developed an automated EIA that was successfully used to monitor the progesterone profiles of four cows. In 2001, Pemberton and coworkers (2001) developed an electrochemical thin-layer continuous-flow progesterone sensor. However, none of these technologies has been successfully commercialised, mainly because they are complex and/or expensive.

2.7 On-line Progesterone Biosensor Requirements

The milking environment is very different from carrying out off-line tests in a laboratory, where biosensing techniques have been developed. This is why attempts to develop an on-line milk-progesterone biosensor have been limited, and not yet commercialised.

Any sensor will need to be simple and robust, cheap to manufacture and run, rapid and accurate. The ability to link to electronic animal identification is a necessity.

2.7.1 Simplicity and Robustness

Most attempts at on-line progesterone measurement have been very complex. The milking environment is generally unsuitable for complex laboratory instrumentation, which is designed to sit on a solid bench in a dry temperature-controlled room. It is nearly always wet, the plant tends to shake during operation and the operating temperature may vary from below 0°C to above 40°C.

The biosensor needs to be designed for the operating conditions. The components need to be robust enough to tolerate vibration and shaking, and operate for long periods (up to a year) without requiring technical input. Therefore, the number of moving parts should be minimal and the design simple.

Any components sensitive to vibration need to be protected and if the assay (or any of the components) is temperature-sensitive, the internal temperature needs to be controlled or compensated for.

2.7.2 Cost

Most New Zealand milking systems are in a herringbone or rotary format, with many sets of teat-cups. For example, a 700-cow farm might have a 50-bail rotary system with 50 sets of teat-cups. If a sample is being collected from a given cow on average every third day (every sixth milking), there would need to be an on-line progesterone sensor at every sixth bail (i.e. a total of eight sensors). This means that the capital cost of the sensor must be very low compared with typical laboratory instrumentation that could process all the samples from many farms.

One potential market sector does not have quite as stringent a requirement on low capital cost. Automatic milking systems (AMS) are becoming more common in Europe and North America. These have a robotic cup-attachment system and milking can occur at any time of day. A single robot can service about 60 cows, each being milked up to three times daily (Australian National Milk Harvesting Centre, 2003). A 700-cow farm would require twelve AMS, only two of which would need a sensor to obtain the milk sample from a specific cow at a three-day average interval. This makes the AMS market a better target sector than the conventional milking parlour. In addition, AMS owners have already demonstrated they are keen to embrace new technology and may be more receptive to the idea of on-line progesterone sensing.

Running costs also need to be low. A 700-cow herd involves over 20,000 tests in a three-month mating season. The cost of each test must be very small to off-set the advantages gained with better reproductive management. Consumables need to have a long-lifetime or be very cheap. The number of reagents needs to be minimised to minimise complexity. Any reagents need to be cheap and minimal quantities should be used. Thus, high-cost protein reagents are undesirable.

2.7.3 Speed

Data from the progesterone sensor should be available before the end of the cow milking so an ovulating cow can be automatically drafted into a holding pen for insemination. If the analysis took longer, the farmer would need to fetch the cow from the paddock or wait until she returned for the next milking before she is drafted, possibly missing the best time for insemination.

In New Zealand, a typical milking may last five to six minutes. Completing an assay in five minutes is very fast by laboratory standards. The sample will need to be taken as soon as possible after milking starts and this may have a detrimental effect on test accuracy.

The fat content of dairy cow milk is lowest at the start of the milking and progesterone has an affinity for milk-fat (Erb *et al.*, 1977) so samples taken at the start of the milking tend to have a lower progesterone content. This makes it more difficult to differentiate high and low progesterone concentrations for an individual cow. However, ELISAs have been used successfully to follow the oestrous cycle using samples of fore-milk (Holdsworth *et al.*, 1980; Claycomb, 1996).

2.7.4 Accuracy

The result from the biosensor does not need to be compared between cows or between farms. The most important requirement is to follow each cow's oestrous cycle so the 'low' level of progesterone can be reliably detected. Thus, the biosensor needs to indicate only a relative value rather than the actual progesterone concentration, so the accuracy of the biosensor can be lower than required in a laboratory assay.

2.7.5 Environmentally-Friendly Waste

To avoid requiring special storage and disposal procedures, any waste from the sensor should be environmentally safe and able to be disposed of with shed waste (cow excrement). Otherwise, running costs will increase.

2.7.6 Electronic Cow Identification and Data Recording

To be commercially successful, it is essential that the assay is linked to electronic cow identification (EID) and data recorded using a herd management system (HMS). Data from a progesterone test needs to be viewed in the context of recent data from that cow. This means that a database needs to be built so the progesterone profiles for individual cows can be analysed. The best way to match the test result to a specific cow is with EID and a HMS. These are becoming more common in conventional milking systems and are

required in AMS. Farmers investing in on-line progesterone sensing are likely to be keen users of technology and already have an EID/HMS system.

More pertinent for applicability of progesterone sensing is having tools to analyse the data and display the profiles so the farmer can make reproductive management decisions. As this would be labour intensive, the analysis software should also be able to make decisions for the farmer, even to the extent of being able to automatically draft cows for insemination.

2.8 Possible Technology Platforms

Table 1: Characteristics of five technologies with potential as an on-line milk-progesterone biosensor.

	SPR (Biacore)	OWLS	TIRF	Traditional ELISA	ELISA as a column of beads
Simplicity	Low	Low	Low	High	Medium
Temperature stability	Low	Low	Medium	Medium	Medium
Vibration stability	Low	Low	Medium	High	High
Suitability for automation	High	High	High	Low	High
Capital cost	High	Medium	Medium	Low	Low
Running cost	High	Medium	Medium	Medium	Medium
Speed	High	Medium	High	High	High
Dynamic range	Medium	High	High	Medium	?
Availability of technical know-how	Low	Low	Low	High	Medium
Multiple analytes possible	Yes	No	Yes	No	No

The traditional way to measure progesterone is with ELISA. In addition, three relatively new technologies identified in the literature have potential as an automatic progesterone biosensor – surface plasmon resonance (SPR), optical waveguide lightmode spectroscopy (OWLS), and total internal reflection fluorescence (TIRF). The characteristics of these technologies, along with the traditional ELISA and ELISA as a column of beads are summarised in Table 1 and discussed in the next sections.

2.8.1 Surface Plasmon Resonance (SPR)

The SPR phenomenon (Figure 3) occurs when polarized light, under conditions of total internal reflection, strikes an electrically conducting gold layer at the interface between media with different refractive indices - the glass of a sensor surface (high refractive index) and an aqueous solution (low refractive index). For a given wavelength at a certain angle of incidence (the resonance angle), a plasmon wave is excited in the conductive surface and propagates along the surface. The resulting decrease in reflected intensity is used to identify the resonance angle and thus changes in refractive index at or near the gold surface (Biacore, 2007). When large molecules such as proteins bind to the surface, the local refractive index increases, which means binding events can be detected in real-time.

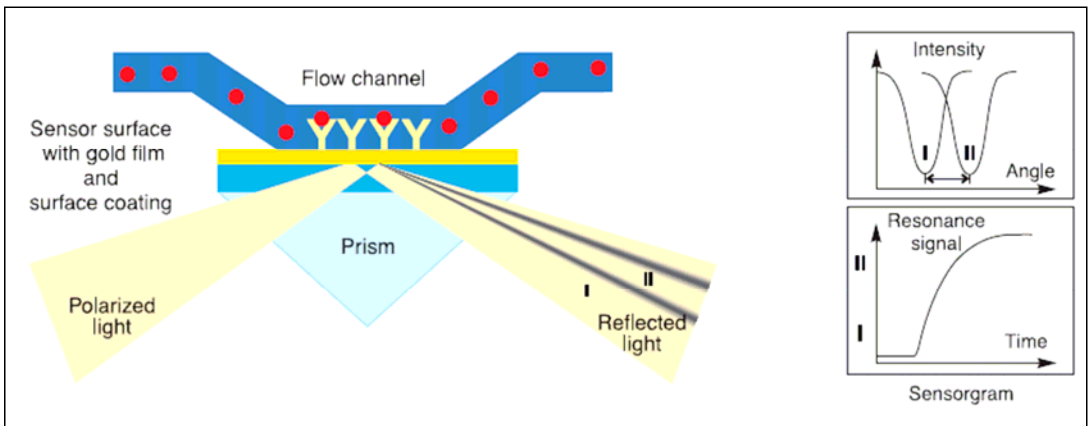


Figure 3: Principles of SPR sensing.
(Source: Biacore, 2007).

To measure progesterone, the chip surface is coated with progesterone and the sample is mixed with antibody and passed across the surface (Gillis *et al.*, 2002). Free antibody (not bound to the progesterone in the sample) binds to the progesterone on the surface causing a measurable response inversely correlated with the progesterone concentration of the sample.

SPR-based biosensors such as the Biacore instrument (Biacore, Sweden) are now widely used in laboratories to quantify a wide range of analytes in a wide range of sample matrices including milk. This technology is very flexible. The

Biacore flow cell has four channels in contact with the gold surface so four analytes could be measured simultaneously with a single sample injection. This means progesterone and other useful analytes such as antibiotics or even bacteria could be measured from a single sample. Furthermore, the instrument is flow-through so it could be easily automated.

The SPR assay developed by Gillis and coworkers (2002) is sensitive enough to follow a cow's oestrous cycle, and fast enough to meet the requirements for an on-line assay. Unfortunately, in its current form the Biacore instrument and associated consumables are too expensive for this assay to be used as an on-line biosensor. Also, the equipment needs to be in a controlled temperature environment because refractive index (and therefore SPR) is temperature dependant. It would also need to be isolated from vibrations in the milking system, which would probably interfere with the ability to accurately measure very small changes in resonance angle. These disadvantages mean SPR may be unsuitable for the farm environment.

2.8.2 Optical Waveguide Lightmode Spectroscopy (OWLS)

Like SPR, OWLS detects mass change at the sensor surface. The basic principle involves coupling linearly polarized light (He-Ne laser) via a diffraction grating into the waveguide layer at a precise angle of incidence, which depends on the refractive index of the medium covering the surface of the waveguide. The coupled light is guided by total internal reflection to the photodiode detector at the end of the waveguide .

An alternative configuration (Figure 4), which determines the resonance angle with a CCD-array, has been used to measure progesterone in whole blood . This assay has a very wide dynamic range and is relatively fast, taking 15 minutes for a full cycle including regeneration. Because the technique has worked with whole blood, it is probably a robust technique that could also

work with milk. The instrument seems to be a relatively straightforward design that could be implanted into an on-line sensor.

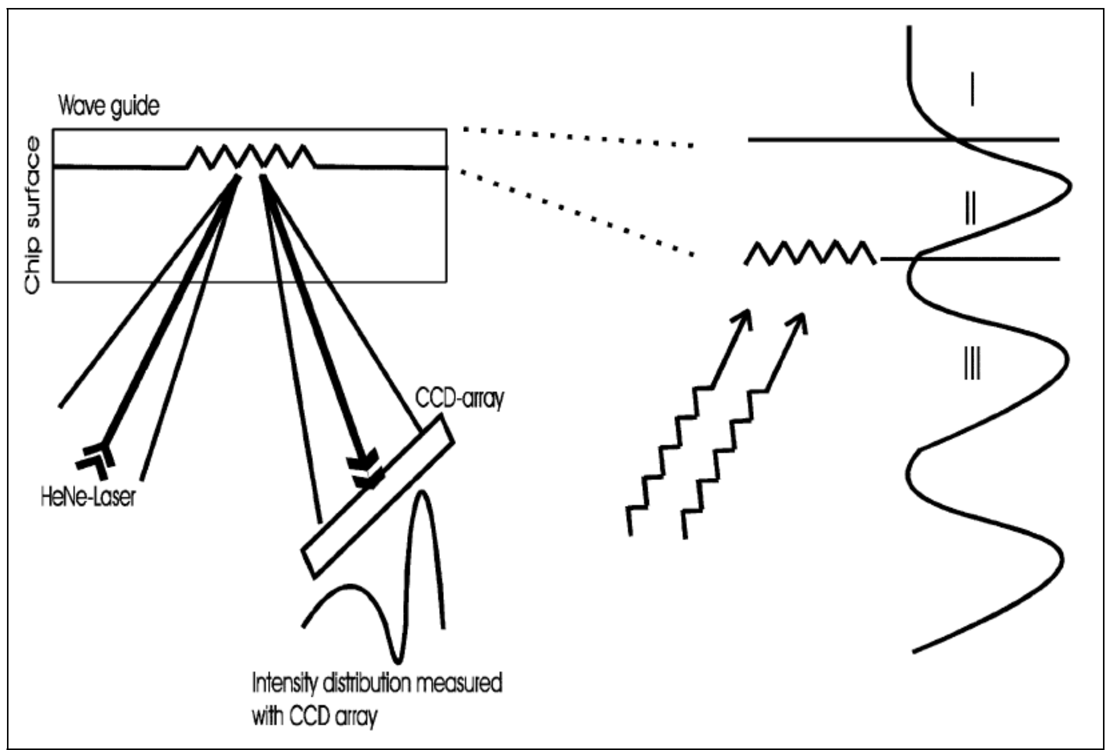


Figure 4: Principles of OWLS.
At the resonance angle, the reflected intensity detected at the CCD-array is minimised. I is the evanescent part of the field above the surface; II is the field distribution in the waveguide; and III is the underlying support.

Optical Waveguide Lightmode Spectroscopy instruments are commercially available. BIOS-1 from Microvacuum (Budapest, Hungary) is an example. As in SPR, OWLS depends on refractive index and resonance angles, so it may also be susceptible to temperature variation and vibrations. Both instruments are flow-through systems suitable for automation but OWLS is probably cheaper and more available for development than SPR (Biacore). However, to develop a satisfactory assay that could be used on-farm probably requires collaboration with the developers.

2.8.3 Total Internal Reflection Fluorescence (TIRF)

The TIRF instrument used by Tschmelak and coworkers (2004) to develop a progesterone assay was a RIANA instrument designed by a consortium of

European universities and industries to measure pesticides and trace-amounts of chemicals in rivers and waterways. A laser beam (excitation light) is coupled into the transducer and propagates by total internal reflection (Figure 5). Fluorescent markers bound to the surface are then excited in the evanescent field and emit light, which is measured perpendicular to the surface (Brecht *et al.*, 1998; Barzen *et al.*, 2002).

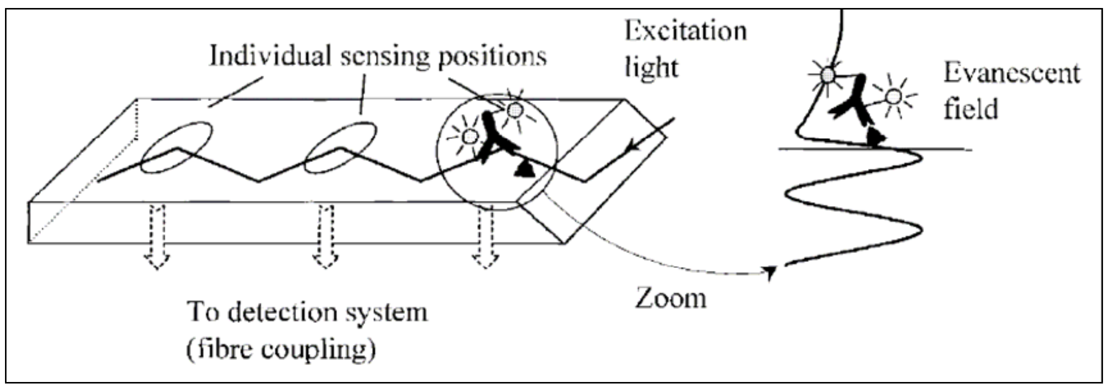


Figure 5: Principles of TIRF sensing.
(Source: Brecht *et al.*, 1998).

Other than the antibody being labelled with a fluorescent marker, this assay is very similar to the SPR and OWLS progesterone assays in that the marked antibody is mixed with the sample and passed across the surface. The SPR and OWLS assays detect bound mass whereas the TIRF assay detects the bound marker. The RIANA can measure multiple analytes because the laser reflects at six spots on the slide. Each of these points could measure a different analyte.

The results reported in the literature for progesterone analysis are very good, with a 5-minute assay time and a wide dynamic range. Like SPR and OWLS, the RIANA is a flow-through system, making it suitable for automation, but the RIANA does not involve precise measurement of a resonance angle or require a stable refractive index of the flowing liquid so this technology is a more robust option for the milking environment than SPR or OWLS. Overall, the RIANA is promising technology platform for on-line progesterone sensing but it is still a complex instrument, so there may be technical issues to overcome, which will require cooperation with the inventors.

2.8.4 Enzyme-Linked Immunosorbent Assay (ELISA)

The development of the ELISA format in the 1970s represented a major milestone in assay technology. Analytes at ng/mL concentrations could be determined in just a few hours without using hazardous radioactive reagents. Furthermore, it led to the development of portable test kits such as home pregnancy tests and cow-side progesterone tests. It is currently the predominant technology for milk-progesterone testing.

ELISAs take many formats, but all involve covalently binding an enzyme to an antigen or antibody and some method of binding the conjugate to a solid surface to allow detection by the addition of a substrate. The proportion bound depends on the analyte concentration.

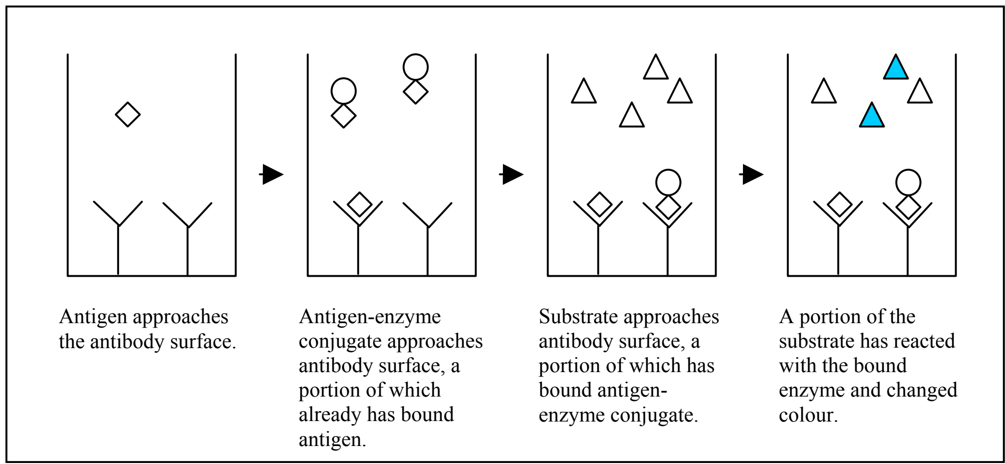


Figure 6: A typical progesterone ELISA.

In a typical progesterone assay (Figure 6), anti-progesterone antibody is immobilised on the surface. The sample is exposed to the surface and the progesterone (antigen) in the sample binds to the antibody. A progesterone-enzyme conjugate is then exposed to the surface and the progesterone already bound limits the amount that can bind to the immobilised antibody. This is known as a competitive assay. A substrate is then exposed to the surface and the colour change depends on the amount of bound enzyme, which is inversely correlated with the progesterone concentration in the sample.

ELISAs are normally carried out on many samples simultaneously in a 96-well microtitre plate. Reagents are pipetted into the wells and manually discarded. The optical density is measured using a plate reader (a spectrometer), which measures the transmission through every well. Although the assays normally take several hours, the manual work involved is only a small part of this time.

The major advantage of ELISA is the relative simplicity of the detection system. While newer technologies such as SPR, OWLS and TIRF use laser light, tightly controlled angles, sophisticated sensor surfaces and advanced optical detection systems, ELISAs simply measure the optical density by shining (non-laser) light through the liquid. This is far better suited to the milking environment.

The main disadvantage is that the traditional plate format is not flow-through, and therefore unsuitable for automation. A second disadvantage is that it requires a substrate. This extra reagent significantly increases assay complexity, which may negate the simplicity of the detection system.

Claycomb (1996) overcame these issues and developed an on-line progesterone biosensor based on this principle. He also modified the assay by adjusting reagent concentrations, which reduced assay time to less than 5 minutes. The biosensor was successfully used to follow the oestrous cycles in three cows, to detect an acyclic cow with cystic ovaries, and the progesterone levels in a pregnant cow. Although the sensor was too complex and expensive for commercialisation, Claycomb's results demonstrate that an ELISA-based system has the potential to meet both the speed and precision requirements of an on-line progesterone biosensor.

2.9 Beads in Immunoassay Systems

Immunoaffinity columns are widely used for purifying and/or concentrating proteins from complex mixtures. Pre-activated media with active groups that readily react to form covalent bonds with a ligand are available from most

media suppliers (for example: Rapp-Polymere, Germany; GE Healthcare-Life Sciences, UK). The ligand can be chosen to interact specifically with the target molecule and separate it from the mixture (Scopes, 1993). Depending on the stability of the bound molecule, the media can be used repeatedly. For example, Acevado and coworkers (2006) immobilised anti-PSA antibody in a Sepharose CL-4B matrix and packed the gel into a column to purify a protein called prostate specific antigen (PSA) from human seminal plasma.

Using beads for immunoassays is not a new concept. Catt and coworkers (1966) covalently coupled antibody to an isothiocyanate derivative of a styrene-polytetrafluoroethylene copolymer and used a suspension of the beads in a radioimmunoassay (RIA) for human growth hormone. Goodfriend and coworkers (1969) entrapped antibody in a cross-linked acrylamide polymer and used a suspension of the resulting granules to determine angiotensin by RIA. Brooker and coworkers (1976) used an anion exchange column to separate bound antigen from free antigen in RIA. This 'Gammaflow' system was completely automated and not analyte specific, so was very flexible. Ismail and coworkers (1978) described the 'Southmead System', which incorporated a Sepharose-linked antibody and was applicable to RIA, EIA and fluoroimmunoassay. Rather than using a column format, they mixed the beads in suspension and later filtered them out. Gray and Keyes (1981) patented a column-based enzyme immunoassay method, which involved pre-saturating the column with enzyme-antigen conjugate. The antigen-containing sample released the bound enzyme, which was quantified using an immobilised substrate.

