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RESEARCH PAPER

Quartz crystal microbalance as an assay to detect anti-drug antibodies for the immunogenicity assessment of therapeutic biologics

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Abstract Because of their biological origins, therapeutic biologics can trigger an unwanted deleterious immune response with some patients. The immunogenicity of therapeutic biologics can affect drug efficacy and patient safety by the production of circulating anti-drug antibodies (ADA). In this study, quartz crystal microbalance (QCM) was developed as an assay to detect ADA. Etanercept (Enbrel®) was covalently grafted to dextran-modified QCM surfaces. Rabbits were immunized with etanercept to generate ADA. Results showed the QCM assay could detect purified ADA from rabbits at concentrations as low as 50 ng/mL, within the sensitivity range of ELISA. The QCM assay could also assess the ADA isotype. It was shown that the ADA were composed of the IgG isotype, but not IgM, as expected. Furthermore, it was shown that QCM surfaces that had been used to detect ADA could be regenerated in glycine-HCl solution and reused. The QCM assay was also demonstrated to detect ADA in crude serum samples. Serum was collected from the rabbits and analyzed before and after etanercept immunization. ADA were clearly detected in serum from rabbits after immunization, but not in serum before immunization. Serum from patients administered with etanercept for rheumatoid arthritis (RA) treatment was also analyzed and compared to serum from healthy donors. Sera from 10 RA patients were analyzed. Results showed one of the RA patient serum samples may have ADA present. In conclusion, QCM appears to be a viable assay to detect ADA for the immunogenicity assessment of therapeutic biologics.

Keywords Immunogenicity · Anti-drug antibody · Quartz crystal microbalance (QCM) · Assay development · Biologics · Biosensor

Introduction

Therapeutic biologics such as proteins and monoclonal antibodies can elicit an unwanted immune response in the patient in a process known as immunogenicity. Immunogenicity is characterized by the patient's production of circulating anti-drug antibodies (ADA) directed against the therapeutic biologic [1, 2]. In some cases, ADA production can result in drastically reduced efficacy and altered pharmacokinetics [3]. In more rare cases, ADA production can lead to more adverse side effects such as organ complications [4]. Immunogenicity is influenced by product-, patient-, and treatment-related factors. Product-related factors can include aggregation during manufacturing, shipping, and storage processes [5]. Patient-related factors can involve whether or not the patients have an active or suppressed immune system or if they have preexisting ADA from past treatment with a similar biologic [6]. Treatment-related factors can include the route of administration. For example, subcutaneous administration is

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believed to have a higher risk of immunogenicity [7]. In summary, there is a need in the biopharmaceutical industry to assess immunogenicity (i.e., to detect ADA) during the development of therapeutic biologics.

In this study, we introduce quartz crystal microbalance (QCM) as a novel assay to detect ADA for the immunogenicity assessment of therapeutic biologics. QCM is an established real-time label-free analytical method that has been used to study biological interactions at the solution-surface interface [8–12]. In QCM, changes in resonant frequency measurements due to the addition of mass on an oscillating quartz crystal surface are recorded, and the magnitude of these frequency changes is directly proportional to the quantity of added mass (assuming no viscous loss) [13]. Although QCM has been used in the past by the biopharmaceutical industry to study the physicochemical properties of therapeutic biologics [14, 15], there are currently no reports that employ QCM as an assay to detect ADA to investigate the immunogenicity of therapeutic biologics.

Recommended strategies to detect ADA generally involve a three-tiered approach: screening, confirmation, and characterization [16–18]. Serum samples are first screened for ADA. Samples that give a signal above the determined cut point then undergo a confirmatory assay. The cut point represents the threshold value to distinguish positive and negative samples, i.e., a signal above the cut point is considered to relate to a positive sample and one below it, to a negative sample. Cut points are determined by analyzing a pool of serum samples from individuals not treated with the biologic under investigation. The confirmatory assay involves spiking the sample with a high concentration of the biologic and redoing the measurement to observe a significant reduction in signal (typically 50%) [19]. Samples confirmed to have ADA then undergo characterization assays. ADA characterization assays can include isotype determination, epitope mapping, biologic neutralization, and binding affinity, to name a few [20–22].

There are multiple well-established assays currently used to detect ADA. The majority (90%) of biopharmaceutical laboratories simultaneously employ multiple assay types [19]. End-point assays include enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence immunoassay (ECLIA). Real-time label-free assays used to detect ADA include surface plasmon resonance (SPR) and biolayer interferometry (BLI). Each assay has its advantages and disadvantages [18]. ELISA and ECLIA platforms offer high sensitivity and throughput, but have limited capability to detect low-affinity ADA because of washing steps. SPR and BLI, on the other hand, provide measurements in real time and because they are label-free, they require less washing/incubation steps and can thus better detect low-affinity ADA [23, 24]. An advantage of BLI over SPR is that BLI offers high throughput. Unfortunately, SPR and BLI have reduced sensitivity in comparison to ELISA and ECLIA. Differences between the sensitivity of ELISA and SPR platforms can be drastic. For example, comparisons show that ELISA can detect positive-control ADA against panitumumab (Vectibix®) at concentrations as low as 10 ng/mL while SPR was only able to detect 1 μg/mL [24]. Even though SPR is less sensitive, it was capable of detecting ADA against panitumumab in a much more reliable manner (4.1%) in comparison to ELISA (0.3%) because of its ability to detect low-affinity ADA [24]. BLI has also been demonstrated to better detect low-affinity ADA in comparison to ELISA, as well as ECLIA [23]. The capacity of SPR and BLI to detect ADA more reliably can be attributed to their capability to detect low-affinity ADA, which can be lost during the washing steps of ELISA and ECLIA. An additional ADA detection assay that has good sensitivity like ELISA and ECLIA, and is capable of detecting low-affinity ADA like SPR and BLI, could be a valuable tool to assess immunogenicity and accelerate the development of therapeutic biologics.

In this study, modified QCM surfaces were developed and employed in a custom-designed QCM instrument to detect ADA as an assay for the immunogenicity assessment of therapeutic biologics. To our knowledge, there are currently no published reports available that use the principle of QCM to detect ADA. The well-established therapeutic biologic etanercept (Enbrel®) was used. Etanercept is a dimeric fusion protein (MW of 150 kDa) composed of two tumor necrosis factor (TNF) receptors linked to the F_c of an IgG_1 immunoglobulin. Etanercept is a TNF antagonist commonly used to treat rheumatoid arthritis (RA), plaque psoriasis, and ankylosing spondylitis [25]. Rabbits were immunized with etanercept to generate ADA. Purified anti-etanercept ADA were reconstituted in buffer and serum-containing solutions, which were analyzed using the QCM assay. The QCM assay was also used to characterize ADA isotypes (IgG, IgM) and regenerate the detection surfaces to determine if they could be reused. Clinical serum samples from patients treated with RA were also analyzed for the possible presence of ADA.

Materials and methods

Generation of anti-drug antibodies (ADA) to etanercept

Anti-drug antibodies (ADA) to etanercept (known by its trade name as Enbrel®, Amgen/Pfizer) were generated in two rabbits. Immunization was performed in the facilities of Biomatik Corporation (Cambridge, Ontario, Canada). Prior to immunization, serum was collected from each animal to serve as a negative control in ADA measurements. Each animal was immunized over a period of 10 weeks with etanercept. Initially, two consecutive 14-day immunizations with complete Freund's adjuvant (Sigma-Aldrich, Oakville, ON, Canada, Cat. # F5881) were performed followed by two

consecutive 7-day immunizations with incomplete Freund's adjuvant (Sigma-Aldrich, Cat. #F5506). Finally, four consecutive 7-day immunizations with 0.9% NaCl were performed. Serum was collected from each animal on the seventh immunization (56 days) for ELISA and QCM measurements. After immunization, each animal was terminally bled. ADA were then separated from the serum of each animal using protein A/G purification (Pierce™, Pierce, Rockford, IL, USA, Cat # 20421) following the manufacturer protocol.

