Biomedical Materials

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Biological and mechanical evaluation of poly(lactic-co-glycolic acid)-based composites reinforced with 1D, 2D and 3D carbon biomaterials for bone tissue regeneration

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Abstract

Considering the fact that life on Earth is carbon based, carbon materials are being introduced in biological systems. However, very limited information exists concerning the potential effects of different structures of carbon materials on biological systems. In the present study, poly(lactic-co-glycolic acid) (PLGA)-based carbonaceous composites were developed by reinforcing 1 wt% of three different carbon-based materials i.e. carbon nanotubes (CNTs-1D), graphene nanoplatelets (GNPs-2D), and activated carbon (AC-3D). The developed composites were characterized for physicochemical, biological, and mechanical properties. Along with their hemocompatible nature, the composites exhibited better swelling ratio, degradation percentage, bioactivity, and tensile strength. The improvement in hydrophilicity and protein adsorption resulted in the enhancement of cell proliferation and differentiation. Overall, sheet-like GNPs showed the strongest effect on the composite’s properties due to their larger exposed area. These results demonstrate the potential of PLGA-based carbonaceous composites for accelerating bone tissue regeneration.

1. Introduction

Bone injuries caused by accidents and trauma are major health issues requiring long treatment for the healing and regeneration of bone. The complications in the treatment of major bone injuries generate the risk of permanent functional disabilities [1, 2]. The inadequate osteogenic response at the place of injury decreases the natural ability of bone to regenerate and thus, requires the bone healing process to be augmented for effective bone repair. Current clinical treatments using autografts and allografts have considerable complications and limitations such as cost issues, donor site injury, deformity, and surgical risks of inflammation and infection [3]. Alternatively, bone tissue engineering offers the elimination of all the risks associated with current treatments. Bone tissue engineering targets the induction of the regeneration of new functional bone by the synergistic combination of biomaterials such as scaffolds, cells, and factor therapy [3]. Although both natural and synthetic polymers have been widely explored for their use in tissue engineering, biodegradable synthetic polymers have attracted attention due to their tunable properties. Poly(lactic-co-glycolic acid) (PLGA), a copolymer of polylactic acid and polyglycolic acid, is one of the most commonly used biodegradable polymers [4]. Its degradation products (lactic acid and glycolic acid) can be easily removed from the body through metabolic pathways [5]. Owing to its biocompatibility and biodegradation properties, PLGA has been widely used in the field of tissue engineering. However, hydrophobicity, weak mechanical strength, and poor bioactivity of PLGA are the critical issues of concern that often limit its application [6]. Many studies have been performed to improve the performance of PLGA as a biomaterial. These include surface treatment, blending with hydrophilic polymers (e.g. polyvinyl alcohol), and the addition of bioactive materials like hydroxyapatite, etc [4, 7, 8]. The improvements obtained from these studies are not yet satisfactory. Thus, adding a suitable reinforcement material into the PLGA matrix
is a valid approach to improve its hydrophilicity and mechanical properties. It has been stated in the literature that the incorporation of carbon into the PLGA matrix tailors its biological and mechanical properties [7, 9].

Carbon-based materials are fascinating due to their nanoscale size and large surface area, and are well accepted by the biological environment. Along with the favorable biological properties, the mechanical properties of these materials are also comparable to those of natural bone. Among the different carbon allotropes, carbon nanotubes (CNTs) and graphene nanoplatelets (GNPs) have drawn much attention, because of their biocompatibility and ability to accelerate bone tissue formation.

Unlike other carbon materials, CNTs are 1D carbon nanomaterials having an aspect ratio greater than 1000 [10]. Owing to their exceptional mechanical and biological properties, CNTs have been reinforced in different polymer matrices to improve their properties. In addition, multi-walled CNTs (MWCNTs) have been found to accelerate the bone formation [11]. However, pure CNTs aggregate, because of their intrinsic van der Waal interactions, which reduce their dispersion [10]. These agglomerated CNTs have shown cytotoxic effects on human cells. Therefore, CNTs can be chemically bonded to different functional groups such as hydroxyl, carboxyl, polyvinyl alcohol, and poly aminobenzoic sulfonic acid, etc to increases their water dispersibility and to remove them from the body by renal excretion. These functionalized CNTs are effectively being reinforced in different polymer matrices for potential use in various biomedical applications. Researchers have shown that the reinforcement of functionalized CNTs in a polycaprolactone matrix improves both the stem cell osteogenesis and mechanical properties of the composite [12]. Lahiri et al showed that the addition of CNTs in polylactide-caprolactone enhanced the osteoblast cell viability and mechanical properties of the composites [13]. Studies have also shown that the functionalized MWCNTs impart osteoinductive properties to the polycaprolactone composite with increased wettability. Furthermore, the incorporation of the carboxylated MWCNTs into PLGA films induced the mesenchymal stem cells to differentiate into osteoblast cells [5].

