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Reduction of severe bovine serum associated matrix effects on carboxymethylated dextran coated biosensor surfaces

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ABSTRACT

Surface plasmon resonance (SPR) based biosensor technology has been widely used in life science research for many applications. While the advantages of speed, ruggedness, versatility, sensitivity and reproducibility are often quoted, many researchers have experienced severe problem of non-specific binding (NSB) to chip surfaces when performing analysis of biological samples such as bovine serum. Using the direct measurement of the bovine protein leptin, present in bovine serum samples as a model, a unique buffering system has been developed and optimised which was able to significantly reduce the non-specific interactions of bovine serum components with the carboxymethyl dextran chip (CM5) surface on a Biacore SPR system. The developed NSB buffering system comprised of HBS-EP buffer, containing 0.5 M NaCl, 0.005% CM-dextran, pH 9.0. An average NSB reduction ($n = 20$) of 85.9% and 87.3% was found on an unmodified CM5 surface and a CM5 with bovine leptin immobilised on the chip surface, respectively. A reduction in NSB of up to 94% was observed on both surfaces. The concentration of the constitutive components and pH of the buffer were crucial in achieving this outcome.

1. Introduction

The need for more samples to be tested for an ever-increasing range of complex substances under more defined performance criteria has resulted in an increased demand for sensitive but robust analytical methods capable of high-throughput and with simple sample preparation procedure. Recent advances in bio-analytical sciences have greatly facilitated this need. One of the most frequently exploited techniques is real-time biospecific interaction analysis (BIA) utilising surface plasmon resonance (SPR) technology that is now widely used in many life science research applications [1–4]. The advantage of SPR biosensor method over other technologies is the ability to deliver rapid and reliable analyte detection in real-time, without the use of labels. The applicability of this technology in the fields of animal health monitoring and food analysis has made a valuable addition to the spectrum of analytical tools available.

A number of different commercial SPR platforms exist, but the technology provided by the GE Healthcare Company, Biacore (Biacore AB, Uppsala, Sweden) is a popular choice for method developers in food diagnostics [5,6]. Biosensor analysis based on carboxymethylated dextran (CM-dextran) surfaces has provided a reliable platform for the rapid determination of a wide range of compounds relevant to food safety and quality, and an equally wide variety of food and related sample types. But while the advantages of speed, ruggedness, versatility, sensitivity and reproducibility are often quoted, many researchers have experienced severe problem of non-specific binding (NSB) in biological samples. It is generally recognized that matrix interference, resulting in interaction between components of biological specimens and the sensor chip surface, is a major technical difficulty in assay development and has, more than any other single factor, limited the success of SPR biosensor system in the study of binding events in complex biological samples such as serum and blood due to an inability to control non-specific adsorption [7].

A number of strategies have been employed to resolve the problem of NSB in real-time BIA applications with various degrees of success. In some cases, worthwhile reductions in NSB have been achieved and workable assays have been developed. For example, Johnsson et al. introduced a simple precipitation step using saturated ammonium sulphate (SAS) to remove proteins of bovine serum and reported the development of a sensitive bioassay for the detection of drug residue (benzimidazole) in bovine serum [8]. However, this technique is not suitable for analysis of proteins since the precipitation step will remove all proteins from the serum sample. No apparent success has been reported in applications where the direct measurement of an analyte present in bovine serum on

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carboxymethylated dextran coated biosensor surfaces. Masson et al. claimed that better performance could be obtained for bovine serum assay by replacing the CM-dextran with other biocompatible polymers [9]. High-throughput analysis of serum samples is highly dependent on an ability to assay samples directly and without the need to perform time-consuming and expensive sample preparation prior to analysis.

The present study used the measurement of bovine leptin protein in bovine sera as a model to investigate the possibility of assembling a buffering system to minimise non-specific binding of bovine serum components to the surface of a CM-dextran coated chip (CM5). Leptin, a 146 amino acid peptide hormone (16 kDa) is produced and released from adipocytes [10], and is one of a group of potential protein biomarkers for the detection of anabolic steroid misuse in cattle.

