Quantification of antibody production of individual hybridoma cells by surface plasmon resonance imaging

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A B S T R A C T
Surface plasmon resonance imaging (SPRi) is most frequently used for the label-free measurement of biomolecular interactions. Here we explore the potential of SPRi to measure antibody production of individual hybridoma cells. As a model system, cells from a hybridoma, producing monoclonal antibodies recognizing epithelial cell adhesion molecule (EpCAM), were used. Recombinant human EpCAM protein was immobilized on an SPR sensor and hybridoma cells were introduced into an IBIS MX96 SPR imager and the SPRi response was followed for 10 h. SPRi responses were detected on the spots of the sensor only where ligands of the produced antibody were present. By measuring the SPRi signals on individual cells the antibody production of the individual cells was measured and production rates were calculated. For 53 single EpCAM hybridoma cells the production ranged from 0.16 to 11.95 pg (mean 2.96 pg per cell, SD 2.51) over a period of 10 h. Antibody excretion per cell per hour ranged from 0.02 to 1.19 pg (mean 0.30, SD 0.25). Here we demonstrate for the first time that antibody production of individual cells can be measured and quantified by SPRi, opening a new avenue for measuring excretion products of individual cells.

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Surface plasmon resonance imaging (SPRi) traditionally is used for the label-free detection of molecular interactions between, for example, antibodies and their ligands [1]. As SPR know-how and microfluidics evolved in the years after the turn of the millennium, various studies have reported the use of SPRi to study cellular responses and interactions. Studies have been performed in which single cells were cultured on an SPR sensor surface and local regions of interest were placed around these cells to monitor their responses to various stimuli [2]. Similarly peripheral blood basophil activation was evaluated using SPRi. In this study basophils were bound to the sensor surface using an anti-basophil antibody, after which the cells were stimulated with various antigens and morphological changes were detected by SPRi. The study concluded that these monitored changes coincided with side-by-side experiments that tested the cells for histamine release upon stimulation [3]. The study, however, did not monitor or quantify the histamine release with SPRi.

When it comes to quantification of functional protein concentration of samples, SPR has turned out to be a suitable method. SPR-based setups were shown to be able to accurately determine analyte concentrations both in offline [4,5] and in at-line settings [6]. But none of these studies reported the ability to quantify excretion at the single-cell level. Recently we have demonstrated the ability to detect the specific binding of cells to antibodies immobilized on a sensor surface by SPRi [7]. In these experiments typical SPR curves were obtained from which the initial signals can be attributed to sedimentation of the cells on the sensor surface followed by signals attributed to the specific binding response between cell surface antigens and their ligands immobilized on the sensor. The magnitude of the SPR responses was proportional to the antigen density on the surface of the cell. From these studies we postulated that it should be possible to measure products excreted by cells using SPRi.

Previously performed studies have shown that SPRi is a suitable technique for analyzing protein excretion by cells. Peterson et al. [8] deposited smooth muscle cells on a fibronectin-covered sensor and quantified their protein deposition as a function of cell density and distance from the cell periphery. However, in this study quantification was not attempted on single cells [8]. Milgram et al. demonstrated real-time monitoring of antibody excretion by hybridoma cells [9]. In this study cells were allowed to sediment nonspecifically onto a biosensor after which their production of anti-hen egg lysozyme antibody was followed. The study proved
that indeed SPRi was capable of following in real time the excretion of the expected antibodies and that it was a much easier and quicker method to confirm antibody production compared to more traditional label-based techniques [9]. Traditionally detection and quantification of single hybridoma cell excretion is a complicated procedure [10–12]. Recently a new fluorescence-based method was introduced, which uses metal-enhanced fluorescence. Although the study showed that the technique was able to be used in real time, there was no quantification done on the excretion [13]. In this paper we demonstrate the feasibility of monitoring antibody production of single hybridoma cells. To investigate this an IBIS MX96 SPR imager was modified so that cellular production could be monitored for prolonged periods of time at 37 °C. As a model system cells from a hybridoma cell line producing VU1D9, an immunoglobulin G1 (IgG1) monoclonal antibody recognizing epithelial cell adhesion molecule (EpCAM), were used. The relatively simple method introduced in this paper allowed us to monitor, track, and quantify the excretion of antibodies label free and in real time from individual cells by SPRi.

