Supplementary Information

Graphene oxide – metal nanocomposites for cancer biomarker detection

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Schematic S1. The pie diagram shows available components of BP with % distribution.



Figure S1. Histogram plot of lethal dose (LD) 50 for various compounds such as (1) G-Pd@rGO (2) G-Pt@rGO (3) G-Ag@rGO, (4) G-Au@rGO and GO.



Figure S2. (a-b) TEM images of green reduced GO (G-rGO).



Figure S3. Pictorial representation of GO, metal salts-GO interactions, and mNPs@rGO sheets interactions.



Figure S4. TEM images of chemical reduction nanocomposites such as C-Pd@rGO (a), C-Pd@rGO (b), C-Ag@rGO (c), and C-Au@rGO (d)



TEM Studies

TEM studies were used to unravel the morphology, shape, and size of the green-reduced GO (G-rGO), metal NPs, and metal-rGO composites [Fig. 1]. As described in Fig. S3, the migration of metal atoms to the most stable sites (B-, T-, and H-sites) on rGO is known to depend on the migration energy (i.e. the energy required for transfer from one site to another) (Chan et al., 2008). HRTEM images of G-Pd@rGO are shown in Fig. 1 (images b-c). After simultaneous reduction of the Pd salt in the GO sheets, the PdNPs perhaps uniformly spread on the G-rGO surface. The most stable sites for the absorption of PdNPs are the B-sites that arise due to the favorable adsorption energy of 1.9 eV. After nucleation of PdNPs, the atoms may move to other B-sites of the rGO *via* migration (migration energy of Pd atom is 0.06 eV) (Chan et al., 2008).

The images [Fig. 1(d and e)] show the results of HRTEM investigations of G-Pt@rGO composites. The different components of BPE are perhaps responsible for the observed different shapes of PtNPs. Simultaneous green reduction of the Pt salt and GO introduces different oxygenated groups into GO providing nucleation site for uniform growth and distribution of PtNPs. Similarly, PtNPs adsorb at the most stable B-sites of rGO sheets and selectively grow on the rGO surface due to the opposite charges of Pt⁴⁺ ions and the oxygen groups of the GO (Fig. S1). The oxygen groups of GO may be attached to Pt⁴⁺ ions either before or after reduction. The migration energy required to move to the B-site is reported to be 0.19 eV for Pt atoms at the rGO surface, whereas the adsorption energy is found to be 2.9 eV, which is higher than that of Pd atoms (Nakada et al., 2011). This may influence the density of nanoparticles on the G-rGO sheet. Some of the PtNPs perhaps interact at the edges of rGO sheets due to presence of the strong electrostatic interaction between the COOH group and Pt ions.

Fig. 1(f-g) shows results of the HRTEM studies conducted on G-Ag@rGO composite after simultaneous green reduction of Ag salt and GO. The AgNPs (size: ~10 nm) are uniformly distributed and attached to the G-rGO sheets. It appears that multilayered GO sheets provide T-sites as the most energetically favored position for stabilization of the Ag NPs after nucleation of Ag seeds(Nakada et

al., 2011). The Ag atoms may adsorb at T-sites of the rGO surface (Schematic S1) due to the low adsorption energy (0.4 eV) and migration energy (0.01 eV).

Fig. 1(h and i) show different 3D structures including triangular, spherical, and hexagonal shapes of AuNPs (image h) after green reduction of Au salt. Anisotropic growth during the nucleation phase may result in formation of these structures due to the presence of specific phytochemicals in BPE. Observed 3D structures of Au NPs on G-rGO sheets are shown in image (i) where AuNPs are attached to rGO sheets. It is apparent that Au NPs are adsorbed at the T-sites of rGO after growth of the NPs at the nucleation sites of G-rGO. The calculated absorption and migration energies in this case are 0.8 eV and 0.03 eV, respectively, which are higher than those for Ag (Nakada et al., 2011). Electron microscopic studies indicate that G-rGO is a suitable candidate for growth of metal NPs since it results in the homogeneous and high-density distribution of mNPs.