Many bead-based immunoassays have been described recently, but the beads are usually suspended in the reaction fluid and then separated magnetically or by filtration. An example is an on-bead assay for identifying substrates for protein tyrosine kinases, which involves slurrying the beads and the reactants (Martin and Peterson, 2002). Such systems are not suitable for a rapid, automated, repeated-use, on-line assay.

2.10 *Project Outline*

2.10.1 Objective

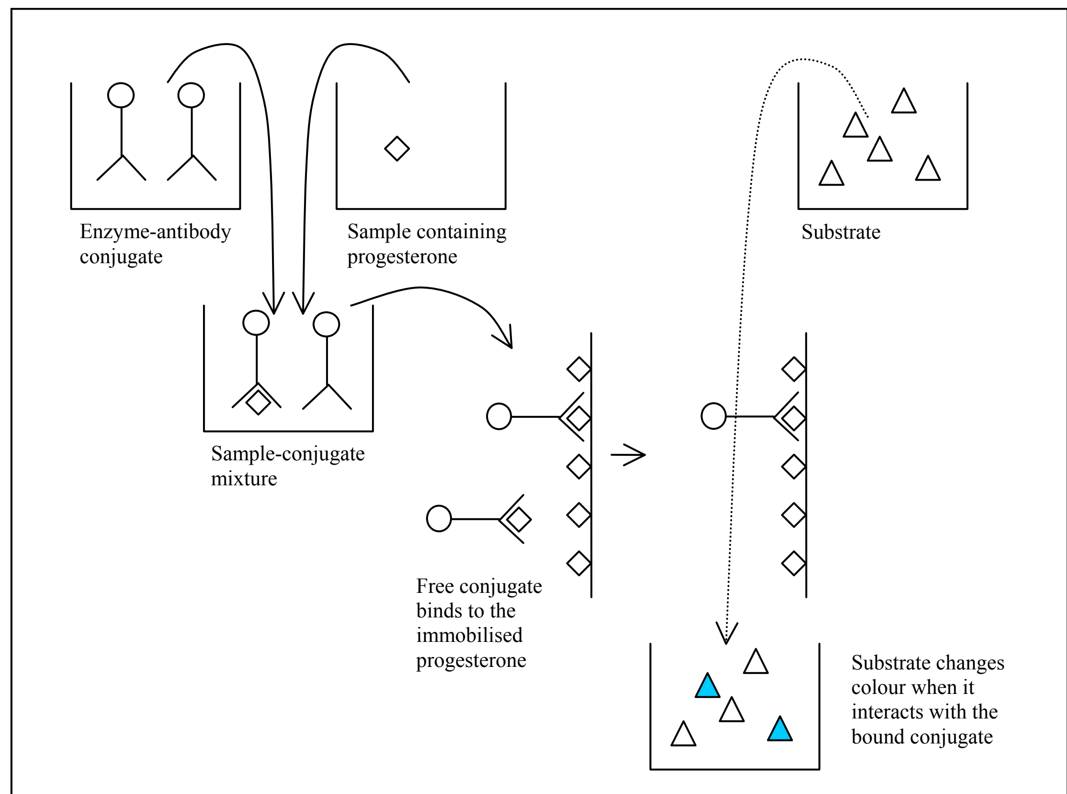


Figure 7: Column-of-beads ELISA concept.

This project aimed to transfer an ELISA for progesterone to a robust, readily-automated flow-through format that maintains the advantage of simplicity of detection (Figure 7). The solid phase, which supports a progesterone-derived antigen, is a column of beads. The milk sample is mixed with a solution containing an enzyme-anti-progesterone conjugate, which effectively binds all the milk progesterone. When this mixture is passed through the column, the remaining free conjugate is bound to the solid phase. A substrate for the conjugated enzyme is then passed through the column and the colour change is inversely proportional to the concentration of milk progesterone.

The advantage of this format over plate-based ELISAs is that the reagents are forced to contact a large active surface. The flow-through nature of the column allows individual samples to be analysed quickly.

2.10.2 Solid Phase

Unlike typical progesterone ELISAs, the assay would immobilise the antigen rather than the antibody. This has the potential to give a more stable and longer-life column because progesterone is very stable compared with antibody. More aggressive regenerants can be used without the risk of damaging the active surface. The immobilised antigens used in the SPR, TIRF and OWLS progesterone assays (Section 2.8) had very long life times (Gillis *et al.*, 2002; Wu *et al.*, 2002; Ehrentreich-Förster *et al.*, 2003; Tschmelak *et al.*, 2004). This suggests that immobilised antigen assays for progesterone are viable and that regeneration is reliable.

A possible disadvantage of a progesterone-coated surface is that steric hindrance might prevent optimal binding of antibody, especially if a blocking agent is required to minimise non-specific binding. This will need to be investigated.

An important consideration for dealing with milk samples is bead size. Bovine milk contains fat globules up to about 10 μm diameter (Rogers, 1928), which could clog a column. Larger beads allow the fat globules to pass through the column, particularly if the temperature is close to cow temperature (38°C) so the fat is liquid. This will minimise clogging, but smaller beads have a larger surface area to volume ratio and are more efficient at capturing antibody. A balance between the two factors is required.

2.10.3 Verifying Antibody-Enzyme and Antigen-Surface Coupling

The primary reagent in the assay is an antibody-enzyme conjugate, which needs to be specially created for this project. Anti-progesterone antibody will be covalently coupled to horseradish peroxidase (described in Section 3.3.2). The other prerequisite chemical reaction covalently couples the antigen to the bead surface (described in Section 3.3.4).

Successful coupling needs to be verified for both these reactions before the products can be used with confidence. Mass-dependent protein purification techniques, such as size-exclusion chromatography or gel-electrophoresis, could be used to qualitatively assess the success of conjugation. But the real test is whether molecules that bind progesterone also catalyse the substrate reaction. It is tempting to think that both the conjugation and the bead-coating can be verified in a single experiment where the conjugate is exposed to coated beads, and then the beads are rinsed and exposed to substrate, which reacts with bound enzyme. However, a more reliable approach would be to first demonstrate a successful bead coating using a system such as Biacore SPR with a surface sensitive to anti-progesterone antibody. The bead-coating can be verified by exposing antibody to the beads and comparing the SPR-response of the eluant with that when antibody is exposed to uncoated beads. Conjugation can be verified by showing that coated beads exposed to the conjugate catalyse the substrate reaction, whereas uncoated beads do not.

2.10.4 Assay Parameters

Once the success of conjugation and bead-coating has been verified, the column can be packed and the assay set up. The column of beads concept can be tested using a laboratory liquid chromatography (LC) system. If an in-line 630 nm detector is not available, the eluted substrate can be collected and analysed in a spectrometer. The LC system will allow approximate assay parameters to be set up and tested in a controlled environment. The parameters to investigate include conjugate concentration, sample to conjugate mixing ratio, substrate concentration, pH and buffer concentration, regenerant, flow rates of each step, and column size.

The LC system can also be used to assess assay robustness against key variables for an on-line milk progesterone sensor. Initial testing would use spiked aqueous samples, but spiked milk samples would eventually be used to adjust the assay parameters for milk and to assess whether milk clogs the column.

Clogging is probably temperature dependent, so temperature would be controlled. The effect of temperature changes on the assay also needs also be determined so temperature tolerances can be specified. In summary, the sensitivity of the assay to all parameters listed above needs to be determined, as well as the effect of milk composition.

2.10.5 Reagent Formulation

At least four reagents are required for this assay: conjugate, substrate, regenerant, and running buffer; as well as the sample. A separate running buffer may also be required for the substrate reaction. Storing and disposing of these reagents is likely to be a major hurdle when transferring the assay from the laboratory situation to the farm, so reagents used in laboratory experiments should be selected with this in mind. Initial experiments should be done using reagents we have confidence in, but we should switch to farm-suitable formulations as soon as possible in the development process.

The conjugate is a protein and therefore susceptible to degradation at elevated temperatures and in the presence of microbial or oxidative contaminants. Losses may be exacerbated at low concentrations. As the concentration will be set to optimise the assay, ideal storage conditions would be approximately 4°C, with a stabilising protein such as BSA, a preservative to minimise microbial growth, and a reducing agent to minimise oxidation. These conditions maximise the storage life of the conjugate and minimise the reagent reservoir refill frequency. However, having a refrigerated storage container increases sensor complexity, so there is a trade-off between a longer storage life and a cheaper, simpler device.

The tetramethylbenzidine (TMB) substrate is light and temperature sensitive and is normally mixed with hydrogen peroxide immediately before use. It can easily be stored in a light-proof reservoir, but the refrigeration issues already discussed are a bigger hurdle. Where possible, commercial, ready-to-use TMB

substrate formulations should be used in the laboratory phase, but they still require refrigeration. Another problem with TMB is disposal. The MSDS (Sigma, 2004) states that TMB should be disposed of by a licensed professional waste disposal service, which will add significantly to the running costs. Alternative common peroxidase substrates (see Section 3.1.2) are no better.

The buffer solutions and regenerant require preservatives to prevent microbial growth. This preservative should be environmentally safe to minimise the volume of waste requiring specialist disposal.

2.10.6 Calibration

Laboratory assays can run a full calibration curve of standard concentrations alongside the test samples, allowing an accurate estimate of the concentration in the samples. This approach is not feasible for the on-line milk progesterone sensor. Firstly, we need to minimise the number of reagents to minimise complexity. Secondly, supply of milk standards is difficult. Standards made from UHT pasteurised milk, could be stored as long as the container remained sealed, but a reliable supply of 'representative' progesterone-free milk is needed to make the standards. This would be difficult.

An alternative to running a full calibration curve is to use positive and negative blanks to normalise the response. The negative blank sample would involve running buffer with no added conjugate. This would emulate a high-progesterone-concentration sample and represents the minimum response of the system. It may not need to be run often. The positive blank would be running buffer mixed with conjugate in the same ratio as for a real sample. This would emulate a low-progesterone-concentration sample and represents the maximum response of the system, thus correcting for variation/degradation in conjugate binding/enzymatic activity, surface activity and substrate activity. These parameters are more likely to change, so the positive blank would need to be run regularly. It is likely that the milk matrix could shift the results

outside the calibrated range, but this should not matter as long as it is repeatable. There must be inter-sensor consistency between the normalised value and the progesterone concentration. This calibration scheme uses no specific calibration reagents and therefore minimises system complexity. The calibration system is a very important component of the assay system so the concept needs to be tested early in the project.

2.10.7 Physical Design

The physical design of the biosensor would be finalised later in the development process, after most of the assay parameters and reagent storage conditions have been optimized/determined. Although the design is outside the scope of this research project, possible designs should be considered, as they will influence the decisions made.

The sample, conjugate and reagents need to be mixed and forced through the column in a controlled fashion. Laboratory pumps such as syringe pumps are expensive and may be unsuitable for the milking environment. Peristaltic pumps are a more robust alternative, but have poorer flow-rate control and the flow is pulsed rather than smooth. Pulsed flow may be detrimental for chromatography, but less critical for our assay.

Mixing the reagents and switching between reagents is not a trivial matter. Liquid chromatography systems typically use expensive, high-pressure valves. The on-farm assay is unlikely to be operating at high pressure so cheaper alternatives may be possible to obtain or custom-design.

Temperature control of the sensor is a major project. The column will probably need to be heated to 38°C during operation. Depending on reagent storage requirements, some reagents may need to be cooled. Temperature control may be obtained using solid-state Peltier devices that act as heat pumps.

The detection system required for an ELISA-based biosensor (Section 2.8.4) is much simpler, more robust and better suited to the milking environment than any of the other technologies discussed. The envisaged system would use a red LED emitting at about 630 nm with a narrow emission angle aimed at a photodiode on the other side of the fluid. The red light would be absorbed by the blue reacted substrate.

Chapter 3 Materials and Methods

3.1 Reagents

3.1.1 Enzyme

Current popular enzyme labels for ELISAs include horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase (Sigma, 2000) and glucose oxidase (Hermanson, 1996). The single-chain polypeptide glycoprotein HRP (Table 2) was selected because it is the smallest and most stable. Its precise carbohydrate and amino acid composition depends on the specific isozyme (Shannon *et al.*, 1966; Clarke and Shannon, 1976). Lyophilised HRP (P8375; Sigma) was supplied by Biolab (Auckland) and stored at room temperature.

Table 2: Properties of HRP.

Property	Value
Molecular weight (including carbohydrate)	40 kDa
Carbohydrate content	18%
No. of isozymes	≥ 7
Disulphide bridges	4
Primary amine groups	2
Isoelectric point (depending on isozyme)	3.0 - 9.0
Optimum RZ* for conjugation to proteins	≥ 3.0
Optimum pH	6.0 - 6.5
Stable pH range	5.0 - 9.0
Storage (powdered)	< 0°C
Storage (suspension)	2 - 8°C

Source: Sigma 2000, Hermanson 1996 pg 100.

* Reinheitszahl (absorbance ratio A_{403}/A_{275}) is a measure of hemin content.

3.1.2 Substrate

HRP readily forms a complex with hydrogen peroxide (H_2O_2), which can then oxidise a wide range of chromogenic hydrogen donors. Several substrates are widely-used in HRP enzyme immunoassays (Table 3). Tetramethylbenzidine (TMB) was chosen for this study because it is the most commonly-used HRP substrate and because it was successfully used in the previously developed plate ELISA (Orchard, 2004).

Table 3: Typical substrates used with HRP.

Substrate	Structure	Characteristics	Measurement absorbance (nm)
ABTS	CH2CH3 CH2CH3 N N HO3S S N—N S SO3H	Light-sensitive More stable in absence of O_2	410 650
OPD	NH2 NH2	Health risks Use within 1 h	450
TMB	H2N NH2 .2HCl	Most sensitive Most common Light-sensitive	370 655
o-dianisidine	H3CO OCH3 H2N NH2	Light-sensitive Health risks	405
5AS	COOH OH H2N		450

ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
OPD - o-phenylenediamine
TMB - 3,3'5,5'-tetramethyl-benzidine
5AS - 5-aminosalicylic acid

Powdered TMB (P8375; Sigma) was supplied by Biolab (Auckland), made to 0.2 mg/mL in 1:3 methanol:water (Appendix 1) and stored in the fridge. Immediately before use, it was diluted to 0.1 mg/mL in phosphate-citrate buffer (pH 5.0) with 0.006% hydrogen peroxide.

In an on-farm situation, it is not feasible to prepare the substrate solution immediately before use without increasing device complexity. Fortunately, ready-to-use TMB formulations that do not require any additives are available. For example, Sigma T0440 is a ready-to-use, one-component liquid substrate solution. It still has the significant drawback that it must be stored at fridge

temperature and used at room temperature. The ideal substrate would be stored ready-to-use at room temperature.

3.1.3 Antibody

Several factors need to be considered when selecting an antibody. Firstly, the antibody must have good selective binding for both the bound and free progesterone. Secondly, it must give relatively repeatable results from batch to batch and there must be some assurance that it will be available commercially. Thirdly, it must be relatively cheap.

Polyclonal antibodies are raised by an immune response in a small mammal (for example a rabbit). Because progesterone is a hapten, some form of conjugated progesterone, normally progesterone-BSA, must be used to induce the immune response. Thus, the antiserum will contain a population of antibodies, many of which will not bind free progesterone. The antiserum is normally purified by immunoaffinity chromatography to select only those antibodies that bind progesterone. Nonetheless, each antiserum batch will have a unique antibody combination.

Monoclonal antibodies are always raised from the same bacterial culture so the antibodies are uniform and identical between batches. Although the initial immune response is initiated by a progesterone conjugate, the antibody selected for immortalisation is specific to progesterone. Monoclonal antibodies have the disadvantage of being more expensive than polyclonal antibodies.

Despite the potential disadvantages of polyclonal antibodies, these were selected for initial trials in this project. The conjugated antibodies will be a high-use consumable in the biosensor, so cost is an important factor.

Another important factor is location of conjugation of both the immunogen against which the antibodies were raised and the progesterone derivative to be immobilised on the column. Counterintuitively, the location of the two may

be different (Munro and Stabenfeldt, 1984), although this may be due to the purification process. For example, polyclonal anti-progesterone produced at UC Davis (USA) is raised against progesterone-11α-BSA and routinely used in combination with progesterone-3-HRP.

This proven combination, sourced from a commercial supplier (Sigma P5289, anti-progesterone (pAb); and P3277, progesterone-3-carboxymethyloxime; both supplied by Biolab, Auckland), was initially selected for the project. Lyophilised polyclonal anti-progesterone powder was stored in the fridge until use.

Later in the project, the monoclonal antibody used in the previously-developed plate ELISA (Orchard, 2004) was used because it was known to interact with progesterone coupled at the 3-carbon. Monoclonal anti-progesterone (mAb1-3 - CL425, batch 9/8/00, UC Davis, USA) was received as a 10.1 mg/mL solution and stored in one of two ways.

- Storage method 1: mAb1 (rec'd 16 Jan 04) and mAb2 (rec'd 1 May 06) were diluted to 252.5 mg/mL in 0.05 M carbonate buffer (pH 9.6) and stored in 0.5-mL aliquots. No preservative was included. The diluent buffer was prepared in 1-L glass bottles used for storing milk for unrelated work. The cleaning regime used for the glass bottles was not aggressive. These aliquots were thawed and diluted further for use.
- Storage method 2: A new storage method was used for mAb3 (rec'd 10 Jul 06) due to concerns about antibody stability. The antibody solution was divided into 10-μL aliquots and stored in the freezer. This meant the antibody was stored in its most concentrated form and not exposed to potentially contaminating buffers during long-term storage. When required, these aliquots were thawed and 490 μL of PBS was added to give a 202 μg/mL solution, which was stored in the fridge for further dilution as required.

3.1.4 Beads

The ideal bead would be large enough to allow fat globules to pass through, small enough to allow sufficient surface area for binding, of a material that does not non-specifically bind antibody or enzyme, pre-activated to allow easy coupling of the ligand, with minimal steric hindrance of antibody-antigen interaction, and cheap.

Tentagel™ Macrobeads (MB 300 02; Rapp-Polymere, Germany) were stored in a desiccator in the fridge. These 280-320 μm diameter polystyrene beads are an order of magnitude larger than beads typically used in immunoassays and were selected so fat globules could pass through. Trials need to be done to determine whether the beads are too large for sufficient binding, but even a very small column will have a much larger active surface area than the well of an ELISA plate. The Tentagel™ Macrobeads are available with a wide range of reactive groups, including amino, which is compatible with the carboxyl group of the P4-3CMO ligand. They cost $14/g, which is relatively cheap for chromatography media, considering a column uses only about 100 mg of beads.

The main disadvantage of the beads is that polystyrene is renowned for non-specific binding (NSB) of proteins, especially antibodies. It is commonly used for ELISA plates partly because of this property. Therefore, some form of blocking agent will probably be required to minimise NSB. This, in turn, may introduce steric hindrance of the antibody-antigen interaction. However, Tentagel™ Macrobeads have long 'molecular tentacles' of about 2000 Da (Figure 8), that may reduce any steric hindrance by 'presenting' the antigen to the antibody away from the blocked bead surface (Rapp-Polymere, 2003).

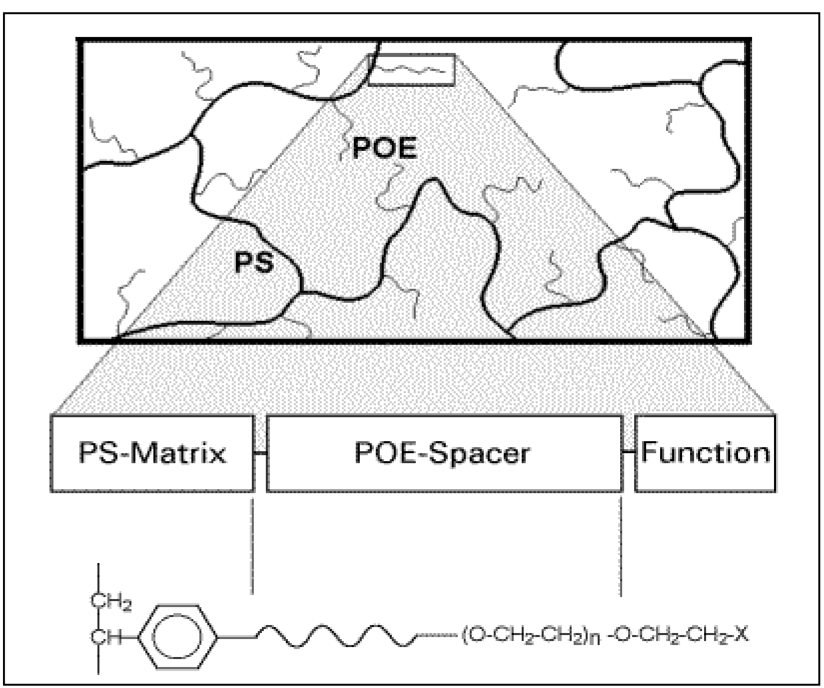


Figure 8: Chemical architecture of Tentagel™ Macrobeads.
(Source: Rapp-Polymere, 2003).

3.1.5 Coupling Reagents

Covalent coupling techniques were used in three parts of this study.

3.1.5.1 Coating Beads

Beads were coated with progesterone-3-carboxymethyloxime (P4-3CMO, Sigma P3277) using an amine coupling technique (Section 3.3.4). The P4-3CMO, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma E7750) and N-hydroxysuccinimide (NHS, Sigma 130672) were supplied by Biolab (Auckland).

