Functionalization Process for Commercial Viability: Oral Leukoplakia Detection Using IL-6 Biomarker

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Functionalization Process for Commercial Viability: Oral Leukoplakia Detection Using IL-6 Biomarker

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Oral leukoplakia (OL) or white patched in the oral cavity poses a diagnostic challenge in oral health due to its white patches on the oral mucosa, affecting 1%-2% of the population, predominantly those over 40 years old. Despite being often benign, OL often precedes potentially malignant disorders and oral cancer, necessitating early detection and intervention. The search for novel biomarkers has intensified, with interleukin-6 (IL-6) emerging as a promising candidate. IL-6 detection levels in saliva offer a non-invasive approach, aiding an accurate risk assessment and treatment planning. Here, we introduce an IL-6-based biosensor for rapid concentration detection. A novel, hour-long functionalization method streamlines mass production, maintaining a low detection limit down to 10^{-15} g ml^{-1}, which is three order lower than current commercial ELISA kits, with a sensitivity around 18/dec. Utilizing a specially designed printed circuit board with double pulse technology ensures precise concentration results, with human sample tests confirming the biosensor's efficacy in real-world applications. This innovation represents a significant advancement in early OL detection, enabling timely intervention to prevent its progression to more severe forms of oral cancer.
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Oral leukoplakia (OL) presents a diagnostic conundrum in the realm of oral health, characterized by white patches on the oral mucosa that cannot be attributed to any other identifiable cause.[1,2] OL occurs in 1%–2% of the population and is prevalent among individuals aged 40 and above.[3,4] While benign, OL is often considered a precursor to oral potentially malignant disorders (OPTMs) and oral cancer, necessitating early detection and intervention for improved patient outcomes.[5–7] Various risk factors contribute to the development of OL, including alcohol and tobacco use, weakened immune systems, long-term immune-suppressing medication use, personal or family cancer history, and cultural practices like the chewing of areca nut and betel leaf.[8–11] Traditional diagnostic methods, including clinical examination and biopsy, while valuable, may lack the sensitivity and specificity required for accurate detection and risk stratification and require surgical and pathology lab availability.[12–17]

In recent years, the quest for novel biomarkers capable of enhancing the precision and efficiency of OL detection has intensified. Several salivary and tissue biomarkers can reveal OL, including IL-6, IL-8, TNF-α, IL-1α, copper, zinc, and LDH.[18,19] Among these, interleukin-6 (IL-6) has emerged as a promising candidate. IL-6, a multifunctional cytokine involved in inflammation and immune regulation, exhibits dysregulated expression patterns in various pathological conditions, including cancer.[18,20–23] Notably, elevated levels of IL-6 in saliva have been implicated in the pathogenesis of oral cancer and other oral mucosal abnormalities, suggesting its potential utility as a diagnostic biomarker for OL.[24–27] Harnessing the diagnostic potential of IL-6 in OL detection holds several advantages. Firstly, IL-6 can be readily detected in biological fluids such as saliva, offering a non-invasive and easily accessible sampling method.[24–27] Moreover, the dysregulated expression of IL-6 in OL and its progression to oral cancer underscores its relevance as a dynamic biomarker reflecting disease severity and progression.[23,28] By integrating IL-6 biomarker analysis into existing diagnostic algorithms, clinicians may achieve more accurate and timely risk assessment and prognostication, facilitating personalized treatment strategies tailored to individual patient needs.[29–31] The typical cost of oral cancer treatment per year varies depending on factors such as cancer stage, treatment modalities, and patient-specific factors. Treatment expenses typically include surgery, radiation therapy, chemotherapy, and supportive care. According to available data on managing oral complications of cancer treatment, costs range widely: from $5000 to $30,000 for mucositis in patients undergoing radiation therapy, $3700 per chemotherapy cycle for mucositis, and over $70,000 for mucositis-related hospitalization in stem cell transplant recipients. These figures underscore the significant financial burden of oral cancer treatment, which can extend to hundreds of thousands of dollars per year, depending on individual circumstances and treatment needs.[32] Hence, there is a necessity to devise a cost-effective method for diagnosing OL at the earliest stage possible, enabling prompt initiation of treatment to prevent its progression to more severe forms of oral cancer.

