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Gelatin methacrylate (GelMA) mediated electrochemical DNA biosensor for DNA hybridization

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ABSTRACT

In this study, an electrochemical biosensor system for the detection of DNA hybridization by using gelatin methacrylate (GelMA) modified electrodes was developed. Electrochemical behavior of GelMA modified Pencil Graphite Electrode (PGE) that serve as a functional platform was investigated by using Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) and compared with those of the bare PGE. Hybridization was achieved in solution phase and guanine oxidation signal changes were evaluated. The decrease in the guanine oxidation peak currents at around +1.0 V was used as an indicator for the DNA hybridization. Also, more interestingly GelMA intrinsic oxidation peaks at around +0.7 V changed substantially by immobilization of different oligonucleotides such as probe, hybrid and control sequences to the electrode surface. It is the first study of using GelMA as a part of an electrochemical biosensor system. The results are very promising in terms of using GelMA as a new DNA hybridization indicator. Additionally, GelMA modified electrodes could be useful for detecting ultra low quantity of oligonucleotides by providing mechanical support to the bio-recognition layer. The detection limit of this method is at present 10^{-12} mol. Signal suppressions were increased from 50% to 93% for hybrid with using GelMA when it was compared to bare electrode which facilitates the hybridization detection.

1. Introduction

Detection of target nucleic acids in biological matrices has attracted considerable attention from many fields, including clinical diagnosis, drug development and environmental analysis (Elliott, 2006; Mohan et al., 2011; Topkaya et al., 2010; Wang et al., 2013). In order to detect target molecules, a great deal of sensing strategies have been developed through the use of various probes coupled with recognition steps, such as nucleic acid hybridization (Ali et al., 2010), antibody/antigen binding (Chen et al., 2011; Yu et al., 2006) or enzyme reactions (Cruys-Bagger et al., 2012; Zhang et al., 2013). Currently, many reports are available for the electrochemical detection of DNA hybridization based on the different materials. However, they generally use expensive nanomaterials such as carbon nanotubes (Niu et al., 2009), gold and silver nanoparticles (Rijiravanich et al., 2008; Zhang et al., 2011) in order to enhance the detected signals. Despite their signal amplification of DNA, pre-preparation process of such materials is highly laborious and it is very difficult to provide the same particle size dimensions and physicochemical properties at every manufacturing step. Recently, integration of electrochemical biosensors with polymer systems for sensitive detection of hybridization is of importance for clinical diagnostic purposes. Polymers in aqueous media offer direct and fast electrochemical detection of DNA hybridization. It is possible to prepare a variety of polymer modified electrodes with highly controllable size, shape, surface charge and physicochemical characteristics. Their tunable properties allow a better immobilization of biomolecules onto the sensing surface (Arslan et al., 2006; Budnikov et al., 2012; Kiilerich-Pedersen et al., 2011; Shimomura et al., 2013) for various recognition species, including enzymes and electrodes (Alizadeh and Akbari, 2013). Moreover, polymer can be readily shaped into various forms each having a different loading capacity, release kinetics and mechanical property due to being prepared in solution (Liu, 2011).

Herein, a new biosensor platform for DNA hybridization detection based on the modification of Pencil Graphite Electrode (PGE) with DNA and Gelatin Methacrylate (GelMA) derived polymer was reported. In order to provide the recognition surface, electrodes were coated with DNA oligonucleotides by passive adsorption. After coating of electrodes with DNA, these electrodes were interacted with GelMA polymer simply by dipping the coated electrodes into the GelMA solution.