Figure S5. EDX spectra for G-Pd@rGO (i), G-Pt@rGO (ii), G-Ag@rGO (iii) and G-Au@rGO (iv) on the surface of indium tin oxide (ITO) coated glass electrodes, while insets show the % atomic and % weight ratio of metal (Pd, Pt, Ag and Au) elements with carbon (C) and oxygen (O).



Energy-Dispersive X-ray Spectroscopy (EDX) Studies

Elemental analysis of various G-mNPs@rGO composites was conducted to evaluate atomic/weight percentages. The EDX spectra of G-Pd@rGO composite indicates 53.3 % coverage by PdNPs on the G-rGO surface (Fig. S5). The ratio of carbon to oxygen species (C/O) is estimated to be 0.78 for the G-Pd@rGO composite. The weight percentages of carbon, oxygen, and Pt were determined to be 53 %, 23 %, and 24 %, respectively. The value of C/O that accounts for the presence of increased numbers of Pt particles at the G-rGO surface compared to that of Pd NPs, was estimated to be 2.2. The

presence of smaller amounts of Ag (17.7 % weight percentage) indicates some coverage on G-rGO surface with Ag NPs. In case of Au NPs, a weight percentage of about 60.6 % is obtained: perhaps indicating that increased numbers of Au NPs interact with G-rGO compared to the other NPs (Pt, Ag, and Pd). The C/O value for G-Au@rGO composite was 1.6, which is higher than that of the G-Ag@rGO composite (1.53).

FTIR Studies

To confirm the formation of green derived noble metal, rGO, and G-mNPs@rGO, Fourier transform infra-red (FT-IR) [Fig. 2]. The results of FT-IR studies reveal the presence of functional groups in chemically exfoliated graphite, natural product derived rGO nanosheets and BPE [Fig. 2(i and ii)]. The IR spectra for BPE (curve a) exhibits characteristic peaks at 1026, 1395, 1593, 2928, and 3552 cm⁻¹. An absorption peak seen at 1026 cm⁻¹ corresponding to the C-O-C stretching confirms the presence of piperine, piperolein B, and piperettine in BPE as this functional group is present in the components. The peak at 1395 cm⁻¹ pertains to the amine (C-N) stretch in piperaide. The stretching of hydroxyls gives rise to the peak at 3552 cm⁻¹ in BPE. In GO, the bands seen at 1614.2 and 1416.2 cm⁻¹ corresponding to C=C stretching and O-H bending (carboxyl group), respectively, are seen.

The small peak found at 1728.9 cm⁻¹ is due to the C=O stretch in GO. The absorption peaks at 1082.7 and 3289 cm⁻¹ are assigned to the C-OH and O-H stretching. In green synthesized G-rGO, the peak appears at 1586.2 cm⁻¹ due to the O=C-N stretch resulting from BPE coating of the GO surface. The peak at 1381 cm⁻¹ (due to C-N stretching in piperaide) is slightly shifted and the intensity of this peak decreases compared to that found in BPE. This may be due to excessive washing after synthesis of G-rGO. The intensity of the peak at 1416.2 cm⁻¹ reflects decreased numbers of O-H groups (e.g. carboxyl group) at the GO surface. This result indicates the conversion of exfoliated GO to G-rGO. After nanocomposite formation with metal (Pd, Pt, Ag, and Au) nanoparticles, the peak positions at 1614.2 and 1235 cm⁻¹ arising from functional groups (C=C and C-N) remain unaltered. However, the additional peak seen at 1318 cm⁻¹ in case of the G-Ag@rGO composite arises due to residual nitrite

ions (NO₃⁻) from the AgNO₃ salt. A small peak at 1046 cm⁻¹ due to C-O stretching is observed in the composites [Fig. 2(b)]. This result suggests the conversion of exfoliated GO to G-rGO.