In this study, we introduce an IL-6-based biosensor capable of indicating relative concentration levels within a minute. A novel functionalization method has been developed with the aim of facilitating mass production within the industry.[33–36] Previous iterations of the functionalization process required two days to complete, involving time-consuming gold plating and complex solution soaking steps.[37–39] These methods were deemed unsuitable for industrial-scale production due to their intricacy. Therefore, optimization of the functionalization process was imperative prior to industry adoption. In our current work, functionalization can be achieved within an hour, involving the simple application of chemicals onto the electrode followed by drying. Despite the shortened process time, the biosensor maintains a limit of detection (LOD) as low as 10^{-15} g ml^{-1} with a sensitivity of 18/dec. We employ a specially designed printed circuit board (PCB) in conjunction with a double pulse design to ensure precise concentration results, thereby mitigating the screening effect. Furthermore, human sample tests involving saliva and tissue demonstrate the efficacy of the biosensor in real-world applications.

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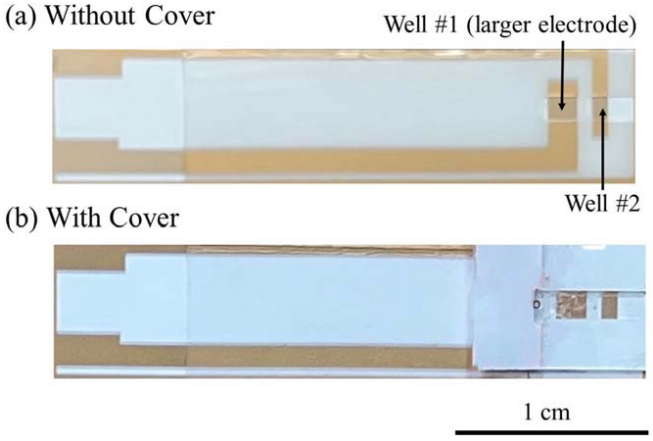

Figure 1. Design of the test strip. (a) The base of the strip, without cover on it. There are two wells created by a transparent film. (b) After functionalization process, the cover will be applied on the strip. There are two holes on it, the smaller one is the air hole, which make the sample solution can be uptake into the channel.

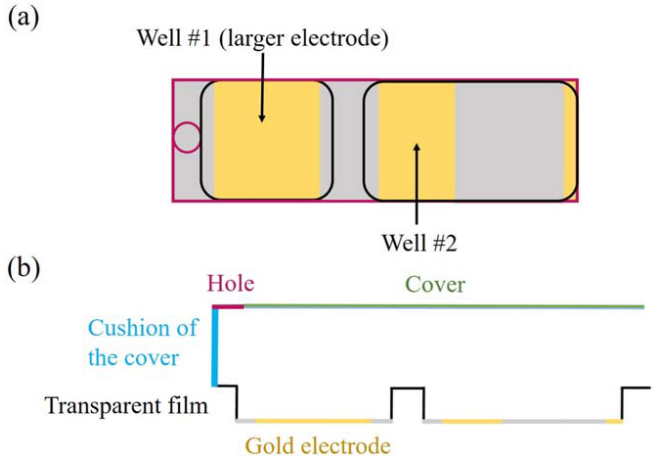

Figure 2. Detailed design of the microchannel on the strip after applying the cover. (a) The top view of channel. (b) The side view of the channel.

Experimental

In this study, the sensor strip manufactured by TaiDoc Technology Corporation in Taiwan. Figure 1 shows the pictures of the test strip. There are two parts: the base with two wells and the cover. The cover will be applied to the base once the functionalization process is completed. Figure 2 illustrates the more detailed schematic of the microchannel. There are two wells on the base with gold electrodes. These strips involved a process of gold sputtering, followed by selective removal of gold to form distinct electrodes, with two gold electrodes, one for signal input and the other for output. The larger electrode was functionalized for the detection of specific biomarkers, enabling the discernment of variations between samples. The sequence of functionalization steps is depicted in Fig. 3 like the first type, the functionalization process began with a 15 min ozone treatment to remove carbon residues. Subsequently, a diluted ammonium hydroxide (NH_4OH) solution is precisely dropped onto the electrode for 1 min with a pipette, followed by three rounds of DI water drops and removal steps to ensure electrode cleanliness. Next, a solution of 10 mM 3-Mercaptopropanyl-N-hydroxysuccinimide ester (NHS ester) is prepared, dissolved in ethanol. This NHS-ester solution, characterized by a three-carbon chain ending in a thiol group with an attached N-hydroxysuccinimide ester, serves as a pivotal agent for bioconjugation. Offering a reactive site for selective coupling with amine-containing molecules, the NHS-ester solution is applied in 1.2 μl drops onto the electrode, allowed to dry for 1 min, and repeated thrice. Subsequent cleaning of the channel with DI water drops precedes the application of 1.2 μl of monoclonal IL-6 antibody (Thermo Fisher Scientific, MA) at a concentration of 20 μg ml^{-1} onto the channel. This step typically requires approximately 20 min to dry. Subsequent rinsing with water drops completed the functionalization process, which could be achieved within an hour.

