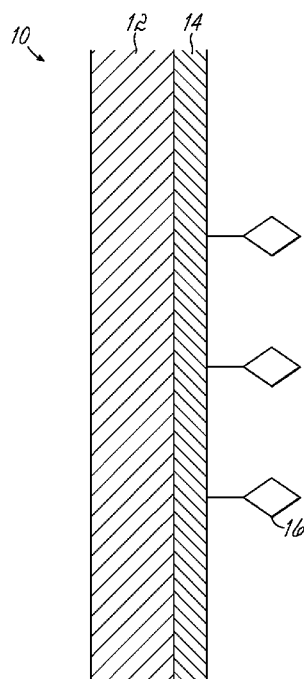




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[Continued on next page]

(54) **Title:** SUPERCAPACITOR-BASED BIOSENSORS AND METHODS OF DETECTING A BIOMARKER**FIG. 1**

(57) **Abstract:** A supercapacitor-based biosensor (10) for use in detecting a biomarker includes an electrode (12) coated with a nanocomposite film (14) including a nanostructured carbon material, such as graphene oxide, and a polymer containing amine functional groups, such as polyethylenimine. The coated electrode (12) is functionalized with an antibody (16) capable of binding with the biomarker. A method of fabricating a supercapacitor-based biosensor (10) includes functionalizing graphene oxide with polyethylenimine to create a nanocomposite, combining said nanocomposite with chitosan to form a blend, coating an electrode (12) with said blend to form a film (14), and functionalizing the film (14) with an antibody (16). A method of detecting a biomarker using a supercapacitor-based biosensor (10) includes adsorbing the biomarker on the supercapacitor-based biosensor (10), measuring a capacitance of the supercapacitor-based biosensor (10), and determining a concentration of adsorbed biomarker using the capacitance.

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SUPERCAPACITOR-BASED BIOSENSORS AND METHODS OF DETECTING A BIOMARKER

Cross-Related Application

[0001] The present application claims priority to U.S. Provisional Application No. 62/240,225 filed October 12, 2015, the disclosure of which is hereby incorporated herein by reference in its entirety.

Technical Field

[0002] The present invention relates generally to biosensor compositions and methods of using same and, more specifically, to supercapacitor-based biosensors and methods of detecting a biomarker.

Background

[0003] Over the last few years, electrochemical biosensors (ECBs) have been the subject of significant scientific research due to their promising applications in biomedical engineering fields. These devices offer some advantages, such as low cost, high sensitivity, and high efficiency, that make them suitable for performing real-time, in-vivo, non-invasive detection of physiological biomarkers related to some medical conditions.

[0004] According to the Center for Disease Control and Prevention, approximately 30% of yearly deaths in the United States occur as a result of diseases linked to high cholesterol concentrations in blood serum such as coronary heart disease, peripheral vascular disease, type 2 diabetes, and atherosclerosis, among others. Considering these bleak figures, an increased interest devoted to the development of Point-of-Care (PoC) devices capable of detecting and quantifying important biomarkers in blood serum have emerged. Among these important biomarkers, lipoproteins, which are blood serum cholesterol carriers, have attracted a focus of attention due to their physiological role and pathological consequences associated with abnormal amounts of these proteins in blood. Specifically, low density lipoprotein (LDL), also known as “bad cholesterol”, is a main cholesterol carrier in blood serum and it has been found to play a major role in atherosclerosis and coronary heart disease (CHD).

[0005] Additionally, venous thromboembolism (VT) conditions, namely, deep vein thrombosis (DVT) and pulmonary embolism (PE), have been responsible for 60,000 to 100,000 deaths per year in the U.S and it has been estimated that around 350,000 to 600,000 Americans suffer yearly of these pathological conditions. DVT is known to be the precursor of PE and, on average, one third of patients with DVT exhibits PE complications. Although these conditions present a significant mortal threat to the patient, the real problem lies in that the early diagnosis of these conditions is easy to overlook because of the large variation in clinical conditions of these diseases and given that about only half of DVT patients present noticeable symptoms. However, conventional detection methods, such as helical computed tomography and ultrasonography, have proven to have slow response times and involve high costs. D-dimer has been identified as an endogenous marker of fibrinolysis and it is known to play a key role in the diagnosis of hemostasis abnormalities such as DVT and PE.

[0006] Given the innovative evolution and development of nanotechnology fields in the last decade, some nanomaterials such as carbon nanotubes and graphene oxide have been used to fabricate novel ECBs in an effort to quantify biomarker levels in blood serum. For example, a carbon nanotubes (CNTs)-based and graphene oxide-based ECBs have been used to detect LDL or D-dimer molecules in solution using electrochemical impedance spectroscopy (EIS). However, potentiometric-based or EIS-based biosensor devices are constrained by the ability to commercialize them in small portable devices. Further, EIS-based biosensors have some drawbacks such as: (1) the use of a redox couple (e.g., $[\text{Fe}(\text{CN})_6]^{3-/4-}$), which can significantly increase materials costs, and (2) a long response time.

[0007] Considering these facts, it could be possible to reduce the costs, equipment complexity and response time of these electrochemical biomarker biosensors using a supercapacitor-based configuration.

Summary

[0008] In accordance with one aspect of the invention, a supercapacitor-based biosensor for use in detecting a biomarker is provided and includes a nanocomposite film including a nanostructured carbon material, such as graphene oxide, and a polymer containing amine functional groups, such as polyethylenimine, an electrode

coated with the nanocomposite film and an antibody capable of binding with the biomarker. The coated electrode is functionalized with the antibody.

