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Electrochemical Sensor for Multiplex Biomarkers Detection

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Abstract

Purpose—Multiplexing assay of biomarkers at the point-of-care is an elusive goal for molecular diagnostics.

Experimental Design—Here we report an electrochemical (EC) sensor for oral cancer detection based on the simultaneous detection of two salivary biomarkers: IL-8 mRNA and IL-8 protein.

Results—Under the multiplexing mode, the limit of detection (LOD) of salivary IL-8 mRNA reaches to 3.9 fM and 7.4 pg/ml for IL-8 protein in saliva. Multiplex assay of these two biomarkers directly from 28 cancer and 28 matched control saliva samples shows significant difference between the two groups. From the receiver operating characteristic (ROC) analysis, the EC sensor yields

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around 90% sensitivity and specificity for both IL-8 mRNA and IL-8 protein, which are very close to the data measured by traditional assays (ELISA and PCR) with the same group of saliva. Combined IL-8 mRNA and protein show better AUC compared with single biomarker.

Conclusions—We demonstrate, for the first time, concurrently multiplexing detection of salivary mRNA and protein biomarkers using point-of-care EC sensor.

Keywords

Salivary diagnostics; biomarkers; oral cancer; electrochemical biosensor; multiplexing

Statement of Translational Relevance

Multiplexing assay of biomarkers at the point-of-care is still an elusive goal for molecular diagnostics. Here we report an electrochemical (EC) sensor for oral cancer detection based on the simultaneous detection of two salivary biomarkers: IL-8 mRNA and IL-8 protein. Under the multiplexing mode, the limit of detection (LOD) of salivary IL-8 mRNA reaches to 3.9 fM in saliva. For IL-8 protein, the LOD is 7.4 pg/ml in saliva. Multiplex assay of these two biomarkers directly from 28 cancer and 28 matched control saliva samples shows significant clinical discrimination. From the receiver operating characteristic (ROC) analysis, the EC sensor yielded 90% sensitivity and specificity for both IL-8 mRNA and IL-8 protein. The good correlation between EC sensor and PCR/ELISA suggests that the EC sensor is promising for clinical diagnostics. We demonstrate, for the first time, concurrently multiplexing detection of salivary mRNA and protein biomarkers using point-of-care EC sensor.

Introduction

Early diagnosis is an important factor to enhance effectiveness of treatment(1,2). The analysis of body fluids offers possibility to shift the detection of cancer to an earlier stage. As we know, molecular markers from the tumors are released into the surrounding and enter into blood and other body fluids. Recent results have shown both cell-free mRNAs(3) and proteins(4) in saliva present diagnostic values for oral cancer and other systemic diseases(5,6). Therefore, developing highly sensitive and accurate assay for salivary mRNA/protein biomarkers makes saliva a valuable diagnostic fluid and offers the potential for the identification of disease in a non-invasive and specific manner (7,8).

Due to the complexity of biological system, especially the human body, single biomarker alone is not effective enough for accurate diagnosis. Medical decision based on single biomarker usually has a high possibility of false positive and false negative. The typical accuracy for salivary biomarkers are from 0.65 – 0.85(9), however it is still far from of real clinical requirement. Recently, research shows that combination of multiple biomarkers generates improved accuracy instead of single biomarker(10,11). In Kozak's work, 3-5 biomarkers shows over 0.94 accuracy compared with single biomarker(11). This fact brings up the importance of multiplexing assay of biomarkers. The combination of multiple biomarkers is not limited to numbers of biomarkers for single type, i.e., proteinomic and genomic. Those biomarkers in the combination could also include nucleic acid(12), protein(13,14) and small molecules(13). Therefore, multiplexing detection of different type of biomarkers is essential for accurate diagnosis. However, due to the difficulties in measuring the low levels of protein/RNA/small molecule with the same condition at the same time, no technology has been reported addressing the multiplexing mode.

Electrochemical (EC) sensor has the advantage of high sensitivity/specificity as well as simplicity of instrumentation, and could be easily expanded into multiplex detection platform (15-17). The performance of the EC sensor have been demonstrated for nucleic acid(18) and protein biomarkers(19) in saliva individually with high sensitivity and specificity. Here we demonstrate for the first the time the use of an EC sensor with the multiplex detection of salivary biomarkers for oral cancer detection: IL-8 mRNA and IL-8 protein. We bench mark the EC detection against conventional assays of ELISA and qPCR and found the EC sensors to be as good as or better.

Materials and Methods

Patient biospecimens

Oral cancer and control subject salivas were obtained from the Gujarat Cancer & Research Institute (GCRI) under approved IRB from the respective institutions (GCRI and UCLA). Saliva collection, processing, stabilization were performed as previously described(6). Patient and control subjects demographics, tobacco habit and frequency, histopathology and staging of the cancer samples are all listed in supplementary material Table 1s.

