Chitosan-Graphene Oxide-Based Ultra-Thin and Flexible Sensor for Diabetic Wound Monitoring

Md. Abdul Kafi, Ambarish Paul, Anastasios Vilouras, Ensieh S. Hosseini, and Ravinder S. Dahiya

Abstract—This paper presents a Chitosan-Graphene Oxide (CS-GO)-based array of ultra-thin biosensors with gold (Au)-based microgap (60 µm) electrode. The cross-linked GO is shown to improve the stability of chitosan substrate in aqueous medium and compatibility with microfabrication steps. The sensor patch has been evaluated for label-free monitoring by immobilizing the CS-GO surface with human dermal fibroblast (HDF) cells. The cyclic voltammetry (CV) of HDF cell immobilized CS-GO surface show quasi-reversible nature with a characteristic cathodic peak at +300 mV and anodic peak at ~300 mV. Both peaks are stable and repeatable up to 50-scan cycle without any potential shift. The device shows a steady-state peak enhancement (1.923-11.195 nA) during the DHF cell growth period (0-96h). The redox peak enhancement correlates with the cell proliferation rates over time, indicating that it could be employed for investigation of the cyto-physiological state against any endo and exogenous stimulation. In addition, the developed sensor-patch was used to detect a wide range of glucose from 1 µM to 20 mM in vitro with a sensitivity of 0.17 µA/µM. Considering these, the presented sensor-patch has a great potential for the detection of glucose level, cell-health proliferation rate at the wound site, and diabetic wound monitoring applications.

Index Terms—Chitosan, graphene oxide, HDF cell, cyclic voltammetry, linear swift voltammetry, wound monitoring.

I. INTRODUCTION

With increasing number of diabetic patients worldwide, the monitoring of non-healing chronic wounds, such as diabetic foot ulcers has attracted significant attention in recent years [1]. In current clinical practice, the chronic wounds are treated with moisture-absorbing or moisture-retentive dressings, which require frequent replacement [2], causing unnecessary pain and hampering the healing process [3]. The passive (i.e. without any sensors to monitor) nature of currently used dressings make it difficult to monitor the progress of wound healing [4]. For this reason, there is a growing demand for the development wearable systems that can monitor the healing of wound and sense the analytes in the wound fluid. While the wearability of such system demands ultra-flexibility and biocompatibility. Thus, flexible and biocompatible functional materials such as Pt/PVDF scaffold and ZnO nanorods composite etc. [5], [6] have been used in active wound dressing to accelerate the healing process through electrical stimulation of wound. Recently, biodegradable piezoelectric glycerine has also been proposed for the fabrication of a flexible self-powered stress sensor for continuous monitoring of applied stress around the wound site under compression bandages [7]. Chitosan (CS) is another material which holds significant promise as besides being biocompatible, it is biodegradable and exhibits antimicrobial properties [8]. It is already being utilized as drug carrier in wound dressings, as scaffolds for tissue engineering etc. [9]. Recently, CS based biosensors have also been reported for measurement of a wide range of analytes from biological [10], chemical [11] and environmental sources [12]. However, the degradability of CS in aqueous medium limits the use of CS-based biosensors to in-vitro measurements. Further, CS as substrate is incompatible with some of the microfabrication steps (e.g. metallization).

If there is a way to prevent the degradability of CS in aqueous medium, for example by tuning the surface properties,
it could be attractive for in-vivo conditions also. For example, using CS-based sensors to monitor the proliferation of cells in vivo tissue engineering applications are attracting much interest [9]. In this regard, recent studies show that cells in vivo tissue engineering applications are attracting ple, using CS-based sensors to monitor the proliferation of it could be attractive for in-vivo conditions also. For exam-

Taking advantage of the above features, herein we present the CS-GO based ultra-thin (2.8µm) and highly conformable array of biosensors made from gold (Au) based electrodes. The micro-gap of 60µm between the electrodes is bridged with cells. The sensors-patch has been used to demonstrate the label free monitoring of human dermal fibroblast (HDF). Further, the HDF immobilized sensor-patch has been used to investigate the in vitro glucose sensing performance. This paper extends the initial results presented at IEEE Sensors 2018 conference [20]. Herein, RGD assisted adhesion process has been demonstrated where RGD nanostructured electrode was achieved with self-assembly of cysteine terminates RGD adhesion motif [21], [22]. The cells firmly adhered on RGD nanostructured electrode were employed for monitoring cell proliferation and glucose sensing in real-time.

