Research Article

Metal-Carbon Interactions on Reduced Graphene Oxide under Facile Thermal Treatment: Microbiological and Cell Assay

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Silver-functionalized reduced graphene oxide (Ag-rGO) nanosheets were prepared by single chemical and thermal processes, with very low concentration of silver. The resulting carbon framework consists of reduced graphene oxide (rGO) sheets or 3D networks, decorated with anchored silver nanoparticles. The Ag-rGO nanosheets were dispersed into a polymer matrix and the composites evaluated for use as biological scaffolds. The rGO material in poly(dimethylsiloxane) (PDMS) has been tested for antimicrobial activity against Gram-positive *Staphylococcus aureus* (*S. Aureus*) bacteria, after exposure times of 24 and 120 hours, as well as in the determination of cell viability on cultures of fibroblast cells (NIH/3T3). Using 1 mL of Ag-rGO in PDMS the antibacterial effectiveness against *Staphylococcus aureus* was limited, showing an increased amount of Colony Forming Units (CFU), after 24 hours of contact. In the cell viability assay, after 48 hours of contact, the group of 1 mL of Ag-rGO with PDMS was the only group that increased cell viability when compared to the control group. In this context, it is believed these behaviors are due to the increase in cell adhesion capacity promoted by the rGO. Thus, the Ag-rGO/PDMS hybrid nanocomposite films can be used as scaffolds for tissue engineering, as they limit antimicrobial activity.

1. Introduction

Several studies have been conducted regarding the four basic pillars of tissue engineering (stem cells, growth factors, scaffolds, and angiogenesis) [1]. The impairment of adhesion, proliferation, and differentiation of stem cells, as well as adequate toxicity, porosity, and degradability, comprises the limitations and challenges to be overcome in the preparation of scaffolds for tissue engineering [2, 3]. However, a determined effort has been made to improve materials so they promote cell colonization, proliferation, and differentiation [4]. In this sense, several materials have been examined in a bid to improve scaffold characteristics [5, 6].

As a material, graphene oxide (GO) has demonstrated promising physical and chemical properties across a wide range of applications, including the development of novel

catalysts and bioactive surfaces. Previous investigations into GO-derived materials (including those functionalized with metal nanoparticles) have shown potential in GO's use in biosensors, water treatment, and advanced drug delivery systems [7–10]. Also, it has been known since antiquity that silver nanoparticles (and salts) are highly toxic to microbial organisms [11], and recent investigations into GO have indicated that the pristine material can inhibit the growth of bacterial colonies [12]. Not least, we emphasize that GO may promote increased cell adhesion and even contribute to paracrine effects [13]. As a result, it is reasonable to assume that a combination of the two into a GO-nanoparticle composite could create a highly effective, yet ultrathin, antimicrobial coating that can subsequently be applied to any given surface and promote increased surface adhesion.

Herein, poly(dimethylsiloxane) (PDMS) was chosen as a scaffold layer for its many desirable characteristics. PDMS possesses good thermal and oxidative stability, low glass transition temperature, low surface free energy, low toxicity, hydrophobic surfaces, and low chemical reactivity [14–16]. The literature shows that when graphite and graphene oxide fillers were used in siloxane material, only the PDMS suspension filled with the graphite oxide showed a drastic change in the viscoelastic properties [17]. This change is directly related to the concentration of graphene oxide in the PDMS and is attributed to aggregation/agglomeration of the sheets within the film. Thus, PDMS has potential as a scaffold for biomedical applications with a flexible and functional surface; these similar polymer nanocomposites have attracted considerable interest in the last decades [18].