[0009] In accordance with one aspect of the invention, a nanocomposite film for use in detecting a biomarker is provided and includes a polymer containing amine functional groups, a nanostructured carbon material functionalized with the polymer containing amine functional groups, and chitosan.

[0010] In accordance with one aspect of the invention, a method is provided for fabricating a supercapacitor-based biosensor includes functionalizing a nanostructured carbon material, such as graphene oxide, with a polymer containing amine functional groups, such as polyethylenimine, to create a nanocomposite, combining said nanocomposite with chitosan to form a blend, coating an electrode with said blend to form a film, and functionalizing the film with an antibody.

[0011] In accordance with one aspect of the invention, a method is provided for detecting a biomarker using a supercapacitor-based biosensor including adsorbing the biomarker on the supercapacitor-based biosensor, measuring a capacitance of the supercapacitor-based biosensor, and determining a concentration of adsorbed biomarker using the capacitance.

[0012] The present invention will be further appreciated in light of the following detailed description and drawings in which:

Brief Description of the Drawings

[0013] Fig. 1 is a cross-sectional view of a supercapacitor-based biosensor according to an embodiment of the present invention.

[0014] Fig. 2 is a schematic representation of a method of making a nanocomposite film according to an embodiment of the present invention.

Detailed Description

[0015] Embodiments of the present invention are directed to electrochemical biosensors based on a supercapacitor configuration for the detection and quantification of a biomarker. Further embodiments of the present invention are directed to methods of detecting and quantifying a biomarker using supercapacitor-based biosensors. With reference to Fig. 1, in one embodiment, a supercapacitor-based biosensor 10 includes an electrode 12 coated with a nanocomposite film 14. The nanocomposite film 14 may

be made of a nanostructured carbon material, such as graphene oxide (GO), functionalized with a polymer containing amine functional groups, such as hyperbranched polyethylenimine (PEI), and chitosan. The nanocomposite film 14 may then be functionalized with an antibody 16.

[0016] One particular nanostructured carbon material is graphene oxide. Graphene oxide provides an excellent platform for the detection of important biomarkers *in vitro*, such as low-density lipoprotein (“LDL”) or D-dimer. GO-based biosensors exhibit an improved sensitivity as well as enhanced specificity and better biosensing capabilities. GO is an attractive material for biosensing applications due to its high surface area, hydrophilic nature and excellent mechanical stability. Furthermore, GO can be easily functionalized via physical or chemical approach, due to the presence of a significant amount of oxygen-containing groups at its surface (i.e., carboxylic groups). The detailed description focuses on graphene oxide. However, it should be recognized that other nanostructured carbon materials may be used such as, for example, carbon quantum dots.

[0017] With reference to Fig. 2, the GO base is functionalized with a polymer containing amine functional groups to create a GO/polymer nanocomposite. The polymer used in the GO/polymer nanocomposite should include amine functional groups and may be, for example, polyethylenimine (PEI) or poly(allylamine). Additionally, the type and size of the polymer may vary. For example, the molecular weight of PEI may range from 10,000 to 30,000 MW. Further, the PEI may be PEI, branched PEI, or hyperbranched PEI.

[0018] In one embodiment, PEI is adsorbed over a GO surface to form a GO/PEI nanocomposite. To that end, GO may be dispersed in an aqueous solution under sonication. Hyperbranched PEI may then be added to the GO dispersion under stirring. Finally, the resultant solids may be separated via centrifugation, washed with DI water, and dried in an oven. The PEI may be added to the GO dispersion in a ratio of 1 g PEI to 100 mg GO. Further, the weight ratio of PEI to GO may range from 0.5 g PEI up to and including 2 g PEI to 100 mg GO. Similar ratios can be used with other polymers and nanostructured carbon material. Then, 1.0 g of PEI was added to the GO dispersion. Hyperbranched PEI contains a high amount of amine functional groups, which facilitates the amide-bond formation with the target-oriented protein (i.e.,

the antibody). Because the protein-amine group interaction is 1:1, the more amine groups over the nanocomposite's basal plane, the more antibodies per unit area there are. Because more antibodies may be bound to the surface, there is a potential to significantly increase the biosensor's sensitivity and enhance detection limits.

[0019] Still referring to Fig. 2, an electrode (not shown) may be coated with a nanocomposite film made of the GO/PEI nanocomposite and chitosan (C). In one embodiment, a first solution of GO/PEI may be mixed with a second solution of chitosan, which is a natural polymer, to create a GO/PEI/C solution. The GO/PEI/C solution may then be cast over an electrode and dried. The electrode may be, for example, a platinum electrode or any other conductive electrode material.

[0020] Next, the coated electrode may be bio-functionalized with an antibody capable of binding to the desired biomarker. In one embodiment, an antibody for LDL specificity is functionalized on the GO/PEI/C film as shown in Fig. 2. Anti-apolipoprotein B-100 ("AAB") has specific affinity for LDL epitopes. N-ethyl-N'-(3-dimethylaminopropyl carbodiimide / N-hydroxysuccinimide ("EDC/NHS") coupling chemistry may be utilized to form amide bonds between the carboxylic (-COOH) terminals of the AAB and the amine (-NH₂) terminals of the chitosan and PEI polymers.

[0021] Those skilled in the art will recognize that supercapacitor-based biosensors according to embodiments of the present invention may be used in other biosensing applications. For example, in an embodiment, an antibody for D-dimer specificity is functionalized on the GO/PEI/C film. Anti-fibrinogen antibody ("AFA") has specific affinity for D-dimer and shares the same isotype as AAB (i.e., the heavy chain ends are constant). EDC/NHS coupling chemistry may be utilized to form amide bonds between the carboxylic (-COOH) terminals of the AFA and the amine (-NH₂) terminals of the chitosan and PEI polymers.