This paper is organized as follows: The detailed discussion about the fabrication and characterization of CS-GO based biosensor is given in the experimental Section II. Various experiments and the analysis of results are presented in Section III. Finally, the key results are summarized in Section IV.

II. EXPERIMENTAL

A. Fabrication of CS-GO Film

The ultra-thin CS-GO film was prepared on cellulose acetate butyrate (CAB) film which acts as the sacrificial layer and prevents mechanical damage of CS-GO during fabrication peel off from the substrate. CS-GO solution was prepared by dissolving 2ml of aqueous solution of GO (ultra-high concentrated single-layer GO solution, Graphene Supermarket, 6.2 g/l) and 0.5 g of CS (high molecular weight, Sigma Aldrich Co., 3050, USA) in 50 ml of distilled water followed by 4.5 ml of 2% acetic acid. The solution was mixed using a magnetic stirrer at 1300 rpm for 12 hours at 40°C until the GO is well dispersed in dissolved CS. The prepared CS-GO solution was spin coated at 1000 rpm for 30 s on pre-coated CAB on Si substrate. A 5 wt.% CAB dissolved in ethyl-L-lactate which was used for pre-coating Si wafer was used as the sacrificial layer. The spin-coated CS-GO on CAB/Si was dried overnight before metallization. Subsequently, a thin layer of gold (Ti/Au) of thickness 10/30 nm was evaporated on the CS-GO substrate using a hard mask for the realization of the micro-electrodes. The CAB carrier substrate was dissolved by immersing the peeled sample in acetone for 30 min leaving the CS-GO substrate floating in the liquid. The floating CS-GO substrate bearing the array of micro-sensors was transferred to Si-wafer and subsequently rinsed with DI water, dried and prepared for cell culture. However, the ultrathin as-fabricated sensor array can also be transferred to substrates with a radius of curvature r> 500 µm for in vivo investigations as shown in Fig. 1d&e. The electrochemical investigations were performed on devices which were transferred on Si wafer. An illustration of the fabrication process steps is shown in figure 1.

B. Fabrication of Au Microgap Electrode

The microgap electrodes were fabricated on CS-GO substrate using nickel hard mask (Ossila Ltd). The Au for micro-gap electrode array was deposited on the CS substrate using an electron-beam evaporator. The fabrication process is illustrated in Fig. 1a and the optical image of the realized microgap electrodes with their thickness characteristics are shown in Fig. 1b, c. The dimensions of the deposited electrodes on the CS-GO film is 1mm² with a separation gap of 60µm. The micro-gap electrode modified substrate showed sufficient stability to cell culture. The fabricated chitosan substrate is thin (2.8µm) enough to conform to different shapes, allowing their use in in-vivo application.
C. RGD Functionalization of CS-GO Film

Establishment of cell on an artificial surface is a challenging step during the fabrication of a cell-based sensor [21]. Significant work is needed at cell-electrode interface to ensure firm adhesion during washing steps during analysis [22], [23]. To this end, several adhesion molecules of extracellular matrix (ECM) proteins such as collagen, fibronectin, and their peptide derivatives like poly L-lysine, RGD peptide etc. have been explored [24]. Though collagen and fibronectin offer good adhesion properties and create in vivo like environment on the artificial surface, their inhibitory roles in electron exchange phenomena at cell-electrode interface makes them unsuitable for application on electrode surfaces [25], [26]. On the other hand, it has been observed that RGD enriched portion of the ECM proteins is actively involved in the cell adhesion process [27]. Thus, RGD peptides have been utilized as nanoscale patterns at cell-electrode interface to enhance the cell adhesion and proliferation [21], [28]. Herein we have utilized cysteine terminated RGD peptide sequences for firm attachment of dermal cell on CS-GO based Au microgap electrode. The functionalization with RGD peptide is discussed elsewhere [21], [23], [28]. The cysteine terminated RGD peptide (Peptron, Daejeon-305-340, South Korea) was diluted in PBS (pH 7.4) and drop casted on a freshly prepared CS-GO based Au microgap electrode and allowed for self-assembly. After overnight incubation at 4°C the CS-GO platform was washed in fresh PBS and dried for morphological investigation.

