HHS Public Access

Author manuscript

Recent Pat Biomark. Author manuscript; available in PMC 2017 June 30.

Published in final edited form as:

Recent Pat Biomark. 2015 ; 5(2): 93–100. doi:10.2174/2210309005666150804195033.

Detection of Lysyl Oxidase-Like 2 (LOXL2), a Biomarker of Metastasis from Breast Cancers Using Human Blood Samples

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Abstract

Metastasis accounts for 90% of the mortality associated with breast cancer. Upregulated expression of members of the lysyl oxidase (LOX) family of secreted copper amine oxidases catalyzes the crosslinking of collagens and elastin in the extracellular matrix. LOXs are linked to the development and metastatic progression of breast cancers. Accordingly, aberrant expression of LOX-like 2 (LOXL2) is observed in poorly differentiated, high-grade tumors and is predictive of diseases recurrence, and for decreased overall patient survival. Therefore, LOXL2 expression may serve as a biomarker for breast cancer. Mechanistically, hydrogen peroxide is produced as a byproduct of LOXL2 when using an appropriate substrate, lysine. We exploited this chemistry to generate a revolutionary gold-based electrochemical biosensor capable of accurately detecting nanomolar quantities of LOXL2 in mouse blood, and in human blood samples. Two different sources of the blood samples obtained from breast cancer patients were used in this study indicating the applicability of detecting LOXL2 in breast cancers patients. Limited numbers of urine specimens from breast cancer patients were also tested. Collectively, all of these tests show the promise and potential of this biosensor for detecting LOXL2 as a surrogate biomarker of breast cancer. This work is described in WO 052962 A1 (2014)

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Keywords

Biomarker; metastasis of breast cancers; lysyl oxidase-like 2 (LOXL2); thin gold film biosensor

1. Introduction

Breast cancer is a significant health concern in the United States, representing the second leading cause of cancer death in women [1]. The lethality of breast cancers reflects their acquisition of invasive and metastatic phenotypes, events that account for nearly 90% of the mortality associated with mammary carcinomas [2–4], while the 5-year survival rate for women diagnosed with localized disease is high at 98%, this rate drops abruptly to 23% for women who exhibit evidence of metastasis at the time of diagnosis [5]. At present, the molecular mechanisms that underlie the development and initiation of metastasis remain incompletely understood, as does the means to effectively monitor patients for disease progression. Along these lines, breast cancer cells often disseminate in patients with small mammary disseminated breast cancer cells often escape clinical detection by acquiring dormant phenotypes, only to reemerge later as aggressive recurrent tumors that no longer respond to the therapeutic regimens used in treating the original tumor [7]. Breast cancer is not a homogenous disease, but is instead a heterogeneous disease comprised of at least 5 genetically distinct subtypes that exhibit disparate (i) histopathological features, grades, and markers; (ii) clinical presentations, prognoses, and outcomes; and (iii) responses to chemotherapies [8]. Collectively, these challenges highlight the need to develop novel diagnostic platforms capable of detecting breast cancers in otherwise seemingly healthy women.

Recent evidence has implicated members of the lysyl oxidase (LOX) family of secreted copper amine oxidases as important mediators of breast cancer development and metastatic progression [9–12]. Several recent patents involved LOXL2 as a potential biomarker of Metastasis of breast cancer [13–16] Recent assessment of the LOXL2 as a potential biomarker of metastasis of breast cancer has been reported. [17–20] related to the metastasis of breast cancer.

LOXs constitute a 5 member gene family consisting LOX and LOX-like (LOXL) 1–4, all of which play essential roles in maintaining cellular homeostasis, and in regulating the composition and biomechanics of the extracellular matrix [13,22–24]. Activated LOXs also function to elicit desmoplastic and fibrotic reactions by catalyzing the crosslinking of collagens and elastin. Amongst individual LOX family members, the inappropriate expression and activity of LOXL2 in human breast cancers correlates with (i) increased malignancy; (ii) the acquisition of invasive and metastatic phenotypes; and (iii) the formation of the premetastatic niche [25]. Furthermore, dysregulate LOXL2 expression in breast cancers is frequently observed in poorly differentiated, high-grade tumors, where its presence predicts an increased likelihood of disease recurrence and decreased overall survival [21–25]. These findings implicate elevated levels of LOXL2 as a biomarker to assess the stage, progression, and recurrence of human breast cancers. The objective of this study was to design, construct, and test a rapid and sensitive electrochemical-based LOXL2

biosensor capable of detecting minute quantities of LOXL2 in blood and/or urine specimens of breast cancer patients. This biosensor and the detection mechanism described herein possess tremendous potential to enhance the early clinical diagnosis of breast cancers, and to monitor metastatic progression and disease recurrence in patients with metastatic disease.

2. Experimental

2.1. Reaction Pathway in LOXL2 Detection

LOXL2 is a member of the LOX family, which catalyzes oxidative deamination of δ-amino group of lysine, resulting in the conversion of peptidyl lysine to peptidyl-α-aminoadipic-δ-semialdehyde (allysine) and the release of stoichiometric quantities of ammonia and hydrogen peroxide (H_2O_2). Therefore, it is possible to develop a method to quantitatively determine the levels of H_2O_2 produced by this peptidyl lysine reaction, and thus to provide a means to assess the relative expression levels of LOXL2 in human biological fluids. Consequently, monitoring of LOXL2 levels will identify patients harboring undetected breast disease, and will also provide an assessment of the stage and likelihood for metastasis of the mammary tumors. An important consideration in exploiting this chemical pathway for LOXL2 detection lies in verifying that the reactant lysine, as well as its co-products allysine and NH_3 do not interfere with the production and detection of H_2O_2. Figure 1 shows the mechanism of this measurement [26].

2.2. Fabrication of the Gold-Based Biosensor Prototype

Figure 2 shows the configuration of the biosensor prototype used in this study. It is a three-electrode configuration electrochemical based biosensor. This biosensor employs thin gold film working and counter electrodes and a Ag/AgCl reference electrode. Different from thick film printing, laser ablation technique is used to fabricate the gold film working and counter electrodes. Thin gold film is deposited on polyethylene terephalate (PET) by sputtering technique. Thus, the gold film is deposited at an atomic level, and without any binder. Consequently, the working and counter electrodes are uniform and reproducible. The laser ablation technique is a mature fabrication technology in micro-electronics industry, but its applications to produce uniform and reducible biosensors have not been fully exploited. The construction of this uniform thin gold film working and counter electrode elements using the laser ablation and roll by roll manufacturing process in creating this biosensor are unique. The deposition of thin gold film on polymeric substrate of a role-to-role process result in the biosensor developed very cost effective and a practical single use, disposable biosensor can be a reality.

In our preliminary design and fabrication of this biosensor prototype, the biosensor was fabricated by the sputtering a gold film onto a polyethylene terephalate (PET) substrate which was 355 × 280 mm^2. Laser ablation technique was employed to define the gold working and counter electrodes. The gold film in this case was 100 Angstrom (10 nanometer) in thickness. A chrome-quartz mask was used and UV-laser ablation to create the electrode features was also be employed. The Ag/AgCl reference electrode, the insulation layer and the silver electrode contacts were printed using thick film printing processing. Multiple, separate masks were used to produce the elements of the biosensor.

The Ag/AgCl reference electrode was thick film printed using DuPont #5870 Ag/AgCl thick film ink. The insulation layer was thick-film printed using Nazdar APL 34 silicone-free dielectric ink (colored in green in Fig. (2). The combined techniques resulted in the well-defined, reproducible and cost effective production of 4 rows of 100 biosensor prototype on each substrate. The overall dimensions of an individual biosensor were 33.0 × 8.0 mm^2 and comprised a three-electrode configuration. Each individual biosensor had a total sensing area of 1.54 mm^2 and a diameter of 1.4 mm which could accommodate a 10–15 μL liquid sample volume. We have identified and worked with an industrial vendor, Conductive Technologies, York, Pennsylvania and they are capable to manufacture this thin gold film biosensor prototype on an industrial scale with the required accuracy and reproductively.