[0022] Another embodiment of the present invention includes a method of detecting a biomarker using a supercapacitor-based biosensor. In that regard, with reference to Fig. 2, LDL may be adsorbed on the surface of the functionalized electrode. The change in double-layer capacitance of the supercapacitor-based biosensor is measurable as a function of LDL concentration *in vitro*.

[0023] A supercapacitor 3-electrode configuration may be used to evaluate the supercapacitor-based biosensor for LDL detection. The supercapacitor-based

biosensor exhibits a consistent non-linear increase of capacitance over LDL concentrations in the range of 10 to 120 mg/dL, which allows for accurate detection and quantification of LDL. Therefore, an LDL concentration may be determined by comparing the measured capacitance to a reference point.

[0024] Those skilled in the art will recognize that supercapacitor-based biosensors according to embodiments of the present invention may be used in other biosensing applications. For example, in an embodiment, D-dimer may be detected using a supercapacitor-based biosensor. Advantageously, the detection time may be less than 5 seconds. For example, the response time may be less than 2 seconds.

[0025] The bio-functionalized electrode acts as a supercapacitor-based biosensor. The biosensor may also be referred to as an immuno-sensor that has been immuno-functionalized with the antibody. Although supercapacitors have been traditionally used as energy storage devices, supercapacitor-based biosensors according to embodiments of the present invention overcome the limitations of EIS-based biosensors. Firstly, a supercapacitor-based biosensor does not require an expensive redox couple to operate. Secondly, a supercapacitor-based biosensor generally exhibits shorter response times than EIS-based sensors because the electrochemical analysis is based only charge/discharge processes that can be much faster than full-range EIS experiments. These improvements over EIS-based biosensors in quantifying biomarkers in blood serum may be employed for faster, accurate, and more economic clinical tests and improved point-of-care devices.

[0026] In order to facilitate a more complete understanding of the embodiments of the invention, the following non-limiting examples are provided.

Example 1

[0027] *Materials.* Chitosan, N-hydroxysuccinimide (NHS), N-ethyl-N'-(3-dimethylaminopropyl carbodiimide) (EDC), lyophilized Low Density Lipoprotein (LDL), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), phosphate buffer saline (PBS), acetate buffer solution (ABS) pH of 4.6, and polyethylenimine (PEI) were purchased from Sigma-Aldrich. Anti-apolipoprotein B-100 (AAB) and Sodium Chloride (NaCl) were obtained from Fisher Scientific. GO flakes were obtained from Graphene Supermarket and used as received.

[0028] *Synthesis of GO/PEI Nanocomposite.* GO was functionalized with PEI via a physical approach. One hundred (100) mg of GO were added to 50 mL of water and sonicated for 1 hour. Then, 1.0 g of PEI was added to the GO dispersion, which was first sonicated for 5 minutes and subsequently stirred at room temperature for 24 hours. Next, a 0.0667M PBS solution was added to the PEI-functionalized GO (G/PEI) dispersion and the resultant mixture was vigorously stirred for 15 minutes. Finally, the solids were separated via centrifugation, washed with DI water and dried in an oven at 70 °C overnight.

[0029] *Synthesis of GO/PEI/C Electrode.* A mixture containing GO/PEI and ABS in a ratio of 5 mg of GO/PEI per 4 mL of ABS was prepared in a beaker and then sonicated for 1 hour (solution 1). In a beaker, 15 mg of Chitosan were mixed with 30 mL of ABS and then stirred at room temperature (solution 2). Next, 0.5 mL of solution 1 and 1 mL of solution 2 were vigorously mixed in a vial. Subsequently, 50 μ L of the GO/PEI/C solution were cast over a platinum (Pt) disk electrode. The obtained GO/PEI/C film was finally dried in an oven at 60 °C overnight.

[0030] *Bio-functionalization of the Electrode.* Ten (10) μ L of a 0.05M NHS and 10 μ L of a 0.2M EDC solutions were uniformly spread through the GO/PEI/C nanocomposite electrode surface. In this case, EDC works as a coupling agent, whereas NHS acts as the activation agent during the peptide bond formation (formation of C-N bonds) that takes place between the carboxylic (-COOH) terminal of the antibody and the amine (-NH₂) terminals in the nanocomposite electrode. After pretreatment, 11 μ L of an antibody solution were cast over the surface. The antibody solution was prepared at a ratio of 1 mg of antibody per 1 mL of a 150mM NaCl and 50mM PBS solution. Then, the electrode was kept in a refrigerator at 4 °C overnight. In addition, a solution was prepared by mixing 2 mg of BSA with 1 mL of a 150mM NaCl and 1mL of 50mM PBS and was used to remove the excess of adsorbed antibody and also to block the non-specific active sites presents in the film.

[0031] *LDL Solutions.* LDL solutions were prepared in concentrations ranging from 20 to 120 mg/dL. This range was selected because is the typical range of concentrations observed when patients undergo clinical lipid profiling tests in blood serum. An aqueous solution was prepared containing 150mM NaCl and 0.01% per weight EDTA (solution 3). An initial LDL solution was prepared by adding under stirring

120 mg of LDL per dL of the aqueous solution. For LSL solutions with reduced concentrations, the initial LDL solution was consecutively diluted using suitable volumes of solution 3 to obtain solutions in the concentration range described above.

