Novel assay format permitting the prolonged use of regeneration-based sensor chip technology

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Received 26 July 2004; received in revised form 1 October 2004; accepted 28 October 2004
Available online 9 December 2004

Abstract

A polyclonal antibody raised against morphine-3-glucuronide (M3G, the main metabolite of heroin and morphine) was used in the development of a novel assay format using a surface plasmon resonance (SPR)-based biosensor. Previously developed assays have generated calibration curves based on differences in the quantity of response units binding to the surface of a chip coated with the analyte. The novel assay described here was based on the development of a standard curve using the slope of a series of consecutive binding interactions. Using this format, regeneration between each assay cycle was no longer required. This increased the useable life span of the chip surface and, as a result, decreased the cost associated with the assay. Thus, at least 15 binding interactions could be carried out before the saturation of antibody on the surface of the chip caused the response to deviate significantly from linearity. After 15 nonregenerated binding interactions, the slope still remained within 1.5% of the slope after a single binding event. Analysis time, and the sample volumes required were also markedly decreased while sensitivity was enhanced. The inhibition assay developed had a detection range of 270 to 17,500 pg ml^{-1}.

Keywords: Morphine-3-glucuronide; Surface plasmon resonance; Biosensor; Inhibition assay

1. Introduction

The development of rapid analytical devices, such as biosensors, for the detection of molecular species has grown enormously over the past few decades (Pancrazio et al., 1999; Rogers, 2000). The use of biosensors has ranged from environmental monitoring (Denisson and Turner, 1995), medical applications (Connolly, 1995) to the detection of coumarins (Keating et al., 1999), mycotoxins (Daly et al., 2000), pesticides (Alcocer et al., 2000), illicit drugs (Dillon et al., 2003) and microorganisms (Leonard et al., 2003).

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The BIACORE biosensor is based on the phenomenon of surface plasmon resonance (SPR; Quinn and O'Kennedy, 1999). This permits the detection of biomolecular interactions in 'real-time' (Jonsson et al., 1991). At the heart of the BIACORE instrument, as with the majority of SPR-based commercially available instruments, is the sensor chip. The BIACORE sensor chip consists of a glass slide with a thin layer of gold deposited on one side. Gold was chosen as it possesses the characteristics of chemical inertness and good SPR response. This gold layer is in turn covered with a covalently bound carboxymethylated (CM) dextran matrix attached by a hydroxyalkyl thiol linker layer. The matrix allows the covalent immobilization of analytes onto the surface of the chip and increases sensitivity by increasing the binding capacity of the surface. The matrix also provides a hydrophilic environment with very low nonspecific binding. The matrix forms one wall of a microflow cell where interactions are monitored. The carboxymethylated side of the chip comes in contact with the solution of interest, while the gold side of the chip is illuminated from the other side, through the glass. SPR is generated through the interaction of the light energy with the gold film and this is used to monitor concentrations of analyte on the surface of the chip.

The BIACORE biosensor is used widely in the development of competitive and inhibition immunoassays. With this system, an inhibition assay is the most commonly used format. In this format, haptens or protein–hapten conjugates are immobilized onto the surface. Standards of free hapten are prepared and premixed with binding ligand and after a suitable incubation time, passed over the protein–hapten surface. The hapten in solution inhibits the binding of the ligand to the immobilized hapten on the surface and the amount of ligand that binds to the surface is inversely proportional to the amount of free hapten in solution. After each sample, the surface is regenerated to remove the bound ligand. Regeneration typically involves the application of agents capable of efficiently disrupting the strong noncovalent interactions between antibody and antigen. This normally involves extremes of pH, and on occasion, the use of surfactants and chaotropic agents. Depending on the strength of the interaction, such conditions can be harsh on the antibodies and conjugates being used and can bring about denaturation and loss of activity.

One of the main problems associated with this assay format is the stability of the immobilized surfaces. In some cases, it may be possible to reproducibly regenerate the surface thousands of times. However, in other cases, the surface may only be able to withstand a relatively small number of regenerations before the surface denatures. If this occurs, more surfaces are required to perform the tests, which increases the expense associated with the assay. The objective of the assay format described here was to greatly increase the number of samples that could be analyzed successfully on one individual surface. By allowing more than one binding pulse between regenerations, it would be possible to analyze more samples on a single surface by shortening the amount of time a sample is passed over the surface and by plotting the slope of the binding interaction versus concentration rather than the traditional method of plotting changes in response units versus concentration. Mass transport-limited binding to the surface follows an exponential saturation binding curve. However, in the early phase of the binding event, the rate of binding is pseudolinear. Continued binding gradually results in surface saturation and deviation from linearity. However, during this linear phase, it is possible to analyze several binding interactions in quick succession. This technique has been applied to the analysis of the pesticide atrazine using a flow-injection-based electrochemical immunosensor (Grennan et al., 2003). In that system, however, only four samples could be applied to the surface before significant surface saturation would occur. It was proposed that the same method would perform considerably better on BIACORE, with its considerably higher sensitivity.

