Surface-Induced in Situ Sonothermodynamically Controlled Functionalized Graphene Oxide for in Vitro Cytotoxicity and Antioxidant Evaluations

Gopal Avashthi,† Shrikant S. Maktedar,† and Man Singh*‡

†School of Chemical Sciences, Central University of Gujarat, Gandhinagar 382030, India
‡Department of Chemistry, National Institute of Technology, Srinagar 190006, Jammu and Kashmir, India

ABSTRACT: Graphene oxide-based advanced functional materials offer an ultimate solution for wider biomedical applications. In situ thermodynamically ultrasound-assisted direct covalent functionalization of graphene oxide (GO) with sulfanilamide (SA) has synthesized f-(SA)GO. Raman spectroscopy, X-ray diffraction, high-resolution transmission electron microscopy, selected area electron diffraction pattern, scanning electron microscopy, atomic force microscopy, Fourier transform infrared spectroscopy, ultraviolet–visible spectroscopy, thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC) have analyzed the f-(SA)GO structure for functional activities, expressed through synergistic impact of heteroatomic domains (SIHAD). The TGA of GO and f-(SA)GO demonstrates their total weight losses of 82.0 and 61.1%, respectively. Enhanced thermal stability of f-(SA)GO infers an exothermic behavior obtained with DSC. The surface-induced in situ thermodynamically controlled nonspontaneous reaction for f-(SA)GO has facilitated calculations for activation energy \( E_a = 2.65 \times 10^8 \text{[kJ mol}^{-1} \text{]} \) and Gibbs free energy \( \Delta G = 8.3741 \text{[kJ mol}^{-1} \text{]} \), energetics for biological activities with sulforhodamine B assay on MCF-7 and Vero cell lines and antioxidant potential by free radical scavenging activity with DPPH (2,2-diphenyl-1-picrylhydrazyl). Cell viabilities are >89.8% for Vero and >90.1% for MCF-7, respectively. Enhanced thermal stability of f-(SA)GO infers an exothermic behavior obtained with DSC. The surface-induced in situ thermodynamically controlled nonspontaneous reaction for f-(SA)GO has facilitated calculations for activation energy \( E_a = 2.65 \times 10^8 \text{[kJ mol}^{-1} \text{]} \) and Gibbs free energy \( \Delta G = 8.3741 \text{[kJ mol}^{-1} \text{]} \), energetics for biological activities with sulforhodamine B assay on MCF-7 and Vero cell lines and antioxidant potential by free radical scavenging activity with DPPH (2,2-diphenyl-1-picrylhydrazyl). Cell viabilities are >89.8% for Vero and >90.1% for MCF-7, respectively. Enhanced thermal stability of f-(SA)GO infers an exothermic behavior obtained with DSC. The morphological effect on MCF-7 and Vero cell lines confirm its structurally stable biocompatibility. The SIHAD of f-(SA)GO scavenges radical activity, and its heteroatomic structure causes valuable physicochemical activities. f-(SA)GO could emerge as an advanced functional biomaterial for structurally and thermally stable biocompatible nanocoatings.

1. INTRODUCTION

Functionalization models of graphene oxide (GO) to derive functional nanomaterials have attracted scientists, industrialists, and researchers globally for biomedical and biochemical applications. Despite several potential properties,1−7 the graphene alone could not significantly contribute toward biomedical applications due to its structural stability and poor processability in its native form.5,6 The graphene-based functional materials are known to be safer and potential for performing the biomedical applications of processed graphene.8−21 The enhanced dispersibility of functional graphene-based materials facilitates smooth processability in various organic solvents.8,9,20 The surface modification of graphene could induce a larger surface area with stronger surface activities for biomedical applications.22−28 Therefore, for transforming the graphene to structurally competent express interacting activities, functionalization becomes a most needed science.

The wet chemical functionalization (f) of GO is widely reported for surface modification30−32 as graphite (Gr) is used as a starting material in the present study for its chemical alteration. Gr also has the same problem of high thermal stability and poor solvent processability.20 So, Gr was converted into graphite oxide (GrO), which was subjected to ultrasound irradiation for exfoliation into thermally active GO. The surplus oxygen-containing GO functionalities are used as a precursor for chemical functionalization in absolute alcohol.33−35 The GO covalent functionalization without using acylating and coupling reagents is a challenge despite innumerable efforts put in using several substances.13 Several attempts are successfully documented to engineer for biomedical applications of routine functionalized GO (f-GO) based materials including in vitro biocompatibility and cytotoxicity.36−46 The researchers have functionalized the GO with various molecules like amino acid2,23 but no studies about GO functionalization with a sulfide drug molecule are reported yet, which has a larger surface area with highest surface activities. We have chosen SA, which has \( \pi \)-conjugation in its benzene ring that makes the ring an active surface for its chemical activities. The \( \pi \)-conjugated stable sulfamoyl functional group (H\(_2\)NSO\(_2\)−) is bonded to aniline. Apart from its active delocalization, its headgroup (NH\(_2\)SO\(_2\)−) keeps exchanging the electron developing temporary \((\text{+})\) and \((-)\) poles, which induce the Coulombic interactions. The GO
functionalization with SA develops a unique class of smart GO-derived functional molecules, which become an extraordinary sensitive thermal sensor due to highly active and receptive surfaces. The chemical energy is conserved in a chemical bond made on sharing or transfer of an electron from one C to another C atom in GO, which has sp² and sp³ hybridization along with −C−O−C−, −C−OH, OH−C−O, and −C−O groups with a lone pair of electrons. Hence, subjecting GO to 30 kHz sonication for 1S min caused cavitation that induce surface stability in GO by inducing atomic oscillations, which lead to disrupt the bond in an alcoholic medium. An initiation of bond disruption generates enthalpy for phase changes, which is used in any secondary chemical process. It is shown that the resultant product with a less energy state causes an exothermic reaction with enthalpy \( \Delta H = -n \varepsilon \) and raises the temperature of GO solution, which controls all thermodynamic parameters. Thus, by the sonication process, the heat holding capacity \( q \) generates robust \( \Delta H \) and \( \Delta S \) (entropy), which make this process thermodynamically robust. Since autogenerated \( \Delta G \), \( \Delta S \), and \( \Delta H \) represent a chemical process itself, the activity is in situ. The in situ controlled raising temperature enhances kinetic energy and lowers \( E_g \), cause spontaneous functionalization of GO with SA to change \( \Delta G \).

The explored thermodynamic parameters \( \Delta G \), \( \Delta S \), and \( \Delta H \) indicate that the surface-induced sonothermodynamically controlled functionalization is exothermic and a nonspontaneous process. \( f-(SA)GO \) due to having highly active surfaces needs less amount of energy for expressing vibrant and visible effects, making it a green science. The present studies successfully reported about \( f-(SA)GO \) formation through the robust in situ thermodynamically controlled sonochemical approach. \( f-(SA)GO \) is subjected to biomedical applications through cytotoxicity profile and free radical scavenging activity. The higher cell viabilities with \( f-(SA)GO \) confirm its excellent biocompatibility. The thermal studies confirm instability of GO accountable for labile surplus oxygen-containing functionality as compared to \( f-(SA)GO \). \( f-(SA)GO \) shows an enhanced thermal stability due to covalently attached SA with a GO surface through an amide bond. The new bond formation causes electronic delocalization in ethanolic medium to enhance \( \Delta G \) of \( f-(SA)GO \) due to synergistic impact of heteroatomic domains (SIHAD) of various functionalities. The higher thermally stable sonochemically controlled thermodynamically favored \( f-(SA)GO \) activities act against higher temperature resist thermophilic bacteria and microorganism, which can be applicable for biological applications to kill thermally stable bacterial cysts. The \( f-(SA)GO \) suitability is for thermally stable biocompatible nanoconjugates and allied applications like antimurfing. The \( f-(SA)GO \) surfaces are accountable to the dipolar head of SA chemically bonded to GO through the −NH \(_2\) of SA at its para position and mechanistically confirmed through the SIHAD. \( f-(SA)GO \) truly emerges as a superb functional bioconjugate for biomedical applications, which could even puncture cysts at a higher temperature.

