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An aptamer based surface plasmon resonance biosensor for the detection of bovine catalase in milk

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ABSTRACT

In this research, we report the development of an aptamer based SPR biosensor for the detection of catalase in milk samples with minimal sample preparation. A biotin tagged aptamer was immobilized onto a gold surface by affinity capture. A limit of detection (LOD) in the nanomolar range (20.5 nM, RSD: 15.2%) was found and a dynamic range of 15–1000 nM was established for catalase in buffer and the aptamer showed good specificity toward catalase. This biosensor has the potential to be used in the detection of catalase in milk samples, a key indicator of mastitis disease in milk.

1. Introduction

Surface Plasmon resonance (SPR) has emerged as a powerful technique in bioanalytical and chemical analyses. It allows for the detection of biomolecules with high sensitivity and selectivity and gives valuable information about the kinetics of bio-interactions.

Aptamers are an emerging class of ligands for SPR based biosensors known as aptasensors. Aptamers are ssDNA that binds to a large variety of targets such as proteins and small molecules. They are an alternative to antibodies and are predisposed for SPR applications due to their stability and small size relative to antibodies. Aptamers are also cheap to produce and can be tagged with a variety of biomolecules and functional groups that allow for easy immobilization onto the SPR surface. Moreover aptamers do not suffer from a loss of activity upon tagging which can occur with antibodies. To date there have been a number of aptasensors developed to detect and quantify proteins. In 2005 Tombelli developed an RNA aptamer based biosensor for quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) to detect HIV-1 Tat protein (Tombelli et al., 2005). Lee et al. developed an aptamer based SPR sensor for the detection of retinal binding protein (RBP4) using an aptamer based system (Lee et al., 2008). They reported a limit of detection (LOD) of 75 nM which was comparable to the conventional immunoassay based methods. A sandwich style SPR aptasensor assay was developed in 2009 for the detection of IgE protein allowing for a detection limit of 2.07 ng/ml (Kim et al., 2009). More recently, a SPR aptasensor was developed for the rapid detection of H5N1 avian influenza with a detection range of 0.128–1.28 hemagglutinin unit (HAU) (Bai et al., 2012). In 2012, Chang et al. developed a SPR based biosensor for the detection of interferon-gamma (IFN-γ) achieving a linear dynamic range of 0.3–333 nM and a detection limit down to picomolar range (Chang et al., 2012). Aptamer based SPR biosensors offer a convenient platform for disease screening and one area that could be of great interest is in the screening for bovine mastitis in milk samples. SPR based biosensors would provide a quick, cheap and sensitive method for screening this disease with minimal sample preparation.

Mastitis is a disease which causes inflammation of the udder tissue in cattle and is the most common disease affecting the dairy industry today. This disease can increase the somatic cell count in milk (an indicator of milk quality) and is caused by a number of pathogens including *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus spp*. A number of methods have been developed to determine the total somatic cell count which can include any bacterial cells (Viguier et al., 2009). In 2009 Koskinen et al. used a PCR based assay to identify mastitis pathogens in milk samples (Koskinen et al., 2009). A chip based method was recently developed to detect mastitis pathogens using a ligation detection reaction (Cremonesi et al., 2009). An online conductivity system for the detection of mastitis has also been developed which

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identified a number of sub clinical quarters (Lansbergen et al., 1994). Recently protein bio indicators of mastitis disease in milk samples have been identified in proteomic studies (Boehmer, 2011). One possible protein identified as a bio indicator of mastitis disease is catalase (Lansbergen et al., 1994). Catalase enters the milk by somatic cells and catalase levels correlated with bulk milk somatic cell counts (Hamed et al., 2008). Futo et al. recently developed an amperometric based biosensor for the detection of catalase in milk samples by measuring the degradation of hydrogen peroxide indirectly (Fűtő et al., 2012). As catalase is a large analyte, SPR would be a convenient method for detecting this analyte in milk.

