Colloids and Surfaces B: Biointerfaces 149 (2017) 312–321

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Real-time label-free detection and kinetic analysis of Etanercept—Protein A interactions using quartz crystal microbalance

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ARTICLE INFO

Article history:
Received 19 April 2016
Received in revised form 5 October 2016
Accepted 19 October 2016
Available online 19 October 2016

Keywords:
Quartz crystal microbalance (QCM)
Ligand-receptor interactions
Biologics
Interaction kinetics
Biosensors
Biopharmaceuticals

ABSTRACT

A quartz crystal microbalance (QCM) was constructed to assess if such a biosensor has value as a complementary real-time label-free analysis platform for the biopharmaceutical industry. This was achieved through modifying QCM crystals with a low-fouling carboxymethyl-dextran layer bearing Protein A, and then injecting solutions containing Etanercept (*i.e.*, Enbrel®) into the QCM chambers. The kinetics of Enbrel® – Protein A interactions was modeled using the Langmuir binding model and Enbrel® concentrations between 0.75–300 ng mL^{-1}. The resulting equilibrium dissociation and association constants (K_D and K_A) were 5.06×10^{-8} M and 1.98×10^{7} M^{-1}, respectively. The association and dissociation rate constants (k_{on} and k_{off}) decreased substantially as Enbrel® concentration, *[C]*, increased, despite that the net binding rate, ($k_{on}[C] + k_{off}$), increased. The decrease in k_{on} and k_{off} was hypothesized to be a consequence of mass transport limitations. To verify this, QCM dissipation measurements were analyzed to provide insight on solution viscosity. As Enbrel® concentration increased, the net change in dissipation, ΔD, became larger. An augmentation of ΔD is associated with a higher solution viscosity, which would result in an increase in mass transport limitations. Therefore, the decrease in k_{on} and k_{off} for increasing Enbrel® concentration can be attributed to mass transport limitations. In conclusion, QCM is a valuable complementary real-time label-free biosensor analysis platform for the biopharmaceutical industry. Unlike the surface plasmon resonance (SPR) platform, QCM allows measuring dissipation, which can provide insight on how mass transport limitations impact interaction kinetics.

1. Introduction

Biosensor assays with real-time and label-free attributes have found use in multiple areas of biopharmaceutical research, development, and production through their ability to assess the kinetics of ligand – receptor interactions. A common application of these assays is assessing the binding affinities of therapeutic biologics to their target molecules [1]. Such assays are also used to evaluate the immunogenicity of biologics [2,3]. In addition, these assays have found use for analyzing protein interactions to develop and optimize effective methods for antibody purification [4]. With such an assay, manufacturing processes can be monitored to measure the quantity of antibodies or proteins produced and their associated bioactivity [5,6].

The most common real-time and label-free biosensor assay used in the biopharmaceutical industry is surface plasmon resonance (SPR) [7]. While SPR has been successfully utilized in each of the aforementioned applications, the technique is not without pitfalls. An intrinsic drawback is that SPR measurements provide no insight on the viscosity of the solution being injected over the biosensor surface. As solution viscosity increases, mass transport limitations become more impactful on measured kinetics. For example, in SPR platforms, because of mass transport limitations, the real kinetic rate and equilibrium constants could be up to two to three magnitudes greater than those observed from other measurements [8].

Quartz Crystal Microbalance (QCM) with dissipation measurements is another real-time label-free biosensor method. QCM has been used to monitor ligand – receptor interaction kinetics [9–12], but to a far lesser extent compared to SPR. An intrinsic feature of QCM that appears to be overlooked in previous kinetic studies is dissipation measurements. Dissipation measurements in QCM, defined as the "half-bandwidth at half maximum" of the resonance curve divided by the maximum frequency of the resonance

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http://dx.doi.org/10.1016/j.colsurfb.2016.10.036
0927-7765/

curve, give valuable information regarding the viscosity of the solution and the viscoelasticity of the film [13]. As already mentioned, solution viscosity can greatly impact kinetic rate and equilibrium constants calculated from SPR data. Thus, unlike SPR, QCM can provide valuable insight regarding the effects of solution viscosity on the calculated kinetic rate and equilibrium constants. In this study, we use both QCM frequency to assess ligand – receptor interaction kinetics and dissipation measurements to hypothesize the effects mass transport limitations play on the observed kinetic rate constants.

In this study, a QCM instrument was designed and constructed in-house to study interactions between Protein A and Etanercept. This is also the first study we are aware of to assess interaction kinetics between Protein A and Etanercept. Protein A was immobilized onto QCM crystals and Etanercept solutions of various concentrations were exposed to the modified crystals. Protein A is a cell-wall protein containing five high-affinity binding sites capable of interacting with the F_c-portion of various IgG antibodies. Protein A was chosen for this study as when immobilized on surfaces, it can be used for the purification of therapeutic antibodies [14] as well as to monitor and optimize bioprocesses (when employing Biacore's commercially available Protein A-modified SPR sensors). Etanercept (known by its tradename as Enbrel®) is a biologic that acts as an antagonist towards tumor necrosis factor (TNF). TNF is a soluble inflammatory cytokine that regulates immune cell signaling. TNF dysregulation is associated with numerous inflammatory diseases such as rheumatoid arthritis, Crohn's, and psoriasis to name a few [15]. Enbrel® was chosen for this study as it is currently used extensively to treat many TNF-associated diseases. Biologics like Enbrel® are very costly. Optimizing and understanding downstream processing of biologics is of importance for the biopharmaceutical industry as it represents very delicate and costly procedures. The fundamentals of Protein A-based separation columns used to purify biologics are not fully mastered, as it can be a challenge to elute Protein A-captured biomolecules; *e.g.*, up to 30% can remain trapped in the column after elution [16], causing financial burden on the process.