2. RESULTS AND DISCUSSION

The feasible greener sonochemical method is applied for GO functionalization with SA to form \( f-(SA)GO \) without using hazardous and acylating reagents starting with Gt flakes as a precursor. The eco-friendly route of ultrasound energy maintains the sustainability of the environment globally. For designing various graphene-based materials, ultrasound cavitation develops the metal-free GO functionalized product to explore exfoliated GO functionalization with an amine-substituted organic frame (ASOF), that is, SA in EtOH medium. In fact, the released \( \Delta H \) has been further used to monodisperse GO for better activation. This has been the reason that a specific self-explanatory surface-induced thermodynamic simulation was made. All the developed GO-based metal-free ASOFs may not necessarily show their exceptional functionality for various applications due to a mismatch in interacting structural units. Therefore, the selection of an ASOF is significant for functionalization with not only homogeneously dispersed GO but also their activities. The sole motto of GO functionalization with SA has been to widen the functionality of GO through −CONH− linkage between GO and SA. In many applications, GO is not that spontaneous in inducing the desired activity. To remove such restrictions, GO was functionalized with the most interactive molecule, that is, SA. The SA has \( \pi \) conjugation, enough lone pair of an electron (LPE) on the sulfamoyl functional group (H\(_2\)N\(_2\)SO\(_2\)), and free amino group at the para position. These electronic moieties additionally enhance the interacting ability of the SA with GO. Thus, GO functionalization enhances the thermal stability, nano coating capability, and dispersibility in wider solvents at varying polarities. During functionalization, the labile oxygen functionality of O═C—OH of GO surfaces is replaced by a more conjugated −NH—C==O of \( f-(SA)GO \) with other conjugated electronic constituents of SA like delocalized benzene ring with conjugated −SO\(_2\)NH\(_2\) and thermally stabilizes \( f-(SA)GO \) for activities. Thus, \( f-(SA)GO \) acts as a whole \( \pi \)-electron-rich conjugated center and synergizes the stability of \( f-(SA)GO \), which has been confirmed by TGA/DSC (thermogravimetric analysis/differential scanning calorimetry) as compared to GO and SA individually. The functionality of \( f-(SA)GO \) depends on the structure—activity relationship due to SIHAD impact, which may be dissimilar for other functionalized products. Recently, a lot of ultrasound-assisted GO functionalizations are reported for various applications, and the functionalized structure is confirmed by various sophisticated analytical methods like Raman spectroscopy, X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, TGA/DSC, scanning electron microscopy (SEM), high-resolution transmission electron microscopy (HRTEM), atomic force microscopy (AFM), and UV–visible spectroscopy. Therefore, the GO functionalization with SA and thermodynamic parameters thereof are initiated first, and hence its detailed structural studies are made and analyzed for biological assay. In cytotoxic assay, \( f-(SA)GO \) is the most balanced molecule manifold localized active site in coordination with other closely placed functional moieties. Therefore, closely placed units with different electronic configurations modulate and tune the optimization of \( f-(SA)GO \) for creating residual force or the van der Waals forces (VWF). Therefore, MCF-7 and Vero cell lines are the most sensitive structures. Their responses are to be studied without their major breakdown. Thus, this was the most potent point to choose \( f-(SA)GO \) to allow study on MCF-7 and Vero cell lines. \( f-(SA)GO \) without destroying them and study of these balanced responses as the SA unit attached to GO through functionalization have further modulated the interacting activities of the GO. Thus, this has a most biocompatible combination of \( f-(SA)GO \) with MCF-7 and Vero cell lines to analyze their responses vis-à-vis. For the past few decades, the prevalence of breast tumor has amplified speedily. GO and its
derivatives have been used for several curative researches, particularly in cytotoxicological evaluations. These studies have been found to offer some positive gestures that can be used in breast tumorous cell treatment predominantly. However, several types of research regarding the applications for graphene-based materials have been investigated for the breast malignant cell line treatment, but no study has been yet examined for the cytotoxic properties and their influences on f-(SA)GO on MCF-7 and Vero cell lines. Hence, in these studies, GO and f-(SA)GO was confirmed on MCF-7 and Vero cell lines as positive cell viable nanomaterials for cytotoxicity effects. Also, GO and f-(SA)GO were undertaken in accordance to noticeable morphological changes against Vero cell lines, which was also a positive viable effect on the cellular uptake relating to its therapeutic exposure. The monkey normal kidney cell line, Vero, is a convenient tool to evaluate the precise toxic influence. It can be promptly altered for higher growth during cytotoxicity assessments. However, kidney is made up by several complex cell types having diverse morphologies and functions, which shows highly sensitive responses to toxic chemicals. Therefore, the selection of Vero and MCF-7 cell lines for in vitro study is primarily feasible for toxic chemical responses to evaluate cytotoxicity and breast malignant cell line treatment, respectively.

2.1. Structural Investigation. Symmetric structures of GO and f-(SA)GO in Figure 1 were confirmed by Raman spectroscopy, with characteristic D and G bands for GO observed at 1359 and 1593 cm$^{-1}$, respectively (Figure 1a). The two less intense bands of 2D and D+G were also analyzed at 2674 and 2931 cm$^{-1}$, respectively. The D band corresponds to structural defects and $A_{1g}$ symmetry, while the G band corresponds to the graphitic domain of $E_{2g}$ symmetry. The D band depicts a formation of sp$^3$ from sp$^2$ carbon due to the covalent attachment of SA with GO. D and G bands for f-(SA)GO were detected at 1359 and 1585 cm$^{-1}$ along with the single broad merged D+G bands at 2845 to 2937 cm$^{-1}$ (Figure 1b). The investigated D/G ratios of GO and f-(SA)GO are 0.85 and 0.86, respectively. The 2D band of GO is redundant in f-(SA)GO in the form of single D+G broadened bands from 2845 to 2937 cm$^{-1}$. Thus, f-(SA)GO has the larger structural defects in crystal lattices as compared to GO due to direct covalent functionalization (DCF) of GO into f-(SA)GO. This has least $\Delta S$ entropy with a higher stability as confirmed by TGA. To break up this stability, f-(SA)GO should have higher enthalpy, and it was ensured by DSC. The sp$^3$ hybridization of the benzene ring of SA along with LPE could also contribute the fundamental shift between sp$^2 \Rightarrow$ sp$^3$.

The powder XRD spectra were obtained with MiniFlex Rigaku Cu Kα radiation $\lambda = 1.54$ Å operating at 40 kV and 15 mA cathodic current and recorded in the $5-80^\circ$ range at 0.1 degrees/s scan rate. The hexagonal GO (Figure 2a; PDF 03-065-1528) was used as a starting material showing a sharp 2θ peak at 9.70$^\circ$ of 100% relative intensities corresponding to the (002) plane having a d spacing of 9.10 Å. Similarly, a second peak is observed at an intensity of 4.95$^\circ$ with a d spacing of 4.53 Å. f-(SA)GO shows two peaks; out of these, one sharp and 100% relative intense peak at 10.42$^\circ$ (Figure 2b) is observed with a d spacing of 4.26 Å. Reduction in the spacing of f-(SA)GO as compared to GO could be attributed to the π conjugation and dipole (−O=S′=O$^-$ or $\pi$-O=S$^-$=O$^-$) of SA. The powder X-ray diffraction pattern of GO and f-(SA)GO (Figure 2a,b) demonstrates intense diffraction peaks at 9.70$^\circ$ and 10.42$^\circ$, respectively. The shift in the position of the diffraction peak and variation in interlayer spacings as compared to the starting material infer f-(SA)GO formation. FWHM (full width at half-maximum) values of GO and f-(SA)GO are 1.21 and 1.07, respectively, and their 91.6 and 94.3 Å crystalline size respectively infer functionalization. The enhanced crystalline size of f-(SA)GO confirms the direct covalent GO functionalization with SA. The calculated strain values are 3.50% for GO and 2.80% for f-(SA)GO and infer more stability in f-(SA)GO. The less strain % in f-(SA)GO supports more stabilization due to SA additional conjugation onto GO. The $-\Delta S$ was also verified for SA-bonded GO with a high-intensity sharp peak, which find an additional stability for f-(SA)GO, was confirmed by TGA, and also favored the reduction in...
strain. This is due to the polar structure of SA and electron delocalization in the amide functionality of f-(SA)GO.

The surplus oxygen-containing epoxy, hydroxyl, carbonyl, and carboxyl functionalities are observed over the GO surface (Figure 3a). The broad peak of –OH appears at 3406 cm\(^{-1}\) and the carbonyl carbon at 1736 cm\(^{-1}\). The 1066 cm\(^{-1}\) characteristic peak infers an epoxy group (C=O–C). The peak at 1626 cm\(^{-1}\) supports the presence of conjugated unsaturated C=C. The peak is observed at 1375 cm\(^{-1}\) and assigned to the C–H bending vibration. Both peaks at 1736 and 1626 cm\(^{-1}\) have nearly equal intensity with 84% transmittance (16% absorbance). The FTIR spectrum of f-(SA)GO comprises a peak for amide at 1629 cm\(^{-1}\), but this peak is absent in GO (Figure 3b). Also, the amide peak is more intense with 25% of transmittance (75% absorbance). The scattered electronic effect (Scheme 1) causes Lennard-Jones repulsion supported by Born–Oppenheimer approximation notated as

\[
E_{\text{total}} = E_{\text{electronic}} + E_{\text{vibrational}} + E_{\text{rotational}} + E_{\text{nuclear}}
\]

In general, the reduction in one lone pair of an electron (LPE) of the O atom of –COOH, which is replaced by –CONH, has activated the structure, which has higher \(\vartheta\) and absorbance (A).

Hence, from Scheme 1

\[
\Delta E_{\text{total}} = 8E_{e-e(LPE)}(HO - C=O)
- 6E_{e-e(LPE)}(HN - C=O)
= 2E_{e-e}
\]

The electron–electron repulsion caused by –COOH in GO is higher than –CONH of f-(SA)GO due to scattering the IR electron cloud of shared as well as LPE. These effects have influenced the stretching frequency (\(\vartheta\)) of –C=O and –OH in COOH than HN–C=O, where its amide \(\vartheta\) gives higher absorbance (75%) with CONH– contrary to 16% for the –COOH group. The analyzed % of transmittance has been also verified with the UV spectra (Figure 8) and shows a higher absorbance for f-(SA)GO, where the peaks of GO along with the amide peak ensures the covalent attachment of SA over the GO surface.

