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Measuring Bone Biomarker Alkaline phosphatase with Wafer-scale Nanowell Array Electrodes

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ABSTRACT

Biosensors that can analyze a single drop of biological fluid can overcome the limitation such as extraction volume from humans or animals, ethical problems, time and cost. In this work, we have developed a highly sensitive electrochemical (EC) biosensor based on a nanowell array (NWA) for the detection of alkaline phosphatase (ALP), a serum indicator of bone formation. The size of electrode is $2 \times 1 \text{ mm}^2$ and has over ten million nanowells (400 nm diameter) arranged uniformly on the electrode surface. For detecting ALP, Anti-ALP was immobilized and oriented on the NWA surface using a self-assembled monolayer and protein G. EC impedance spectroscopy (EIS) was used to determine the amount of ALP in 10 μL of sample. The impedance was calibrated with ALP concentration. The NWA has a linear dynamic range from 1 pg/mL to 100 ng/mL with a limit of detection (LOD) at 12 pg/mL. We used the sensor to measure the ALP in real mouse serum from 4, 10, and 20 weeks old mice and compared the results to the standard photometric assay. This work demonstrates the potential of EC NWA sensors to analyze a single drop of real body fluid sample and can be developed for broad applications.

Monitoring of bone metabolic activity (bone formation and resorption) is critical for determining progression of bone disease or efficacy of treatment. Alkaline phosphatase (ALP) in blood serum is often used as a indicator of bone formation in numerous clinical pathologies: osteoporosis¹⁻², osteosarcoma³, Paget's disease⁴⁻⁵, and diabetes⁶. A recent study has shown that ALP concentrations in human blood serum varies with age, gender, and disease (normal range: 30-115 U/mL)⁷. The ALP level in the blood is high during skeletal growth and periods of bone formation and decreases throughout life⁸. Currently, most test for ALP level in blood serum use the conventional photometric assay, which uses phosphatase reaction chemistry. In this photometric assay, ALP hydrolyzes *p*-nitrophenylphosphatase (PNPP), which is colorless, to *p*-nitrophenol (PNP), which is yellow and absorbs at 405 nm. The PNPP assay has a high limit of detection (LOD) value (10 μ U) which describes the minimum concentration that can be detected when using body fluid samples because hemoglobin and other large cell debris affect optical density. Also, this assay needs fairly large sample volumes (e.g. 100~200 μ L).

Enzyme-linked immunosorbent assay (ELISA) can also be used to measure ALP. This method uses antigen (Ag)-antibody (Ab) affinity and is also measured by a change in color. ELISA has lower LOD value since it has washing step to remove non-specifically bound proteins in the origin sample. However, ELISA consists of several complex steps including a labeling process with secondary antibody and fluorescence measurement, and consumes larger sample volume (100~200 μ L per sample). ELISA assays additionally require high maintenance cost and some multi-flex microplate readers occupy large space, which limits compatibility with the miniaturization. These techniques are therefore suitable for research studies and in clinical blood analysis laboratories but are difficult to adapt to real-time point-of-care measurements. There is a need for a rapid, accurate, easy to use and inexpensive analysis of ALP quantification.

Electrochemical (EC) immunosensors can be used as a point-of-care sensor because measurement is rapid, inexpensive, and has a low LOD⁹. In our previous work, we modified a screen printed electrode for detection of ALP and measured the impedance signal in PBS and serum. The screen printed electrode had a 5 ng/mL LOD in PBS and a 10 ng/mL of LOD in serum¹⁰. Though the electrode successfully detected ALP, the micro-electrode posed two primary limitations: 1) a large surface area still required a larger volume of sample (100 μ L), and 2) the micro-size working area is affected by solution resistance, impurities, and non-specific binding more than a nano-electrode¹¹. These effects resulted in a sensor with low reliability and limited accuracy even though we successfully measured ALP in a range 1.8 to 1000 ng/mL. For these reasons, there is a need for a reliable sensor that allows direct, easy quantification of bone biomarkers with high sensitivity and specificity.

In this paper, we developed an ALP EC immunosensor using a nanowell array (NWA) electrode with high sensitivity, selectivity and signal to noise ratio (S/N ratio) and requiring only 10 μ L of sample volume per sample based on EC impedance spectroscopy (EIS). Finally, NWA's effectiveness was demonstrated by comparing standard colorimetric assay and by detection of ALP with low levels in real mouse serum extracted at different ages.

EXPERIMENTAL SECTION

Materials

Potassium ferricyanide [$K_3Fe(CN)_6$], ethanolamine hydrochloride, 11-mercapto undecanoic acid (11-MUA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), Bovine serum albumin (BSA), sodium citrate dihydrate, phosphate buffer saline (PBS), mouse albumin lyophilized powder, fetal bovine serum (FBS) were purchased from Sigma-Aldrich

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(St. Louis, USA). Protein G (PG) was purchased from Thermoscientific (Cambridge, USA) and C57BL6 mouse serum was purchased from Innovative reserach (Novi, USA). Mouse alkaline phosphatase (ALP) protein (2910-AP-010) and goat polyclonal anti-ALP antibody (AF-2910) was purchased from R&D systems (Minneapolis, USA). Conventional colorimetric (CC) assay was performed by ALP colorimetric assay kit (ab83369) purchased from Abcam (Cambridge, USA)¹²⁻¹⁴.