Quartz crystal microbalance (QCM) instrument

A custom-designed quartz crystal microbalance (QCM) instrument was used in this study. A detailed description of the QCM instrument is available elsewhere [26]. Briefly, QCM frequency measurements are recorded using a network analyzer (Saunders & Associates, Phoenix, AZ, USA, Cat. # 250B). Gold-coated quartz crystals (Inficon, East Syracuse, NY, USA, Cat. # 149273-1) are loaded into QCM chambers (Stanford Research Systems, Sunnyvale, CA, USA, Cat. # O100RH) and capped with fluid injection cells (Inficon, Cat. # 184208). The QCM chambers are screwed at a torque of 0.17 Nm using a customized screw cap. Fluids from syringes are injected at controlled flow rates. The QCM instrument can hold up to four syringes and two QCM chambers. QCM chambers are submerged into a temperature-controlled (± 0.1 °C) water bath. Frequency shift measurements are recorded simultaneously from both chambers from three overtones (5, 15, and 25 MHz). Frequency shift, ΔF, is converted to detected mass, ΔM, using the Sauerbrey equation,

$$\Delta M = -\frac{C_s}{n}\Delta F \quad (1)$$

where C_s is the QCM crystal manufacturer-provided mass sensitivity constant and is equal to 17.85 ng $(cm^2\ Hz)^{-1}$ and n is the overtone. The first, third, and fifth overtones were recorded (n = 5, 15, and 25 MHz, respectively).

QCM surface preparation

QCM crystals (Inficon) were functionalized with amine groups using n-heptylamine plasma polymerization (HaPP) in a custom-designed reactor described elsewhere [27]. The operating parameters of HaPP are also described elsewhere [28]. Carboxymethyl-dextran (CMD) was then immediately covalently grafted to the HaPP-modified QCM surfaces using carbodiimide chemistry as described elsewhere [29]. Etanercept was covalently grafted to the CMD-modified QCM surfaces using a previously developed method [30]. Briefly, etanercept stock solution (50 mg/mL) was diluted into a solution composed of 10 μM $CaCl_2$ to a concentration of 0.22 μM. A CMD-modified surface was then activated for 10 min in 200 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich, Cat. # E1769) and 200 mM N-hydroxysuccinimide (NHS, Sigma-Aldrich, Cat. # 130672). The EDC/NHS-activated crystal was then rinsed one time in Milli-Q water and covered with etanercept solution. The reaction was allowed to proceed for 2 h under gentle agitation. Finally, the modified QCM surfaces were rinsed three times in phosphate-buffered saline solution (PBS, Fisher Scientific, Ottawa, ON, Canada, Cat. # BP6651) and stored at 4 °C until use. See Table S1 (Electronic Supplementary Material, ESM) for a demonstration of the presence of each adlayer via X-ray photoelectron spectroscopy (XPS). Etanercept-modified QCM surfaces were typically used within 3–6 days after modification. If used earlier, they were quenched for 10 min with 1 M ethanolamine hydrochloride (Sigma-Aldrich, Cat. # E6133) solution (pH 8.5). See Fig. S1 (ESM) for a demonstration that quenching is optional after 3 days of storage at 4 °C.

QCM measurements of purified ADA reconstituted in PBS

Etanercept-modified QCM surfaces were loaded into the QCM chambers and manually flooded with PBS solution. The chambers were then equilibrated via a controlled injection of PBS for 10 min at a flow rate of 200 μL/min. Prior to sample analysis, QCM frequency shift measurements were recorded overnight to ensure a flat baseline was achieved. All QCM measurements were performed at 23.0 ± 0.1 °C.

Protein A/G-purified ADA were reconstituted in PBS at concentrations between 500 and 5000 ng/mL. For each analyzed concentration, PBS solution was first injected over the modified QCM surface for 60 min at a flow rate of 27 μL/min. Injection of the ADA-containing sample was then carried out at the same flow rate for 180 min. Finally, there was another injection of PBS for 60 min at a flow rate of 27 μL/min to either close out the experiment or proceed to ADA isotyping and QCM surface regeneration. To confirm the detected mass was attributed to ADA binding to the etanercept-modified QCM surface, a 5000-ng/mL ADA solution spiked with 100 μg/mL of etanercept was also assessed for a reduction in detected mass. The spiked etanercept binds to the ADA in solution and prevents the subsequent binding of ADA to the etanercept-modified QCM surface, so little to no mass should be detected.

To assess ADA isotypes, secondary antibodies to rabbit IgG (Pierce, Rockford, IL, USA, Cat. # 31212) and IgM (Abcam, Cambridge, MA, USA, Cat. # ab97191) were injected at 1/100 dilution in PBS immediately after the second PBS injection. An ADA concentration of 1000 ng/mL was used to assess isotype. Secondary antibody solutions were injected at a flow rate of 27 μL/min for 90 min. After the secondary antibody injection, PBS was injected at a flow rate

of 27 μL/min for 60 min to rinse away any unbounded ADA before data acquisition was stopped.

To regenerate the QCM surfaces, 10 mM glycine-HCl solution (pH 1.5, BioRad Laboratories, Saint-Laurent, QC, Canada, Cat. # 176–2220) was injected immediately after the second PBS injection. Glycine-HCl solution was injected for 90 min at a flow rate of 27 μL/min to dissociate the ADA bounded to the etanercept-modified QCM surface**.** After glycine-HCl injection, PBS was injected at a flow rate of 27 μL/min for 60 min to rinse away ADA that have been dissociated from the etanercept-modified QCM surface before data acquisition was stopped.

QCM measurements of purified ADA reconstituted in rabbit serum

The QCM instrument was prepared and surfaces were analyzed in the same way as for purified ADA reconstituted in PBS. ADA were reconstituted in PBS supplemented with 0.05% Tween®20 (PBST (i.e., PBS with Tween), Fisher Scientific, Cat. # BP337) and 20% normal rabbit serum (Gibco™, Fisher-Scientific, Cat. # 16120-099) at concentrations from 0 to 5000 ng/mL. To reduce nonspecific binding to the QCM surfaces, all solutions were supplemented with 1 mg/mL of CMD. Supplementing solutions with CMD was necessary to reduce nonspecific binding of serum-derived proteins and other molecular species (see Fig. S2 in ESM for demonstration). To confirm detected mass was attributed to ADA binding to the etanercept-modified QCM surface, a 5000-ng/mL ADA solution spiked with 100 μg/mL of etanercept was also assessed for a reduction in detected mass. The spiked etanercept binds to the ADA in solution and prevents the subsequent binding of ADA to the etanercept-modified QCM surface, so little to no mass should be detected.

Enzyme-linked immunosorbent assay (ELISA) of purified ADA

ELISA was performed in the facilities of Biomatik Corporation. Etanercept was diluted in PBS to a concentration of 2 μg/mL and pipetted (100 μL/well) in a 96-well plate (Jet Bio-Filtration Co., Elgin, IL, USA, Cat. # FEP-100-008-1). Wells ("Blank") were also coated with PBS containing no etanercept for controls. With the lids removed, the plates were placed in a 37 °C incubator overnight. All wells were then washed three times in PBST (250 μL/well). Wells were then blocked using 200 μL of 5% milk (Becton, Dickenson and Company, Franklin Lakes, NJ, USA, Cat. # 232100) in PBS for 1 h at 37 °C. Blocking solution was then removed and 100 μL of purified ADA solution was added and incubated for 60 min at 37 °C. Purified ADA solutions at dilutions of 1:8000, 1:16,000, 1:32,000, 1:64,000, and 1:128,000 were analyzed. Stock solutions of purified ADA had concentrations of 4.9 and 5.6 mg/mL for rabbit #1 and rabbit #2, respectively. A 1:1000 dilution of pre-immunized serum ("serum (−)") was also analyzed to confirm adequate specificity. After removal of the purified ADA solutions, wells were washed three times in PBST and incubated with a solution containing a horseradish peroxidase (HRP)-conjugated secondary antibody. The secondary antibody solution was composed of HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA, Cat. # sc-2004) diluted to 1/10,000 in PBST. The secondary antibody solution was then removed and wells were rinsed three times in PBST. Fifty microliters of 0.15% tetramethylbenzidine (Sigma-Aldrich, Cat. # 54827-17-7) was then added to the wells for 10 min. The reaction was stopped by adding 50 μL of 0.5 N sulfuric acid to each well. Optical density readings of the plate were then recorded at 450–655 nm using a Multiskan plate reader (Thermo Fisher, Ottawa, ON, Canada).

