Analytical Biochemistry 608 (2020) 113899

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

In-line detection of monoclonal antibodies in the effluent of protein A chromatography with QCM sensor

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ARTICLE INFO

Keywords:
Quartz crystal microbalance
mAb
IgG
In-line sensor
Protein A
Continuous multi-column affinity chromatography

ABSTRACT

A major drawback of the IgG capture step is the high cost of the protein A resin. For a better utilization of the resin, a continuous multi-column operation was recently proposed. In this method, accurate detection of leaking IgG is crucial to divert the breakthrough fluid from the waste to the next column and prolong the loading step without product loss. The detection of a breakthrough point as a change in UV absorption is based on a relatively small signal addition of IgGs to the bulk signal of host cell proteins. To achieve specificity, we used a quartz crystal microbalance and immobilized protein A as specific ligand on the sensor surface. We integrated the quartz crystal microbalance sensor in-line after the protein A column for real-time detection of IgGs in the breakthrough fluid. We show that this specific IgG detection in the breakthrough fluid can be more sensitive than with the UV detector. The use of the same product-specific ligand in the affinity column and in the sensor allows simultaneous in-line regeneration of column and sensor in a single step. Such a sensor could support cost-efficient load control during the entire continuous multi-column capture step in downstream processing.

1. Introduction

Protein A affinity chromatography is a well-established first step in the downstream IgG purification process [1]. However, the high cost of the protein A resin affects the economics of the whole process and makes it one of the primary targets for optimization. Continuous multi-column protein A chromatography is a new approach in recombinant IgG purification that allows a much better utilization of the protein A resin and thus reduces IgG production costs and the space requirements of the plant [2,3]. The basic idea of continuous multi-column protein A chromatography is to divert the breakthrough fluid, containing unbound IgG product from the first column to the next column where these IgGs are captured. This allows a longer loading of the first column, resulting in very high resin saturation without IgG loss. A prerequisite for efficient continuous multi-column chromatography is a reliable real-time control of IgGs in the flow from the protein A column. This enables timely and possibly automated redirection of the flow from waste to loading of the next column in the series [2].

Real-time, preferably in-line process analytical technology (PAT) is required to support the control of continuous multi-column chromatography. It has been shown that automated online analysis is capable of controlling protein A chromatography. However, the time required for automatic sampling and exhibition of the analysis leads to longer delays between sampling and measured IgG content [4]. The delayed detection of IgG in the breakthrough fluid leads to delayed fluid diversion and product loss in a continuous downstream process. On the other hand, inline concepts based on UV detectors allow continuous monitoring and immediate reaction to changes in the UV signal. The amount of IgGs in the breakthrough fluid can be derived from the signals of UV detectors located before and after the protein A column, which measure the total protein concentration in the loaded harvest and breakthrough fluid, respectively [2,5]. This approach would allow the process control system to react and divert the flow to the subsequent column when the IgG concentration in the breakthrough fluid reaches 3% of the concentration in the loaded cell-free harvest. Another UV/Vis-based method for monitoring and control in protein A chromatography has recently been proposed [6]. It applies UV/Vis absorption spectra and correlates different wavelength absorptions with IgG concentrations in the column effluent, taking advantage of distinct variations in the UV/Vis absorption spectra of individual protein species. Partial least squares modelling of the data enabled good prediction of 5% and 50% of IgG in the breakthrough fluid. Partial least square modelling of the UV/Vis absorption spectra in combination with variable path length spectroscopy was also proposed to distinguish between IgG monomers and higher