Human saliva samples were measured in multiplexing mode. For each saliva sample, both EC and PCR/ELISA were done separately. The details of PCR measurement was described in Hu ZZ's work(20). The details of ELISA detection could be find in Arellano-Garcia MT's work (21).

Electrochemical sensor

All the electrical potentials in the following steps are referred to Au reference electrode. The gold reference electrode has been determined to be +218 mV vs. SCE by measuring cyclic voltammetric curves of 0.1 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (19). In all the steps, the solutions were loaded onto the whole area of electrode region including working electrode and counter electrode. After each step, the EC sensors were rinsed with ultra pure water (18.3 M Ω -cm) then dried by pure N₂.

The electrochemical sensor is a 16 array of gold electrodes chip. For each unit of the array, there are working electrode, counter electrode and reference electrode. Conducting polymer was applied in the EC sensor as the supporting film. To further improve the biocompatibility of conducting polymer, streptavidin modified dendrimer nanoparticles were introduced into the polymer matrix(22). The streptavidin-dendrimer (Genisphere, USA) with diameter of 70-90 nm has 2-4 streptavidin labeling. The basic unit of the dendrimer is oligonucleotide, which is heavily negatively charged and allows the incorporation of dendrimer into polymer matrix. For electropolymerization, the dendrimer was diluted together with pyrrole (Sigma, USA) in 1 \times PBS (pH 7.5, Invitrogen, USA) in the volume ratio of 1:200. The final concentration of pyrrole is 10 mM. After loading the mixture onto the gold electrode, cyclic square wave electrical field (csw E-field) was applied for electropolymerization(23). Each square-wave consisted of 9s at the potential of +350 mV and 1s at +950 mV, and 20 cycles of square-waves were applied. The whole process lasted for 200s.

Probe immobilization

50 μ L of 10 nM biotin and fluorescein dual-labeled hairpin probe for IL-8 mRNA (Operon, USA) in 1 \times Tris-HCl (pH 7.5, Invitrogen, USA), and 50 μ L of 0.005 mg/mL biotinylated human IL-8 monoclonal antibody (Mab) (Pierce, USA) in 1 \times PBS was load separately onto different gold electrode on the same chip array. The hairpin sequence is 5'-GAG GGT TGC TCA GCC CTC TTC AAA AAC TTC TCC ACA ACC CTC-3', whose steric hindrance specifically amplifies the current signal(18,24-27). After loading, csw E-field was applied again for probe

immobilization, with 20 cycles of 9s at -300 mV and 1s at $+200$ mV. The whole process lasts for 200s.

Sample incubation

50 μ L of saliva supernatant were loaded onto the probe-coated electrode. Briefly, Saliva samples were centrifuged at $2,600 \times g$ for 15 minutes at 4°C . The supernatant was removed from the pellet and processed for RNA and protein stabilization(9). Regarding electrodes coated with probes for IL-8 mRNA and IL-8 protein, the same saliva supernatant was loaded. For the concentration calibration process, IVT IL-8 RNA and standard IL-8 protein (Pierce, USA) were spiked into the saliva supernatant first, and then loaded onto the electrodes. For the specificity control, S100A8 RNA(18) and IL-1 β protein(28), which are both biomarkers in saliva were loaded onto the electrodes. Details about preparation of IVT IL-8 RNA /IVT S100A8 RNA could be found in the previous work(18). Protein standard for human IL-8 and IL-1 β are both from Pierce (USA). In the spiking process, the delivery solutions for IVT RNA are RNase-free distilled water (Invitrogen). The delivery solutions for protein standards are $1 \times$ PBS (pH 7.5). After saliva loading, csw E-field was applied again for saliva incubation, with 20 cycles of 9s at -300 mV and 1s at $+200$ mV. The whole process lasts for 200s.

Reporter incubation

Mixture of 150 μ U anti-fluorescein HRP (Roche, USA) and 1:100 dilution of HRP conjugated human IL-8 monoclonal antibody (Pierce, USA) in 1% casein/PBS (Pierce) were prepared. 50 μ L of the mixture were loaded onto the saliva-incubated electrodes separately. After saliva loading, csw E-field was applied again for reporter binding with 30 cycles of 9s at -300 mV and 1s at $+200$ mV. The whole process lasts for 300s.

Signal readout

3, 3', 5, 5' tetramethylbenzidine substrate (TMB/ H_2O_2 , low activity) (Neogen, USA) was loaded and amperometric detection was carried out by applying -200 mV potential to each electrode unit, followed by parallel amperometric signal read-out after 60 s equilibration. TMB acts as a mediator and is reduced at -200 mV; the reduced TMB reduces the oxidized form of HRP. HRP then reduces H_2O_2 to 2 H_2O and the HRP is oxidized.