Analysis of rabbit serum samples

Serum before and after etanercept immunization was collected from each rabbit and diluted to 10% (*v*/*v*) in PBST and supplemented with 1 mg/mL of CMD. To confirm the detected mass was attributed to ADA binding to the etanercept-modified QCM surfaces, serum collected from the rabbits after immunization was spiked with 100 μg/mL of etanercept and assessed for a reduction in detected mass. For each serum-containing solution, an etanercept-modified QCM surface was installed and equilibrated as described earlier. PBST was first injected for 60 min at 15 μL/min followed by injection of the serum solution for 180 min at 15 μL/min. Afterwards, PBST was injected for 60 min at 15 μL/min to complete the data acquisition.

Analysis of serum samples from etanercept-treated patients

Sera from patients treated with etanercept for rheumatoid arthritis (RA) were assessed for the possible presence of ADA. Sera from RA patients treated with etanercept were provided by the Division of Rheumatology (Department of Medicine, Université de Sherbrooke) after written informed consent following the protocol approved by the Institutional Ethical Committee (Dossier # 2015-490). A cut point was first established using serum separated from the blood donated from healthy volunteers. Blood was collected from healthy volunteers after informed written consent following guidelines approved by an Ethical Committee of the Université de Sherbrooke (Dossier # 2015-490). Serum was separated from whole blood via centrifugation in BD Vacutainer® SST™ tubes (Becton, Dickenson and Company, Franklin Lakes, NJ, USA, Cat. # 367985) following manufacturer instructions.

Sera from healthy donors and RA patients were diluted to 10% (*v*/*v*) in PBST and supplemented with 1 mg/mL CMD. Serum samples from healthy donors were also diluted to 25% (*v*/*v*) in PBST and supplemented with 1 mg/mL CMD. For each serum-containing solution, etanercept-modified QCM surfaces were installed and equilibrated as described earlier. PBST was first injected for 60 min at 15 μL/min followed by injection of the serum solution for 180 min at 15 μL/min. Afterwards, PBST was injected for 60 min at 15 μL/min to complete the data acquisition. The cut point was calculated by averaging the detected mass from the serum of all healthy donors. Serum was collected from 4 healthy donors. Serum samples from 10 different RA patients treated with etanercept were analyzed, and those that resulted in detected mass larger than the cut point were further analyzed using a confirmatory assay. The confirmatory assay involved spiking the sample with 100 μg/mL of etanercept and assessing for a reduction in detected mass.

Results and discussion

Purified ADA can be detected

Solutions composed of purified ADA reconstituted in PBS solution at concentrations between 500 and 5000 ng/mL were used to determine if the QCM assay was capable of detecting ADA. Solutions containing purified ADA represent the simplest possible condition to detect ADA because those solutions contain only ADA and no other biochemical species. The ability of the QCM assay to assess ADA isotype and the feasibility to regenerate and reuse the QCM surfaces were also investigated under this solution condition. Figure 1a serves as a pictorial representation of the events occurring at the etanercept-modified QCM surface for ADA detection, isotype analysis, and regeneration.

As shown in Fig. 1b, QCM sensorgrams clearly show that ADA are detected in a concentration-dependent manner at concentrations as low as 500 ng/mL. This is indicated by an increase in detected mass upon injection of the ADA solution. Regulatory agencies recommend that a sample positive for the presence of ADA be subjected to a confirmatory assay [17]. A straightforward approach to confirm specificity is spiking an ADA-positive sample with a known concentration of the biologic and analyzing for a reduction in detection signal. In industry, this is the most common approach (80% of industry survey respondents) where a 50% reduction in detection signal is commonly used to confirm specificity [19]. This confirmatory approach has also been used in the past when employing SPR, another real-time label-free method to detect ADA [24]. To confirm the detected mass was the result of ADA binding to the etanercept-modified QCM surface, a 5000-ng/mL ADA-containing solution was spiked with 100 μg/mL of etanercept and then analyzed. Spiking of the solution with etanercept resulted in the complete (100%) loss of ADA detection confirming that the detected mass was a result of ADA binding to the etanercept-modified QCM surface. Further confirmation that ADA specifically bound with the etanercept-modified QCM surface was demonstrated when no detected mass was observed (see Fig. S3 in ESM for data) following the injection of a 5000-ng/mL ADA solution over bare CMD-modified QCM surfaces (i.e., no etanercept).

ADA isotyping can be carried out

In general, strategies to detect ADA involve a three-tiered approach: sample screening (detection), confirmation (spiking), and characterization [17, 18]. Together, the results of Fig. 1b illustrate ADA detection is sensitive to the ADA solution concentration and confirm that the adsorbed masses corresponded to bound ADA, as when the solution was spiked with etanercept no mass was detected. Once a sample is confirmed to be positive for ADA, further characterization of the ADA such as isotype assessment may be recommended [17]. Immunoglobulin IgG and IgM are typically the most expected ADA isotypes [17]. In past studies using SPR to detect ADA, secondary antibodies against IgG and IgM were injected to characterize detected ADA isotypes [21, 22].

In this study, secondary antibodies against IgG and IgM isotypes were injected immediately after ADA detection to determine if the QCM surfaces were capable of characterizing detected ADA isotypes. As shown in Fig. 1c, when a secondary antibody against IgG (anti-IgG) was injected after ADA detection, there was a drastic increase in detected mass indicating that the ADA contain IgG isotypes. The anti-IgG antibody's binding to the ADA IgG isotype is pictorially presented in Fig. 1a. Etanercept is a dimeric fusion protein made of two TNF receptors linked to an F_c fragment of an IgG_1 immunoglobulin. To ensure that the anti-IgG antibodies were binding to the ADA and not the F_c portion of the etanercept on the modified QCM surface, anti-IgG antibodies were also injected over an etanercept-modified CMD-bearing QCM surface that had not been exposed to ADA. As shown in Fig. 1c (anti-IgG[no ADA]), there was no increase in detected mass when injecting anti-IgG antibodies to the QCM surface with no ADA. This confirms that the anti-IgG antibody binding was due to IgG isotype ADA and not the F_c portion of the etanercept. When antibodies against IgM isotype (anti-IgM) were injected after ADA detection (Fig. 1c), there was no detected mass indicating that there are no measurable IgM isotype ADA. The absence of IgM isotype ADA can best be explained by the fact that the ADA under analysis was generated from 10 weeks of etanercept immunization. IgM ADA are said to be "transient" as opposed to IgG ADA which are said to be "persistent" [31–34]. Optimal sampling to elucidate