Coatings of electrodes were proved with Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) with

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potassium ferri/ferrocyanide as a redox probe. Hybridization between probe and its complementary target caused guanine oxidation signal differences and it was monitored by Differential Pulse Voltammetry (DPV). The selectivity was proven with control studies by using a non-complementary DNA. As a result of the interaction between the DNA oligonucleotides and GelMA, hybridization was detected more sensitively when it was compared with the bare electrodes (without GelMA) due to having more stable and durable sensing surface. The GelMA modified electrodes provided convenient surface for hybridization detection. The most important part of the study was that discovering of the new hybridization indicator: GelMA. GelMA has an oxidation signals at +0.7 V physiological pH and its oxidation signals changed by interaction of different oligonucleotides. GelMA oxidation signal differences enabled us to determine hybridization independently from the guanine oxidation signals. This observation was very important as it offering a new hybridization indicator. Additionally, this is the first study of the use of GelMA within a part of the biosensor system. The designed biosensor eliminates the drawbacks of DNA hybridization detection such as being time consuming, needing radioactive labeling and requiring a high concentration of DNA.

2. Materials and methods

2.1. Apparatus

Fourier Transform Infrared Spectrometer (FTIR) was recorded in a range of wave numbers from 4000 to 650 cm^{-1} for synthesized GelMA. (Spectrum 100, Perkin Elmer, USA). DPV, CV and EIS experiments were performed using the AUTOLAB PGSTAT-10-FRA electrochemical analysis system (μAutolab type III, Eco Chemie, Netherlands). Thermo-Shaker for micro tubes was used for shaking and controlling temperature of the samples (Boeco, Germany). The pH values of the buffer solutions were measured by using inoLab pH Level 1 pH meter (WTW GmbH & Co. KG, Weilheim, Germany). The 3 electrode system consisted of a pencil graphite electrode (PGE) as the working electrode, a reference electrode (Ag/AgCl, Model RE-1 BAS, USA), and a platinum wire as the auxiliary electrode.

2.2. Chemicals

Stock solutions of oligonucleotides were prepared in ultra-pure water and stored at −20 °C until use. Dilute solutions of the oligonucleotides were prepared daily with 0.05 M phosphate buffer (PBS) containing 20 mM NaCl (pH 7.4). 0.5 M acetate buffer (ACB) solution containing 20 mM NaCl (pH 4.8) was used as measurement buffer. Gelatin (Type B) and methacrylic anhydride (MA) were purchased from Sigma-Aldrich Co. (Wisconsin, USA).

2.3. Synthetic oligonucleotides

Synthetic oligonucleotides (as lyophilized powder) were purchased from Alpha DNA (Canada) and used without further purification. Synthetic sequences for probe, target, non-complementary (NC) are

Probe: 5′-TTC GGG GTG TAG CGG TCG TC-3′
Target: 5′-GAC GAC CGC TAC ACC CCG AA-3′
Non-complementary: 5′-TGG AGT ATT GAA GCT TTT GCC GAA GGT-3′

2.4. Procedure

The sensing procedure involves following steps as preparation of GelMA and DNA–GelMA interaction. Fig. 1 shows the schematic representation for the preparation of the biosensor.

2.4.1. Preparation of GelMA

GelMA was produced with a chemical alkaline treatment from Gelatin B. Gelatin was dissolved in PBS at 50 °C. To modify the lysine group on gelatin chains, methacrylic anhydride (0.8 mL per gram of gelatin) was added to the gelatin solution under stirred conditions at 50 °C. The reaction was continued for 2 h. After 2 h,