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https://doi.org/10.1016/j.ab.2020.113899
Received 8 June 2020; Received in revised form 27 July 2020; Accepted 29 July 2020
Available online 4 August 2020

molecular weight variants in the downstream polishing step [7]. Although UV detector-based approaches have the advantage of robustness and easy in-line integration of the detector, they are not product-specific and are therefore based on assumptions or calculations about impurities and IgG product contributions to the overall UV signal of the breakthrough fluid. In addition, the IgG concentration of the breakthrough fluid at the column switch point in continuous multi-column chromatography is very low compared to the concentration of protein impurities. This poor signal-to-noise ratio makes the UV sensor system less accurate in this phase and can lead to suboptimal process control. In addition to UV detectors, the IgG concentration can also be derived from other characteristics of the process fluid. Recently it was shown that osmolality can also be a good predictor for model-based real-time monitoring of IgG concentration [8]. However, osmometers are currently only at-line instruments and the distinction between IgG product and protein impurities is yet to be resolved. An online array of several detectors was used for the model-based estimation of several different quality attributes, including the IgG concentration, in the eluate of the protein A chromatography column in real time [9]. Again, the challenge remains the detection of a low IgG concentration in the mass of host cell proteins in the breakthrough fluid.

As opposed to non-specific UV absorbance method, other approaches have been developed for reliable IgG detection based on specific IgG recognition, such as biolayer interferometry (BLI) [10,11]), surface plasmon resonance (SPR) [12,13] and quartz crystal microbalance (QCM) [14]. BLI has been widely used for screening of clones for IgG production due to its high sample throughput. However, it is suitable for at-line plate mode operation and cannot be readily used for in-line detection. SPR sensors are highly sensitive and distinguished for low sample consumption. The SPR sensor was integrated in capillaries and used as an on-line sensor. However, when analysing raw samples, SPR tends to clog and is therefore less convenient. On the contrary, QCM sensors can operate at higher sample flow rates and larger tube diameters, so that the sensor is less susceptible to clogging and its in-line integration after the chromatography column is easier to achieve.

QCM is a piezoelectric resonator that reacts to the binding of mass to the sensor surface by an inversely proportional change of the resonance frequency [15]. QCM has been used to detect various entities in vacuum, gaseous and liquid environments. It has been used in space research [16], to detect vaporized components in air [17] and to study the surface layer reactions of various materials [18]. QCM has also been used in biomedical studies enabling detection of bacteria, viruses and disease biomarkers [19–23]. The QCM sensor principle is based on a specific molecular interaction between the ligand immobilized on the sensor resonator and the analyte. The detection is not optical and can therefore be performed in turbid samples such as bioprocess fluids. It has been shown that QCM sensors functionalized with protein A specifically bind IgGs [14]. Protein A, used as a specific IgG ligand on the sensor surface,