Graphene, a unique 2D sheet-like carbon nanomaterial, has attracted enormous attention due to its exceptional structural, electrical, and mechanical properties. Moreover, it has the ability to interact with other biomolecules such as proteins, enzymes, and DNA [14]. These astonishing properties of graphene and its derivatives have encouraged researchers to use them in the field of tissue engineering. Graphene is widely being used alone or with polymer matrices to encourage bone regeneration by stimulating cell adhesion, proliferation, and differentiation [15]. Similar to other graphene-based materials, GNPs have also shown great promise as a reinforcing agent for polymer matrix in bone tissue engineering applications. GNPs possess oxygen-containing functional groups at the edges, making their dispersion easy. Furthermore, different functional groups are added to improve the dispersion of GNPs in different polymer matrices. The sheet-like structure of GNPs with more defects on the edges results in more functional groups being introduced during functionalization treatments. GNPs are synthesized in pure form without any metallic impurities; hence, they show no toxicity to adherent cells. The high aspect ratio of GNPs leads to the formation of percolated networks when reinforced in the polymer matrix. The imperfections on the edges of the GNPs endorse the oxidative reaction with a surrounding gas-yielding hydrophilic structure and make GNPs less prone to agglomeration compared to single-layer graphite [16, 17]. Kalbacova et al demonstrated the osteo-conductive behavior of graphene with no toxicity [18]. It was found biocompatible to both human osteoblast and mesenchymal stromal cells. The graphene oxide reinforced PLGA nanocomposites have enhanced surface chemical and mechanical properties [19]. Furthermore, GNPs have also shown positive effects on various biological and mechanical characteristics of composites when incorporated in polylactic acid and hydroxyapatite matrices [20, 21].

Activated carbon (AC) is another form of 3D carbon with highly porous structure, large surface area to volume ratio, large adsorption capacity, and fast adsorption kinetics [22]. Therefore, AC fulfills the space requirements of the cells and helps them to attach to its surface. AC is widely used in plant tissue culture to enhance cell growth. However, much less research has been undertaken on using AC as a biomaterial for tissue engineering. Sandeman et al observed up-regulation of corneal cell growth when cultured on a hydroxyapatite-coated porous carbon matrix [23]. AC fibers and cloth have also been reported to be a good support for cell growth and differentiation [7, 22]. The AC-extracellular matrix composite scaffolds were found to promote neuronal differentiation of human embryonic stem cells and hence, facilitated the regeneration of neural tissues [24].

These findings establish the feasibility of using CNTs, GNPs, and AC as ideal reinforcement materials in PLGA for bone tissue engineering. Thus, the present study focused on using three different types of carbon biomaterials with 1D, 2D, and 3D structures (CNTs—tubular, GNPs—sheet, AC—irregular) to reinforce the PLGA matrix. To the best of authors’ knowledge, no studies have reported the comparison of PLGA–CNT, PLGA–GNP, and PLGA–AC composites for bone regeneration applications. Different structures and aspect ratios of these biomaterials are expected to trigger different biological effects. Therefore, the primary aim of this study is to explore the effects of carboxylated CNTs, carboxylated GNPs, and AC on surface, mechanical, and biological properties of
PLGA composites. With the required enhancement in mechanical and biological properties, the developed PLGA-based carbonaceous composites validated their suitability as a scaffold material for bone tissue regeneration.