2. Experimental

2.1. Instrument and reagents

The SPR biosensor instrument (Biacore® Q), carboxymethylated dextran coated sensor chips (CM5 research grade), HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20, pH 7.4), the amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethyl-aminopropyl) carbodiimide (EDC), and ethanolamine hydrochloride (pH 8.5) were obtained from Biacore AB (Uppsala, Sweden). Biacore control software (version 3.0.1) was used for instrument operation and data handling. Recombinant bovine leptin (purity >95%) was purchased from Oxford Bio-Innovation Ltd (DSL-OBL, Oxon, UK). Synthetic leptin fragment ($_{77}$SNDLENLRDLLHLLAA$_{92}$) was supplied by AgriFood and Biosciences Institute (AFBI, Belfast, UK). Anti-leptin antibody was raised in guinea pigs against recombinant ovine leptin (a gift from Professor Arieh Gertler of the Hebrew University of Jerusalem, Israel) and has been used in radioimmunoassay (RIA) for the analysis of both bovine and ovine leptin. Carboxymethyl dextran sodium salts (MW: 75, 500, and 2000 kDa) were purchased from Ssens (Hengelo, The Netherlands). Sodium dihydrogen orthophosphate and di-sodium hydrogen orthophosphate were purchased from BDH Laboratory Supplies (Poole, UK). Carboxymethyl dextran sodium salt and all other chemicals were purchased from Sigma–Aldrich (Poole, UK). Deionised water was obtained using Millipore reverse osmosis and Milli-Q water polishing systems.

Tris–HCl buffer (pH 7.4) was prepared by mixing 44.2 ml 0.1 M solution of tris (hydroxymethyl aminomethane) and 50 ml of 0.1 M HCl, and then diluted to a total of 200 ml with deionised water. Phosphate buffer (pH 7.2) was prepared by mixing 28 ml of 0.2 M solution of sodium dihydrogen orthophosphate and 72 ml of 0.2 M solution of di-sodium hydrogen orthophosphate. Non-specific binding buffer (NSB buffer) was based on HBS-EP (10 mM HEPES, 3 mM EDTA and 0.005% Tween 20) contained 0.5 M NaCl and 0.005% CM-D500, and was adjusted to pH 9.0 using 1 M NaOH.

2.2. Methods

2.2.1. Preparation of the SPR sensor chip surface

Modified coupling procedures described previously by Johnsson et al. [11] were used for external immobilisation of recombinant bovine leptin or leptin peptide fragment onto the surface of a CM5 sensor chip. Briefly, the chip surface was activated with 50 μl of a mixture of 0.4 M (EDC) and 0.1 M (NHS) (1:1; v/v) for 20 min at ambient temperature on bench. The reactant was removed and 50 μl of leptin peptide fragment (1 mg ml^{-1} in 10 mM sodium acetate, pH 4.5) or whole bovine leptin (500 μg ml^{-1} in 10 mM sodium acetate, pH 4.5) was added and allowed to remain in contact with the chip surface for 2 h (peptide fragment) or overnight (whole leptin) at ambient temperature. Unreacted sites were blocked by the addition of 50 μl of 1 M ethanolamine (pH 8.5) for 20 min at room temperature. The reactant was removed and the chip surface was washed with deionised water and then dried under a stream of nitrogen gas. The prepared chip was stored at 4 °C in the present of desiccant.

2.2.2. Rmax determinations

Rmax is the maximum binding capacity of the surface ligand (peptide) for the anti-peptide antibody, as measured in resonance units (RUs; where 1000 RU ≈ 1 ng mm^2 for proteins). Rmax provides useful information about the ability of immobilised ligand and binding partner to interact. Rmax values were obtained by injecting high concentrations of antibody (e.g. ×10 dilution) over the chip surface at a low flow rate (5 μl min^{-1}) for up to 10 min (injection time). The increase in relative response units over this period is denoted as the Rmax value.

2.2.3. Preparation of bovine serum

Bovine serum was prepared by centrifugation (2500 rpm, 15 min) of clotted bovine blood obtained from local cattle. Serum samples were diluted 10-fold with appropriate buffer prior to analysis.

2.2.4. SPR biosensor assay

All experiments were performed on a Biacore® Q and Evaluation software 1.0 was used for data analysis. Freshly diluted bovine serum was mixed with the same volume of diluted antibody and injected over the sensor chip surface for 4 min at a flow of 20 μl min^{-1}. The surface was regenerated by a 1-min pulse of 50 mM sodium hydroxide at a flow rate of 20 μl min^{-1}.

The leptin assay was designed as a solution competition (inhibition) assay. The high molecular weight (HMW) interactant (anti-leptin antibody) was added to the bovine serum sample containing the analyte of interest. The analyte itself or an analogue (leptin or peptide fragment) was immobilised on the surface and used to determine the concentration of free HMW interactant in solution. Analyte concentration was measured in terms of inhibition of binding of the HMW interactant (antibody) to the surface. A low response indicated a high analyte concentration (the concentration of free interactant in solution was low due to their binding to the analyte in sample). When the concentration of analyte present was known, i.e. bovine serum spiked with a range of analyte concentrations, a calibration curve could be constructed and used to measure the analyte concentration in unknown samples.