In this study we developed a highly sensitive and specific SPR based aptasensor for the detection of catalase protein in milk samples. An aptamer selected from our previous study was used as the transduction element of the biosensor (Ashley et al., 2012). This biosensor was used to measure the catalase directly in milk samples and could allow for rapid determination of mastitis disease in milk.

2. Experimental

2.1. Materials and apparatus

Catalase, casein, β lactoglobulin and bovine albumin proteins were purchased from Sigma Aldrich (Singapore) and dissolved in 10 mM HEPES, 100 mM KCl, 1 mM EDTA and 0.5% BSA buffer pH 7.4 (HKE-BSA). Standardized milk samples were purchased from a local supplier (Meiji, Singapore). For the formation of the self-assembly monolayer (SAM), 11-mercaptoundecanoic acid (11-MUA) and 2-mercaptoethanol (2-ME) were purchased from (Sigma Aldrich, Singapore) and dissolved in absolute ethanol. N-hydroxysuccinimide and ethyl 1-Ethyl-3-(dimethylaminopropyl) carbodiimide were also purchased from Sigma Aldrich (Singapore). Streptavidin was purchased from Merck Chemicals (Singapore) and dissolved in 20 mM sodium acetate buffer pH 5.0. Biotinylated aptamer ligand of sequence CTTCTG CCC GCC TCC TTC CGACCTAG CAGTGGACA TGTGGCAGGGTG AAGTGGCA TCGTCGGAGAC GAG ATAGGC GGA CAC T-3′ biotin was purchased from (1st Base, Singapore) and reconstituted in 10 mM HEPES, 100 mM KCl, 1 mM EDTA, pH 7.4 (HKE) buffer. Aptamers were renatured using a Bio-Rad DNA engine thermocycler (Bio-Rad, Singapore) by heating to 94 °C for 10 min and then cooling down at rate of 0.5 °C/s to room temperature. SIA AU kits containing bare gold chips were purchased from Biacore (Sweden). All experiments were carried out on the Biacore T3000 (Biacore, Sweden). For all experiments, the machine was purged with run buffer prior to analysis. In the immobilization of streptavidin, the flow buffer was 20 mM sodium acetate buffer pH 5.0 and the flow rate was set to 5 μl/min. The flow buffer was then changed to HKE buffer pH 7.4 at a flow rate of 5 μl/min for the immobilization of the aptamer ligand. For the optimization of the catalase assay and real milk analysis, a flow buffer of 10 mM HEPES, 100 mM KCl, 1 mM EDTA and 0.5% BSA buffer pH 7.4 (HKE:BSA) buffer was used and the flow rate was set to 10 μl/min. All buffers were degassed by **Sonication** prior to analysis.

2.2. Preparation of the chip surface

The gold slides were treated with piranha solution, rinsed with water and ethanol and dried with nitrogen. The gold slide was submerged in a solution containing 11-MUA 1 mM and 2-ME 4 mM in absolute ethanol for 24 h. The slide was then rinsed with water and ethanol and dried by nitrogen. The gold slide was then inserted into the chip and docked into the T3000. For the immobilization of streptavidin, 20 mM sodium acetate buffer pH 5.0 was used as a flow buffer at a flow rate of 5 μl/min. A solution of 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was mixed (1:1) and 50 μl was injected onto channels 1 and 2 to activate the carboxylic acid groups on the 11-MUA and 2-ME. 50 μl of streptavidin 50 μg/ml was then injected onto the sensor. The online response was used to characterize the sensor surface.

The remaining unreacted ester groups were blocked using 50 μl of 1 M ethanol amine (pH 8.5). For the biotin tagged DNA, the flow buffer was changed to HKE buffer and the flow rate was set to 5 μl/min. 50 μl of biotin tagged DNA (10 μM) was injected and captured onto the surface of channel 2 only.