Statistical Analysis

The area under the curve (AUC) is based on constructing a receiver operating characteristic (ROC) curve which plots the sensitivity (y-axis in our analysis) versus one minus the specificity (x-axis in our analysis). The area under the curve value is computed by numerical integral of the ROC curve. The typical range for this value is between 0.5, which indicates that the biomarker has no diagnostic utility i.e. that the biomarker is no better than a coin flip, and 1 which would indicate perfect diagnostic accuracy. The cutoff point corresponding to maximum (sensitivity + specificity) -100% was found and used as optimal cutoff in the logistic regression model.

The cutoff points for IL-8 mRNA and protein were separately determined by identifying the cutoff that yields the maximum sum of the sensitivity plus specificity. The ROC curves in Figures 5c were constructed using the ROC package in R (ver 2.70). This function constructs the ROC curve from the continuous markers values by first ordering the marker values then computing the sensitivity and specificity for each possible cut-point of the markers. For the combination of the two, the cutoff point corresponding to maximum of sensitivity and specificity was found and used as the optimal cutoff in the logistic regression model.

Results

Specificity validation of EC sensor

The EC sensors are 16 integrated gold electrode arrays. Probes for mRNA and protein are pre-coated onto different electrodes. Sandwich assay are carried out for both salivary mRNA and protein by spiking the standard *in vitro* translated (IVT) RNA and protein standard into saliva (Fig. 1a).

The specificity experiments are carried out. For IL-8 mRNA probe, the comparison is between complementary IL-8 mRNA and a salivary internal reference gene S100A8 RNA. For IL-8 protein probe, the comparison is between IL-8 protein and another oral cancer marker IL-1 β protein. Both the raw data of amperometric detection (Fig1 b) and the bar charts (Fig1 c) are shown. With the IL-8 mRNA hairpin probe(18), 5 nM IL-8 mRNA generates -904 nA current (mean value). 5 nM S100A8 only results in -103 nA current (mean value), which is very close to the blank control (-98 nA). For protein assay, the current level for 12.5 ng/ml complementary IL-8 protein is -298 nA, while for IL-1 β protein it is only -50 nA (blank control is -33 nA). These results indicate the good specificity of the EC sensors.

Application of cyclic square wave electrical field (csw E-field) during detection

Multiplexing assay of mRNA and protein simultaneously has the advantage of high accuracy (10,11). However, the traditional PCR and ELISA technologies have difficulties in obtain both sensitivity and specificity under the same condition. Instead of applying different buffer and temperature to optimize the assay, cyclic square wave electrical field (csw E-field) provides more effective and versatile way to control the assay. With the csw E-field, both the hybridization and protein binding are finished on the same chip within minutes, while previously these processes have to be done separately and the incubation time varies from 1-24 hours. Meanwhile, the negative potential in csw E-field removes the weak non-specific binding which generate high specificity. csw E-field also results in good mixing during the incubation, which accelerates the binding process as well.

The results of csw E-field with both IL-8 mRNA and IL-8 protein are shown in Fig.2. From the studies in total reaction time, there exists an optimized condition for the csw E-field by spiking the standard *in vitro* translated (IVT) RNA and protein standard into saliva. The signal-to-background ratio (SBR) increases at the very beginning and reach to the highest SBR of about 10. Long time of incubation does not contribute to further improvement in SBR. Detections under DC E-field are also studied. Without the negative potential to remove the non-specific binding, DC E-field only has the low SBR around 3.0 for RNA. In addition, DC E-field could not generate good mixing for protein binding. The highest SBR is only around 3.5 for IL-8 protein binding. With the current saliva sample and EC sensor setup, the optimized condition for both RNA and protein detection in saliva is 20 cycles of 9s at -300 mV and 1s at +200 mV (200 seconds total). However, for each specific clinical sample (serum, urine, saliva, *et. al.*), because of the different properties of targeting molecule and supporting media, the csw E-field condition needs to be optimized independently.

Sensitivity validation of EC sensor

With the csw E-field, only single optimized condition is required for both mRNA and protein with highly sensitive/specific detection. Figure 3 shows the calibration curve of IL-8 mRNA and IL-8 protein by spiking the standard *in vitro* translated (IVT) RNA and protein into saliva under multiplexing mode. The sensitivity is 3.9 fM for mRNA and 7.4 pg/ml for protein with the cutoff at 2 standard deviations. The linearity for the two species are $R^2=0.98$ and $R^2=1$, respectively. These results show the high sensitivity for multiplex salivary biomarker detection. In addition, the dynamic range showed in Figure 3 covers about 4 orders of magnitude. For

IL-8 mRNA, the dynamic range covers from 5 fM to 50 pM (the mRNA concentration profile reach to the plateau at 50 pM level). For IL-8 protein, the dynamic range covers from 10 pg/ml to 12,500 pg/ml. The wide dynamic range provides a powerful tool for variant saliva sample.

