Electrochemical Sensor for Alkaline Phosphatase as Biomarker for Clinical and *in vitro* Applications

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1. Comparative study of ALP activity in different buffers by the gold standard method

The gold standard colorimetric method for ALP activity determination was studied based on the DGKC and SSCC clinical recommendations (1,2). ALP from porcine kidney was used to simulate samples with different activities measured during the kinetic assay. The colorimetric assay consisted in the measurement of the absorbance at 405 nm every 30 seconds in a microplate reader. The absorbance increment is related to the hydrolysis rate of the substrate PNPP (p-nitrophenyl phosphate) to PNP (p-nitrophenol). Enzyme activity in international units per liter was calculated according to Ec. S1 (3,4).

$$\text{Eq. S1} \qquad Act\left(\frac{IU}{L}\right) = \frac{Abs\,(405\,nm)}{minute}.\ \frac{1}{\epsilon\,.\,L}.\ \frac{V_T}{V_E}.1000$$

where:

- Abs/min= slope of the progress reaction plot during the kinetic assay
- L= optic path (cm)
- ε= absorptivity mili-molar coefficient of 4-NP (18.5 $mM^{-1}.cm^{-1}$)
- V_T= total volume of reaction
- V_E= total volume of the sample

ALP activity of the commercial enzyme was compared by the colorimetric gold standard assay to evaluate the influence of the buffer in the activity of the enzyme. As it is shown in figure S1, the buffer has a strong influence in the activity of the enzyme using PNPP 10 mM as the substrate. Buffer DEA-Opt consisted in DEA 1 M, $MgCl_2$ 0.5 mM and pH=9.8, buffer DEA-EC composition was DEA 1 M, $MgCl_2$ 0.5 mM, KCl 0.1M and pH=9.8, and finally buffer TRIS was formulated with Tris 0.1 M, $MgCl_2$ 1 mM and pH=9.8.

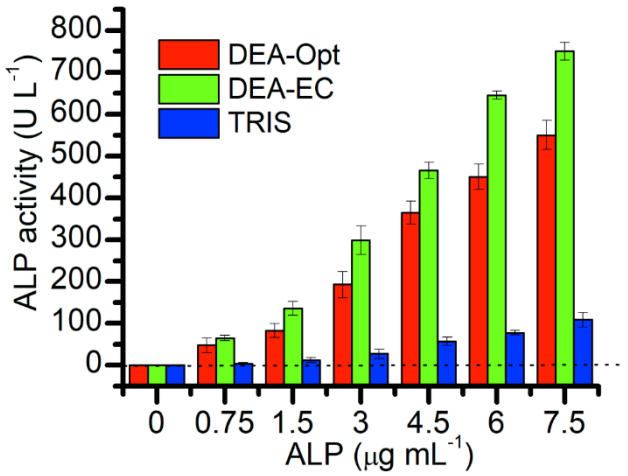

Figure S1. Comparative study of the ALP activities in different buffers performed by the gold-standard colorimetric kinetic enzyme assay using PNPP as substrate in buffer DEA-Opt (red), buffer DEA-EC (green) and buffer TRIS (blue).

2. Electrochemical response on phenol quantification in buffers of interests for ALP activity assay:

The electrochemical response to phenol 100 uM was studied by cyclic voltammetry as depicted in Figure S2, panel "a.". The electrochemical response for phenol presents a single oxidation peak in the first scan, while the redox couple hydroquinone-benzoquinone is present in the following scans. The decreasing of the oxidation current for phenol is due the irreversible process involving its oxidation, and due to a fouling process on the electrode surface by the polymerization of phenoxy radicals.

