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Graphene oxide-based biosensor for detection of platelet-derived microparticles: A potential tool for thrombus risk identification

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ABSTRACT

We report here design of a graphene oxide-based electrochemical biosensor for detection of platelet-derived microparticles (PMPs), a major risk factor for arterial pro-thrombotic pathologies like acute myocardial infarction and stroke. Electrodes were fabricated with immobilized layers of graphene oxide and a specific antibody targeted against active conformation of integrin $\alpha_{IIb}\beta_3$ on PMP surface. Results showed progressive rise in impedance in Nyquist plots with increasing number of PMPs in analyte. The sensor was highly specific for PMPs and did not identify microparticles originating from other cells. Blood obtained from patients diagnosed with acute myocardial infarction exhibited significantly higher values of impedance, consistent with larger number of circulating PMPs in these patients, as compared to samples from healthy individuals, thus validating biosensor as a specific, sensitive, label-free and cost-effective tool for rapid point-of-care detection of PMPs at bedside. Our biosensor is most ideal for mass population screening programs at periphery-level healthcare units with limited resources. It is aimed at early detection of individuals having higher imminent cardiovascular risk, as well as for routine analysis, which in turn would contribute to better management and survival of screened 'high-risk' subjects.

1. Introduction

Global status report of World Health Organization has categorized cardiovascular diseases, comprising of coronary artery disease, stroke, atherosclerosis and heart failure, as leading causes of death resulting in millions of casualties worldwide, followed by deaths due to diabetes (WHO, 2013). Although cardiovascular diseases affect people with advanced age, risk factors may appear relatively early (McGill et al., 2008). One such risk factor is circulating platelet-derived microparticles (PMPs) in blood of patients suffering from myocardial infarction or peripheral arterial diseases (van der Zee et al., 2006). Elevated plasma levels of PMPs have been associated with enhanced risk of coronary heart disease in healthy individuals (Ueba et al., 2010).

Platelet activation is central to the pathogenesis of arterial thrombosis. PMPs are membrane vesicles of less than 1 μm diameter released from stimulated platelets (Siljander, 2011). They play significant role in hemostatic response and their presence in circulation represents serious procoagulant risk (Nomura, 2001; Sinauridze et al., 2007; Skeppholm et al., 2012; Suades et al., 2012; VanWijk et al., 2003). Detection of PMPs at early stage of disease would aid in diagnosis, prevention and management of the pathology. Flow cytometry is the available technique for PMP detection (Lacroix et al., 2010) but is associated with major drawbacks that include underestimation of PMP count, lack of universal standardization, time consuming experiments, high cost of the equipment and need for skilled operator (Barteneva et al., 2013; Dey-Hazra et al., 2010; Leong et al., 2011; van der Pol et al., 2012). In contrast electrochemical biosensing stands far superior chance as an efficient and affordable tool for PMP detection.

'Biosensing' is an emerging concept based on amperometry, potentiometry and impedance analysis, which detects transduction of biological events to electrochemical signals with high sensitivity (Higgins and Lowe, 1987). Nanoscale particles of gold, iron and silicon, as well as graphene and carbon nanotubes have been employed as immobilization matrices during fabrication of electrodes (Chen and Chatterjee, 2013; Hakim et al., 2012; Kuila et al., 2011; Mandal et al., 2012). Material immobilized on exposed surface of electrodes determines its specificity, which include enzymes, antibodies, proteins, aptamers or nucleotides depending upon the nature of the target. Biosensors have been widely designed against molecules like glucose, ascorbic acid, uric acid, proteins, DNA etc. (Kanyong et al., 2012; Luppa et al., 2001; Oliver

Abbreviations: AMI, acute myocardial infarction; FITC, fluorescein isothiocyanate; GO, graphene oxide; PE, phycoerythrin; PMP, platelet-derived microparticle; PPP, platelet-poor plasma; PRP, platelet-rich plasma