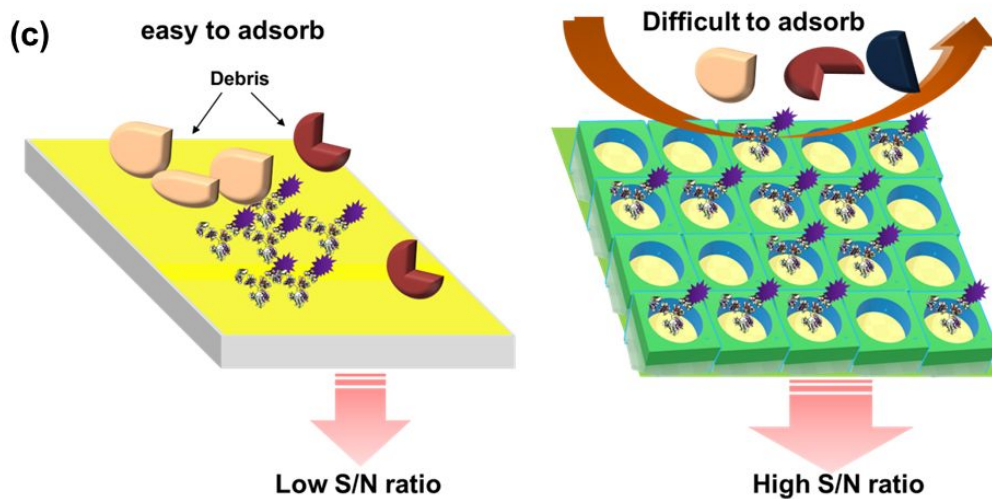
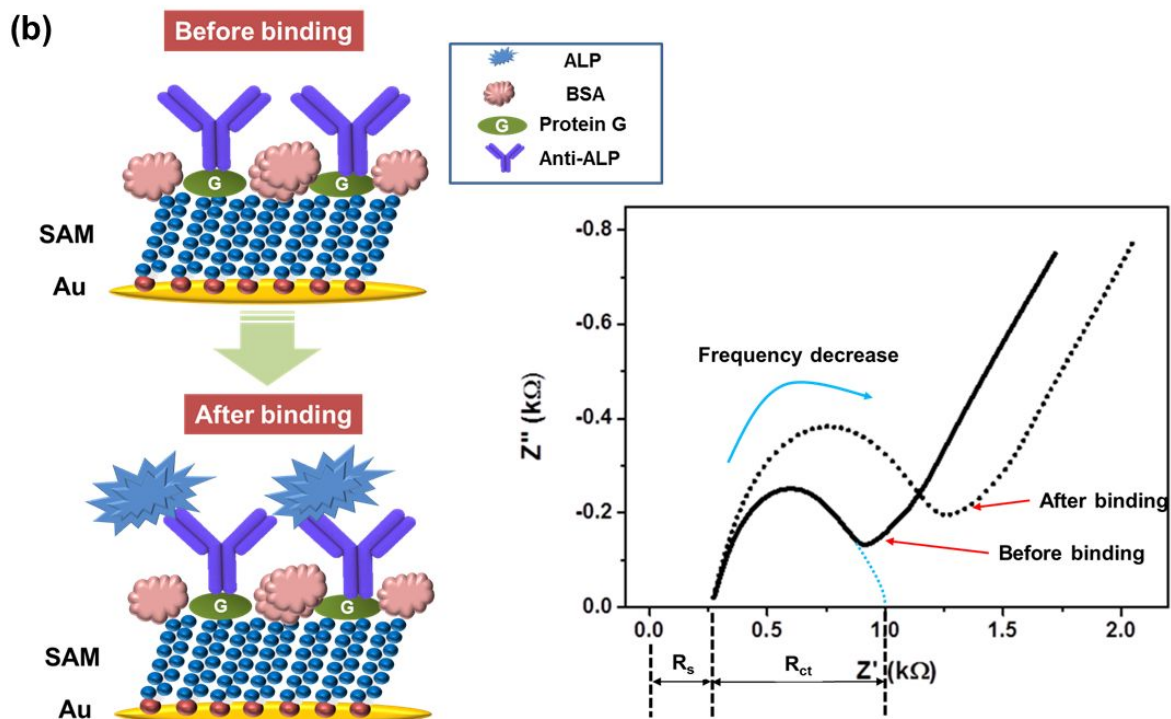
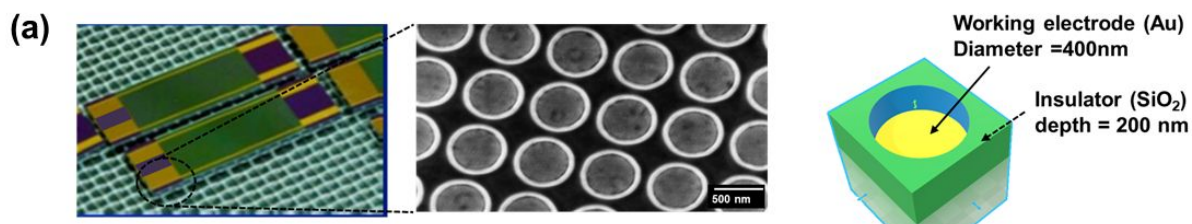


Figure 1. (a) SEM Image of the overall NWA electrode and schematic image of single well. NWA is fabricated on the large square region at the bottom of electrode (black circle). The yellow region is the Au working electrode and the green region is the SiO₂ insulator. The scale bar is 500 nm. (b) Schematic illustration of ALP treatment in serum and impedance measurement. The ALP immunolayer was constructed on the NWA electrode (before binding), ALP binds to the ALP antibody (after binding), increasing the resistance of the electrode. This is measured in the Nyquist plot as an increase in charge transfer resistance value (the diameter of the semi-circle). (c) Non-specific adsorption occurs on flat electrode surfaces, resulting in low signal-to-noise; the small size of the NWA prevents non-specific adsorption.