Raman Studies

Raman spectroscopic studies were utilized to investigate the green reduction of GO [Fig. 2(c)]. The disorder (D)-band seen in GO corresponds to the E_{2g} phonon of sp² atoms; the G-band is known to be the breathing mode of the k-point phonon (A1g symmetry). The peak position of the D-band associated with the graphite edges of GO is obtained at 1340 cm⁻¹. The intensity of the D band in GO is found to be lower than that of G-rGO, and the ratio of I_D/I_G for GO is estimated to be 0.84. The peak position of the D-band in G-rGO is located at the same frequency. The in-phase vibration of the graphite lattice (G-band) in GO and G-rGO is observed at 1583.4 cm⁻¹ and 1579.5 cm⁻¹, respectively. The peak due to the G-band in the Raman spectrum of G-GO is found to be moved to a lower frequency by 2.9 cm⁻¹ suggesting efficient reduction of the functional groups in the GO using the green chemistry approach [Fig. 2(c)]. The double vacancy (C_2) is known to be one of the most stable states arising due to arrangement of the two pentagonal rings and an octagonal ring resulting in a 5-8-5 defect. In addition, the presence of the Stone-Wales defects (5-7-7-5 rings) in G-rGO may lead to improved performance if incorporated into an electrochemical biosensor (Terrones et al., 2012). Green reduction of GO may also be responsible for the observed defects or carbon vacancies (Terrones et al., 2012). The appearance of the 2D peak may be assigned to defects occurring on green reduction of GO. The crystallite size (L_a) of GO was estimated before and after green reduction from the integrated intensity ratio I_D/I_G using Eq. (1) (Cancado et al., 2006).

$$L_a(nm) = (2.4 \times 10^{-10}) \lambda_l^4 \left(\frac{I_D}{I_G} \right)^{-1}$$
(1)

where, λ_l^4 is the laser line wavelength (514.5 nm).

Figure S6 UV-visible studies for various metal salts including (Pd, Pt, Ag and Au), BPE, unreduced GO, G-rGO and mNPs@rGO composites such as (a) G-Pd@rGO, (b) G-Pt@rGO, (c) G-Ag@rGO and (d) G-Au@rGO.



UV-Visible Studies

We conducted the UV-visible spectroscopy measurements at various steps during green synthesis of G-mNPs@rGO composites in order to investigate the nucleation and growth of metal NPs ar rGO sheets. The UV-visible studies further confirm simultaneous, *in situ* reduction of metal salts (Pd, Pt, Ag, and Au) on GO sheets [Fig. 3(d) and Fig. S6].

The peak seen at 278 nm [Fig. S6(a)] is due to presence of the various components (piperine, piperolein B, etc.) in BPE. Fig. S6(a) shows the growth mechanism of PtNPs in the G-rGO sheets. The bare Pt salt and GO show an absorption peak at 377 nm before the chemical reaction. The disappearance of the Pt salt peak and appearance of G-rGO peak indicate the formation of PtNPs on simultaneously rGO sheets after green reduction. Similarly, the absorption peaks at 305 nm and 415 nm of GO-Pd salt found prior to green reduction [Fig. S6 (b)] disappear during G-Pd@rGO nanocomposite formation. However, the small peak found at 270 nm is due to conversion of GO to rGO in the nanocomposite [Fig. S6(b)]. The absorption peaks observed due to growth of AgNPs in G-rGO sheets are shown in Fig. S6(c). No peaks are seen in the Ag salt and GO-Ag salt prior to green reduction [Fig. S5(c)]. A broad absorption peak at 453 nm appears due to the plasmonic properties of Ag nanoparticles produced in the formation of G-Ag@rGO composite. The characteristic peak of rGO (270 nm) is found to be shifted to a lower wavelength in case of the G-Ag@rGO [Fig. S6(c)].

