

An Electrochemical Impedance Spectroscopy Biosensor for Detecting IgG in Human Urine

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Abstract. IgG (immunoglobulin G) is the most abundant serum antibody found in blood circulation in humans and is not present in healthy human urine. The detection of IgG in urine is closely related to valuating the severity and predicting the trend of glomerulitis and other chronic kidney diseases. Electrochemical impedance spectroscopy (EIS) biosensors are highly sensitive and ideal for detecting IgG in urine. This technique uses micro-gap parallel plate electrode (PPE) as the electrode of the EIS biosensor and antifouling Y-shaped peptides as biometric element combined with PPE. The development of an EIS biosensor that provides an expedited and precise method for the detection of IgG in human urine would represent a significant advance.

Keywords: EIS, EIS biosensor, IgG, human urine, PPE, IDE, Y-shaped peptide.

1. Introduction

Proteinuria, as one of the clinical manifestations of chronic nephritis syndrome, has high research value and research space [1]. Proteins are challenging to detect and quantify in urine. However, the injury of the glomerulus or renal tubules can cause proteinuria [2,3]. In addition, the types of proteinuria caused by the two reasons above are different (only proteinuria caused by glomerular damage is described next). When glomerular injury is mild, small-sized and medium-sized molecular weight proteins (mainly albumin) will be excreted with the urine [4]. When the glomerular capillary wall is seriously damaged, large molecular weight proteins such as IgG, IgA will enter the urine, and this condition is known as non-selective proteinuria. IgG (immunoglobulin G), as the smallest immunoglobulin, is the first macromolecular protein that appears and with the highest content in non-selective proteinuria [5]. Additionally, Meng et al. stated that urine IgG exceeding 2.45 mg/L (about 1.63×10^{-8} mol/L) correlates with the onset and progression of diabetic kidney disease and chronic kidney disease [6]. This can be used as a basis for measuring biosensors. Therefore, the accurate detection of the amount of IgG in urine will provide significant assistance in determining the degree of disease and predicting the direction of disease. Human urine is non-invasive and facilitated mass access, commonly employed as a clinical diagnostic specimen [7]. Currently, nephelometry immunoassay is primarily used to detect the IgG content in urine, which is complex. Test paper detection is also used, which is simple but has low

sensitivity and is prone to errors. Electrochemical biosensors are unique because they combine biomolecular identification elements and electrochemical detection [8]. It is used to detect and identify biological molecules and has the advantages of high sensitivity and rapid response. Electrochemical biosensors are suitable for accurately and rapidly measuring the concentration of IgG in complex environments such as urine. So far, electrochemical biosensors to detect the content of IgG in urine have not been invented. The development of an electrochemical biosensor to detect IgG in urine can greatly improve the accuracy of detection, which is expected to subvert clinical treatment.

2. Electrochemical Analysis Technique

Electrochemical impedance spectroscopy (EIS) is an effective and complex technology for analyzing interface characteristics related to biometric events occurring on the surface of electrodes [9]. Compared with other electrochemical analysis technologies such as Coulometric analysis and voltametric analysis, EIS is more conducive to the analysis of small signals and can detect signal relaxation in a wider applied frequency range. EIS is a methodology of subjecting an electrochemical system to a small-amplitude AC sinusoidal potential wave at varying frequencies and measuring the ratio of the AC potential to the current signal (impedance of the system) at each corresponding sine wave frequency [10]. The impedance is a complex value which is represented respectively by the real part (Z_{re}) and imaginary part (Z_{im}) (the impedance modulus Z_0 is the ratio of the applied AC voltage to the detected sinusoidal current, and ϕ is the phase shift between the voltage and current):

$$Z(\omega) = Z_0 e^{j\phi} = Z_0 (\cos \phi + j \sin \phi) = Z_{re} + jZ_{im} \quad (1)$$