Wafer-scale NWA patterning

The NWA patterning was made by KrF stepper as previously detailed¹⁵. Briefly, 1) Si wafer was cleaned using sulfuric peroxide mixture, 2) a 300 nm thick silicon dioxide (SiO₂) layer was deposited, 3) 30 nm Ti (adhesion layer) and 300 nm thick Au layer were deposited, 4) photoresist was deposited, 5) whole electrode morphology was made by mask alignment, 6) Ti/ Au layers were removed by etching, 7) photoresist was removed, 8) a second SiO₂ layer with a thickness 200 nm was deposited, 9) a second photoresist was deposited, 10) the KrF stepper process was used for patterning NWA, and 10) the SiO₂ layer was etched and photoresist was removed.

Preparation of immunolayer on NWA

NWA electrodes were fabricated by the KrF stepper process (briefly outlined above), resulting in an array of 2,500 × 5,000 nano-wells (diameter = 400 nm, depth = 200 nm) (**Figure. 1a**). AFM profiling verified our NWA specification (**Figure S1**). The electrodes were first treated with a self-

assembled monolayer (SAM) to allow covalent bonding with proteins. To create the SAM, NWA electrodes were treated with acetone, cleaned with ethanol and DI water, and dried in a stream of N_2 . Electrodes were then incubated in 10 mM 11-mercapto undecanoic acid (11-MUA) dissolved in anhydrous ethanol for 1 hour at room temperature. Next, equal parts 50 mM EDC and 50 mM NHS in pH 4.5 sodium citrate buffer were used to activate the carboxyl groups on the SAM. Next, 10 μ g/mL protein G (PG) was immobilized on the SAMs by covalent bonding for 1 hour at room temperature (RT) in order to increase antibody (Ab) density and ensure Ab orientation. The unreacted activated carboxyl groups were blocked by 1 M ethanolamine for 10 min at RT. Finally, a solution of 10 μ g/mL anti-ALP antibody was immobilized by PG-antibody affinity, followed by BSA blocking (1 mg/mL for 30 min), which deactivates non-antibody area in the NWA preventing non-specific binding. After that, 5 μ L volume of antigen in different solution (PBS, 10 % diluted FBS, undiluted mouse blood serum) was dropped on the NWA and incubated for 30 min at RT. After washing using 0.05 % PBS tween 20 (3 times repeated), NWA was dried by nitrogen (99 % purity) and performed EC measurement. The schematic of the sensor is shown in **Figure 1b**.

Electrochemical Analysis of NWA

EC assay to quantify ALP based on impedance measurement was performed with a three-electrode system, including a working electrode (WE: NWA), a counter electrode (CE: Pt electrode) and a reference electrode (RE: Ag/AgCl/ KCl). All three electrodes were connected to a potentiostat (ALS 600A, Texas, USA) that measured impedance. All three electrodes were immersed into an electrolytic conducting solution, 5 mM $K_3Fe(CN)_6$ + 0.1 M KCl in pH 7.4 PBS at RT. Impedance spectra were recorded from 1 kHz to 0.1 Hz. 50 mV AC signal amplitudes was applied with fixing the DC potential at 0 V. After measurement, data were fit by using Z-view software (Scribner

Associates Inc, USA). As shown in **Figure 1b**, impedance data can be analyzed with a Nyquist plot. Nyquist plots were generated by plotting the imaginary (out of phase, y-axis component) versus the real (in phase, x-axis component) values¹⁶. The diameter of the semicircle, which corresponds to the charge transfer resistance (R_{ct}), was used for determining the analyte (ALP) concentration. When the ALP was bound to the ALP antibody, the biomolecule layers on the electrode became denser, making it more difficult for electron transfer from electrolyte solution to the electrode. Most biomolecules have low dielectric constants (between 2 and 20)¹⁷, which means they are non-conductive materials. They act like a resistive layer on the electrode, so electrons from the electrolyte cannot easily pass through resistive layer on the electrode surface. The charge transfer resistance, R_{ct} increased (diameter of the semicircle increased) with increasing binding rate between ALP and ALP antibody.

In this work, ΔR_{ct} (%) was calculated to quantify ALP and it was determined by:

$$\Delta R_{ct}(\%) = \frac{\Delta R_{ct}}{R_{ct}^{BSA}} \times 100 = \frac{R_{ct}^{Ab-Ag} - R_{ct}^{BSA}}{R_{ct}^{BSA}} \times 100, \quad \text{where } R_{ct}^{Ab-Ag} \text{ is the value of the } R_{ct} \text{ value}$$

after ALP as Ag binding to ALP antibody on the NWA surface. R_{ct}^{BSA} is the value after BSA adsorption to block non-specific binding as base line. To calibrate EC measurements, concentrations of ALP from 1 pg/mL and 1 μ g/mL were measured in PBS. After calibration in PBS, ALP was spiked in 10 % fetal bovine serum (diluted 1:9 with PBS) also from 1 pg/mL and 1 μ g/mL. Testing in fetal bovine serum (FBS) determined the effects of non-specific binding¹⁸. After measuring the sensor's performance in FBS, undiluted mouse blood serum was tested as "real sample" analysis indicates the potential for direction translation of this technique to the lab. All tests used the CC assay as the photometric validation. CC tests were carried out according to the manufacturer's instructions. The CC calibration curve was estimated using the positive and

negative samples included in the CC kit. ALP concentration was determined by EC measurement (Equation 1) mapped onto the calibration curve. ALP Ab immobilized NWA was used as positive control and NWA without any ALP Ab (only BSA blocking) was used as negative control in mice serum analysis.

