A Simple Surface Plasmon Resonance Biosensor for Detection and Quantification of Recombinant Human Epidermal Growth Factor (rhEGF) in Escherichia Coli Crude Extract

FADZLIE WONG FAIZAL WONG, BENGTI TEY, ZARIDA HAMBALI, SITI MAZLINA MUSTAPA KAMAL, ARBAKARIYA ARIFF, TAU CHUAN LING

Abstract: A reliable detection and quantification assay is important in order to monitor the performance of recombinant human epidermal growth factor (rhEGF) recovery process. Knowing the benefits offered by the surface plasmon resonance (SPR) biosensor, a rhEGF analysis method employing the commercially available BIAcore 3000 equipment was developed by using polyclonal anti-EGF antibody as the capture ligand. The linearity of the assay (determined using the authentic human epidermal growth factor) was found to be in the range of 25 - 250 ng/mL. The performance of the developed assay was further evaluated in terms of accuracy, precision (intra and inter-assay), detection and quantification limit. The practical applicability of the assay was justified as a high accuracy (within 10% recovery of the target) and precision (less than 3.4% CV) were obtained for Escherichia coli crude extract samples. The assay was highly reproducible given that the intra- and inter-assay precision obtained were less than 20% CV. A considerably high sensitivity was also achieved with 8.0 ng/mL of quantification limit.

Key-Words: Quantification assay, recombinant human epidermal growth factor (rhEGF) purification, surface plasmon resonance (SPR), polyclonal anti-EGF antibody, linearity, performance

1 Introduction

Purification stage is an important step in the overall process of biologically active protein production. As the eventual aim is to obtain a final product with high purity and yield, the development of quantification assay is a prerequisite for monitoring the performance of the purification [1]. Thus the progress of purification process beginning right from the crude extract obtained after cell lysis, followed by product concentration process until the final purified product could be monitored. To our knowledge in most of the epidermal growth factor (EGF) production work reported, the calculation of peak area on chromatogram generated from HPLC [2,3,4] and other chromatography system [5] has become a very popular method for the quantification of EGF. However the reliability of the HPLC method is handicapped by its low sensitivity in detection which normally lay in the range of microgram [6]. Generally most of the protein quantification works being carried out during purification steps include radioimmunoassay [7,8], ELISA [9,10,11,12] and the Western blot based densitometry method [13]. These methods are highly impractical as it is time consuming and laborious. They basically operates based on immunochemistry principle and the sample will be reacted with antibody followed by manually done sequential steps like washing and reagent addition to finally reach the colour development stage. With that, about 4-7 hours time will be required to quantify only one sample. Furthermore, the reusability of antibody is not possible thus has made these methods expensive and non-economical.

Generally, the Biacore and other SPR instruments have been used for detecting and monitoring the binding event of wide array of
molecule, for instance antibody–antigen [14,15], toxin [16,17], nucleic acid [18], blood coagulation factor [19,20], enzyme-drug inhibitor [21], vitamin [6], cell-ligand [22] and others. SPR detects the target protein based on the change of the refractive index in the optical signal due to alterations in mass concentrations at the sensor surface [23]. A receptor (eg: antibody) which is specific to our protein is immobilized at an optimum density onto the surface of an activated sensor chip. Standards and test samples are later diluted in the running buffer and injected over the receptor surface. As the target protein binds to the receptor, a change in light intensity occurs and affecting the SPR signal. This signal displayed as resonance units (RU) is proportional to the bound mass on the surface of the chip [24]. A sensorgram is then generated in which the binding data is recorded in real time. The RU are converted into active concentration using a reference standard curve where the RU of standard protein is plotted against the protein concentration.

In the present study, we develop a simple surface plasmon resonance (SPR) method, using the available BIACORE 3000 (GE Healthcare) instrument to quantify our FLAG-tagged recombinant human epidermal growth factor (rhEGF) from the crude Escherichia coli (E. coli) extract. Standard rhEGF was used for the assay calibration and later being applied to our sample. The results are later compared with the rhEGF levels determined using the gold standard, a commercial kit of Quantikine® human EGF enzyme-linked immunosorbent assay (ELISA).