The thermal response profiles of SA, GO, and f-(SA)GO were studied in combination with TGA and DSC (Figure 4a,b) because of their dissimilar electronic structures with various constituents. These structural features establish the structure–activity relationship and generate a scientific ground to compare the structural response of thermal behavior by TGA.
ordered sp2- and ds p3-hybridized functional units, which the SA behavior and inferred very disordered and highly prominent exothermic peak of DSC for f-(SA)GO controlled of thermally labile oxygen-containing functionality. The loss of the weight loss of 45.8% at nearly 318.4 and 336.9 temperature and indicates its thermal stability with a total % loss just above room temperature (RT) (Figure 4a). The total stage GO decomposition, the TGA curve indicates rapid mass in the said domains at 225.3° C and 28.98 mW. However, contrary to f-(SA)GO, the GO induces the exothermic reaction at the active structural state at 197.9 °C and 16.28 mW, which demarcates GO from f-(SA)GO via a covalent bond. The TE on disrupting the binding forces allows the SA molecules to optimize in an ordered form. Initially, SA liberates energy as a heat where its attached hydrogen bonds are broken but their energy is not used but later on; this energy is used to further rearrange a structure, which acquires energy from itself on stabilizing the temperature and shifts from one to another phase. A close look at Figure 4b and the analysis of DSC for SA, GO and f-(SA)GO infer with various structural responses to heat, probably forming separate structures with different T-driven behaviors. The functionalization process is structurally active and inhibits the original T-response or T-driven structural activities of both SA and GO.

The functionalization of GO with SA could act as the temperature sensor and authentically reflects a synergistic effect on both of these. An exothermic peak for f-(SA)GO is shifted from 197.9 to 225.3 °C as the q of the functionalized product is higher than GO and widens temperature-based applications of f-(SA)GO. The pure SA DSC had produced an exothermic curve at 318.4 °C and endothermic at 336.9 °C due to the −SO2NH2 loss. It confirms a para −NH2 of SA attached at GO, which affects delocalization by shifts in electron clouds of −SO2NH2. Such delocalization effects are finished when the −NH2 is used in covalent bond formation for f-(SA)GO stabilization. Hence, f-(SA)GO shows a weight loss at 225.3 °C that indicates the decomposition of oxygen-containing functionalities over the GO surface (Figure 4a). The total % wt loss of f-(SA)GO is 61.1%, which infers its higher thermal stability than GO with a formation of enhanced stabilized residues due to electronic delocalization. A broad exothermic peak of GO at 197.9 °C depicts thermodynamic feasibility. However, f-(SA)GO shows an intense exothermic peak at 225.3 °C (Figure 4b), indicating high thermal stability of graphitic materials. The DSC describes exothermic performance with a lower q of GO with thermal instability, contrary to greater q of f-(SA)GO thermal stability. The increased T of f-(SA)GO as compared to GO (ΔT = 27.4 °C) reveals a strong confinement effect of SA on GO sheets and involves covalent bonding via amide bond formation. Therefore, a quantitative equivalence between f-(SA)GO and GO is developed for a better understanding of the confinement effect.

HRTEM data (Figure 5) was collected by using FEI Model Tecnai G2 S Twin operated at 200 kV. The dispersions of GO and f-(SA)GO were prepared in ethanol and sonicated for 5 min. The 10 μL dispersions of each were uniformly spread over 200 mesh holey carbon-coated copper grid and dried in a
closed Petri dish for 3 h. The TEM and high-resolution images reveal the surface morphology and phase-contrast investigation for GO and f-(SA)GO, respectively (Figure 5a–d). A sheet-like GO surface (Figure 5a) and the formation of wrinkled several small rod-like structure in f-(SA)GO (Figure 5b) infer dissimilar edge morphology. The scrolled edge morphology of f-(SA)GO was evidently investigated and signified the substantial restacking of f-(SA)GO. This significant edge morphological distortions in f-(SA)GO may be due to the chemical functionalization of SA on the GO surface. HRTEM images conclude the inference that f-(SA)GO has more physical consistency (Figure 5d) than GO lattice fringes (Figure 5c). It is because of sonochemically in situ thermally controlled covalent functionalization of GO sheet by SA. The phase-contrast HRTEM imaging based on the higher electronic density of f-(SA)GO and the broad peak in UV spectra at 315 nm was observed for its structural confirmation due to the restoration of π conjugation along with an enhancing electronic delocalization capability (Figure 8). The crystalline behavior of GO and f-(SA)GO are further supported by the corresponding selected area electron diffraction patterns (Figure 5e,f). The ordered arrangements and well-resolved concentric rings with intense spots in the GO electron diffraction pattern confirmed a crystalline GO with a lesser number of layers. The sixfold pattern in selected area electron diffraction (SAED) of GO is consistent with its hexagonal lattice (parameters \( a = b \neq c, \alpha = \beta = 90^\circ \) and \( \gamma = 120^\circ \), \( a = 2.4500 \), \( b = 2.4500 \), \( c = 9.1100 \), and volume = 47.357) and confirmed the graphitic AB stacking order in the lattice even after a higher degree of oxidation. The intense spots in the first ring of GO correspond to the ordered graphitic lattice, and a relatively low-intensity spot in the second ring corresponds to the disordered domain. The interlayer spacing value \( (d = 1/R) \) of the first ring in the SAED pattern of GO is 0.19 nm. In the second ring, the interplanar distance is reduced to 0.11 nm, and the intensity of spots is lowered. It verifies the orientation of oxygen functionalities over the GO surface (Figure 5e). The GO surface consists of sp²- and sp³-hybridized carbon atoms. The SAED pattern of f-(SA)GO reveals the SA signature onto the GO surface. The calculated interlayer spacings for the first and second ring in the SAED pattern of f-(SA)GO are 0.20 and 0.12 nm, respectively (Figure 5f). The enhanced calculated value of interlayer spacing confirms the covalent attachment of SA onto the GO surface. In addition to \( d \) spacing values of GO, f-(SA)GO exhibits a 0.60 nm interplanar distance, attributed to SA (PDF 00-005-0346). The existence of two different crystalline domains in f-(SA)GO rings may be due to GO functionalization with SA.
A chemically active species on the GO surface creates a new material of different electronic configurations that generates new lattice arrangements. These observations are supported by SEM as slightly ordering is seen in planes with higher ordered structure. The higher ordering of f-(SA)GO is further supported by negative values of entropies. The sample was mounted on carbon tape, which was fixed on the stub and exposed for plasma sputtering inside the coating chamber. The target-containing Au and Pd in 80:20 act as a source for coating. After coating, 5 kV for Gt and 7 kV beam voltages were applied to excite the secondary electrons from the sample’s surfaces of synthesized materials. The comparative SEM is observed for pure Gt (Figure 6a), unexfoliated GtO (Figure 6b), exfoliated GO (Figure 6c), and functionalized f-(SA)GO (Figure 6d). The pure Gt shows larger stacked black flakes with highly dense furrows due to a natural alignment. The exfoliated GO surface morphology is investigated with 20 μm scanning area (Figure 6c) with unrestricted threads like crumpled, rippled, and parallel multiple layers as a result of strong hydrogen bonding between two successive layers due to oxygen-enriched carbon functionality at the GO surface in EtOH medium. The exfoliated sheets, which are bound with comparatively least VWF, are not able to hold them firmly (Figure 6c) and have the ability to unobstructed activities for GO functionalization with SA. Unexfoliated GtO (oxygenated Gt) SEM (Figure 6b) shows less dense surface morphology with no loose multiple sheets but strongly bound with strong VWF as compared to the highly dense packet of Gt flakes (Figure 6a), which infer successful oxidation and exfoliation after removing the impurities. The organized loose GO sheet is...
functionalized with SA, which has enhanced the ordered structure for f-(SA)GO (Figure 6d). The hydrogen bonding network controls all the large-scale GO properties in which functional groups of single GO layer and water molecule between the interlayer cavities play a contributory role. The ultrasound mechanical energy enhanced the GO dispersion in EtOH and distilled water to break weak van der Waals forces between layers. The dispersed GO is deformed upon exfoliation and restacking process with more rough surfaces than f-(SA)GO and parallel arrangements of successive layers. SEM elucidates f-(SA)GO sheet formation with smooth surfaces (Figure 6d) than the rough texture of GO (Figure 6c). SEM of the f-(SA)GO structure as condense sheets is credited to π conjugation and −SO2NH2 interaction. These are active and develop stronger intermolecular forces with a stronger covalent bond. The morphological investigation for GO and f-(SA)GO also confirms roughness depicted in AFM.