2.3. Catalase assay and real sample analysis

For the catalase assay and real sample analysis, HKE:BSA buffer was used as the flow buffer and for each cycle the flow rate was set to 10 μl/min. 50 μl of catalase (10–1000 nM) was injected after 180 s. After the end-point of each injection, a dissociation time of 360 s was applied, followed by injection of 50 μl of 0.1 M NaOH and 45 mM glycine in 1.2% ethanol regeneration buffer (see Supplementary data, Fig. S1). A further waiting time of 180 s was applied after each injection to allow the baseline to stabilize. A calibration plot with linear regression of the relative response at 480 s against catalase concentration was plotted and the LOD was determined as three times the standard deviation ($S/N=3$) of three blank samples.

Milk samples were prepared by spiking with different concentrations of catalase (1–0.01 μM). Spiked samples were centrifuged and the supernatant was transferred to a new vial and filtered through a 0.45 μm filter. For each cycle the flow rate was again set to 10 μl/min and injection of 50 μl of each sample (62.5–1000 nM) was performed after 180 s. After each injection of catalase, a dissociation time of 360 s was applied followed by injection of 50 μl of regeneration buffer. A further wait time of 180 s was applied after each injection to allow the baseline to stabilize. The relative response plots were obtained by subtracting the milk only negative control. A graph with linear regression of the relative response at 480 s against catalase concentration was plotted.

3. Results and discussion

3.1. Preparation of the chip surface

A bare gold Biacore chip was coated with a mixture of 11-MUA (1 mM) and 2-ME (4 mM) in absolute ethanol for 24 h. A mixture of thiols was used to achieve easy amine coupling of the protein and to reduce steric hindrance of the protein toward the SAM. The immobilization of streptavidin and the affinity capture of the biotin tagged aptamer were monitored by measuring the relative response (RU). The online response plots for the immobilization of streptavidin and the affinity capture of the biotin tagged aptamer are shown in Fig. 1. For the immobilization of streptavidin, the flow buffer used was sodium acetate (pH 5.0). This is due to the near neutral *pI* value of streptavidin and an attempt to encourage an electrostatic interaction between the activated ester groups on the SAM and the protein leading to preconcentration of the protein onto the surface of the chip.

The tagged DNA was captured by injecting 50 μl at a concentration of 10 μM with a flow rate of 5 μl/min. In general there was no improvement in the response at concentrations >10 μM of tagged aptamer. The binding buffer was changed to HKE buffer (HEPES/KCl/EDTA, pH 7.4) as the sodium acetate buffer would facilitate degradation of the aptamer through hydrolysis in acidic

