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Highly sensitive detection of HER2 extracellular domain (ECD) in the serum of breast cancer patients by piezoelectric microcantilevers (PEMS)

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Abstract

Rapid and sensitive detection of serum tumor biomarkers are needed to monitor cancer patients for disease progression. Highly sensitive piezoelectric microcantilever sensors (PEMS) offer an attractive tool for biomarker detection, however their utility in the complex environment encountered in serum has yet to be determined. As a proof of concept, we have functionalized PEMS with antibodies that specifically bind to HER2, a biomarker (antigen) that is commonly overexpressed in the blood of breast cancer patients. The function and sensitivity of these anti-HER2 PEMS biosensors was initially assessed using recombinant HER2 spiked into human serum. Their ability to detect native HER2 present in the serum of breast cancer patients was then determined. We have found that the anti-HER2 PEMS were able to accurately detect both recombinant and naturally occurring HER2 at clinically relevant levels (>2 ng/ml). This indicates that PEMS-based biosensors provide a potentially effective tool for biomarker detection.

Keywords

Piezoelectric microcantilever sensor (PEMS); biosensor; HER2; biomarker; antibody; antigen

Introduction

Detecting cancer early in its development is one of the largest factors associated with successful treatment outcome[1]. Unfortunately for many types of cancer (e.g., ovarian cancer, pancreatic cancer, etc) the first outward symptoms appear late in disease progression. Therefore, early detection will need to be based upon assays for cancer biomarkers in biological fluids such as serum, sputum or urine. Various serum biomarkers have been identified that can aid in determining the presence of or the progression of some types of cancer. For example, the presence of high levels of CA125 is associated with ovarian cancer[2] and prostate specific antigen (PSA) is associated with prostate cancer[3]. However, it is important to note that each of these biomarkers may also indicate the presence of benign diseases as CA125 is present in patients with endometriosis[4] and PSA can be a sign of

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prostatitis (inflammation of the prostate)[5]. On the other hand, the knowledge of circulatory levels of other biomarkers, such as cytokines released at pg/ml levels as part of an inflammatory response, can also be critically important in assessing adverse responses to biologic therapeutics[6,7]. As a consequence, in order to be clinically useful, future cancer detection assays will likely require the use of panels of relevant biomarkers, as well as the capability of detecting extremely low quantities of biomarkers in blood.

The most commonly employed method for detecting biomarkers in biologic fluids is the enzyme-linked immunosorbent assay (ELISA), a colorimetric antibody-based capture assay. While the ELISA technique is extremely reliable, it is typically only sensitive down to the ng/ml range. Another label-based method, known as Luminex, has been developed to quantitatively detect proteins using antibodies immobilized on polystyrene beads containing fluorescent dyes. Although this method is rapid and sensitive, it's use has sometimes been limited by non-specific binding of protein in human serum[8]. More recently, new classes of biosensors have been developed that are capable of detecting biomarkers in a rapid and label-free manner. Nanowire sensors[9] and optical fluid ring resonator biosensors[10] have been effectively functionalized with antibodies and employed to specifically bind to clinically-relevant antigens. One particular class of sensors, known as microcantilevers, operate based on changes to their resonance frequency as a consequence of binding of proteins or cells[11,12]. Microcantilevers functionalized with antibodies have been reported to be effective for the detection of a number of protein biomarkers including activated leukocyte cell adhesion molecule (ALCAM), a biomarker for pancreatic carcinoma[13] and PSA[14].

We have focused on developing piezoelectric microcantilever sensors (PEMS) as sensitive and rapid biosensors for the detection of cancer biomarkers in human serum. In particular, PEMS composed of a highly piezoelectric layer, lead magnesium niobate–lead titanate $(PbMg_{1/3}Nb_{2/3}O_3)_{0.63}$–$(PbTiO_3)_{0.37}$(PMN-PT)[15] have been shown to exhibit an enhanced detection resonance frequency shift three orders of magnitude larger than could be accounted for by mass change alone due to the polarization switching-induced Young's modulus change in the PMN-PT layer[1617]. In addition, it has also been shown that the length mode of a PEMS can better withstand liquid damping[18] for *direct, in-situ liquid* detection. With Young's modulus change-induced sensitivity enhancement, the length mode of PEMS containing an 8-μm thick PMN-PT layer was shown to be capable of directly detecting white spot syndrome viruses (WSSV) at 100 virions/ml concentration, matching the sensitivity limit of polymerase chain reaction (PCR) in detecting WSSV[19].) While PEMS-based biosensors have the potential for extremely sensitive detection of proteins, their ability to detect biomarkers in the rich mixture of proteins found in human serum has yet to be demonstrated.