2.3. Potential Interference Study of Allysine and Others

As shown in Fig. (1), the formation of an aldehydic-lysine compound from lysine was catalyzed by LOXL2, generating the reaction co-products NH_3, H_2O_2 and allysine. It was necessary to validate that allysine, NH_3 (in fact NH_4OH in an aqueous testing medium), and reactant lysine would not interfere with the electrochemical detection. This verification was accomplished by carrying out the cyclic voltammetric studies of allysine, NH_4OH and lysine ensuring that no oxidation current was produced that could interfere with the H_2O_2 oxidation.

Allysine is not commercially available, so we have to first carry out *in-situ* generation of allysine and its derivatives ensuring that they will not interfere with the electrochemical oxidation of H_2O_2 generated. Commercially available L-allysine ethylene acetal (#215054-80-1, Sigma-Aldrich, St. Louis, MO, USA) was hydrolyzed at room temperature with hydrochloric acid (HCl; #258148, Sigma-Aldrich) in tetrahydrofuran (THF; #494461, Sigma-Aldrich) to generate allysine *in situ*. The reaction mechanism involved in this *in situ* generation of allysine is shown in Fig. (3).

Briefly, one milliliter of 1 M HCl was added to 4.0 mL of tetrahydrofuran containing L-allysine ethylene acetal (0.264 mmol) [27]. The solution was stirred at room temperature under argon atmosphere for 1.25 hr, and then the reactions were neutralized with NaOH (#221465, Sigma-Aldrich) under argon. Afterward, 100 μL aliquots were extracted every 10 min throughout the reaction and tested with 2, 4-Dinitrophenylhydrazine (2, 4-DNP; #D199303, Sigma-Aldrich) for the presence of aldehyde. As early as 10 minutes into the reaction, the presence of aldehyde was detected using the 2, 4-DNP test. On the other hand, ninhydrin (#N7285, Sigma-Aldrich) was used to confirm the presence of the amino acid moieties. These tests could not confirm the sole presence of allysine. We anticipated that the formation of aldol or lysinorleucine was derivative by these rapid condensation reactions. However, these conversion processes occurred spontaneously so it was only necessary to measure the cyclic voltammograms during the *in situ* generation of allysine. Hence, a cyclic voltammetry study was performed during the course of the reaction in order to investigate if allysine or its derivatives would contribute to any oxidation current that may interfere with the electrochemical detection of H_2O_2. Aliquots were tested at t = 0, 10, 20, 30, and 40 min, respectively, throughout the course of the L-allysine ethylene acetal hydrolysis reaction. Figure 4 shows the reaction pathways involving allysine and lysine.

3. Results and Discussion

3.1. Calibration of H_2O_2 Detection of the Biosensor Prototype

The cyclic voltammograms of allysine and NH_4OH are showed in Fig. (5a & 5b) respectively. The generated reactive allysine moieties reacted further with either itself to produce allysine aldol, or with excess lysine to yield lysinonorleucine. These reactions occurred spontaneously, and it was only necessary to measure the cyclic voltammogram during the *in situ* generation of allysine. This would ensure its inability to produce any H_2O_2, or to generate any unwanted oxidation current. Figure 5a shows that the resultant accumulation of allysine, in the presence of tetrahydrofuran (THF) and HCl did not contribute to the measured oxidation current of H_2O_2. Figure 5b shows that NH_4OH also did not contribute to the oxidation current of H_2O_2, nor did lysine until catalytically-active LOXL2 was added to the reaction mixtures.

After validating that the reactants and co-products did not generate any considerable oxidation currents during the detection of H_2O_2 from the enzymatic reaction of LOXL2 reacting with lysine, the biosensor was calibrated to determine its sensitivity to detect the H_2O_2 produced by the LOXL2 based on the reaction pathway illustrated in Fig. (1). Amperometric measurements were performed by determining the oxidation current of H_2O_2 as a means to quantify the level of LOXL2 present. A calibration curve between the biosensor current output and the H_2O_2 concentration in deionized water was established ensuring that any H_2O_2 generated in Fig. (1) can be used to quantify LOXL2 by the biosensor. Thus, known quantities of the substrate, lysine (680 nM) was mixed with LOXL2 to produce H_2O_2 which was electrochemically oxidized. The oxidation current was then used to quantify levels of LOXL2 in the test samples. For these calibration experiments, the currents produced over the concentration range of 60–180 nM LOXL2 were monitored. Various incubation times between 100 seconds to 600 seconds were used in this study. It appeared that the steady study was reached after 400 seconds. Therefore, the reaction was incubated for 400 s prior to placing it on the biosensor for amperometric measurement. Figure 6 shows the calibration curve of LOXL2, which exhibited an outstanding linear relationship with the measured current showing a coefficient of determination of 0.997, and excellent consistency between replicates (n = 3).

For the calibration and testing procedure of this LOXL2 biosensor, typically, lysine solution was prepared by dissolving 10 mg of lysine powder in 1 mL of distilled water, which was subsequently diluted to a working concentration of 680 nM that was used throughout this study. In a typical run, specific concentrations of 60, 120, 180 nM of human LOXL2 (catalog #SRP0179, Sigma-Aldrich) were prepared from a 3 μM LOXL2 solution. The calibration curve for the LOXL2 was established using a total volume of 50 μL of mixed solution of 680 nM lysine solution and the specific LOXL2 concentration solution. This mixed solution was then incubated for 400 s at room temperature, at which point 10 μL of the reaction mixture was placed on the surface of the biosensor for amperometric measurements. The oxidation current output of the H_2O_2 was used to correlate with the LOXL2 concentrations. All measurements were conducted at room temperature, and at least 3 separate measurements were repeated for each LOXL2 concentration. For the detection of

LOXL2 in murine and human biological fluids, namely, serum and urine samples, identical process to the calibration step was used, and the testing procedure of these biological samples was: A 50 μL of 680 nM lysine solution was first placed in a small tube for handling and 5 μL of the murine or human sample was then added and incubated for 400 seconds. Then 10 μL of the incubated test solution was placed on top of the working electrode for measurement.

3.2. Results of LOXL2 Levels in Mouse Blood Samples

Murine metastatic 4T1 breast cancer cells were obtained from Fred Miller (Wayne State University, Detroit, MI, USA) and engineered to stably express luciferase by transfection with pNifty-CMV-luciferase, followed by Zeocin-mediated antibiotic selection (500 μg/mL) as described [16]. Mammary tumor development was initiated by engrafting 4T1 cells (1×10^4 cells) onto the mammary fat pad of 4-week old Balb/C mice. The development and progression of 4T1 tumors was monitored by intravital bioluminescent imaging of the animals on a Xenogen IVIS-200 (Caliper Life Sciences, Hopkinton, MA, USA) as described [24,25]. Upon completion of the studies, the tumor-free and 4T1 tumor-bearing mice were euthanized and whole-blood was immediately collected via the superior vena cava and supplemented with 0.32% sodium citrate prior to storage at −80 °C. Samples were thawed and applied to the biosensor to monitor LOXL2 levels as described above.

Figure 7(a) shows the levels of LOXL2 detected in the mouse whole blood samples obtained from control and 4T1 mammary tumor-bearing mice. The control sample showed a lower LOXL2 concentration, as was expected. Indeed, the electrochemical biosensor clearly distinguished the mice bearing 4T1 mammary tumors from those that were tumor-free (Fig (7a))

3.3. Results of LOXL2 Levels in Human Blood and Urine Samples

Human blood samples from breast cancer patients and healthy controls were obtained from two different sources: University Hospitals Case Medical Center (UHCMC, Cleveland, OH, USA) and its affiliated clinics, and the Hospital of the City of Hope, San Deigo, CA, USA. Briefly, newly diagnosed breast cancer patients were recruited from UHCMC and its affiliated clinics. Controls were recruited from patients receiving normal mammograms at the mammography centers of UHCMC. Blood and urine samples were collected from both patients and controls, and were collected from patients prior to the initation of radiation therapy or chemotherapy. Serum and plasma were isolated the same day of collection, and all serum, plasma and urine specimens were stored in −80 °C until analyzed. For LOXL2 measurements serum, plasma and urine from 10 patient and 10 control samples were randomly selected. Samples were thawed and then placed onto the sensor using the aforementioned reactions conditions. Only blood samples were collected from the patients of breast cancers in the Hospital of City of Hope and four control samples. Typically, 6 mL of blood sample was drawn and placed in serum separation tube (BD#367974, Becton Dickinson). The tubes were centrifuged at 1,100–1,300 rpm for 10 min. The serum was then stored at −80 °C freezer prior to use for test.