For the study of the non-specific binding of serum components on the CM5 sensor chip surfaces, bovine serum diluted 10-fold with appropriate buffer was injected over the chip surfaces, i.e. blank and leptin immobilised surfaces, with or without the presence of anti-leptin antibody. Two sets of data (i.e. relative response units) were subjected to statistical analysis, as other assay parameters (buffer conditions etc.) remained the same. Preparation of calibration curves was not needed for this task.

3. Results and discussion

3.1. Preparation of SPR sensor chip surface

The sensor surface immobilised with recombinant bovine leptin displayed an Rmax of 12.6 kRU while the leptin fragment coated surface gave an Rmax of 17.4 kRU. For concentration measurements utilising small molecule coated surfaces (e.g. drug or peptides) to

determine the concentration of free binding partner available (usually an antibody), an Rmax in the range of 6–12 kRU has been shown to deliver a suitable degree of assay sensitivity (unpublished data). The use of a selected leptin fragment in this study was due to limitations in the quantity of recombinant bovine leptin available, and previous reported that antibodies raised against a synthetic peptide with similar amino acid sequence could recognise not only the peptide fragment but also the native leptin protein molecule. Such anti-leptin fragment antibodies have been successfully used to measure leptin serum concentrations in cows [12,13].

The choice of immobilisation method has to be considered carefully. Preferably, the ligand should be attached covalently to the surface matrix to facilitate reproducibility of the chip surface but the way in which ligand is immobilised onto the sensor surface is of great importance for generating a uniform binding response. For short peptides or terminal peptide fragments, a well-defined orientation coupling (N- or C-terminal attachment) may be needed to ensure the maximum exposure of the ligand molecule to the injected binding partner. Site-directed immobilisation can be achieved by utilising an existing functional group present in the amino acid (AA) residue or by addition of an AA that contains ionisable side chains, e.g. lysine. Controlled orientation of ligand has been shown to enhance ligand recognition by analytes [14,15].

Other factors that need to be considered in the production of the immobilised surfaces include preconcentration of ligands over the surface matrix, the concentration of ligands used, incubation times, and the ionic strength of the coupling buffer. For immobilisation of ligand onto the CM5 chip surfaces, preconcentration is accomplished by electrostatic attraction between negative charges of the carboxymethyl dextran matrix surface (when pH > 3) and positive charges of the ligand at pH values below its p*I*. In the present study, the immobilisation of recombinant bovine leptin (p*I* 8.68) onto CM5 surface was achieved using the standard amine coupling procedure and the ready-to-use coupling buffer from Biacore (10 mM sodium acetate, pH 4.5). For the production of the leptin fragment (p*I* 4.27) surface, the N-terminal residue of serine (p*I* 5.7) contains an additional attachment point at the hydroxyl group; it was therefore targeted as the site of immobilisation using also the amino coupling procedure. The use of commercial ready-to-use coupling buffer (pH 4.5) also favours immobilisation of the N-terminus; due to the positive charge of the N-terminal serine residue under this condition, i.e. the pH value of the coupling buffer (4.5) was lower than the p*I* of the serine (5.7) but higher than the p*I* of the peptide fragment (4.27).

It is the experience of the present researchers that ligand concentrations in the range 0.5–1.0 mg ml^{-1} are sufficient for the vast majority of immobilisation procedures when they are performed externally with a longer incubation time (minimum 2 h), while the range 10–200 μg ml^{-1} is used as recommended by Biacore, when coupling is performed internally within the biosensor system. For leptin peptide surface, 1 mg ml^{-1} was used whilst 0.5 mg ml^{-1} was applied to generate the bovine leptin surface. With regard to the ionic strength of the coupling buffer used, a low concentration (10–20 mM) is recommended and is suitable for most applications but higher ionic strength have also been used routinely (e.g. 60 mM sodium borate buffer) in this laboratory.

3.2. Preparation of bovine serum

A range of assay buffer factors (dilution, composition, pH and ionic strength) were examined for effects on reduction of NSB and assay performance with respect to the ability to detect leptin in bovine serum samples without the need for sample extraction but at sufficiently high sensitivity.