Calibration curves were established by diluting Human IL-6 Recombinant Protein (Thermo Fisher Scientific, MA) to concentrations ranging from 10^{-7} to 10^{-15} g ml^{-1} with artificial saliva (Pickering Laboratories Inc., Mountain View, CA). Fourteen human saliva and tissue samples were procured from individuals, including both oral leukoplakia patients and healthy volunteers, in collaboration with the University of Florida Oral Pathology Clinic and Dental Clinical Research Unit. Both the healthy sample and those with inflammatory oral lesions diagnosed as leukoplakia ranged in age from 40 to 80 years. Comprehensive patient information is presented in Table I. Brush kits (Andwin Scientific, Simi Valley, CA) were employed for tissue sample collection. These specimens were meticulously preserved in a deep-freeze storage unit at −78 °C. The study encompassed two sample groups: saliva and tissue. The OL patients were diagnosed by biopsy results. Prior to testing, both saliva and tissue samples underwent cell lysis. The native lysis buffer, sourced from Thermo Fisher Scientific (Massachusetts, U.S.), was utilized for this purpose. Epithelial cells were harvested by gently swabbing the mucosa inside the oral cavity with the provided brushes. Following collection, the brush heads were excised and transferred into 1.5 ml microcentrifuge tubes. Subsequently, 1 ml of 1x PBS buffer solution was added to the tubes, and tissue samples were suspended in the solution via vortex mixing. In the cell lysis process, the sample solution was mixed with the native lysis buffer at a ratio of 1:10 in a 1.5 ml microcentrifuge tube. Specifically, 50 μl (equivalent to 1 drop) of sample solution was combined with 50 μl of lysing agent and thoroughly mixed using a vortex mixer. The mixture was then incubated at room temperature for 10 min. Finally, centrifugation at 14000 rcf for 15 min at 4 °C facilitated the separation of resulting supernatant and pellet, which were subsequently refrigerated for further analysis. The analysis involved presenting continuous variables as mean (SD) or median [IQR] (range), and categorical variables as N (%). Mann-Whitney tests were used to compare continuous variables, while Fisher's exact tests were employed for categorical variables.

The PCB utilized in this study contains essential components such as the readout block, pattern generator, digitalizer, strip connector, Arduino, display, control switch, and system clock and power management unit (CLK & PMU), which is shown in Fig. 4. Detailed information on its operation has been provided in previous studies.[37,39] Operationally, upon strip connection, the Arduino triggers signal generation, initiating the pattern generator to produce a test pattern for measurement. This pattern traverses the strip, generating varied output signals amplified by the MOSFET in the readout block. These signals are then converted into frequency signals by the (voltage-controlled oscillator) VCO in the digitalizer, providing a digital representation of the readout voltage. This data is processed by the Arduino and displayed on the mini-LCD screen for user interpretation. To ensure measurement accuracy, multiple test patterns are generated and averaged, with the MOSFET's gate terminal grounded after each measurement to dissipate accumulated charge. The device offers adjustability through control switch manipulation, Arduino-controlled digitalizer timing, and PCB potentiometer for voltage adjustment. The result obtained from each measurement in this study is the average of ten readings. Each pulse lasts 1.1 ms, so averaging ten readings will take less than 2 s.

Results and Discussion

To assess the efficacy of the strip fabricated via a novel functionalization process, capable of completion within one hour,

