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Suspended graphene oxide nanoparticle for accelerated multilayer osteoblast attachment

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Abstract: Mimicking bone tissues having layered structures is still a significant challenge because of the lack of technologies to assemble osteoblast cell types into bone structures. One of the promising and attractive materials in biomedical and different engineering fields is graphene and graphene-based nanostructures such as graphene oxide (GO) because of their unique properties. In most studies, GO was synthesized using chemical vapor deposition method, and was coated on the substrate. In this study, we proposed a simple technique for assembly of cells that facilitates the construction of osteoblast-like structures using suspended GO synthesized by graphite powder, H_2SO_4, and $KMnO_4$.Toxicity effects of GO on human mesenchymal stem cells (hMSCs) derived from bone marrow were analyzed. In addition to normal MSCs, toxicity effects of GO on human cancer cell line saos-2 as an abnormal cell line that possess several osteoblastic features, was examined. The attachment and expression of osteoblast cells genes were evaluated after differentiation of MSCs to osteoblast cells in presence of suspended GO by scanning electron microscopy and real time PCR. We found that the toxicity effects of GO are dose dependent and in oseogenic medium containing suspended GO the expression level of osteoblast genes osteopontin and osteocalcin and cell adhesion markers connexin were higher than control group. Interestingly, through this method GO was found to induce multilayer osteoblast cell morphology and enhance the number of cell layer. We expect that the presented method would become a highly useful approach for bone tissue engineering. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 106A: 293–303, 2018.

Key Words: grapheme oxide, osteoblast differentiation, cell assembly, multilayer deposition

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INTRODUCTION

One of the promising and attractive materials in biomedical and different engineering fields is graphene and graphene-based nanostructures because of their unique properties.[1–3] Nano-graphene oxide (GO) is a graphene derivatives and a new class of carbon based materials in a two-dimensional honeycomb structure. One major difference between GO and graphene is the controllable hydrophilic nature of GO. The hydrophilic nature of GO is the result of many hydroxyl groups on its surface (–COOH, –C–O–C–, C–O–H, etc.) which makes it resistant to electron transfer. It was proposed for biomedical applications due to its intrinsic optical properties, small size, easy use, and large specific surface area.[4] These applications include biosensors,[5] drug/gene delivery,[6,7] and antibacterial effects.[8] In addition, GO has thermal, electronic, mechanical, and optical properties.[9–12] In addition, GO has thermal, electronic, mechanical, and optical properties. Biomaterials are used to promote cell differentiation, attachment, and proliferation. Both synthetic and natural biomaterials have been fabricated for bone regeneration therapy using stem cells.

The use of growth factors and inducers is critical for successful direct stem cells proliferation and differentiation. Carbon nanoparticles and recently two dimensional (2D) and 3D graphene have been studied for applications in regenerative medicine. Graphene has proven to be able for directing differentiation of stem cells into specific cell types, based on the type of material (graphene, GO, graphene hybrid scaffolds), as well as the type of stem cell (e.g., neural stem cells and mesenchymal stem cells).[13–15]

These naomaterials have been employed to deliver genes and growth factors into mesenchymal stem cells (MSCs) to manipulate their differentiation.[16]

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Positive effects of graphene on stem cell proliferation, adhesion, and differentiation have been previously confirmed.[17–19] Graphene has drawn attention as a substrate for stem cell culture and has been reported to stimulate the differentiation of multipotent adult stem cells. Recently, Lee and colleagues[20] reported that graphene enhances the cardiomyogenic differentiation of human embryonic stem cells at least in part, due to nano-roughness of graphene.

Nayak and co-workers reported an uncommon behavior of osteoblast cells in the presence of graphene nanoparticles.[13] They showed that graphene does not hamper the proliferation of human MSCs (hMSCs) and accelerates their differentiation into osteoblast cells.[13] Cell adhesion plays a major role for a number of cell functions such as proliferation and formation of mineral deposits[21] and depends on cell–material interface, surface topology, cell number, and time.[14] It is held that graphene can increase focal adhesion, gene efficiency, and proliferation of fibroblast cells.[22]

Recently, Bressan and colleagues[23] reported that graphene nanoparticles with different morphologies, synthesized by various methods have different effects on cell behaviors such as toxicity, proliferation and differentiation. In most previous studies graphene and GO were used in the form of sheets coated on different substrate including SiO_2/Si, gold-coated glass, conventional tissue culture plates, biodegradable polymer, and flexible polydimethylsiloxane.[24]

On the other hand, nanoparticle-decorated graphene sheets, graphene-embedded nanoparticles, and graphene-encapsulated nanoparticles are also the forms most used for biomedical applications in previous studies. Previous reports have confirmed using graphene as a cell differentiation controlling biomaterial, especially for neurogenesis and osteogenesis on 2D substrates.[25] GO has been considered as a carrier for therapeutic proteins due to its highly biocompatible and low cytoxicity effects.[26] Nayak and colleagues found an uncommon behavior of osteoblast cells using graphene coated on SiO_2/Si substrate.[13]

They used graphene coated on cell culture plate substrate by CVD method. The most common method for synthesizing and coating Fe on substrates involved the use of CVD using methane and hydrogen. GO used in the present study was synthesized using graphite powder, H_2SO_4, and $KMnO_4$. Because of the physical and chemical characteristics of GO compared to graphene such as high hydrophilic property (highly oxidized), we used GO in this research. Most studies investigating the effects of nanoparticles on cells differentiation were in forms of coated substrate, and generally, the CVD method was applied to coat nanoparticles on the substrate.

This method allows researchers to coat graphene on the foils of copper, nickel, glass, and so forth, in the form of grapheme foam. Previous reports using graphene as a cell differentiation-controlling biomaterial, especially for osteogenesis on 2D substrates have concluded that graphene induces and enhances MSCs differentiation to osteoblast.

Osteoblast tissue engineering *in vitro* is an essential process for providing artificial bone graft. Until now, the various methods have been proposed for building tissue structures. However, mimicking functional bone tissues having layered structures is still a significant challenge, mainly because of the lack in technologies to assemble multiple cell types into bone structures. In other words, one of the main challenges in the use of biomaterials is improving cell adhesion and proliferation. Because of the different behavior of normal and tumor cells, in this study, besides assessing the effects of nano GO on the differentiation and adhesion of human stem cells, we evaluated its effects on a semi-osteoblastic saos-2 tumor cell line. Our aim was to assess whether the effect of GO is dependent on the cell type as well as whether toxic doses differ in normal and tumor cells.