Our novel biosensor clearly detected LOXL2 in specimens of human serum (Fig. (7b)), plasma (*data not shown*), and urine (Fig. (7c)) from UHCMC. There was a striking linear relationship between LOXL2 concentrations and the biosensor measurements, illustrating that this detection technique was an accurate method for measuring LOXL2 in biological fluids obtained from breast cancer patients without the need to subject them to a complicated biopsy process. There was a trend showing that higher levels of LOXL2 from the biological fluids of breast cancer patients as compared to their cancer-free counterparts (Fig. (7b & 7c)), although there was not a clear cutoff between cases and controls.

Figure 8 shows the test results of a total of 30 patient blood samples and four control samples. It was clearly shown that the LOXL2 levels in the blood samples of the breast cancer patients were higher than those of control samples. These patient samples were collected by a group of nurses. The stage of the sample from each patient was not assessed in this protocol. The LOXL2 concentration level was measured separately four times. Because the stage of the cancer, the age of the patients, and the conditions of collecting the samples were not known, it was difficult to assess the contributing factors in the variation. Therefore, variation of the samples collected existed and any assessment on the collected samples and the stage of the patient individual was beyond the scope of this presentation. However, this would not affect the electrochemical detection of LOXL2 in the blood samples as presented in Fig. (8). Figure 8 shows the current outputs of the blood samples from the breast cancer patients and control collected in Hospital of Hope in San Diego, CA. More importantly, Fig. (9) shows the combined testing results (Fig. (7b) & (8) trend: same order of magnitude of the LOXL2 levels in breast cancer patients and control samples.

It will be necessary to quantify further the LOXL2 in patients with different stages of cancers and possible contributing factors of breast cancers before a definitive assessment of the ability to monitor for progression can be made. However, these findings collectively demonstrated the construction and successful implementation of an electrochemical LOXL2 biosensor as a rapid, sensitive, and noninvasive method for the quantification of LOXL2 in breast cancer patients.

3.4. Inhibition Study of LOXL2 Levels in Mouse Blood Samples

In order to ensure the biosensor detects LOXL2, an inhabitation study of LOXL 2 was carried out using 24 mouse blood samples. β-aminopropionitrile (βAPN) was used in this study. Typically, 10 μL of 2 mM BAPN was added into 5 μL of mouse blood samples. A 48 h of incubation time was chosen based on the results of the studies of various incubation time. After that, 40 μL of 680 nM lysine solution was added and incubated for 400 s, similar to the process decribed above. The steady state current were reachedat 27 seconds. Figure 10 shows the results of the current outputs obtained from whole blood or their clarified counterparts (*i.e.*, centrifuged at 800 rpm for 30 min) incubated with or without βAPN. Each blood sample was measured in triplicate. It was obvious that LOXL 2 was inhibited by the βAPN,thereby ensuring that this biosensor protoype measured LOXL2 as the mechanism suggested.

The mortality of breast cancers decreased by ~24% between 1990–2000 due to the combined actions of screening mammography and adjuvant chemotherapies [26]. However,

nearly 33% of women were subjected to unnecessary invasive secondary examinations due to the high false positive rate of mammography, suggesting that coupling this technique with additional breast cancer biomarker analyses would enhance the effectiveness of mammography. Techniques coupled to mammography to confirm the diagnosis of breast cancer include (i) fine needle aspirate and cytologica analysis, and (ii) surgical biopsy and histopathological analysis. Both secondary measures provide valuable information for disease staging and the likely clinical course and treatment options, but they are nonetheless invasive procedures which can be problematic and painful for the patient. Furthermore, implementing these techniques requires expensive equipment and skilled operators, thereby contributing significantly to the socioeconomic costs associated with treating breast cancer patients. Therefore, it would be highly desirable to develop a rapid, sensitive, and inexpensive test to augment screening mammography potentially reducing the number of women subjected to invasive secondary procedures. Accordingly, a rapid and sensitive diagnostic biosensor which detects nanomolar quantities of LOXL2 was developed. This biosensor measured the oxidation current of H_2O_2 which was generated through the reaction of LOXL2 and lysine. Biological fluids obtained from breast cancer patients such as human serum, plasma and urine samples were used in this evaluation. Moreover, this biosensor prototype was a single-use, disposable, and cost-effective device that readily distinguished tumor-bearing mice from those that were free of disease. The results of this investigation are given. Thus, broad-based application of this manufactured prototype offered new inroads in (i) detecting the development and metastatic progression of breast cancers in otherwise seemingly healthy women, (ii) assessing the efficacy of chemotherapies to elicit tumor regression, and (iii) monitoring the initial stages disease recurrence in women with seemingly stable remission. Future studies would be needed to address these points in a larger cohort of breast cancer patients having varied stages of disease, as well as in normal women to better quantify the range of LOXL2 contained in biological fluids and the causes of changes of these levels in response to aging, diet, menopause, and other noncancerous diseases.

As mentioned, breast cancer is not a single homogeneous disease, but is instead a heterogeneous disease comprised of 5 or more genetically distinct subtypes that collectively are the second leading cancer killers amongst United States women [26, 27]. Amongst individual breast cancer subtypes, those classified as triple-negative breast cancers (TNBCs) are especially lethal due to their highly aggressive and metastatic behavior, and to their propensity to recur following apparent remission [28–30]. TNBCs lacked expression of hormone receptors (estrogen and progesterone) and ErbB2/HER2. However, they typically possess BRCA1- and p53-defects and/or –deficiencies [27–29]. This unique phenotype prevented the development of targeted chemotherapies effectively against TNBCs, which also possess a heightened propensity to acquire resistance to standard-of-care chemotherapeutic agents (e.g., doxorubicin, cisplatin, and taxanes) [31]. Moreover, the diagnosis of TNBC is one of exclusion, not one of inclusion because specific biomarkers for this disease did not exist. Recently, several serum biomarkers for breast cancer were employed in clinical settings. This limited list included the cancer antigens (CA): CA15-3, CA27.29, and carcinoembryonic antigen (CEA), and all lacked the needed sensitivity and specificity [31, 32]. Recent findings linked the aberrant expression of LOXL2 to breast

cancer metastasis and disease progression, particularly in patients harboring late-stage and poorly differentiated TNBCs [33–37]. Similarly, dysregulated LOXL2 expression predicts for disease recurrence and poor prognosis in breast cancer patients [9–13]. Thus, our LOXL2 biosensor could overcome the aforementioned clinical deficiencies by providing a rapid and noninvasive test that detected the presence of breast cancers, and also could assess the disease severity based on the expression of LOXL2, although future studies will be needed to move this into a clinical application.

We recently observed the oncogenic activities of TGF-β and its stimulation of EMT to not only enhance the expression LOXL2 in TNBCs, but to also induce that of LOX [12] and LOXL (data not shown). Moreover, elevated expression of LOX, LOXL, and LOXL3 are associated with the development and metastatic progression of TNBCs [10, 37]. Further studies are needed to determine the extent for which our LOXL2 biosensor detects other LOX family members, particularly LOX and LOX1 which may promote metastasis of breast cancers.

4. Conclusion

A single-use, disposable biosensor prototype uses to detect the biomarker of metastasis of breast cancer, LOXL2, using blood samples from two different sources as well as urine samples from a single patient population was developed and showed very promising. More blood and urine samples from breast cancer patients for testing and validation will be carried out in future work.