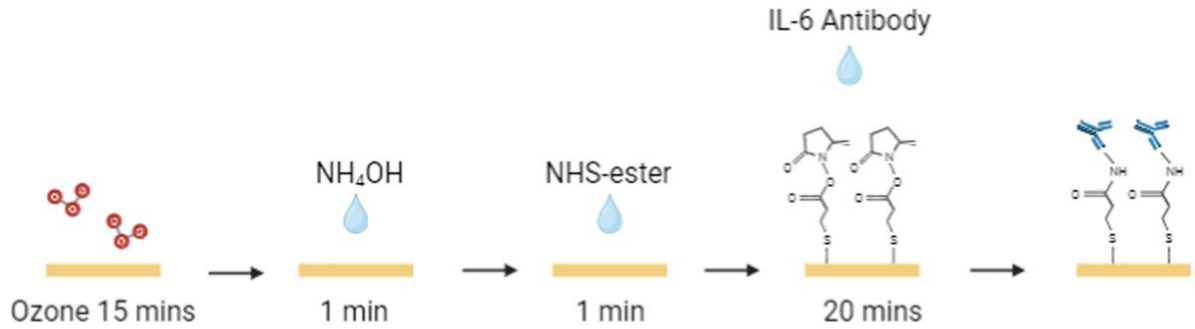

Figure 3. Functionalization step of the test strip. Each drop is 1.2 μl.

Table I. Subject characteristics by pathology status. Continuous variables presented as mean (standard deviation); median [interquartile range]; range; categorical variables presented as N (%). P-values are the results of Mann-Whitney tests (continuous variables) or Fisher's exact tests.

	All subjects (N = 14)	Healthy (N = 7, 50%)	Leukoplakia (N = 7, 50%)	*p*-value
Male	7 (50.0)	4 (57.1)	3 (42.9)	.637
Age	57.8 (13.2); 59 [44,69] (40,76)	52.4 (12.2); 53 [42,59] (40,72)	63.1 (12.6); 69 [59,70] (41,76)	.200
Hispanic	1 (7.1)	1 (14.3)	0 (0)	1
White (vs Asian)	12 (85.7)	6 (85.7)	6 (85.7)	1

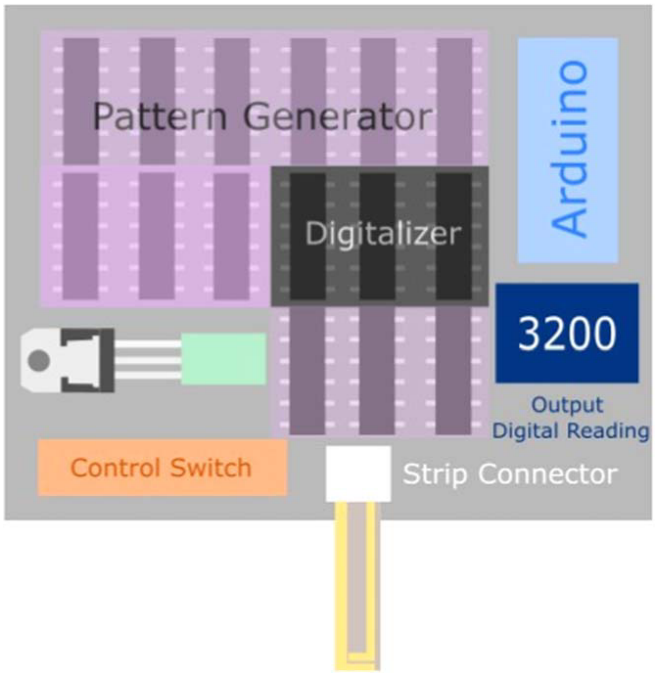

Figure 4. Schematic of the printed circuit board used in this study.

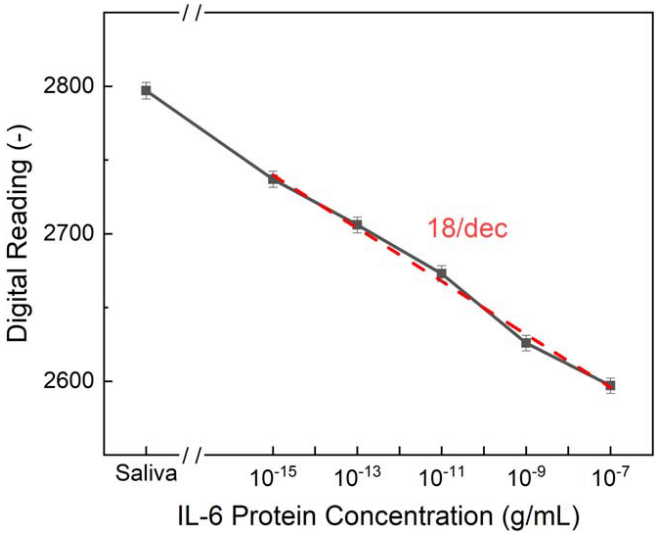

Figure 5. Calibration curve of IL-6 protein. The sensitivity is 18/dec while the limit of detection is 10^{-15} g ml^{-1}.