2 Materials and Methods

2.1 Materials
The BIACore 3000 biosensor together with BIACore 3000 control software (ver. 4.1), amine coupling kit and CM5 sensor chip was supplied by GE Healthcare, Uppsala, Sweden. Anti-EGF antibody used was obtained from Calbiochem®, Merck, Germany. Standard hEGF used was purchased from Peprotech, USA. Chemical like Tris, HCl, NaCl, glycine for buffer preparation were obtained from Fisher Scientific, UK. The ELISA kit for hEGF quantification was purchased from R&D Systems, USA. Bovine serum albumin was obtained from Sigma, USA. The hEGF containing recombinant E.coli (constructed by Faizal et al., 2006) crude extract was prepared by osmotic shock procedure.

2.2 Preparation of sensor surfaces
Amine coupling was conducted using the amine coupling kit according to the manufacturer's instructions. Before the activation, the system was equilibrated with running buffer (Tris buffered saline, 50mM Tris HCl, 150mM NaCl, pH7.4) and maintained at a flow rate of 5 µl/min. An injection of 35 µl NHS/EDC solution [0.05 M N-hydroxysuccinimide/ 0.2 M N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide] activated the carboxymethylated dextran surface of the CM5 chip. Initially pH scouting for immobilization of antibody was first carried out. Then the ligand, anti-EGF antibody was diluted to 2µg/ml in 10mM sodium acetate buffer with the optimum pH and injected at a flow rate of 5µl/mL for 30 minutes. Finally, 35µl of 1.0M ethanolamine-HCl pH 8.5 was injected to deactivate and remove of any non-specifically bound antibody in sensor chip.

2.3 Standard curve construction and sample injection
Prior to sample loading, the anti-EGF coupled chip was preconditioned with TBS buffer by running the automatic prime feature in the wizard for 6 minutes. Standard rhEGF with concentration ranging from 50 to 250 ng/ml and samples were prepared in TBS buffer and stored on ice. A 5 µl of standard solutions and samples were injected across the chip surface. All injections were carried out at a flowrate of 5 µl/min. The measurement of the binding response was taken 100 s after the end of the injection. The chip surface was later regenerated by injection of 10 µl of regeneration solution (10 mM glycine buffer, pH2.5).

2.4 Assay performance evaluation
With regards to the performance evaluation of the developed SPR rhEGF quantification assay, several general validation parameters like precision, accuracy, linearity, detection limit and quantification limit were determined accordingly. These parameters were also compared with the established method of ELISA. The studies were performed using standard rhEGF sample at concentration range of 25 to 250 ng/mL. In determining the accuracy of the assay, E. coli crude
extract with known concentration of rhEGF were used. All experiments were performed in triplicate.

3 Result

3.1 Preparation of sensor surfaces

The antibody (anti-EGF polyclonal) was successfully amine coupled onto the CM5 surface flow cell 2 (Fc2) through the NHS/EDC chemistry as shown in Fig.1. At first pH scouting for immobilization of antibody was performed and pH 4.5 was found to be optimum (Fig.2). Ligand density immobilized at pH 4.5 was at around 18700 RU. The immobilization level was maximized in order to enable lower analyte concentration measurement. For specificity study, immobilized BSA was employed as a control reference surface in flow cell 1 (Fc1). The immobilization level was around 16000 – 17 000 RU. Samples were injected across Fc1 and 2 in sequence during the analysis. Automatic subtraction of any cross reactivity of assay constituents with the BSA surfaces was carried out. The reaction was monitored over time through the sensorgram and the difference of RU between the baseline (set at 30 s before sample injection) and the final response (100 s after end of injection) was determined automatically. It is denoted as Fc 2-1. The typical sensorgram in one analysis cycle is shown in Fig.3.

has completed, ethanolamine was injected to remove any remaining active esters and conditioning with the regeneration solution to be used (not shown).

Fig.1 The sensorgram generated during the preparation of the sensor surfaces. Firstly as the running buffer flowed through the surface, a reference baseline was recorded. Next once the activating solution, EDC-NHS was injected the signal rose to reach point A. Then the signal again dropped as the pulse which has high refractive index passed through. At point B, the anti-EGF polyclonal antibody was injected, so the increment of the RU can be observed here. Lastly once the enrichment

Fig.2 The result of the pH scouting for immobilization of the antibody. pH 4.5 is shown to be the optimum immobilization pH as depicted in the bell-shaped curve.