Several studies have also demonstrated the toxic effects of GO in tumor cells.[27] Saos-2 is a cell line derived from the primary osteosarcoma that "possess several osteoblastic features and could be useful as a permanent line of human osteoblast-like cells." Since they can be fully differentiated in a manner that the osteoblastic cells naturally do, we evaluated their cytotoxicity in different GO doses. Since cell biocompatibility is a prerequisit for the use of graphene in biomedical applications, we first studied the toxic effects of suspended GO in cell culture medium (not coated on substrate) on hMSCs and saos-2 cells. Then attachment and differentiation of hMSCs derived from bone marrow to osteoblast cells were studied in the presence of suspended GO. After 21 days, the control group was compared in terms of connexins gene expression as one of cell adhesion markers and osteogenic markers expression such as osteopontin, osteocacin and alkaline phosphatase. Scanning electron microscope (SEM) and atomic force microscopy (AFM) studies were used for morphological evaluation of osteoblast cells and GO. GO nanoparticles used in the present study obtained from treating graphite with $KMnO_4$, and H_2SO_4 which has a lot of hydroxyl groups (-OH and-COOH) on its surface. MTT assays were carried out, in which cytotoxicity evaluation was based on the activity of enzymes to reduce MTT to formazan dyes, giving a purple color.

MATERIALS AND METHODS

Materials

Natural flake graphite used to prepare GO in the current study was obtained from Qingdao Dingding Graphite Products. We prepared GO out of graphite using H_2SO_4 (98%), H_2O_2 (30%), and potassium permanganate ($KMnO_4$), all from Aldrich Co. Styrene, sodium dodecyl sulfate (SDS), benzoyl peroxide(BPO), and octanol were bought from Sigma–Aldrich. All purchased compounds were used as received, with no further purification except for styrene.

Preparation of water-soluble GO

We made GO out of purified natural graphite with use of modified Hummer's method,[28] in a way that graphite powder (0.5 g) was added to 50 mL of 98% H_2SO_4 in an ice bath, and $KMnO_4$ (2 g) was gradually added while stirring. To avoid a sudden increase of temperature, the rate of addition was carefully controlled. The stirring was kept on for 2 h at temperatures below 10°C, followed by 1 h at 35°C.

Next, the reaction mixture was diluted with 50 mL of DI water in an ice bath while the temperature was kept below 100°C. The mixture was stirred for another 1 h, and further diluted to nearly 150 mL with DI water. Then, 10 mL of 30% H_2O_2 was added to the mixture that turned into brilliant yellow. The resultant was centrifuged and washed several times with 5% HCl aqueous solution, then by DI water until the pH of the supernatant become neutral. Finally, the resulting solid was dried at 60°C for 24 h where a loose brown powder, was prepared.

Characterization

Samples were characterized by X-ray diffraction (XRD, Philips Xpert MPD, Co K irradiation, = 1.78897 A), SEM (Philips XL30 microscope with an accelerating voltage of 25 kV) and ultraviolet–visible spectrometer (UV–vis, Shimadzu UV-2100). In addition, TEM (PHILIPS, EM208S, Netherlands, at 100 kV of acceleration voltage) was employed to observe the morphology of NanoFe_3O_4 at GO hybrids and polymer composites. A radial view Varian Vista-Pro simultaneous ICP-ES (Springvale, Australia) coupled to a V-groove nebulizer and equipped with a charge coupled GO samples were characterized by X-ray diffraction (XRD, Philips Xpert MPD, Co K irradiation, =1.78897 A), SEM, Philips XL30 microscope with an accelerating voltage of 25 kV. In addition, transmission electron microscopy (TEM) (PHILIPS, EM208S, Netherlands, at 100 kV of acceleration voltage) and atomic force microscopy were employed to observe the morphology of Nano GO. Raman spectroscopy is a nondestructive tool used to study the ordered and disordered crystal structures and layers of GO sheets. The Raman spectra of graphene films were obtained at room temperature with a Renishaw Invia Micro-Raman spectrometer in back scattering geometry with the laser excitation of 632.8 nm at a power level of 1.7 mV. Also the few layer GO was characterized by AFM.

Cell culture

Human bone marrow MSCs were obtained from Royan institute. Osteogenic medium used in this study was consisted of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% antibiotic penicillin-streptomycin, ascorbic acid 3-phosphate, dexamethasone, and β-glycerophosphate. All were bought from Gibco company. MSCs at third passage were seeded at a density of 20,000 cells/mL into the osteogenic medium as control group. Experimental group containing osteogenic medium with 1.5 μg/mL suspended GO. Both control and experimental groups were incubated at 37°C and 5% CO_2 for 21 days.

Cell viability study

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out, in which cytotoxicity evaluation was based on the activity of enzymes to reduce MTT to formazan dyes, giving a purple color.

hMSCs were seeded at a density of 20,000 cells/mL into the four well plates containing cell culture medium with 1.5 μg/mL suspended GO as control group and four well plates without GO incubated for 3 days, with a temperature of 37°C, 5% CO_2. After 3 days the remaining medium was removed, and the cells were washed with PBS. Then, 100 μL of culture media and 10 μL of MTT solution were added to each well and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 4 h. After incubation, the cells were washed with PBS solution. Subsequently, 100 μL of isopropanol acid 4% and hydrochloric acid were added to each well, and the cells were incubated at room temperature for 10 min. The absorbance was measured by Elisa Reader (Anthos 2020 ver1.8, Anthos Lab Tec Instruments®, Austria) at 492 nm with 620 nm as reference.

Real time PCR assay

RNA was extracted from sample cells using Trizol (Fermentase, Germany). The cDNA was synthesized using cDNA synthesis kit (Fermentas, Germany) based on the manufacturer's instruction. Primers by using the NCBI website were designed and synthesized by Bioneer Company. Using SYBER Green master mix PCR reactions were performed (Applied Biosystems). QRT-PCR program was performed with a melting cycle for 5 min at 95°C and then 10 s at 95°C by μ cycles of melting, 15 s at 60°C (annealing) and 30 s at 72°C extension. The sequences of primers used for each gene are as follows:

Osteocalcin
forward: 5′-ATGAGAGCCCTCACACTCCTC-3′
reverse: 5′CCCAGCCATTGATACAGG-3′
Osteopontin
forward: 5′–TACAGACGAGGACATCAC-3′
reverse: 5′-TCTACAACCAGCATATCTTC-3′
Connexin
forward: 5′-AACACTCAGCAACCTGGTTGTG-3′
reverese: 5′-ATAGAAGAGTTCAATGATATTC-3′

Scanning electron microscopy

Twenty one days after incubation, osteoblast like cells were fixed in 2.5% gultaraldehyde buffered with a 0.1*M* sodium cacodylate solutions for 2 h at room temperature and then overnight at 4°C. Then the samples rinsed with 0.1*M* sodium cacodylate solutions. For the secondary fixation, 1% osmium tetroxide in 0.1*M* NaCl for 1 h was used. Dehydration of the samples was conducted for 30 min through a graded ethanol series (50, 70, 90, and 100% ethanol at 20 min). Next, the samples were subjected to sputter-coating with gold/palladium and were investigated using a Vega-TEScan (Tescan USA, Inc.) at 20 kV. Cell morphology was performed at different high magnification by SEM.

