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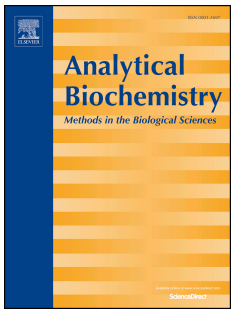

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Modeling single cell antibody excretion on a biosensor

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Abstract

We simulated, using Comsol multiphysics, the excretion of antibodies by single hybridoma cells and their subsequent binding on a SPRi sensor. The purpose was to confirm that SPRi is suitable to accurately quantify antibody (anti-EpCAM) excretion. The model showed that antibody loss by diffusion away from the sensor was less than 1%. Unexpectedly more than 99% of the excreted antibodies were captured on the sensor. This data proves the remarkable phenomenon that the SPRi output of cellular antibody excretion and its subsequent binding, performed under the conditions described here, is directly usable for quantification of single cell antibody production rates.

Keywords: SPRi; EpCAM; Hybridoma cells; Quantification; Simulation.

Introduction

Cells can produce or excrete proteins and other components. We have shown previously that Surface Plasmon Resonance (SPR) can detect cell surface antigens [1] and antibody excretion by hybridoma cells [2]. We postulated that our Surface Plasmon Resonance imaging (SPRi) method can be used for quantifying antibody excretion levels of individual cells. This would enable the assessment of the distribution of antibody production at the single cell level and provide a tool to, for example, select the most optimal conditions for production of therapeutic antibodies. Such a method would require a high affinity capture surface for binding the proteins that are excreted by the cells, ideally this method should be capable of capturing all the produced specific proteins without protein loss due to diffusion that would affect quantification. There are ways to use SPR for at-line quantification where an SPR

machine is connected directly to a cell incubator modified with an external pump that feeds cell culture medium with the excreted antibody directly into the SPR machine for analysis [3], but other than our own method there are no alternatives for accurate label free quantification at the single cell level. If it is feasible that all excreted specific antibody molecules can be measured using our method then we can quantify the production of antibodies by individual cells and measure the distribution of the production per cell. In order to gain insight into the degree of loss of the excreted antibody into the surrounding medium with respect to the amount that will be captured on the sensor surface, we devised a computer model using Comsol multiphysics. In this model various parameters e.g. affinity values of the used recombinant protein and produced antibody were implemented, in order to make an accurate simulation of the event. The simulation was designed to replicate the antibody production per cell attached to the sensor surface as reported recently[2].

Materials and methods

SPR imaging

For the SPRi experiments an IBIS MX96 SPR imager (IBIS technologies bv, Enschede, the Netherlands) and Easy2Spot® pre-activated G-type Senseye® sensors (Ssens bv, Enschede, the Netherlands) were used. Immobilization of Recombinant Human EpCAM (RhEpCAM) (ACRO biosystems, Bethesda, Maryland, USA) protein was performed as described previously [2]. The amount of deposited recombinant human EpCAM after sensor immobilization was determined by SPRi using a so-called R_{LL} determination (Response of the Local Ligand density) prior to any experiments. This R_{LL} determination gives the RU value attributable to the immobilization process compared to a non-affected reference surface directly next to the immobilized protein. The SPR-dip values of the local reference Region of Interest

(RoI) are subtracted from the signal RoI. The MX96 is calibrated in such a way that an output of 1 RU corresponds to 1 pg of bound protein per mm^2, this RU value can be converted into the exact amount of immobilized protein on the surface.