[0032] *Characterization Techniques.* The material properties of the functionalized GO and the electrode coated with the polymer nanocomposite film were characterized using a variety of methods. Fourier transform infrared (FTIR) was performed using a Bruker Vertex80 FT-IR spectrometer. The spectral range used was from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} with 100 scans per sample measured. Raman spectroscopy (RS) was performed using a Bruker Senterra Raman Spectrometer through a 20X objective lens using an argon laser ($\lambda = 532 \text{ nm}$) with 20 mW power. X-Ray diffraction (XRD) was performed using a Rigaku Ultima IV diffractometer with $\text{CuK}\alpha$ radiation (40 KV/44mA) and goniometer rotation rate of 1 $^\circ/\text{min}$. Thermal gravimetric analysis (TGA) was performed using a SDT Q600 T.A with a constant nitrogen flow from room temperature to 600 $^\circ\text{C}$ at a heating rate of 5 $^\circ\text{C}/\text{min}$. Surface LDL adsorption studies were performed utilizing a field emission scanning electron microscope (FE-SEM). Images were obtained using a JEOL-JSM-6930LV SEM employing an accelerated voltage of 10 kV. Images were obtained following standard procedures for low conducting samples.

[0033] *Fourier Transform Infrared (FTIR) Analysis.* FTIR experiments were performed to verify the functionalization of GO with hyperbranched PEI. As expected, the GO spectrum exhibited the characteristic peaks at 1762, 1244 and 1049 cm^{-1} , which correspond to the carbon-oxygen (C=O) stretching vibration of the carboxyl group, the hydroxide (O-H) vibration of the carboxylic acid and to the carbon-oxygen vibrations of the epoxy groups respectively. In contrast, the GO/PEI spectrum exhibited four new bands at 1643, 1566, 1467 and 1226 cm^{-1} , which confirm the successful functionalization of GO with PEI. For example, the band at 1643 cm^{-1} can be related to the grafting of the PEI onto the GO sheets. The band at 1566 cm^{-1} is generally attributed to the bending vibration of the nitrogen-hydrogen (N-H) bonds presents in the PEI structure, whereas the bands at 1467 and 1226 cm^{-1} can be attributed to the stretching vibration of the carbon-nitrogen bonds (C-N) also presents in the structure of this polymer. In addition, the absence of the band at 1762 cm^{-1} is likely

a result of the strong interaction between the polymer chains of PEI and the carboxylic groups present in the GO structure.

[0034] FTIR studies were also performed to characterize the nanocomposite film before and after the bio-functionalization with the antibody. FTIR spectra were obtained for pure chitosan and the GO/PEI/C nanocomposite films. The spectrum of chitosan showed a band at 1500 cm^{-1} , which corresponds to the “-C-C-” stretching vibration. In addition, the spectrum also exhibited a broad band at 3200 to 3500 cm^{-1} , which corresponds to the stretching vibration of the -OH groups present in the chitosan structure. In contrast, the absence of the band at 1500 cm^{-1} in the GO/PEI/C nanocomposite film spectrum indicates the hindrance offered by the GO/PEI filler over the polymeric film. In addition, the noticeable increment in intensity of the band at 2000 cm^{-1} can be also associated with the presence of PEI.

[0035] *Raman Spectroscopy (RS) Analysis.* Chemical characterization of GO and GO/PEI was performed using RS. For the GO spectrum, it is possible to observe the typical D and G bands at 1353 and 1583 cm^{-1} , respectively. The D band was attributed to the structural defects created by the attachment of epoxy and hydroxyl groups on the carbon basal plane, whereas, the G band was attributed to the vibration (E_{2g} mode) of the planar configuration sp² bonded carbon that constitute GO. According to the last, the Raman intensity ratio of D and G bands (I_D/I_G) can be used to estimate the structural disorder of GO before and after its functionalization. In this case, the intensity ratio calculated from the GO spectrum ($I_D/I_G = 0.95$) was lower than the ratio obtained from the GO/PEI spectrum ($I_D/I_G = 1.05$). These results indicate that GO/PEI possesses a more disordered structure than the unmodified GO.

[0036] RS studies were also performed to characterize the nanocomposite film before and after the bio-functionalization with the antibody. Raman spectra were obtained for the GO/PEI/C film before and after the bio-functionalization. As discussed above, EDC/NHS coupling chemistry was utilized to form amide bonds between the carboxylic (-COOH) terminals of the AAB and the amine (-NH₂) terminals of the Chitosan and polyethylenimine polymers. Due to this bond formation, an amide bond characteristic signal is expected to appear in the Raman spectra. A noticeable characteristic peak of the bio-functionalized film from 3100 to 3500 cm^{-1} was directly related to the presence of mono-substituted amide bonds; this peak was not present

before bio-functionalization of the electrode. Peaks present in both spectra (bio and non-bio functionalized film) at near 3000 cm^{-1} showed the presence of carboxylic acids (-COOH) groups over the polymer surface, which was expected because the film was functionalized with GO. The results reveal successful bio-functionalization over the polymeric film.

[0037] *X-Ray Diffraction (XRD) Analysis.* XRD analysis was performed to assess the GO structure before and after its functionalization with hyperbranched PEI. The GO diffractogram shows a peak with high intensity at $2\theta = 11.5^\circ$, which suggests that GO possesses a well-defined structure. Although the GO/PEI diffractogram shows the peak associated to the GO structure, it exhibits a lower intensity and the position of the peak is shifted toward a lower diffraction angle (i.e., $2\theta = 10.5^\circ$). This result suggests that the functionalization with hyperbranched PEI not only disrupts the well-defined structure of GO but also increases its interlayer spacing. Thus, these results suggest the successful modification of GO with hyperbranched PEI and are in agreement with the RS results above.