Materials and methods

SPRi

For SPRi measurements the IBIS MX96 was used (IBIS Technologies B.V., Enschede, The Netherlands). The MX96 combines biosensing with live real-time imaging. It uses a 2-megapixel CCD camera to grab images of the sensor surface; the size of each pixel is 5.5 × 5.5 μm. The fluids in the IBIS MX96 were designed as such that the sample does not pass any valve. The images of the sensor during the cell sedimentation stage were made using a custom-designed image grabber. The standard 100-nm high-flow cell in the IBIS MX96 was replaced with a 300-μm high-flow cell to obtain better conditions for the cells to survive for a longer period of time, because more cell culture medium is available in the flow cell as opposed to the regular-height flow cell. In addition it provides a better sample homogeneity in the flow cell upon injection of the cells. The output of the machine is given in resonance units (RU) and output calibration was set up as such that 1 RU corresponds to 1 pg of bound protein per square millimeter.

Continuous-flow microfluidic spotter

For immobilizing ligands on the sensor surfaces a continuous-flow microfluidic (CFM) spotter was used (Wasatch Microfluidics LLC, Salt Lake City, UT, USA) [14]. Ligand immobilization buffer was used to prime the CFM system and to dilute the desired ligands. The immobilization protocol lasted 60 min. The CFM spotter has the ability to spot up to 48 different ligands in a 6 × 8 array configuration with spot sizes of 500 × 830 μm onto the sensor simultaneously under back-and-forth confined flow. The confined back-and-forth flow increases the efficiency of the spotting and avoids the risk of evaporation for contact and noncontact droplet-based spotting methods.

SPR sensors

Easy2Spot preactivated G-type SensEye sensors (Ssens B.V., Enschede, The Netherlands) were used as SPR sensor surfaces. The sensors have a 100-nm hydrogel-like layer, which enables higher capacity coupling of ligands in the evanescent field and gives the ligands a level of mobility approaching much more in vivo-like circumstances. The sensors are preactivated for easy immobilization without ethyl(dimethylaminopropyl) carbodiimide N-hydroxysuccinimide activation required by the user.

**Antibodies and recombinant proteins**

Human serum albumin (HSA) antibody was acquired from Sigma–Aldrich Corp. (St. Louis, MO, USA). Recombinant human EpCAM/CD326 protein (rhEpCAM) and recombinant human HER2 protein (rhHER2) were acquired from ACR0 Biosystems (Bethesda, MD, USA). IgG1, IgG2a, IgG2b, and IgG3 antibodies were acquired from Rockland Immunochemicals (Limerick, PA, USA).

**Hybridoma cells**

Hybridoma cells producing EpCAM (IgG1, VU1D9) antibodies were used in the experiments (generously provided for these experiments by Immunicon Corp., Huntington Valley, PA, USA). The cell line was cultivated in Iscove's modified Dulbecco's medium (IMDM; Life Technologies Corp., Grand Island, NY, USA) with added 5% fetal calf serum, penicillin, streptomycin, and l-glutamine (Sigma–Aldrich). The cells grow in suspension and can be harvested without the use of trypsin or EDTA. The cells were grown in T75 cultivation flasks in 25 ml of complete cell medium at 37 °C and 5% CO₂.

**Sensor deactivation agent**

A 1% bovine serum albumin solution (BSA; Sigma–Aldrich) in sodium acetate immobilization buffer was used as a deactivation agent. A stock solution of 2-aminoethanol (MP Biomedicals LLC, Illkirch, France) was used to create a 100 mM 2-aminoethanol solution with a pH of 8 and was used as an extra sensor deactivation step after the initial BSA deactivation.

**Ligand immobilization buffer**

A 10 mM solution of immobilization buffer at pH 4.5 was made using anhydrous sodium acetate (Sigma–Aldrich) and acetic acid (Merck Schuchardt OHG, Hohenbrunn, Germany). First a 0.2 M stock solution was made of both components, then from these stock solutions 1.93 parts of sodium acetate was mixed with 3.07 parts of acetic acid, and finally 95 parts of ultrapure demineralized water was added. The pH was checked and if needed adjusted to pH 4.5.

**System buffer**

IMDM complete cell culture medium was used as a system buffer to enable cell survival for several hours inside the IBIS MX96 and to minimize bulk shift differences.