The binding capacity of the recombinant human EpCAM (rhEpCAM) protein spots was determined by flowing purified VU1D9 anti EpCAM antibody (generously provided for these experiments by Immunicon corp, Huntingdon Valley, PA, USA) over the spots in a concentration of 10 µg/ml. Afterwards the analyte response maximum (Rmax) of the spot was determined. The Rmax represents the RU value when all immobilized ligands are fully covered by the analyte (meaning that no binding of specific molecules to the ligands can occur beyond this value) and is a characteristic value for the analyte binding capacity of a spot. Since we used an excess of antibody as an analyte for this experiment, this value represents the maximum amount of antibody that could be bound by the immobilized rhEpCAM. For the affinity experiments, the rhEpCAM was immobilized on the sensor surface with concentrations ranging from 10 µg/ml to 0.002 µg/ml using a Continuous Flow Microfluidic spotter (Wasatch microfluidics LLC, Salt Lake City, Utah, USA). After sensor deactivation the anti-EpCAM antibody was injected in a series of diluted concentrations ranging from 10 µg/ml to 0.002 µg/ml. Association was carried out for 15 minutes with a subsequent dissociation of 2 minutes. System and sample buffer was 1X Phosphate Buffered Saline (PBS) with 0.003% Tween 20 (Sigma-Aldrich Corporation, St-Louis, Missouri, USA). Regeneration of the sensor surface was performed for 1 minute with a 50 mM glycine-HCl solution. Determination of the kinetic/affinity values was done by analyzing the data in SPRintX software v.1.9.3.2 (IBIS technologies B.V., Enschede, The Netherlands). Recently a comparison of various EpCAM antibodies was performed in our group (including VU1D9) using a

novel way (dubbed the Interpolation method) of affinity ranking, similar high affinity values for VU1D9 were found in that study[4].

Computer simulation of cell excretion

One single “average” VU1D9 hybridoma cell on a SPR sensor was modeled in the simulation. The practical experiments were described earlier [2]. We used Comsol multiphysics version 4.3b, released June 2013 (COMSOL, Inc., Palo Alto, California, USA). The module “Transport of diluted species” represented a convenient way to simulate diffusion. It was combined with a mathematical interface on the boundary to model the rate of production on the cell surface and subsequent adsorption on the sensor surface. To build a simulation in Comsol, first, the geometry needed to be defined. In this case, it should be as similar to the flow chamber dimensions as possible. Symmetry in the X-Y dimension was applied in order to reduce computation time. The sensor surface is the bottom of the modeled flow chamber while it is filled with cell culture medium and is heated to 37ºC to keep the cell alive, as was done with the real SPRi experiments. In the model the production of antibodies per cell were simulated by considering diffusion as described by Fick’s second law of diffusion from a point at the surface. The produced antibodies will diffuse in all directions and the part that reach the sensor will eventually encounter their corresponding antigen and consequentially will be captured. As a boundary condition in the model the concentration of diffusing molecules directly at the surface is zero. Transport by convection was zero (no flow has been applied) where the excretion was quantified. As such antibody transportation is solely by diffusion, it can be described by:

$$\frac{\partial \mathrm{c}}{\partial \mathrm{t}} = \mathrm{D}\nabla^2\mathrm{c}$$

In this equation c represents the bulk concentration of the antibodies in the medium and D represents the diffusion constant in m^2/s. D can be described with the Stokes-Einstein relation:

$$D = \frac{k_B T}{6_{\pi\eta r}}$$

The equation shows that diffusion is dependent on the Boltzman constant k_B, temperature T, viscosity η and the radius of the diffusing particle r [5]. The antibody is produced by the cell, which means there is an inflow of concentration P at the cell boundary which is dependent on the constant production rate of the cell.

$$\frac{\partial c}{\partial t} = P$$

And: $$\frac{\partial c}{\partial t} = -\frac{\partial c_{ads}}{\partial t} = -k_a(c_{max} - c_{ads})c + k_d c_{ads}$$

The antibodies that are being produced may bind to the antigen, which is immobilized on the surface or will diffuse in the surrounding medium. The surface concentration per time unit depends on k_a (the adsorption rate), c_{max} (maximum possible adsorbed surface concentration), c_{ads} (surface concentration adsorbed on the sensor) and k_d (The desorption rate). This means that antigen-antibody specificity, ligand density on the surface and bulk concentration in the medium determine the rate at which the surface concentration changes.

As antibodies bind to the surface they are assumed to be bound there. The only way that the antibodies could enter or leave in the model was through the cell or sensor

surface. All other boundaries were impermeable nor were they capable of causing a binding interaction. [6-8].