The model analyte used in the development of this assay was morphine-3-glucuronide (M3G). Following the ingestion of heroin, it is quickly metabolized to 6-monoacetyl morphine and then to morphine, which in turn, undergoes extensive metabolism. Glucuronidation is the main metabolic pathway producing morphine-3-glucuronide (55%) and morphine-6-glucuronide (10%; Zheng et al., 1998). As morphine-3-glucuronide is the main metabolite formed in the body for both morphine and heroin (Moffat, 1986; Coyle, 1987; Gough, 1991), it is used as an indicator

of drug abuse and can be detected in blood or urine. Hence, there is a need for sensitive methods of detection and identification of opiates. This factor, combined with the previous experience within the laboratory for analysis of this drug, suggested that it was the ideal choice of analyte for use in this model assay.

2. Materials and methods

All reagents and chemicals were supplied by Sigma (Poole, Dorset, England), unless otherwise stated. Carboxymethylated (CM) dextran was obtained from Fluka Chemicals (Gillingham, Dorset, England). BIACORE 3000™ and CM5 sensor chips were supplied by Biacore (Uppsala, Sweden). Preparation of hapten drug conjugates and antibody are described in Dillon et al. (2003).

2.1. Coupling reaction of morphine-3-glucuronide-OVA to carboxymethylate-dextran gel

The carboxymethylated dextran (CM-dextran) matrix was activated by mixing equal volumes of 100 mM *N*-hydroxysuccinimide (NHS) and 400 mM *N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) prepared in ultrapure water and injecting the mixture over the sensor chip surface for 7 min at a flow rate of 5 μl min^{-1}. The morphine-3-glucuronide-ovalbumin (M3G-OVA) conjugates to be immobilized (concentration 50–200 μ ml^{-1}) were dissolved in 10 mM acetate buffer, pH 4.3, and injected over the surface for 20 min at a flow rate of 2 μl min^{-1}. The unreacted sites on the sensor chip surface were then capped by injection of 1 M ethanolamine prepared in ultrapure water, pH 8.5, for 7 min.

2.2. Sample preparation for sensor analysis

The antibody (Dillon et al., 2003) was diluted in phosphate-buffered saline solution (PBS—pH 7.3, 0.15 M NaCl) at a dilution of 1/500 (3.5 μg ml^{-1}). The antibody displayed negligible nonspecific interactions for the immobilized protein OVA and the CM-dextran surface thereby negating the need for any preincubation steps with either OVA or CM-dextran. All buffers and solutions used were made up using ultrapure water, degassed and filter sterilized.

2.3. Optimization of polyclonal antibody regeneration conditions on an M3G-OVA-modified chip surface

The regeneration conditions for the removal of the polyclonal antibody from the surface of the chip were optimized. The antibody required three 1-min pulses of 40 mM HCl, 40 mM NaOH and 40 mM NaOH.

2.4. Morphine-3-glucuronide preparation for use in inhibition assay

A 10-mg ml^{-1} solution of free morphine-3-glucuronide was prepared in 50 mM HCl to reduce the formation of insoluble complexes. Standards were prepared in PBS containing 0.5% (v/v) HCl, at concentrations ranging from 122 to 250,000 pg ml^{-1}. Each sample was incubated separately with an equal volume of a 1/250 (7.0 μg ml^{-1}) dilution of antibody for 1 h. A 5-μl sample of each of the solutions was then passed consecutively over the surface of the chip at a flow rate of 20 μl min^{-1}. Samples were applied to the sensor in increasing concentrations. This was followed by regeneration of the surface with the optimized regeneration solutions. This was repeated three times in order to conduct assay variability studies.

The slope of the 15-s injection pulse was used as a measure of response. To normalize the results from individual assays, the binding slope at each M3G concentration (S) was divided by the antibody binding slope determined in the absence of free M3G (S_0) to yield a normalized binding response. This was performed for each of the three sets of replicate samples.

3. Results and discussion

Results presented in this paper clearly show the factors required in the development of a novel assay format using the BIACORE biosensor. A polyclonal antibody raised against morphine-3-glucuronide was used in this model system for the development of the assay. This antibody—already characterized in both ELISA and BIACORE inhibition procedures—was chosen in order to compare the new format to the traditional methods used (Dillon et al., 2003).