To stop these reagents being hydrolysed in the presence of moisture, they were stored under nitrogen in small aliquots in 2-mL plastic vials. The EDC aliquots were stored in the freezer in a desiccator and the NHS aliquots were stored at room temperature in a desiccator.

3.1.5.2 Coating the SPR Chip

The carboxyl surface of the Biacore CM5 chip was coated with P4-3CMO (Sigma P3277) using an amine coupling technique (Section 3.3.1.1). The Amine

Coupling Kit (Biacore BR-1000-50) was supplied by Bio-Strategy (Auckland), and ethylene diamine (Sigma E26266) was supplied by Biolab (Auckland).

3.1.5.3 Enzyme-Antibody Conjugation

The HRP was coupled to pAb and mAb2 using a periodate coupling technique (Section 3.3.2). Sodium periodate (Ajax A695) and sodium borohydride (Ajax A2334) were supplied by Biolab (Auckland).

3.1.6 Other Reagents

Low endotoxin bovine serum albumin (BSA, ABRE-001) was supplied by ICP Bio (Auckland). Progesterone-horseradish peroxidase (P4-HRP) was supplied by UC Davis (USA). All other reagents were research grade. Recipes for the solutions are in Appendix 1 and the suppliers are listed in Appendix 2.

3.2 Equipment

The Biacore 3000 SPR instrument (Biacore, Sweden) in the Mass Spectrometry Suite at the University of Waikato was used for SPR analysis. The Biacore CM5 sensor chip was supplied by Bio-Strategy (Auckland).

A Thermo Multiskan EX plate reader (51118170) supplied by Medica Pacifica (Auckland) with a 630-nm optical filter (1426300) was used to measure the optical density of substrate solutions on a Costar polystyrene microtitre plate (CST3590) supplied by Biolab (Auckland).

Gel electrophoresis was carried out at AgResearch (Ruakura, Hamilton) on a Bio-Rad 4-15% linear-gradient 26-well 15-µL-comb gel (345-0029) in a Bio-Rad Criterion Cell (165-6001) with a Powerpac 200 power supply.

Protein solutions were analysed by size exclusion chromatography at the University of Waikato Engineering Department on an ÄKTA FPLC system

with a Superdex 200 10/30 column, UPC-900 controller, P-920 pump and UNICORN PC software (Amersham Pharmacia Biotech, Sweden).

3.3 Methods

3.3.1 SPR Antibody Activity Test

3.3.1.1 Chip Preparation and Storage

The method of Gillis (2002) was used to couple P4-3CMO to a Biacore CM5 chip surface (Figure 9). Because the flow-cell used for automated coating was sensitive to the dimethylformamide (DMF) solvent, the coupling was done outside the sensor system by applying the solutions directly to the gold surface. The chip surface was first coated with ethylene diamine (EDA) to create an amine surface, compatible with the carboxyl group on P4-3CMO. 40 µL of a 1:1 mixture of NHS solution (115 mg NHS in 10.0 mL deionised water) and EDC solution (750 mg EDC in 10.0 mL deionised water) was placed on the gold surface for 10 min to activate carboxyl groups on the surface. Then, 40 µL of 1 M EDA pH 8.5 was placed on the activated surface for 20 min. One end of the EDA binds to the activated carboxyl groups and the other end provides the amine surface. Remaining active carboxyl groups were capped by applying 40 µL 1 M ethanolamine pH 8.5 for 12 min. The surface was rinsed three times with 10 mM acetate buffer (pH 4.5).

Once the amine surface was created, the progesterone derivative (P4-3CMO) could be coupled. 4.7 mg NHS and 11.1 mg EDC were dissolved in 1 mL 10 mM acetate buffer (pH 4.5) and 450 µL of this activation solution was mixed with 900 µL DMF containing 2 mg P4-3CMO. After 5 min of activation, 40 µL of activated P4-3CMO solution was applied to the surface for 20 min. The finished surface was rinsed three times with acetate buffer and stored in a desiccator at 4°C.

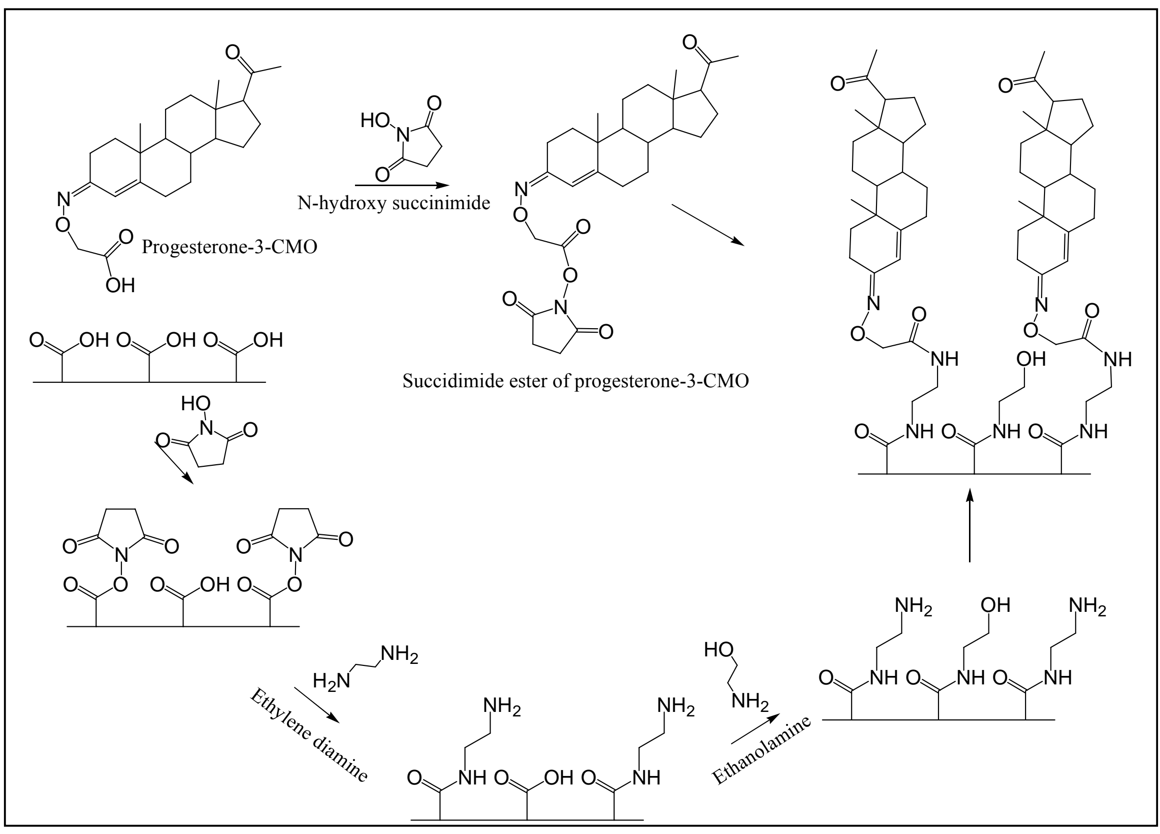


Figure 9: Coating the SPR chip surface with progesterone.

3.3.1.2 Test Routines

Test routines were set up using the Biacore wizard. The entire chip was coated (rather than a pair of channels), so analyses were run through a single channel (channel 2) and no reference channel was used. The first routine used (Table 4) was the most simple and involved an initial baseline measurement, sample injection, a response measurement, and then a regeneration step.

Table 4: Set-up parameters for SPR Test Routine 1.

Flow rate	10 µL/min
Baseline read	30 s before sample inject
Sample inject	30 µL
Response read	30 s before regeneration inject
Regeneration inject	25 µL
Final baseline read	30 s after regeneration inject

This test routine was unsuitable for samples with large amounts of BSA. Some of the BSA would bind non-specifically to the chip surface, creating a much larger response which interfered with measurement of the antibody response. In conventional Biacore SPR procedures, uncoated channels would be run in

series so any response due to non-specific binding could be subtracted from the overall response. This approach could not be used because the entire chip surface was coated with antigen.

Test Routine 2 (Table 5) was developed to cope with high BSA concentrations. It involved extra rinsing to remove the relatively loosely-bound BSA before recording the response, and an extended regeneration to ensure all BSA was removed before applying the next sample. Sections 4.34 and 4.35 describe the development of Test Routine 2.

Table 5: Set-up parameters for SPR Test Routine 2.

Flow rate	10 µL/min
Baseline read	30 s before sample inject
Sample inject	30 µL
Flow rate	30 µL/min
Wait	330 s
Flow rate	10 µL/min
Wait	30 s
Response read	10 s before regeneration inject
Regeneration inject	75 µL
Final baseline read	30 s after regeneration inject

3.3.1.3 Regeneration Routine

To ensure a repeatable baseline before injecting the samples, a stand-alone regeneration routine was set up to run three times at the start of each session (Table 6).

Table 6: Set-up parameters for the SPR Regeneration Routine.

Flow rate	10 µL/min
Baseline read	30 s before sample inject
Regeneration inject	25 µL
Final baseline read	30 s after regeneration inject

3.3.2 Conjugation

3.3.2.1 Method Selection

The carbohydrate moiety of HRP does not participate in the peroxidatic activity (Shannon *et al.*, 1966) and provides a conjugation site that will not inhibit activity. Furthermore, HRP has only two terminal amine groups and is thus less suitable for typical amine coupling protein conjugations (Hermanson, 1996).

It is important not to inhibit the antibody-antigen interaction when coupling with the antibody. Several approaches are in general use, some of which specifically target non-interacting parts of the protein such as the disulphide bridges located in the hinge region. However, non-specific conjugation to amine groups is widely used (Hermanson, 1996).

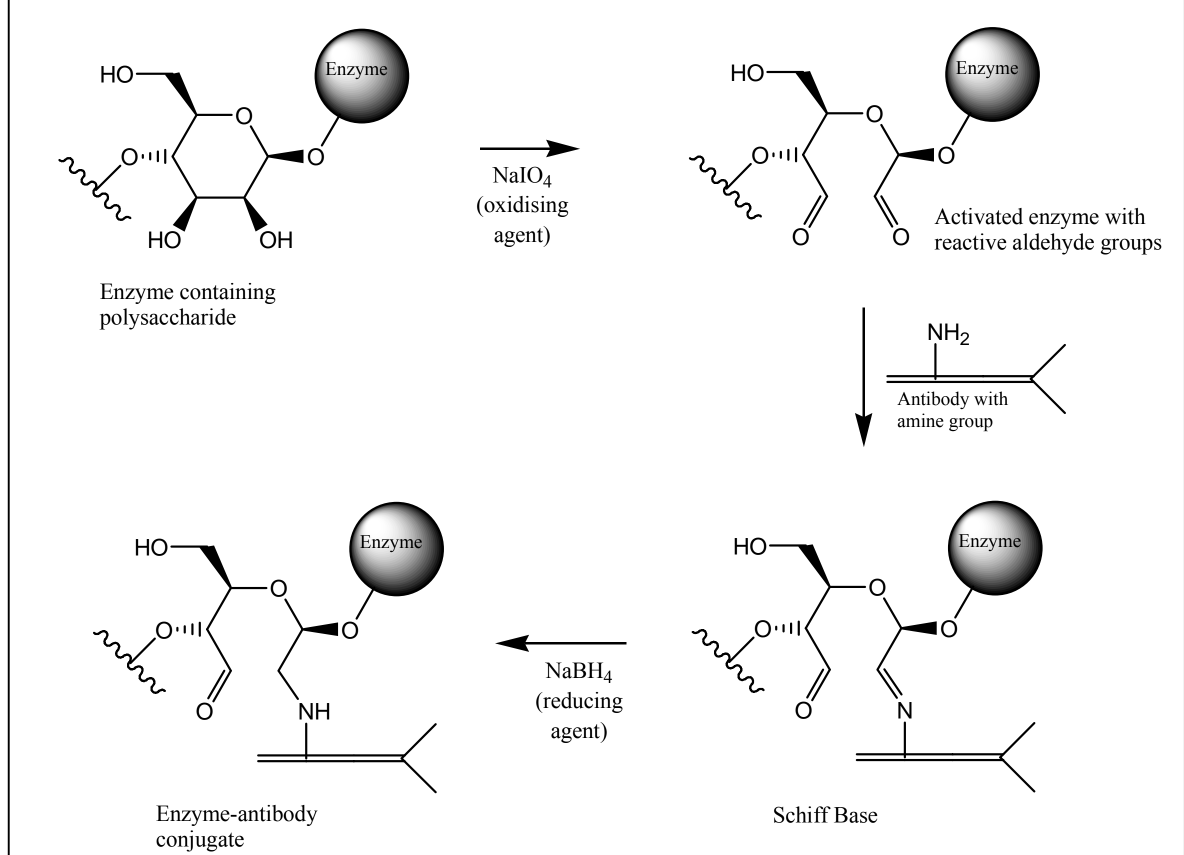


Figure 10: Periodate activated conjugation and reductive amination of HRP-antibody conjugates.

In particular, conjugating HRP to antibodies is a very common activity and several conjugation techniques have been used successfully. This study used a protocol provided by Coralie Munro of UC Davis (USA) (Figure 10). HRP was activated for conjugation by sodium periodate oxidation, which generates reactive aldehyde groups that readily form a Schiff base with terminal amine groups on the antibody. The lack of amine groups on HRP minimises cross-linking. The Schiff base was then reduced with sodium borohydride to form a stable amine linkage. Purification by dialysis was used after activation to purify the activated HRP, and again later to purify the final product.

3.3.2.2 Method

The HRP was coupled to pAb and mAb2 using a periodate coupling method based on the method of Munro and coworkers (1997). Lyophilised HRP (5.0 mg) was dissolved in 1.2 mL deionised water and activated by adding 60 μL freshly prepared 0.1 M sodium periodate (in deionised water) and incubating for 45 min at room temperature. The activated HRP was dialysed overnight with two changes of 1 mM sodium acetate buffer (pH 4.4) at 4°C. After removing the solution from the dialysis tubing, 20 μL of 0.2 M sodium bicarbonate buffer (pH 9.5) was added to adjust the pH to between 9.0 and 9.5. Conjugation was achieved by adding 1.0 mL of 5.0 mg/mL antibody (in 0.2 M sodium bicarbonate buffer (pH 9.5)) to the activated HRP drop-wise with stirring and agitating gently for 2 h at room temperature. The Schiff base was reduced to form a stable conjugate by adding 90 μL of freshly prepared 4.0 mg/mL sodium borohydride and agitating gently for 15 min at room temperature and 90 min at 4°C. The conjugate was dialysed overnight with two changes of 0.01 M phosphate buffered saline (PBS) (pH 7.0) at 4°C.

3.3.3 Conjugate Tests

These methods were used to evaluate the success of conjugation.

- Size Exclusion Chromatography (SEC): Chromatograms were obtained for 40-μL aliquots of HRP-pAb, pAb and HRP using a Superdex 200 10/30 column with PBS (pH 7.0) running buffer at a flow rate of 0.5 mL/min. The protein concentration was about 1 mg/mL.
- SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Samples of HRP-mAb2, mAb2 and HRP were run in parallel on a polyacrylamide gel. The 4-15% Tris HCl gel was run for 50 min at 200 V. Coomassie stain was applied for 2 h followed by destain until the background was clear.
- Surface Plasmon Resonance (SPR): The concentration of HRP-mAb2 was adjusted to an equivalent mAb2 concentration of 10 μg/mL and tested by SPR using Test Routine 1. The results indicate whether there is antibody activity in the HRP-mAb2 solution, but would not confirm conjugation unless the response was significantly higher than for unconjugated mAb2. Otherwise, the response could be caused by free unconjugated mAb2 in the solution.

3.3.4 Coating the Beads

The terminal amine groups on the solid phase are compatible with the carboxyl groups on progesterone-3-carboxymethyloxime (P4-3CMO). A simple amine coupling procedure (Figure 11) was used to permanently immobilise P4-3CMO on the beads. The carboxyl group was activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), forming a succinimide ester. This then reacts with the amine group, covalently bonding P4-3CMO to the solid phase. There is no potential for cross-linking because there are no other carboxyl groups on P4-3CMO or the beads. P4-3CMO was in molar excess to maximise the coating.

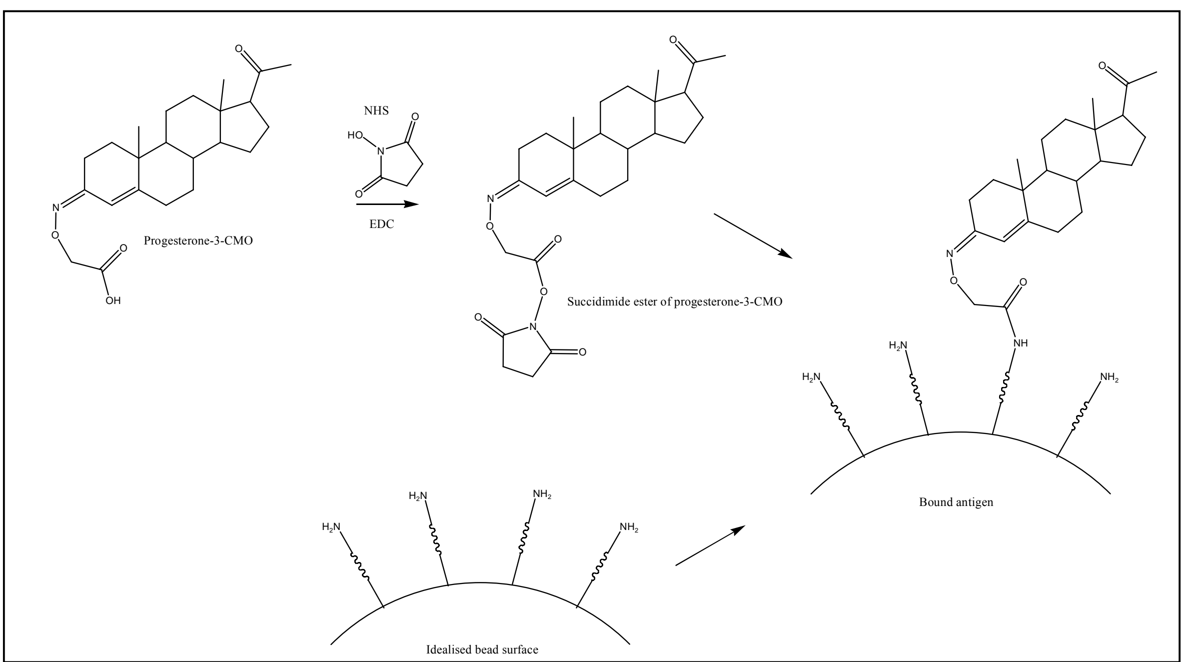


Figure 11: Immobilisation of progesterone-3-CMO to the bead surface.

3.3.4.1 Bead Coating Method 1

P4-3CMO was coupled to amine terminated 'molecular tentacles' on the bead surface with an amine coupling method based on the method used by Gillis and coworkers (2002) to coat a Biacore SPR chip. P4-3CMO (8.1 mg) was dissolved in 3.6 mL dimethylformamide (DMF). 450 µL activating solution (7.8 mg NHS and 20.1 mg EDC in 10 mM acetate buffer (pH 4.5)) was then added. The Tentagel™ Macrobeads (97.8 mg) were added and the slurry was agitated for 40 min at room temperature. The beads were then rinsed with PBS (pH 7.0).

3.3.4.2 Bead Coating Method 2

P4-3CMO was coupled to the bead surface using a method provided by Rapp Polymere (supplier of the beads). It utilised a three-fold higher P4-3CMO to bead ratio and a six to seven-fold higher bead to solvent ratio than Bead Coating Method 1. No water was used.

P4-3CMO (23.5 mg) was dissolved in 600 µL 1:1 DMF:dichloromethane. Then NHS (7.1 mg) and EDC (11.9 mg) were added and the solution was incubated for 5 min to activate the carboxyl groups on P4-3CMO. About 100 mg beads

were added and the slurry was agitated overnight at room temperature. The beads were then rinsed with Regenerant 3. Control beads were prepared with the same method, omitting P4-3CMO, NHS and EDC.

Note: Rapp Polymere recommended diisopropylcarbodiimide and hydroxyl-benzotrizole instead of EDC and NHS respectively, but these were not used due to their hazardous nature.

3.3.5 Bead Tests

3.3.5.1 Transfer-Pipette Column

To quickly evaluate the beads, a crude column was constructed from a 150-mm diameter glass transfer-pipette. Glass wool was packed into the pipette, beads were placed on top of the wool, and a final layer of glass wool was packed on top of the beads (Figure 12). The glass wool did not interact with the reagents and helped (1) prevent beads being washed away as liquid flowed through the column, (2) prevent beads floating up and clinging to the sides of the pipette and (3) to hold them in a packed formation.

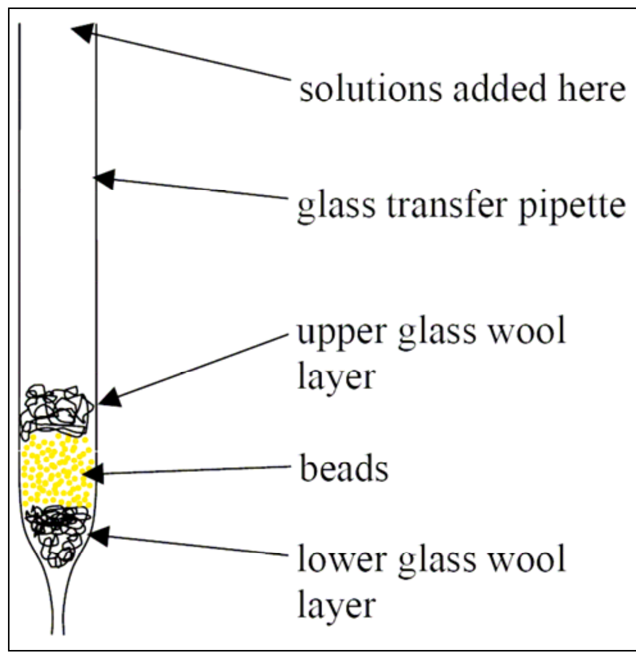


Figure 12: Transfer-pipette column for bead tests.