5. Current & Future Developments

An international Patent Corporation Treaty (PCT) application on this research was filed as PCT/US2013/06262 on September 30, 2013. The application was published as WO 052962 A1 on 3 April 2014 (03.04.2014), titled “System and method for detecting lysyl oxidase-like 2 protein (LOX2) and breast cancer”. The application was nationalized in China, Europe and the United States, with the US national stage application number 14/427,904 filed on 12 May 2015 (12.05.2015). As described in this manuscript, preliminary testing results in blood plasma and urine tests on LOXL2 have been carried out with very promising results. However, more extensive clinical testing on both blood and urine samples from breast cancer patients need to be undertaken in the future development of this single use, disposable biosensor for LOXL2 detection. Also, the stage of the breast cancer and the early prediction of breast cancer using this LOXL2 biomarker detection will be included in future development of this biosensor.

Acknowledgments

This study was funded in part by a grant from the Case-Coulter Translational Research Partnership. Additionally, W.P.S. was supported in part by grants from the National Institutes of Health (CA129359 and CA177069), and the Seidman Cancer Center, while M.K.W. was supported by the National Cancer Institute (CA166140). Support by NSF (Grant No. 61071040) of China, Royal Thai Fellowship, and Taiwan National Central University Fellowship were acknowledged. Finally, we also acknowledge the expertise and assistance provided by the Case Comprehensive Cancer Center Imaging Research Core (P30 CA043703).

References

1. DeSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics, 2011. CA Cancer J Clin. 2011; 61(6):408–18.
2. Parvani JG, Taylor MA, Schiemann WP. Noncanonical TGF-beta Signaling During Mammary Tumorigenesis. J Mammary Gland Biol Neoplasia. 2011; 16(2):127–46. [PubMed: 21448580]
3. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer. 2009; 9:274–84. [PubMed: 19308067]
4. Chiang AC, Massague J. Molecular basis of metastasis. N Engl J Med. 2008; 359:2814–23. [PubMed: 19109576]
5. Altekruse, AF., et al. SEER Cancer Statistics Review 1975–2012. National Cancer Institute; Released on April 23, 2015
6. Donegan, W., Spratt, JS. Cancer of the Breast(book). 5th. London, UK: Elsevier Science Ltd; 2002.
7. Brackstone M, Townson JL, Chambers AF. Tumour dormancy in breast cancer: An update. Breast Cancer Res. 2007; 9:208–215. [PubMed: 17561992]
8. Reis-Filho JS, Pusztai L. Gene expression profiling in breast cancer: classification, prognostication, and prediction. Lancet. 2011; 378(9805):1812–23. [PubMed: 22098854]
9. Erler JT, Giaccia AJ. Lysyl oxidase mediates hypoxic control of metastasis. Cancer Res. 2006; 66(21):10238–41. [PubMed: 17079439]
10. Payne SL, Hendrix MJ, Kirschmann DA. Paradoxical roles for lysyl oxidases in cancer-a prospect. J Cell Biochem. 2007; 101(6):1338–54. [PubMed: 17471532]
11. Nishioka T, Eustace A, West C. Lysyl oxidase: from basic science to future cancer treatment. Cell Struct Funct. 2012; 37(1):75–80. [PubMed: 22453058]
12. Taylor MA, Amin J, Kirschmann DA, Schiemann WP. Lysyl oxidase contributes to mechanotransduction J -mediated regulation of transforming growth factor-β signaling in breast cancer cells. Neoplasia. 2011; 13(5):406–18. [PubMed: 21532881]
13. MeCauley, S., Smith, V. Catalytic domains from lysyl oxidase and LOXL2. US8927700. 2015.
14. MeCauley, S., et al. Antibodies that bind to lysyl oxidase-like 2 (LOXL2). US8680246. 2014.
15. Smith, V., et al. LOX and LOXL2 inhibitors and uses thereof. US 8461303. 2013.
16. MeCurley, S., et al. Catalytic domains from lysyl oxidase and LOXL2. US8512990. 2013.
17. Torres J, et al. LOXL2 is highly expressed in cancer-associated fibroblasta and associates to poor colon cancer survival. Clinical Cancer Research. 2015; published Online First July 23,2015. doi: 10.1158/1078-0432.CCR.14-3096
18. Cano A, Santamaria PG, Moreno-Bueno G. LOXL2in epitheliar cell plasticity and tumor progression. Future Oncol. 2012; 8:1095–108. [PubMed: 23030485]
19. Herranz N, Dave N, Millanes-Romero A, Morey L, Diaz VM, Lorenz-Fonfria V, et al. Lysyl oxidase- like 2 deaminates lysine4 in histone H3. Mol Cell. 2012; 46:369–76. [PubMed: 22483618]
20. Barker HE, et al. LOXL2-mediated matrix remodeling in metastasis and mammary gland involution. Cancer Research. Published Online First January 13,2011.
21. Barker HE, Erler JE. The potential for LOXL2 as a target for future cancer treatment. Future Oncol. 2011; 7(6):707–10. [PubMed: 21675833]
22. Peng L, Ran YL, Hu H, Yu L, Liu Q, Zhou, et al. Secreted LOXL2 is a novel therapeutic target that promotes gastric cancer metastasis via the Sre/FAK pathway. Carcinogenesis. 2009; 30:1660–9. [PubMed: 19625348]
23. Wendt MK, Smith JA, Schiemann WP. Transforming growth factor-beta-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. Oncogene. 2010; 29:6485–98. [PubMed: 20802523]
24. Taylor MA, Parvani JG, Schiemann WP. The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-beta in normal and malignant mammary epithelial cells. J Mammary Gland Biol Neoplasia. 2010; 15:169–90. [PubMed: 20467795]
25. Berry DA, et al. Effect of Screening and Adjuvant Therapy on Mortality from Breast Cancer. N Eng J Med. 2005; 353:1784–92.

26. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. CA Cancer J Clin. 2010; 60:277–300. [PubMed: 20610543]
27. Schneider BP, et al. Triple-Negative Breast Cancer: Risk Factors to Potential Targets. Clinical Cancer Research. 2008; 14(24):8010–8. [PubMed: 19088017]
28. Anders CK, Carey LA. Biology, Metastatic Patterns, and Treatment of Patients with Triple-Negative Breast Cancer. Clinical Breast Cancer. 2009; 9(Supplement 2):S73–81. [PubMed: 19596646]
29. Carey L, Winer E, Viale G, Cameron D, Gianni L. Triple-negative breast cancer: disease entity or title of convenience? Nat Rev Clin Oncol. 2012; 7:683–92.
30. Duffy MJ. Serum Tumor Markers in Breast Cancer: Are They of Clinical Value? Clinical Chemistry. 2006; 52(3):345–51. [PubMed: 16410341]
31. Brooks M. Breast cancer screening and biomarkers. Methods Mol Biol. 2009; 472:307–21. [PubMed: 19107439]
32. Moreno-Bueno G, Fernando S, Alberto M, Alfredo F, Eva PC, Vanesa S, et al. Lysyl oxidase- like 2 (LOXL2), a new regulator of cell polarity required for metastatic dissemination of basal- like breast carcinomas. EMBO Molecular Medicine. 2011; 3(9):528–44. [PubMed: 21732535]
33. Peinado H, Cidcm Del, Olmeda D, Csiszar K, Fong KS, Vega S, Nieto MA, et al. A molecular role for lysyl oxidase-like 2 enzyme in Snail regulation and tumor progression. EMBO J. 2005; 24:3446. [PubMed: 16096638]
34. Peinado H, Portillo F, Cano A. Switching On-Off Snail: LOXL2 Versus GSK3? Cell Cycle. 2005; 4:1749. [PubMed: 16294032]
35. Peinado H, Moreno-Bueno G, Hardisson D, Pérez-Gómez E, Santos V, Mendiola M, et al. Lysyl Oxidase-Like 2 as a new poor prognosis marker of squamous cell carcinomas. Cancer Res. 2008; 68:4541. [PubMed: 18559498]
36. Kagan HM, Li W. Lysyl oxidase: Properties, specificity, and biological roles inside and outside of the cell. Journal of Cellular Biochemistry. 2003; 88:660. [PubMed: 12577300]
37. Lucero HA, Kagan HM. Lysyl oxidase: An oxidative enzyme and effector of cell function. Cell Mol Life Sci. 2006; 63:2304. [PubMed: 16909208]