After entering run time and time resolution, we were able to compute a numerical solution in Comsol. A 3D-plot of the bulk concentration in conjunction with the surface concentration as a function of time was computed. By determining the amount of bound protein on the sensor surface and in the whole volume of the flow cell, absolute values of the amounts of bound and unbound antibody could be compared.

Results

SPRi

After the rhEpCAM was spotted on the sensor surface, the ligand density was determined by SPRi using the R_{LL} method. An average ligand density was determined using SPRintX software based on the average RU measured by the IBIS MX96. The IBIS MX96 was calibrated in such a way that the SPR shift output equals to 1 RU = 1 picogram per mm^2. The ligand density turned out to be 1.0589×10^{-8} mol/m^2. The affinity experiments with the purified EpCAM antibody yielded the affinity values that are noted in table 1. The average production speeds of the VU1D9 hybridoma cell line were determined previously [2].

Computer simulation of excretion

When using the cell parameters as specified in table 1, the model showed that the amount of bound antibody after one hour was the same as was determined in our previous study (1.86×10^{-18} mol), where the amount represented the total amount of produced and bound antibody by a single cell on the sensor surface. A top down view of the simulation can be seen in figure 1. Additionally, the model was also able to calculate the expected amount of antibody, which would not be bound and as such

disappears in the bulk of the system buffer. This amount was 1.67 $x10^{-20}$ mol. This means that only 0.9% of the produced antibody diffuses into the bulk according to the model.

table 1 here

figure 1 here

Discussion

There are some shortcomings in the model. Though we intended to model the loss of the produced antibodies, the diffusion constant D which is used in the model assumes that the antibodies are spherical. This in reality is not the case, however, the antibodies are very small compared to the cells or the domain in which they are modeled and as such model behavior should be similar to reality. For our hybridoma experiments we used a hydrogel sensor surface, the model however does not take this into account but assumes a flat surface, however the parameters which were used in the model were derived from actual practical experiments using a hydrogel sensor surface and as such we don't expect any major differences in the model results, relating to the structure of the sensor surface. The model also doesn't take into account the possibility of an inhomogeneous immobilization of the ligand on the sensor surface. The model currently only assumes a homogenous ligand surface. However in reality this can considerably influence local sensor performance. In our model however we used an average R_{LL} value, which should make the models sensor surface performance come as close as possible to reality. A major difference between the model and the live experiments is that the model assumes that cells have a constant rate of production and this, in reality, is not the case. Hybridoma cells have cycles in which they perform certain activities, such as producing

antibodies and preparing for cell division. Therefore on an individual cell level one can expect that production rates will vary. Cells also age and respond to their surroundings (temperature fluctuations, buffer effects etc.). All of these factors influence the production rates of individual cells and the model in its current state does not account for this. The model simulates only a constant production rate. Thus we decided to use the average production speed of the VU1D9 producing hybridoma as an input parameter. In our previous study we have shown that the VU1D9 producing hybridoma was capable of producing antibodies at an average rate of 3.0 pg per cell per 10 hours (approximately on average: 7.2 pg per cell per day, 0.3 pg per cell per hour, 8.3e-5 pg per cell per minute, 300 antibody molecules per cell per minute [2]). The model showed that with higher production speeds there would be more protein loss (diffusion into the bulk) and as such, depending on the production rates, the SPRi output can possibly not be a reliable value for quantification (see supplemental table 1). In our model we however did not investigate speeds higher than 0.8 pg per cell per hour and as such diffusion into the bulk did not progress to high amounts. We choose not to exceed the 0.8 pg speed in the model as on average hybridoma cells produce at a lower rate and as such increasing the speeds even higher was deemed irrelevant. Since the production speeds are relatively low in our particular case (0.3 pg/h), the model shows that with the used affinity parameters and with the production speed values of the cell, the diffusion into the bulk is not playing a large role. As such the SPRi derived values are accurate enough for quantification purposes of single cell antibody excretion levels, especially when considering that literature shows that production rates of hybridoma cells approximately fall in the 0.2-0.3 pg per cell region [9-12] which agrees with our previous practical findings [2]. The model further emphasizes that the production

speeds are influencing the diffusion into the bulk more than the time duration that a production process is being followed (if the production speeds stay constant) (see supplemental table 2). This would mean that the diffusion into the bulk is heavily influenced by the ability of the ligands immobilized on the sensor to anticipate on the produced antibody. If the production is going to fast the ligands will not have the opportunity to bind the antibody and as such it will diffuse away into the bulk. Even at low production speeds however the capacity of the sensor will reach its maximum and diffusion into the bulk will be higher.