Ag-rGO nanoparticle composites include a carbonaceous shell that appears to play a notable role in stabilizing the metal core of silver and aids with interactions between the particle and the rGO surface. Consequently, these nanoparticles were incorporated into PDMS at different concentrations and the antibacterial activities of the Ag-rGO polymeric hybrid nanocomposites investigated using Gram-positive (G+) bacteria *Staphylococcus aureus* (*S. aureus*). The effects of different Ag-rGO dosages on the antibacterial activity of the nanocomposites are systematically investigated. The biocompatibility of the nanocomposites was also assessed, using a mouse fibroblast cell line (NIH/3T3) in a cytotoxicity assay. A versatile 3D form of Ag-rGO sponge composite was also produced.

2. Materials and Methods

2.1. Ag-rGO Composite Synthesis. The synthesis of the Ag-rGO composite was carried out in two stages: firstly, graphene oxide was synthesized from graphite powder via the Hummers and Offeman process, as described elsewhere [19], followed by the thermal reduction of a polymeric silver precursor to incorporate silver-carbonaceous core-shell nanoparticles into the GO sheets.

The Ag nanoparticles have been produced directly on the surface of GO from a reduction of metal-citrate-polymeric complex solution. Initially, a 50 mL solution of citric acid (CA) (Aldrich) and a 1% solution of silver nitrate were created. To this ethylene glycol (EG), a polyalcohol, was added at a ratio of 20/80 EG/CA, resulting in the formation of a stable complex as described in the literature [10, 20–22]. This occurs through a subsequent condensation reaction between available carboxylic and hydroxyl groups present on the EG and CA, respectively. To this mixture 0.4 g of GO flakes was added and the mixture stirred at 80°C for 1 hour.

The GO flakes were then filtered and dried under vacuum at 50°C for 12 hours, resulting in a dark powder. This powder was subsequently calcined at 450°C for 2 hours under a N₂ stream, promoting the decomposition of the organic component (the resin) and the reduction of the silver salt, resulting in the formation of silver metal nanoparticles anchored on GO flakes, as illustrated schematically in Figure 1.

2.2. Polymer Films Preparation. PDMS was used to produce the polymer nanocomposite, prepared at a ratio of 10:1 base polymer : cross-linker (the PDMS Sylgard® 184 Silicone Elastomer was prepared as described by the manufacturer; the commercial product kit consists of Sylgard 184 Silicone Elastomer base and Sylgard 184 Silicone Elastomer Curing Agent) [14, 23]. Ag-rGO ethanol solution (8 g in 10 mL) was then added at the appropriate volume (0.5, 1.0, and 2.0 mL, resp.) and dispersed via vortex mixing and ultrasonication. The mixture was put into a vessel, and the Ag-rGO-PDMS hybrid film was dried under vacuum at room temperature for 48 h. To examine the morphology of the PDMS matrix and Ag-rGO/PDMS hybrid nanocomposite films, a Veeco Digital Instruments Dimension 3100 atomic force microscope (AFM) was used.

2.3. Ag-rGO Sponge Synthesis. To prepare the GO sponges, GO was dispersed in water at a concentration of 4 mg/mL. To this ascorbic acid was added (at a concentration of 12 mg/mL) and the sample sonicated to uniformity. The solution was then placed in a sealed vessel and heated at 90°C for 1.5 hours to produce a sponge, followed by the impregnation process with Ag polymeric resin precursor [10, 22]. The GO sponge was carefully dried under vacuum at 40°C for 6 hours, resulting in a substantial reduction in the size of the material. Once dried, the sponge underwent thermal treatment at 450°C for 2 hours under a N₂ atmosphere; see illustration in Figure 1.

2.4. Determination of Cytotoxicity. Mouse fibroblasts cells (NIH/3T3) were obtained from Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), purchased from Gibco (Grand Island, NY, USA). Cells were grown at 37°C in an atmosphere of 95% humidified air and 5% CO₂ [24, 25]. The experiments were performed with cells in the logarithmic phase of growth.