Mouse Serum Collection

C57/Bl6 mice (n=18) were obtained from Jackson Labs (Bar Harbor, USA) with 6 mice for each of the following age groups: 4 weeks, 10 weeks, and 20+ weeks. All experiments were approved by the Institutional Animal Care and Use Committee and conducted according to the guidelines of Association for Assessment and Accreditation of Laboratory Animal Care International. After sacrifice, the blood was extracted via cardiac exsanguination and subsequently was centrifuged in order to isolate the serum. The serum was stored at -80 °C until analysis.

Working cycle of the ALP immunosensor

To assess the number of working cycles of the NWA electrode, 100 pg/mL of ALP in serum was applied to the NWA electrode. The current was measured in a 5 mM $K_3Fe(CN)_6$ solution then washed by PBS 3 times. After washing, the electrode was dipped in 10 mM NaOH for 1 minute at RT to remove the ALP but keep the ALP antibody, washed again using PBS, and reused for the same procedure. In this experiment, regeneration was performed by comparing peak current of ALP Ab and peak current of attaching ALP 100 pg/mL in 10 % diluted serum. The electrode was immersed in (1) 10 mM glycine buffer (low pH condition: pH 2.4) for 3 min and (2) 10 mM NaOH (high pH condition: pH 10.3) for 3 min. Then, faradaic current by SWV was measured from 0.6 V

to -0.2 V to observe regeneration efficacy. This process was repeated until regeneration efficiency was less than 80 %.

Regeneration efficiency (%) = $\left(\frac{I_{Ag-Ab} - I_{reg}}{I_{Ag-Ab} - I_{Ab}} \right) \times 100 \%$, Where I_{Ag-Ab} is current signal of electrode which ALP was bind to ALP antibody, I_{reg} is current signal of electrode after regeneration, I_{Ab} is current signal of electrode which ALP antibody only attached, respectively.

Methods in statistics

All assays (EC, CC) were run 5 times and mean and standard deviation were calculated at each concentration to generate the calibration curve. Each replicate was measured with a new NWA electrode. Electrolyte ($K_3Fe(CN)_6$) was newly made at each time measurement to maintain fresh conditions. Nonlinear curve fitting was performed with origin 8.0.

RESULTS AND DISCUSSION

Overall, we found the conformational structure of the NWA allowed sensing of ALP in undiluted serum with high sensitivity and selectivity. We first determined the S/N ratio for the CC and the EC NWA electrode at ALP concentrations of 1, 10, 100 ng/mL ($n=5$). S/N ratio was calculated as the ratio of analyte signal (anti-ALP immobilized electrode) to the blank signal (no analyte in PBS)²¹. As shown in the **Figure 2a**, EC NWA demonstrated higher S/N ratio than the CC assay (CC's S/N ratio is 3.02 ± 0.58 at 1 mg/mL, 4.14 ± 0.43 at 10 ng/mL, 6.32 ± 0.85 at 100 ng/mL. And NWA's S/N ratio is 9.78 ± 1.02 at 1 ng/mL, 11.96 ± 1.98 at 10 ng/mL and 14.35 ± 2.31 at 100 ng/mL, respectively). **Figure 2b** shows the Nyquist plot for the NWA electrodes using ALP spiked in PBS as an antigen at various concentrations. R_{ct} increased with increasing ALP concentration (diameter of the circle increases). R_{ct} indicates the dielectric behavior of the electrode; at high concentration, more ALP is bound to the sensor, making a denser thicker insulation layer that can better store charge than at low concentrations²².