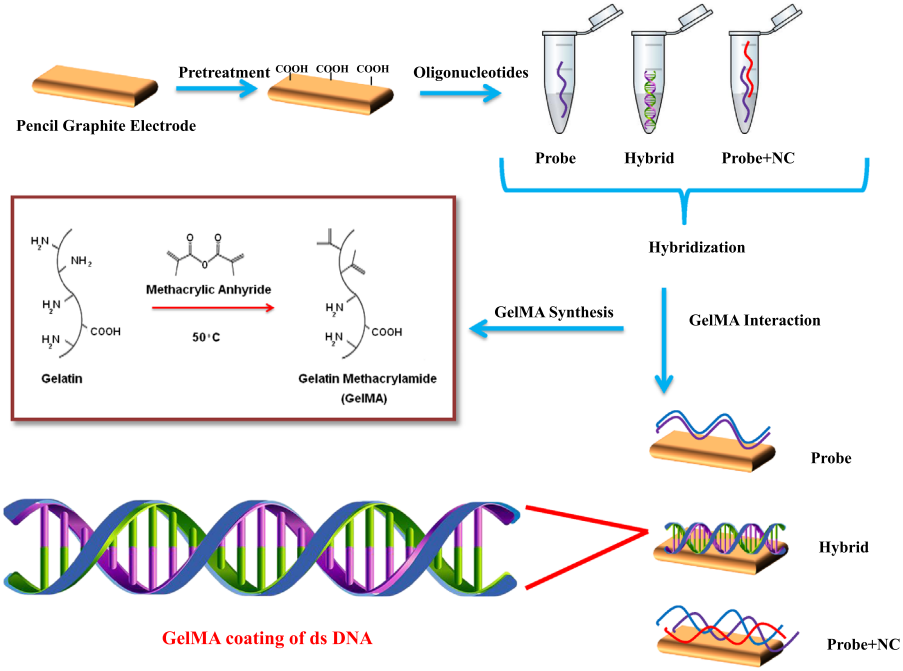

Fig. 1. The synthesis of GelMA and schematic representation of the overall experimental design.

the solution was diluted with PBS to stop the reaction. To remove unreacted methacrylate anhydride, the diluted solution was dialyzed in a distilled water bath for 5 days at 40 °C with mixing. This GelMA solution was filtered (0.2 μm) and then frozen using liquid nitrogen. As shown in Fig. 1, gelatin was introduced methacrylate pendant groups by reacting with methacrylic anhydride.

2.4.2. Characterization of GelMA

GelMA has an intensively characterized polymer (Shin et al., 2013). Additionally, FTIR measurements were performed to evaluate the structure of the GelMA (Fig. S1). ATR-FTIR analysis was recorded at a resolution of 4.0 cm^{-1}. All the spectrums were collected for at least three randomly selected samples.

2.4.3. Interaction of DNA oligonucleotides with GelMA2.4.3.1. Electrode pretreatment. An oxidative pretreatment of carbon surfaces is necessary to enhance the adsorptive accumulation of DNA. For this reason, PGE was pretreated by applying +1.4 V for 30 s in ACB.*2.4.3.2. DNA hybridization.* Hybridization conditions were adapted from our previous study (Topkaya et al., 2012). Probe, probe-complementary target (hybrid) and probe-non-complementary target solutions were prepared in PBS and stirred for 20 min at a mixing speed of 600 rpm. After gentle mixing, probe, hybrid and non-complementary solutions were put into the vials and pretreated bare electrodes were dipped into these solutions for 20 min. The electrodes were then rinsed once with PBS to remove the unbounds.*2.4.3.3. GelMA polymer preparation.* To prepare pre-polymer solution, 5% (w/v) GelMA was dissolved in PBS at 80 °C for 10 min.*2.4.3.4. Fabrication of Modified Electrodes.* In order to provide DNA and GelMA interaction, oligonucleotide coated electrodes were immersed into the plastic tubes containing 40 μL volume of GelMA, and then interacted at 37 °C, 600 rpm mixing speed for 1 h. After 1 h, the electrodes were rinsed once with PBS to remove the residues.*2.4.3.5. Measurements*

DPV: The oxidation signals of guanine (+1.0 V) and GelMA (+0.7 V) were measured by DPV at 50 mV amplitude scanning from +0.4 to +1.4 V in ACB.

CV: CV was carried out in PBS containing 10 mM [Fe (CN)$_6$]$^{3-/4-}$ between a potential window −0.2 V and +0.8 V with a scan rate of 100 mV s^{-1}.