D. Morphological Characterization

The surface morphology and thickness of fabricated CS-GO substrate was analyzed with Scanning Electron Microscopy (SEM) and stylus profilometer respectively. For SEM investigation the substrate was placed on silicon wafer and a thin layer of Au was sputtered on it prior to imaging. Images were obtained with field emission scanning electron microscope (Hitachi S-4700) at an accelerated voltage of 10KV and 10mA current. The profilometric analysis was performed using Bruker Dektak XT height profiler. For optical imaging the CS-GO was placed on glass slide and images were obtained from Nikon Eclipse LV100ND microscope connected with Leica MC170HD camera.

E. Cell Culture and Maintenances

The human dermal fibroblast (HDF) cells were purchased from GIBCO© and seeded at a density of 5×10³ cell/cm² in DMEM supplemented with 10% foetal bovine serum and 1% antibiotic and antimitotic and kept at 37°C in a standard cell culture incubator providing 5% CO₂ and 70% humidity. The cell was fed twice in a week and subculture performed when 100% confluences were attained. Cell from 3rd passage was employed for all experiments in this research.

F. Electrophysiological Investigation

For electrophysiological investigation, the Au microgap patterned electrodes on CS-GO substrate was seeded with HDF cell at a concentration of 1.5×10⁴ cell per device and kept at 37°C in a humidified atmosphere with 5% CO₂ for 48h. Then the device was washed with phosphate buffer saline (PBS, pH 7.4) and CV investigations were carried out with a standard potentiostat (Metrohm Autolab). All experiments were performed in triplicate using freshly prepared device at an identical condition.

G. Cell Proliferation Assay

 Viability of HDF cells attached on to microgap electrode was determined by studying the redox peak intensities. To this end, the cells were seeded on the batches of device and allowed to grow for 0, 24, 48, 72 and 96h. Electrochemical investigation was performed on representative samples from each time point. All the measurements were repeated at least three times and the error bars have been shown in the figure 6b and 8b.

H. Glucose Sensing

For glucose sensing application, batches of HDF immobilized sensor-patch were subjected to various doses of glucose and linear swipe voltammetry (LSV) measurements were performed after 24h post treatment. Glucose sensing performance was evaluated by analyzing and quantifying LSV peak intensities per doses of treated glucose concentrations. All the measurements were performed thrice at identical condition.

III. RESULTS AND DISCUSSION

A. Ultra-Thin CS-GO Substrate

The ultra-thin CS-GO layer was fabricated on silicon wafer by drop casting method (Fig. 1a). The fabricated CS-GO layer attains stability to ordinary solvent. This was achieved by crosslinking of chitosan monomer through interaction between its amine groups (–NH₂) and carboxyl (–COOH) group of GO [13]. This cross-linked CS-GO substrate allows successful metallization to fabricate Au microgap electrodes (Fig. 1b). The micro-gap electrode modified substrate showed enough stability for cell culture. The fabricated chitosan substrate is thin (2.8µm) enough to conform to any shape, allowing their use in in-vivo application (Fig. 1e). In addition, the developed CS-GO based Au microgap electrode employs biocompatible materials and eco-friendly fabrication processes and hence is compatible to living cells or tissues. To prove this, the HDF cells were immobilized on the CS-GO substrate and maintained for electrochemical investigation.