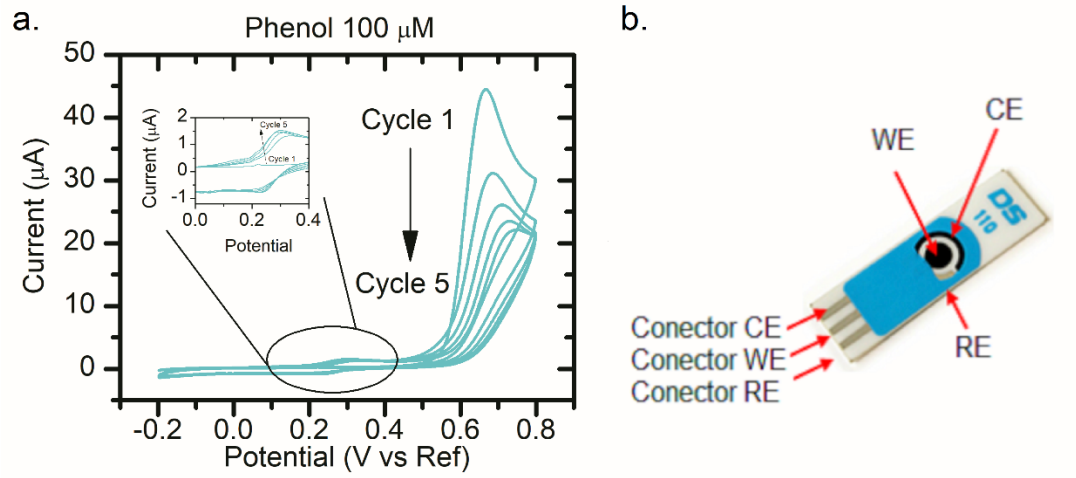

Figure S2. Figure S2. Panel “a.” Voltammogram of phenol 100 uM in buffer DEA using a single graphite-SPE electrode (SR: 50 mVs^{-1}, Potential window: -0.2 to 0.8 V vs Ref, Working volume: 50 µL). Panel “b.” Graphite screen-printed electrode (tripolar configuration). CE=counter electrode, WE= working electrode, RE= Reference electrode.

In figure S3, the electrochemical response for phenol 50 µM in buffer DEA-Opt, buffer DEA-EC and buffer TRIS was assessed by square wave voltammetry (SWV) using disposable graphite SPE electrodes.

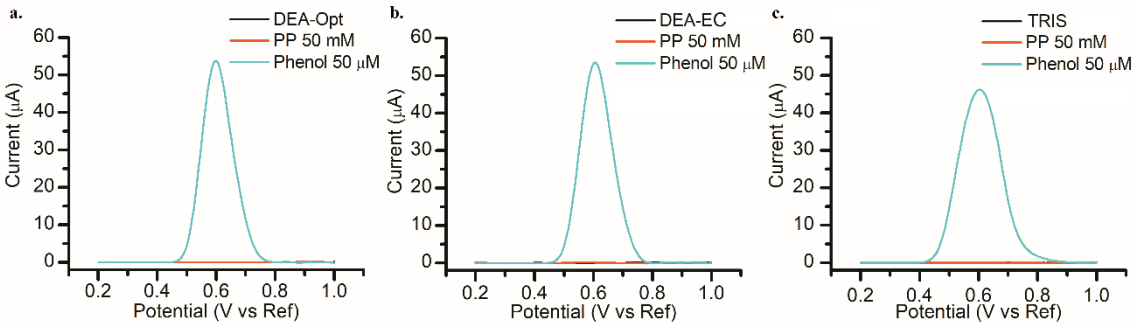

Figure S3. SWV in SPE electrodes of in alkaline buffers, with the addition of phenyl phosphate 50 mM and phenol 50 µM in buffers DEA-Opt (a.), DEA-EC (b.) and TRIS (c.). PP = phenyl phosphate.

The influence of the enzyme substrate PP (phenyl phosphate) 50 mM in the potential window for phenol oxidation was analyzed showing no peaks in the potential window if interest (ca. 0.6 V vs Ref). It is important to notice that the enzymatic substrate PP 50 mM in buffer DEA-EC was stable at least for 2 weeks at 4°C without an evidence of spontaneous hydrolysis measured by electrochemical techniques.