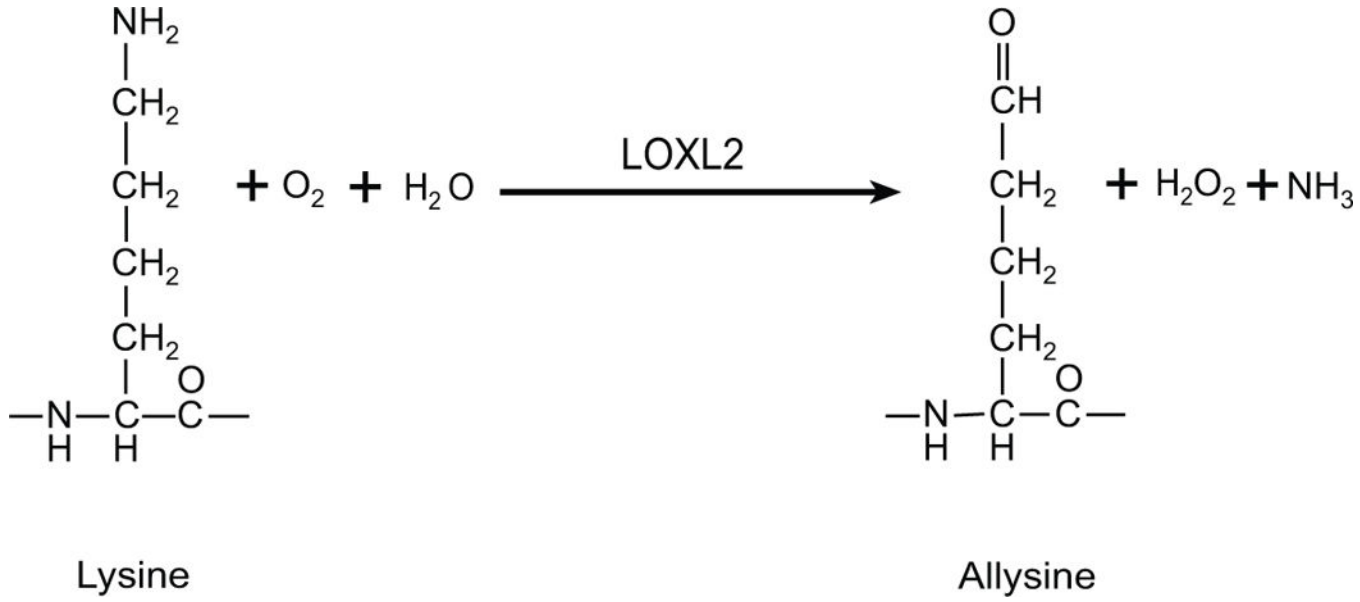


Fig. (1).

Chemistry involved in the detection of LOXL2 using an electrochemical biosensor. LOXL2 catalyzes the conversion of L-lysine to allysine, which produces ammonium hydroxide (NH4OH) and hydrogen peroxide (H2O2) as byproducts. The production of hydrogen peroxide elicits a current response in the biosensor that is proportional to the concentration of LOXL2.

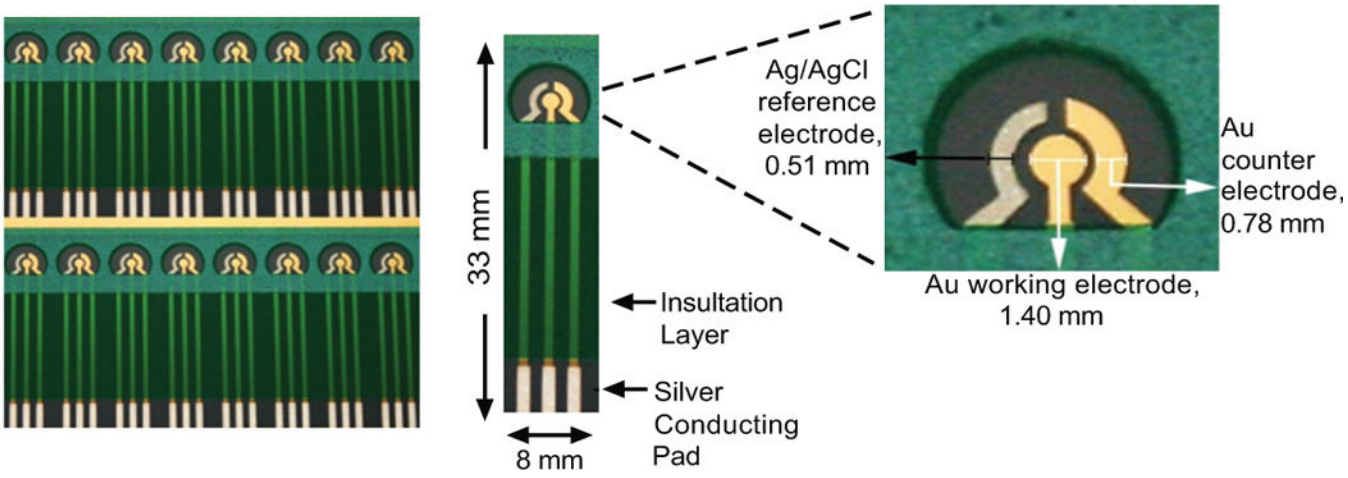
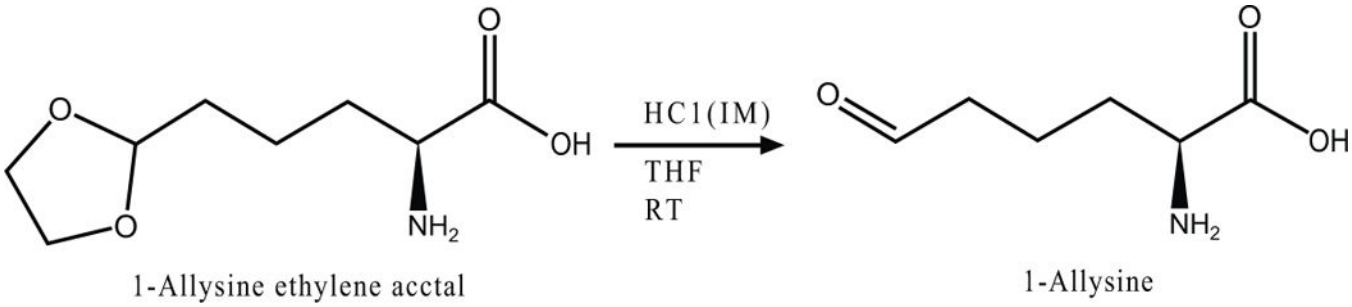


Fig. (2).

The configuration of the biosensor prototype with thin gold working and counter electrodes and Ag/AgCl reference electrode.



1-Allysine ethylene acctal 1-Allysine

Fig. (3).
Reaction mechanism involved in the in situ generation of allysine.