Fig.3 The sensorgram obtained for one analysis cycle of rhEGF concentration measurement by the
prepared sensor chip. The overall event during the analysis can be monitored by the real-time biosensor. (A) is the report point referred as the reference baseline (B) the injection of rhEGF-containing E. coli crude extract sample and followed by the association of analyte to the antibody, (C) the end of injection and replacement by the continuous buffer, (D) the end point for measuring bound rhEGF, (E) the starting of regeneration followed by the dissociation of the ligand-analyte complex and (E) the end of regeneration and the surface is ready for the next analysis cycle.

3.2 Regeneration of the anti-EGF surfaces
The regeneration scouting tool in the BIAcore 3000 control software (ver. 4.1) was used to obtain the optimum regeneration condition (Table 1). The commonly used regeneration solutions are 10-100 mM HCl and 10mM glycine, pH 1.7-2.2. In our case, the optimum regeneration of anti-EGF antibody was achieved by injecting 10 µl of 10 mM glycine-HCl buffer, pH 2.5. In order to check the baseline stability of the sensor chip surface, surface performance test tool was performed with 180 ng/ml rhEGF standard solution as analyte. Baseline stability after each cycle is an important criterion in assay reproducibility. The results of the surface performance test are displayed as a trend diagrams (Fig.4). The absolute baseline level (left) and the analyte response (right) are shown as a function of cycle number for the flow cell 2-1 (Fc2-1) (Fig.4B).

3.3 Standard curve construction
The rhEGF calibration curve was generated by injecting an increasing concentration of standard ranging from 25 to 250 ng/ml constituted in TBS (Fig.5). Flow rate of 5 µL/min was maintained throughout the measurement to ensure that the assay sensitivity is not being compromise. In generating the standard curve, 4 parameter fit function was used for the data regression performed with BIAevaluation software. The calibration curves for 3 days are depicted in Fig.6. Percent coefficient of variance (% CV) of the response unit values were determined to express the intra-precision of the data. The analysis for the concentration range of 25 to 400 ng/mL for the 3 days is shown in Table 1. The result suggests that the assay has a very high repeatability as the entire % CV obtained are less than 2 excluding the result of 25 ng/mL concentration on the 1st day which is 3.4. This is due to the instability of the chip during the early sample application. At the same time, it is also observed that the chip reaches saturation point at 250 ng/mL concentration as the response values remain constant afterwards. On the other hand for the inter-precision determination of the data, % CV was again calculated based on the response values for the 3 days (Table 2). Less than 4 % CV was derived for standards with concentration of more than 200 ng/mL. The % CV is high for the concentration range of 25 to 150 ng/mL (13% to 14%). This is the consequence of low response value obtained during the 1st day measurement compare with the next 2 days. The low stability of chip during early sample application and the degradation of the rhEGF standard protein while being kept in the autosampler might contribute to such an observation. The detection limit (the lowest amount of analyte in a sample which can be detected but not necessarily being quantitated exactly) is calculated by the following approach:

\[
DL = \frac{3.3 \sigma}{S} \quad (1)
\]

where \(\sigma\) = the standard deviation of the blank
\(S\) = the slope of the calibration curve

Meanwhile for the quantification limit described as the lowest amount of analyte in a sample which can be quantitated with suitable precision and accuracy, is also being determined by the above equation but with the numerical constant, 3.3 being replaced with 10 instead. The detection limit and quantification limit are about 2.6 and 8 ng/mL, respectively. The accuracy of the assay was evaluated by including the E. coli crude extract samples with predetermined rhEGF concentration by the established ELISA method. As suggested by International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [25], accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample. The analysis consisted of 9 determinations, covering triplicate analysis for each of the 3 different concentrations. The % recovery data ranges from 97.6 to 108.8 (Table 3). The overall summary of our assay parameter is outlined in Table 4.
Table 1 The intra-assay precision data expressed as % C.V. of the triplicate RU reading for each standard rhEGF concentration measured.