B. RGD Assisted Enhancement of Cell Adhesion

Prior to the HDF cell immobilization, the CS-GO substrate was functionalized with cysteine terminated RGD peptide to enhance the adhesion (Fig. 2a). The RGD nanodot immobilized CS-GO platform, shown in Fig. 2b, offer sufficient adhesion motif for cell anchoring. In this adhesion process, at one end thiol group of cysteine molecule is involved with thiol-gold coupling mediated adhesion with Au electrode. At other end is the RGD-integrin mediated adhesion with cell surface [29]. Thus, numerous adhesion motifs were shown...
on the cell immobilized sensor-patch (Fig. 2c). Whereas such adhesion motifs were absent when SEM images were obtained from non RGD functionalized sensor-patch (Fig. 2d). The HDF cell firmly adhered to RGD functionalized CS-GO substrate showed well spreading morphology and overcame dissociation with several washing steps during the fixation process prior to the SEM imaging [16], [30]. In the absence of RGD, the cells did not withstand such washing force and were dissociated from the electrode, as evident from SEM images shown in Fig. 2d. The Cys-RGD functionalized microgap electrode bridged 60 µm gap with HDF cell is shown in the inset of Fig. 3. This is critical for voltammetric analysis in this study. The nano scale RGD assisted cell adhesion and proliferation are established in the literature elsewhere [21], [22]. Besides this adhesion enhancement, the nanoscale RGD modified metal electrode platform has also showed the enhancement in the electrochemical readout signals [31]. Hence, the presented RGD modified bioplatform holds the promise for better electrochemical read out signals. To prove this hypothesis the electrochemical measurement was also performed.

C. Electrochemical Characterization of HDF Immobilized Substrate

The living cells possess distinct electrochemical redox at the cell membrane, which shows the cell line and cell cycle stage specificity [32]. This cell specific signal has been employed in several sensing applications such as environmental monitoring, toxicity analysis, drug effect study etc. [22], [23], [32]. Recently the cell cycle stage specific signals were also utilized to monitor the potential environmental toxicant in vitro [33]. Considering these background studies, we have hypothesized that this potential analytical method could be utilized to monitor the cell proliferation rates during wound healing process as well as to monitor the analytes in the wound fluid. In view of this, the HDF cell immobilized CS-GO substrate was subjected to electrochemical investigation using a potentiostat controlled with Auto lab software. A standard two electrode setup was employed for cyclic voltammetric (CV) measurement of cell that bridges the microgap electrode on the CS-GO platform to determine the redox potentials. The CV measurements were performed at scan rate of 50 mV/s with a potential window of +500 mV to −500 mV. The cell-immobilized platform was washed thrice with PBS and measurements were performed in PBS (pH 7.4) at room temperature. The CV obtained from cell cultured CS-GO substrate showed a quasi-reversible redox peak with cathodic peak (Ipc) at +300 mV and anodic peak (Ipa) at −300 mV (Fig. 4). Absence of such peak from a device without cell confirms that redox is originated from the immobilized HDF. The quasi-reversibility was confirmed with the peak potential difference ≥100 mV between Ipc − Ipa and the current ratio Ipc/Ipa is ≥1 [34], [35]. The stability of this potential peaks is an essential feature of an ideal sensor. Therefore, a freshly prepared sensor-patch was subjected to
run for 50 scan cycles at similar potential windows and scan rate to verify the stability. The redox peak showed stability to scan rates and scan cycle. There was no significant difference in the current peak up to 50-scan cycles (Fig. 5), indicating stability and repeatability of the electrical signals.

D. Electrical Read-Out to Monitor Cell Proliferation

Considering the potential specificity, stability and repeatability, the electrochemical redox signal of HDF cell was employed to monitor the proliferation in-vitro. For this, several batches of freshly prepared HDF immobilized CS-GO platform were employed for CV measurement at 24h interval and the cell proliferation was monitored over the growth period up to 96h. The CV peak intensities became prominent with increased cell growth period (Fig. 6a). Both \( I_{pc} \) and \( I_{pa} \) peak showed similar trend of enhancement without any peak shift. Hence, both peak values obtained from voltammogram could be considered as sensing values. Herein, a concentration dependent linear plot \( (R^2 = 0.981) \) was obtained between the current intensities \( I_{pc} \) and post seeding periods (Fig. 6a&b). The peak enhancement is due to the redox of increased cell number bridging the microgap electrode [31].