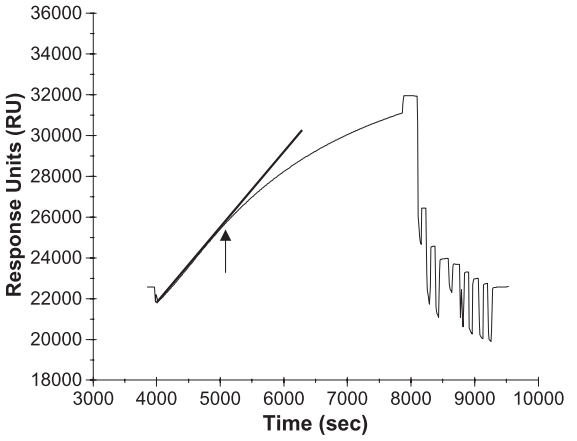

Fig. 1. Illustration of antibody saturation curve on a CM5 chip surface with immobilized M3G-OVA. The sensor surface began to deviate from linearity when approximately 3000 RU were bound to the surface. Up to this point, the binding interaction of antibody to the surface was pseudolinear and may be used in the development of an assay.

One of the main problems associated with the development of BIACORE inhibition assays is the regeneration of the surface. The regeneration solutions required for the antibody by Dillon et al. (2003) showed that this antibody had a high affinity for the conjugate and thus required relatively harsh and time-consuming regeneration conditions. The main aim of this assay format was to see if it was possible to decrease the number of regenerations required when performing an assay. By decreasing the number of regenerations required, the number of assays which could be performed on one chip would increase, allowing a higher number of analyses to be performed on the chip while also lowering the overall cost of the assays.

Previously published results by Dillon et al. (2003) have demonstrated that for this polyclonal antibody, it was possible to regenerate the surface reproducibly at least 50 times with a regeneration cycle between each antibody binding pulse. However, the present work has focused on the development of assays performing a regeneration cycle only after 10–15 antibody binding pulses. In order to do this, calibration curves were not generated by using the response units bound to the surface but by utilizing the slope of each sample binding pulse. The sample injection time was also decreased from 4 min to 15 s for each binding pulse, allowing a higher number of samples to be passed over the surface in the same time period.

In the initial development of the assay format, antibody was passed over the surface of the chip to determine the number of response units required to saturate the surface. Initially, a linear binding response was observed up to approximately 3000 RU of antibody binding to the surface (Fig. 1). This suggested that to develop an assay using consecutive, nonregenerated binding pulses of antibody, the maximum antibody binding response should not exceed 3000 RU. The binding capacities and reproducibility of repeated pulses of the antibody to conjugate immobilized surfaces were determined by injecting a series of binding pulses consecutively without regeneration to assess how many binding pulses were possible before the binding capacity fell below the desired performance parameters (<20%; Wong et al., 1997). Ideally, for this assay format, it was preferable to be able to perform multiple binding

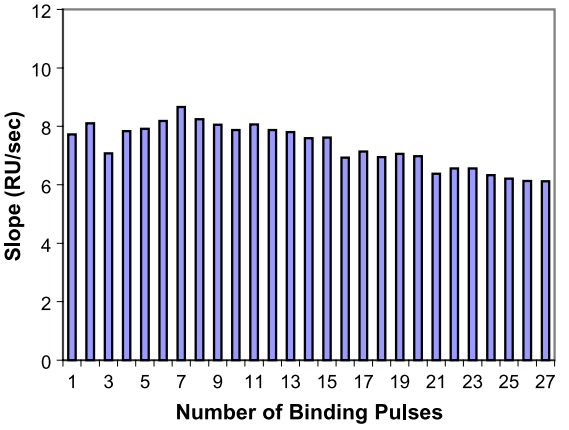

Fig. 2. Typical regeneration profile for 0 to 27 cycles of a 15-s binding pulse of affinity-purified anti-M3G polyclonal antibody to the surface of a chip with immobilized M3G-OVA. A 1/500 (3.66 μg ml^{-1}) dilution of polyclonal antibody was used. After 27 cycles of binding and regeneration, there was approximately 3000 RU of antibody on the surface of the chip and the slope after the injection of the last binding pulse was 6.12 RU s^{-1} compared with the first binding slope of 7.72 RU s^{-1}. The ligand binding capacity was shown to decrease by approximately 20% over the course of the binding pulses. However, between cycles 1 and 15, there was only a 1.5% drop. After the first 15 cycles, the binding capacity of the remaining regeneration cycles dropped by approximately 19%. When performing assays, the first 15 regenerations were regarded as providing the most accurate results.

pulses of antibody on the surface of one derivatised chip. A range of unregenerated antibody binding cycles was investigated and the slope of each binding pulse plotted (Fig. 2). A 20% decrease in the slope occurred after 27 binding cycles. However, after 15 binding cycles, there was only a 1.5% drop in the slope. As a result, for subsequent generation of calibration curves, a maximum of 15 binding pulses were used before regeneration was performed.