EIS: EIS was performed in PBS containing 10 mM [Fe (CN)$_6$]$^{3-/4-}$ at +0.2 V; a frequency range of 10 kHz to 50 mHz and an AC amplitude of 10 mV were applied.

3. Results and discussion

The major challenge of label-free biosensors is to create convenient interface between the sensing layer and the transducer. Ideal surface modifications should be mechanically stable and not change the analytes bioactivity. There are various techniques to immobilize oligonucleotides onto the surface of the electrodes such as passive adsorption, electrophoretic deposition and chemically covalent attachment (Berney et al., 2000; Das et al., 2012; Jung et al., 2004). Each of the mentioned immobilization methods has differences in their adherence mechanisms, intensities of surface density and the stability of attachment. The common expectation from all of surface immobilization methods is that biomolecules should maintain their activity after attachment onto the sensor surface. In this study, DNA-GelMA modified electrodes were prepared by a simple mixing and dipping process, and obtained results were discussed in the following parts.

3.1. Coating of electrodes with GelMA

In this study, electrodes were coated with GelMA and coating was confirmed with DPV, CV and EIS. Fig. 2 shows the coating of electrodes with GelMA layer.

In order to compare the electrochemical properties of GelMA coated electrodes and bare electrodes, cyclic voltammograms of both are investigated and shown in Fig. 2A. A pair of well-defined redox peaks corresponding to the electrochemical response of [Fe (CN)$_6$]$^{3-/4-}$ couple with bare electrodes was observed. When PGE was coated with GelMA, the redox peaks of [Fe(CN)$_6$]$^{3-/4-}$ decreased remarkably which suggested that the electron transfer kinetic of the [Fe(CN)$_6$]$^{3-/4-}$ was hindered by the film of GelMA. This could be attributed to the decline in the electron transfer ability of [Fe (CN) $_6$]$^{3-/4-}$ caused by deposition of GelMA.

EIS can be used as a tool to confirm the electrode surface modification by GelMA. Fig. 2B depicts the Nyquist diagrams of bare and GelMA coated electrodes. Nyquist diagrams (imaginary part Z" vs. real part Z' in the presence of [Fe (CN$_6$)] $^{3-/4-}$ recorded after each modification step at bare PGE and GelMA coated electrodes. The data can be fitted with a modified Randles equivalent circuit (inset in Fig. 2b) where, R_s is electrolyte resistance, R_{ct} is charge transfer resistance, and W is Warburg impedance. The diameter of the semi-circle points out R_{ct} at the electrode surface. It can be seen that R_{ct} of bare PGE was about 355 Ω. The value of R_{ct} obviously increased to 2347 Ω when electrodes were coated with GelMA due to the increase of the thickness of the interface by the polymer layer indicating that the lower conductivity of the modified electrode.This result clearly