Statistical analysis

Statistical analysis was performed using one-way ANOVA and $p < 0.05$ was considered as a significantly different data.

RESULTS AND DISCUSSION

Raman spectroscopy

Herein, we have developed a novel, facile route to study the hMSCs differentiation to osteoblast cells in the presence of suspended few layer GO. Figure 1 shows the schematic

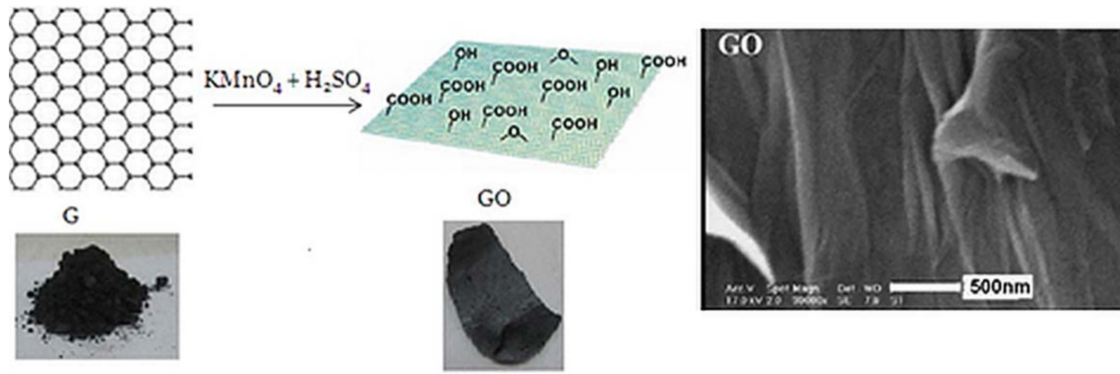


FIGURE 1. (a) Synthesis of the GO nanoparticles from graphite. (b) SEM images of the synthesized GO.

image for synthesis of the GO nanoparticles from graphite and also SEM image of the synthesized GO. GO used in this study was characterized by Raman spectroscopy, a nondestructive tool used to study the ordered and disordered crystal structures and layers of GO sheets.[29] The intensity Raman spectrum of GO displays two characteristic peaks, namely, the D band around 1341 and the G band at 1584 cm^{-1} (Fig. 2). The intensity ratio of D over G band (the R value = ID/IG) which reported as amount of the degree of disorder and average size of the sp^2 domain, was calculated as 1.03 suggesting the presence of localized sp^3 defects within the sp^3 carbon network.[30] The second order Raman feature, namely, the 2D band at 2700 cm^{-1}, is very sensitive to the number of layers. Particularly, the G and 2D bands of single-layer graphene sheets usually locate at 1585 and 2679 cm^{-1}, and the 2D/G ratios of single-, double-, triple- and multi- (>4) layer graphene sheets are typically >1.6, 0.8, 0.30, and 0.07, respectively.[31] In our work, more broadened shape of 2D band (often a doublet) results from increasing number of graphene layers.[30] In Raman spectrum of synthesized GO a weakly smeared 2D band can be seen along with the D + G combination band induced by disorder at 2932 cm^{-1} displaying the multi-layered structure of GO nano-sheets. Moreover, we can use XRD pattern of GO for calculation of the average number of graphene layers in our sample, by Debye–Scherrer equations[32,33]

$$L_a \quad 0.89\lambda/\beta_{002}\cos\theta_{002}$$

$$n \quad La/d_{002}$$

where L_a is the stacking height, β is the full width half maxima-FWHM, n is the number of graphene layers, and d_{002} is the interlayer spacing were obtained by using the data from XRD patterns (Fig. 3). Employing this equation, the number of graphene layers in our sample is calculated as 8. Figure 4 shows TEM and AFM images of the GO used in this study (Fig. 5). The few-layer GO usually had a thickness of 13.6 nm [Fig. 4(c)].

We first investigated the possible toxicity of GO on hMSCs and saos-2 cells using MTT assay at a dose of 1.5 μg/mL. The expression of CD45, CD73, CD90 and CD105 surface markers of hMSCs confirmed by flow cytometry (Fig. 6).

Our data showed nontoxicity effects of suspended GO on hMSCs and saos-2. We found different results at a concentration of 40 μg/mL in MSCs and saos-2 cells (Figs. 7 and

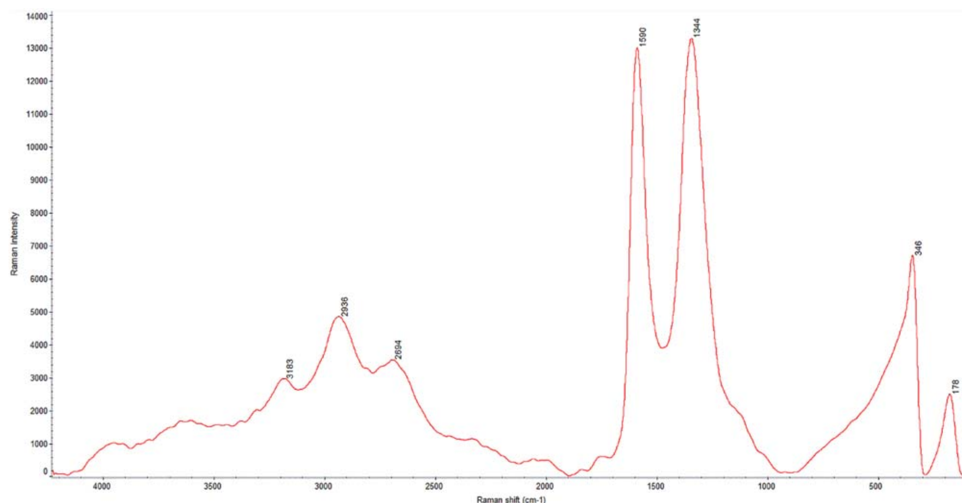


FIGURE 2. Raman spectrum of synthesized GO nano-sheets.