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et al., 2009; Patel et al., 2010; Wen et al., 2012), but with advancement of fabrication techniques electrochemical sensing of individual cells like cervical cancer cells and microorganisms has been reported (Chandra et al., 2011; Deisingh and Thompson, 2004; Mandal et al., 2012; Yanik et al., 2010). Although there has been attempt to detect microparticles using electrochemistry, the method does not differentiate between their cells of origin and types (Lvovich et al., 2010) and thus has limited medical application. Here we describe a simple, quick, sensitive and cost-effective method to detect PMPs circulating in blood of individuals with potential for point-of-care diagnostic at peripheral health care system. The novelty of this product lies in its ease of fabrication and detection method, as no electrochemical sensor has yet been reported which could facilitate quick screening and diagnosis of individuals at 'high-risk' for developing cardiovascular diseases, eliminating requirement of high end lab facilities and experienced technicians as needed in current PMP detection procedures.

2. Materials and methods

2.1. Materials and reagents

Graphene oxide (GO) was purchased from Graphene Supermarket. Ethyl (dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA) and calcium ionophore A23187 were from Sigma. FITC-PAC1 and PE-annexin V and Trucount tubes were obtained from BD Biosciences. Ethanol, sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merck. Heparin was procured from Gland Pharma limited. Glassy carbon electrodes (GCE) (2 mm diameter) were the products of CH instruments.

2.2. Electrode surface modification

GCE were polished with alumina slurry and thoroughly cleansed. Colloidal GO was diluted with a mixture of Milli-Q grade (type 1 deionized, 18.2 MΩ cm, Millipore) water and ethanol (at ratio 2:5:1:: GO:water:ethanol, v/v/v). Four microliter of above mixture was dropped on each electrode and dried in desiccators. Dried GO layer was gently rinsed with phosphate-buffered saline (PBS, 0.1 M phosphate buffer, 0.9% NaCl, pH 7.2). For activation of carboxyl groups, 10 μL each of 100 mM (EDC) and 100 mM (NHS) were deposited over GO layer and incubated for 30 min. Following washing, 5 μL PAC1 antibody (stock concentration, 25 μg/mL; diluted 1:10 with PBS) was dropped on each GO-fabricated electrode and incubated for 1 h. Electrodes were gently rinsed with PBS, blocked with 2% BSA for 30 min (Sanchez et al., 2007; Tran et al., 2012; Wang et al., 2013) inside humid chambers at room temperature, and finally carefully rinsed with Milli-Q water. Electrodes were stored at 4 °C till further use. All incubations were carried out at room temperature under normal ambient illumination in desiccators.

2.3. Sample preparation

Antecubital venous blood was collected from healthy volunteers, as well as patients diagnosed with acute myocardial infarction (AMI) characterized with ST-segment elevation in electrocardiogram, under informed consent as per the recommendations of the Institutional Ethical Committee. Blood was anticoagulated with heparin

