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Immobilization of a HER2 Mimotope-derived Synthetic Peptide on Au and Its Potential Application for Detection of Herceptin in Human Serum by QCM

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Abstract

Therapeutic antibodies are antigenically similar to human antibodies and are difficult to detect in assays of human serum samples without the use of the therapeutic antibody's complementary antigen. Herein for the first time we established a platform to detect Herceptin in solutions by using a small (< 2.2 kDa), inexpensive, highly stable, HER2 Mimotope-derived synthetic peptide immobilized on the surface of Au quartz electrode. We used the HER2 mimotope as a substitute for the HER2 receptor protein in piezoelectrode or quartz crystal microbalance (QCM) assays to detect Herceptin in human serum. We demonstrated that assay sensitivity was dependent upon the amino acids used to tether and link the peptide to the sensor surface, and the buffers used to carry out the assays. The detection limit of the piezoelectrode assay was 0.038 nM with a linear operating range of (0.038 – 0.859 nM). Little non-specific binding to other therapeutic antibodies (Avastin and Rituxan) was observed. Levels of Herceptin in serum samples obtained from treated patients, as ascertained using the synthetic peptide-based QCM assay, were typical for those treated with Herceptin. The findings of this study are significant in that low cost synthetic peptides could be used in a QCM assay, *in lieu* of native or recombinant antigens or capture antibodies, to rapidly detect a therapeutic antibody in human serum. The results suggested that a synthetic peptide bearing a particular functional sequence could be applied for developing a new generation of affinity-based immunosensors to detect a broad range of clinical biomarkers.

Introduction

Therapeutic monoclonal antibodies (MAbs) have been used to treat human disease. Currently more than 20 MAbs such as Bevacizumab (also known as Avastin), Cetuximab (Erbix), Rituximab (Rituxan) and Trastuzumab (Herceptin) are being used worldwide to treat conditions including cancer, autoimmune diseases, allergy, cardiovascular disease and transplant rejection.¹ Around 300 antibodies are undergoing clinical development and 2915 clinical studies involving antibodies are being carried out.² Herceptin, for instance, the first FDA-approved humanized MAb for human cancer therapy³ exhibits the ability to inhibit the proliferation of breast tumors by specifically binding to the extracellular domain IV segment⁴ of HER2(human epidermal growth factor receptor 2) in the membrane of breast cancer cells. Herceptin has been widely used for the treatment of HER2 positive breast cancer since its approval in 1998.^{3,5,6}

However, approximately 1 in 20 patients will produce antibodies to human therapeutic antibodies.⁷ In some human patients, therapeutic antibodies can be rapidly cleared from the body;² and, as such, will not be of benefit to the patient. Additionally, the therapeutic activity of a MAb is dependent upon its serum half-life. MAbs used to kill tumor cells or inhibit cell growth need to have a long half-life, while those used as immune-modulatory agonists should have a shorter half-life. If the MAb serum half-life is too short or too long, then the MAb may not be therapeutically efficacious or may produce deleterious effects. The physician's decision-making process is often dependent upon clinically established protocols that may not be suited to every patient. As such, physicians will need assays to monitor serum therapeutic antibody levels to determine if antibody dosage is appropriate to elicit a positive therapeutic effect.

Typically, immunoassays are used to determine antibody concentrations in human serum. Physicians use the results of such assays to determine how best to treat patients. At present, a rapid, simple, highly sensitive, inexpensive assay is not readily available for the quantification of therapeutic antibodies in biological specimens. To date, enzyme-linked immunosorbent assays (ELISAs) are still the most widely used technique to detect Herceptin in human serum or plasma.^{8–11} However, there are limitations of the ELISA method for detection of Herceptin in solutions. First, an ELISA is costly and time-consuming. Antibodies for completing an ELISA assay are \$200–300 per 100 ug per antibody. These are expensive items. ELISAs require multiple steps and the Herceptin antigen (i.e. HER2) used to carry out the assays is not easily obtained. Genentech, the manufacturer of Herceptin, measures the level of Herceptin in patients by using the extracellular domain of the HER2 receptor as the coating antigen.⁸ Maple and coworkers used a full-length HER2 protein as the coating antigen since Genentech's antigen was not commercially available.⁹ Jamieson, et al described a cell-based ELISA for detection of Herceptin;¹¹ however, cell-based assays are difficult to standardize (i.e. cell growing and plating conditions can vary). Additionally, Herceptin was designed with human IgG₁ constant domains¹² and is immunologically similar to normal human antibodies. As such, it can be difficult to distinguish Herceptin from normal serum antibodies by using traditional immunological reagents and assays. Therefore, new bioassays are needed to detect therapeutic antibodies in human samples.