**Imaging of cell excretion**

The custom image grabber software used with the IBIS MX96 captured images at intervals of 60 s throughout the experiment to analyze the cell excretion process. First an image containing cells on the surface at the initiation of the experiment was selected and an image at the end of the experiment was selected where the cells were presumed to produce their product. Images were taken at the same angle of incidence to enable differential image analysis using Adobe Photoshop (version CS6); with this analysis the “before analysis” image is subtracted from the “after analysis” image leaving only the anticipated products, that were excreted by the cells, visible.
Results

Verification of antibody production by hybridoma cells

The supernatant of the cell culture of the hybridoma cell line was collected. A sensor was made that had rhEpCAM protein immobilized on its surface. Immobilization in the CMF spotter was performed for 60 min. The rhEpCAM protein is the target protein of EpCAM antibodies and as such should specifically bind to it. Additionally anti-IgG1, -2a, -2b, and -3 spots were made, which should show to which isotype the produced antibody belongs, and rhHER2 protein was immobilized as a negative control, as to this protein only HER2 antibodies should bind and EpCAM antibodies should not. The supernatant of the EpCAM-producing hybridoma was flushed over the sensor and indeed showed reactivity only on the spots covered with rhEpCAM and anti-IgG1 (see Fig. 1). These experiments proved that the products excreted by the cells indeed are anti-EpCAM antibodies of the IgG1 subtype. The antibodies show specificity only with their corresponding target recombinant protein and do not show any reactivity with the other recombinant protein.

Real-time SPRi measurement of EpCAM antibody production by hybridoma cells

To determine the feasibility of real-time detection of antibody excretion by EpCAM-producing hybridoma cells, a sensor was spotted with rhEpCAM (10 μg/ml) and anti-HSA (5 μg/ml) spots. Immobilization in the CMF spotter was performed for 60 min. After injection of the cells they were allowed to sediment, after which the unbound cells were washed off. The remaining bound cells were tracked in real time for 60 h without any flow. Regions of interest (ROIs) were placed in such a way that they covered the immobilized ligand spots (the whole spot). In addition smaller ROIs were placed over single cells. This was done so that after the completion of the second part of the experiment differences in antibody production of the individual cells could be observed in the sensorgram, assuming that individual cells within the same population could have excretion rate differences. Fig. 2 shows the sensorgram of the tracking of the hybridoma cells over a 60-h period after their initial capturing. Five sensorgrams are shown in Fig. 2, one representing a spot with anti-HSA showing a nearly flat line, one representing another spot with rhEpCAM showing a gradual increase in the SPRi signal, and the other three representing the smaller ROIs around three individual hybridoma cells on a spot covered with rhEpCAM. ROIs that were placed over individual cells show a difference in the excretion amounts of the respective individual cells. The larger SPRi signal from the individual cells compared to the whole rhEpCAM-covered spot can be explained by the fact that the large ROI spot has a relatively high amount of surface area that was not affected by the excretion of proteins, whereas the small individual ROIs were almost entirely “filled in” with the excreted product.

Quantification of antibody production by individual hybridoma cells by real-time SPRi

To calculate the total excreted protein per single cell, the cells were allowed to sediment onto the sensor surface and attach specifically to their ligands. For quantification it is important that the SPR signal influence of cell sedimentation and interaction with the sensor surface is accounted for and subtracted from the expected excretion signal. When cells sediment on the sensor surface they cause a shift in the SPR angle, just like a specific binding event would between proteins; that is why this influence on the SPR signal needs to be subtracted from the anticipated antibody production signal. Cell sedimentation and settling is complete within the first 20 min, which can be distinguished as a plateau in the SPR sensorgram (Supplementary Fig. 1). The interpretation is that a steady state is reached and that the cells are no longer

![Fig. 1. Culture supernatant of EpCAM hybridoma cells flowed over a sensor on which rhEpCAM; anti-IgG1, -2a, -2b, and -3; and rhHER2 were immobilized. The supernatant interacted only with the rEpCAM and the IgG1 spots, proving that the cells indeed produced anti-EpCAM IgG1 antibodies.](image-url)
sedimenting towards the surface. After this the measurement was stopped and the excess unbound cells were washed off. Smaller ROIs with consistent sizes of 110 x 110 µm (20 x 20 pixels) (the halos of excreted protein by single cells do not exceed 20 x 20 pixels, hence the choice of this size) were then placed as illustrated in Fig. 3A1 and A2. Cells that were too clustered to single out were ignored. Following this a so-called system “prep” was performed during which all the ROI signals were measured and then zeroed, meaning that any contribution of cells within these ROIs is subtracted. This enabled the measurement of SPRi signals attributed

Fig. 2. Sensorgram showing the single-cell production of anti-EpCAM by VU1D9 cells. The rhEpCAM is able to capture the cell specifically and bind the anti-EpCAM product made by the hybridoma cell.