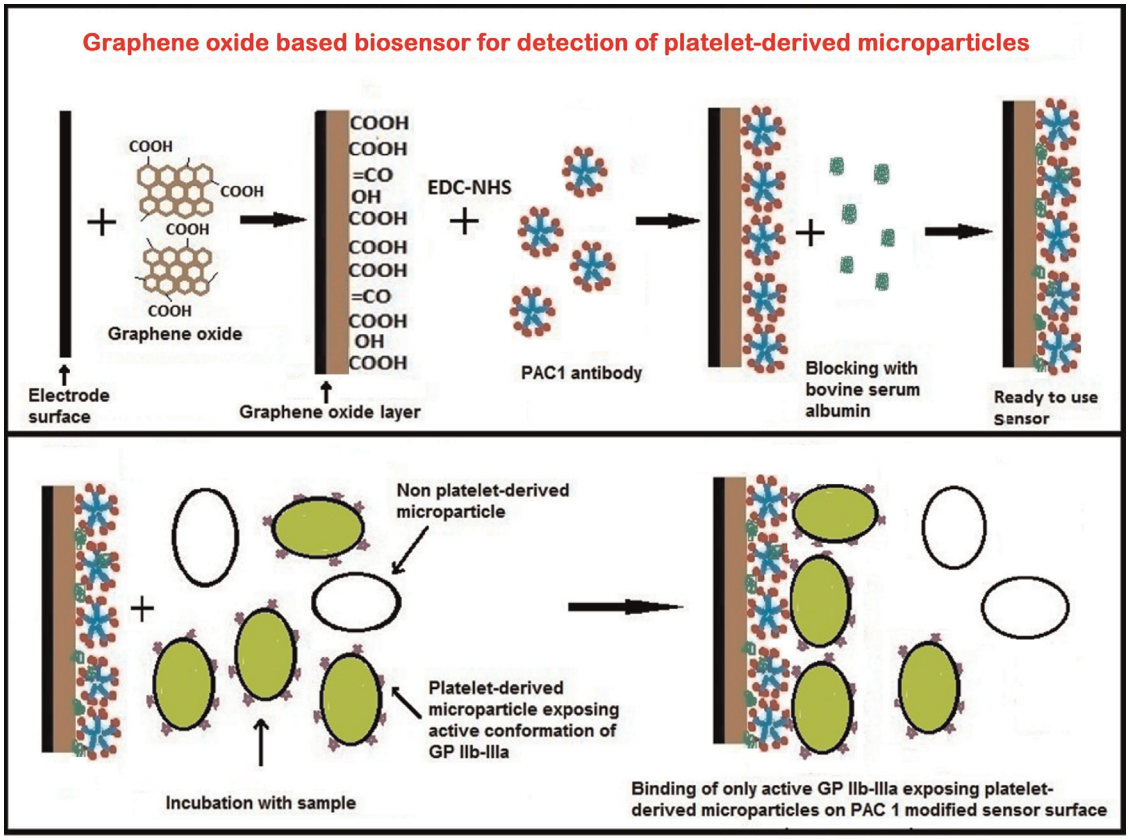

Fig. 1. Schematic design of nano-biosensor for detection of PMPs depicting stepwise immobilization of GO and PAC1 antibody on electrode surface. Subsequent incubation of coated electrode with sample resulted in binding of platelet-derived microparticles, bearing active conformation of integrins $\alpha_{IIb}\beta_3$, to the sensor surface, which can be detected by impedance analysis.

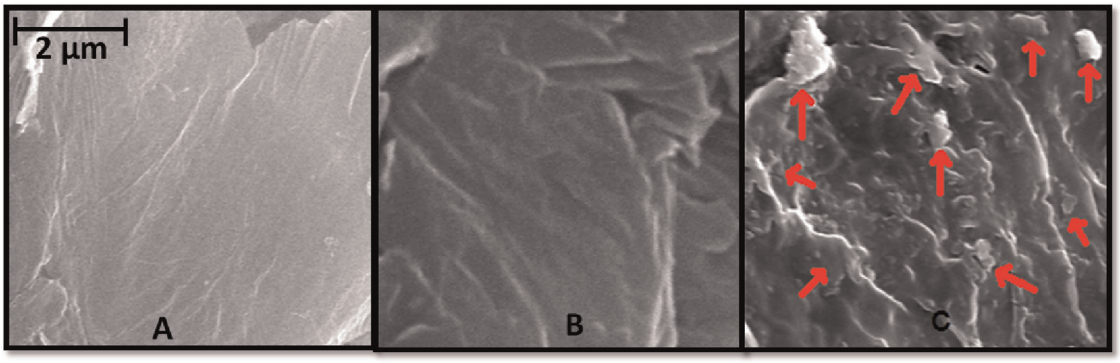

Fig. 2. Scanning electron micrographs of immobilized layers on sensor surface. A, GO; B, GO-PAC1 antibody; C, GO-PAC1-PMPs. Panel C clearly exhibits bound PMPs (marked by red arrows) over modified sensor surface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