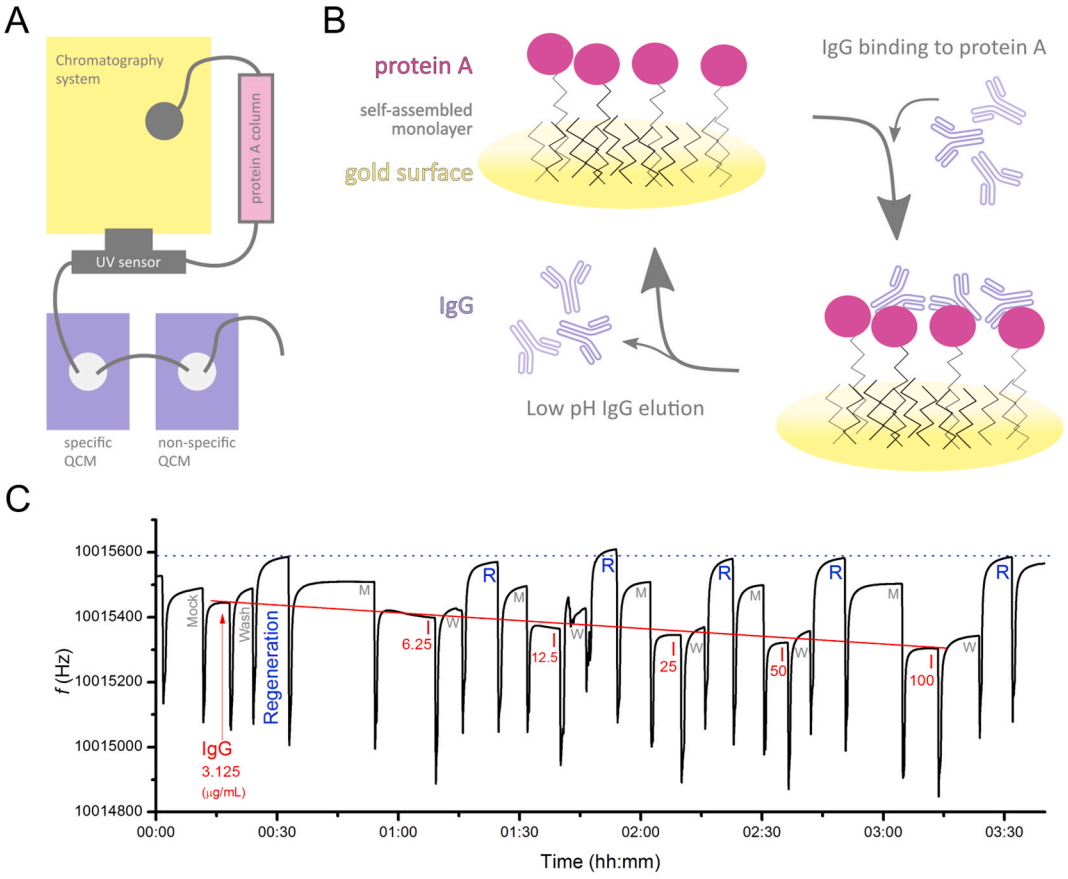

Fig. 1. The principle of IgG detection by QCM and the regeneration of the sensor surface. A) Arrangement of the chromatography system and the QCM sensor. Effluent from the protein A column flows to the UV sensor of the chromatography system. Next there is the specific (protein A) QCM sensor and next is the non-specific QCM sensor. B) IgGs (violet) bind to protein A (purple circles) which is covalently bound to 11-mercaptoundecanoic acid (11-MUA). 11-MUA is a component of the self-assembled monolayer (SAM) on the gold surface of the QCM resonator. The second, shorter component of SAM is 6-mercaptohexan-1-ol (6-MHO). Rinsing at low pH efficiently regenerates the protein A ligands for the next detection cycle. C) Sensorgram of the steps of multiple IgG binding (decreasing frequency) and ligand regeneration (increasing frequency). Injections of increasing concentrations of IgG (also marked I) are interspaced with sensor regeneration (R) and IgG-free mock-harvest injections (M) to saturate the non-specific sensor response. The blue horizontal line represents the regeneration potential of the sensor. The red line represents the concentration-dependent behaviour of the sensor. The red numbers represent the applied concentrations of IgG in μg/mL. The buffer wash (W) indicates a stable IgG binding.

enables repeatable binding and efficient regeneration of the ligand binding capacity for subsequent detection. This principle was also applied in our measurements. QCM devices are commercially available in various forms, both as user-friendly desktop units of relatively high costs, or as simple and inexpensive portable units. The latter are easier to use as integrated sensors in laboratory setups. They are compatible with a range of flow rates, making them easier to harmonize with an HPLC chromatography system as in-line sensor. A schematic presentation of our QCM sensor integration after Protein A chromatography is shown in Fig. 1A.

Our goal was to prepare a robust, specific and cost-effective sensor for the real-time detection of IgGs in downstream process fluids. Here we show the in-line integration of QCM sensor units after Protein A affinity chromatography column and a UV detector. We compare the raw signals obtained from the UV detector with the signals obtained from the QCM sensor. In our setup, the QCM sensor enabled specific detection of IgGs in the breakthrough fluid sooner than the UV detector. We also demonstrate the ability of the sensor to regenerate when integrated into a chromatography system that allows successive detections. We believe that a QCM sensor with these characteristics could support continuous multi-column chromatography by indicating times of IgG product leakage in the breakthrough fluid.