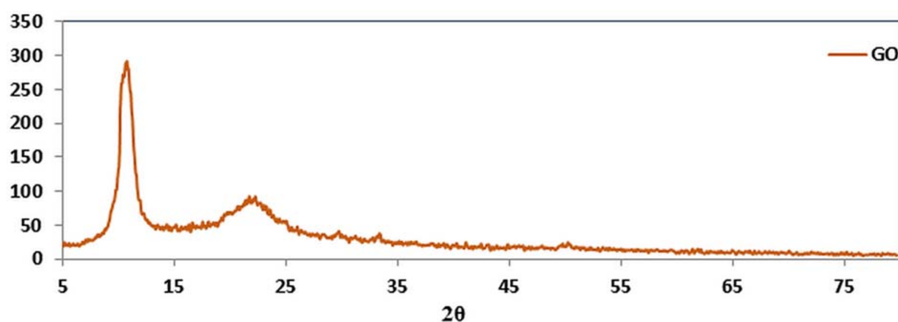


FIGURE 3. XRD pattern of synthesized GO nano-sheets.

8). GO at concentration of 40 μg/mL was toxic on MSCs and nontoxic on saos-2 cells. The toxicological effects of graphene-based nanomaterials are not yet well explained. Several studies showed that graphene is highly biocompatible, has low toxicity and a large dosage loading capacity.[9–16] On the other hand, several pioneers have found GO cytotoxicity in healthy cell lines such as MSCs,[32] erythrocytes,[33] and fibroblasts.[34,35] GO cytotoxicity depends on the specific graphene or GO nano-material used, for example, its physical properties, purity, kind and level of bio-chemical functionalization.[16–18] Kalbacova et al. demonstrated for the first time that graphene coated on SiO_2 substrate is not toxic for hMSCs.[17] When Nayak et al. studied the effects of graphene coated on substrate on hMSCs, they observed no significant difference in control and experimental group in terms of toxicity.[13] Akhavan et al. showed that the cytotoxicity and genotoxicity of graphene sheets and nanoplatelets in hMSCs depended on their concentration, size, and duration of exposure.[36] In another study, it was reported that adipogenic and osteogenic differentiation potential of ADSCs was not adversely affected after treatment with a low (10 μg/mL) or high (50 μg/mL) concentration.[23] The results suggest that GO at concentrations of $<$50 μg/mL could be considered potentially safe incubation conditions for *ex vivo* labeling for MSCs.[23] Based on our data suspended GO unlike GO coated on substrate present toxicity at dose lower than 50 μg/mL on MSCs. The other data of the present research showed cytotoxicity effects of GO on saos-2 cells at a concentration of 80 μg/mL (Figs. 7 and 8). Liao et al. reported a concentration-dependent toxicity of graphene and GO in erythrocytes and skin fibroblasts.[33] Several studies indicated that the factors such as size, concentration, shape, type of dispersants can influence the cytotoxicity of GO.[13] Chen et al. reported that graphene and GO coated substrates are biocompatible with induced pluripotent stem cells and enable cell proliferation and adherence, which supports the notions that they exert low cytotoxicity to mammalian cells.[37] Most studies have indicated reduced or no cytotoxicity of GO in L929 cells[17] and human fibroblasts.[38] GO internalized in cytoplasmic, membrane bound vacuoles of fibroblasts and human lung epithelial cells[38] were found to exert cytotoxicity to cells when applied with high dosage (above 20 μg/mL). On the other hand, when the GO is exerting extra cellular, Chang et al. found that GO presented minimal toxicity even at dose higher than 50 μg/mL on HeLa cells,[37] human hepatoma HepG2 cells, and A549 human lung cancer cells.[39] We found that at dose higher than 50 μg/mL (80 μg/mL) GO had toxic effect on saos-2. Our data showed that in addition to the specific GO material used, cytotoxicity also depends on the cell type, normal or tumor cell. We demonstrated that GO at a dose of 1.5 μg/mL, prepared with presented method has no toxicity effects on stem cell differentiation into osteoblast. Results confirmed that GO elicited a dose-dependent (0–300 μg/mL), but not a time-dependent cytotoxic response on ADSCs.[23] Another researcher showed that GO is safe in the range of

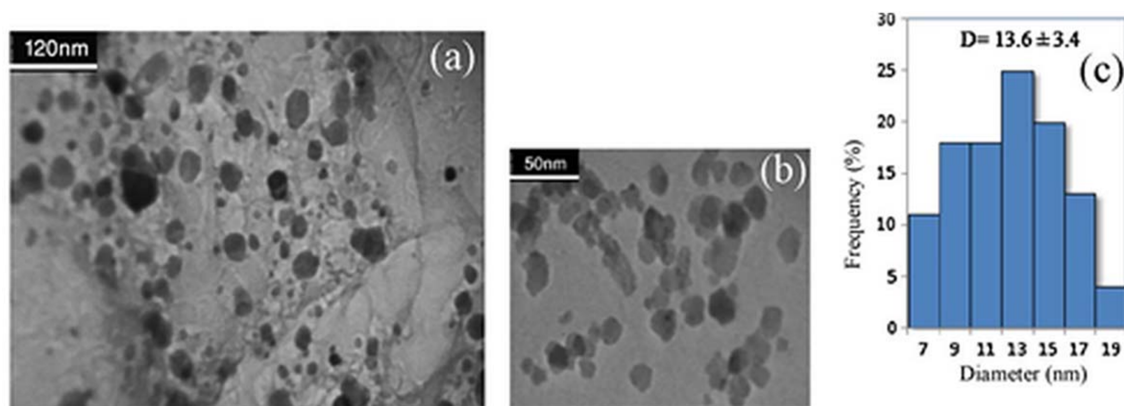


FIGURE 4. (a,b) TEM images at different magnifications and (c) particle size histogram of our sample of GO nanoparticles of GO.