<table>
<thead>
<tr>
<th>Intra-assay precision</th>
<th>Concentration (ng/mL)</th>
<th>25</th>
<th>50</th>
<th>150</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>350</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RU</td>
<td></td>
<td>20.7</td>
<td>45.7</td>
<td>71.8</td>
<td>92.7</td>
<td>97.5</td>
<td>95.0</td>
<td>98.5</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>19.4</td>
<td>46.9</td>
<td>70.4</td>
<td>94.7</td>
<td>97.2</td>
<td>94.8</td>
<td>98.6</td>
<td>98.5</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.7</td>
<td>0.7</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>0.5</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>3.4</td>
<td>1.4</td>
<td>1.2</td>
<td>1.1</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RU</td>
<td></td>
<td>26.0</td>
<td>55.5</td>
<td>93.0</td>
<td>98.5</td>
<td>99.3</td>
<td>100.2</td>
<td>104.2</td>
<td>103.9</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>26.7</td>
<td>56.6</td>
<td>92.5</td>
<td>98.5</td>
<td>99.3</td>
<td>99.8</td>
<td>104.7</td>
<td>103.9</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.5</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>1.8</td>
<td>1.0</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RU</td>
<td></td>
<td>24.6</td>
<td>58.9</td>
<td>89.6</td>
<td>93.8</td>
<td>95.1</td>
<td>95.8</td>
<td>98.1</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td>24.6</td>
<td>57.5</td>
<td>89.6</td>
<td>94.2</td>
<td>95.1</td>
<td>96.0</td>
<td>98.6</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.3</td>
<td>0.8</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>% CV</td>
<td>1.2</td>
<td>1.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>

1 2 EGF 1.00 6 30683.7 130.5 100
1 2 10 mM Glycine, pH2.5 0.90 10 30481.1 49.9 36
1 2 EGF 1.00 6 30541.2 163.0 100
1 2 10 mM Glycine, pH2.5 1.00 10 3075.0 32 -2
1 2 EGF 1.00 6 30023.9 127.9 100
1 2 10 mM Glycine, pH2.5 1.00 10 3059.9 -15.0 -12
1 2 EGF 1.00 6 30483.0 123.1 100
1 2 10 mM Glycine, pH2.5 1.00 10 3040.7 -19.2 -18

B) Baseline Level

Fig.4 The panel of sensorgram report file for the surface performance test shown in tabulated form (A) and trend diagram (B). The regeneration was achieved by injecting 10 µl of 10 mM glycine-HCl buffer, pH 2.5.
Fig. 5 The sensorgrams of the calibration curve. The difference in response was reported 30 s prior injection and 100 s after end of injections. Different concentration is denoted by the varying colour.

Table 2 The inter-assay precision data given as % C.V. for the quantification of rhEGF in *E. coli* crude extract.

<table>
<thead>
<tr>
<th>Inter-assay precision</th>
<th>Concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Day 1 (RU)</td>
<td>19.9</td>
</tr>
<tr>
<td>Day 2 (RU)</td>
<td>26.5</td>
</tr>
<tr>
<td>Day 3 (RU)</td>
<td>24.8</td>
</tr>
<tr>
<td>Mean</td>
<td>23.7</td>
</tr>
<tr>
<td>SD</td>
<td>3.4</td>
</tr>
<tr>
<td>% CV</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Table 3 The accuracy evaluation data of the assay.

<table>
<thead>
<tr>
<th>Accuracy</th>
<th>Mean rhEGF concentration determined by ELISA (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14.65</td>
</tr>
<tr>
<td>Sample</td>
<td>15.38</td>
</tr>
<tr>
<td></td>
<td>16.01</td>
</tr>
<tr>
<td></td>
<td>16.43</td>
</tr>
<tr>
<td>Mean</td>
<td>15.94</td>
</tr>
<tr>
<td>SD</td>
<td>0.53</td>
</tr>
<tr>
<td>% CV</td>
<td>3.32</td>
</tr>
<tr>
<td>% Recovery</td>
<td>108.8</td>
</tr>
</tbody>
</table>
Table 4 The summary of the assay parameter for the detection of rhEGF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>4 parameter curve fit for concentration range from 25 – 250 ng/mL</td>
</tr>
<tr>
<td>Accuracy</td>
<td>&lt; 10 % recovery</td>
</tr>
<tr>
<td>Precision</td>
<td></td>
</tr>
<tr>
<td>Intra-assay</td>
<td>&lt; 3.4 % C.V.</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>&lt; 14 % C.V.</td>
</tr>
<tr>
<td>Detection limit</td>
<td>2.6 ng/mL</td>
</tr>
<tr>
<td>Quantification limit</td>
<td>8.0 ng/mL</td>
</tr>
</tbody>
</table>

