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Multiwalled Carbon Nanotubes Based Immunosensor for Diagnosis of Celiac Disease

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Abstract

Celiac Disease (CD) is an autoimmune disorder, represented by the ingestion of gliadin protein usually found in wheat, barley and rye. CD is closely associated with genes that code HLA-II antigens, mainly of DQ2 and DQ8 classes. Small intestinal biopsy is considered the gold standard for diagnosis of celiac disease. In addition, the most sensitive tests for the diagnosis of celiac disease include the increase in level of IgA isotype of anti-gliadin antibodies, connective tissue antibodies and tissue transglutaminase antibodies. In the present study, an amperometric immunosensor was fabricated for CD diagnosis using multiwalled carbon nanotubes screen printed electrode. Antigen (gliadin) was covalently linked to carbon nanotubes through 1-Ethyl-3-(3-dimethylaminopropyl carbodiimide) (EDC)-N-hydroxysuccinimide (NHS) cross linking chemistry. Different concentrations of anti-gliadin antibodies were added on working electrode surface and change in current was measured by Cyclic Voltammetry using redox indicator Potassium ferricyanide [$K_3Fe(CN)_6$]. Electrochemical studies showed that the immunosensor was able to detect the antibody concentration as low as 0.13ng/µl. Sensitivity of the immunosensor was found to be 119.2µA/ng/cm^2. The present sensor can be used for diagnosis of celiac patients who are sensitive to gluten ingestion.

Keywords: Celiac disease; Gliadin; Immunosensor; MWCNT

Abbreviations: AGA: Anti-Gliadin Antibodies; CD: Celiac Disease; CV: Cyclic Voltammetry; ELISA: Enzyme Linked Immunosorbent Assay; MWCNT: Multiwalled Carbon Nanotubes

Introduction

Celiac disease (CD) is an autoimmune enteropathy characterized by an aberrant immune response to gluten ingestion, which is a component of wheat in genetically susceptible individuals [1]. Gluten is a mixture of wheat storage proteins that is insoluble in alcohol (glutenins), or soluble in alcohol (gliadins). The alcohol-soluble fractions have similar toxic proteins as those present in barley (hordeins), rye (secalins), and in oats (avenins) [2]. Gliadin is the major triggering factors in CD [3] and the mechanism underlying the CD pathogenesis is mediated by deamidation of gluten peptides by tissue transglutaminase (tTG), creating epitopes with increased immunostimulatory potential. Despite the efforts for the development of new therapeutic strategies, the only treatment option for CD patients is strict adherence to gluten free diet [4]. Therefore, an early and accurate diagnosis of CD is extremely important to control the gastrointestinal damage and improve the patient's quality of life.

The presently available diagnostic tests for CD include a series of blood tests and a small bowel biopsy. The most common method for detection of CD biomarkers is enzyme-linked immunosorbent assay (ELISA) [5,6]. However, electrochemical immunosensor appears to be an interesting alternative as it has several advantages viz., an increase in sensitivity due to the electrochemical detection, lower sample volumes and, shorter time of analysis [7-11].

In the recent years, the electrochemical biosensors have witnessed a growing interest in the development of analytical devices for celiac disease diagnosis. The use of electrochemical impedance (EIs) for the detection of specific antibodies for CD was first reported by Balkenhohl and Lisdat [12,13] and they developed an impediametric immunosensor for the detection of antibodies directed against tTG using screen-printed gold electrodes modified with a polyelectrolyte layer of poly(sodium 4-styrenesulfonic acid) [12]. They also developed an immunosensor for the determination of anti-gliadin antibodies (AGA), using 3-mercaptopropionic acid to modify the screen-printed gold electrode surface [13]. Pividori et al. [14] proposed an amperometric immunosensor based on the physical adsorption of tTG from guinea pig liver onto graphite-epoxy composite (GEC) electrodes. Pereira et al. [15] have used a microfluidic immunosensor coupled with electrochemical detection for AGA IgG quantification. In another study, an electrochemical supramolecular platform based on cyclodextrin-modified gold surface electrodes was used to detect anti-gliadin antibodies in serum samples [16]. In spite of the good analytical results, the above used electrochemical devices do not allow a continuous analytical performance and there is a need to develop more simple, miniaturized and portable device for CD diagnosis. Hence, in the present study an electrochemical immunosensor was developed for serological detection of anti-gliadin antibodies in human samples with high sensitivity using gliadin immobilized on screen printed Multiwalled Carbon Nanotubes (MWCNT) electrode surface.