We have selected HER2 (HER2/*neu*; C-ErbB-2) as a proof of concept target to determine the ability of PEMS to detect biomarkers in serum, with the understanding that successful clinical utility of PEMS biosensors will require their future validation in the detection of biomarkers that are present in minute quantities in the serum. HER2 is a trans-membrane tyrosine kinase growth factor receptor in the epidermal growth factor receptor family. HER2 is present at low levels in a number of healthy tissues and is significantly over expressed in approximately 30% of human breast cancer cases[20], classifying it as a tumor associated antigen. HER2 over expression is frequently associated with a poor prognosis in metastatic breast cancer[21] and is the target for the antibody trastuzumab (Herceptin), which has been licensed by the U.S. F.D.A. for the treatment of breast cancer. HER2 over expression is typically assessed by immunohistochemistry (IHC) or fluorescence *in situ* hybridization (FISH) assays performed on tissue samples and not on biologic fluids. However, as the extracellular domain (ECD) of HER2 is enzymatically cleaved (shed) and released into the

circulation, HER2 levels can also be measured in blood samples[22]. The quantity of HER2 in blood can be a reflection of the presence of a tumor as healthy individuals typically exhibit HER2 levels of 2 to 15 ng/ml while breast cancer and prostate cancer patients can exhibit HER2 levels as high as 15 to 75 ng/ml[212324]. Circulating HER2 levels have been reported to be useful for monitoring early disease relapse, cancer progression or response to therapy[25–28]. Furthermore, approximately 3–5 % of breast cancer patients assayed were found to exhibit elevated serum HER2 ECD in the absence of positive IHC results[2930], suggesting a potential role for monitoring biomarkers in disease detection and treatment followup. As with many of the other cancer biomarkers discussed above, the sensitivity limits (ng/ml) and reliability of ELISA-based assays make them more than sufficient for the quantitative detection of HER2 ECD in serum[3121]. While we expect that the true utility of PEMS-based biosensors will be in the detection of biomarkers present at even lower levels, it is first necessary to validate their function in serum using a biomarker like HER2 that can be simultaneously assessed using widely accepted technology (e.g., ELISA).

For the studies we describe here, PEMS functionalized with the anti-HER2 monoclonal antibody (MAb), trastuzumab, were evaluated using recombinant human HER2 ECD spiked into blood samples at concentrations that would be encountered in the clinically setting. The sensitivity of detection was compared to that obtained using a commercial anti-HER2 ELISA kit. The physical presense of HER2 ECD bound by the anti-HER2 PEMS was verified visually using confocal microscopy. To determine the potential clinical relevance of our results, anti-HER2 PEMS and anti-HER2 ELISAs were used side-by-side to determine the concentration of naturally-occuring HER2 ECD biomarker in the serum of women undergoing treatment for breast cancer. Our results indicate that PEMS-based biosensors are capable of reproducible detection of naturally occurring cancer biomarkers in the serum of cancer patients.

Materials and Methods

PMN-PT cantilever production

PMN-PT piezoelectric microcantilever sensors (PEMS) were constructed from freestanding PMN-PT films of 8μm in thickness as previously described[32]. Briefly, a 30-nm thick nickel layer and a 15–30 nm thick chromium/nickel-bonding layer were deposited on one side of PMN-PT freestanding film by evaporation (E-gun Evaporator, Semicore Equipment, Livermore, CA) to serve as an electrode for plating. A 2-μm thick nonpiezoelectric copper layer was then electroplated on to the nickel surface at a rate of 500 nm/min using a plating solution of copper sulfate. A 150-nm thick gold was then evaporated on to both sides of the film. The PMN-PT/Cu bilayer was then embedded in wax and cut into the cantilever shape using a wire saw (Princeton Scientific Precision, Princeton, NJ). Finally, wires were attached to the top and bottom electrodes using conductive glue (XCE 3104XL, Emerson and Cuming Company, Billerica, MA) and the PMN-PT/Cu strips were glued to a glass substrate to form functional microcantilevers. The insulation of the PEMS was performed using mercaptopropyltrimethoxysilane (MPS) as previously described[32]. In brief, PEMS were submerged in 0.1 mM MPS (Sigma Aldrich) diluted in ethanol for 30 minutes and dried in a vacuum-oven (Model 1400E, VWR International) at 762 mm Hg, 37°C, overnight. The PEMS were then submerged in a 1% (volume) of MPS diluted in ethanol (titrated to a pH 4.5 with acetic acid) for a total of 36 hours with the solution changed every 12 hours. The PEMS were then dried in a vacuum-oven (Model 1400E, VWR International) overnight at 762 mm Hg (37°C).