Quartz crystal microbalances (QCMs) have been recognized as a standard tool to detect biomolecular interactions (e.g. antigen or peptide/antibody interactions) in real-time without using labels (e.g. fluorescent dye or enzyme-conjugated secondary antibodies). Based on our previous work, QCM can be used with Au coated quartz crystal as a transducer to detect antibody-binding events.^{13–15} Jiang, et al.¹⁶ used phage display to identify peptides (i.e. mimotopes) that could be used *in lieu* of the HER2 receptor to develop a breast cancer vaccine. We modified one of the HER2 mimotopes (QLGPYELWELSH) and used it to develop a piezoimmunosensor assay to detect Herceptin in solutions. The peptide was redesigned to contain 7 additional amino terminal (CGSGSGS) amino acids to facilitate peptide binding and immobilization on a QCM Au sensor surface. This work demonstrated that a short synthetic peptide (M.W. less than 2.2 kDa, Table 1) could be used to develop an inexpensive, rapid, sensitive, specific and reusable piezoimmunosensor to detect a humanized therapeutic antibody (e.g. Herceptin) in human serum. Moreover, our results suggested that a commercially obtainable synthetic peptide was able to act as a replacement antigen for the HER2 receptor protein in QCM piezoimmunosensor assay to detect Herceptin in human serum with a high sensitivity and specificity.

Chemical and biological reagents

Short peptides, designated as CH-19, RH-18, CH-17, CH-13, and CS-7 (primary sequence shown in Table 1), were chemically synthesized by Bio. Basic, Inc. (Ontario, Canada) and

received in a lyophilized condition. The quality of all the peptides was assessed by high performance liquid chromatography (HPLC) and confirmed through matrix-assisted laser desorption/ionization (MALDI) mass spectrometry analysis (Purity > 95%). Therapeutic MABs such as Herceptin® (Trastuzumab), Avastin® (Bevacizumab), Erbitux® (Cetuximab), and Rituxan® (Rituximab) were provided by Beaumont hospital, Royal Oak, Michigan. The UltraPure™ distilled water (Cat. No. 10977-015) and phosphate buffered saline (PBS, Cat. No. 10010-049) were obtained from Invitrogen Corporation. Hepes buffered saline (HBS Cat. No. BR-1003-69, Br-1003-68) was obtained from GE Healthcare (Piscataway, NJ). Normal human serum samples as well as 3 HER2-positive breast cancer patient samples (collected right before infusion and immediately post-infusion of Herceptin) were all obtained through Beaumont Hospital BioBank. Patient samples were drawn with full informed consent using an IRB-approved protocol. All other related chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and used without further purification.

Characterization methods

Peptide immobilization onto Au transducer sensor surface was performed as described with some modifications.^{13–15,17–19} Briefly, one side of the freshly cleaned and N₂ dried Au quartz crystal (AT-cut 10 MHz, non-polished with ~1000 Å gold, geometric area is 0.23 cm², International Crystal Company) was immersed in a peptide solution at a concentration of 1–1.5 mM in ultra-pure water. After an overnight incubation at 4 °C, the surface of the modified Au electrode sensor was rinsed thoroughly with bio-grade water and placed in 1 ml of PBS, UltraPure™ distilled H₂O, HBS or HBS-EP buffer (pH 7.4). The piezoimmunosensor (also known as Quartz Crystal Microbalance (QCM) immunosensor) was then characterized using an Agilent network/spectrum/ impedance analyzer (Agilent 4395A).