2. Materials and methods

2.1. Materials

PLGA (lactide:glycolide = 75:25), carboxylic acid functionalized CNTs (multi-walled; diameter: 9.5 nm; length: 1.5 μm), Dulbecco’s Modified Eagle’s Medium (DMEM), alkaline phosphatase (ALP) assay kit, alizarin red stain-based (ARS) assay kit, fluorescein isothiocyanate (FITC)-dextran, tetramethylrhodamine (TRITC)-phalloidin stain, Hoechst stain, and fetal bovine serum (FBS) were obtained from Sigma-Aldrich, USA. Carboxylic acid functionalized GNPs (average thickness 6–8 nm, surface area 150 m² g⁻¹) were purchased from Cheap Tubes, USA. AC, antibiotic-antimycotic solution, phosphate buffer saline (PBS), bovine serum albumin (BSA), and 3-(4,5-dimethyl-2-y)-2,5-diphenyltetrazenium bromide (MTT) assay from HiMedia, India, and the MG-63 cells (human osteosarcoma cell line) from National Centre for Cell Science (Pune, India) were procured. All reagents used in this research work were of analytical grade.

2.2. Preparation of PLGA composites

The PLGA–CNTs, PLGA–GNPs, and PLGA–AC composite films were prepared by a solvent-casting technique. To decrease the tendency of agglomeration, carboxylic acid functionalized CNTs and GNPs were used. Briefly, 1 wt% of each of the CNTs, GNPs, and AC in DCM, were sonicated at room temperature. The PLGA (7 w/v%) polymer was dissolved in DCM separately and added to the above solutions. The solutions were then sonicated, poured into glass dishes and vacuum dried overnight. The dried films were then kept in a vacuum oven at 37 °C for 48 h. Similarly, pure PLGA film (PL) was also prepared. Thus, the obtained four different films were coded as PL, PLCN (PLGA–CNTs), PLGN (PLGA–GNPs), and PLAC (PLGA–AC), respectively. To prepare fluorescent carbon-containing composite films, carbon materials were labeled with FITC-dextran. Briefly, 200 μl of FITC-dextran solution (10 mg mL⁻¹ in water) was added to the CNTs, GNPs, and AC dispersions (10 mg/6 ml of water) and kept overnight at 4 °C (in the dark). Then, the solutions were washed, centrifuged (14 000 rpm, 15 min) twice to remove the unbound dye and dried properly. The thus-prepared FITC-dextran labeled fluorescent CNTs, GNPs, and AC were used to prepare PLGA–carbon composite films as mentioned above.

2.3. Characterization

Different structures of the carbon reinforcements were studied with transmission electron microscopy (TEM). The samples were first sonicated using a probe sonicator in ethanol medium until homogeneous dispersion. The dispersed solution was then placed on a carbon-coated copper grid and then observed with a transmission electron microscope (TEM-FEI Tecnai F30). The selected area electron diffraction (SAED) patterns were also observed. The cross-sectional morphology of the composites was evaluated using a field-emission scanning electron microscope (FESEM-FEI NovananoSEM 450). The samples were gold sputter coated prior to SEM imaging to avoid imaging artifacts from electrical charging. To evaluate the dispersion of the different carbon reinforcements in the polymer matrices, confocal microscopy (TCS-SP8, Leica) was performed on the fluorescent carbon-containing polymer composite films. The presence of phase and crystallinity of the PLGA composites was studied using the x-ray diffraction (XRD, RigakuUltima IV Diffractometer, Japan) technique. The XRD over a scan range of 5°–60° was performed with a scan speed of 5° min⁻¹ and a step size of 0.05° using CuKα radiation.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR, AlphaE, Bruker, USA) was done to characterize the functional groups present in the PLGA composites. Transmittance spectra within the range of 600–4000 cm⁻¹ were obtained.

The effect of different carbon-based materials on the hydrophilicity of the composites was evaluated by measuring the contact angles (DSA25, Kruss, Germany) at room temperature with distilled water using the sessile drop method. Ten measurements of contact angle were measured for each sample.

2.4. In vitro swelling and degradation studies

The composite samples (triplicates) were weighed (W₀) initially and submerged in PBS at 37 °C. After 24 h the samples were retrieved from the PBS, and their wet weights (Wᵢ) were measured. The swelling percentage of the composites was calculated from the following equation [25]:

\[ S(\%) = \frac{Wᵢ - W₀}{W₀} \times 100 \]  

where \( W₀ \) and \( Wᵢ \) are the wet and dry weights of the samples, respectively.

To perform degradation studies, samples in triplicate were weighed (Wᵢ) and soaked in PBS for 6 weeks at 37 °C. At regular time intervals, the samples were taken out, dried at 40 °C for 4 days, and weighed (Wᵢ). The pH of the PBS medium was also measured. The degradation percentage (Dᵢ) in the form of weight loss was calculated from the following equation [25]:

\[ Dᵢ(\%) = \frac{Wᵢ - Wᵢ}{Wᵢ} \times 100 \]
where \( W_i \) and \( W_f \) are the initial and final weights of the samples, respectively.