Multiplexed assay of salivary protein and mRNA biomarkers by EC sensor

Totally 56 saliva samples including 28 oral cancer samples and 28 control samples from India were measured using the EC sensor. For each saliva sample, the whole measurement with EC sensor is within 10 min after loading the saliva, and 100 μ L of saliva are required for mRNA and protein detection together.

The EC sensor readouts are shown in figure 4a-b with oral cancer samples in the upper panel and control samples in the lower panel. IL-8 mRNA and IL-8 protein signal are listed together for each sample. Overall, each biomarkers shows higher level of the cancer than those of the control samples. Statistical analysis showed a significant difference for both IL-8 mRNA ($W=810, p = 6.637 \times 10^{-9}$) and IL-8 protein ($W = 820, p = 1.863 \times 10^{-9}$), estimated using the Wilcoxon signed ranks test(29). For the sample size requirements, at $\alpha=0.05, \beta=0.2$, sample size required is 13 for each group. Therefore 28 sample size is sufficiently powered.

Statistical Analysis

An equal number of age- and sex-matched subjects with comparable chewing, smoking and alcohol histories were selected as a control group. Among the two subject groups, there were no significant differences in terms of mean age: OSCC patients, 46 ± 12.8 years; normal subjects, 44.9 ± 11.9 years (Wilcoxon rank sum test, $P > 0.8$); gender ($P = 1$); chewing history ($P > 0.08$); smoking history ($P > 0.27$); or alcohol drinking history ($P > 0.06$). All of the subjects signed the institutional review board-approved consent form agreeing to serve as saliva donors for the experiments (Table 1).

The mean value of IL-8 mRNA is -345 nA with cancer and -116 nA with control (Fig. 4c). The mean value of IL-8 protein is -180 nA with cancer and -66 nA with control (Fig. 4d). However, the broad distribution of two biomarkers should be noticed as well. Despite the wide spread in the of EC values for the markers in the OSCC group we can see from the box plots that there is very little overlap between the groups. For example for IL-8 mRNA 75% of the OSCC cases have lower EC values than the minimum EC value for the controls.

Receiver operating characteristic (ROC) analysis provides a good way to evaluate the accuracy in diagnostic decision making(9). The ROC curves of the EC sensor for both IL-8 mRNA and IL-8 protein are illustrated in Figure 4e. The area-under-curve (AUC) is 0.90 for IL-8 mRNA and 0.91 for IL-8 protein. From the results measured with the same saliva samples by PCR and ELISA, the AUC is 0.91 and 0.87, respectively. The ROC curve analysis showed that the EC sensor for IL-8 mRNA achieved 83% sensitivity (percentage of positive samples correctly identified) and 87% specificity (percentage of negative samples correctly identified). For the IL-8 protein the EC sensor achieved 87% sensitivity and 87% specificity. These results indicate that the EC sensor is an accurate method for measuring salivary biomarkers as good as the conventional PCR and ELISA. It also demonstrates the ability to identify oral cancer subjects using a saliva-based screening.

Logistical regression has also been carried out with combined IL-8 mRNA and protein. The resultant data shows the combined IL-8 mRNA and protein did increase AUC to 0.93. The comparison between individual IL-8 mRNA, IL-8 Protein and combinational IL-8 mRNA +Protein are all illustrated in Figure 4e. The improvement in ROC indicates the combined IL-8 mRNA and protein further increases in area under curve (AUC).

Discussion

The EC sensor shows its advantages to PCR/ELISA in multiplexing assay for salivary diagnostics, in addition to the comparable sensitivity and accuracy. There are several challenges hindering the development of multiple assays with traditional PCR/ELISA. Regarding the assay procedure, most obstacles originate from the sample pretreatment, sample loading, and detection condition.

Sample pretreatment

Regarding the sample pretreatment, currently there are several problems needed to be solved, such as the storage, transportation, pre-amplification and lysis process(30). Those complicate procedures, when stabilizing the sample and amplifying signal, introduce several uncertainties into the saliva sample. For examples, storing the saliva at -80 degree provides long time stability. But the thaw-freeze cycle may probably damage the biomarkers inside the saliva. Another example is that, lysis process is frequently required for the salivary mRNA detection to release the mRNA from binding with macromolecules(30). But the lysis buffer itself will cause the damage of other biomarkers in saliva. Usually, a quality control should be carried out before measuring the saliva with traditional detection methods.

In this work, there is no pretreatment required for the saliva supernatant. The total measurement takes around 10 minutes from the saliva sample loading, which means the saliva could be measured on-site, right after the sample collection. This removes the major hindrance in the sample pre-treatment part.