Functionalization of PEMS with the anti-HER2 MAb, trastuzumab

The anti-HER2 monoclonal antibody trastuzumab (Herceptin) was maleimide-activated by incubation with Sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate (sulfo-SMCC) (Pierce Biotechnology) at a molar ratio of 1:80 (antibody: sulfo-SMCC) for 30 minutes at room temperature. Excess sulfo-SMCC was removed by centrifugation through a microcon filter (Millipore) with a molecular weight cutoff of 10kDa. The MPS-insulated PEMS were then soaked in a solution containing the sulfo-SMCC-linked trastuzumab for 30 minutes, followed by submersion in 3% BSA blocking solution for an additional 30 minutes at room temperature. The PEMS-functionalized with trastuzumab (Anti-HER2 PEMS) were then transferred to a 3.5 ml custom-made flow cell containing PBS-EDTA (1x PBS, 0.5mM EDTA, pH 7.2) circulating at a rate of 0.7 ml/minute via a peristaltic pump (model 77120-62, Cole-Parmer's Master Flex).

Detection of HER2 ECD by anti-HER2 PEMS

The extracellular domain (ECD) of HER2 was cloned and expressed as previously described[33]. A flow system containing a flow cell and a reservoir interconnected with long tubing and driven by a peristaltic pump as described in detail by Capobianco et al.[19] was used for the detection. Anti-HER2 PEMS were immersed in the middle of the flow cell containing PBS-EDTA and were allowed to obtain a stable baseline for at least 20 minutes. Normal human serum was obtained from a healthy male volunteer without breast cancer under an institutional IRB-approved protocol. The serum was assayed by ELISA using the procedures outlined below and was determined to contain 3.68 ng/ml, falling within the reported range for a healthy male[24]. HER2 ECD was prepared, diluted in the serum and injected into the flow cell to reach the indicated concentrations (ranging from 0.05–2.00 ng/ml) and the vibrational frequency of the PEMS was recorded for 90 minutes as previously described[32]. Each concentration was repeated in triplicate. Analysis was performed as described below.

Detection of HER2 ECD in breast cancer patient serum by anti-HER2 PEMS

Serum samples were obtained under an IRB approved protocol from the Fox Chase Cancer Center Biosample Repository. Serum samples from 7 patients with HER2 positive breast cancer and 3 healthy control individuals were included in this study with seven positive and three negative samples (as determined by IHC). Anti-HER2 PEMS were equilibrated in the flow cell as described above until a stable baseline was obtained for a period of at least 20 minutes. 75 μl of patient serum was injected into the flow cell in order to obtain a final dilution of 1:40 (patient serum: PBS-EDTA). The PEMS frequency shift was recorded for 90 minutes. Each sample was tested in triplicate. After each detection cycle, the PEMS were completely stripped by submersion in a 1:100 dilution of piranha solution (two parts of 98% sulfuric acids with one part of 30% hydrogen peroxide) for 30 seconds, rinsing once with deionized water, and twice with 95% ethanol to completely remove the water. The PEMS were then submerged in a sealed container of a 1% (volume) MPS and ethanol titrated to a pH 4.5 with acetic acid for 8 hours, rinsed with ethanol and allowed to air dry before refunctionalizing for reuse as described above.