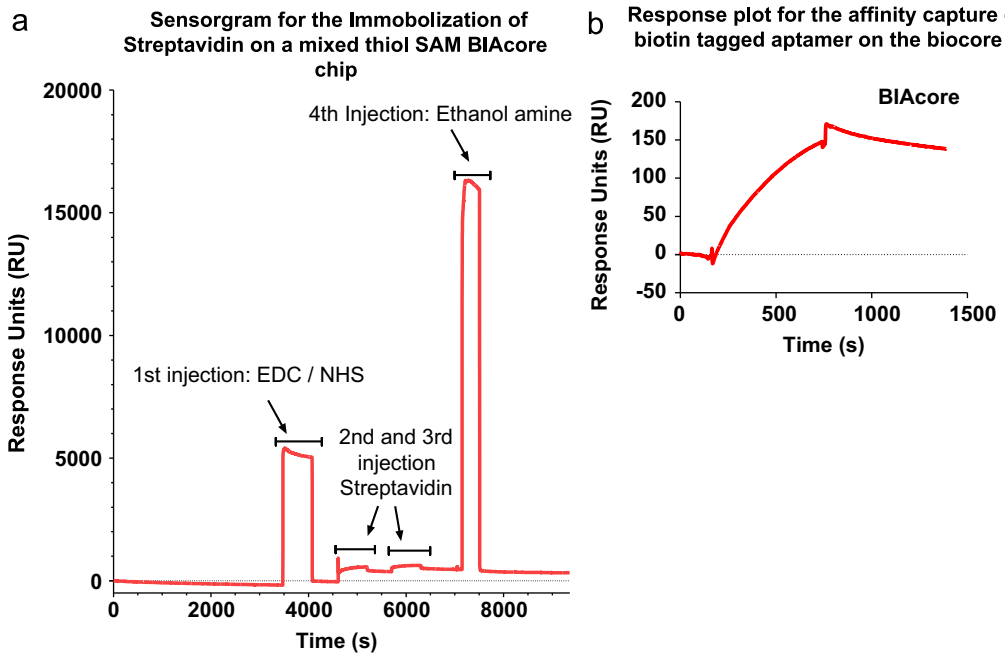

Fig. 1. (a) Immobilization of streptavidin The flow buffer was sodium acetate buffer at 5 μl/min, 50 μl of EDC/NHS solution, 50 μl of streptavidin (50 g/ml), biotin 50 μl of tagged aptamer (10 M) and ethanol amine (1 M) were injected; and (b) response plot showing the affinity capture of 10 μM of biotin tagged CAT aptamer at a ligand density of 150 RU. Flow buffer HKE buffer 5 μl/min.

conditions. A flow rate of 5 μl/min was used again to allow for maximum contact time with the surface. The total immobilization density was about 650RU. This was found to be adequate for the analysis due to the fact that the analyte is much bigger than the ligand allowing for relatively large response changes.

3.2. Optimization and assessment of the catalase biosensor

The performance of the aptamer based SPR biosensor was optimized by altering the injection volume, flow rate, buffer conditions and regeneration conditions. A quick injection volume of 50 μl was chosen to conserve sample and to reduce the time for each injection.

From our initial experiments, we also found that the catalase binds to the aptamer with slow dissociation kinetics due to the fact that the relative response does not return to the base line which could be the result of irreversible binding. This further confirms that the aptamer selected has slow binding kinetics as suggested in our previous studies (Ashley et al., 2012). This made it necessary to employ a regeneration buffer to remove the bound analyte after each injection.

The regeneration buffer was developed by considering the nature of the surface and the analyte. As we were using an aptamer based system we had to avoid the use of acidic conditions which may denature the aptamer. We therefore choose basic conditions for our regeneration buffer and a glycine-NaOH in 1.2% ethanol was used since it had been reported as an effective regeneration buffer for oligonucleotide based aptasensors (Andersson et al., 1999). The effectiveness of NaOH and glycine as a regeneration buffer was tested at various concentrations. If the basic conditions are too harsh then the surface and ligand element can be easily degraded. If the conditions are too weak then the result would be that not all of the analyte would be effectively removed (see Supplementary data, Fig. S1). The regeneration buffer conditions varied a lot and the weaker regeneration buffers (i.e. 10 mM glycine and 100 mM NaOH and 20 mM glycine 100 mM NaOH) were found to give a higher baseline signal suggesting that a lot of the analyte was retained on the aptamer ligand. The stronger conditions (100 mM glycine and 100 mM NaOH) had the opposite effect of removing the ligand from the surface. The compromise was found to be 45 mM glycine, 100 mM NaOH which caused the response to return to the original baseline response after injection.

The flow rate was also optimized by injecting 1 μM catalase at various flow rates (see Supplementary data, Fig. S2). At a flow rate of 5 μl/ min, the curve is a smooth exponential that reaches steady state. As the flow rate is increased, the exponential curve becomes sharper and from 15 μl/ min the curve does not reach steady state suggesting a large mass transport effect. This can be detrimental to analysis as the reproducibility of each cycle can deteriorate. However at lower flow rates, the time for injection was >20 min per injection which would result in a total of 40 min for each cycle including regeneration buffer. We decided to use a flow rate of 10 μl/min which gave reproducible results while allowing each cycle to be completed in 20 min. The flow rate selected allowed the assay to be completed within reasonable time, while allowing for adequate time for a stable baseline to be obtained after each injection and giving reproducible results.