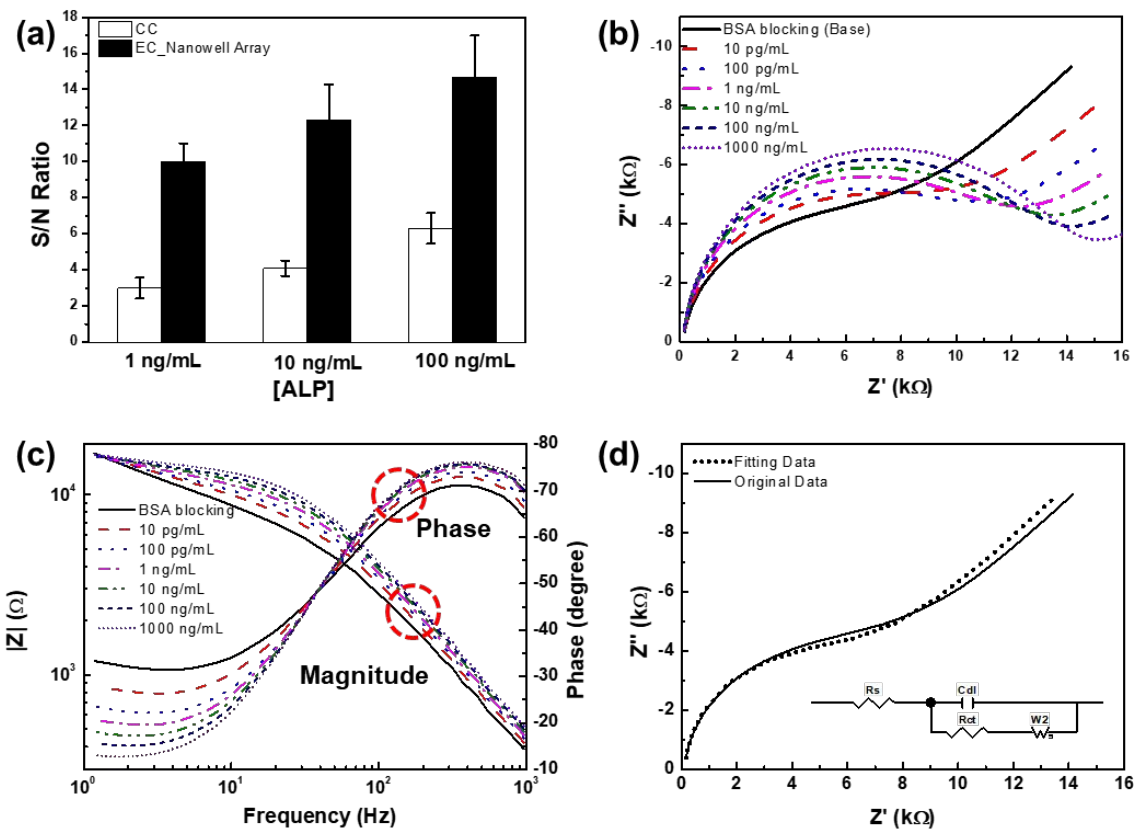


Figure 2. (a) Comparison of the S/N ratio of CC and EC NWA electrode. The EC NWA measurements had a higher S/N ratio compared to CC measurements. (b) Representative Nyquist plots of ALP with sensing range from 1 pg/mL to 100 ng/mL. (c) Bode plot of ALP with sensing range from 1 pg/mL to 100 ng/mL. The curves in the upper circle represent the phase of impedance and in the lower circle represent the magnitude of impedance. (d) Fitting result of impedance plot of ALP antibody immobilized on NWA electrode with a Randles circuit.

The Bode plot in **Figure 2c** shows $|Z|$ increases with increasing ALP concentration, particularly at frequencies below 40 Hz for which the signal is dominated by the dielectric behavior of the electrode surface. These results matched with cyclic voltammetry (CV) and square wave

voltammetry (SWV) data (**Figure S2**). We performed the impedance measurements from 10 kHz (10^4 Hz) to 0.1 Hz however, there was no difference at high frequency region (over 1 kHz). Only at the very low frequency region (< 40 Hz), the impedance increased with increasing concentrations of ALP. We modeled the immunosensor system as a Randles circuit²³⁻²⁴ (a resistor in series with a parallel capacitor and resistor) in order to characterize capacitance (C_{dl}), Warburg's impedance (W), electrolyte's solution resistance (R_s) and charge transfer resistance (R_{ct}). Typically in an EC sensor, C_{dl} and R_{ct} value are used to quantify the analyte because these values change with increasing analyte concentrations. **Figure 2d** shows the experimental data with the model fit. C_{dl} is 477.72 nF, R_{ct} is 5676 Ω , R_s is 157.2 Ω , W is 135.12 Ω , respectively (chi squared = 0.001).

Sensor Sensitivity and Dynamic Range

Figure 3 shows the results of ALP in PBS based on the NWA EC and CC assay. Comparing the NWA sensor and CC responses indicated that the NWA had a lower LOD of 12 pg/mL, $n=3$ (LOD of CC: 5.4 ng/mL, $n=3$) and wider detection range than CC from 12 pg/mL to 1 μ g/mL (range of CC: 5.4 ng/mL~1 μ g/mL). In this calibration curve, LOD is estimated by gathering signal of the zero concentrations value plus 3 times its standard deviation¹⁴.

