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Short communication

Development of biosensor-based SPR technology for biological quantification and quality control of pharmaceutical proteins

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ABSTRACT

We developed and validated a biosensor-based surface plasmon resonance (SPR) technology for the biological quantification and quality control (QC) of pharmaceutical proteins using reference materials as the standard. The surface of the receptors was made homogeneous by covalently immobilizing the receptors onto Au-membrane microchips for use as biosensors for reliably detecting the activity of drug proteins. This assay used only limited amounts of ligands and no additional detection agents. The products were determined to have binding capacity equivalent to that of the reference materials and to exhibit a recovery range of 88.4–115.0%. The binding of analytes to the specific ligand is concentration dependent and parallel. CTLA-4 fusion proteins were quantitatively detectable at concentrations as low as 125 ng/mL. The intra-assay precision was in the range of 1.07–7.27%, and the inter-lot precision was 13.03%. These data proved the usefulness of improved biosensor-based assays in biological quantification and QC of pharmaceutical proteins. This approach is an alternative to traditional assays and offers a potentially significant advantage in that the microchip can be regenerated thus enabling multiple analyses to be performed with a single sensor.

1. Introduction

Several methods have been employed to study drug–target interactions that are involved in the pharmacological action of drugs and to evaluate the biological activity and quality of drugs throughout experimental research procedures and industrial production processes. These techniques involve enzyme-linked immunosorbent assay (ELISA) [1,2], microarrays [3,4], quartz crystal microbalance [5,6], mass spectrometry [7], and biocatalytic precipitation [8]. However, detection of the activity and monitoring the quality of a specific class of agents in real time continue to be major challenges.

Biosensor-based detection of pharmaceutical products presents an exciting alternative to standard immunoassays for the screening and identification of the products qualified for clinical applications [9–11]. Recently, approaches for developing receptor- or antigen-based ELISA using coated ligands (e.g., with receptor, antigen, etc.) in microtiters [1,2] have been employed to detect the binding activity of agents. However, these approaches have to meet the requirements of providing information about the target products, including the labeled second detection reagents employed for signal tests, and ensuring that adequate amounts of the receptors or antigens are available considering that the microtiters of coated ligands are discarded after each detection. During instances wherein there is a lack of necessary materials, analysis of drug–target interactions and quality control (QC) would be of limited use.

We report the development of biosensor-based SPR technology for biological quantification and QC of the pharmaceutical CTLA-4 fusion protein, which is an immunosuppressive agent. The quality of multiple lots of CTLA-4 fusion proteins produced at different times was evaluated by determining their ability to bind to target receptor relative to that of reference materials expressed as a percentage. We examined the statistical data to define the acceptance criteria for this assay and established its validity.

2. Materials and methods

2.1. Reagents and samples

CTLA-4 fusion protein has been shown to have promising immunosuppressive potency against rheumatoid arthritis (RA) [12,13]. Multiple lots of the CTLA-4 fusion protein and reference materials were produced using CHO cells as previously reported [14,15]. B7.1-Ig was used as a co-receptor for detecting the protein agents. Sensor Chip CM5 and a Biacore 3000 system were used for sample testing. An amine coupling kit

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comprising 115 mg *N*-hydroxysuccinimide (NHS), 750 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 10.5 mL ethanolamine–HCl, and HBS-EP buffer [10 mmol/mL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES; pH 7.4), 150 mmol/mL NaCl, 3.4 mmol/mL ethylenediaminetetraacetic acid (EDTA), 0.005% (v/v) surfactant P20, and regeneration buffer [10 mmol/mL sodium citrate, 100 mmol/mL NaCl (pH 4.0)] were purchased from GE Co. (Uppsala, Sweden).