The Ag-rGO/PDMS hybrid nanocomposite films had different concentrations of Ag-rGO (0, 0.5, 1.0, and 2.0 mL). Films were shaped in order to fit into wells of 24-well plates, with 91.6 mm² of surface area per milliliter of culture medium, following the recommendations of ISO 10993-12 standards. Samples were exposed for 40 minutes to UV irradiation on each side and preincubated in DMEM supplemented with 10% FBS at 37°C, pH 7.2, under humidified 5% CO₂ and 95% O₂, for 24 hours as described previously [6].

The viability of NIH/3T3 cells was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to water insoluble formazan. Briefly, cells were seeded at a density of 2×10^4 cells per well under standard conditions for 24 hours. The samples of each Ag-rGO/PDMS hybrid nanocomposite films maintained in culture medium for 24 hours were removed and 200 µL/well aliquots were incubated with the seeded cells for a further 24 hours. Cells were incubated with eluates for 24 hours. After incubation, 20 µL MTT (5 mg MTT/mL solution) was added to each well. The plates were incubated for an additional 4 hours and the medium was discarded.

FIGURE 1: (Top row left to right) a GO sponge in silver resin solution, an Ag resin impregnated GO sponge, and Ag-rGO sponge material after thermal treatment. (Center) Ag-rGO/PDMS composite in 2D structure and a schematic of the Ag decorated rGO in 3D. ((a)-(b)) Field Emission Scanning Electron Microscope (FE-SEM) image of 3D Ag-rGO sponge after thermal treatment.

FIGURE 2: (a) X-ray diffraction of Ag-rGO composite and (b) typical Raman Spectra Ag-rGO composite.

200 μL of dimethyl sulfoxide (DMSO) was added to each well, and the formazan was solubilized on a shaker for 5 minutes at 100 $\times g$. The absorbance of each well was evaluated in a microplate reader (MR-96A, Mindray, Shenzhen, China) at a wavelength of 450 nm. All observations were validated by at least two independent experiments and for each experiment the analyses were performed in quadruplicate.

Cell viability was analyzed by analysis of variance (ANOVA) followed by Tukey post hoc test. The value of $P < 0.05$ was considered significant. Graphic for cell viability was developed using GraphPad Prism Program 4.00 version (GraphPad Software, San Diego, USA).

2.5. Antibacterial Activity. Antibacterial activity of the PDMS-based polymer particles with four concentrations of Ag-rGO (0, 0.5, 1, and 2 mL) was investigated against the bacterium *Staphylococcus aureus* (ATCC19095). All materials tested had dimensions of 0.5 cm $\times$ 1.0 cm and were sterilized with UV light [26]. All bacteria were stored at -80°C until required. For experimental use, the stock cultures were reactivated using the following method: 100 μL was transferred into a sterile tube containing 9 mL of LMW + 1 mL glucose and incubated for 18 hours in CO_2 incubator. After this period, 10 μL of mixture was transferred to agar plate and held on a streak on agar. The plate was incubated for 24 hours and growth was recorded as above. Isolated colonies were collected and transferred to a sterile tube containing 9 mL of LMW + 1 mL glucose and incubated for 18 hours.

The polymer samples were deposited individually in threaded tubes containing 4 mL of tryptone soya broth (TSB). The specimens were incubated at 37°C and sampled at two time periods of 24 hours and 120 hours. At this point, samples were collected in tubes from the solution, followed by addition of 40 μL inoculum of *Staphylococcus aureus*, previously standardized in a spectrophotometer at a 10^7 CFU/mL exponential phase, prior to incubation at 37°C for 24 hours.

Once the samples were fully incubated, they were diluted 7 times with 20 mL saline. A sample from each dilution was plated on tryptone soya agar (TSA) and incubated at 37°C for 24 hours before being analyzed for the presence and number of Colony Forming Units (CFU) [27].

Statistical analysis of microbiological activity was performed using t -test, Kruskal-Wallis test, and ANOVA. The significance was considered at $P < 0.05$, respectively, in analyses. The analysis, with data, is expressed as mean $\pm$ SEM.