2.5. In vitro bioactivity studies

The bioactivity study of the PLGA composites was carried out in simulated body fluid (SBF), an inorganic physiological solution having a similar composition to that of human blood plasma. The SBF was prepared following the standard procedure described by Kokubo and Takadama [26]. The composite samples were immersed in SBF and kept at a constant temperature of 37 °C in a water bath for 21 days. The samples were then removed, rinsed with deionized water and dried. The growth and morphology of the apatite formed were observed by FESEM. Furthermore, energy dispersive x-ray spectroscopy (EDS) was performed to analyze the calcium (Ca) and phosphorous (P) content of the apatite.

2.6. In vitro hemocompatibility studies

The hemocompatibility of the PLGA composite samples was studied by performing hemolysis and anticoagulant assays. To perform hemolysis assay, the samples in triplicates were equilibrated with a physiological saline solution for 24 h. The equilibrated samples were put into test tubes with fresh physiological saline (10 ml) and incubated at 37 °C for 30 min. After the addition of anticoagulant (potassium citrate), goat blood was diluted (v/v: 4:5) with physiological saline. The diluted goat blood (0.2 ml) was added to each tube containing samples with saline and incubated for 1 h at 37 °C. To prepare negative and positive controls, diluted blood was added to physiological saline and distilled water, respectively. After the incubation, the samples were centrifuged (1000 rpm for 10 min) and their absorbance (OD) was taken from the supernatant liquid at 545 nm.

### Hemolysis

The anticoagulant properties of the samples in triplicates were evaluated by a kinetic clotting time method [28]. The clotting reaction was activated using \( \text{CaCl}_2 \) solution. The samples of equal size (1 \times 1 cm) and cover slips (control) were kept in different wells of a 12-well plate. Fresh blood (20 \( \mu l \)) was added to each well followed by the addition of 10 \( \mu l \) of \( \text{CaCl}_2 \) (0.2 M) solution and incubated at 37 °C at regular time intervals (10, 20, 30, 40, 50, and 60 min). Then 5 ml of distilled water was added into each well and incubated for 5 min at 37 °C. The concentration of hemoglobin released was determined by measuring the absorbance at 540 nm.

### Protein adsorption studies

To perform the protein adsorption study, a calibration curve for standard BSA solution was plotted ranging from 200–1000 \( \mu g \text{ ml}^{-1} \). The Bradford assay was used for the quantification of adsorbed protein by the PLGA composite samples. The samples (triplicates) were soaked in 1 ml BSA solution (1 mg ml\(^{-1}\) protein in PBS) for 24 h at 37 °C. The samples were recovered from the solution and then centrifuged at 4000 rpm for 10 min. After centrifugation, 100 \( \mu l \) of the supernatant was mixed with 1 ml of Bradford reagent and 2 ml of distilled water followed by incubation in the dark for 10 min, and its absorbance was taken at 595 nm using a UV-spectrophotometer. The protein concentration was determined using the calibration curve.

2.7. Protein adsorption studies

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2.8. Tensile properties

The tensile properties of the PLGA composites were obtained using a universal testing machine (Electro-Puls E1000, Instron, UK) with a load cell of 250 N at a strain rate of 2 mm min\(^{-1}\). The samples were cut following the ASTM D3039 standard, and the experiment was done in triplicate [29].

2.9. In vitro biocompatibility studies

Human osteoblast like MG-63 cells were cultured in T-25 tissue culture flasks in a humidified atmosphere containing 5% \( \text{CO}_2 \) at 37 °C in DMEM supplemented with 10% FBS, and 100 U/ml of penicillin-streptomycin. Prior to cell seeding, the samples in triplicate were sterilized using UV and ethanol treatment for 20 min each.

2.9.1. Cell viability

The cell viability of osteoblast cells cultured on the PLGA composites was evaluated using MTT assay protocol [30]. The MG-63 cells (1 \times 10^4 cells) were incubated on the samples in a 24-well plate at 37 °C in 5% \( \text{CO}_2 \). After 48 h, 20 \( \mu l \) of the MTT solution at a concentration of 5 mg ml\(^{-1}\) was added to each well, and incubated for 4 h. The removal of the MTT solution from the 24-well plate was followed by the addition of dimethyl sulfoxide (DMSO) to solubilize the formazan crystals. After shaking the plate gently for 15 min, its absorbance was taken at 595 nm.

