Enhanced Apoptosis by Functionalized Highly Reduced Graphene Oxide and Gold Nanocomposites in MCF-7 Breast Cancer Cells

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ABSTRACT: Graphene nanocomposites have gained significant interest in a variety of biological applications due to their unique properties. Herein, we have studied the apoptosis-inducing ability and anticancer properties of functionalized highly reduced graphene oxide (HRG) and gold nanoparticles (Au NPs)-based nanocomposites (AP-HRG-Au). Samples were prepared under facile conditions via simple stirring and ultrasonication. All the samples were tested for their anticancer properties against different human cancer cell lines including lung (A549), liver (HepG2), and breast (MCF-7) cancer cells using doxorubicin as a positive control. In order to enhance the solubility and bioavailability of the sample, HRG was functionalized with 1-aminopyrene (1-AP) as a stabilizing ligand. The ligand also facilitated the homogeneous growth of Au NPs on the surface of HRG by offering chemically specific binding sites. The synthesis of nanocomposites and the surface functionalization of HRG were confirmed by UV−Vis, powder X-ray diffraction, and Fourier transform infrared spectroscopy. The structure and morphology of the as-prepared nanocomposites were established by high-resolution transmission electron microscopy. Because of the functionalization, the AP-HRG-Au nanocomposite exhibited enhanced physical stability and high dispersibility. A comparative anticancer study of pristine HRG, nonfunctionalized HRG-Au, and 1-AP-functionalized AP-HRG-Au nanocomposites revealed the enhanced apoptosis ability of functionalized nanocomposites compared to the nonfunctionalized sample, whereas the pristine HRG did not show any anticancer ability against all tested cell lines. Both HRG-Au and AP-HRG-Au have induced a concentration-dependent reduction in cell viability in all tested cell lines after 48 h of exposure, with a significantly higher response in MCF-7 cells compared to the remaining cells. Therefore, MCF-7 cells were selected to perform detailed investigations using apoptosis assay, cell cycle analysis, and reactive oxygen species measurements. These results suggest that AP-HRG-Au induces enhanced apoptosis in human breast cancer cells.

1. INTRODUCTION

Nowadays, increasing cases of multidrug resistant diseases are a cause of serious concern among scientists and medical professionals, which demands immediate solutions, including the development of novel therapeutic agents.1,2 Among these ailments, cancer is one of the deadliest diseases requiring a challenging task of developing innovative therapeutic strategies.3 Besides resistance to the drugs, the therapeutic treatment of cancer also involves a major obstacle, which is the development of undesired side effects and complications. In this regard, the strategy for inducing apoptosis using novel therapeutic agents has gained considerable attention in cancer treatment.4 Over the past decades, a variety of nanomaterials have shown great potential in clinical studies as anticancer drugs, drug carriers, or diagnostic tools.5,6 From the diverse range of nanomaterials, graphene nanocomposites have gained specific attention due to the excellent physicochemical properties of graphene like the large surface area, superb

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electrical and electronic properties, and its unique two-dimensional geometry, which offer flexible platform for the immobilization of various substances, including drugs, biomolecules, etc.\textsuperscript{7−9}

So far, various studies have highlighted the potential applications of graphene-based materials in cancer therapy, including the benefits of functionalized graphene-based nanocomposites for specific targeting of affected cells to overcome drug resistance and minimize the side effects.\textsuperscript{10} Especially, the fabrication of graphene nanocomposites involving metallic nanoparticles remarkably enhances the anticancer properties of the material due to the synergistic effect and unique physicochemical properties of the combined materials.\textsuperscript{11} Recently, metallic nanoparticles comprising silver and gold have been incorporated on the surface of graphene to attain graphene nanocomposites for unique biological applications like bio-imaging and cancer detection and therapy.\textsuperscript{12,13} Particularly, gold offers several benefits over other nanoparticles owing to its distinctive physicochemical properties like surface plasmon resonance (SPR) and the capability to bind a variety of biomolecules.\textsuperscript{14−16} However, metallic nanoparticle (NP)-based graphene composites generally suffer from aggregation owing to the strong van der Waals interactions, low density, and inhomogeneous distribution of inorganic nanoparticles on the surface, which adversely affect their properties for potential applications.\textsuperscript{17} This is typically avoided through the surface functionalization of graphene.\textsuperscript{18}