3.2.1. Sample dilution

Bovine serum samples were analysed at dilutions of 1:2, 1:5, 1:10, 1:20, 1:50, and 1:100 in HBS-EP buffer (Biacore AB, Sweden). The most substantial reduction of NSB was found at 1:100 dilution (3.8 kRU at 1:2 vs 0.4 kRU at 1:100). However, at this dilution, the ability of the assay to detect the presence of bovine serum leptin at the normal range of 1–20 ng ml^{-1} was compromised, i.e. the detection limit was of the order of 1×10^{-2} ng ml^{-1} using this approach.

3.2.2. Buffer composition

Two different buffering systems were investigated: a Tris–HCl buffer (10 mM, 0.5 M NaCl, pH 7.4) and a commercial HBS-EP buffer. Table 1 shows the effects on binding behaviour of two randomly selected bovine serum samples (10-fold dilution injected over the sensor surface at a flow rate of 20 μl min^{-1}) on three different sensor surfaces, i.e. CM5 chips immobilised with leptin fragment (Leptin 16) or, with recombinant bovine leptin, and a blank CM5 surface. A higher NSB was found with the Tris–HCl buffer in all cases compared to HBS-EP buffer. The highest relative response (RU) was observed on the Leptin 16 surface when compared with the other two surfaces (bovine leptin and CM5 blank) for both HBS-EP and Tris–HCl buffer.

Using the CM5 blank surface, consistent responses were found for the two samples tested with the same buffer but significantly different between the two buffering systems, suggesting that the NSB binding behaviour of the serum matrix towards the CM5 dextran surface was affected by buffer composition.

Moreover, the NSB behaviour was significantly altered on the chip surfaces by differential ligand immobilisation, i.e. decreased NSB was observed with recombinant bovine leptin chip on the surface and increased on the Leptin 16 surface. This effect was probably due to the size and structure of the recombinant bovine leptin molecule through a barrier effect at the surface, preventing binding between serum components and the chip matrix while the linear characteristics of leptin fragment (similar to those of dextran), may favour NSB by creating an extended molecular layer. The Leptin 16 surface was therefore selected for further investigation of a potential buffering system that could lead to substantial reduction in NSB. Similar binding behaviour to that observed using Tris–HCl buffer, was found with phosphate buffer (0.1 M, 0.5 M NaCl, pH 7.2) on a CM5 blank chip in a separate experiment. An increased temperature (up to 56 °C) also had no effect on NSB reduction using the HBS-EP system.

3.2.3. Ionic strength

It has been reported that electrostatic interactions between protein molecules are weaker at high ionic strength [16]. In the present study the optimal ionic strength for the Leptin 16 surface as determined by the degree of NSB reduction was found to be 0.5 M NaCl, over the range 0.15–1.0 M, i.e. a 53% drop in NSB (from 1135 to 538 RU) observed at 0.5 M NaCl (Table 2). A different profile was obtained for the CM5 blank surface, i.e. a higher ionic strength (1.0 M) resulted in a lower NSB (from 816 to 309 RU, reduction by

Table 1
Comparative results of binding behaviour of bovine serum prepared in HBS-EP or Tris–HCl buffer on different surfaces

Bovine serum 1	Leptin 16 (RU)	Bovine leptin (RU)	CM5 blank (RU)
HBS-EP pH 7.4	1168	496	816
Tris–HCl pH 7.4	1907	1285	2088
Bovine serum 2			
HBS-EP pH 7.4	1690	600	818
Tris–HCl pH 7.4	2906	1197	2215

Table 2
Resonance units measured following injection of bovine serum over CM5 blank and Leptin 16 chips at different concentrations of CM-D500 and ionic strengths (NaCl) (HBS-EP, pH 7.4)

CM5 blank				Leptin 16			
NaCl (M)	RU	CM-D500 (%, m/v)	RU	NaCl (M)	RU	CM-D500 (%, m/v)	RU
0.15	816	0.01	248	0.15	1135	0.01	784
0.3	502	0.03	250	0.3	725	0.03	790
0.4	417	0.04	238	0.4	703	0.04	849
0.5	365	0.05	253	0.5	538	0.05	848
0.6	340	0.06	241	0.6	565	0.06	881
0.7	337	0.07	242	0.7	615	0.07	990
0.8	330	0.08	239	0.8	613	0.08	1028
0.9	324	0.09	258	0.9	617	0.09	1055
1.0	309	0.1	265	1.0	577	0.1	1181

62%). The data illustrates that different NSB effects will result from varying the ionic strength (NaCl) on modified sensor surfaces, and this is a buffer parameter that should be considered for optimisation during assay development.