To use the column, 0.5-1 mL of liquid was added to the top, above the glass wool, and allowed to permeate through the column by gravity. Liquid was retained in the column due to surface tension, although the upper glass wool layer nearly completely drained. Eluant from each addition was collected in a vial below the column.

3.3.5.2 Batch Testing Beads in a Test-Tube

One drawback of the transfer-pipette column was that solutions did not contact the beads for very long. Also, contact could not be enhanced with agitation. To overcome this, beads were suspended in fluid in a 10 mL glass test-tube (Figure 13) and agitated on a shaker. This setup allowed the beads to be in contact with the solutions for extended periods.

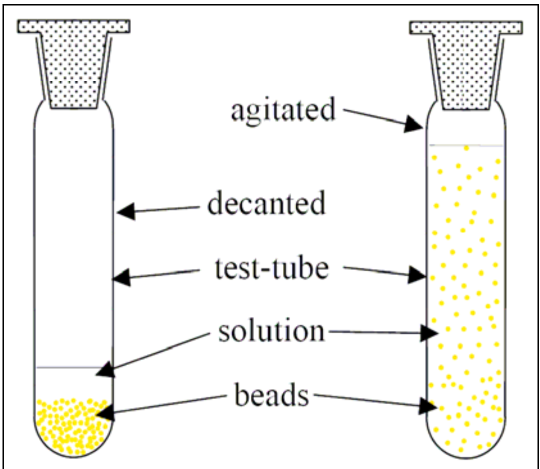


Figure 13: Test-tube set-up for bead tests.

To replace the solution in the test-tube with a new solution:

1. The beads were allowed to settle in the tube.
2. Liquid was removed with a plastic transfer-pipette to the 2-mL line, being careful not to remove any beads.
3. Fresh solution was added to the 10-mL line.
4. Steps 1-3 were repeated several times to ensure the solution was sufficiently replaced.

The number of rinses chosen was based on the similarity between the two solutions and the importance of thorough rinsing. For example, when replacing regenerant with binding buffer, many rinses were required because the regenerant must be completely eliminated or it will damage the antibody or conjugate to be bound. When replacing pH 7.0 buffer with pH 5.0 buffer, the solutions are similar, so less rinses were required.

The beads floated if the solution was very dense (such as Regenerant 1: 5M NaOH) or if air bubbles adhered to the beads. This made decanting very difficult and time consuming and some beads were inevitably lost.

To expose the beads to a critical component (such as conjugate or substrate):

1. The beads were rinsed with the same buffer solution as the critical component to ensure overall conditions after addition were appropriate.
2. The liquid was decanted to the 2-mL line.
3. 2 mL of the critical component was then added.

If the critical component was an interacting component (such as conjugate or enzyme), the current solution was exchanged for PBS (pH 7.0), leaving 2 mL in the tube. Then 2 mL of conjugate/enzyme solution was added and stirred for 10 min. If the critical component was the enzyme substrate (TMB), the current solution (normally conjugate or enzyme) was exchanged for phosphate-citrate buffer (pH 5.0), leaving 2 mL in the tube. Then 8 μL of 3% H_2O_2 and 2 mL of 0.2 mg/mL TMB were added and stirred for 10 min. Duplicate 200 μL aliquots were placed in a microtitre plate well and read in a plate reader at 630 nm. Duplicate readings typically differed by less than 5%.

The following procedure was used to regenerate beads by removing protein from the bead surface:

1. The tube was filled to the 5-mL line with regenerant and agitated for 5 min.
2. The solution was replaced using the solution exchange method above and step 1 was repeated. Four cycles of regeneration were run.

3. The regenerant was exchanged for PBS for storage. The beads were stored in the test-tube in 2 mL PBS at 4°C.

3.3.5.3 Batch Testing Beads in a Syringe

The main problem of the test-tube batch method was the difficulty in separating beads from the solution. The beads are only slightly denser than water (buffer) so they sink slowly, are easily disturbed, and if a bubble adheres to them, they float. Beads were inevitably lost during the decanting process. This problem was overcome using the syringe method, which trapped the beads behind a filter that allowed only the solutions to pass (Figure 14).

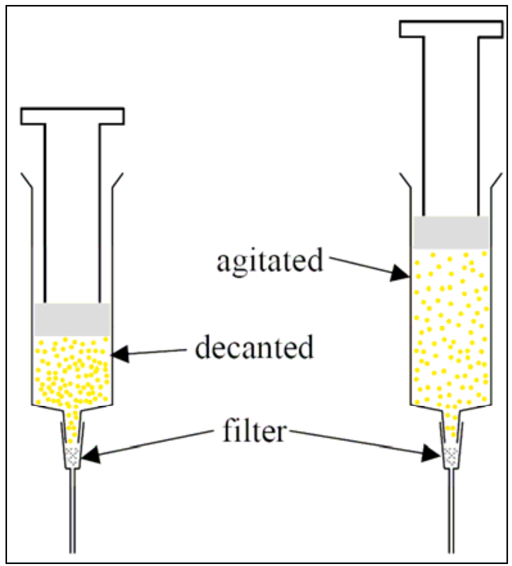


Figure 14: Syringe set-up for bead tests.

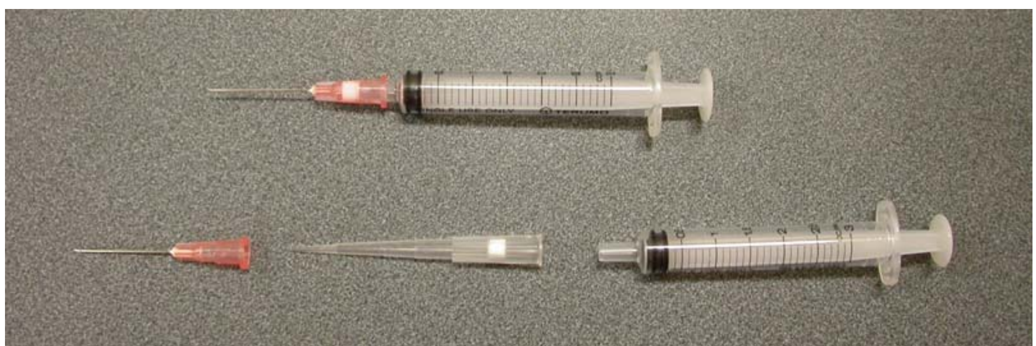


Figure 15: Construction of the syringe bead test device.

The device (Figure 15) was constructed by removing a high-density polyethylene filter from a 200-μL pipette tip (Raylab NZ, Auckland; CPBT200)

and wedging it into the top of a syringe needle (Biolab, Auckland; TER23643422). The modified needle was attached to a 3-mL syringe (Biolab, Auckland; TER23642170), creating a cavity from which beads could not escape. The plunger was removed and beads were loaded through the top of the syringe.

To replace the solution in the syringe:

1. The plunger was pushed down to the 0.5-mL line to eject most of the old solution and leaving the beads loosely packed.
2. The new solution was aspirated by pulling the plunger up to the 2-mL line. This left the plunger in a stable position in the middle of the syringe.
3. Steps 1-2 were repeated several times to ensure that the solution was sufficiently replaced.

As previously, the number of rinses depended on the similarity of the two solutions and the importance of thorough rinsing.

To expose the beads to a critical component (such as antibody or conjugate) in the syringe:

1. The beads were first rinsed with the same buffer as the critical component to ensure conditions after addition were appropriate.
2. The plunger was pushed down to the 0.5-mL line.
3. The critical component was aspirated by pulling the plunger up to the 2-mL line.
4. The syringe was agitated gently by hand for a known time, which depended on the situation, to facilitate interaction between the beads and the solution.

Chapter 4 Assay Development

This chapter describes the methodology used for each individual trial and discusses the results.

4.1 SEC Conjugation Evaluation

Polyclonal antibody (pAb) was conjugated to horseradish peroxidase (HRP) using the periodate conjugation method (Section 3.3.2). To evaluate the success of conjugation, conjugate (HRP-pAb) solution was passed through a size exclusion chromatography (SEC) column (Section 3.3.3). Unconjugated HRP and pAb solutions were run as controls.

The chromatogram (Figure 16) indicated that a large amount of unconjugated HRP and pAb were in the HRP-pAb solution. However, this will probably not affect the performance of the proposed assay. Because excess HRP has no affinity for the antigen, it should not bind to the column and should be eluted with the progesterone-inhibited HRP-pAb. Excess pAb should bind to the column in the same relative proportion as HRP-pAb but, because it has no enzyme activity, it should not contribute to the colour change. However, the excess pAb may inhibit HRP-pAb binding to the column, which could affect the assay. Because there will probably be excess binding sites, this effect should be negligible. Calibrating the system should compensate for any effect of excess pAb. Overall, unconjugated material is probably not a significant problem.

There was also a large amount of higher molecular weight material (Figure 17), presumably pAb and HRP conjugated to varying degrees. The molecular weight difference between pAb and HRP is 110 kDa and their peaks overlap slightly under the column conditions used. Each additional conjugated HRP molecule increased the molecular weight by only 40 kDa, which explains the limited resolution between pAb and HRP-pAb peaks.

This was only a qualitative evaluation. Successful conjugation will only be proven when it is demonstrated that the HRP-pAb solution contains a high enough concentration of species to retain both antibody and enzyme activity.

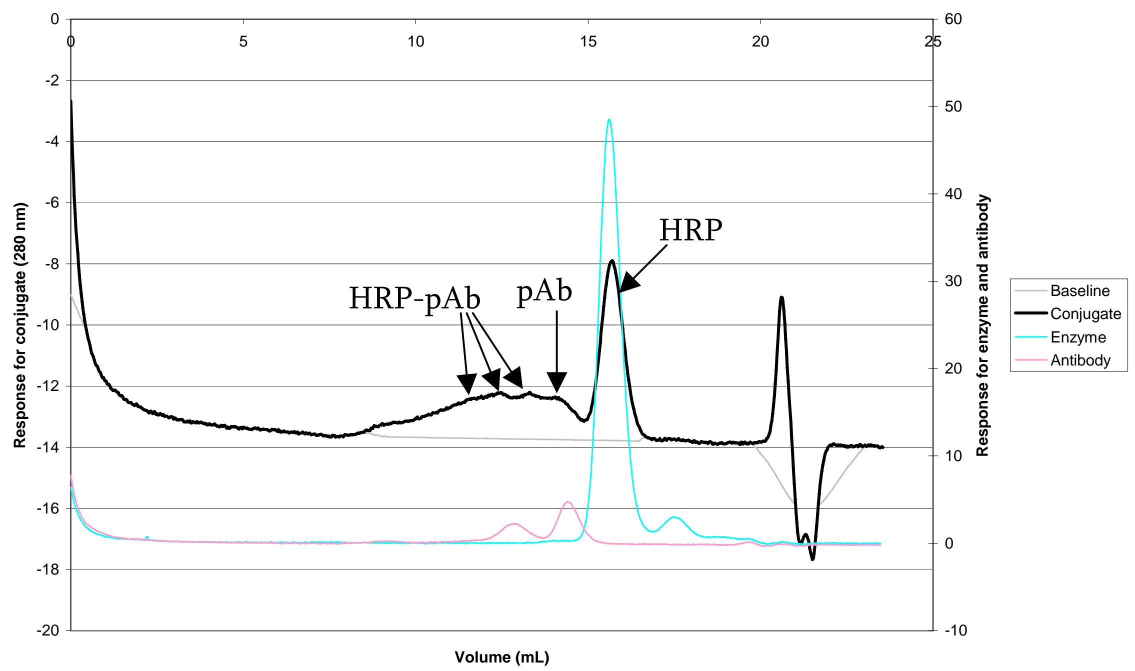


Figure 16: Size exclusion chromatogram comparing pAb, HRP and HRP-pAb.

4.2 Transfer-Pipette Column Bead and Conjugate Test

Tentagel™ Macrobeads were coated with progesterone-3CMO using Bead Coating Method 1 (Section 3.3.4.1). The aim of this trial was to confirm successful bead coating and successful conjugation in a single experiment using the transfer-pipette column (Section 3.3.5.1). It involved rinsing coated beads with PBS, exposing the beads to HRP-pAb, rinsing the beads again, and exposing them to TMB. The intensity of blue in the eluant from TMB was visually evaluated. If there was good binding of HRP or HRP-pAb to the column, there would be good contact between TMB and HRP or HRP-pAb, and the eluant would be an intense blue. Two concentrations of HRP-pAb were tested (1:10,000 and 1:100 dilutions). A control with HRP instead of HRP-pAb would show that binding was due to specific antibody-antigen interaction and not HRP adsorption to the bead surface.

No colour change was observed with the HRP control or the 1:10,000 HRP-pAb. When substrate was applied after the 1:100 HRP-pAb, the solution in the upper glass wool turned blue but the solution in the beads turned a pale almond colour. When the TMB in the upper glass wool layer was flushed with PBS, it turned from blue to pale almond.

A very high HRP-pAb concentration (1:100) was needed to give an observable colour change. HRP-pAb appeared to be retained in the upper glass wool layer as well as in the beads, suggesting either minimal antibody and/or enzyme activity in the HRP-pAb or that any progesterone coated on the beads is not accessible or not sufficiently populated.

Most of the applied solution passes straight through the column minimising the incubation time, which may affect HRP-pAb binding. Beads suspended in a test tube could be incubated for the time required. The disadvantage of this method is that it is more difficult to remove the solution without removing the beads.

The TMB reaction appeared to be 'stopped' by the beads, which may happen if the reaction is pH dependent. The TMB solution was pH 5 while PBS was pH 7. When TMB solution was applied to the column, it remained at pH 5 in the upper glass wool layer (from which most of the PBS had drained) and reacted with any HRP or HRP-pAb to turn blue. When the solution drained into the bead region, there was sufficient PBS to raise the pH and the TMB turned amber rather than blue. The effect of pH on the TMB reaction needs to be investigated.

4.3 pH and Concentration of TMB Reaction

The aim of this trial was to determine whether pH or concentration affects the TMB reaction. The trial involved mixing 0.5 mL of 1:100 (or 1:10000) HRP-pAb with 0.5 mL of PBS (pH 7.0) (or phosphate-citrate buffer (pH 5.0), or no

buffer), then adding three drops (or 0.5 mL) of TMB solution. The colour change was observed for each set of conditions.

Two important characteristics of the HRP-TMB reaction were observed (Table 7). Firstly, if the HRP-pAb to TMB ratio was too high, the colour changed to blue and then quickly to yellow (or almond). This is probably due to blue, reacted TMB undergoing a further reaction in the presence of HRP to form a yellow final product. When only three drops of TMB solution were added to a 1:100 HRP-pAb dilution, the reaction proceeded so quickly that the blue intermediate product was not observed. However, when 0.5 mL of TMB solution was added to a 1:10,000 HRP-pAb dilution, the reaction proceeded very slowly. In a plate ELISA assay (and probably in the proposed column of beads assay system), enzyme is held on the plate wall (or bead surface) and is less accessible and in relatively low concentrations. Therefore, only the blue change is observed.

Secondly, the TMB reaction is pH dependent. At pH 5, the final product is yellow but at pH 7 the final product is almond. This is a secondary issue because the intermediate product appears the same at both pH 5 and pH 7. However, it is recommended that the beads are rinsed with pH 5 buffer before the TMB solution is added to ensure a uniform pH throughout the system. An alternative might be to prepare TMB at pH 7. This would require less rinsing and one less buffer solution - an advantage for an on-line sensor. However, this is an unnecessary deviation from the previous developed ELISA procedure (Orchard, 2004) and from literature upon which the ELISA was based. Improvements could be investigated later when the column of beads assay system is proven.

Data from this trial can explain the results of the previous trial (Section 4.2). The upper glass wool layer contained only a small amount of residual HRP-pAb or HRP so the TMB turned blue. The beads retained a large amount of

HRP-pAb or HRP (whether by non-specific binding or antibody-antigen interaction) and were at pH 7 so the TMB turned almond.

Table 7: Effect of HRP-pAb and TMB concentration and pH on colour formation.

HRP-pAb	Buffer	TMB	Colour
0.5 mL 1:100	0.5 mL PBS (pH 7)	3 drops	Almond
	0.5 mL citrate (pH 5)	3 drops	Yellow
	-	3 drops	Blue → Yellow
	-	0.5 mL	Blue → Yellow
0.5 mL 1:10,000	0.5 mL PBS (pH 7)	0.5 mL	Blue → Almond (slow)
	0.5 mL citrate (pH 5)	0.5 mL	Blue → Green → Yellow (slow)
	-	0.5 mL	Blue → Green → Yellow (slow)

4.4 Test-tube Bead and Conjugate Test I

The aim of this trial was to confirm successful bead coating and successful conjugation in a single experiment, using the batch test-tube setup. The beads were removed from the transfer-pipette column, placed in a 10-mL test-tube (Section 3.3.5.2) and regenerated with two 5-mL rinses of Regenerant 1. To confirm successful regeneration, a development step was completed before adding HRP-pAb or HRP. TMB was exposed to the beads, and the optical density at 630 nm measured. To test the level of NSB, 1:10,000 unconjugated HRP solution (approx. 0.1 μg/mL) with 1% BSA in PBS was stirred for 10 min with the beads. A concurrent control tube contained no beads. The amount of bound HRP was tested by rinsing with phosphate-citrate buffer (pH 5.0), exposing to TMB and measuring the optical density at 630 nm. Beads were regenerated as above. To test the level of HRP-pAb binding, the process was repeated with 1:10,000 HRP-pAb with 1% BSA in PBS.

There was no measurable colour change in any of the conditions used in this experiment (Table 8) and there are several possible reasons for this. The strong regeneration solution (5M NaOH) may have damaged the coating on the beads and prevented HRP-pAb binding; the coating may have been minimal in the first place; the HRP-pAb concentration may have been too low to elicit a

Table 8: Effect of reaction conditions on comparative TMB colour-change with beads exposed to HRP-pAb or HRP in a test-tube.
Data are optical density from the plate reader. Note: due to differences in the development step, values can not necessarily be compared between experiments.

Experiment	Key Differences in Method	Test	Beads				
			Coated A*	Coated B*	Coated C*	Uncoated	None
4.4	1:10,000 HRP-pAb; strong regen.; beads not blocked; BSA in HRP-pAb soln.	Regeneration	0.032	-	-	-	-
		HRP	0.033	-	-	-	0.034
		HRP-pAb	0.037	-	-	-	0.034
4.5	*1:100* HRP-pAb; *mild* regen.; beads not blocked; BSA in HRP-pAb soln.	Regeneration	0.038	-	-	-	-
		HRP-pAb	0.932	-	-	-	-
		HRP	0.274	-	-	-	-
4.6	1:100 HRP-pAb; mild regen.; beads not blocked; BSA in HRP-pAb soln.	Regeneration	0.045	-	-	0.034	-
		HRP-pAb	0.734	-	-	0.541	-
4.7	1:100 HRP-pAb; mild regen.; beads *blocked* with BSA; BSA in HRP-pAb soln.	HRP-pAb	0.458	-	-	0.158	-
4.8	1:100 HRP-pAb; mild regen.; beads blocked with BSA; BSA in HRP-pAb soln.	HRP-pAb	0.262	0.049	-	-	-
4.9	1:100 HRP-pAb; mild regen.; beads blocked with BSA; no BSA in HRP-pAb soln.	HRP-pAb	1.233	-	0.372	-	-
4.10	1:100 HRP-pAb; mild regen.; beads blocked with BSA; no BSA in HRP-pAb soln.; *extra rinse* with pH 5 buffer before 2nd development.	Regeneration	0.052	-	0.035	0.036	-
		HRP-pAb	1.915	-	1.006	1.496	-
		After buffer rinse	0.514	-	0.187	0.352	-

* A, B and C denote batches of beads, all coated using Bead Coating Method 1 (Section 3.3.4.1), but prepared at different times. A: earliest, C: latest.

meaningful response; the conjugation may have been unsuccessful; or the HRP-pAb may have lost either antibody or enzyme activity.

The next trial was done using higher HRP-pAb concentrations and milder regeneration solutions. If it failed, a new batch of beads would be needed to eliminate the possibility that the strong regeneration solution had stripped the binding sites.

4.5 Test-tube Bead and Conjugate Test II

The aim of this trial was to confirm successful bead coating and successful conjugation in a single experiment, using the batch test-tube setup. The same method as described in Section 4.4 was used with the following exceptions:

- Regeneration involved four 5-min rinses with the milder Regenerant 2.
- The HRP-pAb was run prior to the HRP (i.e. the order was reversed).
- The HRP-pAb and HRP dilution was 1:100 with 1% BSA in PBS.
- No control tubes were used.

The results (Table 8) suggest there is good differentiation between the HRP-pAb (0.932) and HRP (0.274), confirming HRP-pAb antibody and enzyme activity and successful bead coating. However, concentrations of stock solutions of the two proteins are only approximate and there is no certainty that their enzyme activities were equivalent prior to exposure. Therefore, not including control tubes with no beads or uncoated beads was a major oversight in this trial. It is possible that the observed response was because free protein had not been properly rinsed between the binding and development steps or because protein was non-specifically bound to the beads.

A better control would be to test uncoated and coated beads concurrently, thus eliminating the need to run HRP. The uncoated beads would indicate NSB or the presence of free-protein.

4.6 Test-tube Bead and Conjugate Test III

The aim of this trial was to confirm successful bead coating and successful conjugation in a single experiment, using the batch test-tube setup. The method described in Section 4.5 was used, but the control was changed. Instead of applying unconjugated HRP to coated beads to measure NSB, the HRP-pAb was applied to uncoated beads in parallel with coated beads.