a series of diluted IL-6 protein samples ranging from 10^{-15} to 10^{-7} g ml^{-1} were utilized to construct a calibration curve. Figure 5 illustrates the resultant calibration curve of the sensor. Each digital reading presented is the average of ten measurements per sample. The limit of detection (LOD) achieved is as low as 10^{-15} g ml^{-1}, with a sensitivity of approximately 18/dec. Sensitivity denotes the decrease in reading corresponding to a one-order increase in protein concentration. The sensitivity of this method is not as high as our previous functionalization technique for other biomarkers.[37–39] This reduction in sensitivity is due to the shorter functionalization time; we reduced it from 2 days to just one hour, which is insufficient for the chemicals to fully bond to the entire electrode surface. Despite this, we were still able to distinguish between standard solutions of different concentrations because the PCB we use is quite sensitive. It employs a double pulse measurement to detect small current differences and amplifies them using a MOSFET. Furthermore, typical IL-6 detection ELISA kits exhibit LODs in the pg/ml range,[40–42] our sensor's LOD enables detection three orders of magnitude lower than that of commercial alternatives.

Besides testing the standard sample solution, IL-6 protein diluted in artificial saliva, human saliva and tissue samples were also investigated. The results are shown in Fig. 6. With the lysed saliva sample. the difference between healthy and the oral leukoplakia group is not that significant as tissue samples. The analytical result is summarized in Table II. Continuous variables are presented as mean (standard deviation), median [interquartile range], and range, while categorical variables are shown as N (%). P-values were derived using Mann-Whitney tests for continuous variables and Fisher's

Table II. Sensor readings by disease group. Continuous variables presented as mean (standard deviation); median [interquartile range]; range; categorical variables presented as N(%). P-values are the results of Mann-Whitney tests (continuous variables) or Fisher's exact tests.

	Healthy (N = 7, 50%)	Oral Leukoplakia (N = 7, 50%)	*p*-value
Saliva measurement	3209 (139); 3147 [3110,3322]; (3051,3402)	2933 (352); 3033 [2754,3201]; (2339,3248)	.128
Tissue measurement	2205 (66.9); 2164 [2156,2252]; (2142,2314)	1784 (91.2); 1812 [1708,1852]; (1661,1895)	.0005

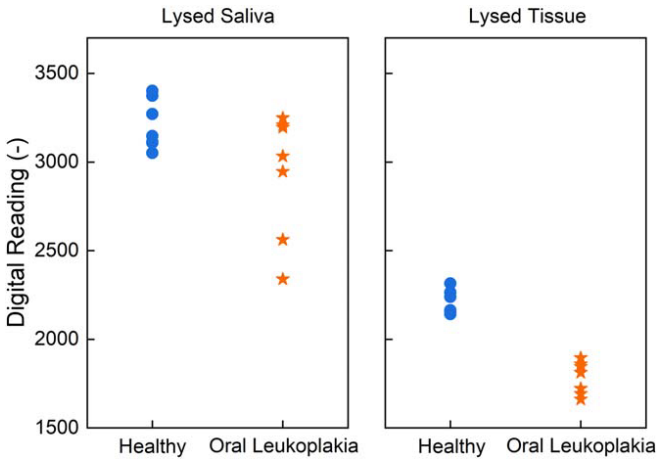

Figure 6. The output digital reading result from the human sample test with strips functionalized by IL-6 antibody.

exact tests for categorical variables. The p-value of the saliva measurement is 0.128, which means the accuracy of our device can do up to 87.2%. On the other hand, tissue measurement demonstrates a much better result with the p-value reaching a significance level of 0.005, indicating the accuracy to be 99.5%. Figure 7 plotted the result of the tests into box plot, showing the analytical result clearer.

The discussion of these findings highlights several key points. Firstly, while the novel functionalization process offers a significant reduction in preparation time, it does come at the cost of reduced sensitivity. This trade-off, however, does not completely undermine the utility of the sensor, as it still achieves a remarkably low LOD and can effectively differentiate between standard IL-6 concentrations. The sensitivity of 18/dec, although lower than that achieved with previous techniques, remains adequate for practical applications given the enhanced speed of preparation.