3. Results and Discussion

3.1. Physicochemical Properties of Nanocomposites. FE-SEM image in Figures 1(a) and 1(b) illustrates a sponge-like 3D matrix with a large surface area dependent on the thermal treatment. According to X-ray diffraction (Figure 2(a)) the hybrid system increased the fraction of reduced graphene oxide as a function of the thermal treatment of GO and the crystalline size of the Ag nanoparticles. Raman Spectra can be used to reveal the nature of the Ag-rGO nanocomposite (Figure 2(b)). The peaks at 1581 cm^{-1} and 1350 cm^{-1} are the characteristic G- and D-bands, which are representative of the sp^2 breathing mode of the graphitic structure and the vibrations of carbon atoms within dangling bonds in plane terminations of the disordered graphite or glassy carbons, respectively. The ratio of intensity of these peaks, ID/IG, suggests a defective structure or a higher degree of graphitization in the Ag-rGO structure.

HRTEM (Hitachi HD2300A STEM and Philips CM200 TEM) micrographs show Ag nanoparticles on a GO sheet which are shown in Figures 3(a) and 3(b). This is corroborated by EDAX analysis (Figures 3(c) and 3(d)), showing that the Ag-rGO contains 99.817 wt% C and 0.183 wt% Ag. It is observed that there is an ultrathin carbonaceous shell surrounding each nanoparticle, this can be attributed to residual organic material arising from the thermal decomposition step of the citrate-polymeric complex and may contribute to the dispersion and attachment of Ag nanoparticles across

FIGURE 3: HRTEM image of (a) Ag nanoparticles on a GO surface, (b) the carbonaceous shell around the Ag nanoparticle core, and (c) EDAX analysis and (d) enlarged circle area.

the surface of the GO sheet (see scheme in Figure 1), and there are no attached silver nanoparticles, this being also observed eventually. This residual carbon could have potential advantages that include the following: high stability (e.g., acid and alkali resistance), nontoxicity [28], and, as has been suggested previously, enhanced integration of Ag nanoparticles with the GO surface.

Thermogravimetric analysis (Figure 4) suggests that there is a slight change in the thermal decomposition in GO and Ag-rGO samples. This is evident above 400°C, where the GO sample is slightly less stable, very likely due to the inclusion of the Ag nanoparticles. These indicate that the amount of Ag coated on the rGO sponge is too small to abruptly change the decomposition behavior, and it suggests sponge with

FIGURE 4: Thermogravimetric analysis (TGA) of GO and Ag-rGO (heating rate = $10^{\circ}\text{C}/\text{min}$ under a nitrogen atmosphere).

higher Ag concentration than presented here will drive a possible dependence of metal phase concentration on the thermal properties of graphene oxide [10]. Further the total weight loss on sponges could be influenced by ascorbic acid treatment on graphene oxide to obtain the sponge shape. Fernández-Merino et al. reported the TGA on graphene oxide samples treatment with several different reducing agents (including ascorbic acid) towards deoxygenation of graphene, which had significant change on TGA plots [29].

Specific surface area measurements of the rGO and the Ag-rGO sponge materials result in surface areas of 0.02 and $103.9\text{ m}^2\text{ g}^{-1}$ and pore volumes of 0.0011 and $0.1273\text{ cm}^3/\text{g}$, respectively. The specific surface area of the Ag-rGO is significantly higher than that for the Ag nanoparticle-free rGO sponge (when treated under similar thermal conditions). Thus, the presence of the nanoparticles may also have an effect on the surface morphology of the samples.