AFM analysis for GO and f-(SA)GO was conducted in a noncontact mode by preparing samples on a cleaned mica sheet, which was cleaned with ethanol and dried with hairdryer, and samples were sonicated for 5 min in ethanol. The dispersions were filtered and poured onto the surface of the mica sheet with a micropipette. Again, the mica sheet was washed with a small amount of ethanol and dried at RT. The surface of each mounted samples was scanned for a topographical view and 3D image (Figure 7). The scanned areas are 6 μm for the GO sheet to generate a 3D topographical view (Figure 7b) and 12.5 μm for f-(SA)GO (Figure 7d). Prominent sheets are haphazardly distributed in GO. The ordered sheet structure with more density is visible in f-(SA)GO and supported by HRTEM (Figure 5d) and UV (Figure 8). It proves GO functionalization with SA into f-(SA)GO and and rough nature of the surface. The values regarding the height, size, and total surface roughness for GO and f-(SA)GO are shown in Table 1. It shows a smaller Ra value for f-(SA)GO due to functionalization of homogeneously dispersed GO sheets.

UV–vis absorbance spectra for GO and f-(SA)GO are depicted in Figure 8, where the GO shows a transition at 235 nm. An absorption peak at 235 nm is attributed to π → π* transition of the C=C bond and a broad peak at 295 nm due to n → π* transition of carbonyl functionality. These observations infer surplus oxygen-containing functionalities over the graphene surface, which endorses a higher degree of oxidation. Consequently, the π conjugation of the GO sheet decreases and the absorbance at a larger wavelength for f-(SA)GO is observed with two sharp absorption peaks at 205 and 265 nm with hyperchromic shift and one small broad peak at 315 nm. This bathochromic shift and broad peak at 265 and 315 nm, respectively, indicate restoration of π conjugation along with an expansion of new functionalities due to covalent GO functionalization with dipolar SA as it expresses activities of π conjugation and dipolar nature. Hence, the UV–vis spectrum of f-(SA)GO infers covalently attached SA on the GO surface.

2.2. Antioxidant Potential (AP). Both GO and f-(SA)GO are active to scavenge free radicals for antioxidant therapy. It has been assessed with DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Figure 9a,b). A 0.1 mM DPPH stock solution in ethanol was made with 2.16 mg/50 mL and UV light absorbed at 520 nm. GO and f-(SA)GO (40, 60, 80, and 100 μg mL−1) are separately prepared to analyze their antioxidant potential. A 100 μg mL−1 solution of l-ascorbic acid (AA) as a standard solution is made to use as a positive control. The 1:1 ratio of sample and DPPH stock solutions are mixed and kept for 1 h. The GO (100 μg mL−1) slightly expresses more scavenging activity (SV) contrary to f-(SA)GO probably because the GO–GO interaction might have catalyzed H+ liberation, but f-(SA)GO decreases SV at 100 μg mL−1. Comparatively, it infers more f-(SA)GO-f-(SA)GO engagement interactions rather than H+ liberation. It happens due to the dipolar π − π interaction between the benzene rings of f-(SA)GO successive layers, which is absent in GO. The HO—C=O, —OH, —C=O, C-O—C groups are unengaged in the case of GO and easily liberate H+ from HO—C=O in EtOH medium during intermolecular in situ ester bond formation as an intermediate between GO and EtOH (Scheme 2a) and scavenge more the DPPH. In f-(SA)GO, all its H+ liberating functional groups (FGs) are engaged in functionalization with SA as an amide bond toward highly stable intramolecular conjugation instead of liberating H+ (Scheme 2b).

Thereby, the GO scavenges by 50.5%, contrary to 43.5% of f-(SA)GO. It also proves the engagement of SA in functionalization with GO.

The percentage scavenging activity (PSA) was calculated by putting A0 and A1 values in eq 25. The relative UV–vis graph for PSA of GO and f-(SA)GO (Figure 9a,b) for their % antioxidant potential is from 40.40 to 43.56% and that for GO is evaluated from 45.8 to 50.57% (Figure 9c).

Figure 9c clearly shows a maximum antioxidant potential at 40 μg mL−1 GO and f-(SA)GO. The highly reactive oxygen of O=—C—OH is accountable for more antioxidant potential of GO due to H+ liberation as the mentioned mechanism, but GO is thermally labile than thermally stable f-(SA)GO, confirmed by TGA (Figure 4a). The intramolecular con-

Table 1. AFM Analysis of GO and f-(SA)GO

| sample   | ΔX (μm) | ΔY (nm) | Rpv (nm) | Rq (nm) | Ra (nm) | angle (°) |
|----------|---------|---------|----------|---------|---------|-----------|
| GO       | 0.075   | 0.054   | 26.762   | 9.634   | 8.916   | 0.042     |
| f-(SA)GO| 0.083   | 0.027   | 13.466   | 3.486   | 2.497   | 0.019     |

Figure 8. Comparative UV–vis spectrum of GO and f-(SA)GO.
jugation in f-(SA)GO hindered to liberate H⁺ but enhanced the thermal stability. Hence, the AP of f-(SA)GO has more potency for thermally stable antioxidant activity.

2.3. In Vitro Biological Evaluation. The different electronic structures of various constituents of the functionalized product make a dissimilar structural response to the biological fauna and flora along with their thermal behaviors because of varying divergent properties of functionalities in f-GO. The biological activities of GO and f-(SA)GO are different because of their dissimilar functionalized surfaces. The in vitro cytotoxicity screening was performed on human breast cancer cell line MCF-7 and monkey normal kidney cell line Vero (Figure 10). The sulforhodamine B (SRB) assay was used for cell growth. In both experiments, 10, 20, 40, and 80 μg mL⁻¹ doses were used in DMSO using adriamycin (ADR) as a positive control. Each experiment was repeated thrice, and a mean reading was considered to plot a graph between % control growth and concentration. The GI₅₀ (drug inhibits 50% of cells), TGI (drug produces total inhibition of cells), and LC₅₀ (drug that kills 50% of cells) were calculated from a mean graph. The GO and f-(SA)GO were screened against

Figure 9. DPPH assay of a (GO) and (b) f-(SA)GO. (c) % AP at varying concentrations.

Scheme 2. (a) H⁺ Liberation by Unengaged HO—C==O of GO in Intermolecular In Situ Ester Formation and (b) Engaged RHN—C==O in Intramolecular Conjugation Hindered the H⁺ Liberation

Figure 10. In vitro SRB assay for biological evaluation of (a) MCF-7 and (b) Vero cell lines.
MCF-7 and Vero cell lines in Figure 10a,b, respectively. The >90.1 GI50 value for MCF-7 confirms the least toxicity and excellent cytocompatibility. The low cytotoxicity of f-(SA)GO probes its biocompatibility with the normal cell line. Their studies were continued to screen against normal Vero cell lines (Figure 10b). The GO and f-(SA)GO both show compatibility >89.8 GI50 as compared to ADR. The morphological effect of f-(SA)GO on the cellular surface of Vero cell lines indicates its biocompatibility (Figure 11). The images were taken under a Nikon-Ti-S inverted research microscope with a magnification of 20× at a 200 pixel scale bar by Eclipse Image processing software NIS-Elements. Figure 11a−c images have shown control, ADR, and GO influences on the morphology of Vero cell lines.50 Figure 11a shows a continuous aneuploid fibroblast-like morphology of Vero cells, which is damaged after applying the ADR, and looks like some typical aberrant morphological cells (Figure 11b). ADR-tested dead Vero cells show discontinued cell division and grew as defected multilayer cellular aggregates, signifying a loss of interacting obstacle or anchorage-self-regulating growth. Figure 11c infers the least damage to Vero cell morphology. The cells also retained their morphology with f-(SA)GO due to their biocompatibility (Figure 11d). In GO and f-(SA)GO, both induce partial aberrations and did not detach the fibroblast-like morphology, not like defected cellular aggregates. The GO and f-(SA)GO both infer no loss of contact inhibition and can possess self-regulatory division.

The GO shows more % of cytocompatibility than f-(SA)GO due to highly reactive oxygen, but due to its thermal instability, it has limited applications. Thus, f-(SA)GO is proven as a biocompatible material with least toxicity and high thermal stability, and it could be used as a biomaterial for thermally stable biocompatible coatings and other biomedical applications.