The response with uncoated beads (0.541) was relatively high compared with coated beads (0.734), indicating a large amount of NSB (Table 8). To reduce NSB, the bead surface could be blocked with BSA.

4.7 Test-tube Bead and Conjugate Test IV

The aim of this trial was to confirm successful bead coating and conjugation in a single experiment, using the batch test-tube setup. The method described in Section 4.6 was used except beads were blocked prior to the binding step by exposing them to 2 mL 2% BSA in tris buffer (pH 8.2) for 1 h with stirring.

Blocking reduced the response with uncoated beads (0.158 cf. 0.541 in Section 4.6), indicating that NSB was much reduced (Table 8). A similar trend was evident with coated beads (0.458 cf. 0.734 in Section 4.6), indicating there was a significant amount of NSB here too. The response with uncoated beads after blocking was still significant (0.158), indicating that NSB was still a factor. The response after blocking was clearly higher on coated than on uncoated beads, indicating the system is valid – the bead coating has been successful and the HRP-pAb has retained both antibody and enzyme activity.

However, one flaw in this experiment casts some doubt on this conclusion. The uncoated control beads had not had the same wear and tear as the coated beads. The coated beads had a longer life and had also been repeatedly exposed to 5 M NaOH during the early experiments. These effects may increase the NSB capacity of the beads. This hypothesis gains further weight because the

HRP-pAb concentration is much higher than that used in the previously developed plate ELISA (Orchard, 2004). It may be that we only see a response (due to NSB) because the HRP-pAb concentration is so high.

4.8 Test-tube Bead and Conjugate Test V

The aim of this trial was to confirm successful bead coating and successful conjugation in a single experiment, using the batch test-tube setup. To eliminate the possibility that the response was due to NSB, fresh beads were coated using Bead Coating Method 1 (Section 3.3.4.1). The experimental method described in Section 4.7 was used with the following exceptions:

- Old coated beads (A) were tested alongside freshly coated beads (B).
- No uncoated control beads were tested.
- Blocking was for 30 min rather than 60 min.
- The HRP-pAb solution contained 1% BSA to help reduce NSB.
- Phosphate-citrate buffer rinsing between the binding and development steps was more thorough, comprising three 5-min exposures with agitation. This would ensure that any free HRP-pAb was removed.

The response with the fresh beads was much lower (0.049) than with old beads (0.262), indicating that either the fresh beads were not properly coated or that the system is not valid and the old beads had higher NSB due to wear and tear. The absolute response cannot necessarily be compared with earlier experiments (see Table 8) because the HRP-pAb solution contained 1% BSA.

The EDC and NHS, coupling agents used in coating the beads are susceptible to hydrolysis in the presence of atmospheric water. If the coating failed, it may be because the EDC and/or NHS used in the preparation had degraded.

4.9 Test-tube Bead and Conjugate Test VI

The aim of this trial was to confirm successful bead coating and successful conjugation in a single experiment, using the batch test-tube setup. A fresh

batch of beads was prepared using Bead Coating Method 1 (Section 3.3.4.1) with freshly purchased and carefully aliquoted EDC and NHS which had been stored under the conditions described in Section 3.1.5.1. The method described in Section 4.8 was used with the following exceptions:

- The original coated beads and freshly coated beads were used. The fresh beads from Section 4.8 were not used.
- Blocking was for 1 h.
- The HRP-pAb solution contained no BSA.

There was a measurable response with freshly coated beads (0.372) giving hope that the coating was successful (Table 8). However, the response ratio between the old (1.233) and new (0.372) beads was large (ratio 3.3) and not much better than the previous trial in Section 4.8 (ratio 5.3). The large discrepancy between old and new beads is most likely from NSB, which is presumed greater in the old beads because of additional wear and tear and exposure to harsh chemicals. The increase in response from Section 4.8 is probably from NSB, because BSA had been omitted from the HRP-pAb solution. This possibility would be eliminated if uncoated beads were run as a control.

4.10 Test-tube Bead and Conjugate Test VII

The aim of this trial was to confirm successful bead coating and successful conjugation in a single experiment, using the batch test-tube setup. The method described in Section 4.9 was used with the following exceptions:

- The uncoated beads from Section 4.9 were used in addition to the original (A) and freshly coated (C) beads from Section 4.9.
- Blocking was for 95 min.
- After the development and reading steps, beads were rinsed with phosphate-citrate buffer (pH 5.0) five times, and additional development and reading steps were completed. The intention was to investigate whether beads were being rinsed properly.

The response with uncoated beads (1.496) was higher than for freshly coated beads (1.006), which is opposite to what would be expected if coating was successful (Table 8). The most likely reason is that NSB is the dominant binding effect. One way to test whether the observed response was due to NSB would be to try to inhibit binding with progesterone. If progesterone inhibits the response, then some antibody-antigen binding is probably occurring.

The response appeared to increase with age of the beads. The original coated beads (A) were oldest (two months old) and gave the highest response (1.915), while the freshly coated beads (C) were newest and gave the lowest response (1.006). This supports the hypothesis that bead wear and tear increases NSB.

Extra rinsing markedly reduced the response for all beads. For example, the response with freshly-coated beads decreased from 1.006 to 0.187, indicating that rinsing before development was not satisfactory and free HRP remaining in solution contributed to the response. Alternatively, weak NSB may be washed off with extra rinsing. This result emphasises the need to eliminate non-specific response from measurements. A more thorough rinse was required.

4.11 Test-tube Bead and Conjugate Inhibition Test I

The aim of this trial was to determine whether the observed response was due to non-specific or antibody-antigen binding. The experiment was carried out with the original coated beads (A) and uncoated beads (A). The stock HRP-pAb solution contained approximately 1.03 mg/mL, which corresponds to 6.87 nmol/mL pAb (including both conjugated and unconjugated pAb), or 13.7 nmol/mL binding sites (two binding sites per molecule). A 1:100 dilution will contain 0.137 nmol/mL binding sites. A 1.27 nmol/mL (400 ng/mL) solution of progesterone has approximately a 9-fold molar excess over the 1:100 dilution of pAb, and should therefore strongly inhibit the antibody-antigen interaction.

1. For uninhibited HRP-pAb, a 1:100 dilution was made using PBS as the diluent. For inhibited HRP-pAb, a 1:100 dilution was made using 400 ng/mL progesterone in PBS as the diluent. No BSA was included. The solutions were stored in glass test-tubes in a refrigerator overnight.
2. The beads were regenerated, rinsed and then blocked with 2% BSA for 90 min.
3. The uninhibited HRP-pAb was exposed to the beads for 15 min with agitation.
4. The degree of HRP-pAb binding to the beads was measured by exposing TMB to the beads and measuring the optical density at 630 nm.
5. Steps 2-5 were repeated for the inhibited HRP-pAb.

Data from the first two inhibition tests (sections 4.11 and 4.12) show that inhibited HRP-pAb (1.612) gave a stronger response than uninhibited HRP-pAb (1.191) (Table 9), which was opposite to what would be expected if the antibody-antigen interaction were the primary binding effect. This appears to confirm that NSB is the dominant effect.

Coated beads gave a higher response than uncoated beads with both inhibited (0.780) and uninhibited (0.885) HRP-pAb. This supports earlier observations and reinforces the hypothesis that beads become more susceptible to NSB as they age. The response with uncoated beads steadily increased throughout the different experiments.

Non-specific binding appears to be a problem with a 1:100 dilution, which is a high concentration compared with the 1:10,000 used for the progesterone-HRP conjugate in the previously developed ELISA (Orchard, 2004). A 1:100 dilution is comparable with the 10 μg/mL mAb1 coating concentration used in the ELISA. The high level of non-specific response may be because the concentration is much higher than if the system were optimum.

Table 9: Summary of results from Test-tube Bead and Conjugate Inhibition Tests. Data are optical density values from the plate reader. Note: due to differences in the development step, data cannot necessarily be compared between experiments.

Experiment	Key Differences in Method	Condition	Beads			
			Coated A*	Coated D*	Uncoated A**	Uncoated B**
4.11	1:100 HRP-pAb; beads blocked with BSA; soln.	Uninhibited	1.191	-	0.885	-
		Inhibited with P4	1.612	-	0.780	-
4.12	*1:1000* HRP-pAb; mild regen. soln.; beads *strongly* blocked with BSA; *extra rinses* with pH 5 buffer before development.	Uninhibited	-	0.047	-	0.050

* A and D denote batches of beads prepared at different times with different methods. A: original beads prepared using Bead Coating Method 1 (Section 3.3.4.1); D: fresh beads prepared using Bead Coating Method 2 (Section 3.3.4.2).
** A and B denote batches of beads prepared at different times with different methods. A: original uncoated beads with no special preparation; B: fresh beads prepared using Bead Coating Method 2 without the coupling agents.

4.12 Test-tube Bead and Conjugate Inhibition Test II

The aim of this trial was to determine whether the observed response was due to non-specific or antibody-antigen binding. Fresh coated beads (D) were prepared using Bead Coating Method 2 (Section 3.3.4.2), recommended by the bead supplier. Uncoated beads (B) were prepared simultaneously using the same procedure, but omitting the activation reagents and the P4-3CMO. This compared coated and uncoated beads, eliminating the effect of bead age.

The method described in Section 4.11 was used with the following exceptions designed to minimise the non-specific response:

- Blocking was more thorough: 4% BSA was used for 2 h.
- A lower HRP-pAb concentration was used: 1:1000 dilution.
- Rinsing was more thorough after the binding step. Beads were rinsed with two changes of phosphate-citrate buffer (pH 5.0), followed by 5 min of agitation. This was repeated twice more before adding TMB.

The experiment was stopped after uninhibited HRP-pAb did not respond with either coated (0.047) or uncoated (0.050) beads (Table 9). The negligible response for both beads indicates that measures taken to minimise the non-specific response were effective.

We also concluded that either conjugation was not effective in combining pAb and HRP activity into a single species, or that the coating was not successful in providing an antigen surface capable of interacting with pAb or HRP-pAb. The inability to obtain a clear conclusion highlights a major flaw in the experiment - trying to validate both components of the system at the same time. Later experiments aimed to validate either the conjugation or the bead coating.

4.13 Microtitre Plate Conjugate Test I

A microtitre plate assay was used to confirm the binding activity of HRP-pAb. This method is based on a successful ELISA (Orchard, 2004) and involved mixing HRP-pAb with a progesterone-HRP conjugate (P4-HRP) to prevent P4-HRP from binding to the antibody surface. The P4-HRP was conjugated at the 3-carbon (the same as the bead coating). If HRP-pAb interacts with P4-HRP, then it is also likely to interact with the bead surface.

1. Wells were coated with mAb1, by placing 100 µL of 10 µg/mL mAb1 in the wells for 2 h.
2. After washing (with ELISA plate wash solution: Appendix A-1.6), wells were blocked with 200 µL of 2% BSA for 1 h.
3. Test solutions were prepared (5 mL each):
    a. 1:500 dilution of HRP-pAb in PBS with 1% BSA.
    b. Positive control: 2 µg/mL pAb in PBS with 1% BSA.
    c. Negative control: PBS with 1% BSA.
4. 12.5 µL 1:200 P4-HRP was added to each test solution to give a final P4-HRP dilution of 1:80,000. The pAb component of test solutions 'a' and 'b' is far in excess of this concentration and so should strongly inhibit P4-HRP

binding to the mAb1 surface of the well. The mixtures were incubated for 15 min at room temperature.

5. After washing the wells, 100 µL of the mixture was placed in the wells and incubated for 1 h with agitation.
6. After washing again, 100 µL of TMB solution was placed in the wells and allowed to develop. The plate was read at 630 nm after about 10 min.

Unfortunately, the plate reader malfunctioned so numerical values could not be obtained. A photograph of the plate (Figure 17) shows a strong colour change in all wells. There is no discernible difference between wells, indicating that neither HRP-pAb nor pAb inhibited P4-HRP. The mixture may not have been incubated long enough before being placed in the wells as some inhibition is expected. Otherwise, it would be concluded that pAb does not interact with P4-HRP, which would explain why the HRP-pAb, conjugated at the same location, would not bind to the beads. mAb1 from UC Davis (CL425) was raised against progesterone-11α-hemisuccinyl-BSA, which earlier ELISA work showed interacted with P4-HRP (Orchard, 2004). pAb was similarly raised against progesterone-11α-BSA, so we would expect the two antibodies to have similar activity towards the antigen.

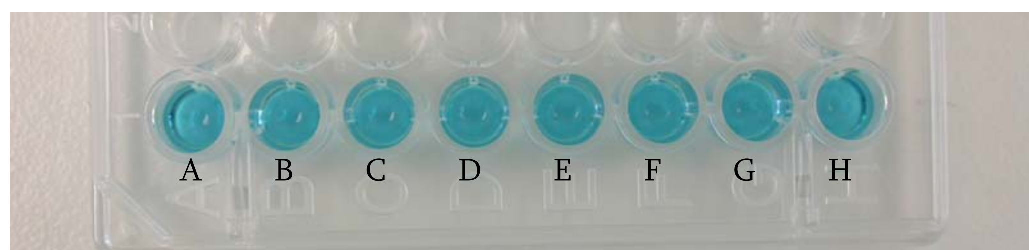


Figure 17: ELISA plate from Microtitre Plate Conjugate Test I.
Wells E, F and H were HRP-pAb, C and D were the positive control (pAb), and A, B and G were the negative control.

4.14 Microtitre Plate Conjugate Test II

This trial compared the binding activity of HRP-pAb, pAb and mAb1. The method described in Section 4.13 was used except mAb1 was included. The

mAb1 solution was made fresh and the other three samples were retained from Section 4.13.

Data from the first five Microtitre Plate Conjugate Tests (sections 4.13-4.17) are summarised in Table 10. Free mAb1, which is known to interact with P4-HRP, failed to inhibit interaction with the immobilised mAb1 (response 1.018) compared to the control (0.997). Therefore, no conclusions can be drawn from this or the previous experiment (sections 4.13).

The P4-HRP may favour the immobilised mAb1 over the free mAb1, pAb or HRP-pAb. Perhaps P4-HRP is effectively removed from solution when it is bound to the immobilised mAb1. Given that bound and unbound states of P4-HRP will be in equilibrium, the long incubation time for binding may allow most of the P4-HRP to be 'removed' from solution by being bound to the surface. This could be tested by reducing binding time.

Table 10: Summary of results from the Microtitre Plate Conjugate Tests
In these experiments, the plate surface was coated with mAb1 and an Ab-based inhibition agent was used to inhibit binding of P4-HRP to the surface. Each value is the average of two replicates. Note: due to differences in the development step the values cannot necessarily be compared between experiments.

Experiment	Key Differences	Inhibition Agent					
		None	**pAb**	**HRP-pAb (1:500)**	**HRP-pAb (1:100)**	**HRP-pAb (no P4-HRP)**	**mAb1**
4.13	Binding 60 min	High	High	High	-	-	-
4.14	Binding 60 min	0.997	1.015	1.016	-	-	1.018
4.15	Binding 10 min	0.467	0.211	0.433	-	-	0.288
4.16	Binding 10 min	0.376	0.338	0.389	-	0.065	-
4.17	Binding 10 min; fresh prep'd HRP-pAb	0.124	0.154	0.149	0.183	-	-

4.15 Microtitre Plate Conjugate Test III

This trial compared the binding activity of HRP-pAb, pAb and mAb1. The previous method (Section 4.14) was used except binding time was reduced from 1 h to 10 min.

mAb1 (0.288) and pAb (0.211) both had lower responses than the control (0.467), indicating they both inhibit P4-HRP (Table 10). It appears that both antibodies were active to progesterone conjugated at the 3-carbon.

HRP-pAb (0.433) had a similar response to the control (0.467), indicating it did not inhibit P4-HRP. Antibody activity appeared to be lost during conjugation and/or subsequent storage. HRP-pAb was stored for several weeks at fridge temperature, which may have contributed to loss of activity.

An alternative, but less likely, explanation for the higher response with HRP-pAb is that it, or free unconjugated HRP in the conjugate solution, non-specifically bound to the surface and interacted with the TMB, causing a colour change.

4.16 Microtitre Plate Conjugate Test IV

This trial compared the binding activity of HRP-pAb and pAb. The method described in Section 4.15 was used except an extra HRP-pAb sample with no P4-HRP was included instead of mAb1. This would indicate whether NSB of the HRP-pAb to the surface was occurring. All protein solutions were prepared fresh from the freezer.

Although the pAb response (0.338) was lower than the control (0.376), the difference was much smaller than in Section 4.15, suggesting that antibody binding ability is not as strong as originally thought (Table 10). The HRP-pAb response (0.389) was very similar to the control (0.376), confirming that HRP-pAb has no antibody activity. The ‘no P4-HRP’ sample had negligible response

(0.065), confirming that the HRP-pAb response was not due to NSB to the plate.

4.17 Microtitre Plate Conjugate Test V

This trial compared the binding activity of HRP-pAb and pAb. The method described in Section 4.16 was used with the following exceptions:

- Freshly conjugated HRP-pAb was used.
- The no P4-HRP control was omitted.
- Two concentrations of HRP-pAb were used: 1:100 and 1:500.

Unexpectedly, the responses of all test solutions, pAb, 1:500 HRP-pAb and 1:100 HRP-pAb were higher (0.154, 0.183 and 0.149 respectively) than the control (0.124) (Table 10). The control had no antibody, and should therefore not inhibit P4-HRP and give the highest response.

The response of the 1:100 HRP-pAb was higher than the 1:500 HRP-pAb, which was also unexpected. The 1:100 has more antibody and should therefore inhibit P4-HRP more and give a lower response than the 1:500 dilution.

These results contradict results from Section 4.15, which demonstrated pAb had antibody activity. In this experiment (as for Section 4.16), antibody activity was questioned. One possible explanation for pAb having low activity may be that the antigen used to purify the pAb was coupled at a different location on the molecule than the antigen used for mAb1.

Based on the uncertain activity of pAb for progesterone coupled at the 3-carbon, it was decided to use mAb1 for conjugation, which is known to interact with progesterone coupled at the 3-carbon. Using this antibody removes one source of uncertainty about the system. The pAb probably has the advantage of being commercially available in larger quantities, but that currently is a low priority. The first priority is to develop a suitable sensor system before beginning to think about commercial considerations.

4.18 ELISA mAb1 and pAb Comparison

This trial used a simple plate ELISA to compare the activity of the two antibodies by coating them directly onto the well (by adsorption), exposing the surface to P4-HRP and developing with TMB solution.

1. A 10 μg/mL solution of each antibody was prepared in carbonate buffer (pH 9.6).
2. A fresh plate was opened and column A was coated by incubating for 2 h with 100 μL mAb1 in each well. Column B had pAb.
3. The wells were rinsed with wash solution (Appendix A-1.6). All the wells were blocked by incubating for 1 h with 150 μL 1% BSA.
4. The wells were rinsed again and antibody activity was tested by incubating for 10 min with 100 μL 1:80,000 P4-HRP with agitation.
5. Wells were rinsed again and P4-HRP bound to the antibody surface was detected by developing with TMB solution for 5 min with agitation.

None of the wells changed colour, indicating that neither the pAb nor mAb1 bound P4-HRP significantly. As mAb1 was known to interact with P4-HRP, the experiment is flawed. This result was attributed to using the same P4-HRP concentration (1:80,000 dilution) as the ELISA (Orchard, 2004) but using a binding time of only 10 min instead of 1 h.

No more comparisons were possible because the supply of pAb had been exhausted. To eliminate doubt about the antibody-antigen interaction, mAb1 with a known interaction with progesterone coupled at the 3-carbon was used in all further trials.

4.19 SPR Surface and Antibody Activity Test I

The aim of this trial was to confirm that there was interaction between an SPR surface coated with progesterone and mAb1. Previous attempts to confirm successful conjugation or coating had been somewhat clumsy. Too many new

parameters and techniques were tested at once so the causes of problems that arose were not clear.

The microtitre plate experiments attempted to overcome this by using established technology to measure antibody activity. If antibody activity could be measured, the bead coating could be tested by its ability to bind antibody. The plate system is designed to measure progesterone concentration by competitive assay against P4-HRP rather than to measure antibody activity. Data from attempts to measure and compare antibody activity by P4-HRP inhibition were poor, so this line of testing was abandoned in favour of the SPR method.

The Biacore SPR system is based on established technology and there are at least two reports of Biacore SPR-based progesterone assays (Gillis *et al.*, 2002; Wu *et al.*, 2002). The system has many advantages over the plate system:

- It measures antibody activity directly.
- Antigen is bound to the chip surface in exactly the same way as to beads so the chip will imitate the bead surface and give a relevant measure of activity.
- Binding events can be viewed in real-time rather than as a single reading at the end of the assay.
- It is automated, so is probably more repeatable.

The chip was coated with P4-3CMO using the method described in Section 3.3.1.1. Two antibody samples were used to test the surface: mAb1 and mAb2. Because stocks of pAb, which was used in the initial conjugation and test-tube bead experiments, had been exhausted, it was not tested.

This was the first time the chip had been used after coating so six regeneration cycles (Section 3.3.1.3) were run to ensure the surface was stable. Regenerant 2 was used for the regeneration cycles and the test routine. Each antibody was diluted to 10 μg/mL in PBS and tested in duplicate by SPR using Test Routine 1

(Section 3.3.1.2). This initial concentration was selected because it was used in the previously developed ELISA (Orchard, 2004).

There was a strong response with mAb1 and mAb2 (2731 and 2439 RU respectively). As the antigen on the surface is the same as that to be immobilised on the beads, this important result implies that mAb1 and mAb2 should also interact strongly with the active surface of the beads.

4.20 Conjugate Antibody Activity Test

The aim of this trial was to confirm that (a) there is an interaction between the SPR surface coated with progesterone and HRP-pAb and (b) that this interaction is inhibited by free progesterone.