A Nyquist plot is a graphical depiction of the impedance spectrum recorded in a certain frequency range, with Z_{re} plotted on the X-axis and Z_{im} plotted on the Y-axis [11]. There exist two types of EIS, namely Faradaic and non-Faradaic. In the case of Faradaic, impedance arises concurrently with a redox reaction, with the primary parameter of interest being the charge-transfer resistance (R_{ct}). In contrast, non-Faradaic pertains to a DC-based impedance spectroscopy that lacks redox pairs in the electrolyte solution and electron charge transfer process. Randles equivalent circuit abstracts the complex electrochemical system into an equivalent circuit model, and describes the electrochemical characteristics of the electrochemical system by the electrical characteristics of the circuit components. The following four elements are commonly used in Randles equivalent circuit: the solution resistance (R_s), the double-layer capacitance (C_{dl}), the charge-transfer resistance (R_{ct}) and the Warburg impedance (Z_w) (Fig.1). Randles equivalent circuit is used to represent Faradaic processes [11]. During electrochemical analysis, initially, a dot Nyquist plot is derived from the experimental data, with each data point signifying the system impedance at a distinct frequency. The Randles equivalent circuit is identified by assessing the characteristics of the Nyquist plot in conjunction with the status of the electrochemical system. Subsequently, the EIS data is refined through fitting utilizing the Randles equivalent circuit, resulting in the determination of the optimal values for the aforementioned four parameters, and generating the Nyquist plot curve.

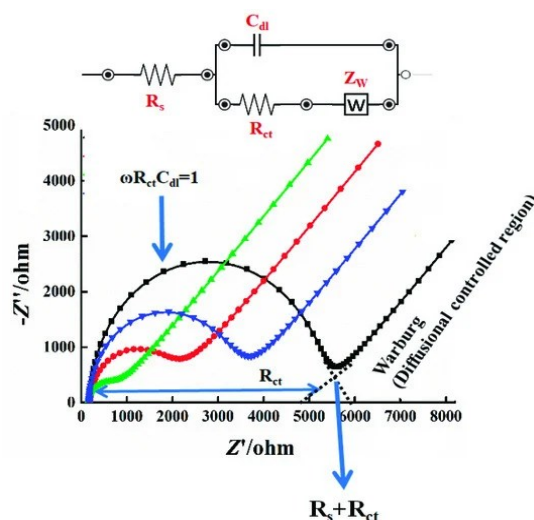


Figure 1. The impedance spectra fitted with the experimental data revealing the simplified Randles equivalent circuit based on the electrochemical system. The Nyquist plot, which is built into two-dimensional X- and Y-axis for the real and imaginary impedances, is used to measure impedance. The X-axis is Z_{re} , which expresses R_s , R_{ct} , and Z_w . The Y-axis is Z_{im} [9].

In Fig.1, it demonstrates the Nyquist plot by showing that the semicircular curve relates to the electron transfer controlling process at high frequencies, while the linear part reflects the diffusion controlling mechanism at low frequencies. Z_w represents the diffusion of molecules or redox agents, which generates additional resistance. At low frequencies, the redox molecules exhibit enhanced diffusivity, leading to an increase in the value of Z_w , which cause the line with a slope of 45° in the Nyquist plot. Conversely, at high frequencies, the value of Z_w is diminished due to the limited distance traversed by the diffusion medium. In addition, the minor intersection point between the semicircle and the X-axis denotes the value of R_s , whereas the other intersection point signifies the sum of R_s and R_{ct} . The calculation of C_{dl} can be derived from the subsequent formula, where ω is equal to $2\pi f$ at the zenith of the semicircle's curve:

$$\omega R_{ct} C_{dl} = 1 \quad (2)$$

A conventional and efficacious method for immobilizing interfacial analytes in impedance detection biosensors involves the measurement of R_{ct} [12]. Commonly in the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ redox probe solution, the interaction between antigens and antibodies at the EIS biosensor interface leads to a modification in the redox transition barrier, which in turn results in an augmentation of R_{ct} . Therefore, R_{ct} changes in a concentration dependent fashion and signifies the extent of binding of the object under examination (IgG in the present article) to the electrode surface.