Sample Loading

Sample loading includes the delivery, mix and incubation. Especially for the sandwich assay, there are multiple steps for loading processes such as the detection sample loading, reporter loading and final read-out reagent loading. It will cause technical problems in multiplexing modes. Increased throughput results in non-linear increase of complexity in the device design by integrating each individual sample loading process. Thus, simplifying the procedure will remove the uncertainty of the device and increase the reliability of the assay. In this work, cocktail reagents are applied during the reporter loading process. Reporters for protein and mRNA respectively are premixed in the same buffer and loading onto the device. During this step, only one cocktail reagent is loaded. No separate control of sample loading is required.

Detection condition

The key of the multiplexing assay is the detection. Usually the protocol for protein and mRNA need to be optimized separately to achieve high accuracy. However, the optimized conditions for measuring protein and mRNA biomarkers are often incompatible. For example, detergent is commonly used in the nucleic acid hybridization process, while it will probably denature the protein biomarker. Another example is, the pH for protein-protein binding is almost around 7.0, while for nucleic acid the optimized pH for hybridization is quite different. In addition, temperature control is important for mRNA hybridization. However, high temperature will definitely denature most of the protein biomarkers. Even for mRNA itself, each mRNA has its specific melting temperature (T_m). Therefore delicate controls of temperature in multiplexing mode are necessary as well with the traditional PCR technique.

In this work, instead of pH, ionic strength and temperature, cycling square wave electrical field (csw E-field) is applied to optimize the assay. Comparing to other conditions, csw E-field has several advantages. First, it is effective in improving the binding efficiency for both mRNA and protein. For mRNA, the positive E-field in the cycles helps the strand to hybridize and the negative E-field removes the non-specific binding. By optimizing the parameters in the csw

E-field, high SBR could be achieved. Meanwhile, the csw E-field generates the mass transportation in the solution which results in good mixing in short time. This effective mixing benefits the protein binding as well. Second, the csw E-field could be easily controlled in multiplexing mode. The profile of csw E-field onto each electrode is versatile, including the high and low voltage, the intervals of each cycle, and the total number of cycles. This advantage will become extremely important when expand into high throughput mode. Since the E-field is high localized, with the microelectromechanical systems (MEMs) technology(31), the EC sensor could be easily expanded to high throughput multiplexing mode with high density array of microelectrodes. The last point is, regarding the damage caused by DC electrical field, here the csw E-field will not cause heavy load to the biological system. The high electrical field is applied for as short as 1 second in each cycle. During the remaining period, the biological system is keeping at very safe E-field (about - 200 mV). Therefore the damage of high voltage E-field could be eliminated.

Base the advantages shown above, EC system is promising in with multiplexing detection. This is the first report for multiplex detections of RNA and proteins for salivary diagnostics.

Conclusion

As a summary, we have developed the electrochemical sensor with multiplexing biomarker detection for salivary diagnostics. The EC sensor shows high sensitivity and specificity. The limit of detection is 3.9 fM for IL-8 mRNA and 7.4 pg/ml for IL-8 protein measured in saliva.

With the multiplexing EC sensor, 56 clinical saliva samples were measured for oral cancer detection. Both IL-8 mRNA and IL-8 protein levels measured by the EC sensors show significant differences between the cancer sample and control sample. From the ROC analysis, the accuracies based on the EC sensor are both around 0.90 for the two biomarkers, which is very close to the results from the same batch of saliva samples measured separately by PCR/ELISA. Combined IL-8 mRNA and protein show better AUC compared with single biomarker. These results indicate that the EC sensor is not only an alternative detection method to PCR/ELISA. It provides fast, effective and accurate multiplexing measurements for real clinical diagnostics. This is the first report for multiplex detections of RNA and proteins for salivary diagnostics, and the EC sensor is promising in miniaturization system with multiplexing detection.

