TOBACCO MOSAIC VIRUS BIOTEMPLATED ELECTROCHEMICAL BIOSENSOR

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ABSTRACT

This work demonstrates the first utilization of virus molecules as nano-scale biotemplates assembled on an electrochemical biosensor, allowing for an 8-times increased signal and an improved biosensing performance of 9.5-fold. The versatile and inexpensive biological *Tobacco mosaic virus* was integrated as a high aspect ratio, low footprint, low-cost, easy to genetically functionalize, nanostructured three-dimensional scaffold for the synthesis of novel multifunctional electrodes. The biotemplated scaffold allows for an increased surface area resulting in higher electrochemical currents, better signal-to-noise ratio and improved sensitivity when incorporated into miniaturized biosensors.

INTRODUCTION

Electrochemical Biosensors

Electrochemical biosensors are based on a bioelectrochemical interaction process, where electrochemical species are consumed or generated. Most electrochemical measurements detect oxidation/reduction of the product generated by biological conversion of the analyte undergoing redox reactions on the sensor's surface and solely respond to electro-active species. Electrochemical measurements are classified according to the variable being measured: amperometry, potentiometry and conductometry [1, 2].

In recent decades, nanostructured materials have received much attention due to unique properties they offer as robust platforms for electronic and optical signal transduction. One of its major contributions is the design of a new generation of miniaturized biosensing devices. Furthermore, biofunctional nanoparticles can produce a synergistic effect between catalytic activity, conductivity, and biocompatibility to accelerate the signal transduction, leading to the quick development of stable, specific, selective and sensitive biosensors in different fields. An important point besides the biosensing applicability of nanomaterials is the use of manipulation techniques for their integration in fabrication techniques [3]. By integrating with electrochemical sensor fabrication, these materials provide interesting properties such as increased surface area. The augmented surface area leads to higher signal-to-noise ratio, increased sensitivity and dynamic range, short distances for mass transport and charge transfer as well as their ability to create complex nano-bio-architectures that allow volume change and unique selective biological functionality. Nanomaterials allow the development of new architectures applied in electrochemical sensing and biosensing devices [4, 5]. For example, Ye and colleagues presented a multi-walled carbon nanotube (MWNT)-based electrochemical biosensor for glucose detection [5]. The higher surface area of the well-aligned MWNT generated higher electrochemical currents that were correlated to a highly sensitive sensor.

Tobacco mosaic virus

Biological nanomaterials provide a versatile and cost effective solution for the fabrication of high surface area nanoarchitectures. One category of these biological nanostructures is plant and

bacteria virus particles consisting of macromolecular assemblies of nucleic acid packaged by many copies of coat proteins. These molecules display some unique advantages as they show exceptional stability in a wide range of temperatures and pH values, in addition to their surface-exposed functional groups, self-assembly and tunability [6].

Among the available plant viruses [7], *Tobacco mosaic virus* (TMV) is the most extensively studied filamentous plant structure for nanoscale applications. The TMV virion is a rigid rod consisting of about 2,130 identical coat protein subunits stacked in a helix around a single strand of plus sense RNA, forming a 4 nm diameter channel through the 300 nm long virion axis. Properties of the TMV system that make it particularly useful as a self-assembling macromolecular template for nanomaterials include: 1) its known three-dimensional structure [8]; 2) a wealth of biophysical information on its self-assembly characteristics [9]; 3) the availability of creating novel virus structures and surfaces via established molecular techniques [10]; 4) a wide range of existing coat protein variants with diverse assembly properties [11], and 5) the ability to easily purify large quantities of virus and coat protein from infected plants. TMV-structured metal and metal-oxide nanowires have been synthesized using several techniques [12, 13], transport properties have been studied [14], and potential applications in nano-scale devices have been investigated through proof-of-concept demonstrations [15].

Previous work with engineered mutations of the TMV has resulted in enhanced particle coatings and templates that can be readily integrated into microfabricated devices and has established a novel patterning process. Efficient templates for metallic coatings have been achieved through the introduction of one (TMV-1cys) or two (TMV-2cys) cysteine residues within the coat protein open reading frame. Cysteines are amino acids with thiol groups that show enhanced metal binding properties based on strong, covalent-like interactions. One and two-step electroless plating methods have been used for the fabrication of TMV-2cys-based wires coated with gold, silver and palladium clusters that show more uniform coating compared to the wild-type virus [16]. Additionally, the rod-shaped viruses can be directionally attached to various surfaces and coated to create high aspect ratio nickel, cobalt and platinum materials (TMV-1cys). Alternative pathways were explored for patterning the viral molecules in microfabricated electrodes as well as controlled environments, utilizing nucleic acid hybridization [17]. Recent work in our team has focused upon the development of novel inorganic structures using the TMV and their application in energy storage devices. A simple and versatile approach for the selective patterning of both metal-coated and uncoated TMV using lift-off processing has been developed [18]. The high aspect ratio of the coated TMV in addition to its robustness was utilized in the development of high surface area nickel-zinc [19] and Li-ion microbatteries [20, 21]. By the integration of high aspect ratio biotemplated TMV scaffold in electrode fabrication process, high surface area electrochemical sensors can be realized improving the overall biosensing performance.