Commonly, graphene oxide (GO), an oxidized derivative of graphene, has been one of the most promising carbonaceous materials for cancer therapy due to its versatile surface chemistry, which allows easy functionalization of materials and high water dispersibility.\textsuperscript{19,20} However, the random presence of a large number of oxygenated groups on the surface of GO limits the prospects of homogeneous and controlled functionalization of the material, which is beneficial in extracting the full potential of graphene-based materials in biological applications.\textsuperscript{21} To overcome this, another derivative of graphene, such as highly reduced graphene oxide (HRG), can be applied, which consists of similar graphitic domains, defects, and residual oxygenated groups on the surface but in far lesser number than GO.\textsuperscript{22} The surface properties of HRG can also be further improved through both covalent and noncovalent functionalization to avoid agglomeration and enhance biological properties including the cellular uptakes, etc.\textsuperscript{23}

Compared to covalent functionalization, which may disrupt the sp\textsuperscript{2} structure of the graphene lattice, noncovalent functionalization does not disrupt the structure and retains the electronic properties of the material while simultaneously introducing new chemical groups on the surface.\textsuperscript{24,25} Noncovalent functionalization of HRG composites through π−π interactions, hydrogen bonding, van der Waals forces, etc. leads to enhanced dispersibility, binding capacity, biocompatibility, and reactivity of the resulting materials.\textsuperscript{26} Among various methods, noncovalent functionalization of HRG through π−π interactions using polycyclic aromatic “π−π stacking” molecules, such as pyrene, anthracene, and coronene as stabilizing agents, has gained interest recently.\textsuperscript{27−29} In our previous study, we have developed a novel approach of employing a hard soft acid base concept to prepare HRG palladium (Pd) nanocomposites (HRG-Pd) using 1-aminopyrene (1-AP) as a stabilizing agent.\textsuperscript{30} In this case, 1-AP not only stabilized the surface of HRG through noncovalent π−π interactions but also facilitated the homogeneous nucleation and growth of Pd NPs on the surface of HRG.

In this work, we demonstrate the effect of functionalization on the biological properties of graphene and its resulting

![Scheme 1. Graphical Representation of the Preparation of Functionalized (AP-HRG-Au) and Nonfunctionalized (HRG-Au) Graphene-Gold Nanocomposites and Their Anticancer Properties](https://doi.org/10.1021/acsomega.1c01377)
nanocomposite. For this purpose, we have applied the concept of noncovalent functionalization to homogeneously anchored Au NPs on the surface of HRG. To study the benefits of noncovalent functionalization in biological applications, the in vitro anticancer activity of the as-prepared 1-AP-functionalized HRG and gold (Au) NP-based nanocomposites (AP-HRG-Au) is obtained (Scheme 1). In addition, for the purpose of comparison, the anticancer properties of pristine HRG, functionalized AP-HRG-Au, and nonfunctionalized HRG-Au nanocomposites have also been investigated. All the samples, namely, HRG, HRG-Au, and AP-HRG-Au, were screened for their antiproliferative effects using an MTT assay against lung (A549), liver (HepG2), and breast (MCF-7) cancer cell lines using doxorubicin as a positive control. To the best of our knowledge, graphene gold nanocomposites have been rarely investigated as therapeutic agents in cancer treatment, and moreover, studies about the benefits of controlled distribution of NPs on the surface substrates in biological applications have not been reported so far.