The absorption spectrum of GO-Au salt has been investigated before and after green reduction [Fig. S6 (d)]. The 278 nm peak may be due to presence of BPE in GO-Au salt. Green reduction of GO-Au salt shows two peaks at 270 nm and 539 nm arising from the formation of G-rGO and AuNPs, respectively.

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Figure S7. Size distribution of synthesis (a) GO, (b) G-rGO, (c) G-Ag@rGO, (d) G-Au@rGO, (e) G-Pd@rGO and (f) G-Pt@rGO.

Dynamic Light Scattering (DLS) Studies

Dynamic light scattering (DLS) measurements were performed to measure the size of the synthesized GO and its green derivatives [Fig. S7 (Supplementary Information)]. The hydrodynamic size (radius) of GO was measured as 308 nm, whereas after reduction using BPE it was 742 nm suggesting that the BPE not only acted as reducing and stabilizing agent but also provided a coating (green corona) on the G-rGO surface. Enhancement of particle size of G-rGO using biological reduction has been reported in the literature (Gurunathan et al.,

2013; Wang et al., 2012). The hydrodynamic size of the other green derivatives (G-Pd@rGO, G-Pt@rGO, G-Ag@rGO, and G-Au@rGO) was found to vary: 618.9, 575.9, 403.3, and 547.4 nm, respectively.

References

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Figure S8. Electrochemical impedance spectroscopic plots of the prepared electrodes are conducted in phosphate buffer saline (pH 7.4, 50mM, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}(5mM)$ at bias potential 0.01 V.



Figure S9. Impedance spectra foranti-ErbB2 functionalized G-Pd@rGO/ITO (a), G-Pt@rGO/ITO (b), G-Ag@rGO/ITO (c) and G-Au@rGO/ITO (d) immunosensors as a function of ErbB2 concentration (1.0 fM-0.5 μ M).



Figure S10. Impedance spectra for anti-ErbB2 functionalized C-Pd@rGO/ITO (a), C-Pt@rGO/ITO (b), C-Ag@rGO/ITO (c) and C-Au@rGO/ITO (d) immunosensors as a function of ErbB2 concentration (1.0 fM-0.5 μ M).



Kinetic Analysis

The binding kinetics of antibody-antigen interactions were analyzed using Hill plots as [Fig. 4(f)] (Segel, 1976). The reaction between the ErbB2 and anti-ErbB2 immobilized on the surface of mNPsrGO composites can be expressed as $nErbB2 + antiErbB2 \Leftrightarrow ErbB2$ antiErbB2 n, where n is the number of binding sites (anti-ErbB2) that react with ErbB2 at the immune electrode surface. The association constant (K_a) represents the binding of ErbB2 with anti-ErbB2. The dissociation of ErbB2 and anti-ErbB2 represented by the K_d value of the fabricated device and is given by $K_d = [ErbB2]^n [antiErbB2]/[antiErbB2n]$. The degree of binding can be determined by slope of the Hill plot, also known as Hill coefficient (n) and represents the protein binding cooperativity. The value of K_d can be calculated from the ordinate intercept of the Hill plot, whereas its inverse yields the value of association constant (K_a) and can be expressed as

$$\log \frac{Y}{1-Y} = \log \frac{1}{K_d} + \text{nlog}[\text{ErbB2}]$$
 Eq.(2)

where Y is the sensor response/maximum response of the sensor calibration curve. The Hill plot is Y

obtained by plotting log of $\overline{1-Y}$ verses log of ErbB2 concentration. The value of K_d follows the order: G-Ag@rGO > G-Au@rGO > G-Pt@rGO > G-Pd@rGO for green and chemically derived composites. The different mNPs on rGO sheets of various shapes and sizes have different affinities for proteins leading to the variation in binding constants. The higher dissociation constant of AgNPs@rGO is due to highly negatively charged AgNPs@rGO (-19.0 mV) composites (Table S2). However, in case of the AuNPs on rGO sheets, the variability in shape (spherical, triangular and hexagonal) leads to higher dissociation constants compared to PtNPs@rGO and PdNPs@rGO composites (Table S2).