2.4. Synergistic Impact of Heteroatomic Domains (SIHAD). The q of biologically active compounds acts against the high-temperature resisting thermophilic bacteria or microorganism. Basically, GO has less thermal stability due to the presence of labile oxygen functionality, but after its functionalization, the labile O=C−OH functionalities transform into O=C−NH of f-(SA)GO with dissimilar q. The new amide bond formation enhances intramolecular conjugation in f-(SA)GO with higher thermal stability. The heteroatomic domains like O, N, and S of f-(SA)GO stabilize the whole structural integrations as a q with more biological activity against the high-temperature resisting thermophilic micro-
organism. Different forms of heteroatoms as carbon functionalities have synergistically contributed for the f-(SA)GO cytocompatibility behavior. f-(SA)GO may be a boon for developing a thermally stable biocompatible coating. The SIHAD in the amide form along with the SA benzene domain has stronger f-(SA)GO structural activities for biomedical applications. In vitro cytocompatibility profiles on MCF-7 and Vero cell lines are >90.1 and >89.8% cell viabilities, respectively. The enhanced cytocompatibility with MCF-7 and Vero cell lines have the least cytotoxicity with higher biocompatibility. The uniqueness of this research work is that the higher q of the f-(SA)GO product contrasts that of GO. f-(SA)GO has SA addition with π-conjugated dipole and GO that engage more and more for oscillating activities. The structural units on getting q initially undergo oscillatory, translatory localized molecular motions, where higher q is held by f-(SA)GO but no such motion causing moieties are with GO, so it could not hold or engage its higher q. GO shows a transition at 197.9 °C contrary to 225.3 °C for f-(SA)GO due to its higher q of f-(SA)GO with additional heat capacity (C). If 1 mol (N) of GO undergoes functionalization with SA into f-(SA)GO at a specific heat (C) of mass (m = 7.726 mg during TGA/DSC analysis) to raise the temperature ΔT (225.3 − 197.9 = 27.4 °C), then the total heat capacity for most of solid crystalline materials is equal to 3R according to Dulong–Petit law noted as

\[
\frac{C}{N} = 3R \quad \text{or} \quad C = 3R
\]

where N = 1, for 1 mol = 6.022 × 10²³ atoms.

The heat added (q) during the phase change is noted as

\[
q = m \times C \times \Delta T = 7.726 \times 10^{-3} \text{g} \times 3 \times 8.314 \times 27.4^\circ
\]

\[
C = 5.280 \text{ J/mol} \quad (4)
\]

\[
q = 5.280 \text{ J/mol} \quad \text{infrers the added TE from its surroundings acting as a remarkable } q \quad \text{sensor for higher temperature application aims to kill thermophilic cysts, which resist at a higher temperature. The contribution of various atomic domains of f-(SA)GO is significant for a biocompatible behavior.}

3. CONCLUSIONS

The Gt → GtO → GO → f – (SA)GO link has been accurately materialized by avoiding hazardous acylating and coupling reagents for GO surface modification. This method shortened the reaction duration to 15 min by ultrasound induced in situ thermodynamically controlled mechanism. The GO functionalization is initially connected to a thermodynamically controlled nonspontaneous reaction with ΔG = 8.3741 kJ mol⁻¹ and Eₜ = 2.65 × 10³ kJ mol⁻¹. The high-end analytical techniques is inferred an f-(SA)GO formation. A peak of 1629 cm⁻¹ in the FTIR spectrum confirms the f-(SA)GO synthesis through an amide bond. The XRD spectrum with shifting in the 2θ peak and a change in the interlayer distance of 8.48 Å establish covalent functionalization of GO. The Raman spectrum of 0.86 D/G validates a larger defect within the crystal lattice due to covalent bonding. The HRTEM and AFM analysis have confirmed f-(SA)GO formation with a smooth surface as compared to GO. An impact of f-(SA)GO is revealed with the thermal and cytocompatibility profile. Total % wt loss of f-(SA)GO is 61.1% with its higher thermal stability than GO. f-(SA)GO with ~90 μg mL⁻¹ GI₅₀ value for MCF-7 and Vero cell lines is found to have an excellent cytocompatibility. These results authenticate the morphological effect. f-(SA)GO has emerged as an advanced functional material for thermally stable biocompatible coatings.

4. EXPERIMENTAL SECTION

4.1. Materials. Graphite flakes, concentrated H₂SO₄ (98%), HCl (37%), H₃PO₄ (85%), sulfanilamide (SA; systematic IUPAC name, 4-aminobenzenesulfonamide (≥99)) were procured from Sigma-Aldrich Co. Analytical grade (AR) potassium permanganate (≥99) was supplied by Rankem. Petroleum ether (40–60) and H₂O₂ (AR, 30%) were purchased from S.D. Fine. Absolute alcohol (≥99%) was obtained from Scvuksmandi Ltd. India. All the chemicals were utilized without further purification.

4.2. Synthesis of GO. GtO was prepared by oxidizing Gt flakes with H₂SO₄/H₃PO₄ in a 9:1 ratio (180:20 mL). A mixture of graphite flakes (1.5 g, 1 equiv weight) and KMnO₄ (9.0 g, 6 equiv weight) was taken into a 500 mL RB flask, and the H₂SO₄ + H₃PO₄ mixture was added dropwise with mild shaking at the raised temperature of 35–40 °C. The reaction was thermodynamically controlled with the enthalpy of formation at a moderate level. This infers Gt → GtO, an exothermic reaction with enthalpy (ΔH) = −ve. Thus, this step naturally invites thermodynamic treatment to ensure the mechanism. Therefore, the success of the reaction depends purely on ΔH as many bonds of oxidizing agents/reagents are broken that released ΔH, and new bonds are also formed along with converting Gt to GtO. Naturally, the thermodynamic process becomes inevitable because chemically bonded impurities (salts) of Gt are detached chemically from Gt during GtO formation. Therefore, several localized chemical activities are initiated in the GtO redox process (GRP) with the number of microstates, which undergo with distinct ΔG, ΔS, ΔH, energy (ΔE), and chemical potential (Δμ). These orders develop an anisotropic situation, which substantially influenced binding of nascent O with Gt and converted into GtO (Scheme 3).

![Scheme 3. Reaction Pathway for Gt to GtO by Reactive Nascent Oxygen](image-url)

Also, bond energy, which depicted by ΔH, becomes an essential factor along with the potential energy denoted by ΔG. The abovesaid intricacies of Gt → GtO naturally connected to eq 5.

\[
\Delta G = \Delta H - T \Delta S
\]

The Gt bonds are broken, which have released ΔH = −ve, because the temperature reached up to 40 °C by detaching intersheet bonds of Gt, which move independently. The surface force attracts the [O] to form −C–O–C–, −C–OH, OH−C=O, and −C≡O functional groups. So, eq 5 is modified as

\[
\Delta G = -\Delta H - T \Delta S
\]

Substantial GtO redox is controlled by ΔH and ΔS, and naturally, a vacancy is generated to use the universal thermodynamic equation ΔG. Since the reaction was

DOI: 10.1021/acsomega.9b01939
ACS Omega 2019, 4, 16385−16401
conducted at atmospheric pressure, that is, constant pressure, heat content released in redox is noted as $\Delta H$, no heat was made from the outside, and $T$ is raised by the reaction only. So, the $\Delta S$ activities also become favorable for the nascent O atom to activate the Gt site to form GtO. Accordingly, the oxoanion (H$_3$PO$_4$ (c), and Gt (d)). Putting the value in eq 8, we get

\[
W = \sum \frac{N!}{N_i!} \frac{(N - N_i)!}{(N - N_i - N_o)!} \frac{(N - N_i - N_o)!}{(N - N_i - N_o - N_b)!} \frac{(N - N_i - N_o - N_b)!}{(N - N_i - N_o - N_b - N_g)!} \times ... \\
W = \sum \frac{N!}{N_i!} \frac{(N - N_i)!}{(N - N_i - N_o)!} \frac{(N - N_i - N_o)!}{(N - N_i - N_o - N_b)!} \frac{(N - N_i - N_o - N_b)!}{(N - N_i - N_o - N_b - N_g)!} \\
\]  
(9)

where $N$ is the total number of microstates, and $N_i$ is the distinguishable microstates in the observable set of free energy associated with Gt $\rightarrow$ GtO $\{i = KMnO_4 (a)$, H$_2$SO$_4$ (b), H$_3$PO$_4$ (c), and Gt (d)$\}$. Putting the value in eq 8, we get

\[
W = \frac{N!}{N_{KMnO_4}!(N - N_{KMnO_4})!} \frac{(N - N_{KMnO_4})!}{(N - N_{KMnO_4} - N_{H_2SO_4})!} \frac{(N - N_{KMnO_4} - N_{H_2SO_4})!}{(N - N_{KMnO_4} - N_{H_2SO_4} - N_{H_3PO_4})!} \frac{(N - N_{KMnO_4} - N_{H_2SO_4} - N_{H_3PO_4})!}{(N - N_{KMnO_4} - N_{H_2SO_4} - N_{H_3PO_4} - N_{Gt})!} \\
\]  
(10)

The GtO synthesis depends on its interacting activities of KMnO$_4$, H$_2$SO$_4$, H$_3$PO$_4$ and Gt, and their distribution pattern could generate the favorable orientation and reorientation to activate the Gt for initially removing the impurities followed by oxidation with the nascent O atom. Hence, $\Delta S$ plays a key role for higher yield of GtO. Since sp$^2$ hybridization exists due to the availability of partial charge holding the few mineral and other impurities. These impurities during GRP are detached from Gt skeleton, which generates vacant forces on the Gt structure. These sites interact with nascent O atoms. The oxidizing medium generates a variety of active chemical species, and their potential unfolds Gt to monodisperse as Gt nanosheets (NS). In this process, the Gt NS gain higher $\Delta S$. The abovesaid mechanism infers increment in $\Delta S$ on breaking the cohesive force of sheets that also partially released $\Delta H$. Since the reaction was conducted, the Gibbs–Helmholtz equation is fitted as in eq 6.