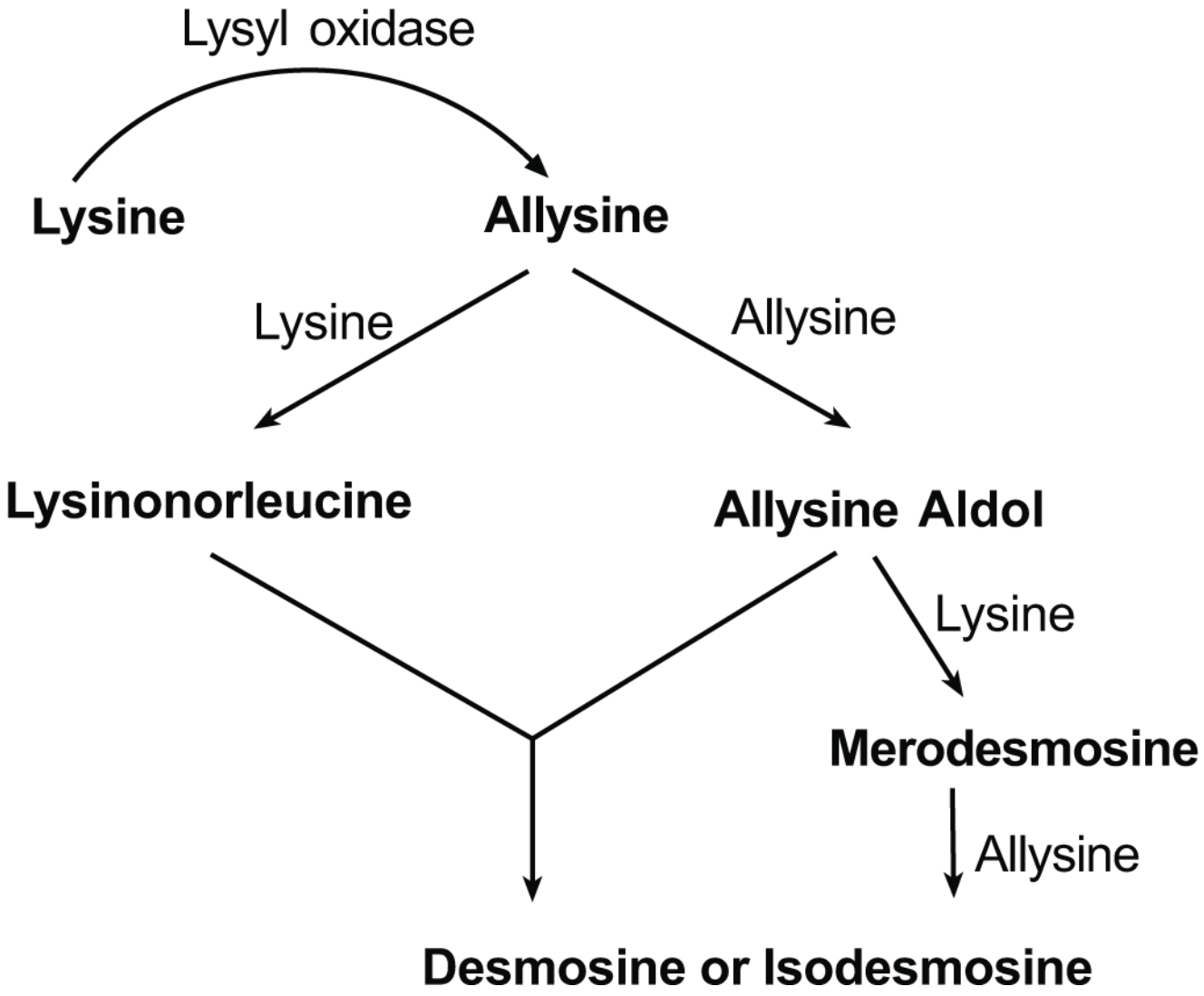


Fig. (4).
Reaction pathways involving allysine and lysine

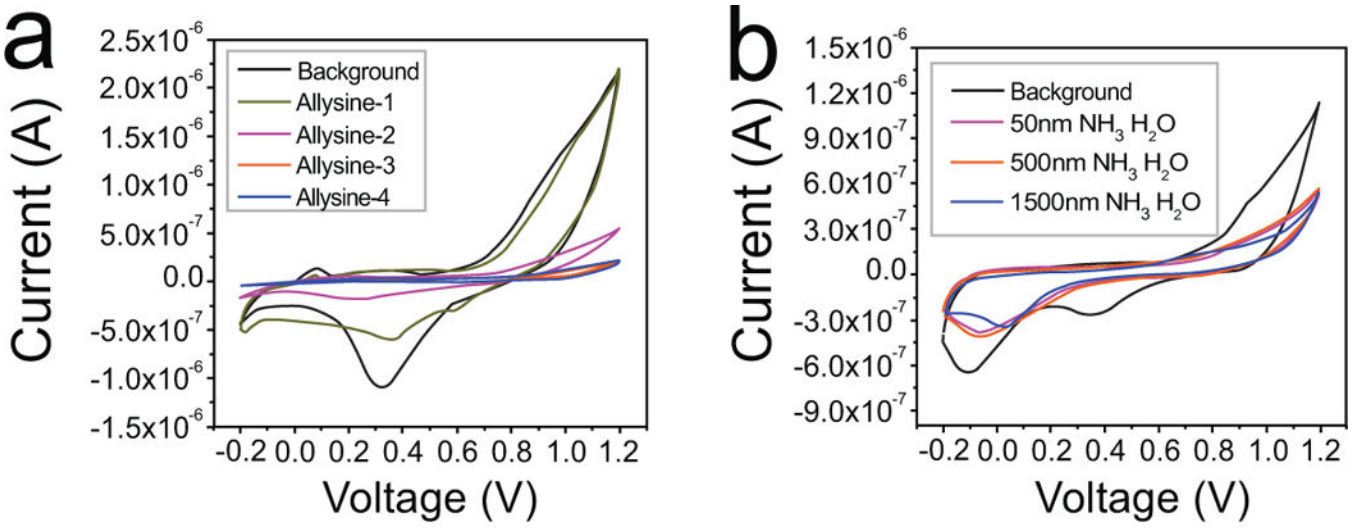


Fig. (5).

Cyclic voltammogram (CV) curves obtained using the LOXL2 biosensor. (a) CV curves measured during the in situ generation of allysine recorded at different time intervals. Background: the mixture of THF and HCl. Allysine 1: the moment of addition of L-lysine into the mixture of THF and HCl. Allysine 2: L-lysine reacted with the mixture of THF and HCl for 10min. Allysine 3: L-lysine reacted with the mixture of THF and HCl for 20 min. Allysine 4: L-lysine reacted with the mixture of THF and HCl for 30 min. (b) CV curves obtained from different concentrations of NH3·H2O.