Moreover, the performance of the sensor in real biological samples further underscores its potential. The ability to detect IL-6 in both artificial and human saliva, as well as tissue samples, demonstrates the sensor's versatility. The relatively high p-value in saliva samples suggests variability that might be attributed to the complex matrix of saliva or the presence of interfering substances. In contrast, the significantly lower p-value in tissue samples indicates a more reliable and accurate detection capability in a less complex matrix. This is expected as the concentration of IL-6 is higher in tissue samples compared to the saliva samples.[28]

Overall, the reduction in functionalization time to one hour represents a substantial improvement in the efficiency of sensor preparation. Despite the decrease in sensitivity, the sensor's performance remains competitive, particularly when considering its extremely low LOD and the practical benefits of rapid preparation. The comparison with commercial ELISA kits further highlights the sensor's superior sensitivity, making it a promising tool for early detection of IL-6 in various biological samples. Future work may focus on optimizing the functionalization process to enhance sensitivity further while maintaining the reduced preparation time, as well as expanding the sensor's application to other biomarkers and complex sample matrices.

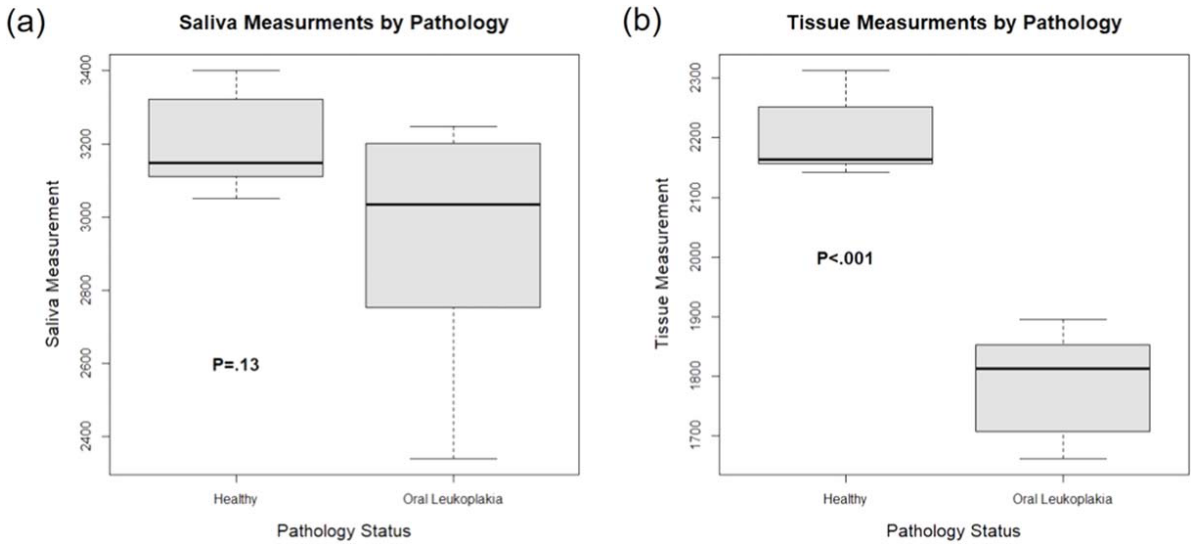

Figure 7. Boxplot showing the test results with (a) saliva and (b) tissue samples. In boxplots, bold center lines mean median, bottom and top edges stand for 25th and 75th percentile, and whiskers are minimum and maximum.

Conclusions

We have developed a novel IL-6 detection strip utilizing a rapid functionalization process that completes within one hour. This innovative approach significantly reduces preparation time while achieving a remarkably low limit of detection (LOD) of 10^{-15} g ml^{-1}, three orders of magnitude lower than conventional ELISA kits. Despite a reduction in sensitivity compared to previous techniques, the sensor demonstrates sufficient capability to distinguish between different IL-6 concentrations, underscoring its practical utility. Our findings show that while the sensitivity of the new method is slightly compromised due to the shorter functionalization time, the sensor's overall performance remains robust. This is particularly evident in the analysis of real biological samples, where the sensor could reliably detect IL-6 in both saliva and tissue samples. The tissue sample measurements yielded highly accurate results, highlighting the sensor's potential for clinical applications.

The study suggests that rapid functionalization does not entirely preclude effective sensor performance, and with further optimization, the balance between preparation time and sensitivity can be improved. This advancement opens new possibilities for quick and efficient biomarker detection, making the sensor a valuable tool for early disease diagnosis and monitoring. Future research will focus on refining the functionalization process and exploring the sensor's application across a broader range of biomarkers and sample types.

Acknowledgments

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