2.2. Immobilization of receptor protein

The ligand (B7.1 Ig co-receptor) was diluted using 10 mmol/mL sodium acetate buffer (pH 5.0) to achieve a surface mass of between 3000 and 9000 resonance units (RU) by using a previously reported method [16,17]. CM5 biosensor microchips were inserted into a Biacore 3000 system (Amersham Biosciences, Uppsala, Sweden) and activated by injecting a mixture (1:1, v/v) of *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (0.4 mol/mL) and *N*-hydroxysuccinimide (0.1 mol/mL) into the microchips. Thereafter, the ligand (dissolved in 10 mmol/mL sodium acetate; pH 5.0) was immobilized covalently onto the surface of the microchips by the amine coupling method. After immobilization, the redundant active groups on the surface of the microchips were blocked by using ethanolamine–HCl (1.0 mol/mL; pH 8.5). As shown in Fig. 1A, to enable immobilization of specific receptors, approximately 5443.0 RU per flow cell were immobilized onto the FC4 tunnel of CM5 for the binding analysis, and bovine serum albumin was immobilized onto the FC3 tunnel as a negative control.

2.3. Standard curve

The reference material was diluted to the target concentrations of the standard curve (0.125, 0.25, 0.50, 1.00, 2.00, 4.00, and 8.00 μg/mL) in 15-mL conical tubes containing HBS-EP buffer. The percentage coefficient of variation (%CV) of the slope values obtained from the experiment performed in triplicate was calculated at each concentration in the standard curve. The %CV (standard deviation/mean × 100) of the triplicate values at each point in the standard curve was ≤15%. This evaluation was performed using the software package Biaevaluation 3.0. The percentage difference between the mean of the calculated values (μg/mL) and the target values was calculated using the following formula: %difference = (mean calculated value − target value)/target value × 100. The mean calculated values (μg/mL) at each standard concentration used to determine the standard curves were within 20% of the target value.

2.4. QC

QC samples were prepared in HBS-EP buffer at the target concentrations of 0.40, 2.50, and 4.50 μg/mL. The reference concentrations in the QC samples were determined by three independent Biacore concentration analysis experiments [18], and the average concentrations were reported as "nominal" QC concentrations. The three QC samples were injected in triplicate in the experiment.

The first set of QC samples was evaluated after the first set of standard curve. If the first set of QC sample results was ±20% of their respective target values, the analytical run was allowed to continue. If the results exceeded ±20% of the target values, the analytical run was terminated and new standard curve samples were prepared as described above; a new set of QC samples were also assayed.

To fulfill the acceptance criteria for the QC samples, the %CV of the triplicate concentration values for each QC sample target concentration was maintained at ≤15%. The percentage difference between the measured and target values of the QC sample concentration was calculated using the formula provided in Section 2.3.