Mental Health Applications

The medical management of mental health care is one of the most debilitating and costly of all disorders. A major unmet need is the ability to have an objective, real time analysis of the disorder at the point-of-care. Schizophrenia is a common mental health disorder, has high social and economic impact, and manifests early – mostly during adolescence and early adulthood. It is frequently preceded by premorbid pattern of rather unspecific but nevertheless handicapping symptoms that may be present for several years before clinical manifestation. Schizophrenia is a complex disorder involving difficulties with reality distortion, cognitive impairments, and work and social dysfunction. Often, it is also associated with symptoms of moodiness and anxiety. Once diagnosed, patients need to remain on antipsychotic medications lifelong. One of the biggest challenges of treatment is that many people discontinue their treatment which can lead to relapse and rehospitalizations, having a major impact on patient and society healthcare costs [22, 23]. Finding a method for continually monitoring patient status would allow for psychiatrists to more effectively treat these mental disorders.

Over the past few years, genetic analysis has found a strong relation between the gene encoding for Neuregulin-1 (NRG1) and schizophrenia [24]. Studies have obtained supporting evidence for NRG1 as a candidate gene for the disorder [25]. Moreover, the identification of NRG1 receptor ErbB4 as an additional candidate risk gene for schizophrenia strongly suggests that this signaling pathway participates in the pathophysiology of the disorder [26]. By monitoring NRG1 concentration during various stages of schizophrenia, mental health care management can be studied and improved.

Microfluidic lab-on-a-chip (LOC) microsystems provide numerous advantages in clinical diagnostics, environmental monitoring and biomedical research fields. These translational technologies hold potential to improve upon the resolution, regulation, sensitivity, flexibility, and cost-savings over more traditional approaches, bringing bench top methods into the point-of-care. By the integration of MEMS sensing devices with mental health analysis, pre-clinical assessments and the overall chance of success of personalized medical care at the point-of-care can be significantly improved.

RESULTS

One of the challenges with miniaturized biosensing devices is the high background signal and low signal-to-noise ratio that decrease the performance of biosensors. By increasing the signal, electrochemical biosensors can improve their bio-detection efficiency resulting in higher sensitivity. Here, we used gold planar square electrodes (20nm Cr/180nm Au, surface area = 0.49 cm²) that were patterned onto a silicon dioxide substrate via DC sputtering and were realized using both wet etching and lift-off techniques. Virus modification of the electrode surface was performed to enhance the total surface area. Fabrication was carried out by submerging the electrodes in a solution of rod-shaped 300 x 18 nm TMV vertical viruses in 0.1M phosphate buffer (pH 7) for 18 hours, allowing for self-assembly of the virus particles on the microfabricated planar gold electrodes via the previously mentioned cysteine residues. Following TMV self-assembly, nickel was electroless deposited for 5 minutes [19]. Sequentially, gold was electroless plated in a gold plating bath (0.007M KAu(CN)₂, 1.4M NH₄Cl, 0.2M Sodium citrate, and 1M NaH₂PO₄, pH 7.0-7.5) for 45 minutes at 90°C to form a conformal gold coating on the virus particles. Fig. 1 shows SEM images of the surface of the electrode modified with nickel-coated TMV-Icys after gold deposition (TMV/Ni/Au modified electrode). TMV-

Icys were observed to attach vertically on the electrode surface. A TEM image of the nanocomposite virus particle (Fig. 2) shows approximately 67 nm nickel and 46 nm gold coatings. The thin films of the nickel and the gold maintain the high aspect ratio of the TMV scaffold after plating, increasing the electrochemically active surface area of the electrode.

Figure 1: Self-assembled TMV-Icys molecules coated with nickel and gold layers on the surface of planar electrode. (A) Cross section schematic. (B) Scanning electron micrograph.

Figure 2: (A) Schematic of the TMV coatings. (B) Transmission electron microscopy analysis of the TMV-Icys coated with nickel and gold layers with EDS profiles of nickel (pink / dark gray) and gold (orange / light gray).

The modified electrodes were electrochemically characterized using cyclic voltammetry (CHI660D single channel potentiostat from CH Instruments, Austin, TX. Commercial Ag/AgCl reference electrode. Pt wire counter electrode) in the presence of a redox couple 5mM ferrocyanide, 5mM ferricyanide, 10mM phosphate buffers saline (PBS) solution. Fig. 3 illustrates the increased signal from the modified electrodes compared to unmodified electrode with cyclic voltammograms of the generated electrochemical reduction and oxidation reactions. The electrodes produced reversible nernstian characteristics where the TMV/Ni/Au modified electrode generated the highest electrochemical current (8-fold higher oxidation peak current in comparison with the unmodified planar electrode) due to the high active surface area. The oxidation and the reduction peaks generated by the TMV/Ni/Au modified electrodes varied in compare to the other types of electrodes. This variation is may be due to increase with the uncompensated resistance in the new high aspect ratio electrochemical interface. Furthermore, the potential presence of

nickel uncoated with gold on the TMV scaffold may result in changes with the standard reduction potential of the redox couple. The increased electrochemical activity of the Ni/Au modified electrode compared to unmodified electrode may be due high surface roughness resulted by the nickel and the gold electroless plating. Furthermore, the TMV modified electrode impeded the generated electrochemical current that was attributed to the non-conductive virus coating on the electrode surface.