Detection of HER2 ECD in breast cancer patient serum by ELISA

The concentrations of both recombinant HER2 ECD spiked into normal serum and HER2 ECD naturally occurring in serum samples obtained from patients with breast cancer were determined using a commercial ELISA detection kit. Assays were performed according to the manufacturer instructions in triplicate using c-erbB2/c-neu Rapid Format ELISA kit (Calbiochem/EMD) which is widely employed by life scientists for the detection of HER2 in

human serum[2131]. ELISA plates were read on a LabSystems MultiSkan Plus plate reader (Fisher).

Visual determination of HER2 ECD binding by anti-HER2 PEMS

In order to verify that HER2 ECD was specifically bound by the PEMS, visual verification was obtained by confocal fluorescence microscopy imaging. The conjugation of the green fluorophore, fluorescein isothiocyanate (FITC) (Cat # 46425 Thermo Scientific), and the red fluorophore, Alexa Fluor 555 (Cat # A20009 Invitrogen), to trastuzumab and HER2 ECD, respectively, were performed according to the manufacturers procedures. PEMS were submerged in 1 μg/ml of trastuzumab-conjugated to FITC for 30 minutes at room temperature, rinsed with deionized water to remove the unbound antibody and submerged in 3% BSA for 30 minutes. Next, the PEMS were submerged in 2.0 ng/ml of HER2 ECD-conjugated to Alexa Fluor 555 for 30 minutes at room temperature. The unbound antigen was removed by rinsing the PEMS with deionized water and the functionalized PEMS were imaged using an Inverted TE2000 Nikon C1 confocal scanhead microscope.

Data analysis

PEMS vibration was measured in the length mode in the 200–1000 kHz range as the peaks in this range exhibit high Q values and data analysis was performed as previously described[15]. Briefly, the relative frequency shift (Df/f) was calculated based on the equation below:

$$Df/f=(F_{sample} - F_{baseline})/F_{baseline}$$

where $F_{baseline}$ is the average of 20 sampled points of the background (before sample injection) and F_{sample} is the average of the last 20 points sampled during the detection period as previously described[32]. The data presented were smoothed with 10-point adjacent averaging to reduce the noise. The quantification limit of PEMS and ELISA were determined from the data generated in assays performed in triplicate based on the equation below:

$$\text{Quantification limit} = 10 \times \text{standard deviation of the blank}^{34}$$

Statistical significant of the differences between the results obtained by ELISA and PEMS was determined using a two tailed t test (Graphpad software). P values <0.05 were considered statistically significant. MedCalc was used to perform a Passing and Bablok regression analysis and a Cusum test. The regression equation and the 95% confidence intervals for the slope and intercept were calculated.

Results and Discussion

Detection of HER2 ECD by anti-HER2 PEMS

PEMS functionalized with the anti-HER2 MAb trastuzumab were employed to detect recombinant human HER2 ECD spiked into normal human serum. Antigenic specificity of the anti-HER2 PEMS was determined by head-to-head measurements of the ability of the PEMS to bind 2 ng/ml recombinant human HER2 or Bovine Serum Albumin (BSA) in normal human serum applied to the flow cell. The results presented in Figure 1A show binding of HER2 ECD and no binding of BSA to the anti-HER2 PEMS. As the 2 ng/ml concentration of HER2 ECD employed in this assay represents the low end of the normal range of HER2 in human serum (2 ng/ml to 15 ng/ml)[23], these results indicate that the anti-

HER2 PEMS were capable of selectively detecting clinically-relevant concentrations of a cancer biomarker.

As many cancer biomarkers are present at lower concentrations than HER2 ECD, we performed a series of assays employing recombinant human HER2 ECD concentrations over a range of 0.05 – 2 ng/ml. The results presented in Figure 1B and the normalized frequency shifts based on these results (Figure 1C) demonstrated that the functionalized PEMS were able to detect antigens below the ng/ml range. The quantification limit of the anti-HER2 PEMS, defined as ten times the standard deviation of the results obtained with a blank solution (a 1:40 dilution of normal human serum), was calculated to be 0.0253 ng/ml.