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to characterize the peptide-modified QCM gold surface. All experiments were carried out using a three-electrode system with a bare or modified gold electrode as the working electrode, a platinum wire as the counter electrode and a Ag/AgCl reference electrode (saturated KCl) incorporating a potentiostat/galvanostat (EG&G Par Model 2263). One ml of 0.1 M NaClO₄ containing 1 mM K₃Fe (CN)₆/K₄Fe(CN)₆ (1:1) was used as supporting electrolyte. The CV potential was scanned from –0.25 to 0.75 V at a scan rate of 50 mV/s. The EIS measurements were obtained by applying a 5 mV amplitude sine wave under bias at open circuit potential within a frequency window range of 0.01 Hz to 100 KHz. Atomic Force Microscopy (AFM) was used to characterize the morphology of the peptide modified surface and its binding with Herceptin. AFM studies were performed using a Molecular Imaging Picoplus microscope from Agilent Technologies, CA, USA. The AFM images were acquired in AC mode with a scan rate of 1.0 line/s in air as well as in buffer solution. Silicon cantilevers with a spring constant of $k = 3.5 \text{ Nm}^{-1}$ and resonant frequency of 75 kHz were used for all the experiments. Au(111) on mica were obtained from Agilent Technologies, CA, USA. Before the AFM imaging, Au(111) surface was annealed using H₂ flame for a few minutes to remove any contaminants.

Results and discussion

HER2 Mimotope-derived peptides modification and immobilization on gold

The HER2 Mimotope-derived peptides, **QLGPYELWELSH** and **GPYELWELSH**, which exhibited specific binding activities to Herceptin, were previously reported by Jiang et al.¹⁶ In order to develop label free piezoimmunosensors for the detection of Herceptin in solution, the peptides were engineered with Cysteine (C) or arginine (R) to be immobilized on gold and correctly orient onto the transducer surfaces (Table 1). As shown in Scheme 1, each

peptide contained three parts: 1) surface coupling amino acids [(**Cysteine (C)** or **Arginine (R)**], 2) a spacer sequence (**GSGSGS** or **RGRGRG**) to eliminate steric hindrance between Herceptin and immobilized peptides, and 3) the functional HER2 Mimotope-derived peptide sequence (red, in Table 1). Except for CS-7, all the other 4 peptides contained the HER2 Mimotope-derived amino acid sequence **GPYELWELSH** bearing either an amino terminal cysteine (peptides CH-13, CH-17 and CH-19) to couple peptides directly (via the cysteine – SH group) to the gold QCM electrode, or arginine (peptide RH-18) for coupling of positively charged arginine to a negatively charged ω -mercapto undecanoic acid (MUA) monolayer. Peptides CH-17 and CH-19 were synthesized with spacer (**GSGSGS**) amino acids while peptides CH-13 and RH-18 were not. Peptide CH-19 contained the amino acids glutamine (Q) and leucine (L) on the amino terminal end of the **GPYELWELSH** peptide to enhance the Herceptin/peptide binding interaction (Table 1).¹⁶ Multiple QCM (Figure 1a), CV (Figure 1b) and EIS (Figure 1c) measurements were carried out to determine the influence of surface amino acid coupling, spacer amino acids, and Herceptin/HER2 Mimotope-derived peptide enhanced binding amino acids (i.e. Q and L) had on sensor assay sensitivity and specificity.

Binding of Herceptin onto QCM sensor surfaces to which HER2 Mimotope-derived peptides were immobilized varied and was apparently influenced by peptide design. Herceptin interacted strongly with peptide CH-19 (**CGSGSGSQLGPYELWELSH**) bearing the surface binding amino acid **C** and the **GSGSGS** spacer, and bound to a lesser extent to the RH-18 peptide (**RGRGRGQLGPYELWELSH**) bearing the surface binding amino acids **RGRGRG**. The QL amino acids presented in CH-19 (**CGSGSGSQLGPYELWELSH**) and absent in CH-17 (**CGSGSGSGPYELWELSH**) dramatically enhanced the Herceptin/HER2 Mimotope-derived peptide interactions. In comparison to CH-19 (**CGSGSGSQLGPYELWELSH**), Herceptin bound poorly to CH-13 (**CQLGPYELWELSH**) that had the same **QLGPYELWELSH** sequence as CH-19, but lacked the **GSGSGS** spacer. Herceptin, Avastin and Rituxan binding to the bare Au sensor surface were negligible, elevated on the CH-13 sensor surface and appreciable for Avastin and Rituxan on the MUA sensor surface. These results suggested that Herceptin was able to bind specifically to the **QLGPYELWELSH** peptide only when immobilized on an inert gold sensor surface via **C** and the **GSGSGS** spacer.