A new batch of conjugate was prepared using mAb2 using the conjugation method described in Section 3.3.2. To confirm that the conjugated mAb2 (HRP-mAb2) had retained activity, it was diluted to an equivalent mAb2 concentration of 10 μg/mL and tested by SPR. Control solutions of 10 μg/mL BSA, HRP and unconjugated mAb2 were also tested. Separate mAb2 and HRP-mAb2 samples were also spiked with progesterone to test for inhibition by adding 200 μL 400 ng/mL progesterone in PBS to 1000 μL of the original 10 μg/mL solution. This amount of progesterone is far in excess of the available antibody binding sites, so there should be strong inhibition. Control solutions were prepared in the same way using 200 μL PBS instead of progesterone. All samples were tested by SPR using Test Routine 1.

If HRP conjugates to mAb2, then each molecule would give a larger SPR response than an unconjugated mAb2 molecule. Therefore, assuming antibody activity is not lost during conjugation, the HRP-mAb2 response should be larger than the mAb2.

mAb2 (1738.8 RU) and HRP-mAb2 (1748.5 RU) had similar responses (Table 11), which could either mean that there was little conjugation, or that loss of

antibody activity during conjugation cancelled out the increased response per bound molecule. Further testing is required to clarify which is most likely. Samples spiked with progesterone gave a much smaller response than those spiked with buffer, indicating (as expected) that free progesterone inhibits the SPR response.

Table 11: Summary of results from the Conjugate Antibody Activity Test.

Sample	SPR response* (RU)	Sample	SPR response* (RU)
mAb2	1738.8	BSA	-29.4
HRP-mAb2	1748.5	HRP	-50.8
mAb2 + PBS	1027.1	mAb2 + P4	60.9
HRP-mAb2 + PBS	1147.3	HRP-mAb2 + P4	72.9

* n=2

4.21 SDS-PAGE Conjugation Evaluation

This trial evaluated the success of conjugation using the SDS-PAGE method described in Section 3.3.3. mAb2, HRP and HRP-mAb2 were tested.

Unfortunately a reducing buffer was used, so the mAb2 was cleaved. The cleaved mAb2 bands were visible at about 25 and 45 kDa and the HRP band was visible at 40 kDa (Figure 18). These bands were also visible in the HRP-mAb2 columns, indicating conjugation was incomplete.

The HRP-mAb2 columns had a smear extending from approximately 45 to 200 kDa, which was probably conjugated material. This may be due to cleaving. If the HRP-mAb2 were not cleaved, we would expect bands at 190, 230, etc. kDa, but with cleaving, we expect bands at 65, 85, 105 etc. kDa, and these may not be easily resolved (although the faint darker patches in the smear from the 420 µg/mL HRP-mAb2 column may be bands).

There was certainly some higher molecular weight material after conjugation, which probably includes some HRP-mAb2, but also a significant amount of unconjugated material. As discussed in Section 4.1, unconjugated material may pose no problem to the performance of the system. The final proof of

successful conjugation would be when HRP-mAb2 was bound to immobilised antigen and still catalysed the TMB reaction, thus demonstrating that the molecules binding to the antigen also have enzyme activity. Using beads would be the easiest way to do this but the bead coating needed to be validated first.

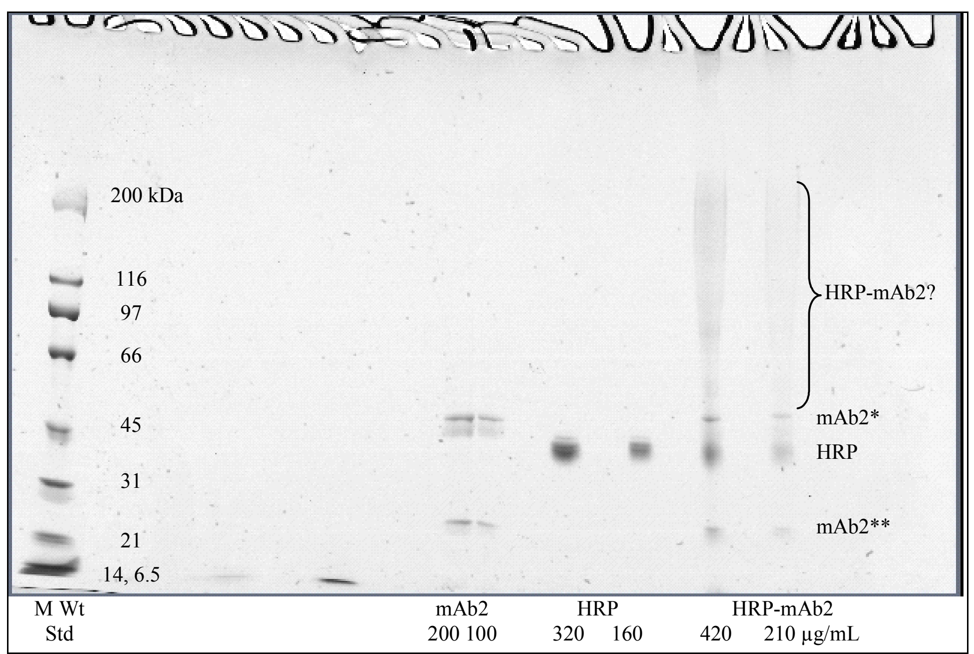


Figure 18: SDS-PAGE gel comparing mAb2, HRP and HRP-mAb2.
* heavy chain; ** light chain.

4.22 Syringe Bead Test I

This trial was to confirm that the coated bead surface interacted with mAb1. New batches of coated and uncoated beads were prepared using Bead Coating Method 2 (Section 3.3.4.2). Each set of beads was loaded into a syringe (Section 3.3.5.3). If the coating was successful, then mAb1 would be extracted from solution when exposed to the beads.

1. The coated and uncoated beads were rinsed five times with PBS to ensure the pH at the bead surface was suitable for antibody-antigen interaction. Each time fresh PBS was aspirated, the syringe was agitated at 400 rpm for 2 min. Finally, the plunger was pushed to the 0.5 mL mark, ready to aspirate the sample.
2. 10 μg/mL mAb1 in PBS was aspirated up to the 2 mL mark for both coated and uncoated beads. The syringes were agitated at 400 rpm for 10 min.

3. The sample was then dispensed from the syringe by pushing the plunger to the 0.5-mL mark and collected in a Biacore vial.
4. The beads were regenerated with three rinses of Regenerant 3, each time agitating for 2 min.
5. Steps 1 to 4 were repeated using the following additional samples:
    a. 10 μg/mL mAb1 with 0.1% BSA. A higher response than the sample without BSA may indicate NSB on the bead surface.
    b. 0.1% BSA as a control.
6. The dispensed samples and control samples not exposed to any beads were tested by SPR using Test Routine 1.

None of the samples gave a response, regardless of whether they had been exposed to beads, suggesting that either mAb1 or the SPR chip coating had degraded.

4.23 SPR Surface and Antibody Activity Test II

The aim of this trial was to reconfirm that there was interaction between the SPR surface coated with progesterone and mAb1. The method used in Section 4.19 was used with the following exceptions:

- Only mAb1 was used.
- 1% BSA was included in the sample.
- A control sample of 1% BSA without mAb1 was included.

A large response with the 1% BSA control sample (754.1 RU) compared with the negligible response observed in the earlier experiment with the 10 μg/mL (0.001%) BSA solution (Section 4.19) indicated that BSA interacted non-specifically with the chip surface and thus, if present in high enough concentrations, would interfere with measurement of antibody activity. There were three ways to overcome this problem:

1. Reduce BSA concentration to a level that would still protect mAb1 yet did not give a significant SPR response.

2. The sensorgram (Figure 19) shows the sensor response due to BSA decreasing steadily after sample injection has finished because the BSA was being slowly flushed by the running buffer. Extending the running buffer period after sample injection so non-specifically bound material was rinsed off should leave only strongly-bound mAb1 to be measured. The drawback of this modification was that cycle time would increase.
3. Normally only two of the four channels on a Biacore SPR chip are coated. The sample is simultaneously run through coated and uncoated channels and the non-specific response for each sample (measured in the uncoated channel) is automatically subtracted from the overall response (measured in the coated channel) in real time. Unfortunately, due to the coating method, the entire surface was coated, so another chip and an alternative coating method would be needed.

Despite the high non-specific response, an antibody-antigen interaction was detected. The response for mAb1 with BSA was 1815 RU compared with 754 RU for BSA only, giving a net response due to antibody of over 1000 RU. Although this is lower than in Section 4.19, it is high enough to allow the beads to be tested. It seems that the antibody and our surface still interact.

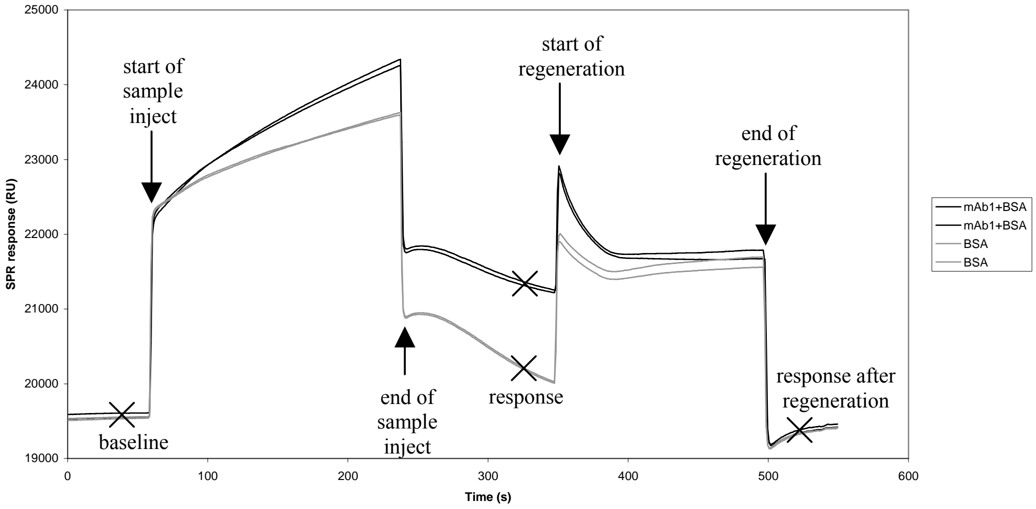


Figure 19: SPR sensorgrams for 10 μg/mL mAb1 with 1% BSA and for 1% BSA.

4.24 Syringe Bead Test II

This trial examined whether there is NSB of mAb1 to the beads.

1. The beads in the syringe from Section 4.22 were rinsed three times with PBS (minimal agitation each time).
2. 10 μg/mL mAb1 in PBS (no BSA) was freshly prepared from stock solution, aspirated to the 2 mL mark with the uncoated beads and manually agitated for 15 min.
3. The sample was dispensed into a Biacore vial and tested by SPR using Test Routine 1.
4. A sample that had not been exposed to the beads was also tested by SPR.
5. The beads were regenerated by rinsing three times with Regenerant 3, agitating gently for 1 min each time.

The response for unexposed mAb1 was 463.5 RU (Table 12), which was lower than observed in previous experiments. This may be because the mAb1 had been out of the freezer for a week, although stability should be better than this.

Table 12: Summary of results from the Syringe Bead Tests.
mAb1 solution was exposed to beads in the syringe and the dispensed solution analysed by SPR (n=2).

[mAb1] (μg/mL)	[BSA] (%)	Blocked Beads	Experiment	SPR response (RU, average of duplicates)		
				Coated Beads	Uncoated Beads	No Beads
10	0	No	4.24	-	43.5	463.5
10	0	Yes (1% BSA)	4.25	-	113.7	-
10	0.01	Yes (1% BSA)	4.26/4.27	11.6	127.3	-
0	0.1	N/A	4.26	-	-	49.7
10	0.01	Yes (2% BSA)	4.28	12.3	211.7	424.1 13.2*
25	0.01	Yes (2% BSA)	4.30	37.0[a]	51.7[b]	107.9[a] 49.4[b]

* The unexposed control sample was repeated at the end of the experiment.
[a,b] Separate batches of solution.

The response for mAb1 exposed to uncoated beads was 43.5 RU, which was much smaller than for unexposed mAb1 and indicates substantial NSB of mAb1 to the beads. Practically all mAb1 was removed from the solution. To minimise NSB, the beads could be 'blocked' by pre-exposing them to a dummy protein such as BSA, or BSA could be included in the mAb1 solution. Alternatively, contact time between mAb1 and the beads could be reduced, limiting the time for NSB.

4.25 Syringe Bead Test III

The aim of this trial was to devise a method to minimise NSB of mAb1 to the beads. BSA was used to block the bead and syringe surfaces.

1. The beads were rinsed five times with PBS (minimal agitation each time).
2. The beads were blocked by aspirating 1% BSA in PBS to the 2 mL mark and agitating gently for 15 min.
3. The beads were rinsed three times with PBS (minimal agitation each time) to remove any unadsorbed BSA, which may interfere with SPR analysis.
4. 10 μg/mL mAb1 in PBS (no BSA) was prepared from stock solution, aspirated to the 2 mL mark with the uncoated beads and manually agitated for 15 min.
5. The sample was dispensed into a vial and tested by SPR.
6. The beads were regenerated by rinsing three times with Regenerant 3, agitating gently for 1 min each time.

The response for the sample exposed to blocked uncoated beads (113.7 RU) was higher than with uncoated beads in Section 4.24 (43.5 RU), suggesting that blocking the beads prevented some NSB (Table 12). However, the response was still very low and not large enough to be useful. Including BSA in the sample solution might further reduce NSB.

4.26 Syringe Bead Test IV

This trial investigated a method to minimise NSB of mAb1 to the beads. BSA was used to block the beads and included in the sample solution. The method described in Section 4.25 was used except the mAb1 sample contained 0.01% BSA. The low BSA concentration minimised the chance of NSB to the SPR chip. A 0.1% BSA sample was also tested by SPR to test NSB to the surface at this concentration.

The response for the sample containing 0.01% BSA and exposed to blocked uncoated beads was 127.3 RU (Table 12). This is very similar to the response obtained in Section 4.25 and indicates that including 0.01% BSA has a negligible effect on loss of mAb1. The 0.1% BSA negative control gave a response of 49.7 RU. This may have been a non-specific SPR response, albeit low, so it was decided to avoid using this concentration if possible.

4.27 Syringe Bead Test V

This trial was to determine whether the coated surface of the beads interacted with mAb1. The method described in Section 4.26 was used except that coated, instead of uncoated, beads were used. Obtaining a much lower response than reported in Section 4.26 would indicate that the coating was successful.

The response for the sample containing 0.01% BSA and exposed to blocked coated beads was 11.6 RU. This was much lower than the corresponding result of 127.3 RU for uncoated beads (Section 4.26; Table 12) and indicated that coating was successful. However, the response for uncoated beads and even for mAb1 not exposed to beads (463.5 RU; Section 4.24; Table 12) was small compared with earlier values of 1500-2500 RU obtained using the same mAb1 concentration. The mAb1 was one week old and may have degraded, which may explain the lower than expected responses. It was decided to replicate the trial using fresh mAb1 that gives a strong response.

The NSB of mAb1 to the beads appeared to be a significant problem. Beads of a different material may have lower NSB.

4.28 Syringe Bead Test VI

This trial investigated whether the coated surface of the beads interacted with mAb1. The method described in Section 4.26 was used with the following exceptions:

- To further reduce the chance of NSB, a higher blocking BSA concentration of 2% was used and the time the sample was in contact with the beads was reduced from 15 min to a single inversion.
- Both coated and uncoated beads were used. A sample that had not been exposed to the beads was also tested by SPR, both before and after samples that were exposed.

The initial results (Table 12) were promising. The mAb1 sample exposed to uncoated beads had a lower response (211.7 RU) than initial samples that had not been exposed to beads (424.1 RU), indicating some degree of NSB. The sample exposed to coated beads gave a negligible response (12.3 RU), indicating that the coating was successful.

However, final retesting of the sample not exposed to beads also gave a negligible response (13.2 RU). All results obtained in this experiment could be explained as being due to antibody degradation, and this raises doubt about the stability of mAb1. The rapid degradation was very surprising because mAb1 had been taken from the freezer and thawed on the day of this experiment.

4.29 Antibody Stability Test I (temperature and dilution)

This trial examined the stability of mAb1 at different concentrations and stored at different temperatures for various times.

1. 2 mL of 252.5 μg/mL mAb1 was taken from the freezer and thawed.

2. Two 1-mL solutions of 10 μg/mL mAb in PBS were prepared, and the remaining 252.5 μg/mL solution was divided into two parts.
3. One sample of each concentration was stored at room temperature and the other was stored in a cold box. Temperature of the cold box was monitored throughout the experiment and ranged from 3.3 to 6.1°C.
4. Solutions were tested periodically throughout the day:
    - The dilute, room temperature sample was stored in the Biacore SPR system sample-rack inside the instrument and sampled directly by the instrument.
    - The dilute, cold sample was stored in the cold box. A 70-μL sub-sample was taken and transferred to the SPR system for each analysis.
    - The non-dilute, room temperature sample was stored on the bench. A small sub-sample was diluted with PBS to 10 μg/mL for each analysis.
    - The non-dilute, cold sample was stored in the cold box. A small sub-sample was diluted with PBS (room temperature) to 10 μg/mL for each analysis.

Although SPR is strongly dependent on temperature in the flow cell, the sample temperature should not have affected SPR measurement. The fluid path in the Biacore is very narrow and any temperature differences in the samples were probably eliminated before they enter the flow cell.

The results indicate that mAb1 was stable even without using BSA as there was no measurable loss of activity for the storage temperatures, times or concentrations used in the trial (Figure 20). This level of stability is good enough for testing the beads.

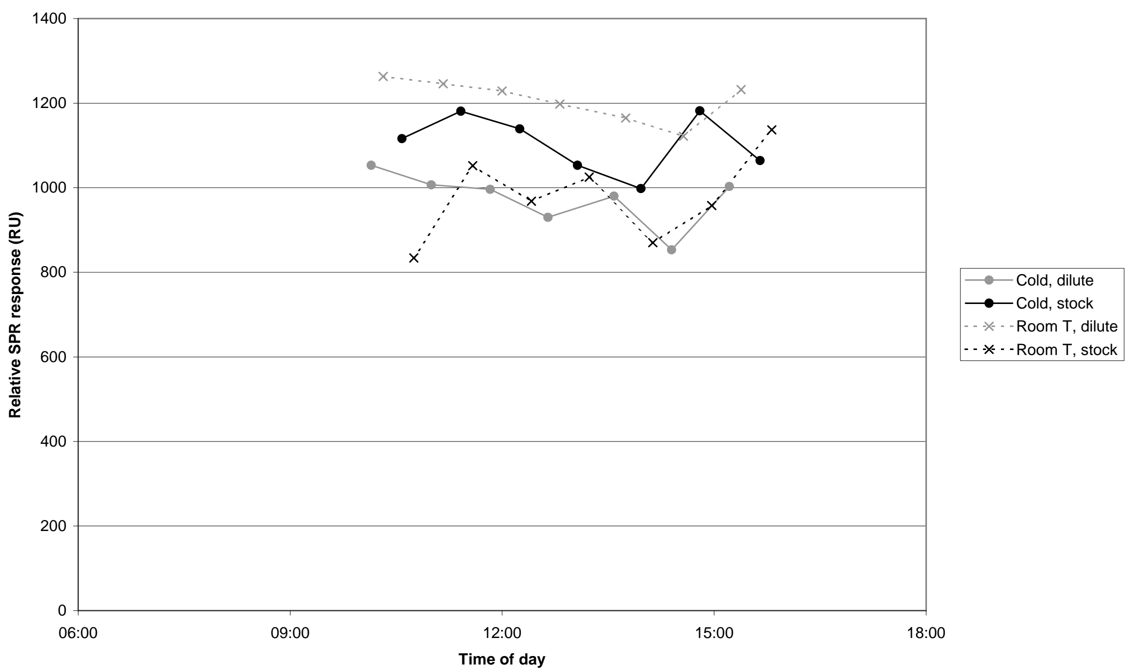


Figure 20: Changes in SPR response of mAb1 under different temperature and concentration storage conditions.

4.30 Syringe Bead Test VII

This trial investigated whether the coated surface of the beads interacted with mAb1.

1. Both the coated and uncoated beads were rinsed three times with PBS.
2. The beads were blocked by aspirating 1% BSA and manually agitating for 15 min.
3. The beads were rinsed a further three times with PBS to remove free BSA.
4. Three batches of 10 μg/mL mAb1 in PBS were prepared. The mAb1 stock solution used was the cold non-dilute mAb1 solution from Antibody Stability Test I (Section 4.29) the previous day, which had been found to be quite stable.
    - Two 2-mL batches had 0.01% BSA to minimise NSB to the beads.
    - One 250-μL batch had no BSA as a control, and was not exposed to the beads.

5. One of the two batches with BSA was exposed to the uncoated beads while the other was exposed to the coated beads. Both samples were exposed for a single inversion of the tube and then dispensed into separate vials.
6. Five vials were tested by SPR: the two dispensed samples; the remainder of the two batches with BSA left after aspiration into the syringe; and the batch without BSA.
7. The beads were regenerated by rinsing three times with Regenerant 3, agitating gently for 1 min each time.
8. The beads were rinsed five times with PBS to remove the regenerant.

The SPR responses for all samples, including those not exposed to the beads, were less than 30 RU. All antibody activity had been lost in the 18 h between the end of Antibody Stability Test I (Section 4.29) and this experiment. All samples from Antibody Stability Test I were retested. Their SPR responses were all lower than 73.8 RU, confirming that all samples had lost activity. This now confirms a serious antibody stability problem. However, even at this level of stability, it should be possible to confirm the success of bead coating with a properly-controlled experiment by using fresh mAb1 from the freezer and completing the experiment quickly.