The cell immobilized platform was also evaluated with another analytical method namely LSV utilizing a wider potential window ranging from 0 to 3V at a scan rate of 50mV/s. This cell growth period dependent peak enhancement was also confirmed with LSV signals measured daily up to 5 days post seeding (Fig. 7). It is well known that living cells have distinct cell line and cell cycle stage specific redox property [15], [36]. Here, the peak is derived from the healthy cell that remained attached with electrode since dead cells are not able to bridge the gap [37]. Hence, the measured redox reflects the viable cell population only. The changes in the peak intensity reflect the state of cell health to any exogenous or endogenous influences on the cell viability. In the wound healing process, many critical steps such as bleeding, inflammation, proliferation and remodeling are also interrupted with many endogenous and exogenous influences [38]. Monitoring of such influences is critically required to predict the prognosis of a wound particularly when a healing patch or bandage is applied. The redox monitoring ability of the presented device could be employed for such purposes. The developed sensor-patch is capable of monitoring healing rate by analyzing and quantifying redox peaks obtained from cells of a proliferative and remodeling stage of a wound. Thus, the developed sensor-patch holds promise for assisting wound healing as well as monitoring healing progress on real-time.

E. Glucose Sensing Performances

The physiological levels of glucose in healthy individual are ranging from 3.0 to 6.0 mM [39]. The cells respond to any change in the glucose level beyond this limit. In particular, the excess blood glucose level of a hyperglycaemic patient interfere the wound healing process and can result into chronic wounds [40]. With this background, the developed
HDF immobilized sensor-patch was validated with various concentrations of glucose and electrochemical measurements were performed for the in-vitro diabetic condition. For this, LSV was employed to monitor the influence of various doses of glucose ranging from 1 µM to 20mM on freshly prepared batches of HDF immobilized sensors and the results are shown in Fig. 8a. The LSV signals from hypoglycaemic (0.001 to 0.01 mM) treated HDF cell immobilized sensor-patch showed no significant difference (with respect to non-treated). The concentration <1mM of glucose was utilized by the HDF cell and hence it did not influence the LSV signals. Whereas, the hyperglycaemic concentration (10-20mM) showed significant changes in LSV signals, particularly when the glucose concentration saturated the cellular demand and remained in the medium to produce the additional peak. This additional peak noticed from glucose concentration >1mM, was further enhanced with increased concentrations. This additional peak enhancement showed linear relation ($R^2 = 0.995$) with the increasing concentrations of glucose as shown in Fig. 8b. The cellular LSV peaks were also increased with the glucose concentration up to 10mM. However, the peak suddenly shifted without any enhancement at a concentration >10mM. This was obvious response from the cells with hyperglycaemic condition [41]. Such potential shift could be used for monitoring of any adverse effects of hyperglycaemic condition of a patient. Thus, the HDF immobilized sensor-patch proved to be useful for monitoring diabetic condition of a patient.

IV. CONCLUSION AND FUTURE WORK

A CS-GO based ultra-thin bio-platform is presented here for cell-health monitoring using a label free electrochemical method. To this end, an array of micro-gap electrodes was fabricated by depositing Au on CS-GO substrate and HDF cell was immobilized on the microgap. The CV of the cell-immobilized sensor shows a quasi-reversible redox with characteristic cathodic peak ($I_{pc}$) and anodic peak ($I_{pa}$) at +300mV, and −300mV, respectively. The intensities of both redox peaks were enhanced (1.923-11.195nA) with the growth period of HDF cell without potential shift. The changes in peak intensities reflect the numbers of healthy cell attached on the electrode (0-96h). A linear plot ($R^2 = 0.981$) derived from $I_{pc}$ values with post seeding period shows that the device is capable of monitoring cell health by analyzing and quantifying the redox peak intensities. Likewise, the linear plot derived from LSV values of hyperglycaemic condition ($R^2 = 0.995$ for 3-points & $R^2 = 0.924$ for 4-point) proved the glucose sensing performances of the sensor-patch. In addition, analysis and quantification of the potential shift in LSV signals could be utilized for monitoring cellular malfunctions at hyperglycaemic condition. Thus, the developed electrochemical sensor holds
promise for forecasting wound healing progress as well as monitoring diabetic condition on real time.