To ascertain the selectivity of the functional HER2 Mimotope-derived peptide sensing region, a negative control QCM real-time measurement was run. As shown in Table 1, the control sequence, CS-7, only consisted of two parts, the surface coupling amino acids **C** and the spacer sequence, **GSGSGS**, with no HER2 Mimotope-derived peptide present. The results of the negative control are shown in green in Figure 1. The CS-7 modified Au electrode surface exhibited a negligible frequency shift response not only to the control drugs (i.e., Avastin, Rituxan) but also to the target MAb, Herceptin, indicating a high degree of selectivity of Herceptin to the HER2 Mimotope-derived peptide attached to the label free piezoimmunosensor. For ease of comparison, the results of QCM, CV and EIS bode plot analysis (Figure S1–2) for all the HER2 Mimotope-derived peptides are summarized in Table S1. Taken together, among all four peptide designs, the CH-19 peptide-modified surface showed the highest binding capacity with Herceptin and the lowest nonspecific binding activity with the control drugs (i.e., Avastin, Rituxan).

AFM Characterization of CH-19 peptide immobilization and binding with Herceptin

QCM, CV and EIS results (Figure 1) clearly demonstrate that Herceptin binds specifically with the synthetic peptide CH-19 on a gold sensor surface. AFM was used to further characterize the immobilized CH-19 binding to Herceptin.

Figure 2a shows the AFM image of the CH-19 peptide on the Au(111) surface in air. It can be seen from the image that CH-19 peptide forms self-assembled monolayers (SAMs) on the Au(111) surface. Sulfur (e.g. cysteine) containing molecules form SAMs on gold due to the strong specific interaction of sulfur with gold.^{20,21} One of the first reports of SAMs on gold involved the self-assembly of alkanethiols on gold.²⁰ Since then, SAMs have been used to study molecular, cellular and biological interactions of other functional groups involving cell signaling, cell adhesion and protein interactions.^{22–25} SAMs of synthetic polypeptides on gold have also been reported.^{26–28} In Figure 2a, self-assembly of CH-19 peptide on the Au(111) surface is through the Au-S covalent bond formation by interaction of the –SH groups in cysteine residues of CH-19 peptide with Au(111) surface. Typical features of SAMs having large numbers of pit like depressions (e.g. Figure 2a arrows) with a depth of 0.5–0.6 nm can be readily observed by AFM imaging.^{29,30} Formation of fewer defects indicates that the monolayers are closely packed.³¹ The AFM image of the CH-19 peptide SAMs in PBS buffer (Figure 2b) are similar to that observed in Figure 2a although it is less clearly resolved due, presumably, to the PBS buffer used for the *in situ* imaging. To investigate the CH-19 peptide-Herceptin interaction on Au(111), Herceptin was exposed to CH-19 peptide SAMs in PBS buffer for 1 hour. After incubation, the surface was washed several times with PBS to remove non-specifically adsorbed Herceptin from the sensor surface. AFM images of the surface were recorded in PBS buffer. AFM images in Figure 2c shows several particles 20–25 nm in diameter with a 0.1 nm average height. The AFM observation of adsorbed particles on the surface suggests that the CH-19 peptide-Herceptin interaction occurred as evidenced by QCM (Figure 1a). To further confirm the interaction and the dimensions of the adsorbed Herceptin particles, AFM images of the surface were taken again after removal of PBS and sensor surface drying by N₂ gas. Figure 2d shows the AFM image of the surface where Herceptin particles of 20–25 nm diameter (as shown by the height distribution profile) are similar to those obtained for CH-19 SAMs (Figure 2c). Surface features of CH-19 SAMs in Figure 2d onto which Herceptin particles are adsorbed are quite similar to those in Figure 2a thus confirming the self-assembly of CH-19 peptide on the Au(111) surface as well as CH-19 peptide and Herceptin interaction.

Analytical performance of the HER2 Mimotope-derived peptide piezoelectrode CH-19 for Herceptin detection