Urine composition in patients with nephropathy is complex, especially in patients with glomerulopathy. EIS biosensor can be used to detect IgG in human urine because of its plentifully advantages, such as unlabeled method, fast response, miniaturization capability, and low detection limit [13]. Additionally, EIS can be employed in conjunction with a diverse array of nanomaterials and nanogap electrodes to fabricate biosensors endowed with superior characteristics, which indicates that EIS biosensor has great potential.

3. The Structure of the EIS Biosensor

3.1. Micro-gap Parallel Plate Electrode (PPE)

With the popularization of EIS, the development of electrode structures more suitable for EIS measurement has become the key to improve the performance of EIS biosensors. Honda et al. invented

a novel micro-gap parallel plate electrode (PPE) using in EIS biosensor to detect IgG in low concentrations [14]. This EIS biosensor has both high sensitivity and reproducibility which can exhibit a linear range from 1×10^{-13} to 1×10^{-7} mol/L with a limit of detection of 1×10^{-14} mol/L as a record in detecting IgG. It is a wise decision to PPE as the electrodes in the EIS biosensor for in vitro IgG detection of human urine.

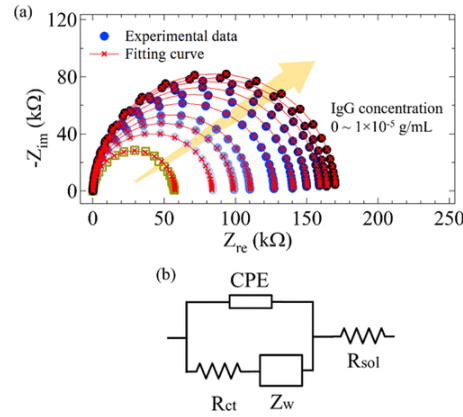


Figure 2. (a) Nyquist plots of EIS measurements produced with a PrG-modified PPE at varying IgG concentrations. The solid semicircular lines are the fitting curves; (b) Randles equivalent circuit diagram fitted with experimental data [14].

The results in Fig.2 (a) were obtained by continuous immersion and measurement cycles in the electrolyte solution with IgG concentrations of $0-10^{-5}$ g/mL. The obtained experimental data were fitted according to the Randles equivalent circuit in Fig.2 (b). It can be observed that the values of R_{ct} increase with the IgG concentration. Through the assessment of $R_{ct}/R_{ct}(0 \text{ g/mL})$, it shows that $R_{ct}/R_{ct}(0 \text{ g/mL})$ increases linearly in the IgG concentration range of 1×10^{-13} to 1×10^{-7} mol/L and the limit of detection is 1×10^{-14} mol/L. The EIS biosensor with electrode structure of PPE in this case has high sensitivity that lays foundation for the development of EIS biosensor for detecting IgG in human urine.