2. Results and discussion

2.1. The principle of IgG detection and regeneration of QCM based sensor

The QCM sensor reacts to the mass change on the surface of the resonator with a corresponding change in the resonance frequency [15]. The specific binding of IgG to the sensor surface was achieved by immobilizing protein A ligand on the sensor surface, as shown before [14,19]. The design of the sensor surface, binding of the IgGs and the regeneration of the sensor are shown in Fig. 1B. In short, the gold electrode on the QCM resonator was coated with the self-assembled monolayer (SAM) of 11-mercaptoundecanoic acid (11-MUA) and 6-mercaptohexan-1-ol (6-MHO) to limit non-specific interactions between the sample and the gold electrode. The 11-mercaptoundecanoic acid was further conjugated to the protein A ligand by amino coupling. Protein A was used because of its specific affinity to IgGs and the ability to release the bound IgGs by lowering the buffer pH to 3.6. In order to use the QCM sensor in several consecutive detections, the sensor surface must be efficiently regenerated each time. Fig. 1C shows the restoration of the initial resonance frequency after each 'sensing – regeneration' step. The sensor reacted depending on the IgG concentration and remained stable for several hours at room temperature or overnight in the refrigerator. We aimed to use the QCM sensor for the detection of IgGs in breakthrough fluid from the protein A chromatography column. Since the protein A ligand on the QCM sensor and the chromatography resin can be identical molecules and are exposed to the same processing conditions, we believe that the sensor could support the entire protein A purification process. To demonstrate sufficient sensitivity of the QCM sensor to detect IgGs in breakthrough fluid from a protein A column at low concentration, we prepared serial dilutions of known IgG concentrations. QCM sensor detected IgGs at about 0.1% of the IgG concentration in the harvest (3.2 mg/mL) that we used for loading the protein A column.

2.2. In-line QCM sensor integration after protein A chromatography column

Two consecutive QCM sensors were integrated into the chromatography system in-line after the protein A column and the UV detector to demonstrate the detection of the emerging IgG product in the breakthrough fluid during the affinity chromatography. The first QCM sensor was functionalized with the protein A ligand for specific IgG detection and the second QCM sensor was without the ligand to detect only signals resulting from non-specific interactions with the sensor surface or physical effects like change in viscosity of the running buffer. The signals of both QCM sensors and the UV detector were recorded continuously during the chromatography at a rate of 1 measurement per second.

The responses of the UV detector and the QCM sensors were compared. An example of responses is shown in Fig. 2. The specific QCM sensor responded to IgGs in the effluent of the protein A column at an earlier time than the UV detector. In our set-up the average difference in breakthrough detection by the sensors was 125 s ± 90 s (n = 4). The responses of the IgG-specific and non-specific QCM sensors to the increased protein concentration was comparable at the breakthrough point of the host cell proteins (Fig. 3A). However, the response of the non-specific QCM sensor was delayed compared to the specific response at the IgG breakthrough point. The response of the non-specific QCM sensor was simultaneous with UV detector suggesting response only to higher IgG concentrations, confirming the ability of the specific QCM sensor to serve as a precise indicator of the IgG breakthrough point. The specific QCM sensor does not operate in a continuous mode and must be regenerated for subsequent detection of IgGs in the breakthrough fluid during the next cycle of column loading. We predicted that the elution of bound IgG from the protein A column by rinsing with low pH buffer could also allow the regeneration of identical ligands on the QCM sensor connected after the column. In our setup we could show the increase in the resonance frequency of the QCM sensor after rinsing with low pH buffer, indicating the release of bound IgG (Fig. 3A). In addition, we could show a successful detection of the next breakthrough point in a subsequent chromatography run immediately after completion of column regeneration. In Fig. 3B the subsequent responses of the UV detector and the specific QCM sensor are aligned to show the ability to repeatedly detect IgG in effluent of the protein A column.