The aforementioned results demonstrated that Herceptin specifically binds to the HER2 Mimotope-derived peptide, CH-19 on a QCM gold sensor surface with high sensitivity (Figure 1). We further investigated the CH-19 Herceptin immunosensor for its potential use in determining Herceptin concentration in simple buffers. In Figure 3a, QCM was also used as the transducer to characterize the CH-19 modified gold QCM electrode and to monitor the binding activity between the immobilized HER2 Mimotope-derived peptide and various therapeutic antibody drugs in real-time. The CH-19 modified gold QCM electrode was exposed, sequentially, to small amounts (e.g. 1 μ l drug spiked into 999 μ l of PBS buffer to give a final concentration of 1 μ g/ml) of different antibodies (Rituxan, Cetuximab and Avastin). There was nearly no response ($\Delta F \sim 0$ Hz) when Rituxan, Cetuximab and Avastin, respectively, were sequentially spiked into the QCM cuvette bearing the CH-19 peptide (Figure 3a). A ΔF response was only seen when 1 μ g of Herceptin was added to the same cuvette. These results further verified that the CH-19 peptide-based piezoelectrode specifically detects Herceptin. Once Herceptin was spiked into the QCM cuvette bearing the CH-19 peptide, a large frequency shift ($\Delta F \sim 225$ Hz) was observed. According to Sauerbrey's equation,³² the interfacial changes in mass (Δm) caused by the molecular deposition onto a sensor surface are directly related to the resonant frequency shifts (ΔF) on the gold QCM electrode surface. The equation can be simply expressed as $\Delta F = -C \Delta m$, where C is a constant. And, based on our previous studies,^{33,34} a fitted ΔF of 1 Hz corresponds to about 1 ng of Δm for the 10 MHz quartz crystal which was employed in this

work. Through this equation, we can roughly estimate that about 225 ng of Herceptin bound to the CH-19 modified gold QCM electrode. This indicated that the CH-19 modified gold QCM electrode had fairly high affinity for Herceptin ($K_a = 7.96 \times 10^8 \text{ M}^{-1}$) (Figure S3). Furthermore, the results of EIS and CV confirmed the QCM results (Figure 3b–c). The redox peaks of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ probe gradually disappeared with the surface mass deposition of CH-19 and Herceptin (Figure 3b). The Nyquist plots (semi-circles in Figure 3c) reflecting electron transfer resistance of the redox probe enlarged step-by-step after the surface modification from bare Au to CH-19 and binding with Herceptin later on.

The CH-19 peptide interaction with Herceptin was rapid. Herceptin could be detected within minutes with changes in frequency varying as the concentration of Herceptin varies (Figure 4a). Traditionally, the higher the concentration of analyte (e.g. Herceptin) in solution, the larger the CH-19 QCM sensor frequency would shift. The frequency change terminated once the CH-19 sensor surface became completely bound by, and saturated with, Herceptin. As shown in Figure 4b, the CH-19 peptide-based piezoelectrode exhibited a sensitivity of $0.52 \text{ Hz} \cdot \text{ml}/\text{ng}$ and a detection limit of $10.70 \text{ ng}/\text{ml}$ (deduced according to $S/N = 3$, $N \sim 1.85 \text{ Hz}$) in PBS buffer.

HER2 Mimotope-derived peptide immunosensor optimization

In order to optimize the binding capacity of CH-19 sensor, we investigated the binding activity between CH-19 to Herceptin under different buffer conditions. One μl of Herceptin at a concentration of $1 \mu\text{g}/\mu\text{l}$ was spiked into a CH-19 bearing QCM cuvette containing 999 μl of H_2O , PBS or HBS-EP buffer. Herceptin binding to the CH-19 sensor surface varied and was dependent upon buffer conditions with binding (varying from high to low) as follows: $\text{HBS-EP} > \text{PBS} > \text{H}_2\text{O}$ (Figure 5a). Buffering effects on the Herceptin/CH-19 interactions were similar when the concentration of Herceptin spiked into 999 μl of buffer was lowered 10 fold to $0.1 \mu\text{g}/\text{ml}$ (Figure 5b). Based on these results, it appears that different buffers can influence the Herceptin/CH-19 interactions, and that buffers optimal for use in immunoassays can be quickly determined using QCM.

Traditionally, HBS-EP buffer contains 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20. As a zwitterionic organic chemical, Hepes has been widely used and categorized as a “Good” buffer for maintaining physiological pH.³⁵ EDTA is considered as a masking agent to sequester metal ions that would interfere with the analyses within the detection environment. Moreover, the addition of appropriate amount of surfactant Tween-20 in the buffer system may assist in preventing non-specific binding. Presumably, HBS-EP buffer facilitates proper CH-19 peptide conformational folding, orientation and immobilization on the gold QCM surface for efficient Herceptin binding.