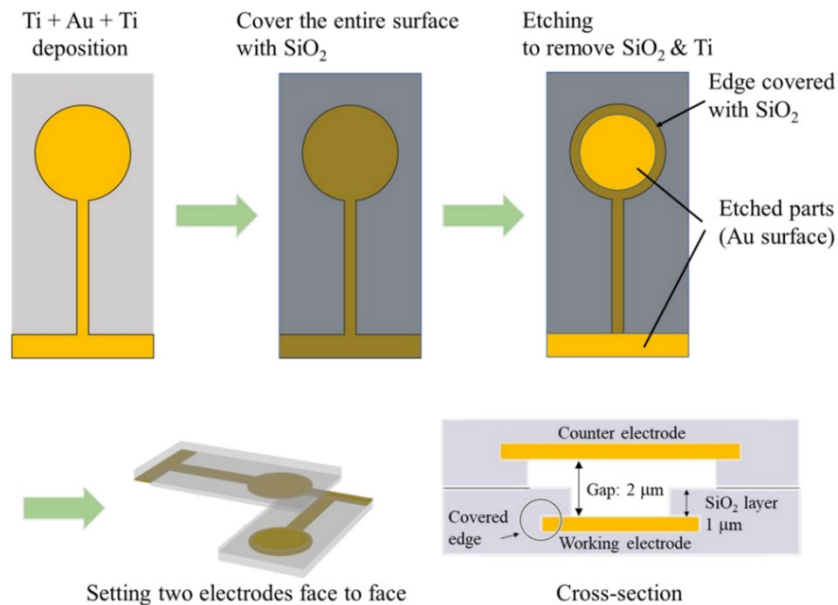


Figure 3. Process of fabricating PPE [14].

Interdigitated electrode (IDE) as a double electrode system widely used in EIS biosensor has plenty advantages, such as the double electrode system more conducive to EIS detection, smaller volume and

higher sensitivity [11]. However, the close and abreast arrangement of adjacent electrodes in IDE makes the current density at the electrode edge corner significantly higher than that at other places and such discrepancies in current distribution are detrimental to the accuracy of EIS measurements. Moreover, the lithography process can result in the rough edge angles of IDE electrodes, thereby introducing instability and a decrease in the reproducibility of EIS measurement outcomes. Therefore, PPE is constructed with insulated electrode edges (see Fig.3), allowing electric current to be distributed uniformly over the electrode surface. Furthermore, two electrode plates of PPE are arranged face-to-face with a micro-gap to ensure that the current passes down the shortest path between the electrodes (see Fig.3). This is another main reason for the uniform distribution of the current. The evenly distributed current on the electrode surface is conducive to more stable EIS results which have high reproducibility. Given the widespread adoption of IDEs in EIS biosensors, and considering that PPE can offer superior reproducibility alongside the identical benefits as IDE, it is both rational and imperative to integrate PPE into EIS biosensor manufacture.

Apart from that, Singh et al. found that the sensitivity of IDE decreases with the increase of clearance between the devices [15]. Given that the current within the PPE traverses the minimal normal distance between the electrode plates, a judicious reduction in electrode separation could potentially elevate the homogeneity of current distribution and intensify the redox cycle occurring across the electrodes, thereby enhancing the sensitivity of the EIS biosensor.

3.2. *Y-shaped Peptide*

In the research from Honda et al., the PPE biosensor works only in simple environments and yields impressive outcomes. Further endeavors are required to facilitate the capability of the EIS biosensor to sustain minimal limit of detection and maintain exceptional precision within more intricate sample matrices such as urine. The nonspecific adsorption and accumulation of proteins especially albumin on electrode interfaces is still a severe challenge for detecting IgG in human urine sensitively and accurately. In addition, the concentration of albumin is much higher than IgG in non-selective proteinuria. Therefore, the design of antifouling bioreceptor is crucial for the development of an electrochemical biosensor to detect IgG in human urine. Although polyethylene glycol and oligo (ethylene glycol) are commonly used to build antifouling layers, they are more prone to oxidation and are not easily chemically adjusted or modified. As a result, peptides are supposed to be used as antifouling materials for the outstanding biocompatibility, lower synthesis cost, high chemical and thermal stability, and tunable structures [16-18].

The fouling caused by nonspecific proteins adsorption on electrode surfaces is largely attributed to the hydrophobic interaction and charge attraction. It is because that hydrophilic surfaces inhibit protein adsorption through hydrophobic interactions, while neutrally charged surfaces prevent protein adsorption through charge attraction [19]. To obtain a high-performance antifouling bioreceptor, peptides should be designed with highly hydrophilia and charge neutrality. Chen et al. state that the specific Y-shaped peptide has two branches (see Fig.4): one branch has antifouling function with the peptide sequence of EKEKEKE, while the other can specifically detect IgG with the peptide sequence of HWRGWVA [20,21]. Besides, the trunk with a sequence of (CPPPP) for anchoring and linking is a benefit for peptide immobilization on electrode surfaces.