4 Discussion

Anti-EGF antibody which is commercially available with high purity is immobilized on the dextran surface of sensor chip to act as a receptor for the target analyte, hEGF. The rabbit produced antibody recognizes the targeted analyte, hEGF protein (~6.8 kDa). Even though the analyte hEGF consisting an additional 8 amino acids FLAG tag sequence for the ease of purification, anti-EGF antibody is preferred over the versatile M2 anti-FLAG antibody for the immobilization. This approach allows the direct quantification based on the binding with EGF portion of the fusion protein alone. Hence, the EGF protein can still being quantified if in the case where the FLAG sequence of the protein is cleaved off. In fact, Mersich and Jungbauer (2007) [26] have reported a generic method for quantification of FLAG-tagged fusion proteins using this biosensor technology.

The amount of antibody being immobilized onto the chip is important to ensure rapid binding of analyte and favoring the mass-transport limited binding between receptor and the target ligand [19,26]. Here the dextran surfaces was activated by amine coupling chemistry with the addition of mixture of 0.2 M EDC and 0.05 M NHS reagents to form succinimide esters that will react with primary amine groups in protein. Contact time and concentration of ligand basically are the 2 major factors influencing the immobilization level [24]. With the recommended flow rate of 5 µL/min we have performed preliminary optimization of contact time during the activation and immobilization stage. For instance we increased the activation time from 7 to 10 min and from 12 to 30 min for immobilization to finally achieve about 18700 RU for the immobilization of polyclonal anti-EGF. Irrespective of ligand molecular weight, this result is comparable with other reports [17,20,23,26] in which the achieved RU is ranging from 10000 to 20000. However the ligand concentration for immobilization RU improvement was not increased due to the presence of preservative (sodium azide) in the antibody stock. Additionally instead of having blank surface as reference [27,28] we employed BSA in flow cell 1 to obtain the RU caused by protein-protein unspecific binding. The protein-protein unspecific binding might occur between impurities in sample and the immobilized ligand in flow cell 2.

The objective in regeneration is to break the non-covalent bonds between the antibody paratope and the antigen epitope without permanently affecting the antibody-antigen binding characteristics [29]. Here the regeneration was achieved with regeneration solution (10 mM glycine-HCl buffer, pH 2.5). The stability is demonstrated by the surface performance test in which the baseline decreases while the response is constant (Fig.4). In an ideal situation, both the baseline and response should be constant between cycles. A decreasing baseline is generally not a problem if the response trend is constant. This indicates that the regeneration is complete and no material is accumulating on the surface. Furthermore, the ligand remains intact and its EGF-binding capacity is also maintained within these cycles.

The assay response to the increment of EGF concentration is non-linear. With that, 4 Parameter Fit (used to model complex curves where the data does not show a linear trend) curve fitting model has come into place to generate the standard curve [30] as shown in Fig.6. The operational range examined extended from the concentrations of 25 to 250 ng/mL.
ng/mL. It is noted that the sensitivity of assay is 1000-fold lower than the existing ELISA method (quantification range: 3.9 to 250 pg/mL). Basically we have expected this result since ng and higher quantity is the common threshold reported in SPR quantification [23,26,31,32]. An important parameter, precision (inclusive of intra-assay and inter-assay) evaluated are within the acceptance criteria (<20% CV) and highly comparable with the established ELISA assay (Tables 1 and 2). It should be pointed out that the sensitivity of this assay is determined by the limit of quantification calculated which is 8 ng/mL. Quantification limit is chosen as the determinant of sensitivity instead of detection limit as the value is consistent and reproducible through multiple assays. So any value lower than 8 ng/mL is reported as ‘below the limit of quantitation’ [31]. Another important parameter, accuracy, defined as the closeness of agreement between the value of accepted reference value and value found was demonstrated by running the 3 known concentrations. The recovery for all samples are within 10% of the target concentration (Table 3).