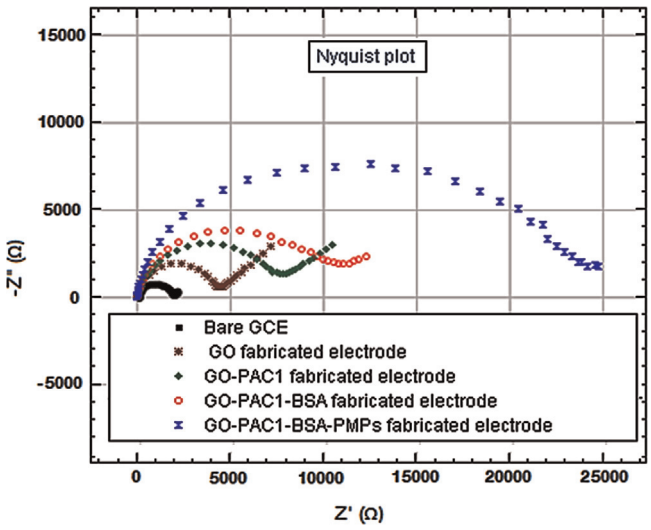

Fig. 3. Impedance analysis after each step of sensor fabrication. Nyquist plots exhibit rise in impedance after successful stepwise immobilization of GO, PAC1 and PMPs on sensor surface.

(1 IU/mL) and centrifuged at 200 g for 10 min to acquire platelet-rich plasma (PRP). Platelets in PRP were pelleted down at 800 g for 10 min to obtain platelet-poor plasma (PPP), which was subjected to another short spin (12,000 g for 1 min) to eliminate possibility of platelet contamination.

2.4. Generation of PMPs

Platelets were induced to shed microparticles by treatment of PRP with 1 µM A23187 at room temperature for 30 min in the presence of 2 mM $CaCl_2$. Platelets were then sedimented and PPP containing microparticles was separated as described above.

2.5. Flow cytometry

For microparticle labeling, PPP samples (100 µL each) were incubated either with FITC-PAC1 or PE-annexin V (8 µL each), as indicated, for 30 min in dark, and then diluted with 300 µL FACS sheath fluid. At time of analysis, appropriate gate in dot plot was drawn to differentiate microparticles from noise and fluorescence data were collected using four-quadrant logarithmic amplification with flow cytometer (Becton Dickinson FACSCalibur) using CellQuest Pro software. Fluorescence positive events were collected by running each sample under 488 laser excitation and mean fluorescence intensities were recorded. Absolute counting of PMPs in PPP samples was carried out using BD Trucount tubes following recommended standard protocol. Briefly, PPP was incubated with FITC-PAC1/PE-annexin V (that bind with microparticles) and