Fig. 3. (A1) Schematic illustration of the ROI setup. The white dot is the hybridoma cell and the gray field diminishing in intensity around the cell is the anticipated excreted product of that cell. ROIs are placed centered over the cell and have a size of 110 x 110 µm (20 x 20 pixels). (A2) An SPRi camera image of the actual placement of an ROI on a cell prior to the initiation of the real-time following of cellular excretion. (B) Actual image of EpCAM hybridoma cells after sedimentation. (C) The same cells shown after a 60-h SPRi experiment; the halos seen around the cells are the anti-EpCAM antibodies that were produced by the cell line. (D) Image showing the difference between the images in (B) and (C); only the produced antibody is shown, whereas the rest of the spot remains black, as there were no changes on those surfaces of the spot.
to protein excretion by the cells without the “interference” of a cell signal. The experiment was then restarted and the cells were followed for 10 h. The spikes in the curves in the beginning are most probably attributable to the smaller sized ROIs, being able to detect fine movements and settling of the cells that just underwent a wash that caused them to get unsettled again. We see that after the first hour the sensorgram smoothens (see Fig. 4), implying that the cells have now fully settled again. After the completion of the experiment, the measured RU signal represents the amount of bound protein in picograms per square millimeter. However, since ROIs of a much smaller size were used, a correction for the size of the ROI was performed. One pixel in the MX96 system has a size of $5.5 \times 5.5 \mu m$, resulting in a size of $1.21 \times 10^{-2} \text{mm}^2$ for an ROI of $110 \times 110 \mu m$. This resulted in a correction factor of $1/(1.21 \times 10^{-2}) = 82.6$. In this experiment the cells were monitored for 10 h. The RU value, which was measured after 10 h, was then divided by the correction factor. The value resulting from that calculation was the actual excreted amount of antibody by the hybridoma cell(s) present in the $110 \times 110-\mu m$ ROI within the 10-h time frame.

**Imaging of cell excretion**

Fig. 3 shows images of the cells on one of the 48 spots on a SPR sensor covered with rhEpCAM, which were captured using the camera that was also used for the capturing of the SPR signals. Fig. 3B shows the cells after sedimentation and binding to the sensor and Fig. 3C shows the same cells after being in the SPRi instrument for 60 h. At this time EpCAM antibodies excreted by the cells show up as so-called “halos” around the original cell location. Fig. 3D shows the differential image of the first two images, showing only the excreted product. Clear differences in antibody production between some of the cells can be seen. This is further illustrated in Fig. 4 showing the SPRi sensorgram of seven VU1D9 cells producing different amounts of product in their respective $110 \times 110-\mu m$ ROIs. The production of antibody from 53 individual hybridoma cells was measured and the results are shown in Table 1.

**Discussion**

The ability of SPRi to detect excretion products of cells was explored. We decided to use hybridoma cells as a model system for cell excretion since these types of cells are engineered to produce monoclonal antibodies and are relatively easy to maintain in culture. First, the supernatant of the cell culture of the hybridoma cells producing the VU1D9 EpCAM antibody was tested to verify that the excreted monoclonal antibodies could be detected specifically without any cross-reactivity by SPRi. The supernatant of the cell culture was passed over a sensor with immobilized rhEpCAM, rhHER2, and antibodies recognizing Ig subtypes. This confirmed that antibodies of the IgG1 subclass recognizing rhEpCAM were produced.