3.2.4. Optimising ionic strength and CM-D500 concentrations

The addition of a range of CM-dextran with different molecular weights (12–2000 kDa) to HBS-EP (0.15 M NaCl, pH 7.4) was examined. CM-D500 (MW 500 kDa) exhibited the most notable effect on NSB reduction (data not shown). This could be due to similarities between the molecular weight of this dextran and that used on the CM5 sensor chip surface.

Addition of CM-dextran at 0.01–0.1% (m/v) had no significant effect on the CM5 blank chip characteristics. However, an increase in NSB on the Leptin 16 surface was observed as the concentration of CM-D500 increased (Table 2). Further investigation using lower concentrations of CM-D500 in combination with 0.5 M NaCl, showed the greatest degree of NSB reduction (76%; from 1296 to 307 RU) at 0.007% CM-D500. The combination of 0.005% CM-D500 and 0.5 M NaCl (75% reduction of NSB) was chosen in order to assure lower NSB and least addition of dextran.

3.2.5. Change of pH value

The degree of NSB reduction was also examined over the pH range 5.3–9.9. A gradually decrease in NSB was observed as pH was increased and was lowest at pH 9.5 (67% reduction). Bovine serum albumin (BSA) is a highly abundant protein and is believed to contribute primarily to the NSB of blood components towards CM-dextran sensor surfaces. The interaction between BSA and the charged dextran can be induced by the primary force of electrostatics, i.e. the negative charges on the CM-dextran and the residual positive charges on the BSA. The isoelectric point for BSA is pH 4.8. At pH 9.5 (>p*I*), BSA acquires a negative charge, i.e. the same as of CM-dextran surface, and hence electrostatic repulsion between the protein and CM-dextran would prevent the formation of complexes. Thompson and McKernan reported no detectable complexes of BSA and dextran sulphate above pH 8.5 [17]. It has been suggested that BSA can partially denature at pH 9 [18]. Moreover, a higher pH (>9.5) may lead to a conformational change of the protein and result in an altered binding behaviour towards the sensor chip surface.

The data outlined previously showed the effects of alterations to the composition of the buffering systems used on an individual basis. The combined effects of each of the altered parameters were then examined. It was found that the greatest degree of NSB reduction was obtained using the NSB buffer derived from the HBS-EP buffer but with increased ionic strength (0.5 M NaCl), addition of CM-D500 (0.005%, m/v) and a pH of 9.0 (Fig. 1). Reductions of up to 95%, 88%, and 81% reduction of NSB were observed on the blank CM5 chip, the Leptin 16 chip, and bovine leptin chip, respectively. Addition of CM-D500 in the buffer system appeared to act as a 'competitor' to the CM-dextran on chip surface for binding of BSA, most notably at pH 9.0. The synergistic effects of this buffering system were found to be highly effective in minimising the non-specific binding properties of bovine serum components to the CM5 sensor chip surfaces.

During this investigation, it was noted that the degree of NSB measured varied between individual serum samples. The NSB buffer that was developed during the study was further tested against a panel of 20 randomly selected bovine serum samples on a CM5 blank chip and bovine leptin surfaces. An average of 85.9% (±4.76) and 87.3% (±5.71) of NSB reduction was found on the CM5 blank chip and bovine leptin surface, respectively. Statistical analysis (Paired-samples test, $t = 0.87$) indicated no significant difference between the two means and it was therefore concluded that, using the developed NSB buffering system, a substantial degree of non-specific binding towards the CM5 chip surface had been removed.

Sample matrix effects or sample interference is well known as a major cause of bias in analytical procedures especially when dealing with complex samples such as blood or other biological fluids. The problem is magnified in the case of real-time BIA technology in which the detection principle is based on changes in mass concentration at the surface as molecules bind or dissociate, since any binding to the chip surface (specific or non-specific) is recorded and reflected as part of the total SPR response (RU). In the case of bovine serum, an overwhelming signal of up to 100-fold greater intensity than the signal produced from the analyte may be generated on CM-dextran sensor surfaces. Replacing the CM-dextran with different biocompatible polymers (e.g. CM-hyaluronic acid and alginic acid) could greatly improve biosensor performance when using serum as a matrix on an optical fiber SPR sensor (7). McArthur et al. also claimed that the electrostatic effects of CM-dextran with different degrees of carboxymethyl substitution on protein adsorption varied towards different proteins and they concluded that elimination