3.2. AFM Analysis. Figure 5 shows AFM micrographs of a pristine PDMS sample and a sample modified by the addition of the Ag-rGO nanocomposite. It can be observed that there is a significant change in the surface morphology between the pristine polymeric matrix and the hybrid material. This is shown by the progressive increase in roughness (4.7, 4.1, 11.7, and 54.9 nm) in the PDMS samples containing 0, 0.5, 1.0, and 2.0 mL of Ag/RGO (previously dispersed in ethanol solution), respectively. This fact is attributed to the successful incorporation of the Ag-rGO into the PDMS film. This composite surface can help explain the bioactivity in our polymeric hybrid material. Allied to the characteristics of the PDMS elastomer (flexibility, conformal contact to surfaces, and low hardness) [16], this improves the functionality of our material.

3.3. Determination of Cell Viability. The cell viability of NIH/3T3 cells was tested against Ag-rGO/PDMS hybrid

nanocomposite films containing Ag-rGO in different concentrations (0, 0.5, 1.0, and 2.0 mL). As shown in Figure 6, in relation to the time of figure exposure, all groups showed significant increase in cell viability after 48 h ($P = 0.000$), as could be expected since the period of culture was higher. When groups were compared, the PDMS/Ag-rGO (1 mL) group showed a statistically significant difference in cell viability after 48 h of exposure, with a higher cell viability than the other groups tested ($P = 0.0032$). In addition, our data showed that Ag-rGO/PDMS hybrid nanocomposite films, with the different concentrations of Ag-rGO, were not cytotoxic to NIH/3T3 cells, since the cell viability was not decreased in the test groups when compared to controls.

Therefore, beyond the limitation of our study, the results indicate that the Ag-rGO and PDMS hybrid nanocomposite films can be used as a new composite for tissue engineering. To this end, many researchers have investigated the development of new approaches, such as the association of different composites for the improvement of scaffolds [7]. Recently, experimental work has demonstrated that the association between nano/microfibers of titanium dioxide and hydroxyapatite to alginate hydrogel scaffolds provides an increase in cell viability in both the long and short term, respectively. This association is interesting for tissue engineering, since it enables cell adhesion to the hydrogel scaffold [5, 6].

Our results showed a significant increase in cell viability when 1 mL of Ag-rGO/PDMS was used, and this could possibly be attributed to the Ag-GO presence. In this sense, studies have suggested that GO could adsorb extracellular matrix proteins, creating a more adapted environment for cell adhesion and favoring its adjustment in a hostile setting, like those generally provided by an ischemic circumstance [30]. In agreement, recently it was reported that the incorporation of GO in poly(lactic-co-glycolic acid) nanofibrous mats optimizes cell adhesion and proliferation and stimulates the differentiation of human mesenchymal stem cell [31]. Other researchers argue that a GO film coated on glass could promote attachment and proliferation of mammalian colorectal adenocarcinoma cells [32, 33]. Graphene has also shown the capacity to stimulate human mesenchymal stem cells to strongly adhere to the substrate and differentiation into bone cells at the same rate as Bone Morphogenetic Protein-2 (BMP-2), a molecule that has been shown to induce bone formation [33].

Therefore, in relation to cell viability, it seems feasible to use this hybrid material for tissue engineering. However, this statement must be analyzed with caution, since there is no consensus in regard to Ag toxicity in vitro [34]. In this context, we suggest that the cytotoxic potential of Ag nanoparticles is possibly cell dependent. Samberg et al., in agreement with our results, showed that human adipose-derived stem cells exposed to $100\text{ }\mu\text{g}/\text{mL}$ of Ag nanoparticles (between 10 and 20 nm diameters), did not present a loss of cell viability [35]. Moreover, Hackenberg et al. reported loss of human mesenchymal stem cell viability, when exposed for 1 hour to $10\text{ }\mu\text{g}/\text{mL}$ of silver nanoparticles [36]. Therefore, it seems that, in our study, the 1 mL concentration of Ag-rGO combines the best qualities of Ag and rGO for NIH/3T3

FIGURE 5: AFM micrographs of PDMS and modified GO hybrid materials consisting of PDMS with 0, 0.5, 1.0, and 2.0 mL Ag-rGO, respectively.