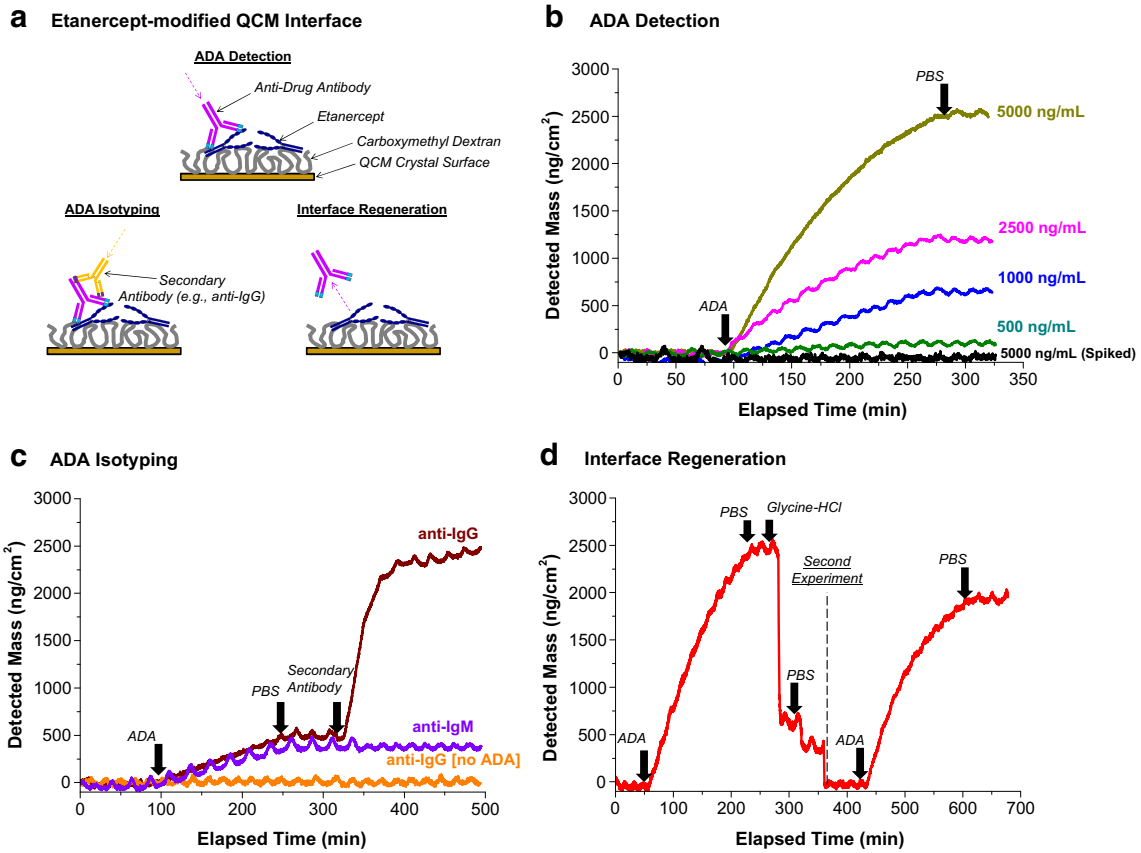

Fig. 1 **a** Pictorial presentation of anti-drug antibody (ADA) interactions at the etanercept-bearing CMD-modified QCM surface during "ADA detection," "ADA isotyping," and following "surface regeneration." **b** QCM sensorgrams for ADA detection. For purified ADA in PBS (500–5000 ng/mL), there is a clear concentration dependence on detected ADA mass. The detected mass was confirmed to be a result of specific interactions between ADA and the etanercept-bearing QCM surface as spiking a 5000-ng/mL ADA solution with 100 μg/mL of etanercept resulted in complete loss of detected mass. **c** QCM sensorgrams showing ADA isotyping. Secondary antibodies against IgG (anti-IgG) and IgM (anti-IgM) isotypes were injected immediately after ADA detection (1000 ng/mL). Injection of anti-IgG antibodies resulted in a drastic increase in detected mass indicating that there is a considerable quantity of ADA of IgG isotype. Specificity of this detected mass being because of binding between the anti-IgG secondary antibody to the detected ADA was confirmed as when injecting anti-IgG antibodies to a bare (no ADA) etanercept-bearing CMD-modified QCM surface, there was no observed detected mass. Injection of anti-IgM antibodies resulted in no observed detected mass indicating there are no measurable quantities of ADA of IgM isotype. **d** Regeneration and reuse of QCM surfaces to detect ADA using glycine-HCl. When a QCM surface that had detected ADA was exposed to glycine-HCl (pH 1.5), there was an immediate loss in detected mass indicating dissociation of the detected ADA. The fact that the baseline returned to zero before the second experiment was initiated indicates that all detected ADA from the first experiment have been dissociated and rinsed away from the QCM surface. The regenerated detection surface was reusable as a second injection of ADA also resulted in a pronounced increase in detected mass

a primarily IgM ADA immunogenic response should be taken between 1 and 2 weeks after exposure to the therapeutic biologic. Since the samples in this study were taken after 10 weeks of exposure and IgM ADA are "transient" in nature, it is not surprising that no detectable levels of IgM isotypes were observed. Not observing IgM isotypes may also be because the ADA were purified using protein A/G. Because of the pentameric structure of IgM, it does not bind well to proteins A and G.

Biosensor surfaces can be regenerated

Previous studies using SPR to detect ADA for the immunogenicity assessment of therapeutic biologics employ a regeneration solution to reuse the biosensor surface [21, 22, 24, 35]. Regenerating and reusing the biosensor surface has two advantages. First, biosensor surfaces are costly and using a surface only once and then discarding it likely makes the experiment unsustainable from a financial standpoint. Second is that reusing the biosensor surface keeps, in theory, the same surface ligand density for each sample making data analysis and sample comparisons more practical and reproducible. We therefore attempted to regenerate and reuse the etanercept-modified CMD QCM surfaces to detect ADA.

A glycine-HCl solution (pH 1.5) was injected after using the QCM surface to detect ADA to determine if the surface could be regenerated and reused. As shown in Fig. 1d, upon injection of glycine-HCL after ADA detection, there is a drastic decrease in detected mass indicating dissociation of ADA from the etanercept-CMD-modified QCM surface. This

dissociation is pictorially presented in Fig. 1a. When PBS was injected to remove the regeneration solution, there was a modest but considerable further decrease in detected mass. This additional loss in detected mass can be attributed to differences in viscosity between glycine-HCl and PBS, as QCM measurements can be sensitive to changes in solution viscosity [26]. This observation shows that glycine-HCl solution is more viscous than PBS. Upon returning to PBS, the solution with which the initial baseline was established, the detected mass did not completely return (Fig. 1d). This indicates that not all of the ADA were dissociated from the QCM surface. Therefore, some of the ADA likely have a high-binding affinity. Prolonged and repeated exposure to an immunogenic antigen has been known to result in affinity maturation of the antibodies that are continually produced to bind to the antigen [31–33]. Since the ADA analyzed were generated after 10 weeks of exposure to etanercept, it can be hypothesized that some of the ADA are of high-enough-binding affinity that glycine-HCl cannot dissociate them from the etanercept bound to the CMD-modified surface.

After regeneration with glycine-HCl, the QCM surface was re-used to assess if it could still detect ADA. The same concentration (5000 ng/mL) of ADA was injected over the regenerated surface. As shown in Fig. 1d, there was a drastic increase in detected mass upon exposure of the regenerated surface to ADA. However, the increase in detected mass was noticeably smaller after regeneration. Challenges inherent when regenerating any detection surface best explain this observation [36]. First, because there appears to be ADA remaining on the surface from the first injection (Fig. 1d), there is less available etanercept for ADA detection relative to the first injection and thus less detected mass. Second, the regeneration solution itself being of such low pH may have compromised the bioactivity of the etanercept on the surface thus resulting in less ADA binding relative to the first injection. Determining the optimal regeneration conditions requires scouting for appropriate chemicals, solution concentration, and solution contact time with the sensor surface [36]. Our results show that the QCM surfaces can be regenerated and reused. However, for this particular experiment, the regeneration condition requires further optimization. This could mean trying regeneration solutions other than low-pH glycine-HCl. A common regeneration solution is high-pH NaOH. Other common solutions include NaCl or $MgCl_2$ at varying concentrations and thus varying ionic strength, and detergents such as sodium dodecyl sulfate.

Purified ADA reconstituted in serum can be detected

Purified ADA were reconstituted in a serum-containing solution composed of PBS solution supplemented with 20% rabbit serum, 0.05% Tween®20, and 1 mg/mL carboxymethyldextran (CMD), and analyzed with QCM. Now that it is demonstrated that the QCM assay can detect ADA in PBS (Fig. 1b), analysis of serum-containing ADA solutions is necessary as these conditions are more typical when analyzing pre-clinical and clinical samples to predict and assess the immunogenicity of therapeutic biologics. Supplementation with CMD was necessary to minimize nonspecific binding of serum proteins (see Fig. S2 in ESM), and supplementing with Tween®20 detergent helped to rinse away the serum-containing solution in the QCM chamber after sample injection.