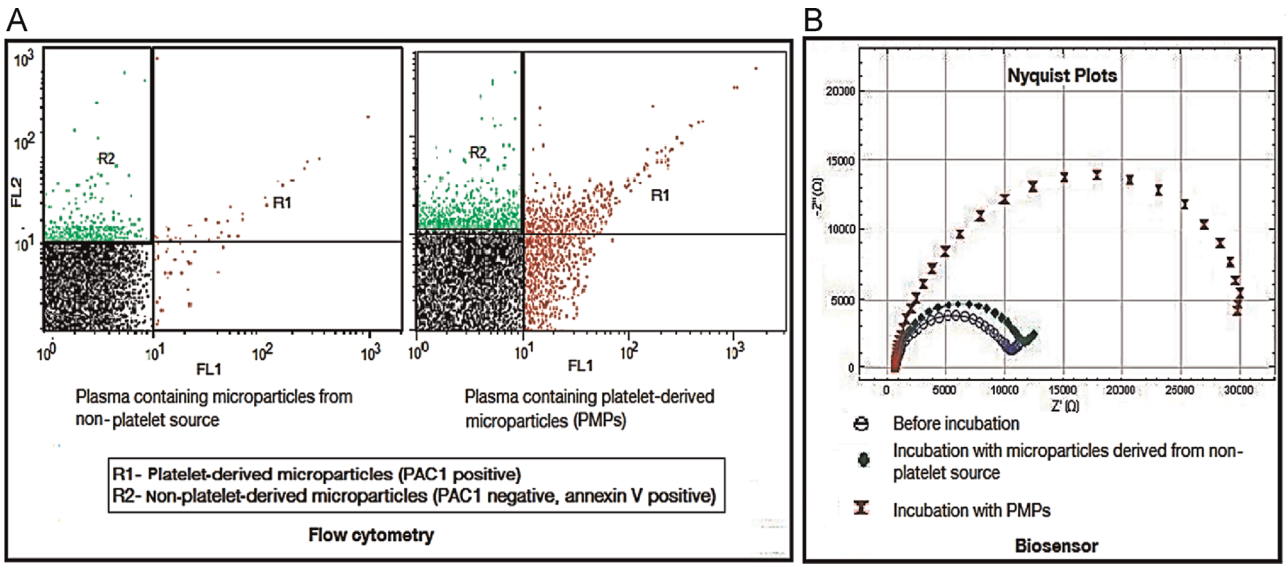

Fig. 4. Detection of PMPs in plasma by flow cytometry (panel A) and electrochemical biosensing (panel B).

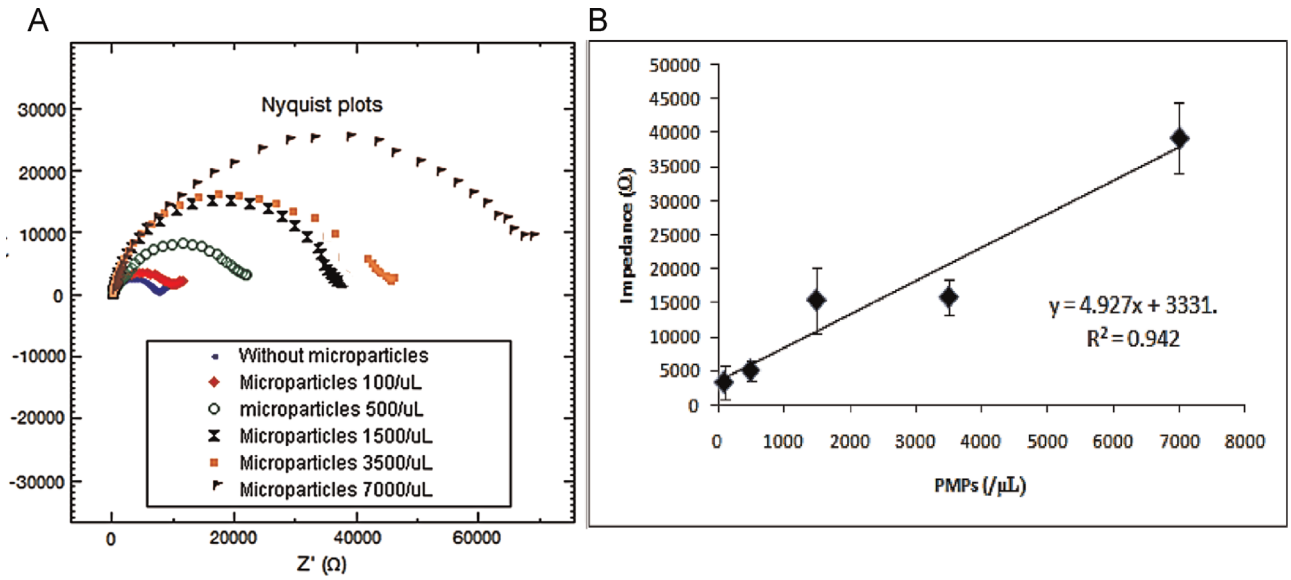

Fig. 5. A, Nyquist plots in presence of increasing number of PMPs; B, standard dose-response relationship between PMP number and impedance (average of repeat experiment results) with standard deviations.

known number of fluorescent beads inside the Trucount tubes and subjected to flow cytometry. Microparticle number was calculated by comparing microparticle-associated fluorescence events to bead events.