The results obtained with the anti-HER2 PEMS biosensors were validated using a commercial ELISA kit capable of detecting human HER2 ECD in serum. HER2 ECD standards ranging from 0 – 3 ng/ml, (provided by the manufacturer) were employed to generate a standard curve. The quantification limit of ELISA assay was determined from the standard curve to be 0.123 ng/ml. Accordingly, the quantification limit of these early-generation anti-HER2 PEMS was approximately 5-fold more sensitive than that of the ELISA (Figure 2). It is our expectation that increased in the sensitivity of the PEMS can be achieved through immunological methods commonly employed to improve the sensitivity of ELISAs such as the use of higher affinity antibodies or signal amplification based on the use of mixtures of polyclonal antibodies that target multiple epitopes on the target biomarker molecule.

Visualization of HER2 ECD bound to anti-HER2 PEMS

We employed confocal fluorescence microscopy to verify the binding of recombinant human HER2 ECD by the anti-HER2 PEMS. FITC (green fluorophore) conjugated anti-HER2 MAb, trastuzumab, was immobilized on the PEMS surface as described above and Alexa Fluor 555 (red fluorophore) conjugated recombinant human HER2 ECD or Alexa Fluor 555 (red fluorophore) conjugated BSA was allowed to bind to the anti-HER2 PEMS. The confocal image stack presented in Figure 3A obtained using a green filter reveals that FITC-trastuzumab (green fluorophore) is conjugated uniformly across the surface of the PEMS. The localization of the Alexa Fluor 555-HER2 ECD (red fluorophore), obtained using a red filter, is shown in Figure 3B. The overlaid image presented in Figure 3C reveals yellow fluorescence where the red fluorophore co-localized with the green fluorophore indicating that the HER2 ECD is bound at the sites occupied by the anti-HER2 MAb. An overlay of the negative control Alexa Fluor 555-BSA (red fluorophore) on the FITC-trastuzumab (green fluorophore) conjugated PEMS is presented in Figure 3D. The lack of red fluorescence in this control image indicated that the BSA control was not bound by the anti-HER2 PEMS.

PEMS-based detection of HER2 ECD in the serum of breast cancer patients

In order to determine the clinical potential of PEMS, we employed the functionalized PEMS to detect the naturally occurring HER2 ECD concentrations in a series of serum samples obtained from 7 patients with breast cancer and 3 healthy control individuals. Patient samples were assayed by anti-HER2 PEMS at a final dilution of 1:40 in the flow cell. All samples were assayed in triplicate in a blind manner in order to eliminate observational bias. The normalized frequency shift was then compared to the calibration curve shown in Figure 1B in order to calculate the concentration of HER2 ECD injected into the flow cell. The concentration of HER2 ECD present in each original patient serum sample (Figure 4) was then calculated by adjusting this number to account for the dilution employed (multiplied by 40).

Each patient sample was assayed by ELISA to independently determine the concentration of HER2 ECD. A comparison of the serum HER2 ECD levels measured by both methods is presented in Figure 4A. P values for all five of the 7 paired PEMS and ELISA measurements were greater than 0.05, indicating that the differences between these measurements obtained using the two technologies were not significant. A possible explanation for the lack of concordance in the measurements obtained for these two samples is the prototype nature of the sensor. Variability was likely present in a number of areas including material properties, surface topography, and geometric shape. Future work will focus on PEMS design and fabrication to address these issues. The correlation between PEMS and ELISA measurements of HER2 ECD in serum samples was determined using a Passing and Bablok regression analysis (Figure 4B). The slope and the intercept were determined to be 0.5023 to 1.2046 and −2.5460 to 9.3446 for the PEMS and ELISA methods, respectively (95% confidence interval). A Cusum test indicated no significant deviation from linearity ($P>0.10$). The regression analysis therefore suggested agreement between the ELISA and PEMS-based measurement of HER 2 ECD in the sample serums.”

Conclusions

We have demonstrated rapid and sensitive measurements of both recombinant human HER2 spiked into human serum and naturally occurring HER2 present in serum isolated from breast cancer patients using PEMS functionalized with the anti-HER2 MAb, trastuzumab. These results were in close agreement with those obtained using widely accepted ELISA techniques. To our best knowledge, this is the first report of the use of PEMS for the detection of naturally occurring cancer biomarkers in serum. Our results suggest that PEMS biosensors incorporating antibodies specific for tumor biomarkers have the sensitivity necessary for clinical applications. Our future efforts will focus on improving assay reproducibility by using automation to better standardize the dimensions of the PEMS sensors, developing PEMS arrays that simultaneously assay samples in triplicate and including reference and control channels. We will also attempt to extend these studies to the detection of other clinically relevant biomarkers that are present at very low levels and are therefore difficult to quantitate using ELISAs. In conclusion, our results suggest that PEMS offers potentially a sensitive, rapid, label free technology for the detection of relevant cancer biomarkers.