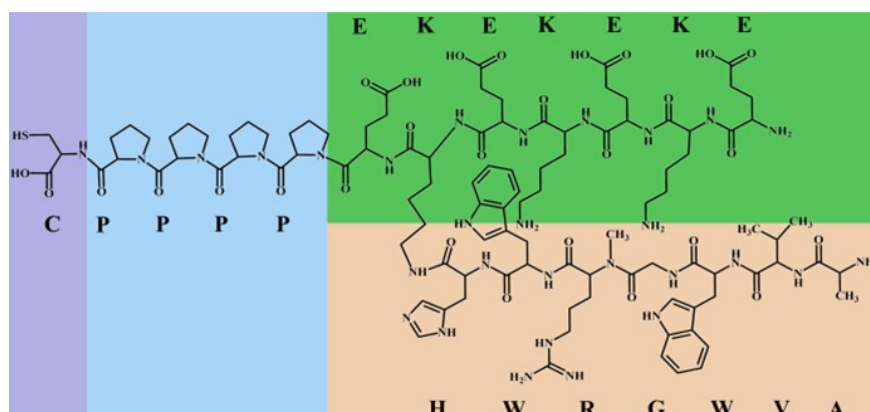


Figure 4. The Y-shaped peptide designing with chemical structural formula of CPPPPEK (HWRGWVA) EKEKE (HWRGWVA) EKEKE sequence [20].

The antifouling function of Y-shaped peptide is based on the sequence of EKEKEKE. Previous research found that zwitterionic peptide sequences including alternating positively charged lysine (K) and negatively charged glutamic acid (E) were very resistant to protein adsorption [21]. The hydrophilic property is tested by the water contact angle, and GCE/PEDOT-citrate/AuNPs/Y-shaped peptide is $(30.1 \pm 0.2)^\circ$ (see Fig.5), which proves the high hydrophilicity of Y-shaped peptide. It shows that the isoelectric point of the Y-shaped peptide is 7.05 and the zeta potential (ζ) is +2.259 mV (see Fig.6A) that is close to electrically neutral. This result indicates rationale is followed in designing antifouling peptides. Circular dichroism spectroscopy is used to analyze the secondary structure of peptides. The high peak (–) near 200 nm and the low peak (+) at 226 nm show the exist of a helix structure of the amino acid proline (P) (see Fig.6B), which aids in graft density. This capability enables the construction of Y-shaped peptide upon the working electrode of PPE.



Figure 5. Contact angle photographs of the electrode surfaces of GCE/PEDOT-citrate/AuNPs/Y-shaped peptide [20].

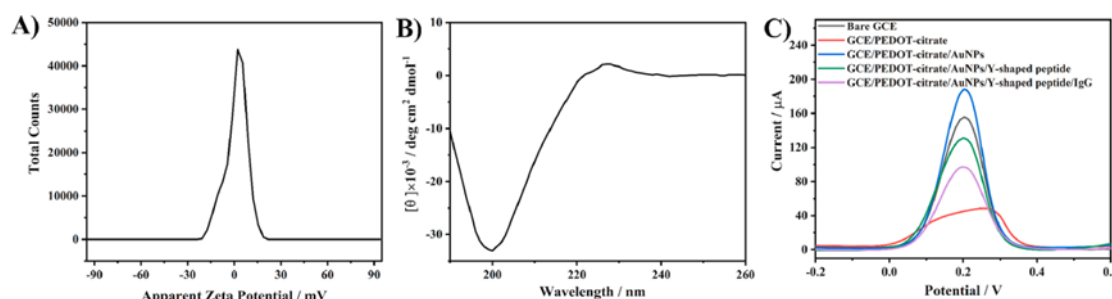


Figure 6. A) ζ of the Y-shaped peptide; B) Circular dichroism spectrum in PBS of 0.2 mg mL^{-1} Y-shaped peptide; C) Differential pulse voltammetry results in $5.0 \text{ mM } [\text{Fe}(\text{CN})_6]^{3-/-}$ electrolyte solution of various electrodes [20].