Peptide-based piezoelectrode to quantify Herceptin in human serum sample

In order to quantify Herceptin in human blood samples, we established a Herceptin standard curve in HBS-EP buffer first. Six calibrators containing 0, 5, 10, 62.5, 100, and 125 $\text{ng}/\mu\text{l}$ of Herceptin in HBS-EP buffer were prepared prior to each QCM assay. During QCM measurement, a 1-microliter volume of each calibrator was spiked into the assay cuvette containing 999 μl of HBS-EP buffer and diluted 1:1000 times. Consequently, as shown in Figure 6, Herceptin standard concentration curve was established and ranged within 0–125 ng/ml . The comparison of analytical parameters of the CH-19 piezoelectrodes in different buffering systems was summarized in Table S2. The detection limit of the CH-19 piezoelectrode assay could be as low as 0.038 nM with a linear operating range of (0.038 – 0.859 nM).

Typically, an immunoassay would be used for detection of antibody concentration in human blood samples. In order to validate the practicability of CH-19 piezoimmunosensor for analyzing a clinical sample, we further applied it to determine Herceptin concentration in human serum. However, sugars, fats, amino acids, urea, and a myriad of proteins, especially IgGs in human serum can bind non-specifically and interfere with or inhibit ligand/analyte interactions to produce false positive assay results. In general, normal human serum contains 4–16 g/l of IgG. The IgG subclass levels in the WHO-reference serum were assessed at 5.0–9.0 g/l for IgG1, 2.6 g/l for IgG2, 0.4 g/l for IgG3 and 0.5 g/l for IgG4, respectively^{12,36}. These values have been recommended by the International Union of Immunological Societies (IUIS) as the human IgG subclass levels' standard. For this concern, non-specific binding effects within human serum sample particularly on the surface of CH-19 piezoimmunosensor was evaluated and determined by comparing frequency changes upon human serum sample addition followed by free Herceptin injection. As shown in Figure 7a, the free Herceptin could be detected by the CH-19 piezoimmunosensor in simple buffer easily ($\Delta F \sim 275$ Hz). However, it could hardly be recognized ($\Delta F \sim 5$ Hz) once an undiluted pooled normal human serum is applied prior to the sensor surface (Figure 7b). Instead, if a diluted normal serum replaced the undiluted one, non-specific binding reduced and Herceptin binding increased significantly (Figure 7c).

Most likely, the sensor surface was fouled by the non-specific binding of components in undiluted serum; hence we decided to test the level of Herceptin in real patient samples by using serum diluents. In addition, based on current clinical data, therapeutic antibody concentration in patient serum could be in the range of 10–80 $\mu\text{g/ml}$ or 60–500 nM.³⁷ Also, previously published methods for Herceptin detection by Enzyme-Linked Immunosorbent Assay (ELISA) reported a Herceptin concentration range of 10–200 $\mu\text{g/ml}$ in human serum⁹ and 21–441 $\mu\text{g/ml}$ in plasma¹¹, respectively. We analyzed both serum validation samples and real serum samples from patients who were treated with Herceptin therapeutic MAb drugs. In detail, human serum samples were diluted 1:10 in buffer. One microliter of the diluted human serum sample was applied to the QCM cuvette bearing the mimotope and containing 1 ml of buffer. Herceptin concentrations were then determined by measuring the frequency change by using an Agilent network/spectrum/impedance analyzer (Agilent 4395A), and then comparing measured results to the calibration curve in Figure 6. The results are summarized in Table 2.

Validation samples were prepared from the Herceptin standard in normal human serum at three concentrations (10 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$ and 40 $\mu\text{g/ml}$ in 10% normal human serum). The ΔF of each validation sample was obtained by using normal human serum on the surface of CH-19 modified Au as a blank to calibrate the nonspecific binding. The ΔF s for all 3 patient samples were obtained by calculating the differences between frequency shifts in pre- vs. post-Herceptin infusion. According to the calculations, the concentration of Herceptin in the 3 patient sera varied between 43.34 $\mu\text{g/ml}$ and 193.18 $\mu\text{g/ml}$ (Table 2). These results were in agreement with previously published results in which Herceptin concentration (10–200 $\mu\text{g/ml}$) in human serum samples was determined using an ELISA.⁹