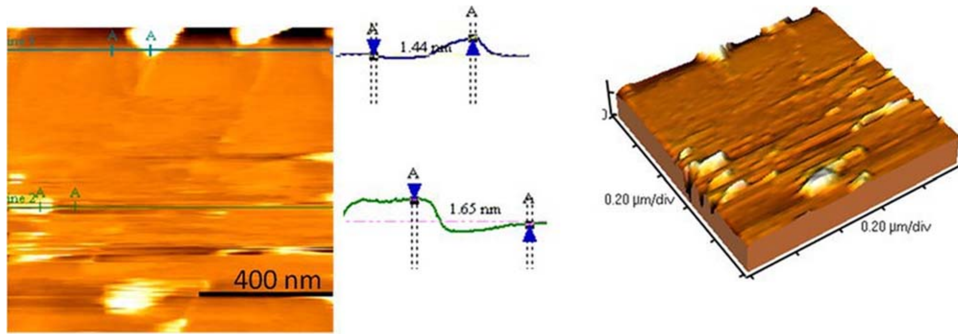
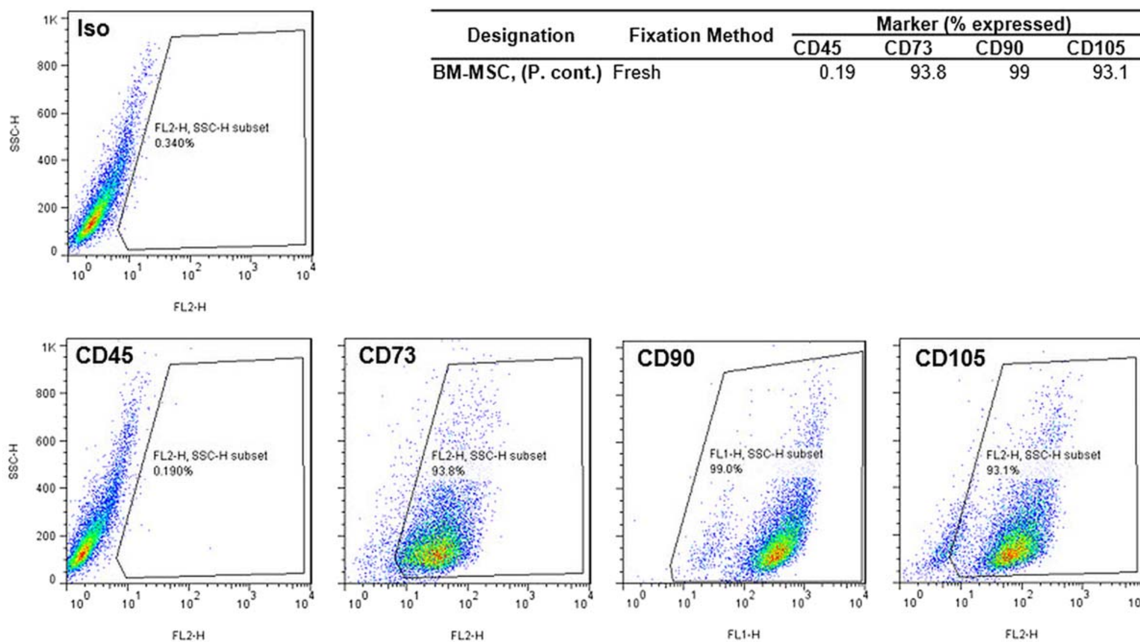


FIGURE 5. AFM images of GO.

50–1000 μg/mL[40,41] and they concluded that Go cytotoxicity is dose-dependent. It seems regardless of the substrate, cell growth and differentiation was not affected by the presence of graphene-nanomaterials and unless cells are treated with high dosage of the graphene-based nanoparticles, it could be deduced that they will not impose harmful effect on mammalian cells.

Our data showed that in the presence of GO the expression of osteoblast genes were increased (Figs. 9, 10 and 12). Also the attachment of cells to each other were increased in the presence of suspended GO Fig. 11. Shi et al. showed that the surface oxygen content of few layer GO (eight layer) coated on substrate by chemical vapor deposition method has a strong influence on cellular behavior, with the best performance for cell attachment, proliferation and phenotype being obtained in moderately reduced few layer GO.[42] They found that cell performance decreased as the few layer GO was highly reduced significantly. This highlight the important role of physicochemical characteristics of graphene and its derivatives in their interactions with bio-components. Reactive oxygen species generated by graphene is proposed as one of the mechanisms for the cyto-geno toxic effects of graphene at high concentrations (100 μg/mL),[34] although cell apoptosis was also reported as the other mechanism.[43] It can be concluded that suspended GO nanoparticle at very low concentrations of 1.5 μg/mL is not only nontoxic, but also is highly useful for increasing osteoblast gene expression, which expands the potential of suspended GO as key materials for osteoblast differentiation from stem cells. The direct contact interaction of sharp edges of graphene with membrane of cells is one of the mechanisms involved in cyto-geno toxicity of graphene sheets.[34] Reduced GO nano platelets with an average lateral dimension of 11 ± 4 nm have a very high genotoxic potential and ability to penetrate into the nucleus of hMSCs, causing chromosomal aberrations and DNA fragmentation, even at very low concentrations of 0.1 μg/mL[36] but GO had no significant cytotoxic effects in A549 cells.[44] Some modifications of GO, causing changes in surface charge, had genotoxic and cytotoxic effects in human lung fibroblasts.[45]



Designation	Fixation Method	Marker (% expressed)			
		CD45	CD73	CD90	CD105
BM-MSC, (P. cont.)	Fresh	0.19	93.8	99	93.1

FIGURE 6. The expression of CD45, CD73, CD90, and CD105 surface markers of hMSCs confirmed by flow cytometry.

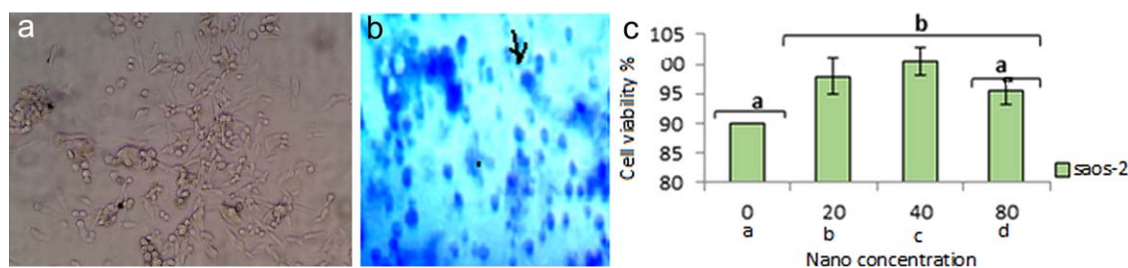


FIGURE 7. (a) The saos-2 cells phase contrast microscopy image cultured in control medium. (b) The saos-2 cells images of trypan blue staining. (c) The saos-2 cells survival rate in the studied groups with 630 nm wavelength for 72 h. (a) Phase contrast microscopy image of saos-2 cultured in 80 g/mL dosage. It concluded that the highest absorption was in 20 g/mL dosage and around 90% of cells were alive in this group, showing nontoxicity of GO at this dosage ($p < 0.05$).