The most common sensors currently used to determine protein concentration in chromatography are UV absorbance detectors. Recently, great progress has been made in the model-based prediction of various quality parameters in chromatographic processes in real time, including IgG concentration [8,9]. The model, which was trained using data from commercial online sensors and offline analysis data from several runs, provided a good prediction of the protein A column elution step in the test run, thus enabling pooling decisions in real time. An even higher accuracy in predicting IgG concentration was achieved by Rüdt et al. [6] by partial least squares modelling of the UV/Vis absorption

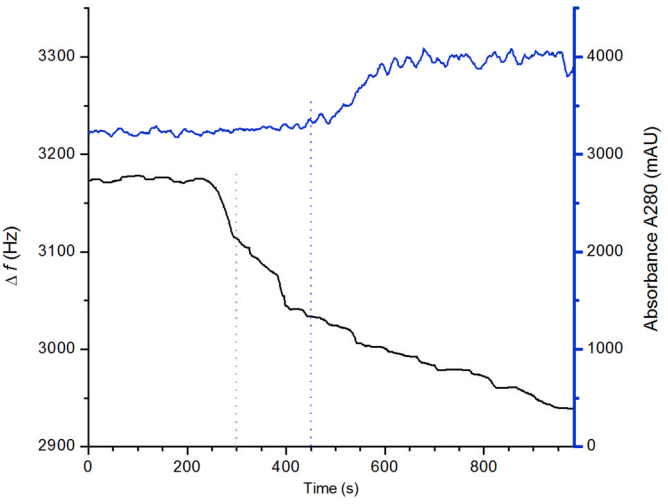

Fig. 2. Detection of IgGs in the protein A column effluent. QCM sensors (black line) responded to IgGs earlier than the UV detector (blue line). QCM signal is a corrected response (the non-specific signal subtracted from the specific signal). The grey dotted vertical line marks the breakthrough detection time by QCM sensor and the blue dotted vertical line marks the breakthrough detection time by UV detector.