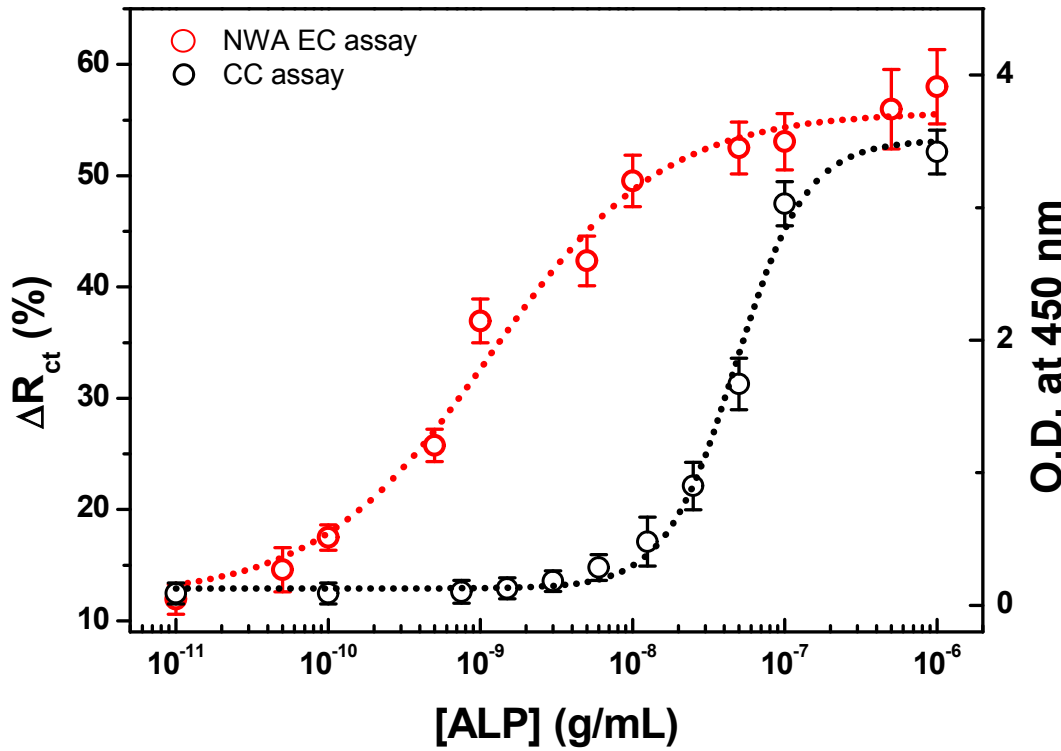


Figure 3. (a) Calibration curve of ALP by NWA EC measurement (red) and CC measurement (black). Both of them, ALP is spiked in pure buffer (10 mM PBS, pH 7.4). In EC measurement, 5 mM $K_3Fe(CN)_6$ in PBS was used as electrolyte ($n=5$).

The EC sensor’s selectivity was determined in serum in order to demonstrate sensor’s effectiveness in the presence of other proteins, cell debris, and albumin, which are large component in serum. We used mouse leptin as a non-specific binding protein against ALP specific binding protein. R_{ct} did not change with incubation of leptin in serum on the NWA (**Figure S3a**). **Figure S3b** is the R_{ct} ratio in the presence of ALP and in the presence of leptin in serum at 0.1, 1, 10, 100 ng/mL, respectively. There were no significant differences between the antibody (ALP) layer with and without leptin, indicating that the NWA EC sensor has high selectivity even in serum. ALP was additionally calibrated in 10 % FBS (in which there are other proteins but insignificant amounts

of ALP)²⁵. R_{ct} increased with increasing ALP and it shows the similar result of ALP measured in pure PBS buffer (**Figure S4**). Comparing buffer and serum calibrations, there are two differences 1) diffusion region (linear region with upward slope) at low frequencies disappeared in serum. We assumed this effect occurs for cell debris effect. Serum contains cell debris, which is adsorbed on SiO_2 region by physical adsorption. This does not affect the signal because SiO_2 is an insulator. However, the diffusion process is affected by the cell debris found in serum because most of cell debris has a negative surface charge because of the phospholipid membrane. Electrons in the electrolyte solution cannot pass through easily to the electrode (repulsion). Cell debris may additionally adsorb on the electrode creating a blocking layer so, electrons cannot easily pass through, 2) R_{ct} of Ab layer is increased ($10.46\text{ k}\Omega \rightarrow 21.87\text{ k}\Omega$), which is likely caused by non-specific binding. Even in these disadvantages of serum analyzing, our NWA shows enough selectivity as already shown in **Figure S3**. In serum conditions the NWA EC still maintained linear range from 50 pg/mL to 10 ng/mL range with sensitivity $[0.0647]\Delta R_{ct} \times (\log [g/mL])$ despite having lower than the sensitivity in PBS (**Figure 4**).

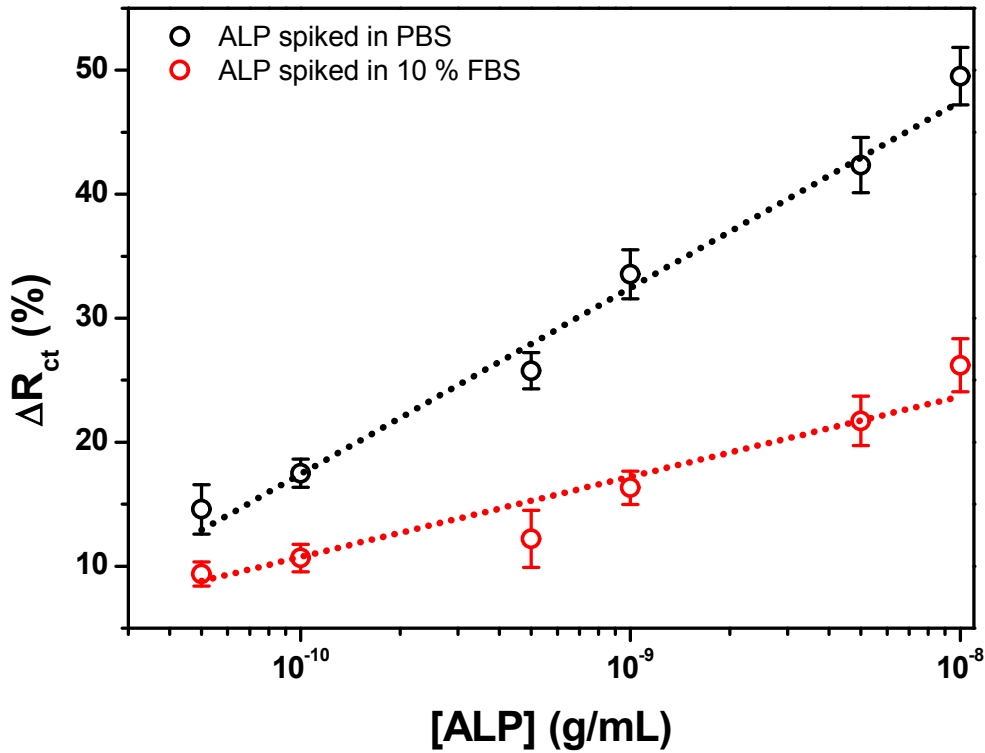


Figure 4. Calibration curve and linear property based on NWA EC measurement with range from 50 pg/mL to 10 ng/mL. ALP spiked in PBS (black circle) and 10 % diluted FBS (red circle) (n=5).