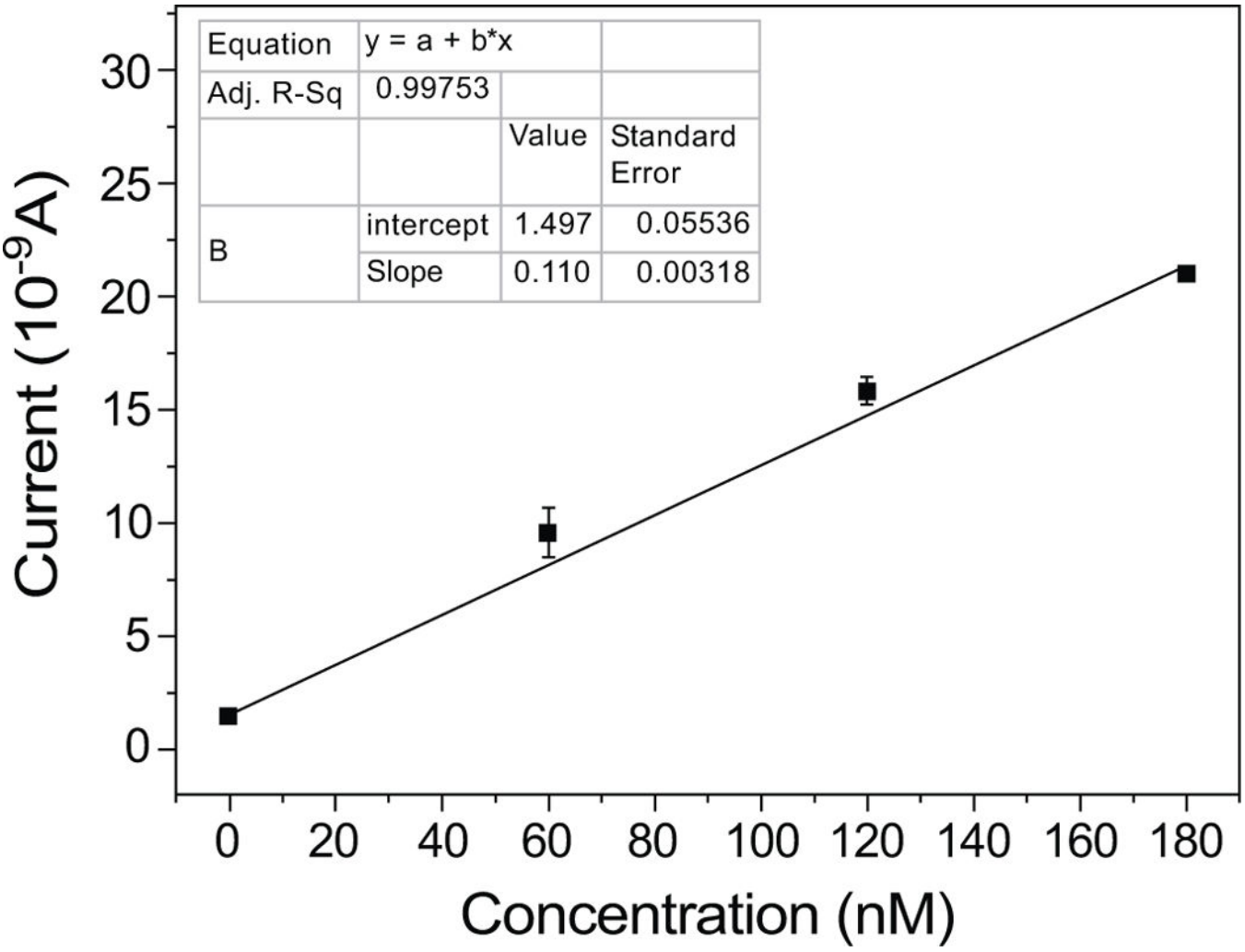


Fig. (6).

Calibration of the LOXL2 biosensor. The calibration curve was of current output versus the LOXL2 concentration covering 0–180 nM. The data were analyzed using an ordinary least squares fit of a simple linear regression model and are the mean ± SD (n = 3).

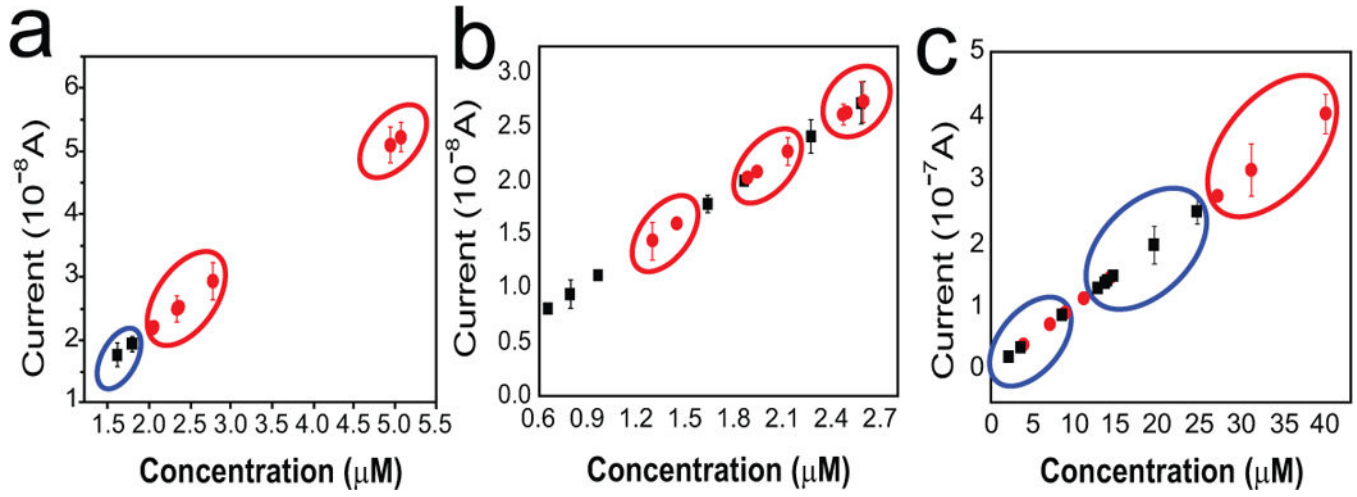


Fig. (7).

Detection of LOXL2 Activity in Murine and Human Biological Fluids. (**a**) The application of whole-blood samples to the LOXL2 biosensor clearly delineates tumor-free mice (blue circles) from those harboring mammary tumors (red circle). Date are the mean ± SD (n = 3). (**b** & **c**) The LOXL2 biosensor readily detects LOXL2 activity in serum (**b**) and urine (**c**) collected from normal controls (blue circles) and breast cancer patients (red circles). Data are the mean ± SD (n = 3).

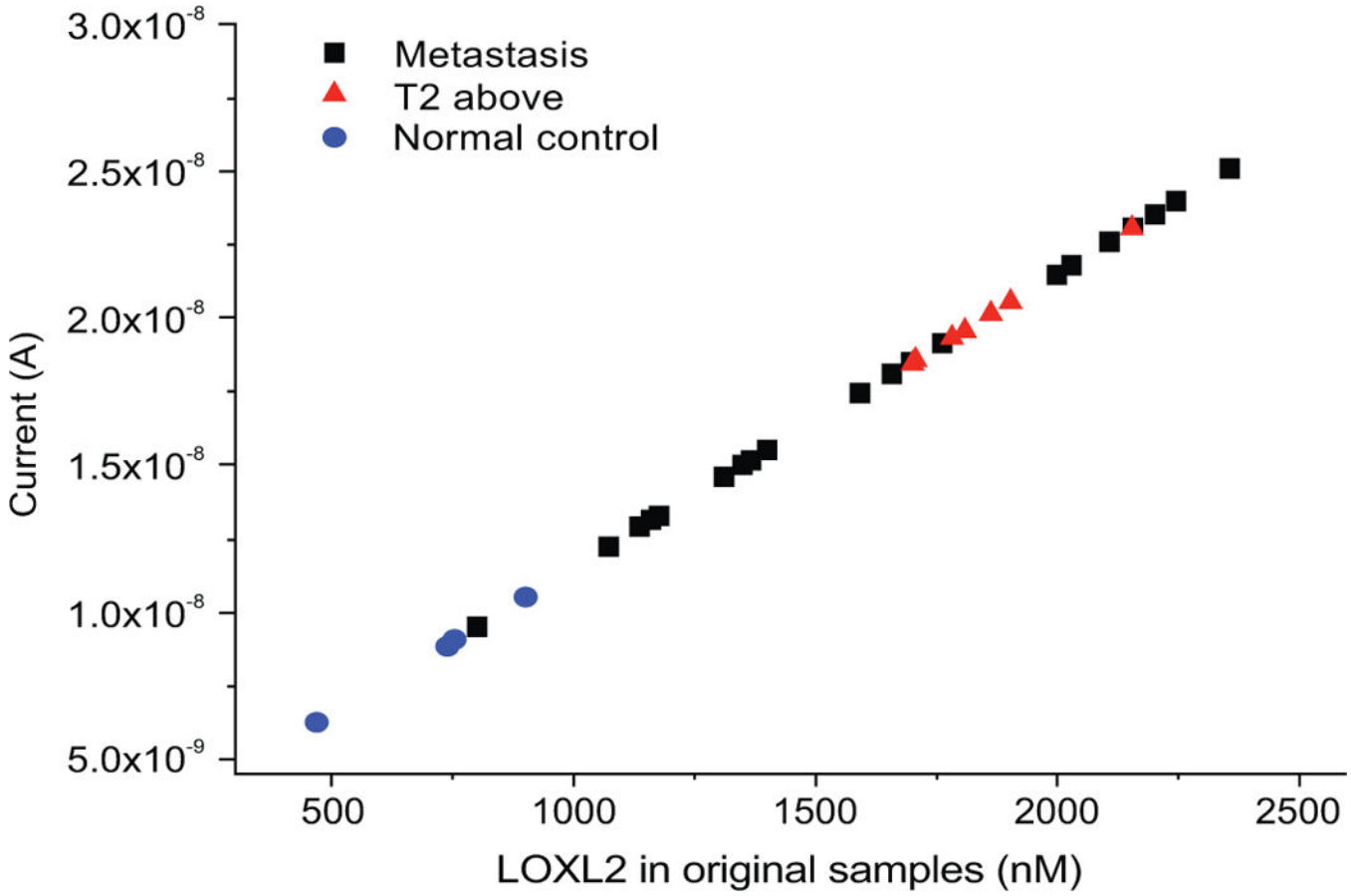


Fig. (8).
Detection of LOXL2 in the blood samples of breast cancer patients and control from the Hospital of City of Hope, San Diego, CA.