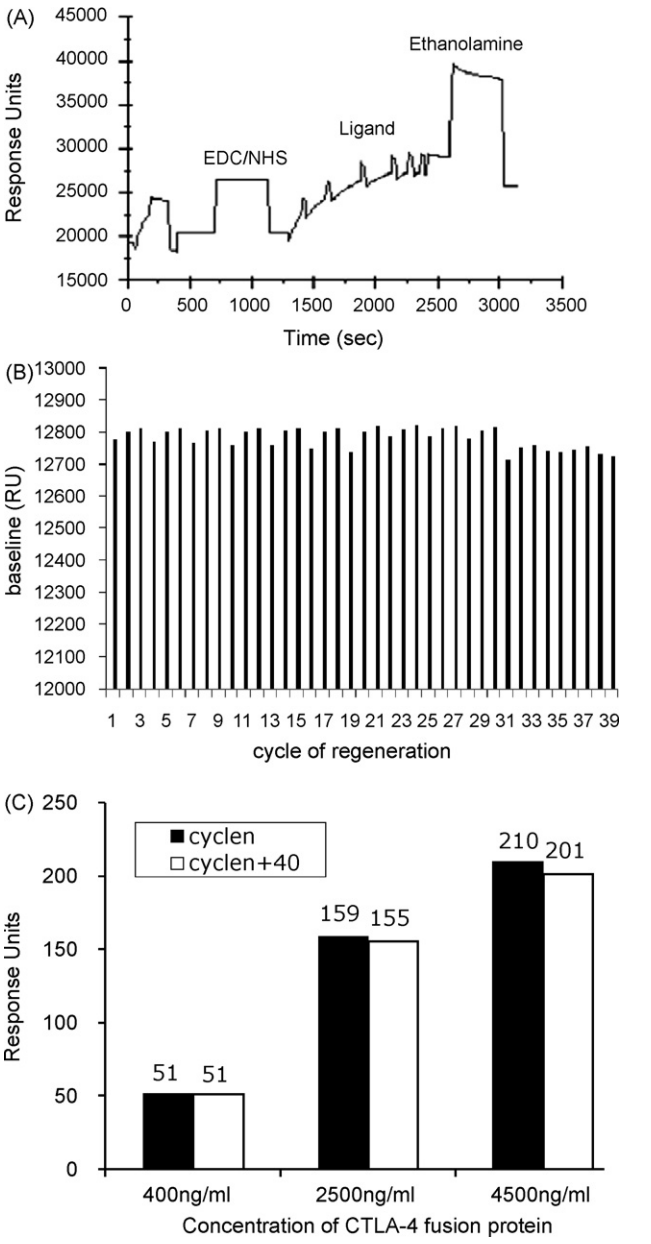

Fig. 1. Stability of sensorchip for CTLA-4 fusion protein binding to immobilized receptor. (A) Immobilization of co-receptor onto microchip surface. The procedure of immobilization comprises three steps: activation using EDC/NHS, coupling of ligand and blocking by ethanolamine in turn. The desired level of immobilization at 5443 RU was achieved. (B) B7.1 Ig co-receptor was immobilized onto the microchip surface and was regenerated with 10 μL of buffer (10 mmol/mL sodium citrate, 100 mmol/mL NaCl pH 4.0) after each sample addition. Baseline measurements in response units were obtained after each regeneration for at least 40 cycles. (C) The binding capacity of immobilized receptor was demonstrated by measuring the binding of the three different concentrations of references for CTLA-4 fusion protein initially and throughout 40 regeneration cycles using 10 mmol/mL sodium citrate and 100 mmol/mL NaCl.

The QC sample results were within 20% of their respective target values enabling at least 70% QC determinations. If the obtained values for the QC concentration did not meet the acceptance criteria, the assay was considered to be unacceptable and was repeated.

2.5. Analytical procedure

The concentration of the test sample (CTLA-4 fusion protein) solution should lie within the range defined on the basis of the

reference standard curve. The test sample was diluted to an approximate target concentration of 2.00 μg/mL using HBS-EP buffer. Sample dilutions of 200 μL were prepared in test tubes for analyses; these samples were injected in triplicate. The ability of the samples to bind to target receptors expressed as a percentage were determined by using the same 2.00 μg/mL dilutions from three different sets of test samples.

The acceptance criteria for the test samples were examined. The %CV of the triplicate observations obtained for each test sample was ≤20% and was calculated using the above-mentioned software package. If two or more mean slope values (RU/s) could not be calculated because they fell outside the QC sample concentration range (QC1–QC3, 0.40–4.50 μg/mL), the samples were re-assayed at different concentrations.

2.6. System suitability

The mass of receptors immobilized on activated flow cells are expressed in RU. The surface mass of the ligands should lie between 3000 and 9000 RU for optimal assay performance. The baseline drift was calculated manually for each analytical run as the percentage change in the baseline values (absolute response values; RU) between each cycle relative to the immobilized surface mass of the ligand. The percentage difference was determined by the following formula: baseline drift = (highest/lowest RU − mean RU)/mean RU × 100. The percentage of change between the cycles of the analytical run was ≤5.0%. In order to further evaluate the extent of surface-receptor activity, the binding values at the start and end of all runs were measured and the percentage difference was determined.

2.7. Calculations

The calculated mean concentration values for each of the test samples were analyzed and divided by the reported protein concentrations of the samples as determined using a BCA kit; the resultant value was multiplied by 100. This calculation could be denoted by the following formula: binding relative to the reference material = mean concentration/reported concentration × 100. The result was denoted as a percentage value.

3. Results and discussion

3.1. Effect of regeneration and stability

Since covalently immobilized ligands were used for multiple assay runs, the regenerative stability of the sensor chip-based assay was an area of concern [19]. We analyzed this and observed that the co-receptor ligand remains essentially homogeneous and active throughout the lifetime of the sensor chip surface as reflected by parameters such as baseline stability and binding capacity [19,20].