2. RESULTS AND DISCUSSION

Typically, the agglomeration of nanoparticles in biological systems seriously alters their mobility, size, and surface properties, often complicating their phase behavior and biodistribution. Agglomeration of nanoparticles results in the formation of slow or even immobile materials, which promotes their local accumulation and can significantly affect their overall cellular uptake. Aggregation of nanoparticles is typically inhibited through various surface functionalization approaches. In this study, to achieve nonaggregated, homogeneously distributed gold NPs on the surface of graphene, polycyclic aromatic hydrocarbon, namely, 1-aminopyrene (1-AP), is used, which noncovalently functionalizes the sample through π–π interactions. In this case, 1-AP has displayed a dual role, and not only has it stabilized the surface of HRG but its suspended amino group (NH2) also provided active sites for the homogeneous nucleation and growth of gold NPs on the surface of HRG. The biological application of surface-functionalized AP-HRG-Au is demonstrated through a study of anticancer properties of the sample against various cell lines. To investigate the effect of functionalization on its biological properties, the anticancer properties of the functionalized sample (AP-HRG-Au) was compared with its nonfunctionalized counterpart (HRG-Au) and pristine HRG.

Initially, the effect of functionalization of HRG on the dispersion quality of the resulting AP-HRG-Au is assessed and compared with other two samples including pristine HRG and nonfunctionalized HRG-Au. To do this, dispersions of the samples were prepared via sonication by taking 5 mg of each sample in 10 mL of deionized water. Among all the samples, functionalized AP-HRG-Au demonstrated superior dispersion quality, as the dispersion remained stable even after several hours. However, pristine HRG and HRG-Au exhibited poor dispersibility, as the samples settled down after some time (cf. Figure 1).

The functionalization of HRG with 1-AP and the formation of HRG-Au and AP-HRG-Au were initially confirmed by UV analysis. For this, the UV–vis spectra of pristine 1-aminopyrene (1-AP), HRG, Au-HRG, and AP-HRG-Au were compared (cf. Figure 2). 1-AP exhibits three characteristic absorption peaks at ~242, ~285, and 360 nm (black line, Figure 2), while the characteristic peak of HRG appears at ~270 nm (red line, Figure 2). All peaks of 1-AP and HRG exist in AP-HRG-Au (green line, Figure 2), which confirms the successful functionalization of HRG with 1-AP. In addition, the spectrum of AP-HRG-Au also exhibits an absorption peak at ~540 nm, which is a typical SPR peak of gold nanoparticles, and this points toward the formation of a functionalized HRG-Au nanocomposite. On the other hand, the spectrum of nonfunctionalized HRG-Au (blue line, Figure 2) only exhibits characteristic peaks of HRG and Au NPs. Similarly, the successful functionalization and formation of nanocomposites have also been confirmed by FT-IR spectroscopy by comparing the respective IR spectra of 1-AP, HRG, HRG-Au, and AP-HRG-Au, as shown in Figure 3. The FT-IR spectra of pristine 1-AP (black line, Figure 3) and AP-HRG-Au (green line, Figure 3) closely resemble each other, as majority of IR peaks belonging to 1-AP, such as the IR peaks corresponding to the aromatics of 1-AP between 800 and 1700 cm\(^{-1}\), are also present in the AP-HRG-Au spectrum. Meanwhile, the corresponding IR spectrum of HRG-Au (blue line, Figure 3) demonstrates different characteristics due to the absence 1-AP
in the composite. This clearly confirms the functionalization of HRG with 1-AP in AP-HRG-Au.

In addition, XRD analysis also indicated the formation of HRG-Au and AP-HRG-Au nanocomposites besides confirming the crystallinity and phase purity of the samples. The XRD patterns of HRG, HRG-Au, and AP-HRG-Au are shown in Figure 4. Pristine HRG typically exhibits a broad reflection at 22.4° (red line, Figure 4), which is present in the XRD patterns of both HRG-Au (blue line, Figure 4) and AP-HRG-Au (green line, Figure 4). Apart from this reflection, both nanocomposites also demonstrate various reflections in their corresponding XRD patterns, which are similar to the XRD reflections of pure gold nanoparticles. For example, the XRD peaks at 38° (111), 44° (200), 64° (220), and 77° (311) represent Bragg’s reflections of the cubic structure of metallic gold (JCPDS no. 04-0784). The mean size of Au NPs on the surface of HRG in AP-HRG-Au was found to be 3.27 ± 0.02 nm (Table 1), which was calculated using the Debye–Scherer’s equation

\[ d = \frac{\lambda}{\beta \cos \theta} \]

where “hkl” are the Miller indices and “a” is edge lengths.