3. Effect of stopping solutions in the optical and electrochemical assays for ALP activity and phenol quantification:

Two solutions were studied to stop the enzymatic reaction in the optimization of the end-point electrochemical ALP assay. These solutions consisted of NaOH 5 M and H_3PO_4 2

M which were added in a 10 and 20% of the total reaction volume Samples with 1000 $U.L^{-1}$ were stopped by the addition of either NaOH or H_3PO_4. After the addition of each stopping solution, samples were transfer to a 96 wells micro plate and the kinetic assay was performed for 15 minutes while measuring the absorbance at 405 nm. The possible interference of phosphoric acid and sodium hydroxide in the electrochemical determination of phenol by SWV was also evaluated. As it is shown in figure S3, the slopes of the progress reaction curves are plotted for each case. The addition of NaOH 5M (figure S4, panel a) and H_3PO_4 (figure S4, panel b) at a 20%v are able to provide the same value of the background, being thus able to stop the enzymatic reaction with the same effectiveness.

The election of the stopping solutions was based on previous results found in the literature. The kinetic reaction can be stopped by adding an agent that denaturalize the conformation of the enzyme (in the case of NaOH) or adding high concentration of the enzymatic product (as in the case of phosphoric acid) (5-7).

The effect of the stop solution in the electrochemical detection of phenol was evaluated in buffer DEA-EC by SWV. As can be observed in figure S4, no change in the oxidation peak was observed upon the addition of H_3PO_4 but only a slight decrease due to the dilution of phenol. On the contrary, NaOH as stop solution produced a dramatic change in the oxidation signal. Moreover, the addition of NaOH 5 M changed the pH from 9.8 to 13, while the addition of H_3PO_4 did not cause any decreasing in the pH. Any change of pH introduced for any stop solution was not desirable in order to maintain the conditions for phenol detection by SWV.

The effect of the stopping solutions in the optical assay and electrochemical determination of phenol is shown in figure S5. Samples of commercial ALP with 1000 $U\ L^{-1}$ were added to a solution with PNPP (p-nitrophenyl phosphate) 10 mM for 3 minutes and the stopping

solutions were added to each samples. The optical absorbance was monitored after 30 second in a microplate reader and the slopes of the reaction curves were plotted. The slope of the blank corresponded to the response when the activity of ALP is zero (buffer DEA-Opt + PNPP 10 mM).

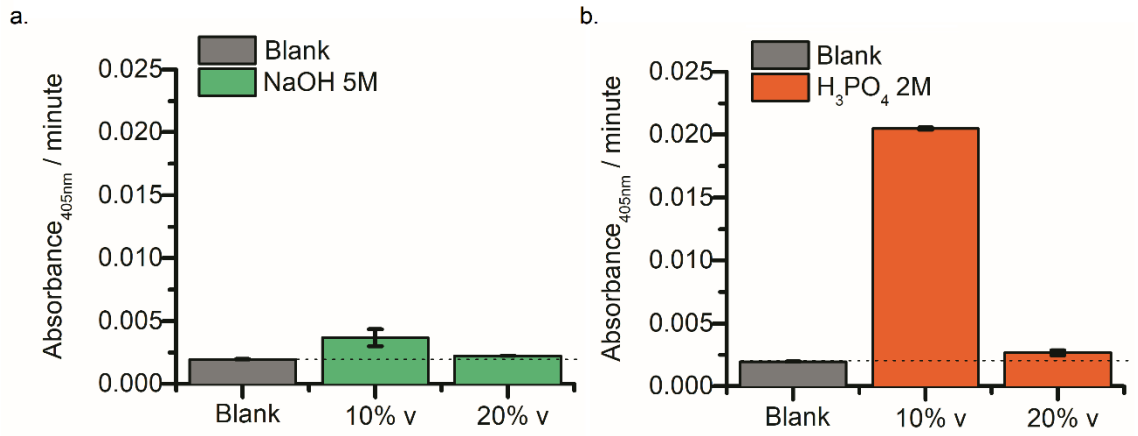

Figure S4. ALP activity measured on a microplate reader of samples with 1000 $U.L^{-1}$ after the addition of different volumes of sodium hydroxide (panel "a.") and phosphoric acid (panel "b.") as stopping solutions.

The electrochemical response for the addition of H_3PO_4 and NaOH was analyzed for phenol 20 µM determination by SWV. In Figure S5 the response is plotted for 50 µl of phenol 20 µM in DEA-EC buffer was added and phenol in the same concentration in the presence of phosphoric acid 2M added as a 20% of the volume of phenol.