4.31 Syringe Bead Test VIII

This trial investigated whether the coated surface of the beads interacted with mAb1. Two freshly-thawed aliquots of mAb1, diluted to 10 μg/mL, gave responses of 301.2 and 137.2 RU when tested by SPR. Both these responses are low so a higher concentration was used for the experiment. The method described in Section 4.30 was used except mAb1 concentration was 25 μg/mL and 2% BSA was used for blocking. A BSA-free control was included.

The response for the sample exposed to coated beads was lower (37.0) than when the sample was not exposed (107.9). There was no difference in response for the corresponding samples exposed to uncoated beads (51.7 versus 49.4),

indicating that the coated surface may be interacting with mAb1 (Table 12). However, due to the low response for all samples, it is not reasonable to draw this conclusion. The observed differences are smaller than variations in response occasionally observed between theoretically equivalent antibody samples.

Antibody stability in the two freshly-thawed samples used in this experiment was much worse than for Antibody Stability Test I (Section 4.29). Within a few hours of being removed from the freezer, nearly all activity had been lost.

It is useful to review the antibody storage procedure (Section 3.1.3). This storage procedure was reasonable but not perfect. Proteins are normally most stable when concentrated. Diluting the antibody increases its susceptibility to several modes of activity-loss (Deutscher, 1990). Furthermore, diluent buffer was prepared in glassware used for storing milk for unrelated work so it may have been contaminated with microbes with their associated degradative enzymes to the antibody.

Due to these concerns, the storage procedure was modified. A new shipment of mAb was received (mAb3) and stored in very small aliquots of the original concentrated 10.1 mg/mL solution so it would only be exposed to potentially-contaminated buffers immediately before use.

4.32 SPR Surface and Antibody Activity Test III

This trial investigated whether there was interaction between the SPR surface coated with progesterone and the mAb3. Stored in concentrated form (10.1 µg/mL), mAb3 was first diluted to 202 µg/mL and then to 10 µg/mL in PBS before being tested by SPR using Test Routine 1 (Section 3.3.1.2).

The average SPR response was 1587.7 RU, which is similar to the good responses observed in the initial SPR work (Section 4.19). Because of the new

storage procedure and the relative freshness of mAb3, there was confidence that the bead coating could be tested successfully.

4.33 Syringe Bead Test IX

This trial investigated whether the coated surface of the beads interacted with mAb3.

1. The coated and uncoated beads were rinsed three times with PBS.
2. The beads were blocked by aspirating 2% BSA and manually agitating for 15 min.
3. The beads were rinsed three times with PBS.
4. Two 2-mL samples of 10 μg/mL mAb3, 0.01% BSA in PBS were prepared from the same thawed aliquot of mAb3 used in SPR Coating and Antibody Activity Test III (Section 4.32) earlier the same day.
5. A 1.5 mL aliquot from one sample was exposed to the uncoated beads and one from the other sample was exposed to the coated beads for a single inversion of the tube and then dispensed into separate vials.
6. The dispensed sub-samples and the un-aspirated samples were tested by SPR using Test Routine 1 (Section 3.3.1.2).
7. The beads were regenerated by rinsing three times with Regenerant 3, agitating gently for 1 min each time.
8. The beads were rinsed five times with PBS to remove the regenerant.

The SPR response of the control samples was too low (13.7 RU) so no conclusion could be made about the bead coating. This experiment was carried out within 2 h of the good result that was obtained in SPR Coating and Antibody Activity Test III (Section 4.32). Antibody activity had decayed from about 1600 to about 10 RU indicating that the antibody stability problem was not resolved by using a new shipment of mAb and improving the storage procedure.

4.34 Modifying SPR Test Routine for 1% BSA I

This trial investigated whether a SPR test routine with a longer buffer run-time after sample injection could be developed to sufficiently rinse away non-specifically bound BSA. One way to protect proteins from degradation is to include a high concentration of a 'dummy' protein such as BSA, which necessitates a change to the test routine.

The SPR response for samples with 1% BSA, immediately after injection, was higher than for samples without BSA (Figure 21) and gradually decreased as buffer rinsed away the BSA.

The total rinse time for Test Routine 1 (Section 3.3.1.2) was 107 s with a flow-rate of 10 μL/min. The response was measured 30 s before the end of rinsing, so the effective rinse time was 77 s. The test routine was modified by incrementally increasing the effective rinse time and the rinse flow rate until the measured relative response for 1% BSA in PBS was insignificant. After defining a suitable test routine, a 10 μg/mL mAb3 sample containing 1% BSA was tested by SPR to show that mAb3 was not also rinsed away.

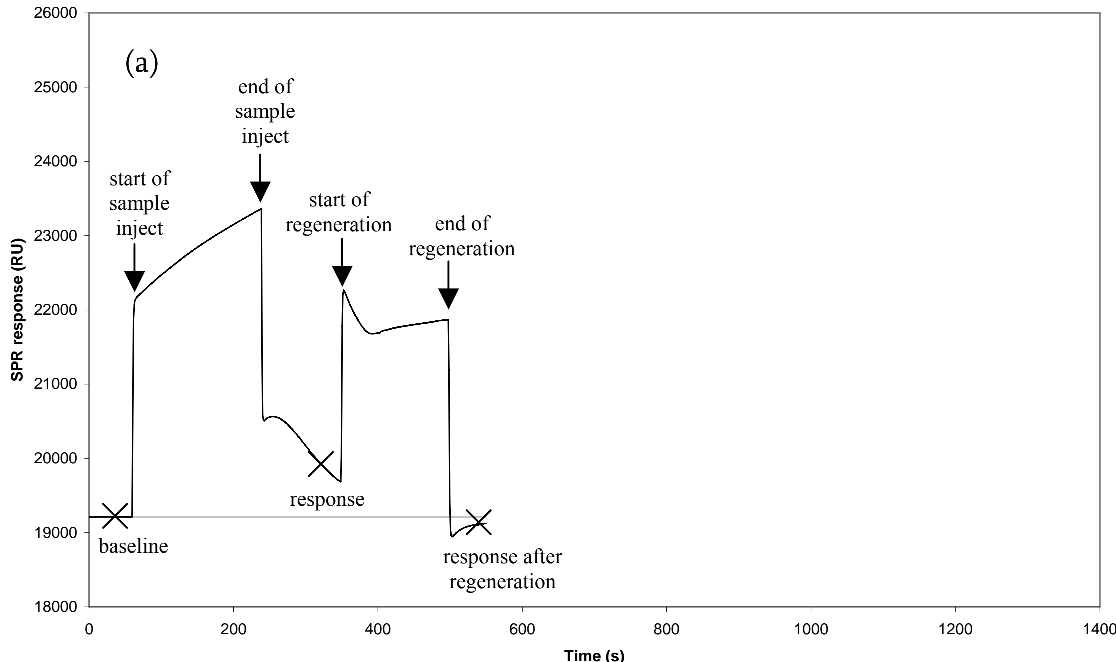
Improving the rinsing procedure successfully reduced the relative response in the absence of mAb3 to insignificant levels (38.8 RU, Figure 21, Table 13). The effective rinse time was 427 s with a rinse flow rate of 30 μL/min.

Table 13: Effect of rinse time and flow rate on relative response with 1% BSA.

Effective rinse duration (s)	Rinse flow rate (μL/min)	Relative response for 1% BSA (RU)
77	10	745.7
222	10	140.8
401	10	71.8
427	30	38.8

However, when mAb3 was included in the sample, the response did not return to the baseline after regeneration (Figure 22). The response after regeneration was 313.5 RU higher than the baseline response before sample injection.

Further improvements to the procedure were needed to ensure the surface was adequately regenerated and ready for the next sample.



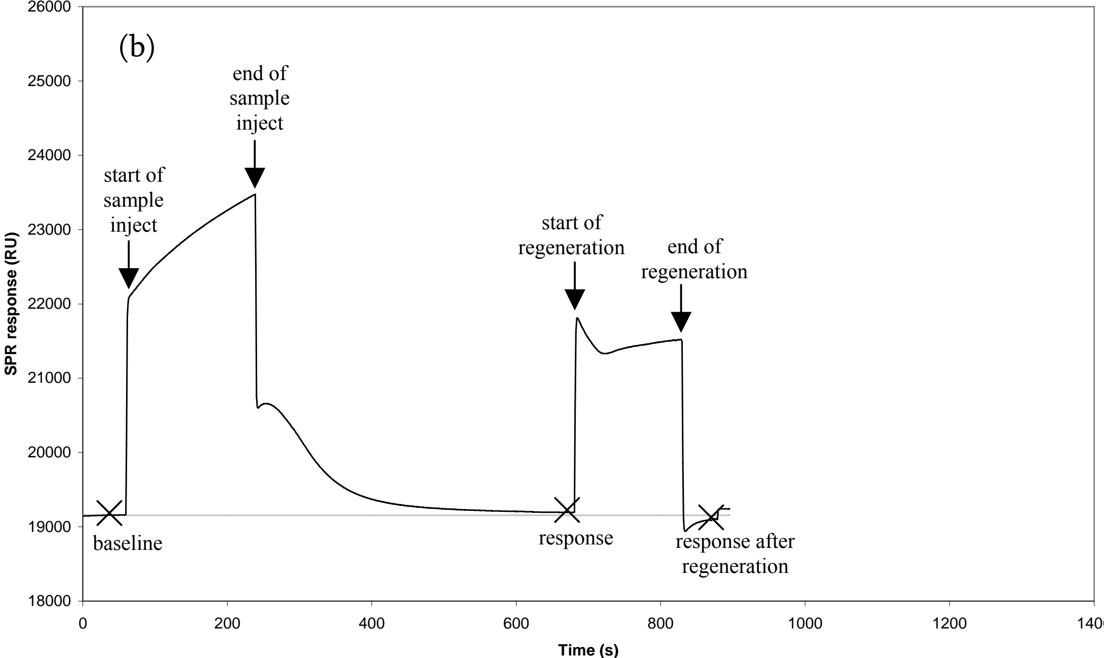


Figure 21: SPR sensorgrams with 1% BSA for (a) Test Routine 1 and (b) a modified routine with longer rinse time and faster rinse flow-rate.

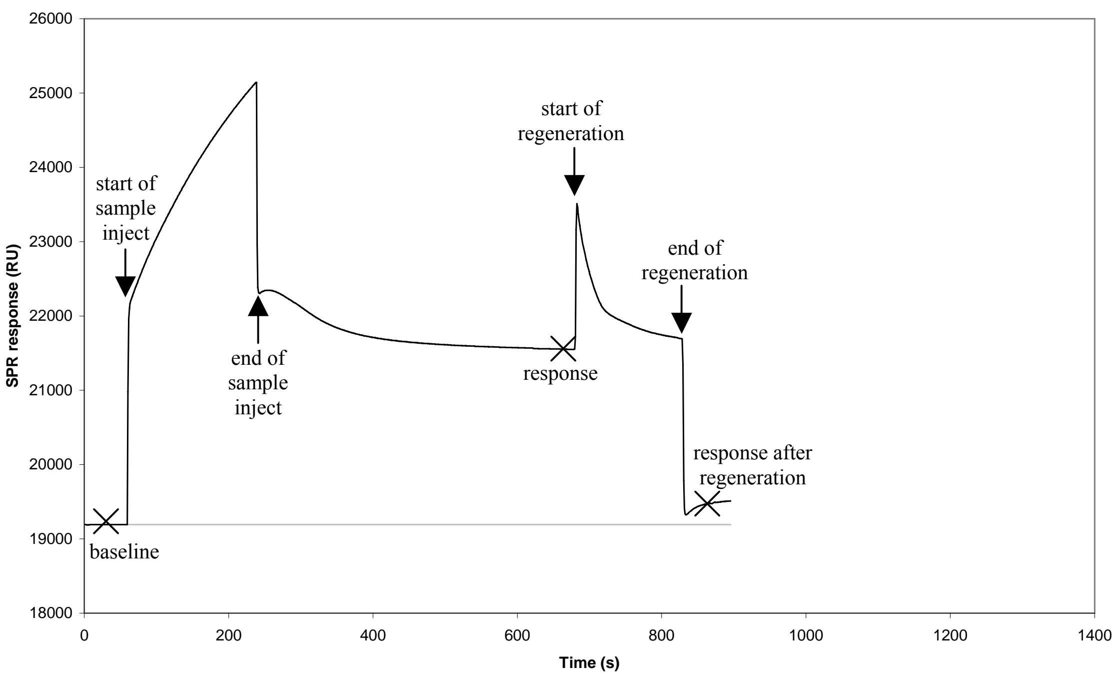


Figure 22: SPR sensorgram for 10 µg/mL mAb3 with 1% BSA using the modified test routine with an increased rinse time and increased rinse flow rate.

4.35 Modifying SPR Test Routine for 1% BSA II

This trial investigated how to improve the regeneration part of the test routine so the response would return to the baseline after injecting mAb3 with 1% BSA. During regeneration, the response gradually decreased as the SPR surface was cleaned (Figure 22). The duration and flow rate of Regenerant 3 were increased incrementally until the response after regeneration when testing 10 µg/mL mAb3 was insignificant.

Improvements to the regeneration step successfully regenerated the chip surface. The response after regeneration was 97.6 RU lower than the baseline before sample injection (Table 14, Figure 23). The final routine (Test routine 2) had a regeneration time of 449 s, which is three times longer than Test routine 1. Regeneration flow-rate did not affect the success of regeneration so it was concluded that contact time between the regenerant and the surface was the most important factor in regeneration. Now a test routine that could handle 1% BSA had been developed, solutions containing such high BSA concentrations could be used to test the beads.

The antibody stability problem was again observed in this trial. The very large relative SPR response due to mAb3 (2185.1 RU) had decreased to only 165.9 RU when tested later in the day.

Table 14: Effect of modifying the SPR Test Routine to handle 1% BSA.

Regeneration duration (s)	Regeneraton flow rate (μL/min)	Relative response after regeneration for 10 μg/mL mAb3, 1% BSA (RU)
149	10	313.5
149	30	360.9
449	10	-97.6

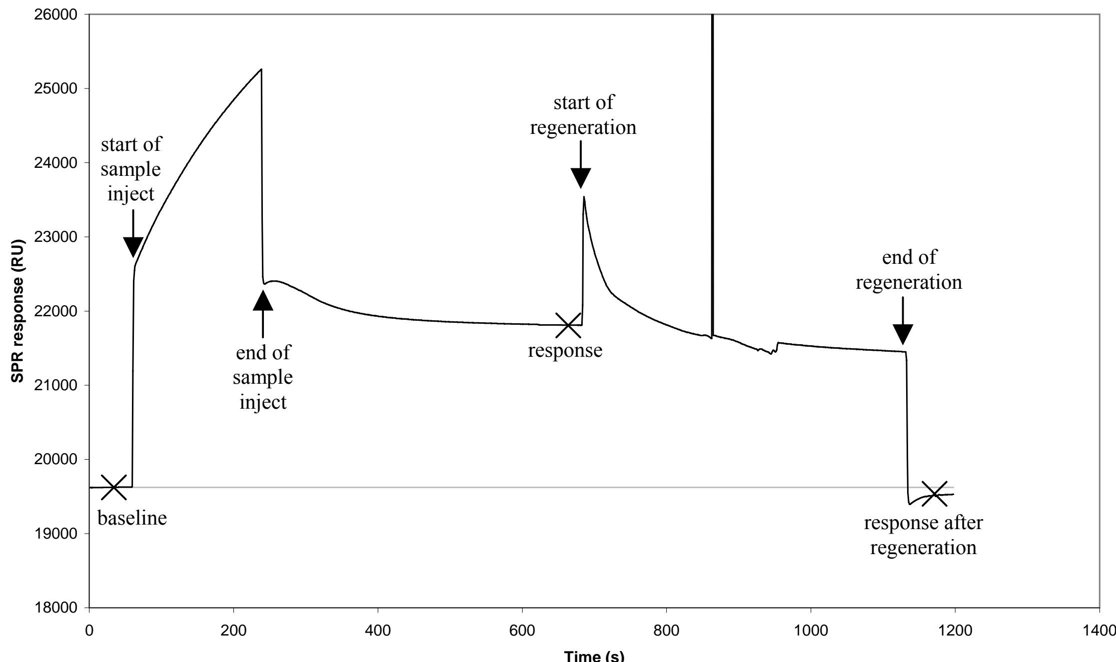


Figure 23: SPR sensorgram for 10 μg/mL mAb3 with 1% BSA using Test Routine 2.

4.36 Antibody Stability Test II (pH)

This trial investigated whether pH affects mAb3 storage stability.

1. Four buffer solutions with various pH values were prepared:
    a. pH 4.5: acetate buffer (10 mM, Biacore BR-1003-50).
    b. pH 7.0: PBS (100 mM).
    c. pH 8.0: tris buffer (50 mM) prepared from a ready-mix sachet.

d. pH 9.6: carbonate buffer (50 mM) prepared using the method described in Appendix A-1.3.

2. 1% BSA solutions were prepared with each buffer. The approximate pH of each solution was confirmed with pH test paper.
3. Four aliquots of frozen mAb3 were taken from the freezer. One aliquot was thawed and diluted to 202 μg/mL with each buffer. Note: the four thawed aliquots were not combined before dilution (only 10 μL was frozen in each aliquot).
4. The solutions were stored in a cold box at about 5°C.
5. A sub-sample of each stock solution was taken from the cold box and diluted to 10 μg/mL using the corresponding buffer containing 1% BSA. The solutions were tested by SPR using Test Routine 2.
6. Step 5 was repeated about 3 h later and 4 d later (solutions were stored in a fridge between sampling).

Initial measurements for samples prepared at different pH values were different (Table 15). The response for pH 8.2 (3096.9 RU) was over twice that for samples stored at pH 4.5 (1257.4 RU). The pH may affect antibody-antigen interaction so more mAb3 binds to the surface at pH 8.2 than at other pH values. Even though the SPR running buffer was pH 7.0, most of the sample will contact the surface within the sample slug. With hindsight, we should have diluted to 10 μg/mL with running buffer to eliminate these differences. Alternatively, because aliquots of mAb3 were not combined before being diluted in specific buffers, the observed difference could be due to inter-aliquot differences in activity from frozen storage.

Compared to previous experience, mAb3 was stable at all pHs. The same batch of mAb3 had been used in SPR Surface and Antibody Activity Test III (Section 4.32) and Syringe Bead Test IX (Section 4.33), yet in those trials the SPR response decreased from about 1600 RU to about 10 RU in a few hours. The only significant difference between the two experiments (Table 16) is that the earlier experiment had no BSA, which may be the critical factor. Some earlier

experiments did contain BSA, but only at 0.01% rather than 1%. The lower concentration may not prevent degradation, especially if a suboptimal storage protocol is used.

The overall conclusion is that mAb3 is relatively stable over the entire pH range (4.5-9.6), but maximum antibody-antigen interaction may occur at about pH 8.

Table 15: Effect of storage pH and time on mAb3 activity.

Time	pH 4.5	pH 7.0	pH 8.2	pH 9.6
Initial	1257.4	2338.5	3096.9	2548.0
3 h	1245.7	2050.0	2890.6	2003.9
4 d	1256.2	1986.1	2827.6	1870.8

Table 16: Storage conditions for two experiments with differing mAb3 stability.

Parameter	Early experiment (Section 4.32 and 4.33; unstable)	This experiment (stable)
Antibody	mAb3	mAb3
Ab concentration	202 μg/mL	202 μg/mL
BSA concentration	0%	1%
Temperature	5°C	5°C
Container	Polypropylene	Polypropylene

Chapter 5 Discussion

This project had the ambitious objective of developing an assay technology platform that could be automated and used as an on-line progesterone biosensor. Unfortunately, due to the many problems encountered, a suitable, workable procedure was not found.

The first two major milestones in the project were to coat the beads with a progesterone derivative and to conjugate HRP to anti-progesterone. The coupling procedures used were not novel. The bead manufacturer provided the procedure for coating the beads and a laboratory that had performed exactly the same conjugation, and similar conjugations on a regular on-going basis, provided the procedure for enzyme-antibody conjugation. Although none of the experiments in this study demonstrated conclusively that either coupling procedure was successful, some results indicated successful coupling and very few were negative. Given enough time, successful coupling would probably have been confirmed.

5.1 Conjugation

Data from size exclusion chromatography (Section 4.1) and SDS-PAGE (Section 4.21) indicate some conjugation. Both these analyses show a population of higher molecular weight proteins, which are probably conjugated material. This conjugated material has a wide range of molecular weights and there is also a significant amount of unconjugated enzyme and antibody, casting doubt on the completeness of conjugation. However, the presence of unconjugated enzyme and/or antibody should not compromise the final assay. Unconjugated enzyme should not bind to the antigen surface, and excess antigen on the surface should ensure that unconjugated antibody will not inhibit the amount of conjugate that can bind.

Although the surface plasmon resonance (SPR) response of conjugate and unconjugated antibody solution were similar (Section 4.20), this does not

indicate whether the antibody binding to the surface has enzyme attached. Conclusively demonstrating successful conjugation first requires confirmation of an active antigen bead surface. A colour change if first conjugate and then, after rinsing, enzyme substrate were exposed to the beads would indicate enzyme had bound to the beads. Using uncoated beads, which should not bind the conjugate and therefore give no colour change, in a parallel experiment would confirm the enzyme causing the colour change is conjugated to the antibody. It would be prudent to also demonstrate that free progesterone in the conjugate solution inhibits surface binding as this is the practical function of the conjugate.

Experiments to demonstrate these effects (sections 4.4-4.12) were confounded by a combination of doubts about activity of the polyclonal antibody for progesterone coupled at the 3-carbon, non-specific binding to the beads, and the fact that the antigen surface had not been independently confirmed.

5.2 Bead-Coating

The Syringe Bead Tests (sections 4.22-4.28, 4.30-4.31; data summarised in Table 12) tended to indicate successful coating. The SPR response of antibody solutions exposed to coated beads was consistently lower than for solutions that had not been exposed to beads or had only been exposed to uncoated beads. However, due to problems with antibody stability, the response in these control solutions was too low to eliminate doubts that the results were due to random fluctuations in SPR response.