The specificity was tested for the aptamer surface toward catalase by comparing its response to the three other abundant proteins found in milk. The protein content in milk can be put into 2 main groups, namely caseins and whey proteins. For this experiment we choose casein (52 μM), beta lactoglobulin A (54 μM whey protein) and bovine albumin (15.4 μM, whey protein) for comparison. 50 μl of each protein was injected and the relative response was measured at just before the endpoint of the injection to take into account both the refractive index changes and binding that occurs. From Fig. 2, it can be observed that catalase gave an average response of 820 RU which was much greater when compared to the other proteins which gave <200 RU. This confirmed that the aptamer ligand was specific toward catalase. It is also noted that from the response, the relative response after injection for the 3 control proteins went back down near to the base line, although this could be because of rapid dissociation of the protein after binding and may suggest that the other proteins have a very high k_{off} rate to the aptamer

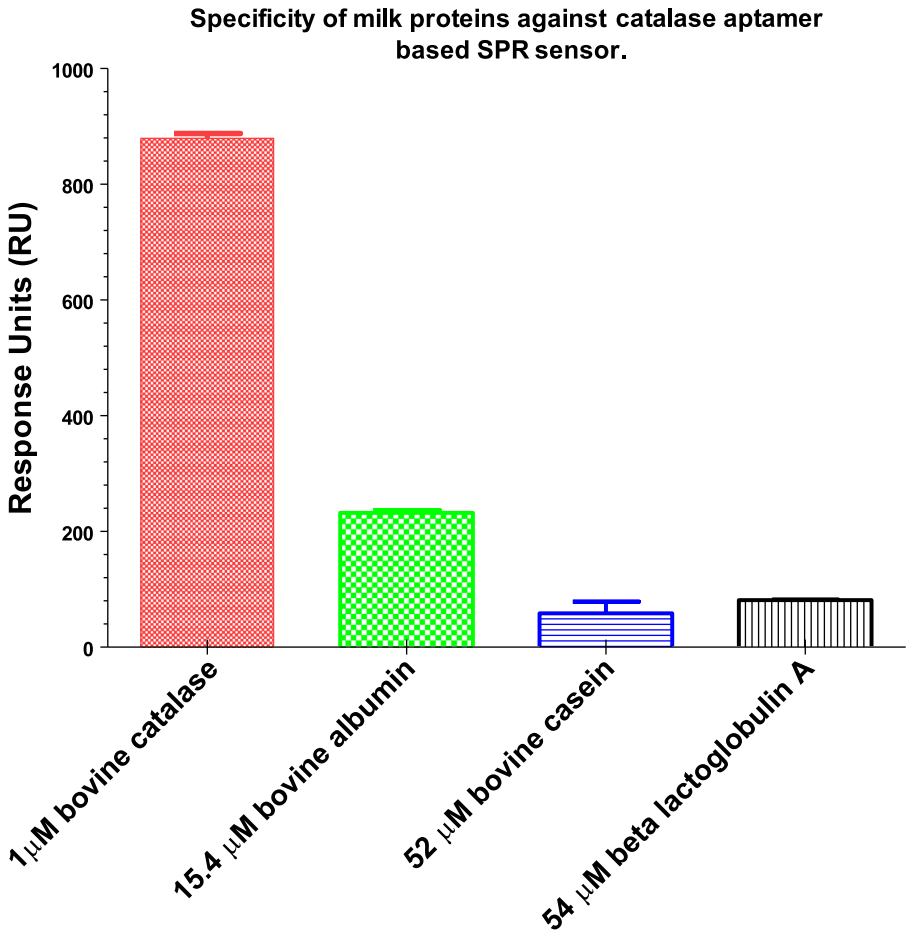

Fig. 2. (a) Graph showing specificity of the sensor chip surface toward different proteins found in milk. 50 μl of bovine catalase (1 μM), bovine albumin (15.4 μM), bovine casein (52 μM) and beta lactoglobulin (54 μM) were injected, run buffer of HKE, 0.5% BSA at a flow rate of 10 μl/min regenerated using 45 mM glycine, 100 mM NaOH in 1.2% EtOH.

(see Supplementary data, Fig. S3). Nevertheless we can account for the non-specific binding and matrix effect of the milk by using a milk only blank injection as the negative control.