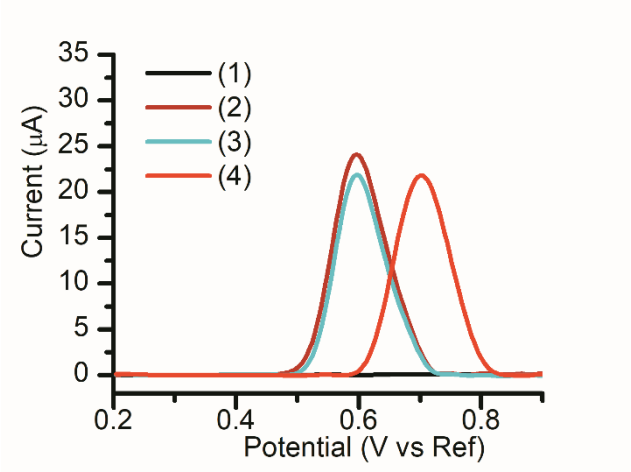

Figure S5. SWV of buffer DEA-Opt (1), phenol 20 μM (2), after the addition of H_3PO_4 2 M (3) and NaOH 5 M (4) to study the influence of the stopping solutions in the electrochemical determination of the enzymatic product

4. SWV of human serum samples in absence of the enzymatic substrate

Five human serum samples were evaluated by SWV in absence of the enzymatic substrate phenyl phosphate. 10 μL of each sample was mixed with 90 μL of DEA-EC and incubated for 10 minutes at 37ºC. Then, 20 μL of phosphoric acid 2 M is added to each tube and 50 μL are used for the electrochemical determination of phenol. As it can be observed in figure S6, HS did not interfere in the potential window of interest for phenol oxidation.

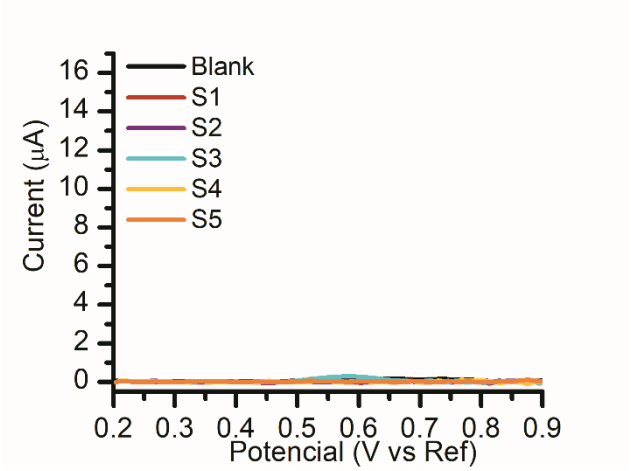

Figure S6. SWV of HS samples in absence of PP 50 mM. HS are incubated with DEA-EC under the pre-established conditions for the ALP electrochemical assay. HS=Human Serum

5. Human serum sample spiked with ALP to reach pathological levels of activity

HS2-Spk sample with an ALP activity of 64 $U.L^{-1}$ was spiked with commercial ALP in order to reach pathological levels. As it can be seen in figure S7, ALP was determined by the electrochemical ALP assay in real samples up to ca. 550 $U.L^{-1}$. The analytical response of the electrochemical method is compared with the optical standard assay in Table S1.

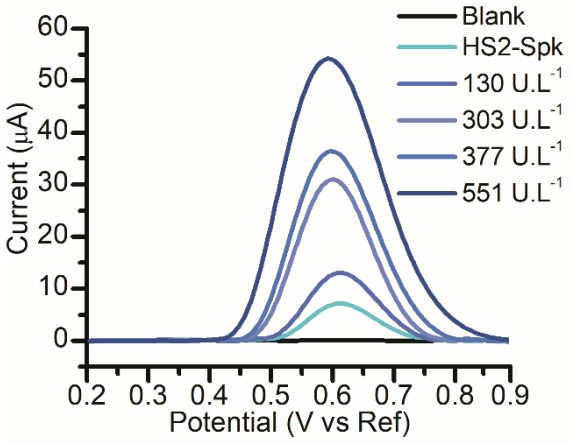

Figure S7. SWV of human serum (sample HS2-Spk) spiked with commercial ALP to reach pathological levels up to 538 $U.L^{-1}$.