After this confirmation the VU1D9 hybridoma cells were injected in the IBIS MX96 SPR imager and followed overnight by taking images of the sensor spots and the measurement of SPRi signals. During the measurements the cells either interacted with the rhEpCAM or excreted a product that interacted with the rhEpCAM on the sensor spots. No interaction was measured on the sensor spots covered with other ligands, indicating that the observed response was due to the production of antibodies that were actively being excreted. To prove that individual cells that actually produce VU1D9 antibody can be detected we decided to reduce the number of cells injected into the IBIS MX96 SPR imager, from approximately $1 \times 10^6$ cells to $2.5 \times 10^5$ cells, to be able to easily distinguish single cells on the sensor spots. Smaller ROIs were placed around individual cells, as we expected that this would give higher response rates as illustrated in Fig. 2. It has to be noted however, that because the experiments were done over such a long period of time the cells could have contributed to the increased signal in a different way. The cells might have settled in and continuously interacted with the sensor surface and the ligands; additionally the cells might have been dividing, causing an increase in the sensorgram. We, however, have seen that cell division did not influence the sensorgram in this particular experiment. In a separate experiment we noticed cell division and the sensorgram showed a sudden drastically higher slope (see Fig. 5), which was
In the antibody production quantification experiments, the differential image as bright round white dots around the initial deposited cells. In these experiments, however, this was not seen, hence our conclusion that the measurements were not influenced by cell division. During the loading of the cells into the SPR imager, we saw only an initial increase in the slope of the sensorgram, which then transitioned into a plateau (see Supplementary Fig. 1), indicating that the cells were no longer actively sedimenting toward the sensor surface. Also seen in Supplementary Fig. 1 is the fact that approximately at the 12-min mark the sensorgram increases slightly again; this in our opinion is indicative of the immediate excretion and binding of small amounts of protein. Additionally noteworthy is the fact that there was no nonspecificity seen on the blank sensor surface with any of the components of the cell sample (i.e., cell products, complete cell culture medium). The mere presence of cells thus does not interfere with the data by interacting with the sensor surface or the immobilized ligands. The introduced method can be used to quantify the antibody production of individual hybridoma cells and by doing so determine the heterogeneity of the antibody production. For the hybridoma cell line producing the VU1D9 antibody recognizing EpCAM we showed a production ranging from 0.02 to 1.19 pg/h (mean 0.3, SD 0.2); the average values agree with previous findings in the literature relating to antibody production by hybridoma cells per cell per hour [15–18]. Using this knowledge the growth conditions can be optimized to arrive at a more uniform and higher antibody production. This is especially of importance, for example, for the production of therapeutic antibodies. Antibody-producing cells can be monitored in real time over the course of a few hours to identify the best antibody-producing cell with the highest amount of production. Preferably one would like to recover/isolate the highest producing cells from the measurement to obtain high-producing cell clones. Potentially the IBIS MX96 SPR imager can be modified in such a way that cells that are present in specific regions of the sensor can be isolated, after which they are collected and deposited in a vial containing culture medium for the sake of expanding the cells with the highest production rate.

Here we have demonstrated that the production of antibodies by individual hybridoma cells can be measured and quantified in real time using SPRi. This opens a new avenue to measure products excreted by cells, thereby increasing our understanding of cellular processes. One can envision the simultaneous measurement of a variety of products excreted by cells using SPRi, such as hormones, neurotransmitters, cytokines, and exosomes.

**Conclusion**

Our experiments have shown that it is possible to detect and quantify cell-excreted products using SPRi. EpCAM hybridoma cells were capable of excreting their antibodies while they were bound on an SPRi sensor. The recombinant protein ligands on the sensor enabled the binding of the cells and the subsequent detection of their excreted product. By placing single-cell ROIs after the initial binding of the cells we were able to distinguish individual cell differences in excretion activity. By further refining this method we were able to quantify the individual cell product excretion. We have managed to perform quantitative excretion analysis on 53 single EpCAM hybridoma cells and have calculated an average production rate of 0.30 pg per cell per hour (SD 0.25), with a maximum production rate of 1.19 pg per cell per hour. Because the SPRi apparatus used combines imaging with sensing we were able to use sensor surface images created during the measurement to compare the results with the sensorgrams. The images show that the hybridoma cells were surrounded by halos of excreted product of varying size, confirming the single-cell responses seen in the sensorgrams and the notion that individual cells might have differences in production rates even though they stem from one and the same clone.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.06.018.

References


Fig. 5. SPRi sensogram showing cell division and subsequent cell removal. At $t = 266$ min one cell is clearly seen in the highlighted area, which represents the approximate area of the ROI that was placed on the sensor. At $t = 381$ min the cell has apparently increased in size and changed its morphology into what looks like two cells next to each other. Directly prior to the time that image was taken the SPR sensogram shows a steep increase. At $t = 700$ min the cells detach from the surface and immediately thereafter the SPR sensogram shows a sharp decrease in response.