FIGURE 6: Effect of Ag-rGO/PDMS hybrid nanocomposite films, with differing concentrations of Ag-rGO (0, 0.5, 1.0, and 2.0 mL), in NIH/3T3 viability after 24 h and 48 h of exposure. Data are expressed as the means $\pm$ SEM. Letters indicate significant differences between times of exposure and * indicates significant differences between groups. A P value < 0.05 was considered significant (Tukey test).

cells, increasing their viability. The roughness surfaces may be affecting the cell proliferation, where the samples with 1 mL (composite) hit the optimal roughness.

The sterilization process of scaffolds is a prerequisite for in vitro culture and in vivo implantation, considering the

possible occurrence of bacterial contamination in scaffolds used for bioengineering tissues [37]. Thus, some sterilization methods are required (autoclaving, irradiation, and ethylene oxide treatment); however these can cause various negative effects on the scaffolds, for example, the impairment of the

FIGURE 7: Mean viable bacteria (CFU/cm² plate) in 24 and 120 h ($n = 7$). Within a panel, group values that were identified using similar lower case letters were not significantly different ($P > 0.05$).

mechanical properties alterations in the adsorption rate and its proper performance [38]. Therefore, the incorporation of materials that perform antibacterial activity in scaffolds, as the GO and Ag, was investigated.

3.4. Antibacterial Activity. The antibacterial activity of the pure PDMS and that incorporated with Ag/rGO, at different concentrations, was assessed after 24 and 120 hours of contact with the bacterium *Staphylococcus aureus* (Figures 7(a) and 7(b)). Our data suggest that, after 24 hours, PDMS/Ag-rGO (1mL) was the least effective group against the bacteria, whereas the PDMS and PDMS/Ag-rGO (0.5 mL) groups showed greater antibacterial activity. There is also a tendency that this behavior is maintained after 120 hours of contact.

According to literature reports, the association between GO and the Ag metal exhibits strong antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, Gram-negative and Gram-positive bacteria, respectively [39]. However, with regard to the antibacterial profile of the hybrid composite investigated, it can be affirmed that our results suggest no reduction of CFU/cm². In particular, when adding 1mL of Ag-rGO/PDMS, within 24 hours the amount of CFU/cm² increased with respect to the control group. We suggest that this conduct is contrary to that recommended in the literature and is a reflection of the surface adhesion capacity promoted by the GO.

There are expectations that the PDMS/Ag-rGO would be an effective antibacterial medium in Gram-negative bacteria (not investigated here), since in liquid systems Gram-positive antibacterial efficacy is less than that observed in Gram-negative bacteria, justified by the difference in structure and

thickness of the cell wall, due to the amount of peptidoglycan [39].

Thus, considering the above parameters and despite the material limitations in not promoting an antibacterial effect, the scaffolds produced here could avoid contamination risk, and the literature leads us to believe that the hybrid material developed would be a good choice for use in tissue engineering, due to the increase in cell adhesion on the surface of the scaffold.

4. Conclusion

Throughout the synthesis here, low resin concentrations were used in order to reduce the residual amorphous carbon in the final Ag-rGO composite. Despite this, it is currently believed that a low concentration of amorphous carbon plays an important role in the formation and stabilization of the Ag particles and also promotes a strong interface between the nanoparticles and the GO layer. PDMS/Ag-rGO composites have been produced and tested for their viability as tissue scaffolds. The observation of residual carbonaceous shell around the silver particles (as a result of the thermal treatment) suggests optimized deposition and a strong interaction with the graphene surface. The PDMS/Ag-rGO hybrid nanocomposite films showed no loss of cell viability, and indeed the 1% Ag-rGO-PDMS material enhances cell viability, which is possibly related to an increase in cell adhesion. There is a limitation to the material, since this concentration allows increased CFU/cm²; however it is believed that with proper management of contamination this limitation will

not interfere in the materials use to produce bioengineered tissues.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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