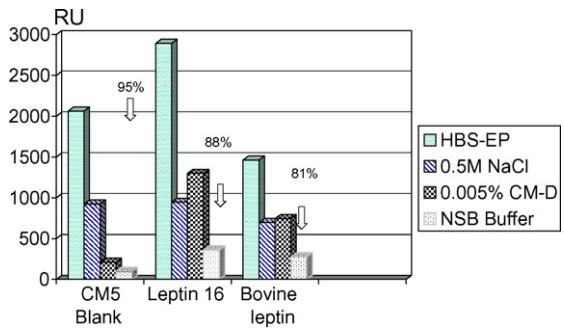

Fig. 1. Effect of various conditions on the binding behaviour of bovine serum matrix on different CM5 chip surfaces. HBS-EP: ready-to-use buffer; 0.5 M NaCl: HBS-EP buffer contains 0.5 M NaCl; 0.005% CM-D: HBS-EP buffer contains 0.005% dextran; NSB buffer: HBS-EP buffer contains 0.5 M NaCl, 0.005% CM-dextran and pH 9.0.

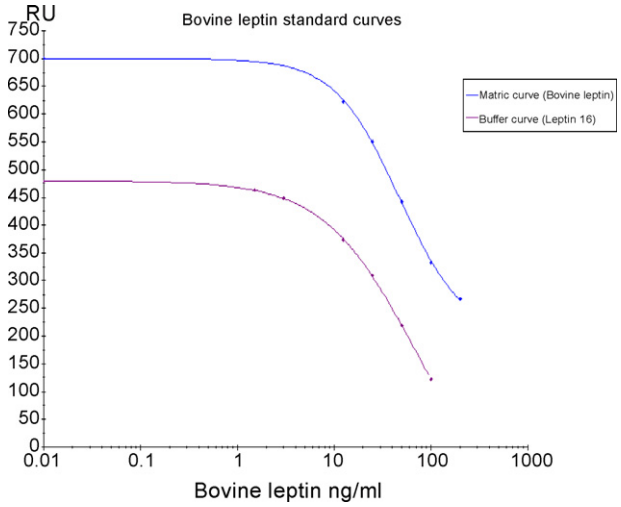

Fig. 2. Typical calibration curves constructed in buffer or serum matrix for bovine leptin on Leptin 16 and bovine leptin surfaces.

of adsorption of a broad spectrum of proteins is not straightforward with negatively charged polysaccharide coatings [19].

3.3. SPR biosensor assay

In the optimised leptin assay, bovine sera were diluted 10-fold in NSB buffer (10 mM HEPES, 0.5 M NaCl, 0.005% CM-D500, 3.4 mM EDTA, 0.005% P20, pH 9.0) and mixed with an equal volume of diluted antibody solution. At a flow rate of 20 μl min^{-1}, the mixture was injected over the sensor chip surface for 4 min, followed by a 1-min pulse of 50 mM sodium hydroxide to allow surface regeneration. Report points were taken before and after each injection and the biomolecular interaction was recorded as a sensorgram. HBS-EP was used as the running buffer. Typical calibration curves (Fig. 2) were constructed using 10-fold diluted serum on bovine leptin chip and buffer only on Leptin 16 surface. The mid-points (IC_{50}) of the calibration curves were 38.8 ng ml^{-1} (matrix curve) and 26.5 ng ml^{-1} (buffer curve), indicating slightly decreased assay sensitivity due to matrix. Work is ongoing to improve the sensitivity by screening a panel of anti-leptin antibodies. Serum samples from hormone-treated and non-hormone-treated cattle will be analysed for leptin to determine if this protein is a potential biomarker for growth promoter abuse in cattle.

4. Conclusion

Using a leptin SPR assay as the model system, a NSB reduction buffering system has been developed to tackle the significant problem caused by interference of bovine blood components on carboxymethylated dextran coated sensor chips (CM5) for biotechnological applications using BIACORE biosensors. The current study has shown that up to 94% of the NSB can be eliminated by appropriate use of the buffering system developed in this study. It is believed that this buffering system will have a major impact in reducing the non-specific protein fouling of sensor chip based SPR immunoassays for complex biological solutions, especially when the scope for test sample dilution is limited. Similar effects on NSB reductions in serum samples of other species have currently been observed with the developed buffering system. It may be that for the development of assays for other bovine serum proteins (or other blood based analyse of interest) the parameters determined to give maximum reduction in NSB in the present study may have to be altered in terms of concentration and pH. However the present study should serve as a model for those faced with extreme NSB problems in SRP based analytical systems.

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