Purified ADA from two rabbits immunized with etanercept were reconstituted in serum-containing solutions at concentrations between 50 and 5000 ng/mL. As shown in Fig. 2a, for both rabbits, there was clear concentration dependence on ADA detection in serum-containing solutions. Spiking the solutions with 100 μg/mL of etanercept resulted in complete loss of detected mass, confirming ADA detection specificity (Fig. 2a). The QCM assay was capable of detecting ADA in serum-containing solutions at concentrations as low as 50 ng/mL. This is much lower than the lowest detectable concentration of 500 ng/mL for ADA reconstituted in PBS (Fig. 1b). Similar observations have been reported in past studies for ADA detection [37]. Because the detected mass at 50 ng/mL is noticeably higher than the detected mass with no ADA (0 ng/mL), the detected mass at 50 ng/mL is clearly not due to nonspecific binding of serum proteins.

The improved sensitivity using serum-containing solutions in comparison to PBS has two possible explanations. First, serum contains a plethora of proteins which include albumin. Through binding of ions comprising PBS to charged epitopes of serum proteins, the ionic strength of the solution decreases [38]. A decrease in solution ionic strength has been known to result in an increase of the association rate of antigen-antibody reactions [38]. This increase could explain our observed increase in ADA detection sensitivity in comparison to PBS. Second, the presence of a large flexible steric polymer such as CMD can also increase the sensitivity of an antigen-antibody assay. It has been speculated in the past that steric exclusion from PEG in an antibody solution resulted in a higher probability of contact with the antigen, thereby appearing as if the antibody was more concentrated [38, 39]. CMD and PEG have similar steric properties [40]. Therefore, it is reasonable to hypothesize that the addition of CMD can explain the increase in ADA detection sensitivity for serum-containing solutions in comparison to PBS.

Comparing QCM and ELISA sensitivities to detect purified ADA

ADA from each of the etanercept-immunized rabbits were also analyzed using ELISA. ELISA is the most common technique used in the biopharmaceutical industry to detect ADA for the immunogenicity assessment of therapeutic biologics

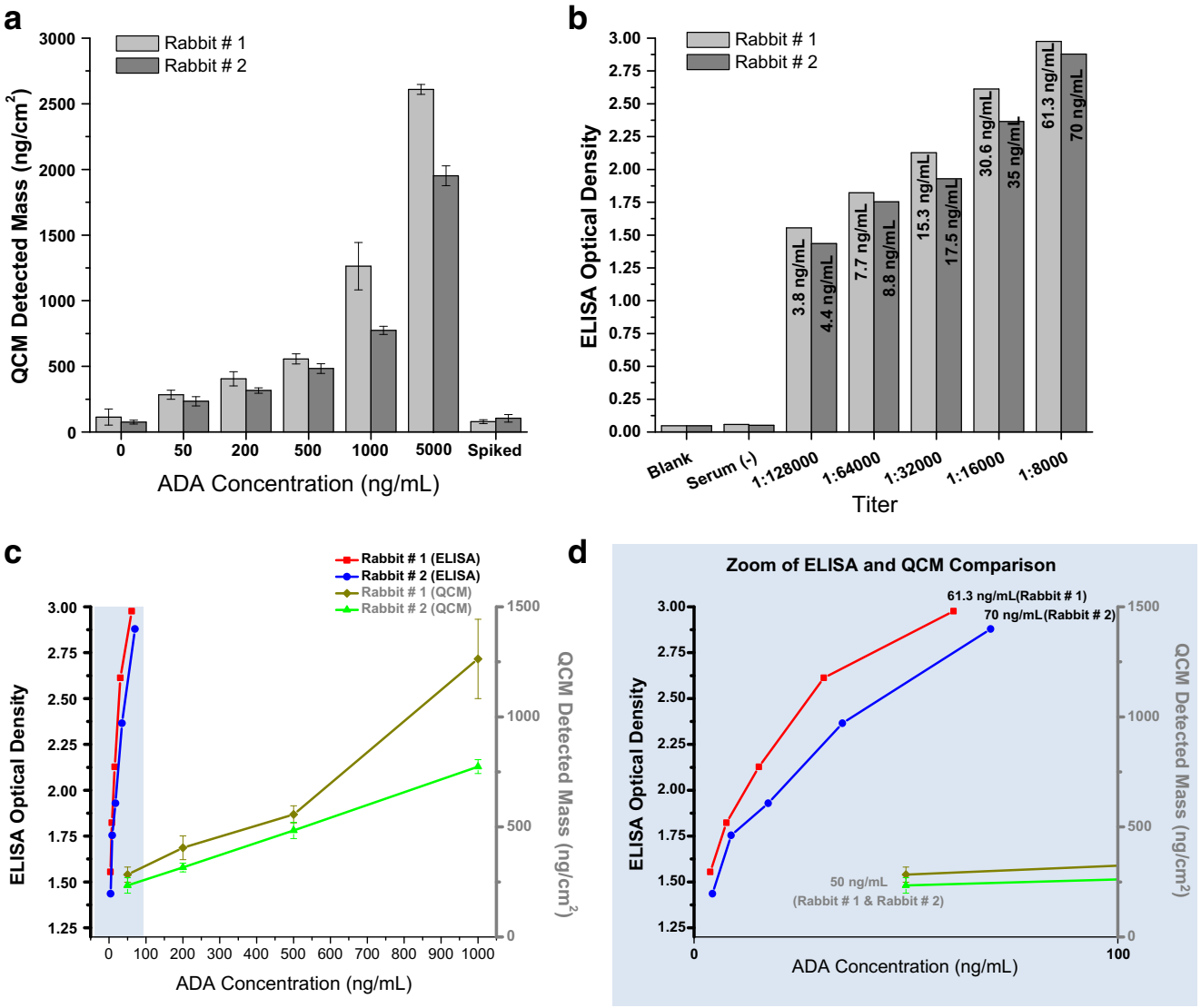

Fig. 2 **a** Measured QCM detected mass using purified ADA reconstituted in serum-containing solutions. Serum-containing solutions were composed of 20% (*v*/*v*) rabbit serum in PBST supplemented with 1 mg/mL of CMD. The QCM surfaces were capable of detecting ADA at concentrations as low as 50 ng/mL. Detection specificity was confirmed via analyzing a 5000-ng/mL ADA concentration spiked with 100 μg/mL of etanercept, which resulted in nearly complete loss of detected mass. **b** ELISA was able to detect ADA at concentrations of 61.3 and 70.0 ng/mL for rabbit #1 and rabbit #2, respectively. **c** QCM clearly detects ADA within the same concentration range of ELISA. **d** Zoom of QCM-ELISA comparison (part **c**, shaded) exemplifying the detection overlap of QCM and ELISA. All data are presented as the average ± standard deviation of frequency recordings of $n = 3$ overtones (5, 15, and 25 MHz)

[19]. As shown in Fig. 2b for each animal, optical density measurements clearly increase as ADA concentration increases. The highest detectable concentrations of ADA before reaching the threshold (optical density = 3.00) were 61.3 and 70.0 ng/mL.

As shown in Table 1, the lowest ADA concentration that could be detected using QCM was 50 ng/mL for each animal. The highest ADA concentration that could be detected using ELISA was 61.3 and 70.0 ng/mL (Table 1). When plotting optical density measurements from ELISA and detected mass, ΔM, measurements from QCM as a function of ADA concentration in a single graph (Fig. 2c, d), it can be clearly seen that QCM is capable of detecting ADA within the concentration range of ELISA. These results are similar to those of a previous study that compared ELISA and SPR assays to detect ADA to panitumumab (Vectibix®), where it was observed that the detection ranges of each assay overlapped [24]. However, in that study, for SPR, the lowest concentration of ADA detected from serum-containing samples was 650 ng/mL. This ADA concentration is much higher than 50 ng/mL, which was the lowest concentration we detected with the QCM assay in serum-containing samples. Our lowest detectable concentration of 50 ng/mL ADA is also lower than that of another previous study that was able to detect ADA to romiplostim

Table 1 ELISA optical density (OD) measurements and QCM surface detected mass, ΔM, measurements for purified ADA

Rabbit #	ELISA		QCM assay	
	Concentration (ng/mL)	OD	Concentration (ng/mL)	ΔM (ng/cm^2)
1	3.8	1.555	50[b]	283
	7.7	1.823	200	405
	15.3	2.128	500	556
	30.6	2.613	1000	1263
	61.3[a]	2.976	5000	2609
2	4.4	1.436	50[d]	234
	8.8	1.754	200	315
	17.5	1.929	500	483
	35.0	2.365	1000	773
	70.0[c]	2.879	5000	1952

[a] Highest ADA concentration detected in rabbit #1 using ELISA

[b] Lowest ADA concentration detected in rabbit #1 using QCM assay

[c] Highest ADA concentration detected in rabbit #2 using ELISA

[d] Lowest ADA concentration detected in rabbit #2 using QCM assay

(Nplate®) at concentrations as low as 200 ng/mL using SPR [41]. Our lowest detectable concentration is also below that of another SPR study which detected ADA to epoetin alfa (EPOGEN®) at concentrations as low as 80 ng/mL [21]. Together, this indicates that the QCM assay may be a more sensitive immunogenicity assay to detect ADA than SPR.