An important note to this study is that we have demonstrated that the saliva oral cancer biomarkers initially discovered based on a US population can also discriminate oral cancer in an Indian population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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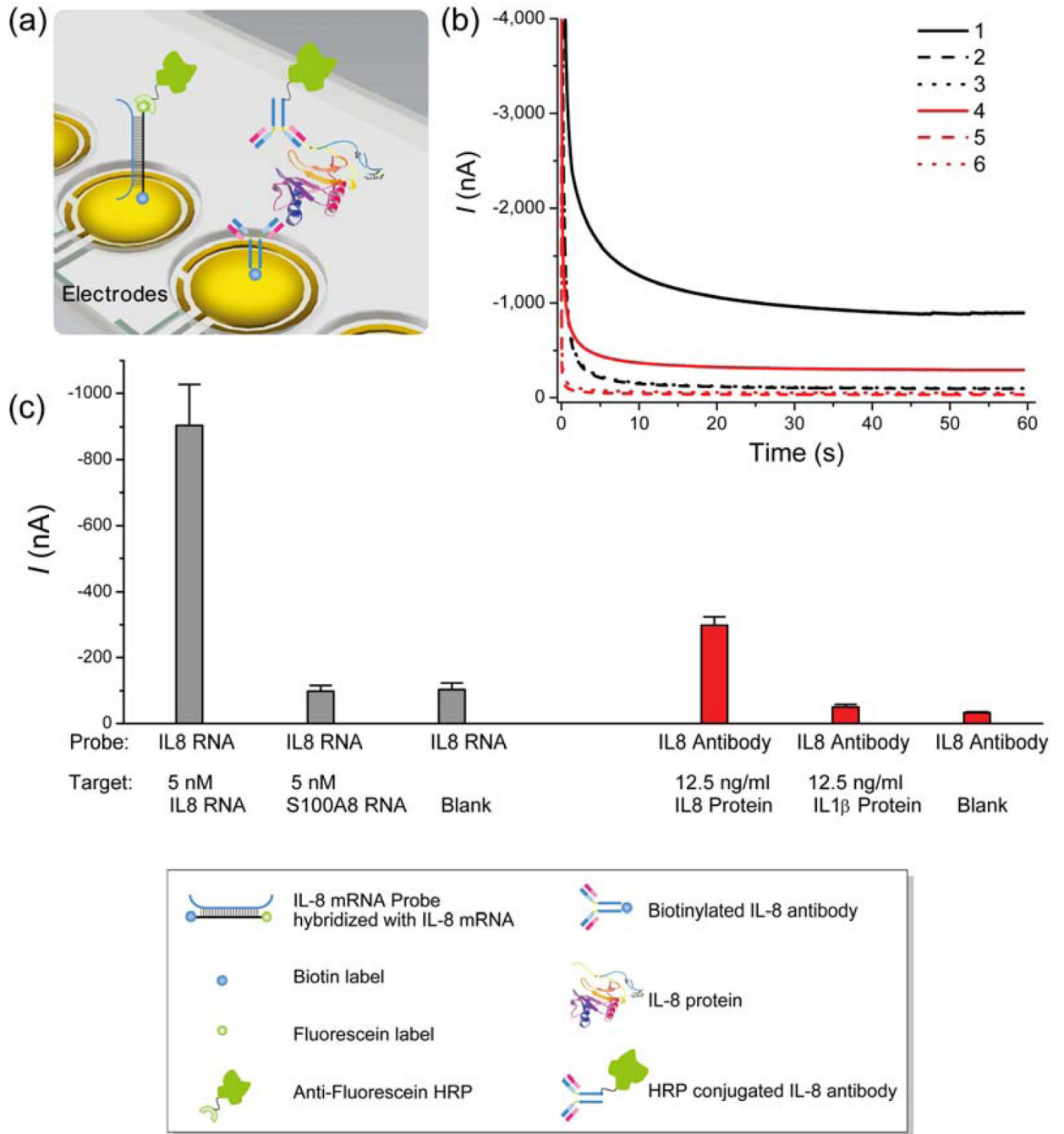


Figure 1. Electrochemical (EC) sensor for multiple salivary biomarker detection. (a) Illustration for array of electrodes with both mRNA (left) and protein (right) detection. (b) Amperometric detection of IL-8 mRNA probe with (1) 5 nM IL-8 IVT RNA, (2) 5 nM S100A8 IVT RNA, (3) blank control; and IL-8 protein probe with (4) 12.5 ng/ml IL-8 protein standard, (5) 12.5 ng/ml IL-1 β protein standard and (6) blank control. (c) Bar chart of IL-8 mRNA and IL-8 protein in saliva with control experiment from S100A8 and IL-1 β respectively. Mean value and standard deviation are illustrated with triplet experiment.