The size and morphology of Au NPs on the surface of HRG in AP-HRG-Au were determined by HRTEM (high-resolution transmission microscopy), as shown in Figure 5a−c. The prepared composites were subjected to HRTEM analysis by dispersing the prepared composites in hexane and using a sonicator water bath, and then, the dispersed solution was dropped on the TEM grid, which was dried for 6 h at 60 °C in an oven. The size ( <4 nm) of spherical-shaped Au NPs can be seen on the surface of HRG in AP-HRG-Au. These NPs are homogeneously and densely distributed on the surface of HRG, which is facilitated by the dual function of 1-AP including the stabilization of HRG and promotion of nucleation and growth of Au NPs. The π−π interactions mediated noncovalent functionalization of HRG involving the pyrenyl ring of 1-AP and the basal plane of HRG, which largely prevented the aggregation of HRG nanosheets, whereas the amino group offered active nucleation sites for the effective growth of NPs. This resulted in considerable enhancement in dispersibility of the nanocomposite (AP-HRG-Au) nanocomposite.

### 2.1. Anticancer Activity

To compare the biological activity of as-prepared HRG, nonfunctionalized HRG-Au, and functionalized AP-HRG-Au, an MTT assay was performed, which assessed the reduction in cell viability by nanocomposites. It was observed that both HRG-Au and AP-HRG-Au induced a concentration-dependent reduction in cell viability in all tested cell lines including lung (A549), liver (HepG2), and breast (MCF-7). However, after 48 h of exposure, a significantly higher response was observed in MCF-7 cells compared to other cells. Notably, HRG did not display any activity against all tested cells, which may be due to the low

![Figure 3. FT-IR spectra of 1-AP, pristine HRG, nonfunctionalized HRG-Au, and 1-AP-functionalized AP-HRG-Au nanocomposites.](image)

![Figure 4. XRD patterns of pristine HRG, nonfunctionalized HRG-Au, and 1-AP-functionalized AP-HRG-Au nanocomposites.](image)

![Table 1. XRD Results and Crystallographic Data of the Au Nanoparticles Existing in the AP-HRG-Au Nanocomposite](table)

| 2θ (°) | FWHM (°) | hkl | a (nm) | sin θ (radian) | cos θ (radian) | d (nm) | D (nm) |
|--------|----------|-----|--------|-------------|---------------|--------|--------|
| 38.1   | 2.6      | 111 | 0.4    | 0.326443    | 0.9452        | 0.24   | 3.22   |
| 44.3   | 2.9      | 200 | 0.4    | 0.360957    | 0.89263       | 0.20   | 3.28   |
| 64.5   | 3.2      | 220 | 0.4    | 0.5334254   | 0.845847      | 0.14   | 3.29   |
| 77.5   | 3.2      | 311 | 0.4    | 0.6257055   | 0.780059      | 0.12   | 3.22   |
| 81.6   | 2.6      | 222 | 0.4    | 0.6536024   | 0.756838      | 0.12   | 3.32   |

*Note: X-ray wavelength \( \lambda \) (nm) = 0.15406.*
dispersibility of the sample in aqueous solution and/or due to the absence of gold NPs. The IC_{50} (concentration reduced the viability of the cells to half) values are shown in Table 2. The dose–response curve for HRG-Au and AP-HRG-Au are presented in Figure 6. Since MCF-7 cells were the most responsive to HRG-Au and AP-HRG-Au, therefore it was selected for the remaining assays.

2.2. HRG-Au- and AP-HRG-Au-Induced Cell Cycle Arrest. In order to determine whether cell growth inhibition was associated with cell cycle arrest, cell cycle analysis was performed against MCF-7 cells. Upon treatment with AP-HRG-Au and HRG-Au, the percent of cell populations in the G1 phase increased from 50.7 to 62 and 55.1%, respectively. This increase was accompanied by a decrease in the percentage in the phases of G2M and S cell population for AP-HRG-Au and HRG-Au, respectively (Figure 7).