The analytical response for the spiked samples is analyzed in Table S1. The recovery of the added enzyme was calculated as the ratio of ALP measured with the electrochemical assay with the activity of the enzyme measured with the gold standard colorimetric method added to the serum samples.

Table S1. Analytical results for the standard addition assay of ALP in sample HS 6

Gold standard colorimetric ($U.L^{-1}$)	Alp added ($U.L^{-1}$)	Activity measured ($U.L^{-1}$)	%RSD	%REC

64	0	67	4.2	103
120	60	130	1.5	108
295	200	303	3.1	103
370	300	377	2.9	102
530	450	551	1.2	104

6. Electrochemical determination of bone ALP isoform in human sera incubated with the lectin WGA:

The method for bone ALP determination in human serum using the lectin WGA precipitation assay, consisted in the incubation of serum from 3 patients with the lectin solution at 37ºC for 30 minutes. After the binding of the lectin with the bone ALP fraction, the solution is centrifuged for 15 minutes at 3000 g and the ALP activity determined by the optical and electrochemical assays. The analytical response of the electrochemical assay was compared with the gold standard colorimetric assay for the samples incubated with the lectin WGA and it is shown in Table S2 of Supporting material.

Table S2. Human serum of patient with low, normal and pathological level of bone ALP determined by the electrochemical assay of the supernatants after the incubation with WGA.

Sample	**ALP Activity in SN* ($U.L^{-1}$)**	**Total ALP in serum ($U.L^{-1}$)**	**Bone isoform ALP ($U.L^{-1}$)**
B1	184	315	131
B2	74	152	78
B3	55	72	17

* SN= Supernatant ALP activity after incubation with WGA and centrifugation.

7. Routine assays of hFOB 1.19 osteoblastic human cells culture:

Cell viability and total protein content of cells lysates were assessed on hFOB cells seeded in 24 wells TCP plates after three days of culture under controlled conditions. The results can be seen in figure S8.

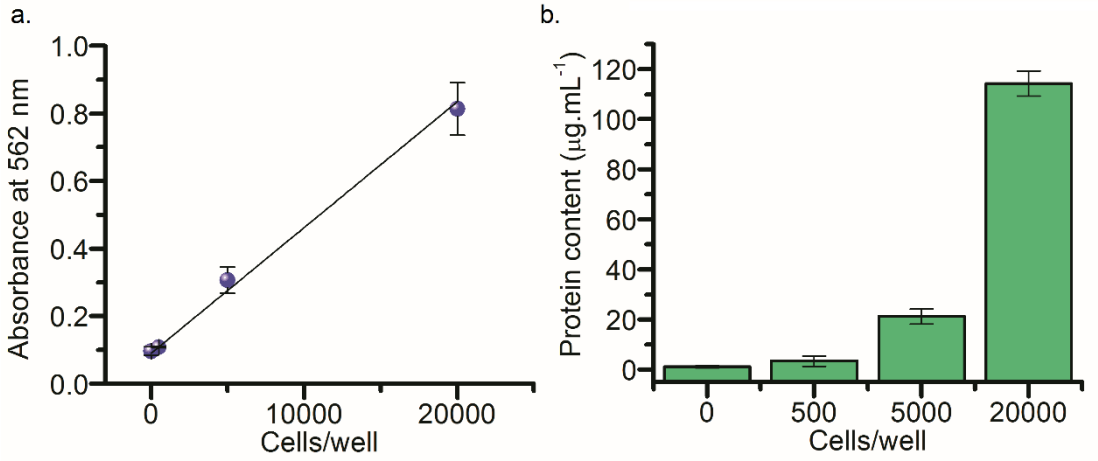

Figure S8. a. Cell viability assay with the MTT kit in cell at day 3 of culture. b. Total protein content of cell lysates obtained at day 3 of culture with different cell seeding density (expressed as µg of protein in 1 ml of cell lysate).

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