Concluding remarks

Generic biological (e.g. bio-similars or follow-ons) are currently under development to replace and reduce the healthcare costs associated with brand name biological therapeutics (e.g. Bevacizumab). Generic biologicals will undoubtedly increase the number of clinical trials and uses for therapeutic antibodies in humans. Therapeutic antibodies have been engineered or developed to assume most, if not all, of the immunological features of a normal human antibody so that they will not be overly immunogenic in most humans and will not be readily eliminated from the body after administration. Thus there will be

increasing need for rapid, accurate low cost assays to determine the clinical efficacy of these drugs for therapy. Fast, highly sensitive, stable and inexpensive assays to detect therapeutic antibodies in human serum samples do not exist. In this study, we demonstrated for the first time that a short synthetic peptide mimotope could be used as a “surrogate antigen” in a piezoimmunosensor assay development to detect a therapeutic antibody in human serum samples. The CH-19 HER2 mimotope could be immobilized on the surface of Au and function as a substitute for the HER2 receptor protein to detect Herceptin in serum samples. The CH-19 piezoimmunosensor assay was rapid (approximately 20–30 minutes) and capable of detecting Herceptin with a detection limit of 0.038 nM (Table S3) both in simple buffers and in human serum samples. Short synthetic peptides (15–20 amino acids) are generally more stable and easier to chemically synthesize in comparison to antibodies or recombinant proteins that need to be produced using prokaryotic or eukaryotic cells. . Therefore, synthetic peptides can be more readily synthesized on a large scale under controlled conditions to ensure peptide quality and batch-to-batch reproducibility. As such, the cost to produce a peptide-based immunosensor can be significantly reduced. The novel piezoimmunosensor possesses the potential to be used in clinical diagnostics to determine if the level of a particular therapeutic antibody such as Herceptin is sufficient to be clinically efficacious for a breast cancer patient. And, since peptide CH-19 could be easily immobilized on the surface of Au, it can also be used to develop a SPR (Surface Plasmon Resonance) Herceptin immunosensor. Furthermore, peptide CH-19 is also suitable for developing a new type of ELISA if the peptide is conjugated to maleimide activated carrier protein. The carrier protein bearing the peptide can be immobilized onto the micro-titer plate for use as a surrogate antigen. We believe our approach using a small peptide *in lieu* of an antigen for immunosensor development represents an improvement on the application of affinity-based biosensor technology for clinical diagnostics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

HER2	human epidermal growth factor receptor 2
QCM	quartz crystal microbalance
Mab	monoclonal antibody
ELISA	enzyme-linked immunosorbant assay
scFv	single chain variable fragment recombinant antibody
HPLC	high performance liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
PBS	phosphate buffered saline
HBS	Hepes buffered saline

HBS-EP	HBS containing 3 mM EDTA, and 0.005% Tween-20
CV	Cyclic voltammetry
EIS	electrochemical impedance spectroscopy
AFM	atomic force microscopy
MUA	ω -mercapto undecanoic acid
SAM	self-assembled monolayer
IgG	Immunoglobulin G antibody

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Figure 1.

(a) The frequency change vs time curve: bare gold electrode (black), CS-7 (green), CH-13 (dark cyan), RH-18 (dark yellow), MUA (magenta), CH-17 (blue), and CH-19 (red) modified Au QCM electrodes were exposed to small amount (1 μ l spiking in 1 ml PBS) of various MAb drugs (1 μ g/ μ l) sequentially. (b) CVs of 1 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ in 0.1 M $NaClO_4$ on bare gold electrode (black) and other 6 modified electrode surfaces. Scan rate, 50 mV/s. (c) EIS Nyquist plots. Frequency range was 0.01 Hz-100 kHz, ac amplitude 5 mV.

Figure 2.

Sequentially obtained AFM images of CH-19 peptide monolayer (a) self-assembled on Au(111) surface (in dry condition), (b) in PBS buffer, (c) after addition of Herceptin in PBS buffer, and (d) after removal of PBS buffer and drying under N_2 atmosphere. Scan area for all the images $1 \times 1 \mu m^2$. 40 μl of 1 mM CH-19 peptide solution (prepared in double distilled water) was put on the freshly annealed Au(111) surface at $4^\circ C$ for overnight incubation. After incubation, the Au(111) surface was thoroughly washed with double distilled water followed by PBS to remove non-specifically adsorbed peptide. The surface (d) was dried under N_2 atmosphere before AFM imaging to eliminate PBS/salt imaging interference effects..

Figure 3.

(a) The frequency change vs time curve: CH-19 modified Au QCM electrode was exposed to small amount (1 μ l spiking in 999 μ l PBS) of various MAb drugs (1 μ g/ μ l) sequentially. (b) CVs of 1 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ in 0.1 M $NaClO_4$ on bare gold electrode (black), CH-19 modified electrode (red), and CH-19 binding with Herceptin (blue). Scan rate, 50 mV/s. (c) EIS Nyquist plots. Frequency range was 0.01 Hz-100 kHz, ac amplitude 5 mV.