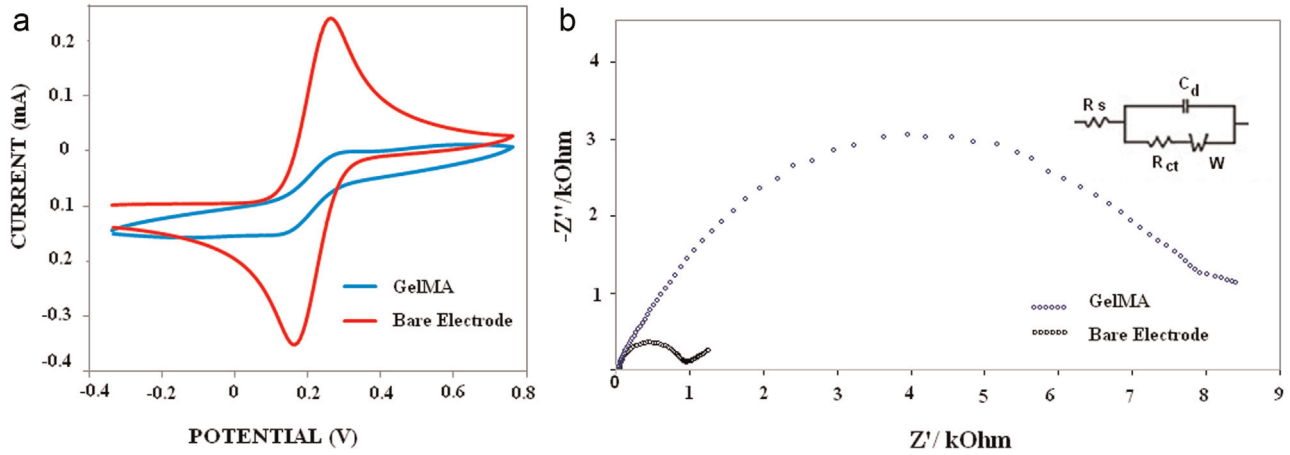

Fig. 2. Coating of electrodes with GelMA with CV (A) and EIS (B) in [Fe (CN) $_6$]$^{3-/4-}$.

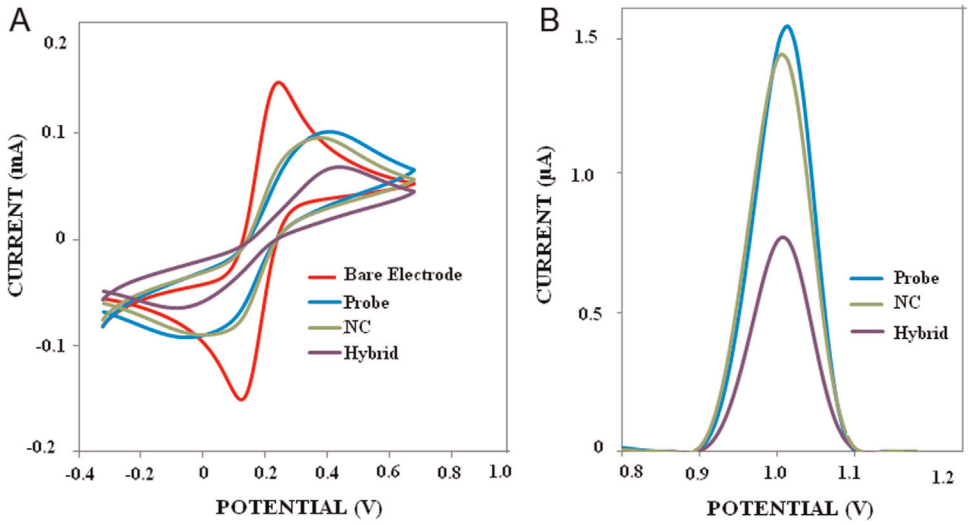

Fig. 3. CVs (A) and DPVs (B) of oligonucleotides on PGE with only probe, after hybridization with complementary target (hybrid), after hybridization with non-complementary target. Experimental conditions: PGE pretreatment, at +1.4 V in ACB for 30 s; 2.5 μg/ml probe and 3.5 μg/ml target/non-complementary target hybridization for 20 min; immobilization of oligonucleotides to the bare electrode for 20 min; rinsing of electrodes once with PBS; Measurement; −0.4 V to +0.8 V in 10 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ for CV and +0.8 V to +1.2 V in PBS for DPV.

demonstrates that GelMA has been successfully immobilized onto the PGE surface. Furthermore, according to the difference value of R_{ct} (ΔR_{ct}) between on GelMA coated electrode and bare electrode, the surface coverage (θ) of GelMA on PGE surface was calculated by the following equation (Sabatani et al., 1987):

$$(\theta) = 1 - \left(R_{ct}^{bare}/R_{ct}\right) \quad (1)$$

From the impedance spectra displayed in Fig. 2B, charge transfer resistances of the bare electrode and the GelMA coated electrode are 355 Ω and 2347 Ω, respectively. According to the equation above, the corresponding surface coverage values of with GelMA was calculated as 85%. As a summary of Fig. 2, it is very obvious that bare electrodes were coated with GelMA layer.