Materials and Methods

Chemicals and reagents

1-Ethyl-3-(3-dimethylaminopropyl carbodiimide) (EDC), N-hydroxysuccinimide (NHS), anti-gliadin (wheat) antibody, Potassium ferricyanide $K_3[Fe(CN)_6]$ were purchased from Sigma Aldrich, USA. Gliadin was purchased from MP Biomedicals, India.

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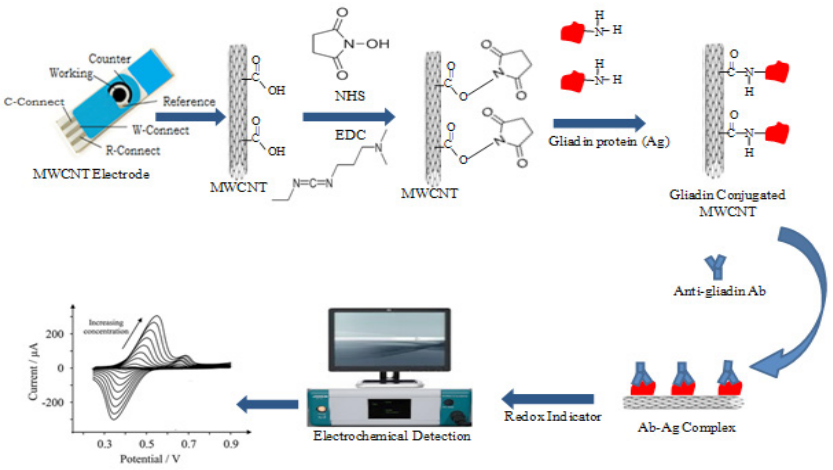

Figure 1: Schematic representation of gliadin conjugated MWCNT based immunosensor for celiac disease diagnosis.

Gliadin IgG ELISA kit was purchased from Diagnostics and Scientific Suppliers, Chandigarh, India. All the chemicals used in the study were of molecular biology and analytical grade. All the reagents were prepared in Milli-Q water, the solutions and glassware's were autoclaved prior to being used. Screen printed Multiwalled Carbon Nanotubes (MWCNT) electrodes and a specific connector were purchased from DropSens, Spain.

Fabrication of immunosensor

Three electrode system based screen printed electrode (MWCNT) was used for the fabrication of immunosensor. The electrode was washed with autoclaved Milli-Q water and PBS buffer (pH 7) respectively and was dried properly at room temperature. MWCNT electrode was then treated with the mixture of 10 mM EDC and 10mM NHS (1:1, v/v in PBS, pH 7) for 1.5h to activate the carboxyl groups at the electrode surface. Electrode surface was then washed with PBS (pH 7) to remove excess of reagent and was allowed to dry at room temperature. The working electrode was then coated with 6µL of 1mg/ml gliadin solution and left to incubate overnight at 4°C followed by washing using PBS (pH 7). 5mM $K_3[Fe(CN)_6]$ was used as an artificial electron donor at each step of fabrication.

Hybridization and characterization of immunosensor with human serum samples

The human serum samples were hybridized onto the surface of gliadin fabricated MWCNT electrode and left to incubate for 10mins followed by a washing step. The process was repeated for different dilutions of anti-gliadin antibodies from human serum samples. Hybridization studies were characterized using cyclic voltammetry (CV). Thereafter the electrode was washed using PBS- tween 20 buffer to remove the unbound anti gliadin antibodies.

Comparison of developed immunosensor with ELISA

Samples were assayed for human autoantibodies against gliadin in serum using ELISA. 1mg/ml gliadin was prepared in 70% ethanol and left to incubate overnight at 4°C. Different antibody concentrations ranging from 2.7ng/µl to 650ng/µl prepared in serum were pipetted out onto the ELISA strip and left to incubate for 30mins each followed by washing. Thereafter enzyme conjugate (peroxidase labelled) was added onto the wells and left to incubate for 30mins followed by washing. The substrate 3,3',5,5'-Tetramethylbenzidine/Hydrogen peroxide (TMB/H_2O_2) was then added and left to incubate for 30mins. The reaction was halted by the addition of stop solution (0.5M sulphuric acid) that turned TMB yellow.

Results and Discussion

The schematic fabrication of the MWCNT electrode, immobilization of the antigen (gliadin), hybridization with anti-gliadin antibodies and its electrochemical detection is shown in Figure 1.