The recognizing function of Y-shaped peptide is based on the sequence of HWRGWVA. V_H and V_L regions (see Fig.7) of the Fab region are involved in antigen recognition. Human IgG is made up of many distinct antigen-specific antibodies, or isotypes. All of these isotypes have a reasonably conserved region (Fc) on their IgG molecules. Y-shaped peptides achieve specific binding to IgG by recognizing Fc region so that all isotypes of IgG in urine can be detected. The differential pulse voltammetry measurement shows that the current signals continue to decrease sequentially (see Fig.6C). It is thought that immobilized peptides and captured IgG impeded the electron transport to the electrode after the Y-shaped peptides combined with the specific IgG targets. The interfacial reaction associated with IgG binding provided signals for EIS measurements, indicating the possibility of Y-shaped peptides as bioreceptors. In addition, the gold-sulfur bond (Au-S) constitutes the fundamental requirement for the binding of Y-shaped peptides and PPE.

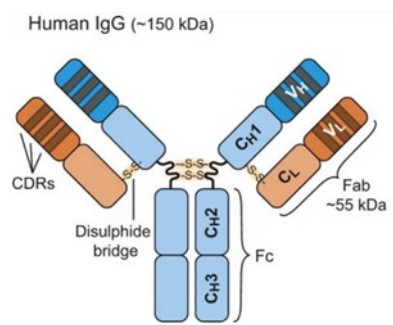


Figure 7. The structure and size of IgG [22].

4. Results and Discussion

EIS biosensor is selected for detecting IgG in human urine because of its outstanding attributes such as high sensitivity and accuracy. According to the previous research, PPE is very suitable as the electrode for this specific EIS biosensor. In order to use it in more complex environments, Y-shaped peptide was adopted. It can specifically identify IgG while antifouling, so that it is more likely to be applied to complex environments such as urine. Due to the electrode structure of PPE and the optimization methods of Y-shaped peptide, it is predicted and expected in theory that the limit of detection can be at 1×10^{-14} mol/L. However, it is only based on theoretical speculation and provides a possible solution for the development of biosensors for detecting IgG concentrations in human urine. Actual experiments are needed to invent the biosensor and adjust its parameters so that it can be used in clinical testing and other application scenarios.

EIS biosensors are capable of operation without the necessity of labeling the target entity. However, labeling IgG in human urine may amplify the signals which is beneficial to the detection. Singh et al. researched and found that EIS biosensors utilizing nanomaterials (NMs) in conjunction with Protein-A (target entity) for IDE can markedly amplify the analytical signal, as evidenced by substantial alterations in the response R_{ct} and C_{dl} [23]. Consequently, the utilization of an analogous technique for the labeling of IgG within urine specimens using NMs would appear to be advantageous for the detection via EIS biosensors. Nonetheless, it is imperative to consider that the presence of a Y-shaped peptide does not alter the IgG capture process.

5. Conclusion

The development of an electrochemical biosensor for the detection of IgG in human urine has significant implications for monitoring the progression of glomerular diseases. An EIS biosensor is designed in theory with PPE as the electrode and antifouling Y-shaped peptides as the biometric element to detect the content of IgG in human urine. This EIS biosensor is supposed to have excellent advantages such as high sensitivity, low limit of detection and easy miniaturization. Additionally, labeling IgG with NMs might be an effective method to amplify the signals which is conducive to EIS measurements. This

research has the potential to be used in the management of kidney diseases, offering more rapid and accurate diagnostic capabilities. Furthermore, this design provides perspectives and ideas for subsequent related development.

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