Figure 5 and **Table 1** show the results of NWA EC and CC assay using blood serum from different age mice. Both NWA EC and CC results indicated that the 4 weeks old mice had higher ALP serum concentration than older ages, which is expected due to skeletal growth at the young ages. The concentration by both methods in mice serum with different ages matched to within 10 %.

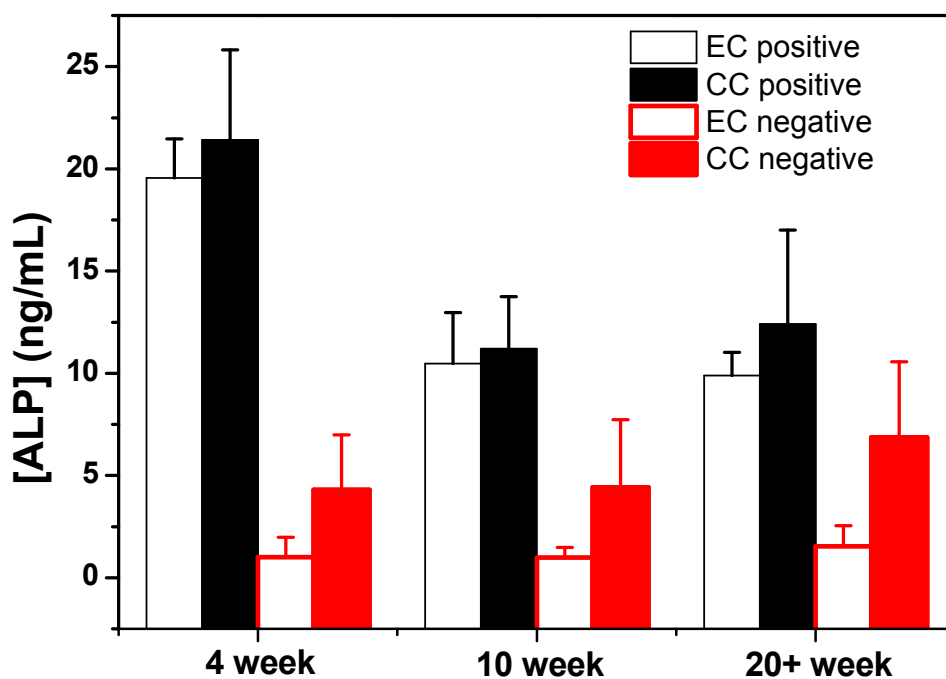


Figure 5. ALP quantification using undiluted mouse serum which was collected from specimens at 4, 10, 20 week ages. Unfilled bar is the result of NWA EC and filled bar is the result of CC assay (n=5).

Sample	[ALP] by CC (ng/mL)	[ALP] by EC (ng/mL)	RSD (%)
4 week	21.41 ± 3.41	19.56 ± 1.96	9.46
10 week	11.23 ± 2.54	10.47 ± 2.51	7.26
20+ week	8.42 ± 4.59	7.89 ± 1.13	6.72

Table 1. Comparison of results from NWA EC and CC assay.

Previous studies about the relationship between ALP and age have shown that ALP is higher in young mice than in old mice²⁶. Our results agree with these findings, with higher ALP in the 4-week group than any other groups. The two methods we used measured similar ALP concentration but their operation procedure is clearly different: 1) NWA EC assay uses specific binding of ab-ag while CC assays use dephosphorylation method by inserting labeling chemical (*p*-NPP), 2) a washing step which can remove non-specific binding is performed in EC but not in CC assay, and 3) NWA EC used 5 μ L of volume per test but CC used 200 μ L per test. These differences affected the negative control data as shown in **Figure 5**. Negative control data of CC assay is higher than the NWA EC control data, which could be due to the non-specific binding that is not washed away in the CC test. The ratio of positive control to the negative control (P/N ratio) in 4 weeks was 10.87 NWA EC vs 5.08 CC, in 10 weeks was 13.51 NWA EC vs 2.53 CC, and in 20+ week was 5.16 NWA EC vs 2.18 CC. The NWA EC sensor maintained high S/N ratio since it used specific binding of Ab-Ag and the presence of a washing step to remove non-specific binding.

Compared to other type of ALP NWA EC sensing, not just CC assay^{10, 25, 27-32}, this NWA EC method has the advantages of a simple procedure, consuming extremely low volume of sample, low LOD and wide linear range and applicability to blood serum (**Table 2**).

Analysis Method	Method	LOD	Linear Range	Ref
8-quinolyl phosphate	F	1 U/L	1~16 IU/L	27
Enzyme linked immunosorbent assay (ELISA)	F	5 U/L	5~180 U/L	28
Graphene QDs	F	0.017 nM (0.3 U/L)	0.05~2.5 nM, 5-10 nM	29
Screen printed Au electrode	EC	5 ng/mL (PBS) 10 ng/mL (serum)	10^{-9} ~ 10^{-5} g/mL	10
CNT electrode	EC	0.25 U/L	0.5~600 U/L	30
Glassy carbon electrode	EC	0.03 U/L	0.03~300 U/L	31
Au flat micro electrode	EC	1 ng/mL	1~400 ng/mL	32
CeO ₂ nanocrystal	EC	0.002 U/L	0.01~150 U/L	33
Au nanowell array electrode	EC	12 pg/mL	10^{-12} ~ 10^{-9} g/mL, 10^{-8} ~ 10^{-5} g/mL	This work

Table 2. Fluorescent and EC assay reported in the literature for the determination of ALP (ITO:

Indium tin oxide, CNT: Carbon nanotube, F: Fluorescent, DNA: Deoxyribonucleic acid).