2.6. Electrochemical analysis

Electrochemical impedance spectroscopy was performed on Autolab potentiostat-galvanostat model PGStat 302NFRA2 (Metrohm Autolab B.V, The Netherlands) employing a three-electrode system (modified GCE as working electrode, platinum wire as counter electrode and Ag/AgCl as reference electrode) operated through software Nova 1.7. Microparticle suspension (15 μL) was dropped on the fabricated working electrode. After short incubation for 15 min, electrode was gently rinsed with type 1 deionized water and electrochemical analysis was carried out in presence of the electrolyte (5 mM K_3FeCN_6 in PBS). Electrodes were scanned for 50 points within frequency range 0.1–10,000 Hz at 0.2 V (half-wave potential of the mean of the anodic and cathodic peak potentials obtained from cyclic voltammograms of electrolyte solution used) with 10 mV AC signal amplitude and Nyquist plots were obtained. Parameters were optimized by repeated experiments to acquire stable system. Impedance values were measured from Nyquist plots of electrodes at different stages of preparation. Each experiment was repeated for at least 3–5 times.

3. Results and discussion

3.1. Confirmation of successful electrode surface modification and binding of PMPs

Schematic representation of nano-biosensor analytical system for detection of PMPs is shown in Fig. 1. GO was employed as the matrix as it can be easily functionalized while providing stable and thin conductive layer on electrode surface. PAC1 antibody was immobilized on GO to make the electrode target-specific, as PAC1 exclusively binds to active conformation of integrins $\alpha_{IIb}\beta_3$ (glycoproteins IIb–IIIa) present on the surface of activated platelets as well as PMPs (Lecut et al., 2004; Tomer, 2004). Fabricated electrodes were characterized by scanning electron microscopy. Fig. 2 shows electron micrographs of successively immobilized layers of GO (panel A), PAC1 antibody (panel B) and PMPs (panel C) on electrode surface during different stages of electrode fabrication. Frequency response analysis (FRA) following each step of sensor fabrication exhibited progressive rise in impedance in Nyquist plots suggestive of successful immobilization of the layers on sensor surface, where as incubation with PMPs was associated with drastic rise in impedance (Fig. 3).

In addition to negligible presence of platelet-derived microparticles, plasma possesses circulating microscale vesicles derived from non-platelet sources (RBCs, endothelial cells, leukocytes and megakaryocytes) (Barteneva et al., 2013; Cui et al., 2013; Jy et al., 2013; Lacroix et al., 2013). To test whether our sensor exclusively detects microparticles derived from platelets only and not from other originating cells in the physiological fluid, platelet-poor plasma (PPP) was subjected to analysis by flowcytometry. Platelets in PRP were stimulated to shed PMPs by treatment with A23187 (1 μM). Cells were then sedimented down to obtain supernatant (PPP) enriched with PMPs. In control experiments A23187 was replaced with dimethylsulfoxide (DMSO) (Gyulkhandanyan et al., 2013). Presence of microparticles in plasma was validated flow cytometrically by dual staining with annexin V-phycoerythrin (PE) (binds globally to microparticles from diverse cellular origins including platelets) and PAC1-fluorescein isothiocyanate (FITC) (binds exclusively to PMPs). Flow cytometry data were consistent with the presence of PMPs (PAC1-positive particles, shown as red dots in Fig. 4, panel A) in sample pre-treated with A23187 and not in vehicle (DMSO)-treated counterparts, while both the samples possessed microparticles derived from non-platelet sources (PAC1-negative, annexin V-positive, shown as green dots in Fig. 4, panel A) in circulation.