[0038] *Thermal Gravimetric Analysis (TGA).* TGA experiments were performed to corroborate the PEI functionalization of GO. The GO thermogram showed three major weight losses steps. The first step ($T < 100^\circ\text{C}$) was generally attributed to evaporation of moisture adsorbed on the graphene oxide sheets. The second step ($100^\circ\text{C} < T < 220^\circ\text{C}$) was associated with the pyrolysis of the oxygen-containing functional groups present in the GO. The last step ($220^\circ\text{C} > T > 300^\circ\text{C}$) was assigned to the decomposition of more stable oxygen-containing functional groups. At temperatures between 300°C and 500°C , the material was almost stable. After functionalization, GO exhibited a different thermal behavior. The thermogram exhibited four weight loss steps. As mentioned before, the first weight loss step was associated with moisture evaporation. The second ($150^\circ\text{C} < T < 200^\circ\text{C}$) and third ($200^\circ\text{C} < T < 300^\circ\text{C}$) weight loss steps were assigned to the decomposition of the oxygen-containing functional groups, as well as to the initial stage of the PEI decomposition. The weight loss steps observed at temperatures higher than 300°C were attributed to the complete thermal decomposition of the PEI. These results suggest that GO/PEI is thermally more stable than GO, which is probably because of the partial reduction of GO during the functionalization process. The appreciable differences between GO and GO/PEI in

terms of thermal behavior suggests the successful modification of GO with hyperbranched PEI.

[0039] *Surface LDL Adsorption Analysis.* Field emission scanning electron microscope (FE-SEM) images were obtained to complement the physical characterization of the fabricated nanocomposite film electrode before and after LDL adsorption onto its surface. After LDL adsorption, white spots of micrometric size were randomly distributed across the film surface, which were not observed before entering in contact with the LDL solution. These spots most likely correspond to the traces of LDL adsorbed onto the electrode during the electrochemical experiment.

[0040] *Capacitance Measurement Techniques.* Capacitance measurement methods were used to assess the electrochemical performance of the nanocomposite electrode. Capacitance measurements were obtained from cyclic voltammetry (CV) experiments performed at room temperature on a VersaSTAT 3 potentiostat from Princeton Applied Research and using a supercapacitor 3-electrode configuration in order to determine the system's capacitance as a function of the low density lipoprotein (LDL) concentration.

[0041] The working electrode was the bio-functionalized nanocomposite electrode, the reference electrode was an Hg/HgO electrode obtained from Koslow, and the counter electrode was a titanium foil electrode. The specific capacitance measurements were calculated by determining, using cyclic voltammetry (CV), a curve of potential (V) vs. current (i) and the mathematical relation described by Equation 1:

$$C_s = \frac{q}{\Delta E \times A}$$

where C_s , q , ΔE , A are: specific capacitance ($\mu\text{F}/\text{cm}^2$), electric charge (C), potential window (V), and electrode's specific area (cm^2), respectively. The selected scanning rate was of 1 V/s to avoid response from mass transport limitations. The voltage window at which CV tests were performed was 0 to 0.6 V. The prepared curve was obtained by analyzing and calculating the area under the curve on a CV potential (V) vs. Current (i), then, the specific capacitance was obtained by utilizing Equation 1.

[0042] *Capacitance Analysis.* The CV results showed a consistent non-linear increase in capacitance as the concentration of LDL is increased from 10 to 120 mg/dL. The performed electrochemical studies presented a detection range from 10 to

120 mg/dL of LDL. An average healthy human's LDL levels usually don't exceed 100 mg/dL. Deviations from this value are usually within the range of 100 ± 20 mg/dL. Certain patients may display LDL levels higher than 120 mg/dL. It is believed that due to the extent amount of amine groups over the surface, more antibodies per unit area are fixed over the surface, which may lead to increased detection limits beyond 120 mg/dL. Capacitance measurements at LDL concentrations below 10 mg/dL are not reported because clinical studies to date have not found a person with such extreme low levels of LDL. A slight curve deviation was not observed as the concentration approaches 120 mg/dL, suggesting that, possibly, detection limits may be increased beyond 120 mg/dL. Further, the supercapacitor-based biosensor exhibited excellent response times. At a scan rate of 1 V/s, the supercapacitor-based biosensor exhibited response times of 3 to 5 seconds, while a typical EIS biosensor may take up to 250 seconds in response time.

[0043] From 10 mg/dL on, the results were proved valid by triplicate experiment runs. Measurements were taken in duplicates. The results showed the same consistent non-linear behavior, validating the results. The results demonstrated the feasibility of using the biofunctionalized supercapacitor as electrochemical biosensors. Furthermore, these results indicate that supercapacitor biosensors can be used as substitutes for the commonly used EIS technique-based biosensors.

Example 2

[0044] *Materials.* In addition to materials used in Example 1, D-dimer from human plasma and monoclonal Anti-Fibrinogen antibody ("AFA") (clone 85D4, isotype IgG1) were purchased from Sigma-Aldrich.

[0045] *Bio-functionalization of the GO/PEI/C Electrode.* A GO/PEI/C electrode was fabricated according to the method described in Example 1. Next, 10 μ L of a 0.05M NHS and 10 μ L of a 0.2M EDC solutions were uniformly spread over the GO/PEI/C nanocomposite electrode surface. In this case, EDC works as a coupling agent, whereas NHS acts as the activation agent during the peptide bond formation (formation of C-N bonds) that takes place between the carboxylic (-COOH) terminal of the antibody and the amine (-NH₂) terminals in the nanocomposite electrode. After pretreatment, 35 μ L of an antibody solution were cast over the surface. The antibody solution was prepared at a ratio of 1 mg of antibody per 1 mL of a 150mM NaCl and

50mM PBS solution. Then, the electrode was kept in a refrigerator at 4 °C overnight. In addition, the excess antibody solution over the electrode's surface was removed by gently washing with deionized water.