We have to stress however that the results shown here only apply to cases where production rates are in the ballpark of our used model cell line and the affinities of the proteins involved are comparable. We therefore encourage additional research to be done on characterizing and modeling other "producing cell lines" and evaluate the applicability of SPR based quantification in their particular case.

Conclusion

We created a computer model in which we simulate the production of antibodies by a single high producing hybridoma cell immobilized on an SPR sensor surface. By implementing affinity and production values of the excreted antibody of the VU1D9 cell line, the model was able to accurately simulate the binding process of the anti-EpCAM antibody on the sensor surface as a function of time. The simulation that we ran showed that only 1.67 $x10^{-20}$ mol of produced antibody diffused away from the sensor surface into the bulk medium, while 1.86 x 10^{-18} mol was bound locally on the sensor surface after 1 hour. This means that only 0.9% of antibody is lost due to diffusion. Our previously performed live experiments and this computer model show

that SPRi can indeed be used as an accurate single cell antibody production quantification tool.

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Table 1: Overview of the parameters used in the Comsol model.

Parameter	Value in SI-units
Radius of the cell	$6 \cdot 10^{-6}\ m$
Production rate of a single cell	$0.3\ \frac{pg}{h}$ $(1.86 \cdot 10^{-18}\ \frac{mol}{h})$
Adsorption rate constant	$2.6 \cdot 10^{8} \frac{\mathrm{m}^3}{\mathrm{mol} \cdot \mathrm{s}}$
Desorption rate constant	$3.3\ \cdot 10^{-5}\ \frac{1}{\mathrm{s}}$
Capacity of the sensor surface (ligand density)	$1.0589 \cdot 10^{-8}\ \frac{mol}{\mathrm{m}^2}$
Temperature	$310.15\ K\ (37ºC)$
Viscosity	$0.692\ mPa \cdot s$
Radius of antibodies	$12\ nm$
Height of flow cell	$200\ \mu m$
Width of flow cell	$60\ \mu m$
Maximum amount of captured antibodies	$1.699 \cdot\ 10^{-6}\ \frac{kg}{\mathrm{m}^2}$
Weight of antibodies	$160000\ Da$

Figure 1: Top down view output of the Comsol model. The white circle in the middle represents the cell, the colored halo around it represents the bound protein to the sensor surface. The color scale ranges from red (high amount of protein) to dark blue (low amount of protein). Insert A shows an actual cell on an SPR sensor upon binding. Insert B shows the same cell with the excreted antibody bound on the sensor surface around the cell.

Supplemental table 1: Simulated model data at various speeds of production

Production rate of single cell (pg/h)	Antibody concentration bound on sensor surface (mol)	Antibody concentration in bulk (mol)	% ratio of antibody diffused into the bulk
0.02	1.23E-19	1.12E-21	0.91
0.05	3.08E-19	2.80E-21	0.91
0.1	6.17E-19	5.60E-21	0.91
0.2	1.23E-18	1.12E-20	0.91
0.3	1.86E-18	1.67E-20	0.90
0.5	3.16E-18	2.80E-20	0.89
0.8	3.69E-18	4.47E-20	1.21

Supplemental table 2: Simulated model data at various time intervals, at a production speed of 0.3 pg per cell per hour.

Time interval	Antibody concentration bound on sensor surface (mol)	Antibody concentration in bulk (mol)	% ratio of antibody diffused into the bulk
1 hour	1.86E-18	1.67E-20	0.90
5 hours	8.83E-18	1.68E-20	0.19
10 hours	1.94E-17	1.68E-20	0.09