3.3. Catalase assay and real sample analysis

Response curves were recorded by injecting 50 μl of bovine catalase in HKE BSA buffer at different concentrations (0–1000 nM) on the SPR sensor and the surface was subsequently regenerated using regeneration buffer. The relative response was taken by subtracting the streptavidin only channel (channel 1) from the aptamer surface (channel 2). Each concentration was injected in triplicate and a calibration plot of the relative response at 480 s was plotted against catalase concentration. The relative response was measured at 480 s because this was the point after injection where the baseline stabilizes. The response plot and calibration plot are shown in Fig. 3. The LOD ($S/N=3$) was determined to be 20.5 nM (RSD: 15.2%) and the dynamic linear range was determined to be between 20.5 and 1000 nM. The linear regression for the aptamer catalase interaction was $y=0.4750x+1.048$ ($R^2=0.94$).

The relative standard deviation of injections was found to be higher at higher concentrations (i.e. higher than 1 μM) which may be due to the fact that the sensor was reaching a point of saturation which can be a source of systematic errors. At lower concentrations the standard deviation was lower and within $<10\%$ RSD. The reproducibility of the biosensor was comparable to other methods reported for SPR. The sensor performance was then assessed in real milk samples. For this study standardized milk was used. Samples of milk were prepared by spiking the samples with catalase, centrifuging to remove the fat layer and filtering using a 0.45 μm filter. The centrifugation and filtering steps are necessary to remove particulates from the sample as this can spoil the machine. The spiked samples were injected at different concentrations (0–1000 nM) and the response plot and graph of spiked milk response at 480 s against the spiked sample concentration are shown in Fig. 4. A milk only blank response plot was used as the negative control and subtracted from each signal to give the relative response. Spikes in the signal were observed at the start and end of the injection due to the slight differences in the times of injection. The catalase recoveries in milk are shown in Table 1. Going from higher concentrations, the recoveries of catalase significantly improved as lower concentrations of spiked milk were injected with the 67.7 nM catalase giving a recovery of 104%. Since the levels of catalase in milk will be in the lower nanomolar concentrations, the sensor should successfully detect increases in catalase levels in milk with adequate accuracy and specificity.

These results are comparable to previous studies on aptamer based SPR sensors such as by Lee et al. who developed an aptamer based biosensor for the detection of RBP in human serum, reporting a similar K_D (0.2 μM) for the aptamer and LOD (75 nM) although the binding affinity K_D for the aptamer developed in this study was determined using non equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) and fluorecence intensity, giving a K_D of 0.237 μM and 0.430 μM respectively.