[0046] *D-Dimer Solutions and BSA Solutions.* D-dimer solutions were prepared at concentrations ranging from 50 ng of D-dimer per mL of a 0.01% per weight EDTA and 150mM NaCl, to 500 ng/mL. This D-dimer concentration window was selected given that a sufficiently wide range that covered enough to diagnose healthy and non-healthy levels of this biomarker was desired. The highest numerical value a person can have of this biomarker to be considered as safe from VT-related conditions is 250 ng/mL. An average healthy human may present concentrations as low as 50 ng/mL, while other non-healthy humans may present concentrations up to greater than 400 ng/mL.

[0047] BSA solutions were also made to determine if the AFA interacted consistently to the presence of D-dimer or if other proteins would trigger the same response. The BSA solutions were prepared in the range of 100 to 600 ($\times 10^3$) ng/mL by dissolving the corresponding amount of BSA in the previously prepared 0.01% per weight EDTA and 150mM NaCl solution.

[0048] *Characterization Techniques.* The material properties of the functionalized GO and the electrode coated with the polymer nanocomposite film were characterized using the methods described in Example 1.

[0049] *Raman Spectroscopy (RS) Analysis.* Raman spectra were obtained for the GO/PEI/C film before and after the bio-functionalization with the antibody. Once EDC/NHS treatment has been done over the GO/PEI/C electrode's surface, amide or peptide bonds will form between the carboxylic (-COOH) terminals of the antibody and the amine (-NH₂) terminals of the electrode's surface. This type of bond was not present before the bio-functionalization took place, therefore, a characteristic signal of this type of bonds in the Raman spectra will corroborate that the AFA was successfully immobilized over the surface. Before bio-functionalization took place, a characteristic peak near 3000 cm⁻¹ corresponds to the presence of carboxylic acid (-COOH) groups. No other major characteristic peaks are noticed before bio-functionalization in the area of interest. After functionalization, however, a peak at 3000 cm⁻¹ is still present while another high-intensity peak is noted in the 3100 to 3500 cm⁻¹ region, which

corresponds to the presence of mono-substituted amide bonds. The results reveal successful bio-functionalization over the polymeric film.

[0050] *Capacitance Analysis.* The capacitance measurement method described in Example 1 was used to assess the electrochemical performance of the nanocomposite electrode. The CV results suggested that the material's capacitance is mainly attributed to double layer and non-faradaic processes such as adsorption of the biomarker over the electrode's surface. Other curves showed slight deviations which indicated that non-significant faradaic processes are taking place in the electrode, such as the partial oxidation of the electrode surface. The specific capacitance of the system ($\mu\text{F}/\text{cm}^2$) increases non-linearly as the D-dimer concentration increases as well in the range of 50 to 500 ng/mL. The increase in capacitance as the concentration of D-dimer increased is mainly due to surface modification by the proportional adsorption of the biomarker to the electrode's surface (a non-faradaic process) which finally influences in the overall double layer capacitance. Also, the results suggested that the electrode has the ability to store charge across the electrode/solution interphase. The biosensor showed remarkable accuracy over successive scans and surpassed potentiometric-based biosensors in that it took around 2 seconds to detect and respond. Additionally, the ease of reducing size and developing portable-biosensing devices according to the present invention is much feasible than with potentiometric-based biosensors. Finally, the biosensor according to the present invention surpassed EIS-based sensor due to not needing a redox couple, and the direct correlation of capacitance to concentration, there is no need to model an equivalent circuit which introduces doubt about the validity and accuracy of the model.

[0051] From 50 to 500 ng/mL, the results were reproduced in triplicates. The results showed the same consistent non-linear behavior, validating the results. Further, the results showed that detection limits might not have been reached in the performed experiments (either from the low concentrations or the high concentrations side). The results demonstrated the feasibility of using the biofunctionalized supercapacitor as electrochemical biosensors.

[0052] *Capacitance Analysis using a Non-functionalized Biosensor.* Specificity studies were also conducted to validate the affinity of the biosensor for its corresponding antigen. In this case, the prepared working electrode was not

functionalized with the EDC/NHS treatment or the antibody exposure. The results exhibited a non-proportional nor consistent capacitance response as the D-dimer concentration varies. By comparing these results with the ones obtained from the functionalized biosensor, it was concluded that the presence of the antibody (AFA) over the surface plays a major role in the proportional and direct adsorption of D-dimer over the surface which, furthermore, has a significant effect on the capacitance of the system and double layer arrangement. In short, the comparison supports the idea that the specific capacitance and its proportionality to the biomarker concentration is tightly related to the specific adsorption of D-dimer over the electrode's surface. This property of the system allows for the easy measurement and detection of D-dimer by high rate cyclic voltammetry.

[0053] *Capacitance Analysis using a Biomarker Other Than D-Dimer.* Specificity studies were also conducted to validate the affinity of the biosensor for its corresponding antigen by exposing it to a foreign protein with foreign epitopes, in this case, bovine serum albumin (BSA). From 100 to 300 ng/mL BSA, the system appeared to have a consistent increase in response. After 300 ng/mL BSA, the capacitance drops and increases in a random matter. Such results were interpreted as that the system does not consistently nor proportionately attach the BSA over the surface (results that are expected), but that the capacitance response is being affected by secondary/parallel surface interactions such as electrolyte-electrode. These results further confirm the specificity of the developed biosensor in that it is not consistently responding in terms of capacitance when is exposed to BSA—a foreign protein.