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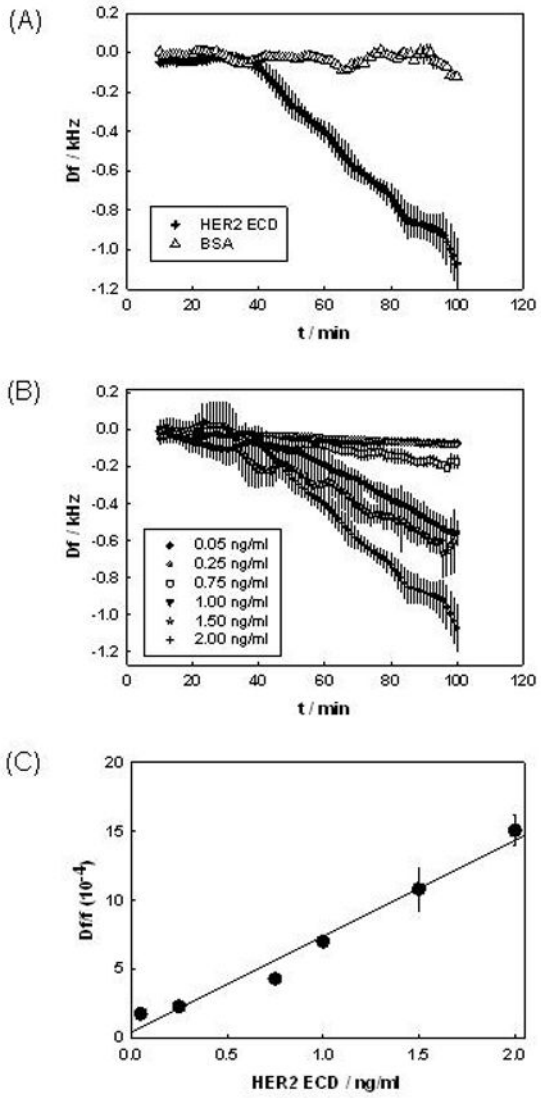

Figure 1.

The ability of PEMS functionalized with the anti-HER2 monoclonal antibody, trastuzumab (anti-HER2 PEMS) to specifically detect HER2 ECD in serum was determined as described in the Methods Section. (A) The specificity of the detection of the HER2 ECD (plus signs) by the anti-HER2 PEMS is indicated by the frequency shift, which was not present when BSA (triangles) was injected into the flow cell. The data plotted is an average of two assays. (B) The ability of the anti-HER2 PEMS to detect low concentrations of biomarkers in serum was assayed by injecting a range of concentrations of HER2 ECD into the flow cell. These concentrations were the actual concentrations in the flow system. The frequency shifts indicate that the PEMS were able to detect HER2 ECD in the low ng/ml concentration range. Data plotted are an average ± standard deviation of three independent tests. Df/f was calculated using the 20 points prior to sample injection (background points) and the average of the final 20 points measured after sample injection. (C) The normalized frequency shift plotted as df/f vs. concentration indicates a nearly linear relationship between these two parameters ($R^2 = 0.963$). Data presented are the average ± standard deviation of assays performed in triplicate.