Figure 4.

(a) The comparison of frequency change vs time curve: CH-19 modified Au QCM electrode was exposed to small amount (1 μ l spiked into 1 ml PBS) of Herceptin at 5 different concentrations. (b) The frequency change vs concentration of Herceptin in PBS buffer.

Figure 5.

The frequency change vs time curve of Herceptin detection in different solutions: **(a)** 1 μ l Herceptin stock solution (1 μ g/ μ l); **(b)** 1 μ l Herceptin stock solution (0.1 μ g/ μ l) was spiked in CH-19 immunosensor system containing 999 μ l H₂O (black), PBS (red) and HBS-EP (blue) buffer, respectively.

Figure 6.

Herceptin standard concentration curve. Herceptin was spiked into QCM cuvette bearing HER2 mimotope-derived peptide (CH-19) containing 999 ul of HBS-EP. Results depict QCM frequency change (ΔF) with changes in Herceptin calibrators' concentration.

Figure 7.

The frequency change (ΔF) vs time curve of Herceptin binding capacity evaluation in different solutions: (a) 1 μ l Herceptin stock solution (1 μ g/ μ l) (black arrow); (b) 1 μ l undiluted pooled normal human serum first, Herceptin stock solution (1 μ g/ μ l) second (red arrows); (c) 1 μ l diluted pooled normal human serum first, Herceptin stock solution (1 μ g/ μ l) second (blue arrows) were spiked in CH-19 immunosensor cell containing 1 ml HBS-EP buffer, respectively.

Scheme 1.

Schematic illustration of surface assembly of HER2 Mimotope-derived peptide-based piezoimmunosensor for the detection of Herceptin.

Table 1

List of HER2 Mimotope-derived peptide designs.

Designated Name	Purity (%)	Primary sequence ^{<i>i-iii</i>}	M.W. (g/mol)
CH-19	> 95.81	CGSGSGSQLGPYELWELSH	2007.15
CH-17	> 96.16	CGSGSGSGPYELWELSH	1765.86
CH-13	> 95.15	CQLGPYELWELSH	1574.76
RH-18	> 96.30	RGRGRGQLGPYELWELSH	2111.33
CS-7	>98.31	CGSGSGS	553.55

Notes:

^{*i*} Surface coupling amino acids are **C** or **R**;^{*ii*} Linker amino acid sequence used as a spacer is **GSGSGS** or **RGRGRG**;^{*iii*} HER2 Mimotope-derived sequence is in red.

Table 2

Herceptin sample assay by QCM measurements.

Validation sample analysis						
Sample number	Prepared Concentration of standard Herceptin (µg/ml) in 10% normal serum	Frequency shift readout (Hz)	ΔF (Hz)	Tested C of Herceptin (µg/ml) in 10% normal serum ⁱ		
1	10	236.18	7.80	8.81		
2	20	247.34	18.96	19.97		
3	40	267.95	39.57	40.58		
Blank	0	228.38	0	0		

Breast cancer patients' sample analysis						
Patient	Gender	Age	Disease information	Collection time	Frequency shift readout (Hz)	Tested C of Herceptin (µg/ml) in undiluted serum ⁱⁱ
#1	female	52	Stage IV metastatic breast cancer, ER PR HER2/neu positive	Pre-infusion	223.97	121.96
				Post-infusion ~2hrs	235.16	
#2	female	61	Stage IV positive HER2/neu breast cancer, ER PR negative	Pre-infusion	168.38	43.34
				Post-infusion ~2hrs	171.71	
#3	female	44	Metastatic ER PR Her2/neu positive, invasive ductal adenocarcinoma of breast with sarcomatoid differentiation	Pre-infusion	226.64	193.18
				Post-infusion ~2hrs	244.95	

Notes:

ⁱThe concentrations of Herceptin in each validation sample were calculated based on the equation $C = (\Delta F + 1.0022) \div 0.9997 \mu\text{g/ml}$;

ⁱⁱThe concentrations of Herceptin in each patient sample after infusion of Herceptin were calculated based on the equation $C = (\Delta F + 1.0022) \div 0.9997 \times 10 \mu\text{g/ml}$