[0054] While all of the invention has been illustrated by a description of various embodiments and while these embodiments have been described in considerable detail, it is not the intention of the Applicants to restrict or in any way limit the scope of the appended claims to such detail. Additional advantages and modifications will readily appear to those skilled in the art. The invention in its broader aspects is therefore not limited to the specific details, representative apparatus and method, and illustrative examples shown and described. Accordingly, departures may be made from such details without departing from the spirit or scope of the Applicants' general inventive concept.

What is claimed is:

1. A supercapacitor-based biosensor for use in detecting a biomarker comprising:
an electrode coated with a nanocomposite film including a nanostructured carbon material and a polymer containing amine functional groups to form a coated electrode,
wherein the coated electrode is functionalized with an antibody capable of binding with the biomarker.
2. The biosensor of claim 1, wherein the nanostructured carbon material includes graphene oxide.
3. The biosensor of claim 1, wherein the polymer is polyethylenimine.
4. The biosensor of claim 3, wherein the polyethylenimine is branched or hyperbranched.
5. The biosensor of claim 1, wherein the polymer is poly(allylamine).
6. The biosensor of claim 1, wherein a weight ratio of the polymer to the nanostructured carbon material ranges from 0.5 g per 100 g to 2 g per 100 g.
7. The biosensor of claim 1, wherein the nanocomposite film includes chitosan.
8. The biosensor of claim 1, wherein the antibody is an anti-apolipoprotein antibody.
9. The biosensor of claim 1, wherein the antibody is an anti-fibrinogen antibody.
10. A nanocomposite film for use in detecting a biomarker comprising:
polyethylenimine;
graphene oxide functionalized with the polyethylenimine; and
chitosan.

11. The nanocomposite film of claim 10, wherein the coated electrode is functionalized with an antibody capable of binding with the biomarker.
12. The nanocomposite film of claim 10, wherein the polyethylenimine is branched or hyperbranched.
13. A method of fabricating a supercapacitor-based biosensor comprising:
functionalizing graphene oxide with polyethylenimine to create a nanocomposite;
combining said nanocomposite with chitosan to form a blend;
coating an electrode with said blend to form a film; and
functionalizing the film with an antibody.
14. The method of claim 13, wherein functionalizing includes N-ethyl-N'-(3-dimethylaminopropyl carbodiimide / N-hydroxysuccinimide coupling chemistry.
15. The method of claim 13, wherein the polyethylenimine is branched or hyperbranched.
16. The method of claim 13, wherein the nanocomposite film includes chitosan.
17. The method of claim 13, wherein the antibody is an anti-apolipoprotein antibody.
18. The method of claim 13, wherein the antibody is an anti-fibrinogen antibody.
19. A method of detecting a biomarker using a supercapacitor-based biosensor comprising:
adsorbing the biomarker on the supercapacitor-based biosensor;
measuring a capacitance of the supercapacitor-based biosensor; and
determining a concentration of adsorbed biomarker using the capacitance.