3.2. Hybridization of DNA oligonucleotides

In order to investigate the electrochemical behavior of the electrodes, the CV responses of

$[Fe(CN)_6]^{3-/4-}$ at the bare and different modified electrodes were studied and shown in Fig. 3A.

The highest redox peaks for $[Fe(CN)_6]^{3-/4-}$ were obtained with the bare electrodes. Deposition of the probe DNA onto the bare electrode surface decreases the reversibility and increases the ΔEp ($\Delta Ep = Ep_{anodic} - Ep_{cathodic}$) value. We obtained ΔEp: 106 mV for the probe even though the bare graphite electrode was ΔEp: 73 mV. The peak currents of $[Fe(CN)_6]^{3-/4-}$ belonging to bare electrodes decreased because probes immobilized on the electrodes prevented the ion exchanges between the electrode and solution species during the redox reaction process.

Complementary/non-complementary targets were interacted with probe. The highest current decrease and highest ΔEp value [ΔEp: 149 mV] was obtained with hybrid formation, which can be attributed the negatively charged target DNA repelling the negatively charged $[Fe(CN)_6]^{3-/4-}$.

Non-complementary sequences showed similar peak currents and ΔEp values as probe [ΔEp: 110 mV] due to not being hybrized with probe. These results demonstrate that DNA can be successfully immobilized on electrodes and retain the ability to be hybridized with its complementary sequences.

The intrinsic electro-activity of guanine bases can be used for the direct measurement of nucleic acids in a label-free assay. The principle of guanine based detection system is based on measuring changes in oxidation current properties when a target binds with the sensing element on the electrode surface. Fig. 3B shows the voltammograms obtained from the guanine oxidation signals at probe, hybrid and non-complementary coated electrodes in solution phase. Hybridization was detected by the decrease in the magnitude of the guanine oxidation peak as measured by DPV with the highest guanine signal obtained from the probe coated electrode. The reason for this is that all guanines in the probe DNA were partly closed to oxidation after hybridization. To determine sensor specificity, non-complementary oligonucleotide was used under the same hybridization conditions. In the presence of non-complementary, higher guanine signal than complementary was observed. This clear difference between full match and non-complementary indicated that hybridization did not occurred with a non-complementary sequence. Signal suppressions were 50% for hybrid and 7% for the non-complementary sequences when it was compared to probe.

3.3. Interaction of DNA oligonucleotides with GelMA

After having obtained hybridization, the next step was the interaction of the oligonucleotides with GelMA. Observed results are shown in Fig. 4. GelMA oxidation signals were obtained at +0.7 V with DPV while guanines were at +1.0 V.

As shown in Fig. 4, GelMA oxidation signals changed with probe, hybrid and NC sequences. When hybrid interacted with GelMA, the oxidation signal of the GelMA had a larger decrease compared to probe. These differences allowed hybridization detection without labeling or using redox probes. Signal suppressions were 93% for hybrid and 19% for the non-complementary sequences when it was compared to probe.

GelMA is involved the category of non-conductive polymers that do not support electron transfer. It can be used for the mechanical protection of the DNA layer by providing mechanical support for the immobilized DNA. Through manipulation of gelatin macromere length and degree of methacrylate functionalization by adjusting experimental parameters, GelMA matrix provides multiple features to tune local structural properties. In modified electrode systems, electrodes are generally coated with the polymer layer and following this coating, the electrodes are interacted with the bio-recognition layer. However, in this case electrodes