Stability and Selectivity Tests

The stability of the G-mNPs@rGO based bioelectrodes was determined by measuring R_{ct} values at regular intervals. Among the various electrodes, the G-Au@rGO based immunoelectrode was found

to exhibit maximum stability of up to 75 days with 93% of the initial response. The enhanced stability of the fabricated bioelectrode is assigned to corona-assisted immobilization of anti-ErbB2 on the G-mNPs@rGO composites. These G-mNPs@rGO biosensors exhibit good reproducibility for detection of cancer biomarker (ErbB2). These antibody-conjugated G-mNPs@rGO composites show negligible interference with normal concentrations of human IgG, cholesterol, PSA, and glucose, i.e., good selectivity.

	Factor	PdNPs	PtNPs	AgNPs	AuNPs	
1	SMaterials	5 nhWHM(cm ⁻¹)	15 nm RWH	M(om n)	Beaknposition (cm ⁻¹	⁻¹)
2	_{Shap} မြှု	Spherica ^{1,6} 3D	3D topped and 1	455herical	Spherica ^{1,583}	
_	G-rGO	top shaped	other 3D	14	Triangular,79.5	
			irregular		hexagonal	
3	Charge on composite (zeta potential)	-14.7 mV	-16.4 mV	-19.0 mV	-2.31 mV	

Table S1 The peak positions of G-band, left and right widths at half-maximum.

Table S2. Size, shape and zeta potential for nanoparticles and synthesis composite composites

Table S3. Various electrochemical parameters obtained for different electrodes.

Electrodes	Peak	Electrochemical	Diffusion Co-	HET rate constant	Time constant
	Current	Area	efficient	(cm/s)	(τ) s
		(cm²)	(cm²/s)		
GO	435 μA	0.498	3.35 ×10 ⁻⁵	1.59 ×10 ⁻³	0.065
G-rGO	462 μA	0.544	3.88 ×10 ⁻⁵	1.76 ×10 ⁻³	0.081
G-Au@rGO	656 μA	0.811	7.73 ×10 ⁻⁵	2.40 ×10 ⁻³	0.08
G-Ag@rGO	689 µA	0.846	8.52 ×10 ⁻⁵	2.80 ×10 ⁻³	0.12
G-Pt@rGO	547 μΑ	0.648	5.26 ×10 ⁻⁵	2.25×10 ⁻³	0.103
G-Pd@rGO	519 μA	0.622	4.94 ×10 ⁻⁵	1.99 ×10 ⁻³	0.065

Transducers	Biomarkers	Sensitivity	Low detection	Test range	Ref.
			range		
Hydrazine–Au NPs–aptamer	HER2	1.117 μAmL/ng	0.037 pg/mL		Zhu et al., 2012
ZnO	DNA based	6.36 μΑ/μΜ	3.32 μM	10.0 - 100.0 μM	Mansor et al., 2014
rGO-	HER2		1 pM	1 pM ⁻¹ μM	Myung et al., 2011
SiO ₂ NPs					
CNTs	Anti-breast	8–925 nA/μM	13.8 μM	40-160 μM	Baj-Rossi et al.,
	cancer agent				2012
G-Pd@rGO		86.0 Ω/M		1fM-1nM	
C-Pd@rGO		644 Ω/M		100fM-1nM	
G-Pt@rGO		41.48 Ω/M]	1fM-1nM	
C-Pt@rGO	ErbB2	676 Ω/M	1.0 fM	100fM-1nM	Present Work
G-Ag@rGO		35.88 Ω/M		1fM-0.5μM	
C-Ag@rGO		260 Ω/M		100fM-0.1µM	
G-Au@rGO		157.6 Ω/M]	1fM-0.1µM	
C-Au@rGO		544 Ω/M		1fM-1nM	

Table S4. Sensing characteristics of the various immunoelectrodes along with those reported in literature for breast cancer biomarker detection.

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