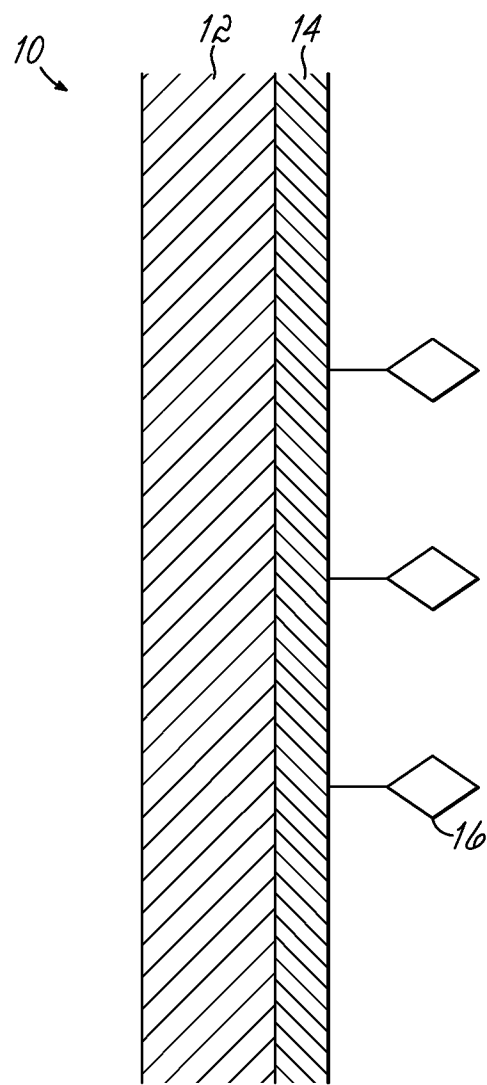


FIG. 1

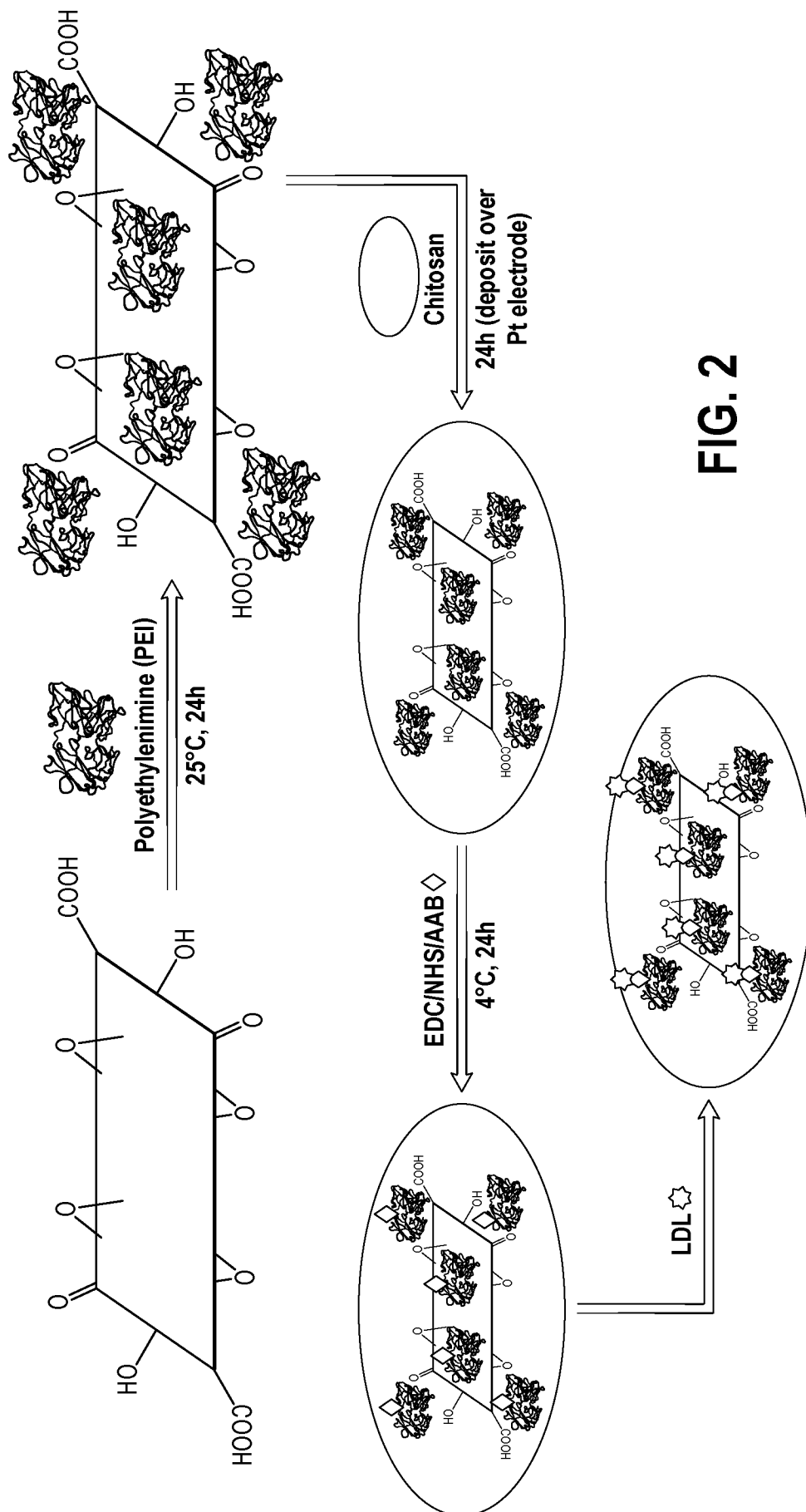


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/56570

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - G01N 27/00; G01N 33/543; C12Q 1/00 (2016.01)
 CPC - C01N 33/5438; C12Q 1/001

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC(8): G01N 27/00; G01N 33/543; C12Q 1/00 (2016.01)
 CPC: C01N 33/5438; C12Q 1/001

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 USPC: 436/518; 436/149 (key word limited; see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 PatBase, Google Patents, Google Scholar
 Search terms used: Supercapacitor-based biosensor, coated electrode, nanocomposite film, graphene oxide, polyethylenimine, poly(allylamine), chitosan, antibody, biomarker

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No. ***
Y	WO 2015/038570 A2 (INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION) 19 March 2015 (19.03.2015); pg 12 para 1; pg 15 para 1; pg 16 para 1; pg 17 para 2-3; pg 18 para 1	1-9, 13-19
Y	US 2014/0315213 A1 (NAGRATH et al.) 23 October 2014 (23.10.2014); para [0002], [0004], [0006], [0078], [0085], [0105]-[0106]	1-18
Y	WO 2015/039555 A1 (SUZHOU INSTITUTE OF NANO-TECH AND NANO-BIONICS, SINANO) 26 March 2015 (26.03.2015); [translation attached separately]: pg 2 para 4; pg 5 para 11	10-18
Y	US 2012/0186997 A1 (LI et al.) 26 July 2012 (27.07.2012); para [0066], [0097]	3-5, 12, 15
Y	US 9,091,640 B1 (MOHAPATRA et al.) 28 July 2015 (28.07.2015); col 1 ln 14-15; col 7 ln 27-30	7
Y	US 2010/0136572 A1 (ATAMAN-ONAL et al.) 03 June 2010 (03.06.2010); para [0078], [0085]-[0086]	8, 17
Y	US 5,976,466 A (RATNER et al.) 02 November 1999 (02.11.1999); col 13 ln 39-40	9, 18
Y	US 2012/0238835 A1 (HYDE et al.) 20 September 2012 (20.09.2012); para [0101], [0150]-[0151], [0153], [0327]	19
Y	US 9,011,779 B1 (ANGLIN et al.) 21 April 2015 (21.04.2015); col 2 ln 15-16; col 9 ln 21-24; col 15 ln 6-8	19
Y	US 2010/0047815 A1 (CHEN et al.) 25 February 2010 (25.02.2010); para [0009], [0068], [0071]	14

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

29 November 2016

Date of mailing of the international search report

19 JAN 2017

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