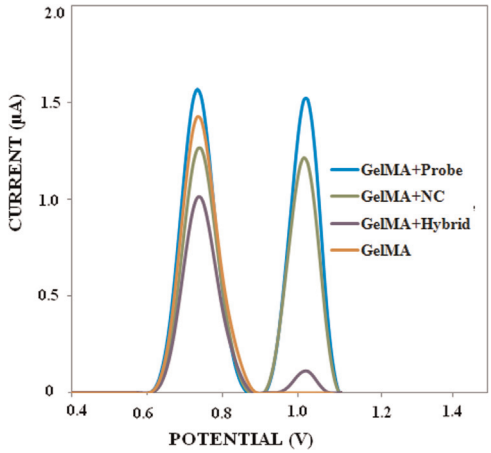

Fig. 4. DPVs of GelMA (+0.7 V) and guanine (+1.0 V) oxidation signals obtained from GelMA, GelMA-Probe, GelMA-Hybrid and GelMA-NC coated electrodes.

were coated with the bio-recognition layer and interacted with polymer. The approach did not prevent DNA layer activity and the hybridization efficiency by comparing other immobilization methods as mentioned in the Mascini's paper (Lucarelli et al., 2004). We suggest that the negatively charged phosphate backbone of DNA most likely interact electrostatically with the positive amino groups of a polymer chain. The molecular forces stabilizing the structure which is formed with DNA and GelMA are sufficient and covalent attachment was not needed. Because it is biocompatible and bio-degradable, GelMA modified electrodes system can be adapted for in-situ biomarker detection within living cells.

One of the advantages of the designed system is that during the process, organic solvents for deposition of the polymer were not used which is damaging for the biomaterials such as DNA, RNA or cells. Additionally, DNA has been substantially used as non-viral vectors for the delivery of genes or siRNA (Han et al., 2014). Owing to the strong and long-ranged electrostatic interaction, the structure and property of the DNA complex should remain stable over time. The GelMA film could also cause DNA to be more stable during the process.

The most notable advantage of the sensor is that GelMA can be used as an indicator of the hybridization due to its intrinsic oxidation signal. By taking advantage of this, hybridization can be detected by monitoring the changes in the electrochemical characteristics of GelMA resulting from its interaction with the DNA probe and/or target oligonucleotide. In the literature, methylene blue (Gu et al., 2002), meldola blue (Aydinlik et al., 2011), cobalt complexes (Wang et al., 2012) and anthraquinone derivatives (Kowalczyk et al., 2010) are some examples of the hybridization indicators. Among them, meldola blue and methylene blue are light sensitive and meldola blue is toxic. It is also important to use non-toxic redox indicators for both human health and environment. In our previous study, GelMA has been widely examined in terms of toxicity especially in 2D and 3D tissue engineering applications and found to be non-toxic to living cells (Shin et al., 2013). Furthermore, in order to immobilize the polymer film to the surface, covalent modifications or reactions such as sulfamide coupling (Wang et al., 2014) were not used.

4. Conclusion

In summary, this is the first report to use GelMA as modified covering material and a new hybridization indicator for DNA hybridization detection within the electrochemical biosensor. GelMA oxidation signal differences enabled us to determine hybridization independently from the guanine oxidation signals which would provide to detect hybridization even with the guanine free sequences. Guanine based detection methods are convenient however; the interest of the sequence may not contain the guanine base in some cases. Also, this protocol provides an effective assay that takes advantage of electrochemical methods such as being facile, fast and low-cost. The use of the GelMA is of great advantage, because no labeling of the analytes is required. This offers a novel way to create lab-on a chip system without using covalent attachment steps or labeling of the bioanalyte. Covalent attachment was also not preferred ın this case, because our sequences were not linked with special groups and we did not use nanomaterials such as gold or silver. So, we did not need to use specific immobilization. The polymeric indicator is applicable to the discrimination of complementary DNA from non-complementary sequences sensitively. Also, GelMA can be removed from the electrode surface by rinsing the electrode with mixing, and thus, the polymeric indicator-based DNA sensor can be used repeatedly and applied to different electrode materials such as gold or platinWe anticipate that this novel covering material will provide a suitable platform in electrochemical sensors for highly sensitive and sequence-specific detection.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.060.

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