The effective surface area of the unmodified and the modified electrodes were calculated from Bard and Faulkner [27]:

$$I_{peak} = 0.4463(F^3/RT)n^{3/2}AD^{1/2}C^*v^{1/2} \quad (1)$$

Where F [$C \text{ mol}^{-1}$] is the Faraday constant, R [$J \text{ mol}^{-1} \text{ K}^{-1}$] is the gas constant, T [K] is the temperature, n is the stoichiometric number of electrons involved in an electrode reaction, A [cm^2] is the effective surface area of the electrode, D [$\text{cm}^2 \text{ s}^{-1}$] is the diffusion coefficient of the electro-active species, C^* [mol cm^{-3}] is the bulk concentration of the electro-active species, v [$V \text{ s}^{-1}$] is the linear potential scan rate, and I_{peak} [A] is the peak current. Effective surface area calculations demonstrated that TMV/Ni/Au resulted in the highest area (Table 1), a characteristic important for the high performance of electrochemical sensors due to the surface area limited reaction kinetics.

Figure 3: Cyclic voltammograms of the planar electrode modified with either the TMV, the Ni/Au electroless deposition, or both. Inset shows only the unmodified and the TMV modified electrodes.

Table 1: Measured effective surface area and bio-detection efficiency analysis for the unmodified and the modified electrodes.

Electrode modification	Effective surface area [cm^2]	Bio-detection efficiency [%]
Unmodified	0.42	1.7
TMV	0.32	2.1
Ni/Au	1.05	-1.1 (poor efficiency)
TMV/Ni/Au	3.34	16.1

In this study the target NRG1 polymorphism ssDNA (SNP8NRG243177) bio-detection efficiency was electrochemically analyzed using thiolated ssDNA probe to NRG1 assembled on the high surface area TMV/Ni/Au modified electrode. In short, electrodes were incubated in a solution containing 10 mM PBS, 100 mM NaCl, 10 μM Tris (2-carboxyethyl) phosphine (TCEP) and 1 μM probe ssDNA for 3 hour followed by rinsing with PBS. Afterwards, the electrodes were incubated for 24 hour in PBS solution containing 1 mM of 6-mercapto-1-hexanol (MCH). MCH is used to passivate any

exposed regions on the surface to reduce non-specific binding during DNA hybridization. Incubation with either the non-complementary or the target sequences was performed in a 4x saline sodium citrate (SSC) buffer containing 1 μM of the target DNA for 1 hour.

Biosensing performance was studied with cyclic voltammetry in the presence of the previously described redox ferrocyanide/ferricyanide solution (Fig. 4). All electrodes demonstrated reversible nernstian characteristics in the presence of complementary NRG1 target and non-complementary ssDNA. Moreover, the TMV/Ni/Au modified electrode demonstrated the largest current differentiation upon DNA hybridization. DNA hybridization causes stronger electrostatic repulsion forces with the negatively charged electro-active species in the electrolyte, hence impeding the electrochemical reaction at the electrode [28]. Bio-detection efficiency (ratio of oxidation peak current decrease between non-complementary and complementary ssDNA) analysis (Table 1) demonstrated 9.5-fold improved bio-detection performance for the 3D TMV/Ni/Au modified electrode compared to the unmodified electrode. This increase is not only due to the higher effective surface area (factor of 8), but also due to the inherent properties of nanomaterials improving biosensing performance, e.g. augmented amount of probes functionalizing the surface of the electrode and heightened target concentration near the electrochemically reactive surface.

Figure 4: Cyclic voltammograms of the (A) planar unmodified electrode, (B) TMV modified electrode, (C) Ni/Au modified electrode, and (D) TMV/Ni/Au modified electrode following introduction of ssDNA NRG1 probe (black solid), non-complementary ssDNA (red dashed), and complementary NRG1 target ssDNA (blue dashed-dot). Arrow indicates current reduction upon DNA hybridization.

CONCLUSIONS

This work demonstrated the first utilization of TMV molecules coated with nickel and gold as nano-scale biotemplates assembled on an electrochemical biosensor for schizophrenia analysis. The biotemplated TMV modified sensor generated heightened electrochemical activity that improved the biosensing performance. Given the large set of biological recognition events that can be detected electrochemically and the innately high compatibility of TMV-biotemplate integration with conventional microfabrication technology, we believe this work has broad applications in biological electrochemical sensing and transduction. Specifically, we envision the utilization of TMV as a high aspect ratio nano-scale 3D scaffold for high surface area microelectrodes integrated in MEMS sensing devices improving

dramatically their performance. We believe TMV-biotemplated LOC devices leverage electrochemical biosensing reactions that will lead to the creation of new families of low-cost, rapid and sensitive miniaturized biosensors for point-of-care analysis.

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