5.3 Antibody Stability

The key to confirming successful bead coating is to overcome the antibody stability issue. Two major problems occurred with antibody during this project. Firstly, there were doubts about the binding activity of polyclonal antibody (pAb) towards progesterone coupled at the 3-carbon. Lack of activity

was never demonstrated in a comparative experiment with proven mAb1. Data from both experiments comparing the two antibodies showed similar activity (sections 4.14 and 4.15). However, doubts about antibody activity were raised in the subsequent experiments (sections 4.16 and 4.17), which used the same procedure as in Section 4.15 but without mAb1 as a positive control (at that time, pAb was considered the positive control). The pAb may have activity towards progesterone coupled at the 3-carbon, but lost it in much the same way that mAb1-3 did later in the project. Hindsight suggests that either an antibody with a known interaction should have been used, or tests for interaction with the antigen should first be done before using the antibody. This would have been easy to test using SPR, but by that time, stocks of pAb had been exhausted.

The second issue with antibody was mAb1-3 stability. Antibody was used to confirm successful bead coating but it did not retain activity long enough to allow conclusive results. Experiments demonstrated that dilution, temperature (Section 4.29), pH (Section 4.36), and storage regime (sections 4.32 and 4.33) did not affect stability. Data were contradictory; solutions used in the dilution/temperature stability trial were stable throughout the day of the experiment but had lost all activity the next day (Section 4.30); solutions that had been stored under a new regime lost all activity within a few hours; but solutions used in the pH trial lasted 4 days without significant loss of activity.

The latter trial used a BSA concentration of 1% (Section 4.26-4.28) rather than no BSA (other stability trials: sections 4.30-4.31) or 0.01% (the latter bead tests: Section 4.33). Many modes of protein degradation can be minimised by adding a stabilising protein so it is plausible that this could be the key to the problem. For example, oxidising agents or degradative enzymes will normally attack any protein so adding a large concentration of a dummy protein protects the active protein. Likewise, if protein adsorbs to the sides of the container, the dummy protein will be adsorbed in proportion to its concentration, thus retaining most of the active protein in solution. Adding 0.01% BSA may be

insufficient to adequately protect the antibody. Higher concentrations were not initially used because of NSB to the SPR surface but this was overcome by incorporating longer rinsing times into the SPR test routine (sections 4.34 and 4.35). An intermediate concentration such as 0.1% would not require such long rinsing times but may still provide adequate antibody protection.

5.4 Non-Specific Binding to Beads

The polystyrene Tentagel™ Macrobeads tend to enhance NSB. This was best demonstrated when antibody exposed to uncoated beads gave a much lower SPR response than antibody not exposed to beads (Section 4.24). To test whether this had been caused by the syringe, an antibody exposure test should be run in an empty syringe. If there is NSB to the syringe, a glass syringe could be used. Polystyrene is renowned for NSB. If NSB to the Macrobeads is a serious issue, sepharose beads (EAH Sepharose 4B, GE Life Sciences, UK) could be used. Sepharose is commonly used in protein purification columns and should have minimal NSB. The NSB may be substantially reduced by adding higher BSA concentrations in the protein solutions (e.g. 0.1% or 1%).

5.5 pH of Substrate Reaction

The substrate reaction can be carried out at pH 7 (Section 4.3), which increases the simplicity of an on-line biosensor because the whole assay could be done with a single pH 7 running buffer.

Chapter 6 Conclusions and Recommendations

Despite poor progress in this project, there is still optimism. SPR is a fast and reliable technique for measuring antibody activity. Using the purpose-built coating apparatus to coat only two of the four channels (Section 4.3) will allow the non-specific response to be measured on the uncoated channel in real-time and subtracted from the response in the coated channel. This would allow higher BSA concentrations to be used without compromising activity measurements or extending the analysis time.

Once the antibody stability issue is overcome (which is achievable), it should be relatively simple to confirm conjugation and bead coating as both are based on well-understood and commonly-used chemistry.

The next step of proving the assay format is the major hurdle in the project. After loading the coated beads into a column, it is necessary to show: (a) conjugate is bound to the column as solution passes through; (b) substrate, after binding, measurably changes colour; and (c) free progesterone at naturally-occurring levels measurably inhibits conjugate binding to the column. Because many parameters may affect the assay, these are not simple tasks. Once these parameters are set, the economic feasibility of the assay (e.g. running cost) will be better understood so a decision on continuing the project could be made.

In summary, many problems were encountered but these did not undermine the project's potential. There is a good chance of success if work were to continue.

References

Acevedo, B., Perera, Y., Torres, E., Pentón, D., Ayala, M., and Gavilondo, J. 2006. Fast and novel purification method to obtain the prostate specific antigen (PSA) from human seminal plasma. Prostate 66:1029-1036.

At-Taras, E.E., and Spahr, S.L. 2001. Detection and characterization of estrus in dairy cattle with an electronic heatmount detector and an electronic activity tag. J Dairy Sci 84:792-8.

Australian National Milk Harvesting Centre. 2003. Cow time quick note 5.8: automatic milking installations (robotic milking) [Online] http://www.cowtime.com.au/technical/QuickNotes/Quick_Note_5_8.pdf (posted Jan 2003; verified May 20).

Biacore. 2007. Technical note 1: surface plasmon resonance [Online]. Available by Biacore http://www.biacore.com/lifesciences/service/application_notes/index.html.

Brecht, A., Klotz, A., Barzen, C., Gauglitz, G., Harris, R.D., Quigley, G.R., Wilkinson, J.S., Sztajnbok, P., Abuknesha, R., and Gascón, J. 1998. Optical immunoprobe development for multiresidue monitoring in water. Anal Chim Acta 362:69-79.

Britt, J.H. 1985. Enhanced reproduction and its economic implications. J Dairy Sci 68:1585-1592.

Brooker, G., Terasaki, W.L., and Price, M.G. 1976. Gammaflow: a completely automated radioimmunoassay system. Science 194:270-6.

Catt, K., Niall, H.D., and Tregear, G.W. 1966. Solid-phase radioimmunoassay of human growth hormone. Biochem J 100:31c-33c.

Claycomb, R.W. 1996. Biosensor for on-line measurement of bovine progesterone during milking. PhD thesis, University of California, Davis.

Deutscher, M.P. 1990. Maintaining protein stability, p. 83-89, *In* M. P. Deutscher, ed. Methods in Enzymology, Vol. 180. Academic Press, New York.

Edgar, D.G. 1953. The chemical assay of progesterone. Biochem J 54:50-5.

Engvall, E., Jonsson, K., and Perlmann, P. 1971. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin g, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim Biophys Acta Protein Struct 251:427-434.

Erb, R.E., Chew, B.P., and Keller, H.F. 1977. Relative concentrations of estrogen and progesterone in milk and blood, and excretion of estrogen in urine. J Anim Sci 45:617-26.

Esslemont, R.J., Kossaibati, M.A., and Allcock, J. 2001. Economics of fertility in dairy cows, p. 19-29, *In* M. Diskin, ed. Fertility in the High Producing Dairy Cow, Vol. Occasional Publication No 26. British Society of Animal Science.

Fricke, P.M., and Lamb, G.C. 2005. Potential applications and pitfalls of reproductive ultrasonography in bovine practice. Vet Clin North Am Food Anim Pract 21:419-36.

Frobenius, W. 1999. Ludwig Fraenkel: 'spiritus rector' of the early progesterone research. Eur J Obstet Gynaecol Reprod Biol 83:115-119.

Gil, Z., Szarek, J., and Kural, J. 1997. Detection of silent oestrus in dairy cows by milk temperature measurement. Anim Sci 65:25-29.

Gillis, E.H., Gosling, J.P., Sreenan, J.M., and Kane, M. 2002. Development and validation of a biosensor-based immunoassay for progesterone in bovine milk. J Immunol Methods 267:131-138.

Goodfriend, T., Ball, D., and Updike, S. 1969. Antibody in polyacrylamide gel, a solid phase reagent for radioimmunoassay. Immunochemistry 6:481-4.

Gray, D., and Keyes, M. 1981. Enzyme immunoassays using immobilized reagents in a flowing stream. US Patent 4,277,560.

Henderson, K.M., Camberis, M., Simmons, M.H., Starrs, W.J., and Hardie, A.H. 1994. Application of enzymeimmunoassay to measure oestrone sulphate concentrations in cow's milk during pregnancy. J Steroid Biochem Mol Biol 50:189-96.

Hermanson, G.T. 1996. Bioconjugate Techniques. Academic Press, San Diego, USA.

Hillerton, E.J. 2000. Detecting mastitis cow-side(ed.). National Mastitis Council Annual Meeting Proceedings, 2000. Atlanta, USA.

Holdsworth, R.J., Booth, J.M., Sharman, G.A., and Rattray, E.A. 1980. Measurement of progesterone levels in whole and fore-milk from dairy cows. Br Vet J 136:546-554.

Humblot, P. 2001. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology 56:1417-1433.

Ismail, A.A., West, P.M., and Goldie, D.J. 1978. The "Southmead System," a simple, fully-automated, continuous-flow system for immunoassays [Appendix: application to serum thyroxine radioimmunoassay]. Clin Chem 24:571-9.

Koelsch, R.K., Aneshansley, D.J., and Butler, W.R. 1994. Milk Progesterone Sensor for Application with Dairy Cattle. J Agric Engng Res 58:115-120.

Laitinen, M.P.A., and Vuento, M. 1996. Affinity immunosensor for milk progesterone: identification of critical parameters. Biosens Bioelectron 11:1207-1214.

Martin, S.E., and Peterson, B.R. 2002. A colorimetric enzyme-linked on-bead assay for identification of synthetic substrates of protein tyrosine kinases. J Pept Sci 8:227-233.

Miller, S., Morton MS, and A, T. 1988. Chemiluminescence immunoassay for progesterone in plasma incorporating acridinium ester labelled antigen. Ann Clin Biochem 25:27-34.

Morais, R., Valente, A., Almeida, J.C., Silva, A.M., Soares, S., Reis, M.J.C.S., Valentim, R., and Azevedo, J. 2006. Concept study of an implantable microsystem for electrical resistance and temperature measurements in dairy cows, suitable for estrus detection. Sens Actuators, A 132:354-361.

Munro, C., and Stabenfeldt, G. 1984. Development of a microtitre plate enzyme immuno-assay for determination of progesterone. J Endocrinol 101:41-49.

Munro, C.J., Laughlin, L.S., Illera, J.C., Dieter, J., Hendrickx, A.G., and Lasley, B.L. 1997. ELISA for the measurement of serum and urinary chorionic gonadotropin concentrations in the laboratory macaque. Am J Primatol 41:307-22.

Nebel, R.L., Whittier, W.D., Cassell, B.G., and Britt, J.H. 1987. Comparison of on-farm and laboratory milk progesterone assays for identifying errors in detection of estrus and diagnosis of pregnancy. J Dairy Sci 70:1471-1476.

Nebel, R.L., Altemose, D.L., Munkittrick, T.W., Sprecher, D.J., and McGilliard, M.L. 1989. Comparisons of eight commercial on-farm milk progesterone tests. Theriogenology 31:753-764.

Orchard, R.G. 2004. Development of a progesterone EIA. Directed Research Report, University of Waikato, Hamilton, New Zealand.

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

Rajamahendran, R., Ambrose, D.J., and Burton, B. 1994. Clinical and research applications of real-time ultrasonography in bovine reproduction: a review. Can Vet J 35:563-72.

Rapp-Polymere. 2003. TentaGel resins: a brief summary and overview [Online]. Available by www.rapp-polymere.com www.rapp-polymere.com/preise/tent_s_d.htm.

Rathbone, M.J., Macmillan, K.L., Inskeep, K., Burggraaf, S., and Bunt, C.R. 1998. Fertility regulation in cattle. J Control Release 54:117-48.

Ribadu, A.Y., and Nakao, T. 1999. Bovine reproductive ultrasonography: a review. J Reprod Dev 45:13-28.

Rogers, L.A. 1928. Fundamentals of Dairy Science. Chemical Catalog Company, New York, USA.

Romano, J.E., Thompson, J.A., Kraemer, D.C., Westhusin, M.E., Forrest, D.W., and Tomaszweski, M.A. 2007. Early pregnancy diagnosis by palpation per rectum: Influence on embryo/fetal viability in dairy cattle. Theriogenology 67:486-493.

Sananikone, K., Delwiche, M.J., BonDurant, R.H., and Munro, C.J. 2004. Quantitative lateral flow immunoassay for measuring progesterone in bovine milk. Trans ASAE 47:1357-1365.

Scopes, R. 1993. Separation by adsorption - affinity techniques, p. 187-204 Protein purification: Principles and Practice, 3rd ed. Springer-Verlag, New York.

Shannon, L.M., Kay, E., and Lew, J.Y. 1966. Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. J Biol Chem 241:2166-72.

Sheldon, I.M., Wathes, D.C., and Dobson, H. 2006. The management of bovine reproduction in elite herds. Vet J 171:70-8.

Sheldon, M., and Noakes, D. 2002. Pregnancy diagnosis in cattle. In Pract 24:310-317.

Short, R.V. 1958. Progesterone in blood. I. The chemical determination of progesterone in peripheral blood. J Endocrinol 16:415-25.

Sigma. 2000. Peroxidase from horseradish [Online]. Available by Sigma http://www.sigmaaldrich.com/Area_of_Interest/Asia__Pacific_Rim/Australia.html (product number P2088) (posted Nov 2000; verified 30 Oct).

Sigma. 2004. MSDS: 3,3',5,5'-Tetramethylbenzidine dihydrochloride (T8768) [Online] http://www.sigmaaldrich.com/Area_of_Interest/Asia__Pacific_Rim/Australia.html (product number T8768).

Tschmelak, J., Proll, G., and Gauglitz, G. 2004. Sub-nanogram per litre detection of the emerging contaminant progesterone with a fully automated immunosensor based on evanescent field techniques. Anal Chim Acta 519:143-146.

van Weemen, B.K., and Schuurs, A.H.W.M. 1971. Immunoassay using antigen-enzyme conjugates. FEBS Lett 15:232-236.

Williamson, N.B., Morris, R.S., Blood, D.C., and Cannon, C.M. 1972. A study of oestrous behaviour and oestrus detection methods in a large commercial dairy herd. I. The relative efficiency of methods of oestrus detection. Vet Rec 91:50-8.

Appendices

Appendix 1 Recipes for Solutions

A-1.1 Buffers

0.2 M sodium phosphate (used in the preparation of PBS)	27.8 g $NaH_2PO_4.H_2O$ Up to 1000 mL with distilled water
0.2 M sodium biphosphate (used in the preparation of PBS)	28.4 g Na_2HPO_4 Up to 1000 mL with distilled water
0.1 M Phosphate Buffered Saline (pH 7.0) (PBS)	195 mL 0.2 M sodium phosphate 305 mL 0.2 M sodium biphosphate 8.7 g NaCl Dilute to approx. 950 mL with distilled water pH to 7.0 with 5 M HCl or 5 M NaOH Store at 4°C
0.05 M carbonate buffer (pH 9.6)	1.59 g Na_2CO_3 2.93 g $NaHCO_3$ Dissolve in approx. 950 mL distilled water pH to 9.6 with 5 M HCl or 5 M NaOH Up to 1000 mL with distilled water Store at 4°C
Phosphate/citrate buffer (pH 5.0)	4.668 g anhydrous citric acid 7.309 g Na_2HPO_4 Dissolve in approx. 950 mL distilled water pH to 5.0 with 5 M HCl or 5 M NaOH Up to 1000 mL with distilled water

A-1.2 Enzyme Substrate Solutions

0.2 mg/mL tetramethylbenzidine dihydrochloride (TMB) solution	10-11 mg TMB 12.5 mL methanol Up to 50 mL with distilled water
3% hydrogen peroxide solution	100 μL 30% H_2O_2 900 μL distilled water Prepare immediately before use
TMB solution	5 mL phosphate citrate buffer (pH 5.0) 5 mL 0.2 mg/mL TMB solution 20 μL 3% hydrogen peroxide solution Prepare immediately before use Colour develops with absorbance at 630 nm

A-1.3 Regenerants

Regenerant 1	5 M NaOH
Regenerant 2	20 g 5 M NaOH 20 g NaCl (2% w/w) Up to 1000 g with water pH 12.5
Regenerate 3	2 g 5 M NaOH 100 g Acetonitrile 10 g 10% sodium benzoate Up to 1000 g with water

A-1.4 Progesterone Standard Solutions

Progesterone stock standard solution (10 μg/mL)	On a 5 d.p. balance (0.01 mg resolution) weigh out 1.00 g progesterone Make up to 100 mL with absolute ethanol Taking care to minimise evaporation of ethanol (eg Use a syringe), transfer the standard to 2 mL glass vials (approx. 2/3 full) Store in freezer
Progesterone secondary standard solution (400 ng/mL)	Pipette 1 mL 10 μg/mL stock standard into 25 mL volumetric flask Blow down ethanol standard to dryness Make up to 25 mL with PBS Store in 0.5 mL aliquots in glass vials in freezer

A-1.5 SDS-PAGE solutions

These solutions were kindly provided by AgResearch, Ruakura, Hamilton

5X Sample loading buffer (reducing)	2.5 mL 0.5 M Tris buffer (pH 6.8) 8 mL glycerol 1 g SDS 5 mL H2O (MQ) 5 mL B mercaptoethanol Some grains of bromophenol (to colour dark blue) Aliquot 1 mL, store -80°C
Coomassie Stain 1L	0.2 g Coomassie R250 100 mL acetic acid (glacial) 300 mL methanol (drum) 600 mL water
Coomassie Destain 1L	100 mL acetic acid (glacial) 300 mL methanol (drum) 600 mL water

A-1.6 Other Solutions

ELISA plate wash-solution	Stock solution: 87.66 g NaCl 5.0 mL Tween 20 Up to 1000 mL with distilled water Add 100 mL stock solution to 900 mL distilled water
P4-HRP stock solution (1:200)	0.250 mL conjugate stock Up to 50 mL with PBS with 1% BSA Store in 1 mL aliquots in glass vials freezer

Appendix 2 Suppliers

Item	Manufacturer	Supplier	Product No.
Acetonitrile	Merck	Biolab, Auckland, NZ	1.00030
Albumin, bovine serum	ICP Bio	ICP Bio, Auckland, NZ	ABRE-001
Antibody, polyclonal (pAb)	Sigma-Aldrich	Biolab, Auckland, NZ	P5289
Antibody, monoclonal (mAbX)	UC Davis	UC Davis, USA	CL425 (9/8/00)
Beads, 300 μm Tentagel Macrobeads™ (-NH_2)	Rapp Polymere	Rapp Polymere, Germany	MB 300 02
Beads, 90 μm (-NH_2) EAH Sepharose 4B	GE Life Sciences	GE Life Sciences, UK	17-0569-01
Biacore Amine Coupling Kit	Biacore	Bio-Strategy, Auckland, NZ	BR-1000-50
Biacore CM5 chip	Biacore	Bio-Strategy, Auckland, NZ	BR-1003-99
Buffer, acetate pH 4.5	Biacore	Bio-Strategy, Auckland, NZ	BR-1003-50
Buffer, HBS-EP pH 7.4	Biacore	Bio-Strategy, Auckland, NZ	BR-1001-88
Citric acid	BDH	Biolab, Auckland, NZ	277814N
Dimethylformamide	BDH	Biolab, Auckland, NZ	103224J
Ethanol, 100%	-	InterAg, Hamilton, NZ	-
Ethylene diamine	Sigma-Aldrich	Biolab, Auckland, NZ	E26266
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide	Sigma-Aldrich	Biolab, Auckland, NZ	E7750
N-hydroxy succinimide	Sigma-Aldrich	Biolab, Auckland, NZ	130672
Horseradish peroxidase	Sigma-Aldrich	Biolab, Auckland, NZ	P8375
Hydrochloric acid, 5M	Pauling Industries	Pauling Industries, Auckland, NZ	HCl 5.0N
Hydrogen peroxide	BDH	Biolab, Auckland, NZ	103665H
Methanol	BDH	Biolab, Auckland, NZ	10158BG

Item	Manufacturer	Supplier	Product No.
Progesterone	Sigma-Aldrich	Biolab, Auckland, NZ	P8783
Progesterone-3-carboxymethyloxime	Sigma-Aldrich	Biolab, Auckland, NZ	P3277
Progesterone-horseradish peroxidase	UC Davis	UC Davis, USA	-
Plate reader (Multiskan EX)	Thermo	Medica Pacifica, Auckland, NZ	51118170
Plate reader, optical filter 630 nm	Thermo	Medica Pacifica, Auckland, NZ	1426300
Sodium benzoate	BDH	Biolab, Auckland, NZ	103974R
Sodium bicarbonate	Merck	Biolab, Auckland, NZ	1.06329
Sodium biphosphate	Scharlau	Biolab, Auckland, NZ	So 0337
Sodium borohydride	Ajax Finechem	Biolab, Auckland, NZ	A2334
Sodium carbonate	Merck	Biolab, Auckland, NZ	1.06392
Sodium chloride	Labserv (Biolab)	Biolab, Auckland, NZ	BSPSL944
Sodium phosphate	BDH	Biolab, Auckland, NZ	102454R
Sodium hydroxide	BDH	Biolab, Auckland, NZ	102524X
Sodium periodate	Ajax Finechem	Biolab, Auckland, NZ	A695
Tetramethylbenzidine (powder)	Sigma-Aldrich	Biolab, Auckland, NZ	T8768
Tetramethylbenzidine (formulation)	Sigma-Aldrich	Biolab, Auckland, NZ	T0440
Tween	APS Chemicals	Biolab, Auckland, NZ	2509