ADA can be detected directly from serum of etanercept-immunized rabbits

Crude serum samples were collected from the immunized rabbits and analyzed for the presence of ADA. These serum samples from animals fully represent samples that biopharmaceutical laboratories would analyze for ADA during pre-clinical drug development. Serum was collected from the animals prior to etanercept immunization (pre-immunization). These samples were used to determine the assay cut point or background signal. Serum samples were also collected after the 10-week etanercept immunization (post-immunization). For an animal to be considered to perhaps have ADA present as a result of an immunogenic reaction to etanercept, the signal from the "post-immunization" serum sample must be higher than that of the "pre-immunization" serum sample.

The results of serum collected from each animal before (pre-immunization) and after (post-immunization) etanercept immunization are presented in Fig. 3. QCM sensorgrams from each animal clearly show more detected mass in serum after the animals were immunized with etanercept in comparison to before immunization. This indicates that each animal potentially has ADA produced because of immunogenicity to etanercept. To confirm the increased detected mass in the serum after etanercept immunization was due to the presence of ADA and not from nonspecific binding to the QCM surface, each sample was spiked with 100 μg/mL of etanercept and reanalyzed. Such a confirmatory assay is recommended by regulatory agencies [17]. As shown in Fig. 3, there was a drastic reduction of detected mass when spiking the post-immunization serum samples with etanercept. The reduction was greater than 50%, which is typically the quantity necessary to confirm a sample has ADA present [19]. It can be confirmed that each animal had ADA present after immunization and thus had an immunogenic reaction to etanercept. In conclusion, the QCM assay is capable of detecting ADA in crude serum samples and can be used to study the immunogenicity of biologics during pre-clinical drug development.

Analysis of serum samples from etanercept-treated rheumatoid arthritis patients

To further confirm the utility of the QCM assay to assess the immunogenicity of therapeutic biologics, serum samples from rheumatoid arthritis (RA) patients treated with etanercept were also analyzed (Table 2). These serum samples from RA patients represent samples that the biopharmaceutical industry would analyze for ADA during clinical drug development.

Determining the cut point of a clinical ADA assay is more challenging than that of pre-clinical as most often it is not possible to collect serum from patients prior to treatment with the therapeutic biologic [19]. Therefore, biopharmaceutical companies use a pool of sera from individuals that have not been treated with the therapeutic biologic. Ideally, the pool of sera includes both healthy individuals and individuals in the disease state that have not been treated with the biologic. In many cases, serum samples from disease-state individuals are not available [19]. When this is the case, using only healthy individuals to determine the cut point can be acceptable,

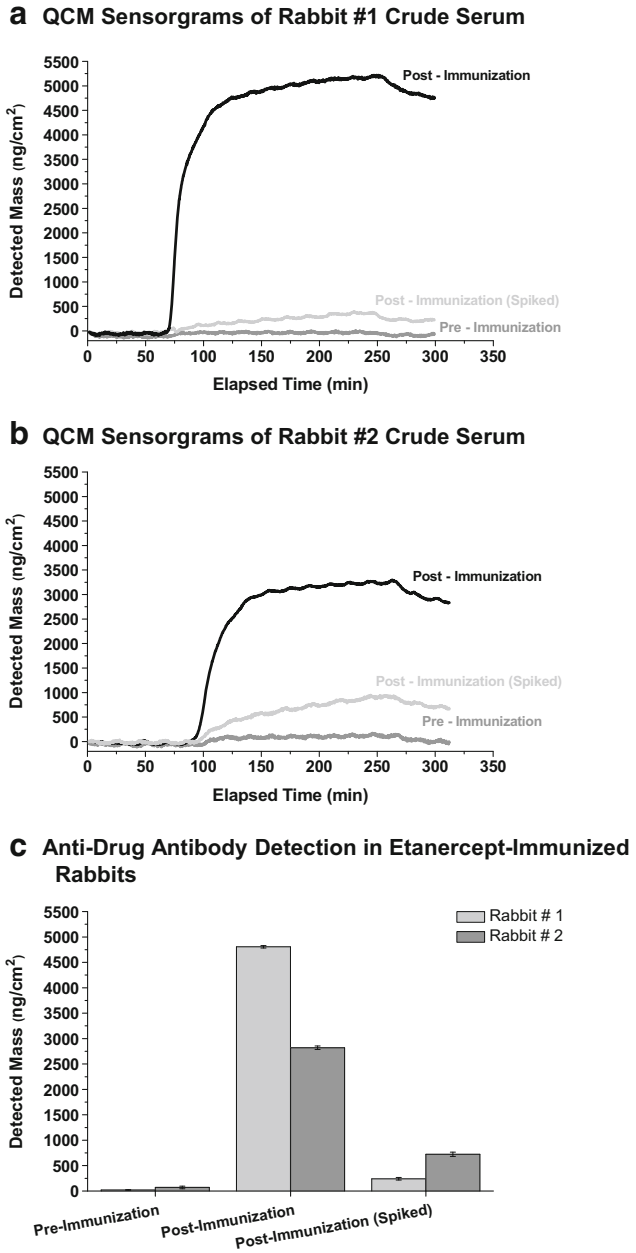

Fig. 3 **a**, **b** QCM sensorgrams of crude serum samples from rabbits before (pre-immunization) and after (post-immunization) etanercept immunization. Samples were diluted to 10% serum (*v*/*v*) in PBST supplemented with 1 mg/mL of CMD. Both animals show negligible levels of detected mass before etanercept immunization. After etanercept immunization, serum from both animals showed considerable detected mass indicating the possible presence of ADA as a result of an immunogenic reaction to etanercept. **c** Spiking post-immunization serum from each animal with 100 μg/mL of etanercept resulted in more than a 50% loss of detected mass, confirming ADA detection specificity. Data are presented as the average ± standard deviation of frequency recordings of $n = 3$ overtones (5, 15, and 25 MHz)

particularly during the early stages of ADA assay development [17]. In this study, we analyzed serum samples from healthy individuals only, as sera from disease-state patients not treated with etanercept were unavailable.

Serum components can bind nonspecifically to the QCM surfaces thereby obscuring ADA detection. This typically occurs when serum samples are undiluted [17]. It is thus recommended that serum samples be diluted to minimize the impact of interfering serum components. Determination of the appropriate dilution usually involves serially diluting serum from healthy individuals until there is minimal background signal [17]. In this study, serum from healthy individuals was analyzed to determine the appropriate dilution. A small number of serum samples may be used to estimate the cut point [17]. In this study, sera from a total of four healthy donors were analyzed to estimate the cut point.

Serum samples should ideally be minimally diluted to keep any possible ADA presence at a detectable concentration. The initial dilution tested was 25% (*v*/*v*) serum (diluted in PBST supplemented with 1 mg/mL of CMD). As shown in Fig. 4a, there was considerable detected mass, ΔM, at 25% (*v*/*v*). This average detected mass of 542 ± 76 ng/cm^2 was comparable to that of 500 ng/mL for purified ADA in serum-containing solutions (Fig. 2a). It was thus concluded that 25% serum resulted in too much nonspecific binding. The next dilution tested was 10% (*v*/*v*) (serum diluted in the same solution as 25%). As shown in Fig. 4b, the average detected mass was 134 ± 46 ng/cm^2. This level of detected mass was on average below that of the lowest detectable ADA concentration of 50 ng/mL in serum-containing solutions. It was therefore decided that serum from RA patients would be diluted to 10% and the assay cut point would be 134 ± 46 ng/cm^2. Sera from a total of 10 patients diagnosed with RA and treated with etanercept (Table 2 for details) were subjected to a screening step using the QCM assay. As shown in Fig. 4c, some RA patient sera resulted in detected mass above the cut point and others below the cut point. Serum samples above the cut point may be positive for ADA and thus should be subjected to a confirmatory assay. Because of the limited availability of sera from RA patients treated with etanercept, it was not possible to perform a confirmatory assay on all serum samples that had detected mass above the cut point. However, we were able to perform confirmatory assays on four of the serum samples that may have ADA present based on the results of the screening step.