3.2. Specificity of sensor

In order to test sensor specificity, PPP, either with or without platelet-derived microparticles, was added onto PAC1-fabricated electrodes, and subjected to FRA (Fig. 4, panel B). Strikingly, electrodes exposed to control PPP exhibited hardly any change in impedance in Nyquist plots, whereas the PPP sample possessing platelet-derived microparticles evoked dramatic rise in impedance, indicating that sensor surface was able to exclusively bind and detect PMPs having active conformation of integrins $\alpha_{IIb}\beta_3$ on their surface. Minor PAC1-positive signals in control

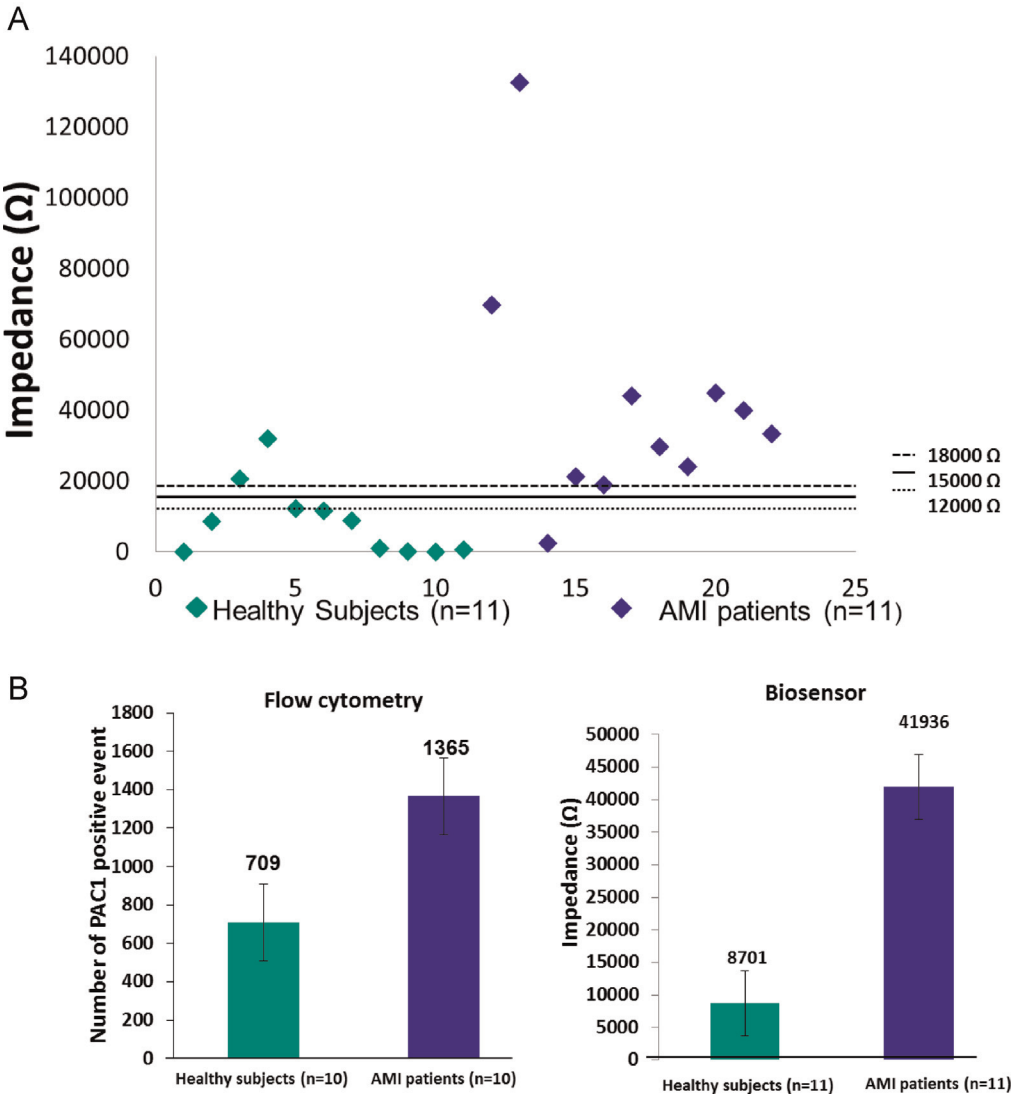

Fig. 6. A, Biosensor-based analysis of circulating PMPs in healthy subjects and in patients with AMI. The solid straight line parallel to *X* axis represents the cut off value of 15,000 Ω, while the dashed lines represent 20% above and below the threshold (18,000 and 12,000 Ω, respectively). Majority of healthy subjects revealed impedance below ~12,000 Ω, while most of the AMI patients had values greater than 18,000 Ω, indicative of higher number of circulating PMPs. B, PMP detection by both flow cytometry and biosensor in healthy subjects as well as AMI patients. The numbers represent the mean values.