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Md. Abdul Kafi received the DVM degree and the M.S. degree in microbiology from BAU, Bangladesh, and the Ph.D. degree from Sogang University, South Korea. During the Ph.D. research, he developed cell-based sensor environmental monitoring with level-free electro-chemical methods.

Afterward, he received the Japanese Society for the Promotion of Science (JSPS) Fellowship for working at Kyushu University, Japan, where, he developed peptide functionalized chitosan scaffold suitable for hMSC adhesion and proliferation. Dr. Kafi received one of the prestigious Marie Curie Individual Fellowship (BEND-704807) under Horizon-2020 program of European Commission for worked as a Marie Curie Individual Fellow at the University of Glasgow, U.K., on BIOPOLYMER-based platform for biocompatible, biodegradable, and bioresorbable electronics for wearable/implantable applications. He is currently a Professor with the Department of Microbiology, Hygiene Bangladesh Agricultural University, Memnsingh, Bangladesh.

Ambarish Paul received the bachelor’s (Hons.) degree in physics from Calcutta University, Kolkata, in 2004, the M.Sc. degree in physics from Banaras Hindu University (BHU), Varanasi in 2006, the M.Tech. in advanced materials science and technology from the National Institute of Technology (NIT), Durgapur, in 2008, and the Ph.D. degree from the Indian Institute of Technology (IIT), Kharagpur in 2015.

He received the prestigious Newton International Fellowship from the Academy of Medical Sciences, The Royal Society, U.K., when he was with the University of Glasgow in 2016. His research interest includes physical and chemical sensors for environmental and medical applications.

Anastasios Vilouras (S’16) received the B.Sc. degree in physics from the Aristotle University of Thessaloniki, Greece, in 2014, and the M.Sc. degree in bioelectronics and biosensors from the University of Edinburgh, U.K., in 2015. He is currently pursuing the Ph.D. degree with the Bendable Electronics and Sensing Technologies (BEST) Group focusing mainly on CMOS-based bendable sensors for chemical and bio-chemical measurements.

He joined the Centre for Doctoral Training in Intelligent Sensing and Measurement (CDT-ISM), University of Glasgow, U.K., in 2016.

Ensieh Hosseini received the B.Sc. and M.Sc. degrees in chemistry from the Sharif University of Technology, Tehran, Iran, in 2009 and 2011, respectively, and the Ph.D. degree in nanoscience and nanotechnology from the University of Averio, Portugal, as a Marie Curie Early Stage Researcher in the NANO MOTION project in 2015. Her Ph.D. research focused on synthesis and investigation of new class of biopiezoelectric/bioferroelectric materials for various bioelectronic and biomechanical applications.

Afterward, she was a Post-Doctoral Researcher with the University of Glasgow and Strathclyde, U.K., and subsequently she received the prestigious Marie Curie Fellowship (ELECTROHEAL-based) from the University of Glasgow. Her current research focuses on fabrication of bioelectric dressing using bioelectroactive materials for acceleration of the wound healing process in the chronic wounds.

Ravinder S. Dahiya (S’05–M’09–SM’12) is a Professor of Electronics and Nanoengineering with the University of Glasgow, U.K. He is the Leader of the Bendable Electronics and Sensing Technologies (BEST) Research Group. His group conducts fundamental and applied research in the multidisciplinary fields of flexible and printable electronics, tactile sensing, electronic skin, robotics, and wearable systems. He has authored over 200 research articles, four books (including three in various stages of publication), and 11 patents (including seven submitted). He has led several international projects. He is a Distinguished Lecturer of the IEEE Sensors Council and is serving on the editorial boards of the Scientific Report and the IEEE SENSORS JOURNAL. He holds the prestigious EPSRC Fellowship and received the Marie Curie Fellowship and Japanese Monbusho Fellowship. Among several awards, he recently received the 2016 Microelectronic Engineering Young Investigator Award and the 2016 Technical Achievement Award from the IEEE Sensors Council. He was the Technical Program Co-Chair of IEEE Sensors 2017 and IEEE Sensors 2018.