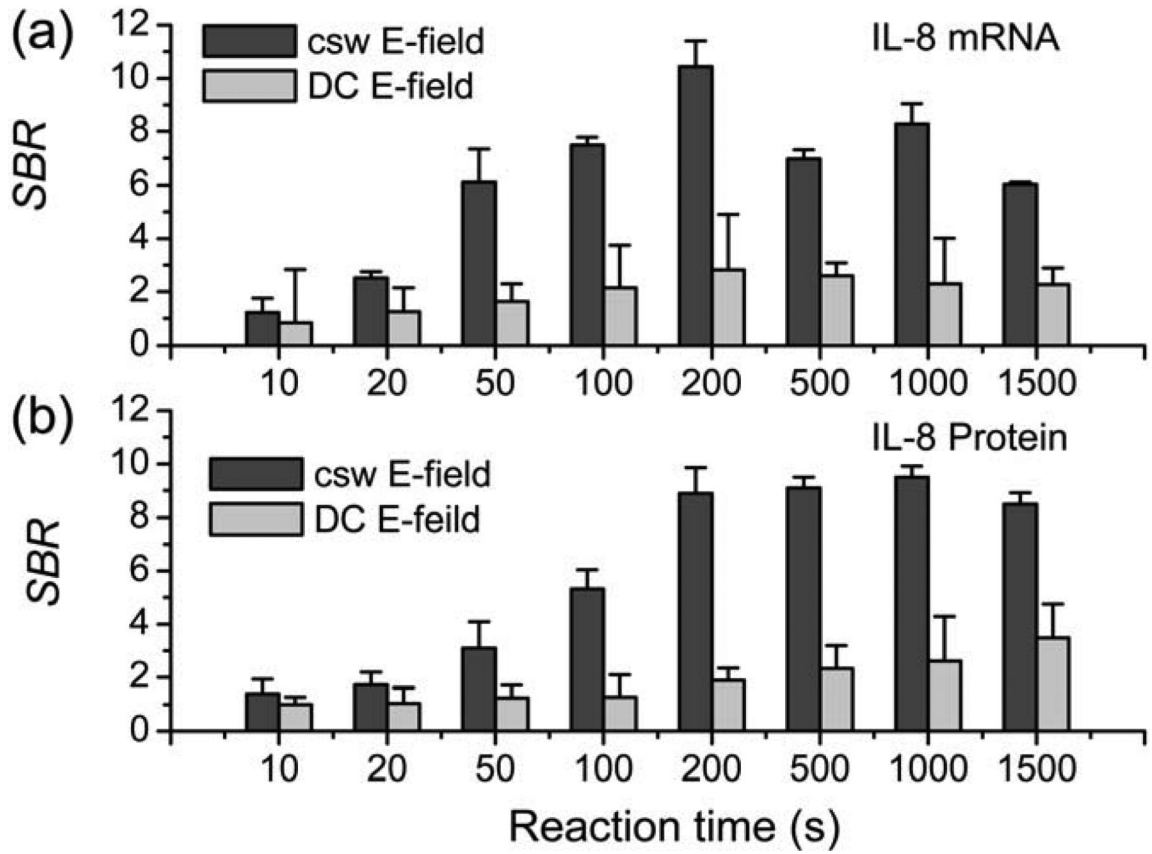


Figure 2. SBR with different reaction time under csw E-field and DC E-field assisted bio-sensing with (a) 5 nM IL-8 IVT RNA and (b) 12.5 ng/ml IL-8 protein standard. Mean value and standard deviation are illustrated with triplet experiment.

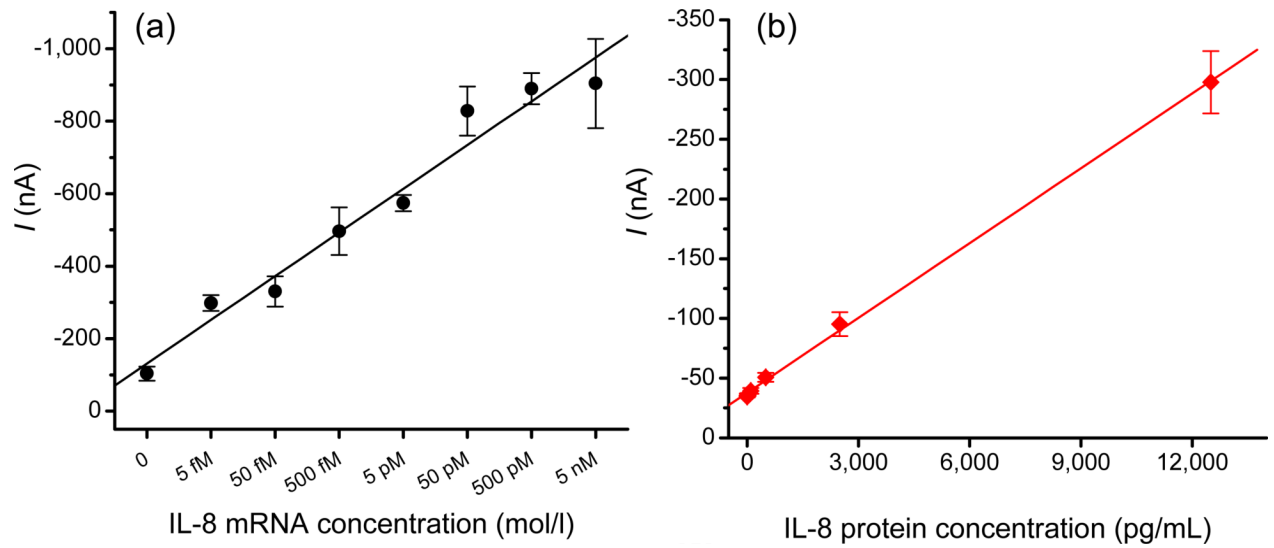


Figure 3. Sensitivity of EC sensor for multiplexing detection of salivary biomarkers under csw E-field with linear fit. (a) IL-8 mRNA ($R^2=0.98$). (b) IL-8 protein ($R^2=1$). Mean value and standard deviation are both illustrated with triplet experiments.