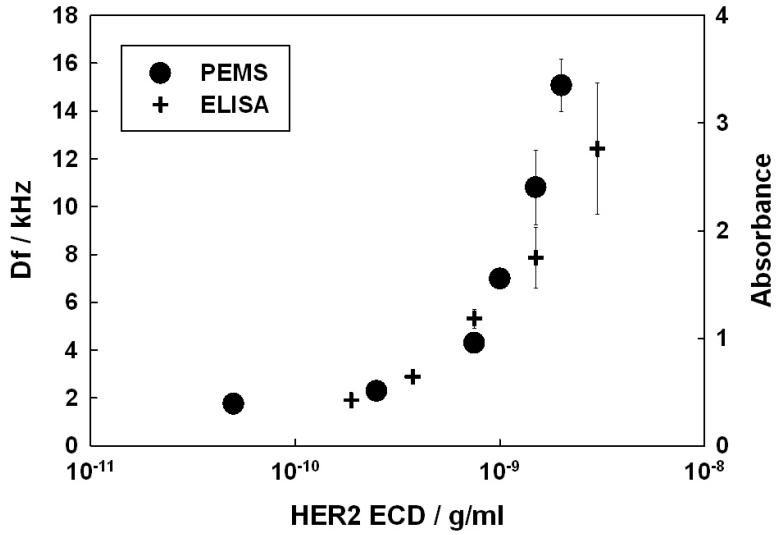

Figure 2.
A comparison of HER2 ECD detection using standard ELISA (plus signs) and anti-HER2 PEMS (circles).

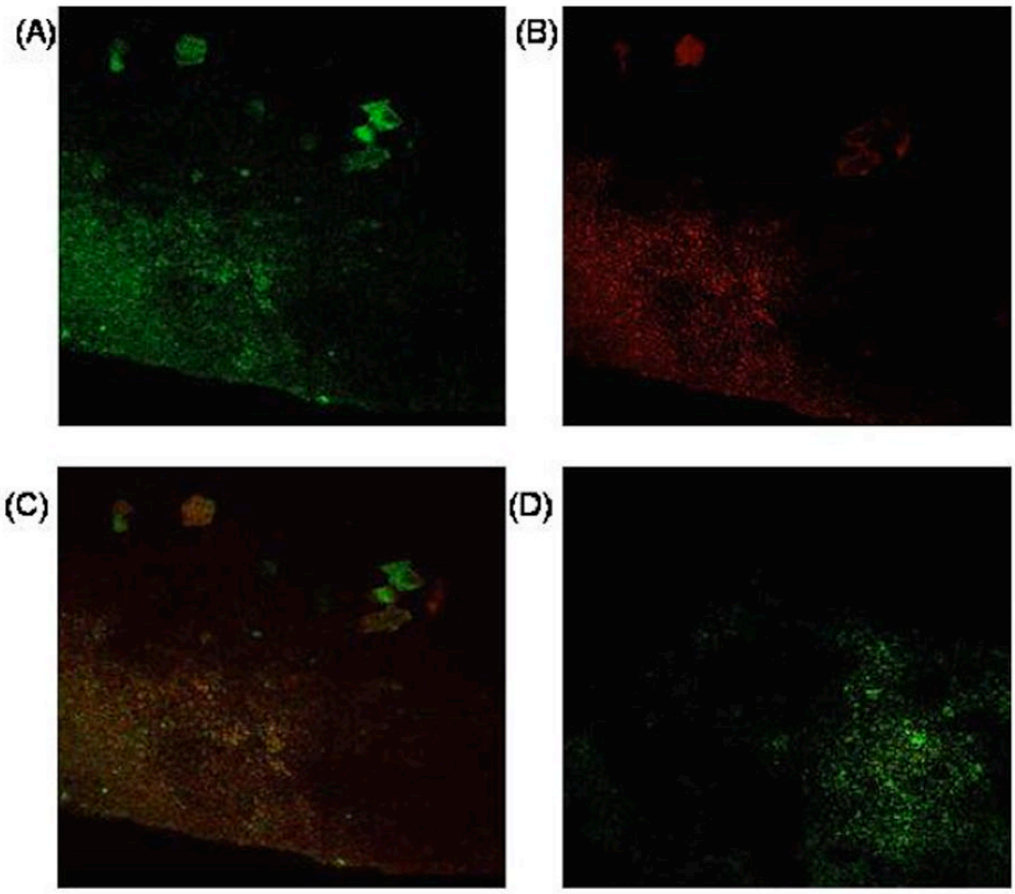

Figure 3.

Fluorescence imaging was performed to verify the binding of HER2 ECD by the anti-HER2 PEMS. The anti-HER2 MAb, trastuzumab, was labeled with FITC (green) and immobilized on the PEMS surface. HER2 ECD labeled with Alexa Fluor 555 (red) was allowed to bind to the PEMS surface. Examination of the PEMS by confocal microscopy under a green filter (A), red filter (B) and an overlay of green/red filter (C) revealed yellow fluorescence where the trastuzumab (green) and HER2 ECD (red) are co-localized. An overlay from a similar study performed with Alexa Fluor 555-labeled BSA (negative control) revealed a lack of BSA binding and minimal co-localization (D), suggesting that the HER2 ECD was specifically bound by the anti-HER2 PEMS.