Serum samples selected for a confirmatory assay were spiked with 100 μg/mL of etanercept and reanalyzed using the QCM. Typically, when the confirmatory assay has a 50% reduction in comparison to the screening step, the sample is said to be ADA positive [19]. The results of the confirmatory assay for the four selected samples are presented in Fig. 5. Results of the confirmatory assay show that only one of the four selected serum samples (patient #6) had a greater than 50% reduction in detection signal upon spiking with etanercept. Excluding other factors that affect the assessment of ADA levels in RA patients (to be discussed below), this result implies that this patient had an immunogenic reaction to etanercept resulting in the production of ADA. Previous

Table 2 Treatment history and information for rheumatoid arthritis patients treated with etanercept (Enbrel®). One patient was treated with another biologic prior to etanercept treatment. Etanercept treatment was discontinued in favor of other biologics for most patients. Serum analyzed was collected after "Etanercept treatment duration"

Patient #	Treatment before etanercept	Etanercept treatment duration	Etanercept treatment termination	Treatment(s) after etanercept
1	None	17 months	Yes	Abatacept (Orencia®)[a]
2	None	17 months	Yes	Adalimumab (Humira®)[a]
3	None	36 months	Yes	Abatacept (Orencia®)[a]
4	None	48 months	Yes	Abatacept (Orencia®)—3 months rituximab (Rituxan®)[a]
5	None	9 months	Yes	Adalimumab (Humira®)—32 months, adalimumab (Humira®)[a] + duloxetine[a] (Cynbalta®)[a]
6	None	9 months	Yes	Adalimumab (Humira®)—6 months, rituximab (Rituxan®)—4 months, abatacept (Orencia®)—26 months, tocilizumab (Actema®)—19 months
7	Infliximab (Remicade®) —39 months	19 months	Yes	Tocilizumab (Actema®)—21 months, abatacept (Orencia®)[a]
8	None	45 months	No	n/a[a]
9	None	37 months	No	n/a[a]
10	None	47 months	No	n/a[a]

[a] Treatment still active as of January 2016

studies have reported etanercept to have an immunogenicity incidence between 0 and 13.5% [42–45].

Additional factors other than ADA binding can be attributed to observing what may appear to be a patient's

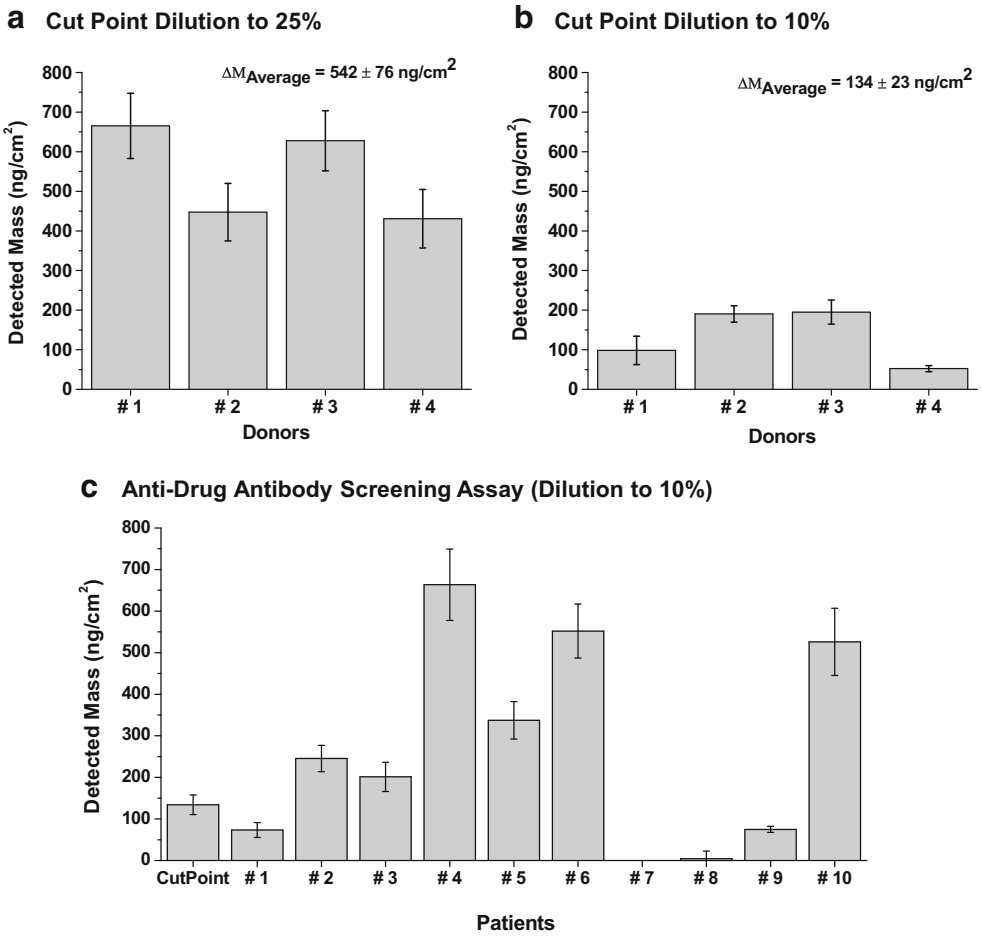

Fig. 4 **a** Analysis of sera from healthy donors using 25% (*v*/*v*) serum (serum diluted in PBST supplemented with 1 mg/mL CMD). Results show considerable levels of detected mass, ΔM, indicative of nonspecific binding and thus insufficient dilution. **b** Analysis of sera from healthy donors using 10% (*v*/*v*) serum. QCM results show negligible levels of detected mass indicating little to no nonspecific binding. Clinical serum samples from RA patients treated with etanercept were thus diluted to 10% (*v*/*v*). The average detected mass was 134 ± 24 ng/cm^2. This average was used as the cut point. **c** Screening assay using sera from 10 RA patients treated with etanercept. Results show that sera from 6 of 10 patients resulted in detected mass above the cut point indicative of the possible presence of ADA. All data are presented as the average ± standard deviation of frequency recordings of $n = 3$ overtones (5, 15, and 25 MHz)

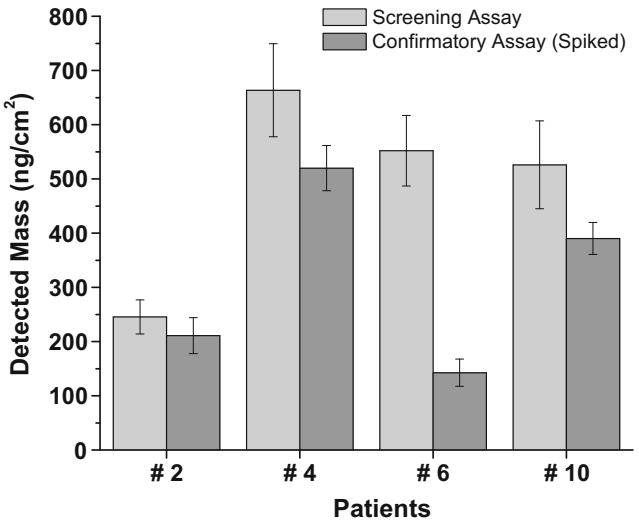

Fig. 5 Confirmatory assay of selected serum samples from RA patients treated with etanercept that resulted in detected mass above the cut point during the screening assay (Fig. 4c). Serum samples were spiked with 100 μg/mL of etanercept and reanalyzed. Results show that only one of the patient serum samples (patient #6) had a noticeable (> 50%) reduction in detected mass. Data are presented as the average ± standard deviation of frequency recordings of $n = 3$ overtones (5, 15, and 25 MHz)