$\Delta G$ is positive, which infer robust Gt functionalization as per general norms $\Delta G$ predicts the successful reaction. Fundamentally, the Gt $\rightarrow$ GtO $\rightarrow$ GO involves sequential steps before the GO functionalization. After reacting the nascent O atoms with Gt, GO is an only reactive material, which play a key role in the functionalization. The generated chemical active sites of Gt were also occupied by impurities due to partially charge states with sp$^2$ hybridization on subjecting Gt to redox mixture (RM), and the occupied sites become free by detaching the impurities as the chemical species of RM are highly active and have higher chemical affinities toward the cationic impurities to form their salts and get separated from the Gt structure-based cationic $\pi$-interactions and oxidized into GtO followed by GO exfoliation. Here, the generation of active sites on the Gt surface converted into GtO to exfoliated GO followed by robust functionalization with SA via a sonochemical assisted in situ thermodynamically favorable mechanism.

The exotherm produced was cooled to RT to avoid an excess release of heat to avoid an explosion. The oil bath was heated over a magnetic stirrer with a hot plate for 50 °C. The RB flask holding the reaction mixture was kept inside an oil bath heated to 50 °C with constant stirring and reflux for 12 h. After completing the reaction, the workup, the reaction was cooled to RT and poured onto ice-cold water (~200 mL) containing 30% H$_2$O$_2$ (1.5 mL) and smoothly shaken to homogenize with a precautionary measure to avoid O$_2$ escape. The reaction mixture was centrifuged at 6000 rpm for 0.5 h to separate larger aggregates, and the supernatant containing inorganic solids was decanted away from the sediment. The complete washing of the remaining solid was made several times, in succession of 200 mL of water, 150 mL of 30% HCl, and 100 mL of ethanol. For each wash, the filtrate was centrifuged at 6000 rpm for 0.5 h, and the supernatant was decanted. After multiple washing, the remaining material was coagulated with 100 mL of petroleum ether as an antisolvent. The brownish colored solid sediment was obtained, which was vacuum-dried overnight at 1.05 bar and 50 °C. A generally brown colored GtO is reported for well-oxidized Gt. In a complete Gt oxidation to brownish GtO is inferred by a presence of OH$\rightarrow$C$\equiv$O$\rightarrow$C$\equiv$O$\rightarrow$C$\equiv$O$\rightarrow$C$\equiv$OH. The GtO synthesis method is slightly modified to obtain highly oxidized GtO, a precursor for surface modification. Since a sequential method from Gt to GtO is applied by a wet oxidation method, it converts into homogeneously dispersed GO by applying ultrasound energy at 30 kHz for 3 h.

4.3. GtO: Precursor in the Synthesis of GO. GtO was exfoliated through an intense ultrasonication for 180 min into homogeneously dispersed GO as a precursor for chemical functionalization. The sound waves play a major role in eco-friendly routes for a GO-based covalent bonded nanostructure without any side product except inducing oscillation of wanted atoms. The ultrasound generated mechnochemical energy to break the weaker van der Waals forces and active between layered GtO. Thus, uniformly dispersed GO in ethanol is used as a precursor for covalent functionalization with an amine-substituted organic molecule as SA. The process of GO sheet unfolding is seen to be controlled in ethanol. The GtO sheets are bound by weak van der Waals forces due to differently localized electrons in the form of sp$^2$ and sp$^3$ hybridization. The ethanol with two lone pair of electrons can have a delocalized potential energy of GtO to assist functionalization.

These GtO chemical activities vis-à-vis ethanol activities had to exfoliate GtO intersheet arrangements. Considering the potential of the ethanolic medium versus GtO, the study with solvents other than ethanol is being pursued in the laboratory. The science that generates GtO combinatorial activities versus medium constitute functional rheology, so functionalization could act as the remarkable industrial fluid with unique heat
dissipation, holding O₂ activities to be used in diamond or metal cutting industries along with integrated chips.

4.4. Thermodynamically Controlled Nonspontaneous Functionalized GO. Ultrasound assists GO nanosheets formation by exfoliation of multilayered GtO through an oscillatory mechanism. A 0.04 g of GtO and 40 mL of absolute ethanol solution in 100 mL of RB was subjected to ultrasonic energy (USE) by using Oscar ultrasonic (Microclean-103) at 30 kHz for 3 h. The USE enhances the rate of kinetics and lowered E₉₁ to form GO nanosheets. The light brown colored dispersion was observed on an abovesaid sonochemical treatment. A reactive nucleophile, SA (0.2 g), was added directly into high in situ kinetically energetic GO in an ethanolic medium. The in situ GtO → GO process at a fixed rate is controlled by cavitation energy. Simultaneously, the GO forms into f-(SA)GO and seems to follow the Lindemann reaction mechanism via the RRKM (Rice–Ramsperger–Kassel–Marcus) theory noted as in eq 15. Since the model of active mass of reacting species vis-à-vis, their Eᵣ becomes a foundational mechanism for an initial success of reaction. So, Gt, which initially holds the highest potential energy, does not undergo in the reacting mode. Hence, its few molecules gain Eᵣ and take part in the reaction, and hence this kinetic model of Gt → GtO → GO → f – (SA)GO becomes most relevant and self-explanatory. Gt → GtO is a slow reaction, which infers the activation of the Gt molecule with time and initiates the reaction with a specific reaction rate. Thus, there is a very complex change in the methods of Gt → GtO and GtO → GO, where, for the latter, the chemical energy is used, while in the former case, the sonochemical energy is applied. Therefore, Eᵣ acquiring the ability of Gt, GtO, GO, and f-(SA)GO is not the same, and hence they get activated with different times and reaction rates. Therefore, k₁, k₂, and k₃ are most relevant for applying the RRKM theory (eq 15). For Gt → GtO, the conversion chemical potential is applied as

$$\mu = \mu^0 + RT[H_2SO_4]$$

$$\mu = \mu^0 + RT[H_3PO_4]$$

$$\mu = \mu^0 + RT[KMnO_4]$$

For GO → f – (SA)GO, functionalization is accomplished by sonochemical energy as

$$E = h\theta or \frac{h\nu}{\lambda}$$

Hence,

Gt → GtO → GO → f – (SA)GO

where k₁, k₂, and k₃ are rate constants for each step of reactions noted as

$$r_1 = k_1 = \frac{[GtO]}{[Gt]}, \quad r_2 = k_2 = \frac{[GO]}{[GtO]}, \quad r_3 = k_3 = \frac{[f – (SA)GO]}{[GO]}$$

where r₁, r₂, and r₃ are rates of reactions. If the process is in equilibrium (r₁ = r₂ = r₃), then

$$k_1 \frac{[GtO]}{[Gt]} = k_2 \frac{[GO]}{[GtO]} = k_3 \frac{[f – (SA)GO]}{[GO]} \text{ (steady state)}$$

k₁ = k₂ = k₃ connected to free energy due to bond breaking, making the mechanism

$$\Delta G = -RT \ln k_1 \text{ or } \Delta G = -2.303RT \log k$$

R is the gas constant, T is the temperature in Kelvin, and k is the equilibrium constant. The yield of f-(SA)GO at standardized reaction conditions is 38 mg in 15 min (900 s) at 45 °C (45 + 273.15 = 318.15 K). Hence, a calculated rate of reaction is noted as

$$r = \frac{dn}{dt} = \frac{m_2 - m_1}{t_2 - t_1} = 0.04222 \text{ mg s}^{-1} = k$$

By putting the k value in eq 18 gives ΔG = 8.3741 kJ/mol. The positive ΔG value infers GO functionalization and is a nonspontaneous process. The Arrhenius equation is applied for the calculation of Eₐ as

$$K_{eq} = Ae^{-\frac{E_a}{RT}}$$

where Kₑₒₗₑₚₑ₉ is the rate constant at equilibrium, and A is the frequency factor. By applying eq 20, a further calculation is noted as log Kₑₒₗₑₚₑ₉ ≈ kT and connects the reaction mechanism to constitutionally make up of GtO → GO → f – (SA)GO by surface-induced oscillation, which raises the temperature to 45 °C. However, a reaction was conducted by inducing the USE at 30 kHz, which directly connects to constitutional makeup and nature of the arrangement and linkage in Gt → GtO → GO → f – (SA)GO. ln Kₑₒₗₑₚₑ₉ = ln A – Eₐ/RT is the most operative tool or mechanism to modulate an output or yield of products. The sequential steps k₁, k₂, and k₃ are defined and modulated through the distribution of average thermal energy (E = 1/kT = 0.014 eV, where k is the Boltzmann constant) and the cavitation caused by USE of 30 kHz, which are equilibrated to induce atomic oscillations supported by causing a favorable bond twisting to break them to facilitate the sequence of the modular reaction mechanism. The USE at 30 kHz was calculated with the equation E = hθ, where h is Planck’s constant, θ is the frequency, and E = 1.24 × 10⁻¹⁰ eV. Elaborating eq 20 for calculation of Eₐ, hence

$$\ln k = \ln A – \frac{E_a}{RT} \text{ or } \ln k = -\frac{E_a}{R} \left(\frac{1}{T}\right) + \ln A \text{ or } E_a$$

$$= (\ln A – \ln k)RT$$

where ln A = ln k/R and the slope has been formulated as

$$\frac{E_a}{R} = (\ln k – \ln A) \times T.$$ The graphical representation of the Arrhenius equation for f-(SA)GO is shown in Figure 12.