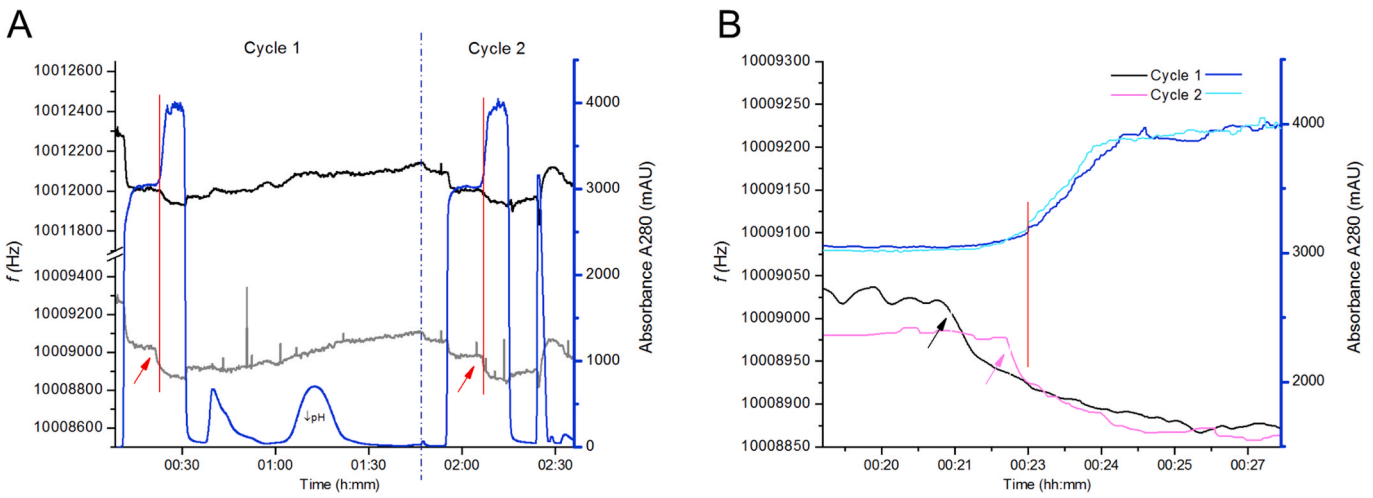

Fig. 3. Detection of IgGs in breakthrough fluid with inline integrated QCM sensors after protein A chromatography column. A) The first increase of the UV signal (blue) from 0 to about 3000 mAU is due to the breakthrough of unbound host cell proteins from the column. The second increase of the UV signal is due to breakthrough of IgGs due to saturation of the chromatography column. Both breakthrough events are well pronounced in corresponding QCM signals as a decrease in resonant frequency. Superimposed recordings of the UV detector, the QCM sensor without protein A ligand (black) and the IgG specific QCM sensor functionalized with protein A (grey) show responses to changes in total protein concentration at host cell protein breakthrough point. IgG breakthrough points are marked with red lines. Response of IgG specific QCM sensor is enhanced at this point and precedes other responses (arrow). The figure shows responses of sensors in two subsequent chromatography cycles. The last blue peak (↓pH) in cycle 1 indicates low pH rinsing, leading to the specific QCM sensor (grey) regeneration. Note increase in resonance frequency indicating release of bound IgG. B) Subsequent responses of the UV detector are aligned at the IgG breakthrough point and specific QCM sensor responses are added on the same time scale. Arrows indicate IgG breakthrough points in the signal from the specific QCM sensor as in panel A.

data in protein A column breakthrough fluid with a root mean square error of the prediction of only 0.01 g/L. A disadvantage of the model-based approach is the sensitivity to unforeseen variations in the process, which would lead to suboptimal decisions. The prediction of IgG concentration in protein A chromatography is less accurate at the breakthrough point where most of the signal is derived from the impurities and only a small fraction from the IgGs. On the other hand, the IgG breakthrough point can be specifically and precisely detected by our QCM sensor with a protein A ligand at very low IgG concentration (Fig. 1 C). In contrast to UV detector and model-based approaches, which allow continuous monitoring of the IgG concentration throughout the entire process, our biosensor has to be regenerated after each individual measurement and therefore cannot be used for continuous monitoring of the chromatography process. However, the specific real-time response of our QCM detector to the emerging IgGs in the breakthrough fluid can serve as a reliable signal required for the change in continuous chromatography to divert IgG-containing breakthrough fluid from the waste to the subsequent column.

In continuous multi-column protein A chromatography, the columns are repeatedly regenerated and reused. With regeneration of the protein A column and the sensor in a single step, the complexity of the chromatography system is kept low. However, the addition of the specific and sensitive sensor contributes to the clarity and precision of process control.

3. Conclusions

The in-line integration of the QCM sensor after the protein A capture chromatography step allows a specific and very precise determination of the breakthrough point of the IgG product. The use of the protein A ligand in the QCM sensor enables specific detection of IgGs. It enables sensor regeneration during column elution without additional processing steps. The principle of column and sensor ligand matching could be used even more widely for other affinity chromatography approaches. The QCM sensor used is inexpensive, simple and can be adapted to high fluid flows in the purification process. We see the precise and clear determination of the breakthrough point with the QCM sensor as a good solution that can improve the control of continuous multi-column chromatography.

4. Experimental procedure

The IgG containing harvest and mock harvest without IgG product were obtained from Lek Pharmaceuticals d.d. (Mengeš, Slovenia) and stored at −80 °C until use.

4.1. Functionalization of QCM sensor

QCM instruments and 10-MHz quartz crystal chips were purchased from Novaetech S.r.l., Italy. 11-mercaptoundecanoic acid (11-MUA), 6-mercaptohexan-1-ol (6- MHO), sulfur-*N*-hydroxysuccinimide (*S*-NHS) and dibasic sodium phosphate were purchased from Sigma. *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimidehydrochloride (EDC) and *Staphylococcus aureus* protein A were purchased from Thermo Scientific. The hydrogen peroxide solution was sourced from VWR. All other chemicals were sourced from Merck.