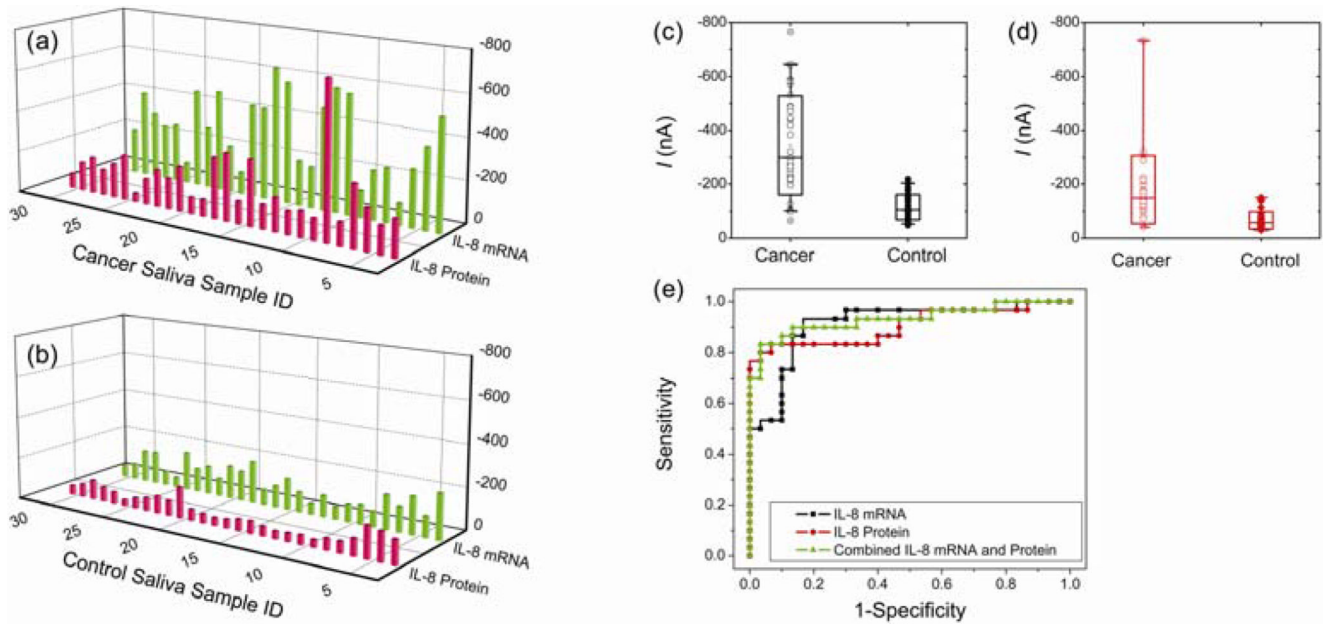


Figure 4. Multiplex detection of IL-8 mRNA and IL-8 protein with EC sensor of 56 saliva samples. (a) Results from cancer samples and (b) results from control samples and statistical analysis for clinical saliva samples tested by EC sensors. Box of standard deviation and mean value of (c) IL-8 mRNA and (d) IL-8 protein are shown. (e) ROC analysis on ROC curve with individual IL-8 mRNA with area-under-curve (AUC) of 0.90, IL-8 protein respectively, with AUC of 0.91, and combinational IL-8 mRNA+Protein with AUC of 0.93.

Table 1

Statistical evaluation of correlation between oral cancer and control subjects

		Normal Group (28)	OSCC Group (28)	<i>p</i> -value
Age (years)	Mean	44.9	46.0	0.83
	Standard deviation	11.9	12.8	
Habbits/factors	chewing (Yes)	28	22	0.08
	Smoking (Yes)	7	12	0.27
	Alcohol (Yes)	1	7	0.06
Gender	Female	4	5	1
	Male	26	25	
Tumor Stage ^a	T (1, 2, 3, 4) %	N/A	(14, 61,14,11)	N/A
	N (0, 1, 2, 3) %	N/A	(29, 46, 25, 0)	N/A
	M (0, 1) %	N/A	(100, 0)	N/A

^aTNM stands for "Tumor, Node, Metastasis", which is tumor staging system