Jaworski et al. demonstrated dose-dependent geno-cytotoxicity of pristine graphene nanoplatelets on glioblastoma multiforme cells.[46] Hinzmann et al. showed that pristine graphene, graphite, and reduced GO caused DNA damage and were genotoxic in cells, whereas GO was not.[47] It seems there are still not enough reteaches comparing the bioactivity of the different graphenes to determine their potential application in cell biology due to the high diversity of graphene-related structures. Trapping microorganisms within aggregating reduced graphene sheets can be considered as another mechanism for explaining cytotoxicity of graphene particles.[47] GO has hydrophilic properties and smoother edges and, therefore seems to be less potent in terms of interacting with DNA and penetrating cell compartments, resulting in the absence of cyto and geno-toxic effects.

In the current study, we developed GO through treating graphite with $KMnO_4$ and H_2SO_4 which has higher number of -OH and-COOH groups compared to graphene. There has been no investigation yet on stem cell differentiations into osteoblast in the presence of suspensed GO. Since GO has more polar groups of OH and COOH compared with graphene, we used GO for its immobility on the underlying surface (via negative charge) to facilitate the absorption of growth factors by the cell. We assumed GO can be considered as an excellent material for stem cell sustainable growth, expansion, and differentiation *in vitro* and *in vivo*. GO sheets strongly hydrophilic, causing them to swell readily and disperse in water.[48] Noncovalent bonds between GO and others molecules are mainly governed by hydrogen bonding or electrostatic interactions due to the high electronegativity character of its surface.[49] The oxygenous groups in GO introduce electronegative and charged regions to the surfaces and enable the formation of hydrogen bonds with growth factors and proteins. The possible reason for strongest adsorption in GO may be due to a mixture of, hydrogen bonding, electrostatic forces, and hydrophobic interactions in GO to biomolecule. Although more oxygenous groups in GO enhance the hydrogen bonding adsorption of some grow factors, also they induce more electrostatic repulsion to these factors from negative charges of oxygenous groups. Adsorption on carbon surfaces is suitable for molecules with low solubility, positive charge, or partial hydrophobicity (for the common case of negatively charged GO).

One of the main challenges in the use of biomaterials is improving cell adhesion and proliferation. We showed that in the presence of suspended GO when hMSCs were cultured for 21 days, the expression of osteoblast markers of osteopontin, osteocacin, and cell adhesion marker connexin were higher than control group significantly ($p < 0.05$) (Fig. 12). On the other hand, SEM data analysis showed that the layer number of the cells were increased in compared to control group with monolayer cells (Fig. 11). Recently, graphene was found to be useful as enhancing the therapeutic efficiency of stem cells[25] and plays a key role in cell adhesion and prevents cell death of implanted stem cells.[23] Another study reported that when MSCs were mixed with GO prior to *in vivo* injection, GO to be absorbed on extracellular matrix proteins, resulting in protection of implemented MSCs from cell death.[26]

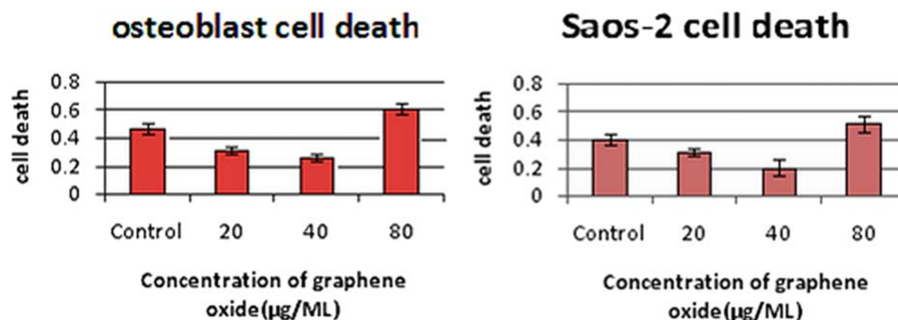


FIGURE 8. The hMSCs and saos-2 cells' death rate were stained with trypan blue after being exposed to different concentrations of GO with 200× magnification: (a) control group, (b) 20 g/mL group, (c) 40 g/mL group, and (d) 80 g/mL group ($p < 0.05$).

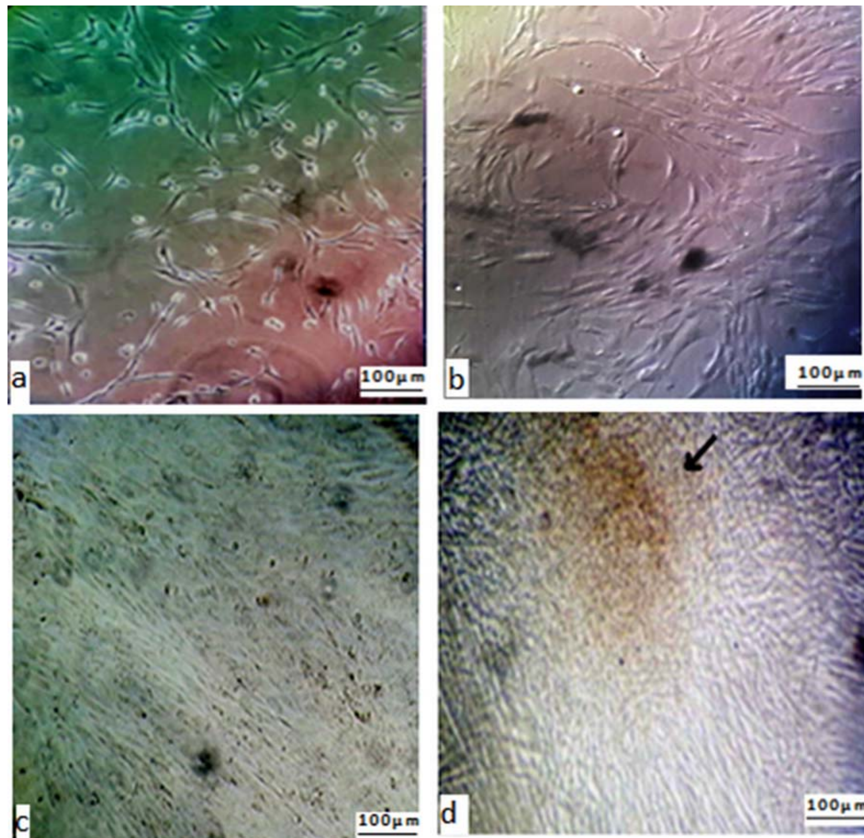


FIGURE 9. (a) Phase contrast microscopy image of hMSCs at passage 1 and (b) passage 3. (c) hMSCs in differentiation cultured in osteogenic medium without GO after 21 days. (d) hMSCs cultured in osteogenic e medium containing 1.5 μg/GO after 21 days. Multilayer cells are observed in center of plate specially (star) clear.