The gold surface of the QCM sensor was functionalized in a similar way as in Ref. [23]. The QCM chip was first cleaned with piranha solution (H_2SO_4: 30% H_2O_2 was 7:3 (v/v)) for 2 min and then rinsed with ultrapure water and ethanol. The chip was then incubated for 24 h at room temperature in a solution of 2.5 mM mercaptoundecanoic acid and 7.5 mM 6-mercaptohexan-1-ol in ethanol to form a mixed, self-assembled monolayer on the gold surface. After rinsing with ethanol and ultrapure water, the chip was inserted into the QCM instrument and the carboxyl groups on the mercaptoundecanoic acid were activated with 100 mM EDC/50 mM S-NHS solution for 2 h at 22 °C, followed by rinsing with ultrapure water and PBS. Protein A (1 mg/ml) was injected into the detection chamber and incubated overnight at 4 °C. The reference resonator was prepared identically, only the last step with binding of protein A was omitted.

4.2. QCM sensor characterization

The QCM resonance frequency was recorded by computer using software supplied by the manufacturer (OpenQCM version 1.2). The QCM responses were determined after injection of 100 μL IgG dilutions

into phosphate buffer or into mock harvest with a 1 ml syringe. Before and after each sample injection the sensor was rinsed with PBS buffer and/or IgG-free mock harvest. The difference in resonance frequency in the rinsing steps before and after sample injection was considered as sensor response to the binding of the analyte. After each sample measurement the sensor was regenerated by injection of acetate buffer pH 3.6.

4.3. Protein A affinity chromatography

Chromatography experiments were performed on the chromatographic system AKTA Purifier (GE Healthcare, USA). The QCM modules were mounted one after the other immediately after the chromatographic UV detector. The delay of sample flow between UV sensor and IgG-specific QCM chip was in the range of 20–30 s, as determined by major changes in both signals when the host cell proteins started to leave the column during the loading step. This delay was accounted for during the analysis of the data.

The 1 ml column with Poros Mab capture resin (Thermo Scientific™) used in the experiments was packed in-house in a column with a diameter of 0.5 cm and a length of 5.5 cm.

The chromatography flow rate was constant at 0.5 mL/min. The chromatography column was first equilibrated with 20 mM Na-phosphate buffer, pH 7.2. After equilibration, IgG-containing cell-free medium was applied at the same flow rate. The column was then rinsed with 20 mM Na-phosphate buffer, pH 7.2, followed by a washing step with 100 mM Na-citrate buffer, pH 5.5. The column was then rinsed with 20 mM Na-phosphate buffer, pH 7.2, followed by an elution step with 100 mM Na-acetate buffer, pH 3.6.

4.4. Data analysis

The IgG breakthrough point was determined as the point of a significant change in the recorded value. Each measured resonance frequency point of the IgG-specific QCM sensor was compared with the average of the recordings made during one minute, which ended 10 s before the evaluated point. If the value deviated from the one-minute average by more than 3 standard deviations of the averaged values, it was assumed to be significantly different and assigned to the IgG breakthrough point. In the same way, the data from the UV sensor were processed to determine the breakthrough point determined by the UV detector. The data was processed and visualized using Origin 8.1 software (OriginLab Corporation, USA).

CRediT authorship contribution statement

Matic Kisovec: Data analysis, QCM experiments, Writing - original draft, Visualization. **Gregor Anderluh:** Data evaluation, Writing - review & editing. **Marjetka Podobnik:** Data curation, Data evaluation, Writing - review & editing. **Simon Caserman:** Writing - original draft, Conceptualization, Data curation, Formal analysis, Project administration, Final data analysis and evaluation, Paper preparation, Project overview.

Declaration of competing interest

None.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635557 and program grant by the Slovenian Research Agency, P1-0391. We thank Urška Dečko for her excellent technical assistance.

The datasets generated during and/or analysed during the current study are available from the corresponding author upon request.

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