2.3. Apoptosis Detection. Flow cytometry was further employed to quantify apoptotic or necrotic death after treatment with AP-HRG-Au and HRG-Au for 48 h. As shown below (Figure 8), approximately 12.2% of the cells stained positive for apoptosis events (5% early apoptosis, ±7.2 late apoptosis) after AP-HRG-Au treatment. Cell treatment with HRG-Au also caused changes in the percent of cells undergoing apoptosis compared to the control. A necrotic cell death was also observed after treatment with both particles (Figure 8).

2.4. AHRG and APHRG Stimulate ROS Production. Next, we examined the cellular ROS levels in MCF-7 cells to determine whether AHRG and APHRG induced oxidative stress. It was found that MCF-7 cell populations differ in fluorescence intensity. Nontreated cells displayed low ROS content, while treatment of the cells with AHRG and APHRG leads to an increase in cell number with relatively higher ROS content, which mediated cell damage and initiated apoptotic cell death (Figure 9).

3. EXPERIMENTAL DETAILS

3.1. Chemicals. Graphite powder (200 mesh) was purchased from Alfa Aesar. 1-Aminopyrene, gold(III) chloride trihydrate, concentrated sulfuric acid, potassium permanganate, sodium nitrate, hydrogen peroxide, hydrazine hydrate, and...
other organic solvents were purchased from Sigma-Aldrich and used without purification.

3.2. Preparation and Functionalization of HRG with 1-Aminopyrene. HRG was prepared according to the method presented in our previous literature.36−39 HRG (50 mg) was dispersed in 20 mL of methanol via sonication for 30 min. Separately, 1-AP (50 mg) solution was prepared in methanol (20 mL) using equivalent weight percent of HRG.
The HRG dispersion and 1-AP solution were mixed and stirred at room temperature (48 h). After this, stirring was stopped and the mixture was subjected to sonication at a lower temperature (20 °C) for 6 h. The resulting mixture was centrifuged at a lower speed (4000 rpm) for a period of 3 h to remove excess of 1-AP. To remove the remaining unadsorbed content of 1-AP, the as-obtained black powder was again dispersed in 20 mL of fresh methanol and sonicated at a lower temperature (20 °C) for 30 min. Later, the black dispersion was isolated via centrifugation (9000 rpm, 15 min), and the product was separated by simple decanting of suspension. The process continued many times until the leftover liquid turned colorless. Finally, the as-obtained solid was dried overnight in vacuum.

### 3.3. Preparation of 1-AP-Functionalized Graphene and Gold Composites (AP-HRG-Au)

AP-HRG-Au was prepared using equivalent (1:1 weight ratio) contents of freshly functionalized HRG and gold precursors. To begin with, 10 mg of HAuCl4·3H2O was dissolved in 10 mL of ethanol, and separately, 10 mg of H[AuCl4·3H2O was dissolved in 10 mL of ethanol. Both the solutions were mixed together and sonicated for 1 h, and subsequently, the product was isolated via centrifugation (9000 rpm). The resulting product was dispersed in 20 mL of deionized water for further use. HRG-Au was also prepared in a similar manner; however, in this case, nonfunctionalized HRG was used instead of functionalized HRG.

### 3.4. Characterization

The preparations of AP-HRG-Au and HRG-Au nanocomposites were confirmed by UV–vis spectrophotometry (Perkin Elmer Lambda 35 (Waltham, MA, USA)), HRTEM and EDX (JEM 2100F (JEOL, Tokyo, Japan)), FT-IR (Perkin Elmer 1000 FT-IR spectrometer) spectroscopy (Agilent (single quadrupole) MSD-5975C detector, Agilent Technologies Inc., USA); MS was acquired in EI mode (scan range: m/z 45–600, ionization energy: 70 eV)), and XRD (D2 Phaser X-ray diffractometer (Bruker, Germany), Cu Kα radiation (k = 1.5418 Å)).