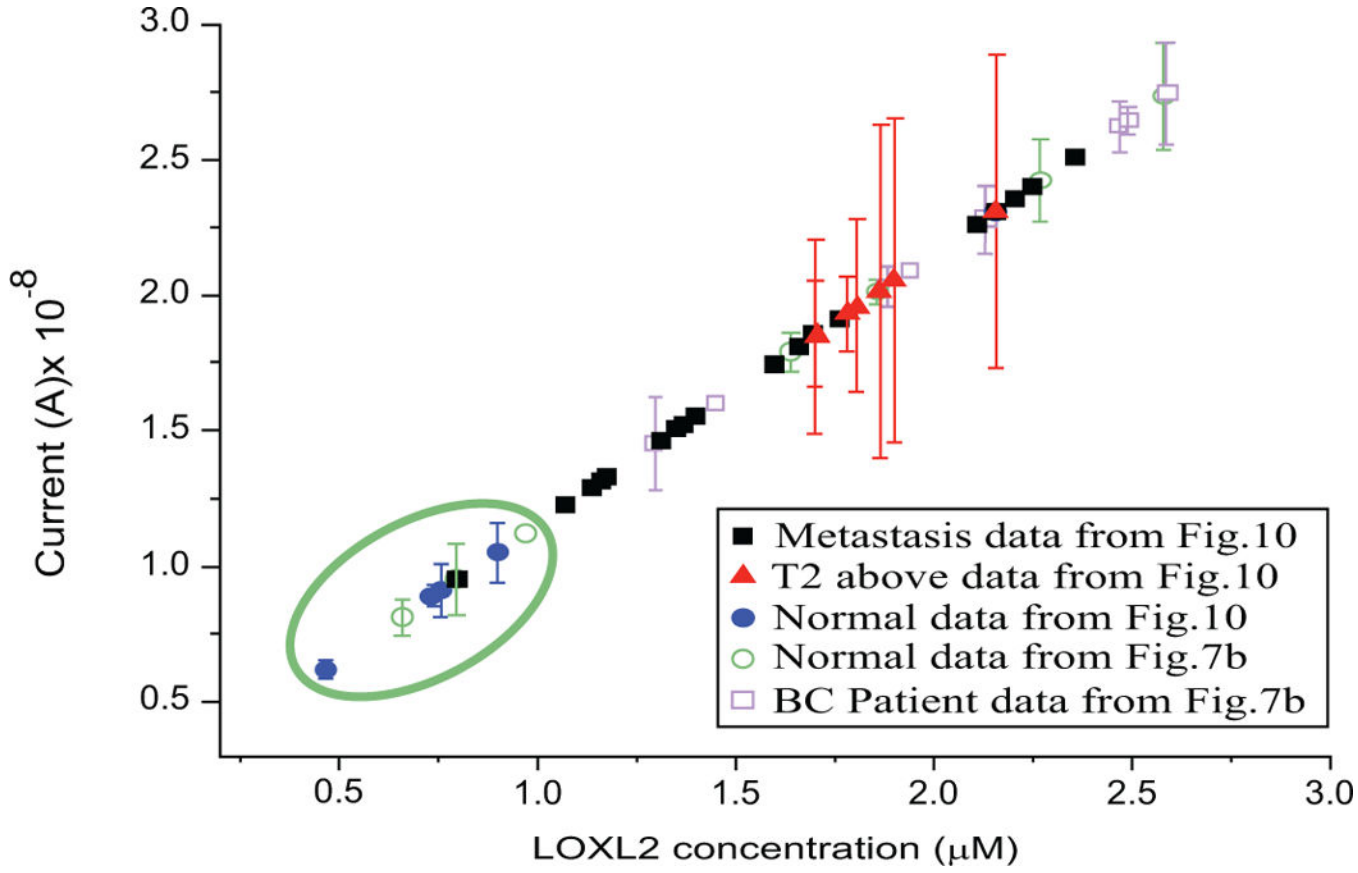


Fig. (9).
LOXL2 concentrations and the biosensor current outputs from two different sources of blood sample collections from breast cancers patients and control.

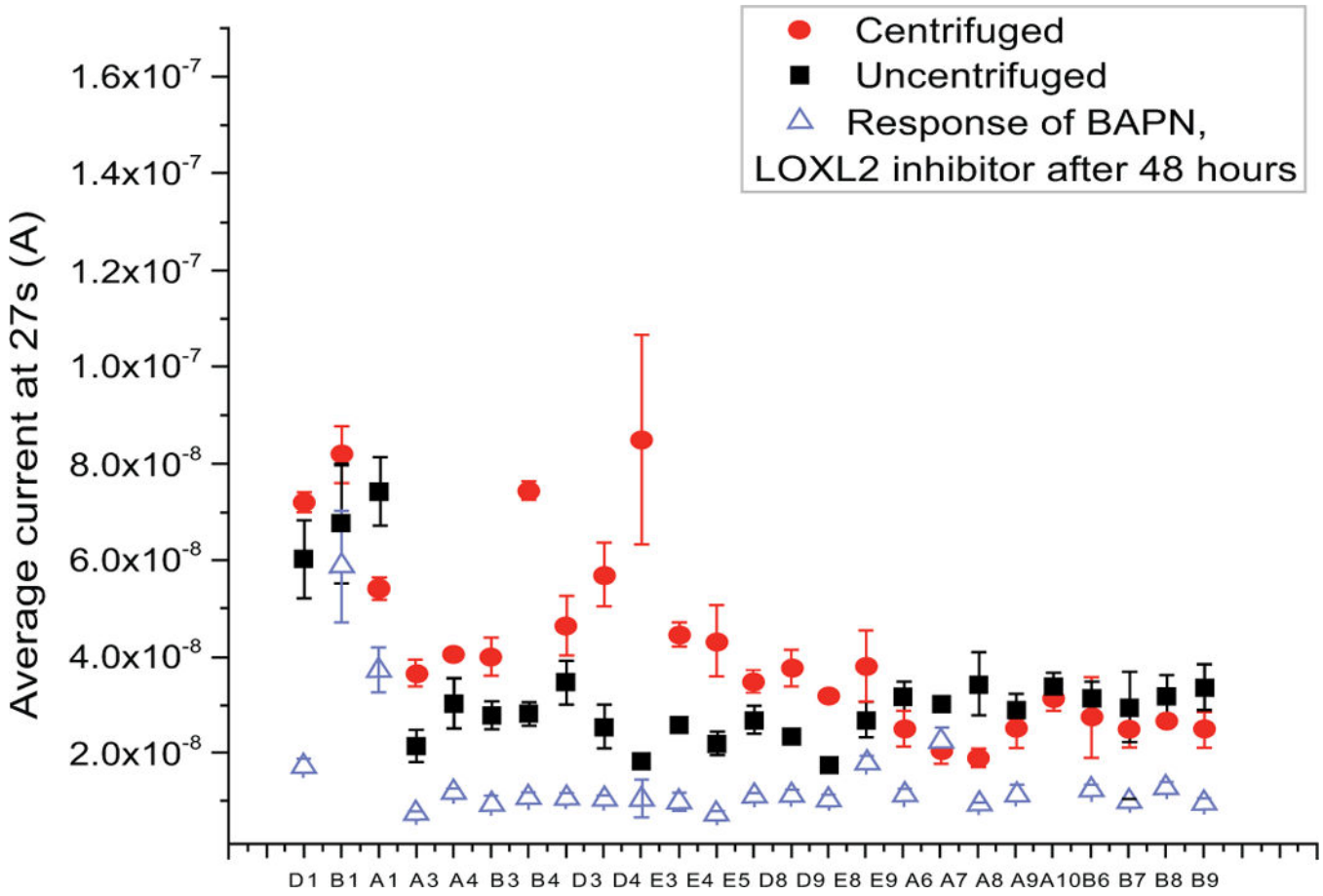


Fig. (10).
Inhibition study of LOXL2 in mouse blood samples using BAPN as the inhibitor