samples could be due to microparticle release from platelets activated during preparatory stages or residing in healthy donor's blood in insignificant low counts. It may be noted that, electrochemical sensing exhibited remarkably higher sensitivity and specificity towards PMPs compared to flow cytometry and obviated the need for fluorescence staining/labeling of the particles.

3.3. Absolute count of PMPs and dose-dependent response of sensor

For the proof-of-concept sensor application, PPP enriched with platelet-derived microparticles were prepared as above. Absolute counting of PMPs was carried out by flow cytometry using BD Trucount tubes. PPP samples with known number of PMPs were incubated over fabricated electrodes. Changes in impedance were evaluated from Nyquist plot (Fig. 5A), which demonstrated linear relationship between microparticles (in range from 100 to 7000 per μL) and impedance (Fig. 5B). The values in Fig. 5B (*Y* axis) were derived by subtracting the impedance results without microparticles from the same obtained in presence of indicated number of microparticles. It may be mentioned here that flow cytometry has its own limitations in counting PMPs, which may explain high variability in impedance values. We also recommend repetitions for building analytical curve as standard deviations are high for lower concentrations of PMPs.

3.4. Test of sensor for clinical application

Next we asked whether our proposed biosensor can be applied for point-of-care testing in routine clinical practice. We examined blood from patients ($n=11$) diagnosed with AMI (exhibiting ST segment elevation in electrocardiogram) for the presence of PMPs and compared the results with samples obtained from matched healthy controls ($n=11$). As reported earlier (Stepien et al., 2012; van der Zee et al., 2006), significantly higher number of circulating PMPs was detected in patients of AMI by flow cytometry. The graphene oxide-fabricated biosensor, too, successfully identified greater presence of PMPs in blood from AMI patients compared to the healthy counterparts (Fig. 6A). Impedance never rose beyond 15,000 Ω in 80% healthy individuals, while majority of AMI patients consistently exhibited higher values even up to 1,35,000 Ω, suggestive of greater number of PMPs in circulation (Fig. 6A). Higher impedance in few apparently healthy individuals might be

indicative of imminent cardiovascular risk, as they reflected activated platelets in circulation capable of shedding microparticles. Based on this report the 'high-risk' group should be isolated and subjected to further specific investigations as well as preventive measures, and may be kept under prolonged observation. Individuals with lower impedance may thus be declared 'safe'. Fig. 6B represents comparison of mean values and standard deviations of PMPs detected through flow cytometry and biosensing, which underscores the fact that biosensor is endowed with greater specificity and sensitivity as a screening method for early detection of 'high-risk' individuals, which in turn would contribute to better management and survival of subjects.

4. Conclusions

Many complicated sensor fabrication methods have been described previously, but in this work our main objective was to find a simple method with easy available reagents and techniques. Graphene oxide and PAC1 antibody fitted best to the criteria, and electrochemical impedance analysis was easiest to perform with good results. The graphene oxide-based biosensor offered many advantages over conventional flow cytometry in detection of PMPs in clinical samples. It requires no expensive instruments, fluorescence labeling or laboratory setup, is quick, sensitive, cost-effective and easy to operate. This method can be applied on spot/bedside by patient himself or care provider. We believe that biosensing will prove highly expedient at peripheral health care level as a screening method to identify individuals at high risk to develop coronary artery diseases, which include people having positive family history, history of hypertension, diabetes mellitus, and smoking habits or sedentary life styles. The nano-biosensor design can be easily fabricated over screen printed electrodes as well and devised as a ready-to-use kit with commercial scope and ready availability.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.10.056.

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