immunogenic reaction to etanercept. This is particularly true when evaluating the serum of RA patients as was done in this study. RA patients have a high incidence (63–79%) of rheumatoid factor (RF) present in their sera [43, 46, 47]. RF can exist in many Ig-isotypes but it is most commonly an IgM antibody that recognizes IgG isotypes [48]. It is thus possible that RF, if present, could bind to the IgG-Fc portion of etanercept [17]. Consequently, this could result in the false perception of ADA being present in patient #6 (Fig. 5). Since the presence of RF cannot be ruled out of patient #6, we cannot decisively conclude that the serum of patient #6 is ADA positive. However, the objective of this study was not to evaluate the immunogenicity of etanercept, but to evaluate QCM as a novel assay to detect ADA for the immunogenicity assessment of therapeutic biologics. Our analysis of RA patient sera demonstrates the QCM assay is a viable method that could be used to evaluate the immunogenicity of therapeutic biologics. Using the QCM assay, we were able to analyze sera from healthy donors at multiple dilutions to estimate a cut point, perform a screening assay using sera from RA patients treated with etanercept, and perform a confirmatory assay on samples that during the screening resulted in detected mass above the estimated cut point. Carrying out each of these steps regardless of the ADA detection method is necessary when evaluating the immunogenicity of therapeutic biologics during clinical drug development.

Fully demonstrating the utility of the QCM assay to evaluate the immunogenicity of therapeutic biologics during clinical drug development would require multiple additional tests. A better cut point needs to be estimated by analyzing a larger number of serum samples from a more diverse population of subjects. Ideally, a cut point is estimated from both healthy male and female volunteers, and from treatment-naive subjects suffering from the disease. A previous clinical immunogenicity study used sera from 20 healthy male, 20 healthy female, and 20 treatment-naïve subjects to estimate their cut point [24]. In this study, sera from only 4 healthy male subjects were used to estimate the cut point. A larger number of patients treated with the therapeutic biologic should also be analyzed for ADA to more accurately determine the rate of immunogenicity. In this study, sera from only 10 RA patients treated with etanercept were analyzed for ADA. Previous studies that assessed the clinical immunogenicity of etanercept for RA treatment analyzed between 40 and 297 samples [42–45]. Additional tests should also be performed to determine if factors other than ADA are contributing to the detected mass. These factors could include RF (in the case of RA patients) and pre-existing antibodies to name a few [49, 50]. Finally, a direct comparison between the QCM assay and another more common ADA detection assay should be performed. More specifically, the comparison should determine if the QCM assay detects ADA in clinical serum samples at a greater, equal, or lesser incidence in comparison to other more common assays such as ELISA, SPR, or ECLIA.

The detection of ADA can be difficult when an excess of the therapeutic biologic is present in the serum. Should circulating ADA bind to excess therapeutic biologic forming an ADA-biologic complex, ADA could possibly go undetected [6]. Should there be ADA-etanercept complexes in any of the RA patient serum (Table 2) analyzed by the QCM assay, ADA could possibly go undetected resulting in false negatives. To reduce the possibility of such false negatives, an acid dissociation procedure has been developed to free ADA from ADA-biologic complexes allowing for detection [51, 52]. An acid dissociation step has been developed for SPR ADA detection assay [53]. Such an acid dissociation step could possibly be developed into this QCM assay to further demonstrate the utility to evaluate the immunogenicity of therapeutic biologics.

Conclusions

There is a need in the biopharmaceutical industry to assess immunogenicity during the development of therapeutic biologics. The present study demonstrates the QCM assay as a novel method to detect ADA for the immunogenicity assessment of therapeutic biologics. QCM sensorgrams clearly showed the detection of ADA in a concentration-dependent manner. The QCM assay detected ADA at concentrations between 50 and 5000 ng/mL. This range falls within the *United States Food and Drug Administration*'s (*US FDA*) recommendation that ADA assays must have a sensitivity of at least

100 ng/mL [17]. ADA detection specificity was also confirmed as when 100 μg/mL of etanercept was spiked to an ADA-containing sample, a noticeable (> 50%) reduction in detected ADA was observed. In addition, the QCM assay is also capable of assessing ADA isotype, a characterization also recommended by the US FDA [17]. In addition, the QCM assay surfaces can be regenerated and reused, allowing for better comparison of samples and reduction of experimental costs. Furthermore, the QCM assay yields reproducible detected mass with the same ADA concentration and consistent detected mass with a single ADA concentration in various serum dilutions. Data highlighting such reproducibility and consistency is available in Fig. S4 (ESM).

The QCM assay offers similar advantages to those of SPR and biolayer interferometry (BLI). Like SPR, QCM provides label-free real-time measurements. It is believed that because SPR and BLI require fewer rinsing and incubation steps in comparison to ELISA, SPR better detects low-affinity ADA [23, 24, 54]. Because QCM requires a similar number of rinsing and incubation steps as SPR and BLI, it is reasonable to hypothesize that QCM is also capable of detecting low-affinity ADA. The major drawback of SPR and BLI in comparison to ELISA is a reduced sensitivity [23, 24]. In this study, the lowest ADA concentration the QCM detected was 50 ng/mL. This lowest concentration of ADA is below that of previous studies that used SPR to detect ADA. The lowest reported ADA concentrations detected using SPR and BLI are 80 and 130 ng/mL, respectively [21, 23]. Direct comparisons of QCM to SPR and BLI would have to be performed to more decisively determine which real-time label-free assay is the most sensitive to detect ADA.

QCM is not without its disadvantages. Intrinsic disadvantages of QCM are described in detail elsewhere [26]. Briefly, QCM is sensitive to temperature and the Sauerbrey equation underestimates the detected mass. Even though temperature in this study was controlled to ± 0.1 °C, QCM is so sensitive to temperature that small oscillations can be observed in many of the QCM sensorgrams (Figs. 1 and 3). The Sauerbrey equation assumes that the added mass is completely rigid [55]. Added masses on the QCM crystals in this study including CMD grafting, etanercept immobilization, and ADA detection are certainly not completely rigid. As a result, the Sauerbrey equation underestimates detected mass [56]. Extrinsic disadvantages of QCM are similar to those of SPR. QCM has modest throughput in comparison to BLI, ELISA, and ECLIA making it more time consuming and expensive. Therefore, QCM is likely a less attractive option when assessing clinical immunogenicity in which thousands of samples can require analysis.

Despite the drawbacks, QCM is a promising novel assay to detect ADA that complements other techniques for the immunogenicity assessment of therapeutic biologics. Additional tests to further confirm the full potential of QCM to assess immunogenicity should be performed. One additional test could be to assess the drug tolerance limit. The biologic drug itself can interfere with an assay's capacity to detect ADA resulting in false negatives [49]. In this study, only one concentration (100 μg/mL) of drug (etanercept) present in a sample was tested to confirm assay specificity. It would be interesting to test a number of drug concentrations in an ADA-positive sample to compare the drug tolerance of QCM to that of other immunogenicity assays, as has been done previously [23]. Another test could be to directly compare the QCM's capability to detect low-affinity ADA in comparison to other immunogenicity assays using ADA of known affinity as also done previously [23]. This test would confirm if QCM can detect low-affinity ADA as effectively as other real-time label-free immunogenicity assays such as SPR and BLI. Finally, another additional test could be to compare the incidence of ADA detection in QCM to that of other immunogenicity assays using a large number of clinical samples, as was done in a previous study [24]. This would compare the performance of QCM to detect ADA in ill-defined samples where drug interference and ADA of varying affinities may or may not be present. Such a study would evaluate the QCM's potential to producing false negatives. Together, QCM may be a valuable assay to detect ADA.

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Compliance with ethical standards

Ethical approval The collection of all serum samples was done in accordance with approval of the Ethical Committee of the Université de Sherbrooke (Dossier # 2015-490). Work with rabbits was performed in accordance with internal ethical guidelines of Biomatik.

Conflict of interest The authors declare that they have no conflict of interest.

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