Due to the low efficiency of MSC in injection for the treatment of myocardial infarction, MSC mixed with GO prevents transplanted cells death and increases the rate of transplant success.[26] The potential of GO to protect MSCs from ROS was observed *in vitro* experiments when H_2O_2, as a ROS-generator, was used to damage MSCs.[25] One possible mechanism to increase osteoblast and adhesion markers is related to some functional indicators such as cell secretion of growth factors which increased in the presence of GO. It seems that suspended GO in osteogenic medium protect MSCs from any unpredictable damage and also increase absorbing cellular matrix proteins such as precursor connexin, which provide a possible strategy to increase cell attachment and change the layer number cell. It was shown that hMSCs coated on the graphene-calcium (GC) silicate composite have a better attachment than coated on pure calcium silicate composite.[23] They showed that osteogenesis-related gene expression of the hMSCs on the GC coating did not deteriorate with the adoption of graphene and even better attachment of the hMSCs was observed on the GC coated than on the pure calcium silicate coated.[23] We found that GO without any additional composite increased attachment of the osteoblast differentiated from hMSCs cultured in osteogenic medium after 21 days. It was reported that graphene penetrated into the cell membrane through receptor or nonreceptor-mediated endocytosis.[39] We suggest that while GO comes in contact with the cell membrane, it gains entry into the cell and small-scale of GO is able to cross the cell membrane and establish molecular cascades in the signaling pathway in the cytoplasm.

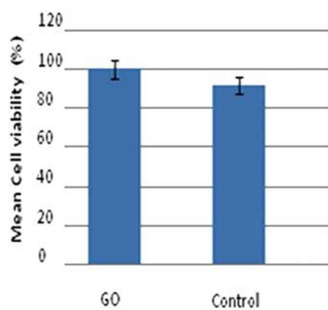


FIGURE 10. Cell viability of hMSCs grown on medium with and without Fe oxide. Graph showing the percentage of cell viability of hMSCs after 72 h exposure to GO suspended in normal stem cell medium as determined by MTT assay.

It seems that suspended GO might have stronger ability to get in contact with all sides of cell membrane than coated GO with one side of cell membrane (under line side). It is possible through absorbing necessary molecules for osteogenesis, crossing the cell membrane, and entering cytoplasm, GO provides an opportunity for these molecules to improve osteoblastic differentiation. We believe that GO

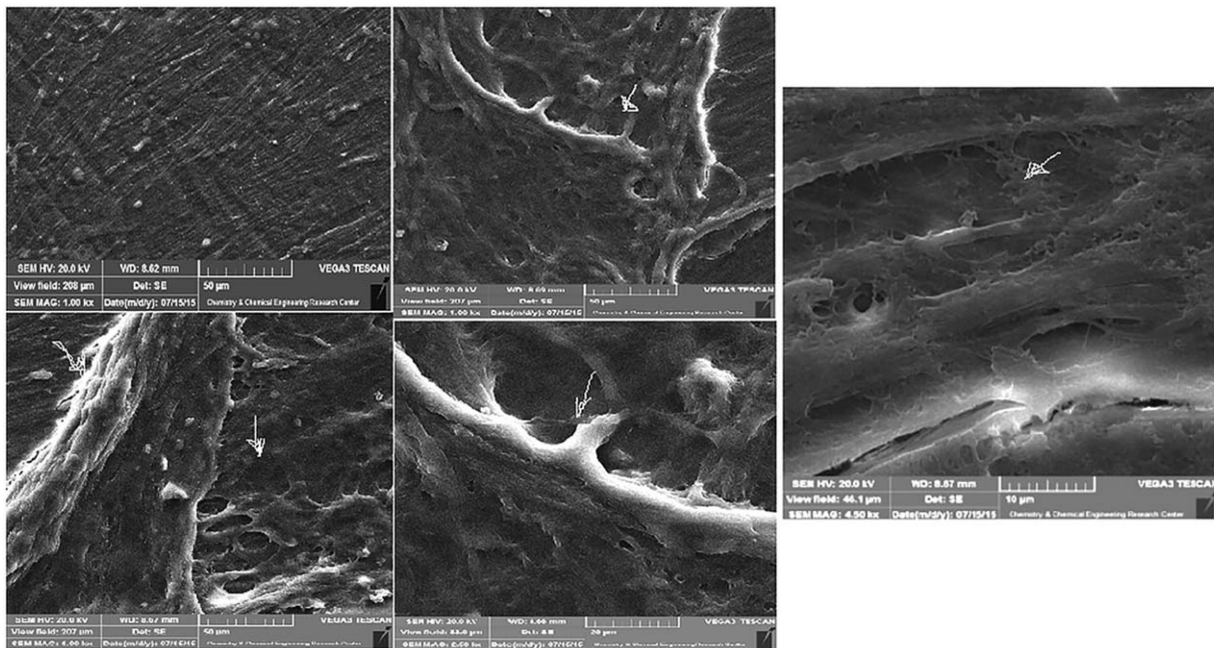


FIGURE 11. SEM images of hMSCs cultured in osteogenic medium with and without nano-GO after 21 days. (a) Mono layer cells are observed in osteogenic medium without GO (magnification: 1000). (b, c) MSCs cultured in osteogenic medium with 1.5 μg/mL GO. Monolayered cells are converted into multilayer cells (magnification: 1000). (d) Magnification 2500 of cells cultured in osteogenic medium containing 1.5 μg/mL GO. In (b, c), stars indicate different cell layers in each view. (e) Large number of connections (stars) is observed well among cells in GO group with a magnification of 4500.

coated on substrate has fewer opportunity to get in contact with the cell membrane compare to suspended GO. It is known that GO stimulates stem cells electrically and alters the intracellular calcium ion concentrations.[24] Even if we assume that there is no possibility for suspended GO enter the cytoplasm, it has more time to contact the cell membrane for