The NWA EC assay has a low LOD (NWA EC: 5.4 pg/mL vs CC: 5.8 ng/mL) and wider range (NWA EC: 10 pg/mL ~ 1 µg/mL vs CC: 8 ng/mL~14 µg/mL) than CC assay. However, in 8 ng/mL~30 µg/mL range (which is the physiological range), the conventional method is more sensitive than NWA EC assay. These results demonstrate that the NWA EC is more effective at extremely lower range and CC method is effective at higher concentration range. Recently, many research groups have developed biosensors for detecting biomarkers in urine, saliva, sweat as body fluid, which are more easily obtained, but contain vary low concentrations of analyte. The NWA EC holds potential for biomarker sensing in such applications as it is sensitive at low concentrations and uses small sample volumes.

ALP NWA Biosensor Regeneration

Regeneration in NaOH dissociates the ALP antibody-ALP antigen complex. Most of antibody-antigen interactions can be disrupted by low pH condition, or high salt and high pH conditions. We found the glycine wash (low pH) did not change the binding resistance, indicating there was likely no ALP-antibody dissociation. However, the NaOH wash restored the resistance to its pre-binding value. As shown in **Figure S5 (a), (b)**, Δ peak current of 5 times regeneration is similar to the initial Δ peak current in 10 mM NaOH. However, Δ peak current is dramatically increased after 5 or more times regeneration (**Figure S5(c)**). The NaOH wash possibly to affected the protein G and BSA protein on the NWA electrode after 5 times of regeneration (**Figure 6**). Our NWA can be reused for 5 working cycles.

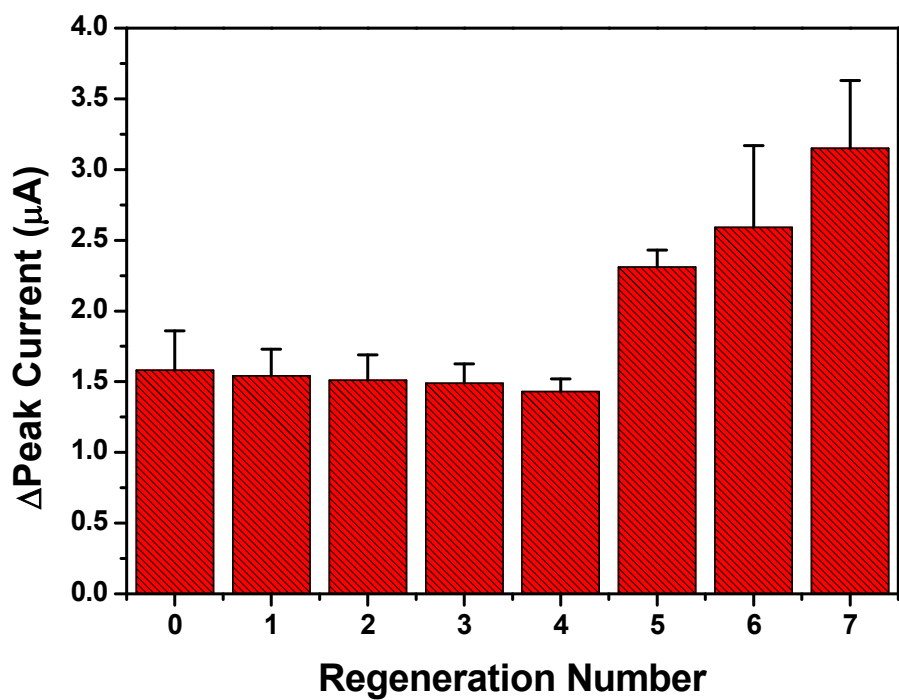


Figure 6. Regeneration of NWA EC sensor by immersing in 10 mM NaOH for 3 min. Δ Peak current is calculated by SWV before and attaching ALP at 100 pg/mL in 10 % diluted serum, repeatedly. SWV was performed in 5 mM $K_3Fe(CN)_6$ in PBS (n=5).

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CONCLUSIONS

We demonstrated the NWA EC enables detection of ALP at low concentrations in undiluted blood serum. Currently, most of ALP detection assays use optical methods such as fluorescence or ELISA, which typically require sample volumes of 200 μ L, cannot be used with whole blood, and are currently not portable given the equipment required. The advantages of the NWA EC sensor are the short analysis time, high S/N ratio, low LOD, and use of very little sample (5 μ L). In addition, the potential for miniaturization of required electronics could allow a portable, and simple point-of-care analysis system. This demonstrates the potential of NWA EC sensors to be used in detection of other biomarkers in blood, serum, and urine or even sweat or saliva as the LOD is so low. NWA EC sensors will become a powerful tool for point-of-care biomarker assessment.

SUPPORTING INFORMATION

Atomic force microscopy (AFM) image of NWA and their line profile was shown at **Figure S1** and CV and SWV data was shown at **Figure S2**. Selectivity of NWA EC was shown at **Figure S3**. Nyquist plot by NWA EC assay using ALP spiked in FBS was shown at **Figure S4**. Regeneration signal was shown at **Figure S5**.

AUTHOR INFORMATION

Author Contribution

Sandra J. Shefelbine and HeaYeon Lee contributed equally as corresponding author.

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Notes

The authors declare no competing financial interest

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