### 3.5. Anticancer Activity (MTT Assay)

Pristine HRG, HRG-Au, and AP-HRG-Au were assessed for their anticancer activity in a panel of three human cancer cell lines, namely, lung (A549), liver (HepG2), and breast (MCF-7) using the MTT method as described in a previous study.40

Briefly, different concentrations of each sample were added to the cells (5 × 10^4/well) after 24 h incubation in a 96-well plate and doxorubicin was used as a positive control. After incubation for 48 h, 10 μL of the MTT solution (5 mg/mL, prepared in PBS) was added to each well. The cells were further incubated for another 4 h. Excess medium was removed and replaced by 100 μL of acidified isopropanol in each well to dissolve the formazan crystals. Optical densities were determined using a microplate spectrophotometer (BioTek, USA) at 570 nm. IC_{50} (50% inhibition of cell growth) was calculated using OriginPro 8.5 software.

### 3.6. Cell Cycle Analysis

The effects of HRG-Au and AP-HRG-Au on the cell cycle distribution of MCF-7 cells were determined by flow cytometric analysis as earlier reported.41 Briefly, treated or untreated cells were harvested and washed with cold PBS twice. Following fixation with 70% ethanol at 4 °C for 4 h, cells were stained with freshly prepared DNA-staining solution containing propidium iodide (PI, 50 μg/mL) and RNase A (100 μg/mL). Stained cells were then subjected to analysis using a flow cytometer (flow cytometry, Beckman Coulter, USA). CXP software V. 3.0 was used to determine cell phase distributions.

### 3.7. Apoptosis Assay (Annexin V-FITC/PI)

Apoptosis detection was performed according to a double stain apoptosis detection kit protocol (BioLegend, CA, USA). In brief, MCF-7 cells were cultured in six-well plates for 24 h before treatment with IC_{50}. After 24 h incubation, treated and control cells were collected and washed with cold PBS. Annexin-binding buffer (1×, 100 μL) was used to resuspend cell pellets, which were then stained with both Annexin V-FITC and propidium iodide (PI) dyes for 15 min in the dark. To this mixture, 400 μL of 1× Annexin buffer was added before analysis using a Cytomics FC500 flow cytometer (Beckman Coulter, USA). Data obtained from the flow cytometer were analyzed using CXP software V. 3.0.

### 3.8. ROS Measurement

ROS detection reagents (cat. no.: 88-5930, Invitrogen) were utilized to detect the level of ROS in MCF-7 cells. In brief, a working solution (1×) was prepared from ROS assay stain solution. MCF-7 cells were seeded in six-well plates and exposed to HRG-Au and AP-HRG-Au for 48 h. Cells were then harvested and washed twice with PBS and resuspended with 100 μL of working solution. Thereafter, cells were incubated for 60 min and fluorescence was read at 490 nm excitation and 520 nm emission using the Cytomics FC500 flow cytometer (Beckman Coulter, USA).
4. CONCLUSIONS

Herein, we have investigated the apoptosis-induced anticancer activity of 1-AP-functionalized Au-HRG nanocomposite (AP-HRG-Au). The anticancer property of the functionalized nanocomposite is also compared with those of pristine HRG and its nonfunctionalized counterpart. Functionalization of HRG enhanced the aqueous dispersibility of the sample due to the dual role of the 1-AP ligand, which effectively prevented the aggregation of HRG nanosheets and also offered specific nucleation sites for the growth of Au NPs on the surface of graphene. Due to this, functionalized AP-HRG-Au demonstrated slightly better anticancer properties when compared to the nonfunctionalized nanocomposite (HRG-Au). Flow cytometry and the results of ROS generation tests point toward the apoptosis-induced cell death by both AP-HRG-Au and HRG-Au, while the former was found to be more effective against MCF-7 cells.

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M. Khan, M.R.S., and S.F.A. designed the project. M. Khan, S.F.A., M.R.H., and M.R.S. helped to draft the manuscript. M.R.S. carried out the experimental part and some part of characterization. M. Kuniyi, and A. Almutairi carried out formal analysis. F.A.N., A.S.A., M.Z.A., and W.Q. carried out the anticancer activity. A. Alwarthan, M.R.H.S., and M.R.H. provided scientific guidance.

Notes
The authors declare no competing financial interest.

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