![Figure 12. Representation of Arrhenius constant (ln A).](Image)
The intercept $\ln A$ is obtained by plotting a slope between $\ln k$ versus $1/T$ (K$^{-1}$) or $(1/318.15 = 0.0031431$ K$^{-1}$). From eq 19, calculated $k = 0.04222$; therefore, $\ln k = -0.31648$. Putting all the values in eq 21 gives $E_a = -2.65 \times 10^6$ J mol$^{-1}$ or $-2.65 \times 10^3$ kJ mol$^{-1}$. Therefore,

$$\Delta H = E_a - 2RT = -2.66 \times 10^6 \text{ J mg}^{-1}$$

and

$$\Delta S = \frac{\Delta H - \Delta G}{T} = -8.372 \times 10^3 \text{ J K}^{-1}$$

Hence

$$T\Delta S = \Delta H = -2.66 \times 10^6 \text{ J mg}^{-1}$$

$E_a$ for GO functionalization infers a thermodynamically controlled reaction as a rate of reaction on increasing the temperature by ultrasound methods. The sp² and sp³ GO functionalization with SA generates so many suborders, which could also neutralize intermolecularly, leading to more order to have more −ve entropy. This is supported by the least value of $\Delta S = -8.372 \times 10^3$ J K$^{-1}$. However, the sp² and sp³ GO functionalization with SA confirmed was by Raman spectroscopy (Figure 1) with variation in their D/G ratio. The dipolar solvents do not destabilize ISF to form an ester, as it does not have stronger dipolar interactions, while contrary in H2O. The value of 30 kHz seems adequate to activate ethanol in the microwaves form, which can easily enter or interact with intersheet forces. The solvent, which has the highest friction ability, could yield good exfoliation and ester formation as an intermediate due to robust percolating ability. The reaction mixture was centrifuged at 6000 rpm for 10 min, the supernatant was decanted away to remove the unreacted SA, and the solid coagulates were washed and centrifuged twice with absolute ethanol (10 mL each time). f-(SA)GO was dried at RT then under vacuum for 48 h at RT at 1.05 bar. The GO functionalization with SA is analyzed with various analytical techniques. The functionalized nanosheets are to be reported yet, and hence we initiated this process with the dipolar SA molecule.

4.5. Analysis and Characterization. Synthesis of GO and f-(SA)GO were made by using an REMI 1MLH magnetic stirrer with the hot plate and Oscar ultrasonic (Microclean-103). GO was purified by using an Eltek centrifuge (MP 400). The functional activities over GO and f-(SA)GO surfaces are analyzed with a Perkin Elmer spectrometer (version 10.00.00). The hyperchromic and bathochromic shifts for f-(SA)GO were measured with an EXSTAR TG/DTA 7300 analyzer in N2 liquid. The X-ray diffraction spectra are recorded with MiniFlex Rigaku. The topographical and morphological analysis is carried out by using Park System XE-70 AFM and Carl Zeiss Evo-18 SEM, respectively. HRTEM data along with SAED patterns are collected with FEI Model Tecnai G2 S intermediate, so ethanol efficiently exfoliated GtO into GO and the carboxylic group (O═C−OH) of GO to form an ester as an intermediate through in situ condensation mechanism (Scheme 4), which is most feasible for nucleophilic substitution with free −NH₂ group of the SA and functionalized with GO as stable amide bond (Scheme 5). In situ exfoliation occurs as the H2O is in situ generated, which was verified by Karl Fischer testing. Ethanol was used to make the solvent as its higher percolation power and in situ ester formation as an intermediate, which is proven by its boiling point of ~78 °C and surface tension of ~22 N/m, respectively, than H2O, having stronger cohesive force. For a biological system, the most significant property of functionalization and most suitable for a biological system for anchoring activities, making GO and f-(SA)GO as an ideal nanomaterial.

**Scheme 4. EtOH-Assisted In Situ Ester Formation as an Intermediate**

![Scheme 4](image-url)
concentrations of GO and f-(SA)GO solution in a 1:1 ratio. Scavenging activity, pure DPPH solution was mixed with various dilutions of drugs to evaluate their free radical scavenging activities with DPPH as reference. The 0.1 mM DPPH absorbance was read on a plate reader at 540 nm with 690 nm reference. Percentage of growth was calculated on a comparative humidity for 24 h prior to adding of testing drugs. Testing drugs (100 mg/mL) were initially solubilized in DMSO and were diluted up to 1 mg/mL using water and stored in frozen prior to use. At a time of drug addition, an aliquot of frozen concentrate (1 mg/mL) was thawed and diluted to 100, 200, 400, and 800 μg/mL with a medium containing the test article. An aliquot of 10 μL of each dilution was added to each microtiter well. An aliquot of 10 μL of the various diluted drugs was supplied to microtiter wells, which were measured at 520 nm. The respective scavenging activities were determined with the following equation:

\[
\text{scavenging activity (%) } = \frac{A_D - A_S}{A_D} \times 100
\]

Now, these samples were kept in the dark after vigorous shaking to incubate for 1 h. The scavenging activity was measured as comparatively % decrease of absorbance of pure DPPH at λ = 520 nm with a 1:1 ratio of DPPH and samples mixtures at a similar wavelength. The respective scavenging activities were determined with the following equation:

\[
\text{scavenging activity (%) } = \frac{A_D - A_S}{A_D} \times 100
\]

A_D and A_S are absorbances of DPPH and samples, respectively, which were measured at 520 nm.

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: mansingh50@hotmail.com. Tel: +91-079-23260210. Fax: +91-079-23260076.*

**ORCID**

Man Singh: 0000-0002-0706-3763

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Authors are thankful to the Central University of Gujarat, India for infrastructural support. Dr. Vasant Sathe, UGC-DAE CSR Indore, India is acknowledged for providing the Raman facility. Dr. Jyoti A. Kode, ACTREC, Tata Memorial Centre, Mumbai, India is acknowledged for providing the in vitro cytotoxicity screening facility.

**REFERENCES**

(1) Geim, A. K.; Novoselov, K. S. The rise of graphene. Nat. Mater. 2007, 6, 183–191.

(2) Georgakilas, V.; Otyepka, M.; Bourlinos, A. B.; Chandra, V.; Kim, N.; Kemp, K. C.; Hobza, P.; Zboril, R.; Kim, K. S. Functionalization of graphene: covalent and non-covalent approaches, derivatives and applications. Chem. Rev. 2012, 112, 6156–6214.

(3) Dai, L. Functionalization of graphene for efficient energy conversion and storage. Acc. Chem. Res. 2013, 31, 42–49.

(4) Park, J.; Yan, M. Covalent functionalization of graphene with reactive intermediates. Acc. Chem. Res. 2013, 46, 181–189.

(5) Chng, E. L. K.; Pumera, M. The toxicity of graphene oxides: dependence on the oxidative methods Used. Chem. – Eur. J. 2013, 19, 8227–8235.

(6) Yin, S.; Chen, P.; Sun, H.; Sun, K.; Wu, Y.; Shi, C.; He, Y.; Fu, Y.; Guo, X. Fabrication of the graphene honeycomb structure as a scaffold for the study of cell growth. New J. Chem. 2018, 42, 6299–6304.

(7) Pinto, A. M.; Gonçalves, C.; Sousa, D. M.; Ferreira, A. R.; Moreira, J. A.; Gonçalves, I. C.; Magalhães, F. D. Smaller particle size...
and higher oxidation improves biocompatibility of graphene-based materials. Carbon 2016, 99, 318–329.

(8) Kumar, A. M.; Suresh, B.; Ramakrishna, S.; Kim, K.-S. Biocompatible responsive polypropylene/GO nanocomposite coatings for biomedical applications. RSC Adv. 2015, 5, 99866–99874.

(9) Hasanzadeh, M.; Mokhtari, F.; Shadjou, N.; Eftekhar, A.; Moharrame, A.; Jouybar-Gharamaleki, V.; Mahboob, S. Poly arginine-graphene quantum dots as a biocompatible and non-toxic nanocomposite: layer-by-layer electrochemical preparation characterization and non-invasive malondialdehyde sensory application in exhaled breath condensate. Mater. Sci. Eng., C 2017, 75, 247–258.

(10) Barua, S.; Chattopadhyay, P.; Phukan, M. M.; Konwar, B. K.; Islam, J.; Karak, N. Biocompatible hyperbranched epoxy/silver−reduced graphene oxide-curcumin nanocomposite as an advanced antimicrobial material. RSC Adv. 2014, 4, 47797–47805.

(11) Barahuei, F.; Saiulfah, B.; Dorniani, D.; Fukaruzi, S.; Karthihaswan, G.; Hussein, M. Z.; Elfigh, F. M. Graphene oxide as a nanocarrier for controlled release and targeted delivery of an anticancer active agent chlorogenic acid. Mater. Sci. Eng., C 2017, 74, 177–185.