B7.1-Ig was immobilized as a co-receptor onto the microchip surface for detecting the protein agents. The procedure of immobilization comprises three steps: activation using EDC/NHS, covalent coupling of ligand, and blocking by ethanolamine. The desired level of immobilization (5443 RU) was achieved as shown in Fig. 1A. To determine whether it can be modified to be reused after regeneration after each run without significant loss of assay activity, we calculated manually the baseline drift (RU) for each run as the percentage change in the baseline between each cycle relative to the immobilized surface mass of the ligand. As shown in Fig. 1B, the co-receptor immobilized onto the surface of the microchip could withstand over 40 regeneration cycles without loss of activity and any significant change in the baseline in the presence of 10 mmol/mL sodium citrate and 100 mmol/mL NaCl (pH 4.0). The highest and lowest percentage changes in the run were 0.91 and 0.28%, respectively. The determined %CV was 0.31%; this represents the fine differences observed in baseline drifts between cycles. Fig. 1C shows the extent of binding activity demonstrated toward three different CTLA-4 fusion protein concentrations. The binding capacity of the fusion protein changes by less than 5.0% from the start to the end of the analytical run; this indicates the stability of the microchip assay.

Table 1
Determination of the range of quantitation for standard curve.

Reference (μg/mL)	No.	Mean ± SD (μg/mL)	%CV	%Recovery
0.125	3	0.111 ± 0.005	4.47	89.120
0.250	3	0.250 ± 0.019	7.44	99.994
0.500	3	0.504 ± 0.030	6.03	99.112
1.000	3	1.014 ± 0.068	6.66	98.619
2.000	3	2.016 ± 0.144	7.13	99.200
4.000	3	4.109 ± 0.305	7.43	97.273
8.000	3	8.285 ± 0.801	9.67	96.433

The range of quantitation and limit of quantitation (LOQ) were determined by diluting the reference materials in HBS-EP buffer at concentrations of 0.125, 0.25, 0.50, 1.00, 2.00, 4.00 and 8.00 μg/mL. The samples were examined in triplicate and the mean, standard deviation, %CV and %recovery were calculated for each concentration.

3.2. Limit of quantitation

The standard curve for the CTLA-4 fusion protein quantitative assay was constructed using the standard reference material. The range of the standard curve of the protein in HBS-EP buffer was 0.125–8.00 μg/mL. The examined range and limit of quantitation (lowest concentration that could be accurately quantified; LOQ) when the %CV was within 15%. For a quantitation assay to be considered valid, the response units for the lowest standard concentration should be greater than 15 RU based on instrument validation [18]. Due to the large molecular weight of the CTLA-4 fusion protein (92,300 Da), the sensitivity of this assay as defined by the LOQ was 125 ng/mL, which corresponded to the binding capacity determined previously (15.37 RU). This indicates that at least 125 ng/mL of CTLA-4 fusion protein could be accurately determined quantitatively, as shown in Table 1.

Furthermore, to prove whether the result was accurate over a wide range of target values and could be used for quantitative testing of samples, a duplicate analysis was performed to measure the

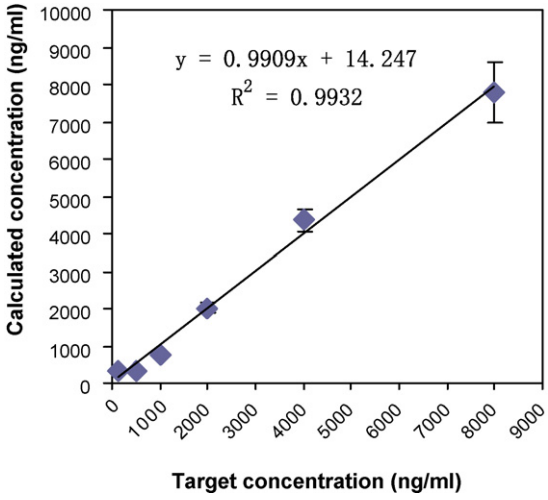

Fig. 2. Analysis on comparing calculated concentration values against target concentration values from different dilutions of references for CTLA-4 fusion protein. The concentration fit plot was showed. The correlation for linear assay was expressed as R^2 = 0.993, which indicates over a wide range of values. The "—" represented the trend line, standard deviations were shown as error bars and a regression equation was shown in the figure.

Table 2
Intra-assay precision and accuracy determined by QC samples.