Fig.6

A) Day 1

B) Day 2

C) Day 3

Fig.6 The calibration curves for determination of rhEGF concentration on the three days fitted using the 4 parameter fit function.
The main concern here is the low maximum RU reached during the analysis of small 6.8 kDa analyte (rhEGF). From the data obtained, the sensor surface reaches early saturation level, at only 100 RU. This phenomenon also has been encountered by Myszka [21] in which at saturating concentration for the small analyte, methylsulfonamide (95Da) only 144 RU is reached. Gao et al. [6] highlighted the difficulty in measuring small analytes such as vitamins (<1000 Da) as they generate only small changes in mass. In the meantime for our case, the potential binding capacity might have been affected due to partial loss of ligand activity, inactive starting material (ligand) or the antibody itself being immobilized through the antigen binding site as encountered by Pflegerl et al [20].

Nevertheless this limitation is compensated by several advantages over the existing methods like ELISA and densitometry in that it is fast, avoiding the time consuming incubation and washing step. In the case of ELISA, up to 5 hours is required in getting the test outcome while with our simple SPR method only about 7 minutes is needed per analysis cycle. In economic point of view, sandwich ELISA method can be expensive and tedious as it requires two antibody-epitope systems. The reusability of the sensor chip indeed giving an edge over ELISA method as the single-use antibody surface is usually being discarded once being used. On the other hand, in SPR method, the target molecule do not required the use of enzymatic or radioisotope labelling [6,17,33]. In our procedure, only small volume which is 35 µL of sample is needed per injection during the analysis by SPR. Such benefits are further boosted by the automated running of analysis from sample injection to data analysis and reporting through the wizard drive software. On the other hand, if densitometry method is used the standard protein applied in the SDS-PAGE and Western blot should be the same with the target protein in order to yield an accurate result [26] and we could not fulfil this criterion as our standard protein, rhEGF is distinct from the FLAG-tagged rhEGF. However most importantly the applicability of this assay is justified by its capability to detect rhEGF in E. coli crude extract sample with high accuracy (within 10% recovery of the target) and precision (less than 3.4% CV).

Future works will be focused on the improvement of the sensitivity and data quality as outlined by the previous researcher. As an example, Myszka [21] has proposed the usage of the advanced Biacore S51 instrument, designed with a change related to the introduction of hydrodynamic-addressing flow cell for an improved sensitivity platform. Interestingly, some researchers reported the running of SPR through an inhibition mode principle. In this circumstance, the target analyte is being immobilized onto the surface while the ligand (larger molecular mass) is added into the sample. This means that the higher the analyte concentration in the sample, the higher the inhibition level and lower response. In addition, an antibody-antigen-antibody sandwich format can also be applied to enhance the response of the original method [17,19,23]. Here the secondary reagent is crucial in increasing the response specificity, thus overcoming any non-specific and background noise signal. Lastly, optimization of the flow rate with probably as low as 3 µL/min could increase the interaction time between ligand and analyte thus producing higher magnitude of signal during analysis [17].

5 Conclusion
Given the assay performance: accuracy, repeatability, intermediate precision, limit of detection, quantification limit and measurement range, we have demonstrated the ability of the SPR system to quantify our target molecule, the ~6.8kDa FLAG-tagged recombinant human epidermal growth factor by immobilizing the polyclonal anti-EGF antibody onto the biosensor chip surfaces. The measured sample rhEGF concentration value lay significantly above the quantification limit allowing the quantification to take place. The developed SPR (Biacore 3000) method has showcased a full repertoire of practicality and benefits to make it as a powerful quantification method of rhEGF. With that the performance of the upcoming rhEGF purification process right from the crude extraction level up to the final purification stage would be able to be monitored.

Acknowledgements
This study was supported by the IRPA grant (03-02-04-0562-SR0008/05-3) and ScienceFund grant (02-01-04-SF0895) from the Ministry of Science, Technology and the Innovation, Malaysia (MOSTI).

References:


[25] ICH Topic Q 2 (R1), Validation of Analytical Procedures: Text and Methodology