The Langmuir isotherm was fit to experimental data obtained from QCM frequency shift measurements to derive the equilibrium constants (K_D, K_A), and kinetic rate constants (k_{on}, k_{off}) at varying Enbrel® concentrations. It is shown that while the net binding rate increases with increasing Enbrel® concentration, k_{on} and k_{off} in fact decrease. The decrease in k_{on} and k_{off} can be attributed to mass transport limitations. For the first time, we demonstrate how *in situ* QCM dissipation measurements can be used to provide valuable insights regarding mass transport effects on ligand – receptor interaction kinetics.

2. Materials and methods

2.1. Quartz crystal microbalance (QCM)

The design of the QCM instrument is shown in Fig. 1A. The QCM instrument is housed on a frame composed of chemically resistant type I PVC (McMaster Carr, Cleveland, OH, U.S.A., cat. # 87545K651). The QCM chambers were purchased from Stanford Research Systems (Sunnyvale, CA, U.S.A., cat. # O100RH) and FC-550 flow cells from Inficon (East Syracuse, NY, U.S.A., cat. # 184208). The QCM instrument is computerized using a user interface that was programmed in Labview (National Instruments, v2013). Electronics of the QCM instrument are controlled using a microcontroller unit (Digi-Key, Thief River Falls, MN, U.S.A., cat. # ATMEGA1281-16AURCT-ND). The QCM instrument is integrated to the computer using a USB, type 2 connection (Digi-Key, cat. #MUSBD11130-ND). Power is supplied to the QCM instrument using a 24 V, 180 W AC/DC desktop power supply (Digi-Key, cat. # 1470-1046-ND).

QCM frequency and dissipation measurements are made using a network analyzer (Saunders & Associates, Phoenix, AZ, U.S.A., cat. # 250B). All frequency measurements are acquired using a 10 kHz sweep centered around the 1st, 3rd, and 5th fundamental (*i.e.*, harmonics) frequencies (5 MHz, 15 MHz, and 25 MHz, respectively) of the QCM crystal. The input power is 80 mW. The resulting resonant curves are fit to a Lorentzian function upon which the frequency that had the maximum conductance, f_{max}, was recorded. An algorithm is used to compute the frequency shift and dissipation. A single frequency shift data point is calculated by subtraction the f_{max} at the initial time, t_0, from that of an f_{max} at a later time, t_n of the data acquisition: $f_{max}(t_n) - f_{max}(t_0)$. A single dissipation data point is calculated by computing the half-bandwidth at half-maximum and dividing that value by $f_{max}(t_0)$. The QCM instrument measures both frequency and dissipation simultaneously in real time. Up to 60 measurements can be recorded per minute. When measuring frequency and dissipation from all three harmonics (5 MHz, 15 MHz, 25 MHz) simultaneously using one QCM crystal, it takes 0.1 s to make the three 10 kHz sweeps and record the maximum frequency and calculate the dissipation. Likewise, when using two QCM crystals, it takes 0.2 s to make the six 10 kHz sweeps and record the maximum frequency and calculate the dissipation.

The QCM chambers and flow cells rest inside a stainless steel water reservoir (Fig. 1A). The water reservoir is heated using 4 polyimide flexible heater strips (Omega, Laval, QC, Canada, cat. # KHLV-104/10P). Water reservoir temperature is measured with electronic sensors (Digi-Key, cat. # ADT7310TRZ-REEL7CT-ND). Two sensors are secured to the outside of the water reservoir at opposite ends. The temperature sensors and heating strips are fastened to the stainless steel water reservoir using high-thermal conductivity epoxy (Omega, cat. # OB-101-16). The recordings from each are averaged to yield the temperature measurement. This temperature measurement is fed back into the computer where it is compared to the user set-point. Current through the heater strips is switched on if the measured temperature is below the set-point or switched off if it is above. The water reservoir temperature is displayed on the user interface and on the front of the QCM instrument (Fig. 1A).

Injection of fluids into the QCM chambers is done using syringes. Up to four different syringes can be used for QCM measurements (Fig. 1B). Each syringe is driven using a linear actuator (Electrocraft, Dover, NH, cat. # APPS17). Motion of the linear actuator is controlled using two integrated circuit motor drivers (Digi-Key, cat. # 497-3641-1-ND) and one integrated circuit motor controller (Digi-Key, cat. # 497-2945-5-ND). Flow rates of the electronically-controlled linear actuators are entered through the user interface.

The fluid circuit presented in Fig. 1B was constructed for this experiment. There are a total of two independent QCM chambers, each coupled to two syringes, which are loaded with different fluids. Check-valves (Cole Parmer, Montreal, QC, Canada, cat. # 30505-92) are coupled to the syringes to ensure one-way directional flow. Fluid from each syringe is tied into a chamber using Tygon® tubing (Cole Parmer, cat. # 95666-01) and a Y-connector (Cole Parmer, cat. # 40726-41). Fluids leaving the chambers are collected into Septa-Jar® containers (Fisher Scientific, Ottawa, ON, Canada, cat. # 05-719-313).

2.2. Quartz crystal surface modification

Chromium/gold-plated 5 MHz Maxtek QCM crystals (Inficon, cat. # 149273-1) were modified with functional amine groups using a custom built *n*-heptylamine plasma polymerization (HaPP) reactor [17]. The operating parameters of the HaPP reaction are described elsewhere [18]. Low-fouling carboxymethyl-dextran