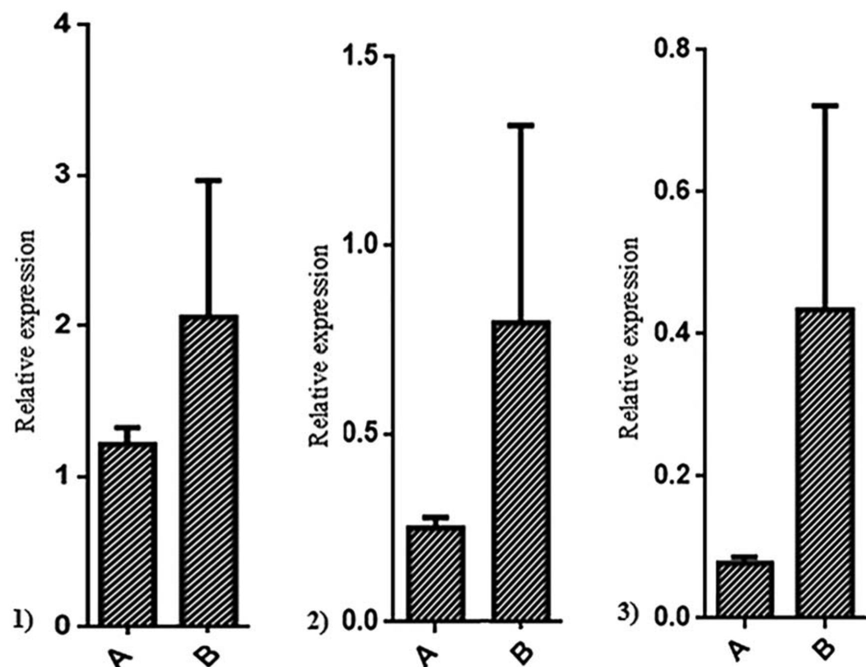


FIGURE 12. Comparison of osteocalcin, osteopontin, and connexin genes expression in hMSCs cultured in osteogenic medium with (B) and without (A) GO after 21 days. (1) Osteocalcin gene expression in MSCs cultured in osteogenic medium with 1.5 μg/mL GO (B) is higher than without GO group (A) significantly. $p < 0.05$. (2) Osteopontin gene expression in MSCs cultured in osteogenic medium with 1.5 μg/mL GO (B) is higher than without GO group (A) significantly.

osteogenic induction due to its high hydrophilic activity. Go is highly stable and a good material for a differentiation process that takes >14 days such as differentiation of MSC to osteoblast.[25] SEM images showed that the used dosage in the form of GO created multi-layer cells of osteoblast compared to monolayer control group (Fig. 11). SEM images confirmed the increase of the layer number of cells in the treatment of GO. Ryoo et al. showed that graphene improves gene efficiency, adhesion, and proliferation of fibroblast cells.[22]

On the other hand, cells can sense the lower layers up to several micrometers.[32,41] Given that the hardness of substrate is an important factor in connecting the cells to each other; the cells can sense the hardness of the underlying layer and connect to cell substrate due to GO nanometer thickness. In our study because of suspending GO in the medium, the cells were in contacting with GO in all of their surface area not only in their under layer side. Therefore, the cells contacted with each other with a higher speed. This provides multilayer cells formation. In fact, cells in the first layer in association with GO suspension on the same layer are considered as a hard bed for cell surface of the second layer. In another study, it was reported that GO can increase vinculin protein for cell connection.[50] The increase of integrin-associated signaling (such as focal adhesion kinases) by graphene has been also reported. In other words, cells not only established a stronger connection with lateral cells in the presence of GO suspension but also they connected to upper and lower layer. Differences in cellular connection may be due to absorption of cell adhesion proteins such as vinculin, fibronectin, and connexin.[40]

The GO synthesis method in the present project is different from the other studies. Most studies investigating the effects of graphene on cell differentiation were in forms of coated substrate and generally CVD method was applied to its coating on the substrate.[13] This method allows researchers to coat graphene on the foils of copper, nickel, glass, and so forth, in form of graphene foam. Besides graphene-modified composite materials, graphene coated on substrate without using any other supporting materials were used in the last investigation.[25] We obtained GO with a size of 14 nm using a particular way which has large number of COOH and -OH groups. We used GO without any connection to other materials and in the form of suspension in the culture medium. Nayak and colleagues have already reported the power of graphene on MSCs differentiation into osteoblast on day 15 (complete differentiation is occurred on day 30).[13] They observed the osteogenic potential of graphene coated on substrate in the osteogenic medium in the presence of additional growth factors such as BMP-2 to achieve differentiation through a synergistic effect. In none of graphene coated substrates studied in their research, the osteogenic medium alone was sufficient to lead to osteogenic differentiation over the whole duration of the experiment (15 days). They introduced CVD method as the best synthesis method for increasing osteogenesis. Using graphene coated-substrate synthesized by CVD method, they demonstrated that stem cells have higher bone differentiation in osteogenic medium compared to substrate without graphene. Since GO has more polar groups of OH and COOH compared with graphene, we used GO for its immobility on the underlying surface (via negative charge) to facilitate the absorption of growth factors by the cell. We assumed GO can be considered as an excellent material for stem cell sustainable growth, expansion, and differentiation *in vitro* and *in vivo*.

CONCLUSION

In this article, we confirmed that in addition to graphene and its derivatives coated on different substrates, noncoated GO as suspended in culture medium with special dose did not have toxic effects on MSC differentiation to osteoblast and accelerate osteogenesis. Furthermore, the present study reports a new approach for constructing multilayer cell deposition using suspended GO nanoparticle made of treating graphite with $KMnO_4$ and H_2SO_4. We successfully obtained multilayer cell inside the osteogenic medium cell culture containing suspended GO. We showed that suspended uncoated GO greatly influenced the cell attachment behavior, resulting in increase in the layer number of cells compared with monolayer cells in the control group. It seems that depending on dose and synthesis method, GO affects stem cell behavior in a different manner. Our results indicated that suspended GO could be potentially used as a scaffold to fabricate various biomedical materials to improve osteoblast adhesion. Interestingly, besides graphene-polymer hybrid scaffolds, graphene itself was reported to be able to form 3D structures that are suitable for long-term cell growth without showing cytotoxicity. GO fabricated on bone implant components would also decrease the healing time after surgery. We proposed a new method developed for preparing tissue models. We expect that the presented method would become a highly useful approach for bone tissue engineering. It seems that the next generation of bone implants coated with GO layer can have better adhesion properties and reduce bone healing time.

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