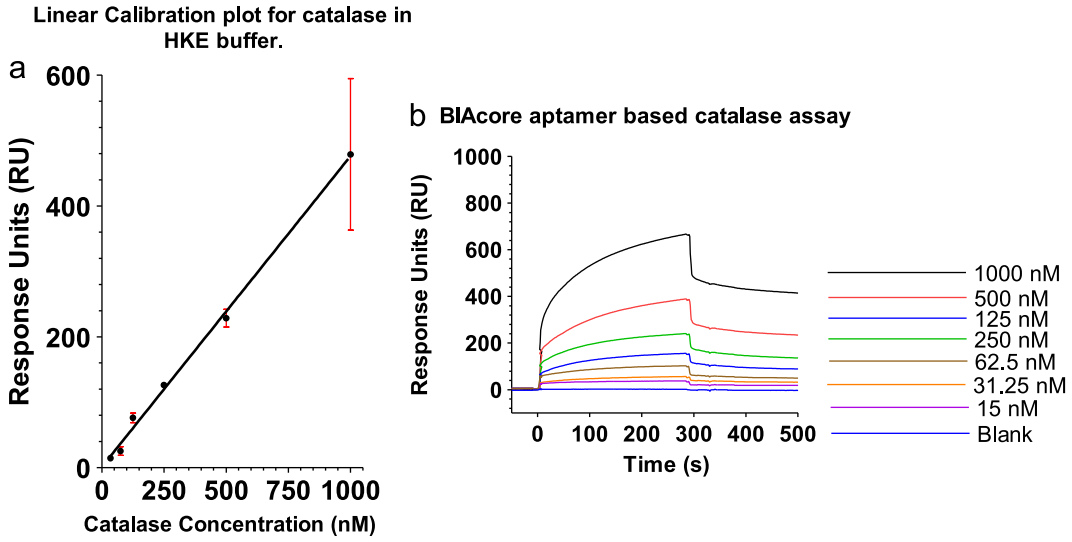

Fig. 3. (a) Calibration plots for the aptamer based catalase SPR biosensor. Injections of 50 μl of catalase (15–1000 nM), flow rate: 10 μl/min HKE buffer (LOD=20.5 nM, $S/N=3$, $n=3$, RSD: 15.2%); and (b) response plot for the aptamer based catalase SPR biosensor. Injections of 50 μl of catalase (15–1000 nM), flow rate: 10 μl/min HKE buffer 0.5% BSA, regenerated using 45 mM glycine, 100 mM, NaOH in 1.2% EtOH.

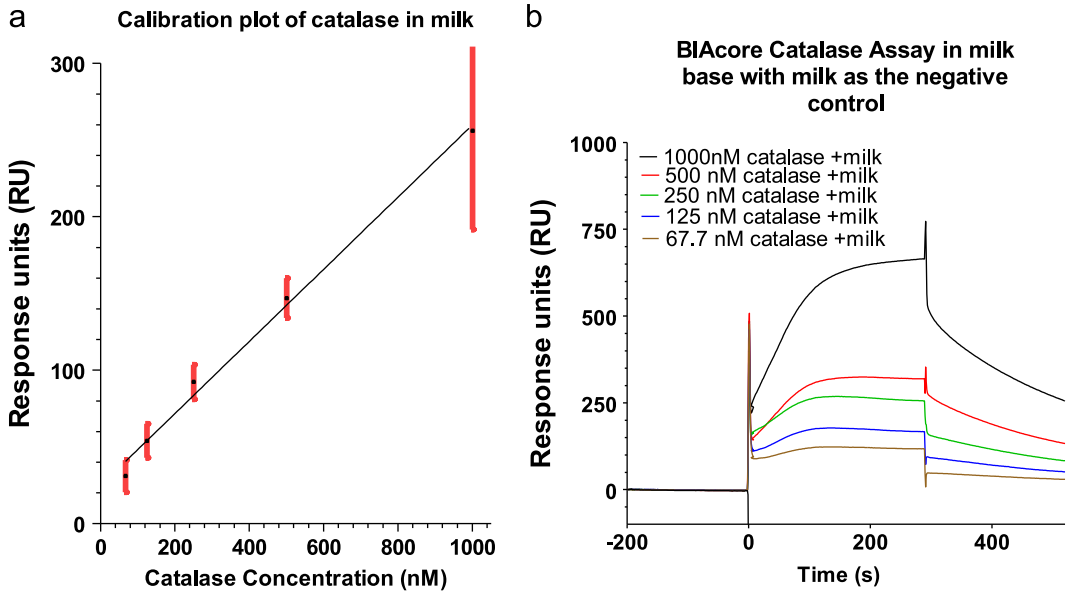

Fig. 4. (a) Calibration plots for the aptamer based catalase SPR biosensor. Injections of 50 μl of catalase (15–1000 nM); flow rate: 10 μl/min HKE buffer; and (b) corrected response plot for the aptamer based catalase SPR biosensor in spiked milk samples. Injections of 50 μl of catalase (0–1000 nM), flow rate: 10 μl/min HKE buffer, regenerated using 0.1 M NaOH in 1.2% EtOH. The relative responses of the spiked samples were corrected by subtracting the milk only reference.

Table 1
A summary of the percentage catalase recoveries from spiked milk samples (67–1000 nM).