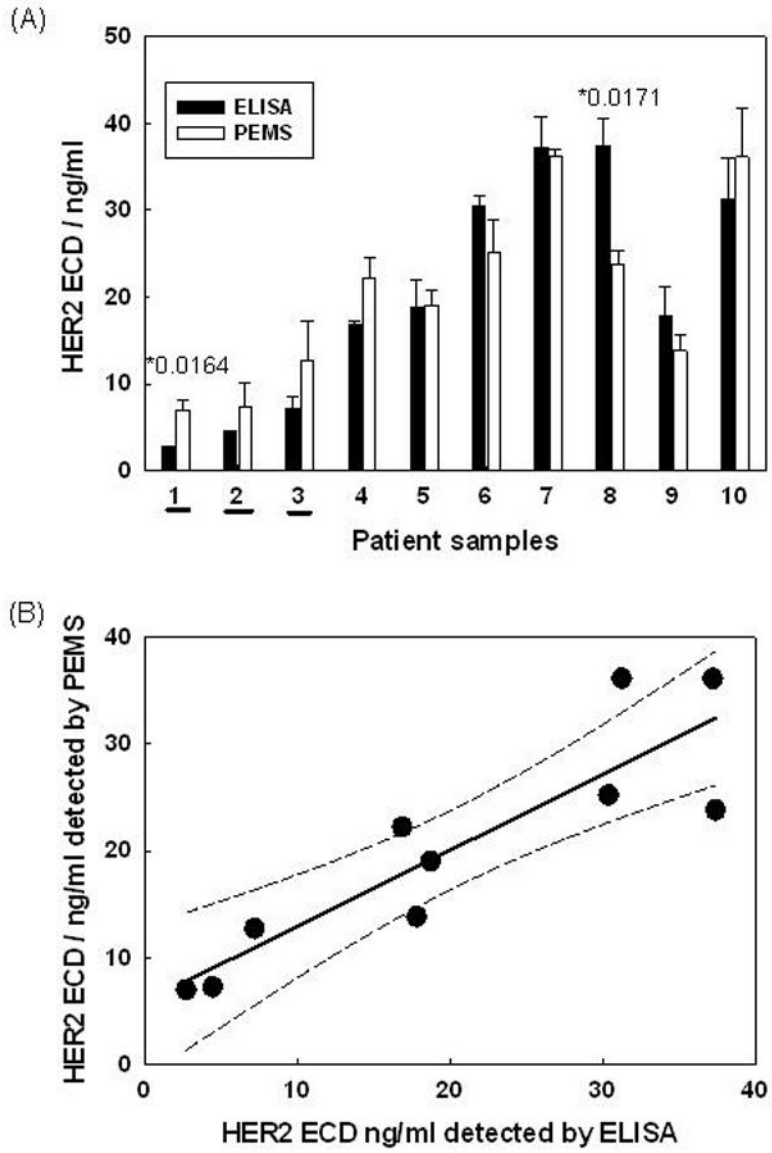

Figure 4.

(A) Detection of HER2 ECD in the serum of breast cancer patients and healthy controls by HER2 ELISA and anti-HER2 PEMS. Negative control serum samples (#s 1–3, underlined) and serum samples from patients with HER2 positive breast cancer (#4–10) were assayed head-to-head by ELISA and anti-HER2 PMES in triplicate. Data values were plotted as average ± the standard deviation. P values for the paired analysis are indicated in the figure with values greater than 0.05 indicating a lack of significance difference of the PEMS and ELISA based measurements. Measurements in which a significant difference was observed are indicated by an asterisk. Each assay was performed in triplicate. (B) A Passing and Bablok regression analysis was performed on the data demonstrated agreement of ELISA and PEMS-based measurements. The regression line (solid) and the confidence interval for the regression line (dashed) are indicated (regression equation: y= 4.0665 + 0.8454x). A Cusum test indicated no significant deviation from linearity (P>0.10), suggesting that the results obtained with the two methods are comparable.