CV studies

Voltammetric measurements of immobilized antigen and antigen antibody interaction studies were carried out using potassium ferricyanide that act as a redox indicator. The cyclic voltammetry (CV) peak current (Ip) of MWCNT/gliadin/antigliadin antibodies (Figure 2) was lower than that of bare MWCNT. It may be due to the decrease in the surface area of MWCNT electrode at each step of fabrication. Peak current (Ip) of negative control was found to be 250 µA and after interaction with different antibody concentrations, dip in the current was observed. For antibody concentrations 2.7ng/µl, 10.15ng/µl, 81.25ng/µl, 650ng/µl the observed peak currents were 175 µA, 100 µA, 75 µA and 50 µA respectively. With increase in the concentration of antibody, a decrease in the current response was recorded which is attributed to their non-electrochemical activity which partially blocked the electron transfer between $K_3Fe(CN)_6$ solution and the electrode surface. For calculation of sensitivity and limit of detection (LOD) the concentrations of control as zero (0), 2.7 and 10.15 ng/µl anti gliadin antibodies were considered to achieve best regression coefficient. The plot followed the linear equation I_p (µA) = 15.02 (µA/ng) x anti-gliadin antibodies (ng) + 0 (intercept) with regression coefficient (R^2) 0.991 (Figure 2 inset B). The Sensitivity (S) of the immunosensor was 119.2µA/cm^2/ng which was calculated using the formula S = m/A where, m is the slope of the linear equation and A is the area of the working gold (0.126 cm^2) electrode. The limit of detection (LOD) was found approximately 0.13 ng/µl using the formula LOD = 3(σ/S) where σ is the standard deviation and S is the sensitivity.

Comparison of the immunosensor with ELISA

ELISA of immobilized antigen and interaction studies of antibody with antigen at different concentrations is shown in the Table 1. ELISA assay was performed for different antibody concentrations ranging from 2.7ng/µl to 650ng/µl. It was observed that the assay could detect antibody concentration upto10.15ng/µl whereas the immunosensor

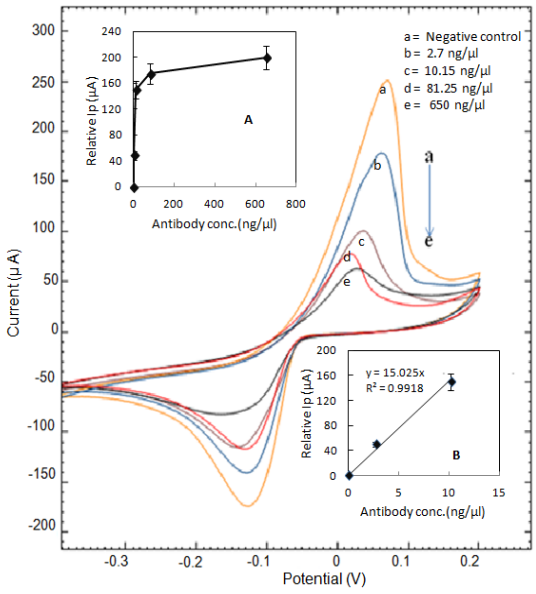

Figure 2: Cyclic voltammetric studies of gliadin based immunosensor using different antibody concentrations ranging from 2.7 ng/µl – 650 ng/µl at 50 mVs^{-1} using 5 mM $K3[Fe(CN)_6]$. The inset A shows hyperbolic curve from 0 – 650 ng/µl with linear peak current (I_p) up to 81.25 ng/µl of anti- gliadin antibodies. Inset B shows the linear plot from 0 – 10.15 ng/µl anti-gliadin antibodies for the calculation of sensitivity and LOD.

Anti-gliadin (Antibody) (ng/µl)	Comparative methods used for anti-gliadin detection	
	Immunosensor	ELISA
650	+	+
325	+	+
81.25	+	+
40.1	+	+
20.3	+	+
10.15	+	+
5.14	+	-
2.7	+	-
[+] = Positive, [–] = Negative		

Table 1: Comparison of ELISA based and Immunosensor based detection of anti-gliadin antibodies ranging from 2.7ng/µl – 650 ng/µl.

could detect the antibody concentration as low as 2.7ng/µl, thereby, confirming the immunosensor sensitivity.

Conclusion

The sensitivity of the immunosensor was 119.2µA/cm^2/ng and LOD was 0.13 ng/µl with the regression coefficient (R^2) 0.991 using CV. The MWCNT based immunosensor can detect as low as 2.7ng/µl concentration of antigliadin antibodies in 30 min confirming that the immunosensor is sensitive analytical tool for the detection of antigliadin antibodies raised in response to the ingestion of gliadin in patients suffering from Celiac disease.

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