For the determination of the optimal range of detection of M3G for a BIACORE inhibition assay, a range of M3G standards was prepared. The affinity-purified anti-M3G polyclonal antibody was premixed with the corresponding concentration of free M3G and allowed to equilibrate for 1 h at 37 °C. The samples were then passed consecutively over the surface of the sensor chip immobilized with M3G-OVA, followed finally by one regeneration of the surface using the appropriate regeneration solution (Fig. 3). This was repeated three times for variability studies.

A calibration curve for the anti-M3G polyclonal antibody was constructed by plotting the ratio of mean binding slope of each standard of free drug to the sample standard with no free drug (S/S_0) against the concentration of M3G. The range of detection for the polyclonal antibody using this method was found to be between 273 and 17,500 pg ml^{-1} (Fig. 4). At 273 pg ml^{-1}, the average slope of 4.88 (standard deviation of 0.01, n=3) was greater than three standard deviations below the control slope (S_0) of 4.96. All samples had standard errors below 5%, except for the high concentrations of 17,500 and 8750 pg ml^{-1}, which were 16.2% and 21.7%, respectively. This novel assay format was more sensitive compared to the original assay developed (Dillon et al., 2003), which had a range between 976 and 62,500 pg ml^{-1}. However, a flow rate of 20 μl min^{-1} was used in the present work as compared to 10 μl min^{-1} (Dillon et al., 2003). A higher flow rate was used as only a very short sample pulse (15 s) was used for this assay. In addition, it was possible to use the surface up to 15 times longer resulting in considerable saving on the cost of each assay. Samples were now only passed over the surface for 15 s instead of 4 min, resulting in the need for markedly less quantity of sample and decreased assay time. These factors may be of great significance where sample volumes and availability of expensive standards are severely limited. In conclusion, we believe that this finding may have significant utility in the design and use of chip-based assay formats. However, it remains to be seen whether this

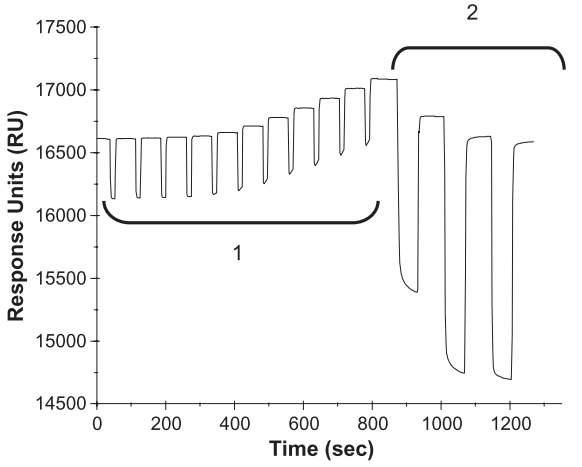

Fig. 3. (1) Illustration of samples of antibody premixed with different concentrations of free drug (range between 136 and 70,000 pg ml^{-1}) injected over the surface of a chip immobilized with M3G-OVA. Each dip was a different concentration of hapten premixed with antibody injected over the surface. (2) Illustrating the effect of the solutions (three 1-min pulses of 40 mM HCl, 40 mM NaOH and 40 mM NaOH) used to regenerate the surface. The total amount of antibody bound to the surface after the 11 mixtures were passed over was approximately 470 RU, which was well below the 3000 RU required to bring about significant deviation from linearity.

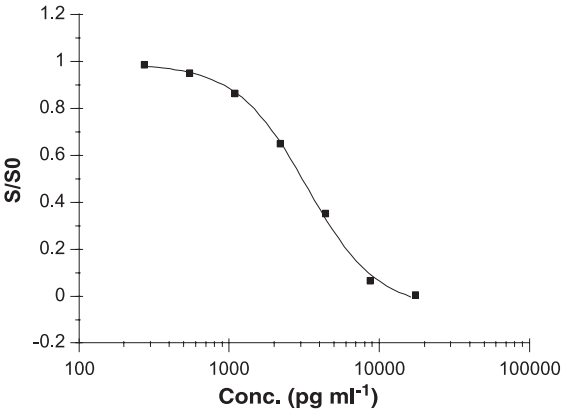

Fig. 4. Intraday assay curve for M3G using the anti-M3G polyclonal antibody on an M3G-OVA immobilized chip surface. The results shown are the average of triplicate results and the range of detection of free M3G was between 273 and 17,500 pg ml^{-1}. The binding slope at each M3G concentration (S) was divided by the antibody binding slope determined in the presence of zero M3G concentration (S_0) to give a normalized binding response (S/S_0).

technique is applicable to the analysis of M3G and other analytes in real sample matrices, such as blood or urine.

Acknowledgements

The financial support of Enterprise Ireland is gratefully acknowledged.

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