	QC1 0.40 μg/mL	QC2 2.50 μg/mL	QC3 4.50 μg/mL
RU	48.500	155.400	201.400
	50.600	159.000	206.900
	51.400	161.200	209.500
Mean ± SD (RU)	50.167 ± 1.498	158.533 ± 2.928	205.933 ± 4.136
%CV	2.986	1.847	2.008
Nominal (μg/mL)[a]	0.46	2.63	4.85
%Recovery	114.218	105.364	107.700

[a] The reference concentrations in the QC samples are determined in three independent Biacore concentration analysis experiments and the average concentration results are reported as the "nominal" QC concentrations.

Table 3
Intra-assay and inter-lot precision and accuracy for detecting test samples in binding activity.

Three different lots of samples at target 2.00 μg/mL			
RU	Mean ± SD	%CV	%Recovery[a]
Lot 1			
160.60			
140.90	148.23 ± 10.77	7.27	115.04
143.20			
Lot 2			
126.70			
128.90	128.63 ± 1.82	1.41	88.45
130.30			
Lot 3			
139.00			
140.70	140.57 ± 1.50	1.07	103.90
142.00			
Inter-lot precision			
Lot 1 calculated (μg/mL)		2.30	
Lot 2 calculated (μg/mL)		1.77	
Lot 3 calculated (μg/mL)		2.08	
Mean ± SD (μg/mL)		2.05 ± 0.27	
%CV		13.03	

[a] The recovery from three different lots of CTLA-4 fusion protein products at the same concentration of 2 μg/mL was 2.300, 1.769 and 2.078 μg/mL, corresponding to percent binding activity as 115.0, 88.4 and 103.9%, respectively.

correlation between the actual and calculated values of various concentrations of reference proteins. The concentration fit plot shown in Fig. 2 illustrates the linearity of the assay (R^2 = 0.993) over a wide range of values.

3.3. Precision and accuracy of QC test

The accuracy of the assay for the quantitation of the CTLA-4 fusion protein was determined by calculating the percentage recovery of known values for three different concentrations of QC samples. The percentage recovery for QC samples of concentrations 0.4, 2.5, and 4.5 μg/mL ranged from 105.4 to 114.2% (Table 2). Precision was expressed as %CV; this reflects the agreement among the tests performed in triplicate. As shown in Table 2, the intra-assay %CV ranged from 1.85 to 2.99% for the same three concentrations of QC samples.

To further demonstrate the stability of the assay system, the percentage difference between the first and the last injections of the QC2 sample was determined and observed to be as low as 3.73%. The accuracy of data from the QC tests for the reference standard curves led to the conclusion that this assay system could be used for the evaluation of the binding capacity of the fusion proteins in the test samples.

3.4. Effectiveness of multiple lot samples in binding to immobilized co-receptor

A sensor chip-based assay was performed to further analyze the effectiveness of the test samples with three different lots of samples from the same manufacturer by detecting the extent of binding to the immobilized co-receptor. The percentage binding activity of the test samples was expressed as percentage recovery or accuracy. A known concentration of CTLA-4 fusion protein test samples was prepared in HBS-EP buffer; Table 3 presents the measurement data. The recovery from three different lots of CTLA-4 fusion protein products at the same concentration of 2.000 μg/mL was 2.300, 1.769, and 2.078 μg/mL, which corresponded to the percentage binding activities of 115.0, 88.4, and 103.9%, respectively. The %CV for the intra-assay (variation within an assay) was in the range of 1.07–7.27% (Table 3). Further, the mean percentage recovery was 102.43%, and the inter-lot precision (variation between lots of products) was 13.03%, which was consistent with the value for the reference material. Our data showed that the binding of various lots of CTLA-4 fusion proteins to specific receptors was equivalent with no significant differences.

4. Conclusion

A biosensor-based SPR method was developed and validated for the biological quantification and QC of pharmaceutical proteins using reference materials as the standard. This approach is an alternative to traditional assays and offers a potentially significant advantage in that the microchip can be regenerated thus enabling multiple analyses to be performed with a single sensor, which has higher stability and precision and accuracy associated with previous enzyme immunoassays (EIAs) [21,22]. The method allows prompt online evaluation on the activity of drugs via the detection of their ability to bind immobilized ligands.

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