Spiked concentration (nM)	1000	500	250	125	67.7
Calculated recovery (nM)	514.26	308.42	193.04	112.20	65.057
% Recovery	51.4	61.7	77.2	89.8	104
% RSD	14.5	5.07	6.99	11.6	19.4

The results obtained from this study show that aptamers are viable alterntive to antibodies. Furthermore due to the large size of the analyte in comparison to the aptamer, the results imply that nanomolar detection limits can be accomplished with relatively low levels of immobilization. This feature suggests that aptamer based SPR sensors can potentially be a good method for detection of large analytes at low concentrations. However it is also noted that further improvements in aptamer based SPR technology need to be made, e.g. to improve the reproducibility to less than 10%, if it is to be accepted as a main stream technique for bio sensing.

4. Conclusion

An SPR based aptamer biosensor was successfully developed for the non-labeled detection of bovine catalase. A bare gold

surface was immobilized with the aptamer using a streptavidin: biotin interaction. The sensor was demonstrated to have good specificity toward bovine catalase when compared to other milk proteins such as casein, β lactoglobulin and bovine albumin and is sensitive down to the nanomolar range (LOD = 20.5 nM). The biosensor also allows for the detection of catalase in milk samples with minimal sample preparation and without much effect on the LOD or standard deviation. The linear range of the biosensor was between 20.5 and 1000 nM. The recoveries at low concentration were near to 100% compared to higher concentrations suggesting that SPR based biosensors have an upper limit on detection of high molecular weight targets. The use of aptamers in SPR based biosensors shows great potential and has obvious advantages over other types of ligands such as antibodies. This sensor could be potentially used in the detection of catalase in milk samples, a key indicator of mastitis disease in milk. We are currently considering testing the SPR based aptamer sensor on clinical samples of milk to see how the biosensor performs.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.04.009.

References

Andersson, K., Hämäläinen, M., Malmqvist, M., 1999. Analytical Chemistry 71 (13), 2475–2481.
Ashley, J., Ji, K., Li, S.F.Y., 2012. Electrophoresis 33 (17), 2783–2789.
Bai, H., Wang, R., Hargis, B., Lu, H., Li, Y., 2012. Sensors (Switzerland) 12 (9), 12506–12518.
Boehmer, J.L., 2011. Journal of Mammary Gland Biology and Neoplasia 16 (4), 323–338.
Chang, C.-C., Lin, S., Lee, C.-H., Chuang, T.-L., Hsueh, P.-R., Lai, H.-C., Lin, C.-W., 2012. Biosensors and Bioelectronics 37 (1), 68–74.
Cremonesi, P., Pisoni, G., Severgnini, M., Consolandi, C., Moroni, P., Raschetti, M., Castiglioni, B., 2009. Journal of Dairy Science 92 (7), 3027–3039.
Fűtő, P., Markus, G., Kiss, A., Adányi, N., 2012. Electroanalysis 24 (1), 107–113.
Hamed, H., El Feki, A., Gargouri, A., 2008. Comptes Rendus Biologies 331 (2), 144–151.
Kim, Y.H., Kim, J.P., Han, S.J., Sim, S.J., 2009. Sensors and Actuators B: Chemical 139 (2), 471–475.
Koskinen, M.T., Holopainen, J., Pyörälä, S., Bredbacka, P., Pitkälä, A., Barkema, H.W., Bexiga, R., Roberson, J., Sølverød, L., Piccinini, R., Kelton, D., Lehmusto, H., Niskala, S., Salmikivi, L., 2009. Journal of Dairy Science 92 (3), 952–959.
Lansbergen, L.M.T.E., Nielen, M., Lam, T.J.G.M., Pengov, A., Schukken, Y.H., Maatje, K., 1994. Journal of Dairy Science 77 (4), 1132–1140.
Lee, S.J., Youn, B.-S., Park, J.W., Niazi, J.H., Kim, Y.S., Gu, M.B., 2008. Analytical Chemistry 80 (8), 2867–2873.
Tombelli, S., Minunni, M., Luzi, E., Mascini, M., 2005. Bioelectrochemistry 67 (2), 135–141.
Viguier, C., Arora, S., Gilmartin, N., Welbeck, K., O'Kennedy, R., 2009. Trends in Biotechnology 27 (8), 486–493.