(12) Zhang, H.; Grüner, G.; Zhao, Y. Recent advancements of graphene in biomedicine. J. Mater. Chem. B 2013, 1, 2542–2567.

(13) Shi, S.; Chen, F.; Ehlerding, E. B.; Cai, W. Surface engineering of graphene-based nanomaterials for biomedical applications. Bioconjugate Chem. 2014, 25, 1609–1619.

(14) Pattnaik, S.; Swain, K.; Lin, Z. Graphene and graphene-based nanomaterials: biomedical applications and biosafety. J. Mater. Chem. B 2016, 4, 7813–7831.

(15) Gulzar, A.; Xu, J.; Yang, D.; Xu, L.; He, F.; Gai, S.; Yang, P. Nano-graphene oxide-UCNP-Ce6 covalently constructed nanocomposites for NIR-mediated bioimaging and PTT/PDT combinatorial therapy. Dalton Trans. 2018, 47, 3931–3939.

(16) Zhang, W.; Zhao, K.; Banks, C. E.; Zhang, Y. Antibody-modified hydroxyapatite surfaces for the efficient capture of bladder cancer cells in a patient’s urine without recourse to any sample pre-treatment. J. Mater. Chem. B 2017, 5, 8125–8132.

(17) Kostarelos, K.; Novoselov, K. S. Materials science. exploring the interface of graphene and biology. Science 2014, 344, 261.

(18) Kavitha, T.; Abbhi, S. I. H.; Park, S.-Y. pH-Sensitive nanocarrier based on smart polymer functionalized graphene oxide for site-specific drug delivery. Phys. Chem. Chem. Phys. 2013, 15, 5176–5185.

(19) Kenry, K.; Lim, Y.-B.; Nai, M. H.; Cao, J.; Lim, C. T. Chemical properties and biocompatibility. Inorganic/organic interpenetrating hydrogels with excellent mechanical properties and biocompatibility. ACS Omega 2019, 4, 16385–16401.

(20) Teo, W. Z.; Cheng, E. L. K.; Sofer, Z.; Pumera, M. Cytotoxicity of halogenated graphene nanosheets. Nanoscale 2014, 6, 1173–1180.

(21) Dong, H.; Li, Y.; Yu, J.; Song, Y.; Cai, X.; Liu, J.; Zhang, J.; Evring, R. C.; Shi, D. A versatile multicomponent assembly on graphene via β-cyclodextrin host-guest chemistry on graphene for biomedical applications. Small 2013, 9, 446–456.

(22) Chen, J.; Shi, X.; Ren, L.; Wang, Y. Graphene oxide/PVA inorganic/organic interpenetrating hydrogels with excellent mechanical properties and biocompatibility. Carbon 2017, 111, 18–27.

(23) Duan, G.; Kang, S.-g.; Tian, X.; Garate, J. A.; Zhao, L.; Ge, C.; Zhou, R. Protein corona mitigates the cytotoxicity of graphene oxide by reducing its physical interaction with cell membrane. Nanoscale 2015, 7, 15214–15224.

(24) Zhang, P.; Wang, H.; Zhang, X.; Xu, W.; Li, Y.; Li, Q.; Wei, G.; Su, Z. Graphene film doped with silver nanoparticles: self-assembly formation, structural characterizations, antibacterial ability, and biocompatibility. Biomater. Sci. 2015, 3, 852–860.

(25) Sayyar, S.; Murray, E.; Thompson, B. C.; Gambhir, S.; Officer, D. L.; Wallace, G. C. Covalently linked biocompatible graphene/polycaprolactone composites for tissue engineering. Carbon 2013, 52, 296–304.

(26) Hirsch, A.; Englert, J. M.; Hauke, F. Wet chemical functionalization of graphene. Acc. Chem. Res. 2012, 46, 77–86.

(27) Johns, J. E.; Hersam, M. C. Atomic covalent functionalization of graphene. Acc. Chem. Res. 2012, 46, 77–86.

(28) Xu, H.; Suslick, K. S. Sonocchemical preparation of functionalized graphenes. J. Am. Chem. Soc. 2011, 133, 9148–9151.

(29) Marcano, D. C.; Kosynkin, D. V.; Berlin, J. M.; Sinitskii, A.; Sun, Z.; Silesave, A.; Alemany, L. B.; Lu, W.; Tour, J. M. Improved synthesis of graphene oxide. ACS Nano 2010, 4, 4086–4104.

(30) Clause, A.; Plass, R.; Bohem, H.-P.; Hofmann, U. Untersuchung zur Struktur des graphitoxid. Z. Anorg. Allg. Chem. 1957, 291, 205–220.

(31) Scholz, W.; Boehm, H. P. Untersuchung am graphitoxid. VI. betrachtungen zur struktur des graphitoxid. Z. Anorg. Allg. Chem. 1969, 369, 327–340.

(32) Kenry, Lim, C. T. Biocompatibility and nanotoxicity of layered two-dimensional nanomaterials. ChemNanoMat 2017, 3, 5–16.

(33) Majeed, W.; Bourdo, S.; Petibone, D. M.; Saini, V.; Yang, K. B.; Nima, Z. A.; Alghazali, K. M.; Darrigues, E.; Ghosh, A.; Watanabe, F.; Casciano, D.; Ali, S. F.; Biris, A. S. The role of surface chemistry in the cytotoxicity profile of graphene. J. Appl. Toxicol. 2017, 37, 462–470.

(34) Crisan, L.; Crisan, B.; Soritau, O.; Baciu, M.; Biris, A. R.; Baciu, G.; Lucaciu, O. In vitro study of biocompatibility of a graphene composite with gold nanoparticles and hydroxyapatite on human osteoblasts. J. Appl. Toxicol. 2015, 35, 1200–1210.

(35) Bitounis, D.; Ali-Boucetta, H.; Hong, B. H.; Min, D.-H.; Kostarelos, K. Prospects and challenges of graphene in biomedical applications. Adv. Mater. 2013, 25, 2258–2268.

(36) Farshid, B.; Lalwani, G.; Sitharaman, B. In vitro cytocompatibility of one-dimensional and two-dimensional nanostructure-reinforced biodegradable polymeric nanocomposites. J. Biomed. Mater. Res., Part A 2015, 103, 2309–2321.

(37) Zhang, H.; Peng, C.; Yang, J.; Lv, M.; Liu, R.; He, D.; Fan, C.; Huang, Q. Uniform ultrasmall graphene oxide nanosheets with low cytotoxicity and high cellular uptake. ACS Appl. Mater. Interfaces 2013, 5, 1761–1767.

(38) Liu, X.; Ma, D.; Tang, H.; Tan, L.; Xie, Q.; Zhang, Y.; Ma, M.; Yao, S. Polymidoamine dendrimer and oleic acid-functionalized graphene as biocompatible and efficient gene delivery vectors. ACS Appl. Mater. Interfaces 2014, 6, 8173–8183.

(39) Li, D.; Meng, D.; Yu, Z.; Liu, W.; Zhou, G.; Li, W.; Wang, X.; Yang, D.-P.; Zhang, W. Biocompatible and stable GO-coated Fe3O4 nanocomposite: A robust drug delivery carrier for simultaneous tumor MR imaging and targeted therapy. ACS Biomater. Sci. Eng. 2018, 4, 2143–2154.

(40) Zhuang, W.; He, L.; Wang, K.; Ma, B.; Ge, L.; Wang, Z.; Huang, J.; Wu, J.; Zhang, Q.; Ying, H. Combined adsorption and covalent linking of paclitaxel on functionalized nano-graphene oxide for inhibiting cancer cells. ACS Omega 2018, 3, 2396–2405.

(41) M.; Sousa, M.; Visani, L. A.; Fosso, L. C.; Giorgi, Alves, O. L. Folic acid-functionalized graphene oxide nanocarrier: synthetic approaches, characterization, drug delivery study and antitumor screening. ACS Appl. Nano Mater. 2018, 1, 922–932.

(42) Xu, L. Q.; Yang, W.; Neoh, K.-G.; Kang, E.-T.; Fu, G. D. Dopamine-induced reduction and functionalization of graphene oxide nanosheets. Macromolecules 2010, 43, 8336–8339.
(47) Hashemi, H.; Namazi, H. Sonochemically synthesized blue fluorescent functionalized graphene oxide as a drug delivery system. Ultrason. Sonochem. 2018, 42, 124–133.

(48) Shen, B.; Zhai, W.; Lu, D.; Wang, J.; Zheng, W. Ultrasound-sonication-assisted direct functionalization of graphene with macromolecules. RSC Adv. 2012, 2, 4713–4719.

(49) Rubio, N.; Au, H.; Leese, H. S.; Hu, S.; Clancy, A. J.; Shaffer, M. S. P. Grafting from versus grafting to approaches for the functionalization of graphene nanoplatelets with poly (methyl methacrylate). Macromolecules 2017, 50, 7070–7079.

(50) Maktedar, S. S.; Avashthi, G.; Singh, M. Understanding the significance of O-doped graphene towards biomedical applications. RSC Adv. 2016, 6, 114264–114275.