2.9.2. Cell morphology

The MG-63 cells (1 \times 10^4 cells) were seeded on the sterilized samples into a 24-well plate and incubated (at 37 °C in 5% \( \text{CO}_2 \)). After 7 days, the samples were washed gently with PBS. For fixing the samples, 2.5% glutaraldehyde solution (3 h) was used followed by dehydration in a graded series of ethanol solution (30%, 50%, 70%, 90%, 95%, and 100%). The samples were dried (vacuum), and gold sputter coated before observing the cell morphology and adhesion in FESEM. To further evaluate the cytoskeletal
organization of the cells, double staining was performed on the PLGA composite samples. After 48 h of cell seeding, the cells on the samples were fixed with paraformaldehyde (4%) for 15 min, and permeabilized with Triton-X 100 (0.25% in PBS) for 10 min. The actin cytoskeleton and nuclei of the cells were stained with TRITC-phalloidin and Hoechst stain, respectively. The stained samples were washed with PBS, followed by the addition of lysis buffer and then p-nitrophenyl phosphate (pNPP) substrate for 1 h at 37 °C. To stop the reaction, 0.5 N NaOH was added and the absorbance was measured at 405 nm.

2.9.3. Alkaline phosphatase (ALP) activity
The alkaline phosphatase activity of osteoblast cells incubated on PLGA composites was evaluated using ALP colorimetric assay. After 7 days of cell seeding, the samples were washed with PBS, followed by the addition of lysis buffer and then p-nitrophenyl phosphate (pNPP) substrate for 1 h at 37 °C. To stop the reaction, 0.5 N NaOH was added and the absorbance was measured at 562 nm. A calibration curve was drawn for known concentrations of ARS.

3. Results and discussion

3.1. Characterization
TEM analysis was performed to investigate the different structures of the three carbon-based materials used in this study to reinforce the PLGA matrix. The TEM micrographs (figure 1) revealed the typical morphology of the CNTs, GNP, and AC. The CNTs and GNP showed their respective tubular and sheet-like (platelets) structure. However, AC exhibited irregular structure. The SAED patterns indicated the amorphous nature of the CNTs, whereas the GNP displayed crystalline nature. The lack of distinct reflections in the SAED pattern indicated the poorly ordered structure of AC, which is consistent with a turbostratic structure [31].

The cross-sectional surfaces of the composites were examined to investigate the dispersion of different carbon reinforcements in the PLGA matrix. The FESEM images (figure 2) showed that the PL surface was smooth and flat. The addition of reinforcements in the PLGA matrix made the composite surfaces relatively rough, which is expected to improve the biological responses on the composites. The FESEM images at higher magnification showed the well-dispersed CNTs (figure 2(e)), GNP (figure 2(f)), and AC (figure 2(g)) in the polymer matrix. Generally, nanomaterials like CNTs and GNP tend to aggregate in polymer matrices due to van der Waals forces. However, in the present study, carboxylic acid functionalized CNTs and GNP were used. Therefore, the reinforcements were well dispersed in the PLGN and PLGN samples and no agglomeration was observed. Furthermore, owing to the presence of oxygen-containing groups, no agglomeration of AC in the PLAC confirmed their well distribution throughout the matrix. Hence, the FESEM results confirmed the successful reinforcement of carbon biomaterials in the PLGA matrix.

The dispersion of the carbon reinforcements was further evaluated by performing confocal microscopy (figure 3). The FITC-dextran labeled (fluorescent green colored) reinforcements were found to be well dispersed in all the samples. The PLN sample showed good distribution of CNTs with few small aggregates. On the other hand, PLGN, with a comparatively fewer number of small aggregates, showed the best dispersion, which is further expected to improve its mechanical properties. As shown in FESEM micrographs, AC particles of various sizes were also found in the confocal micrographs.

The XRD patterns of the CNT, GNP, AC, PL, and PLGA composites are shown in figure 4(a). The CNTs and GNP showed characteristic peaks at 25.6° and 26.5°, respectively. The AC exhibited broad peaks around 25° and 44° corresponding to amorphous
graphite phase and a sharp peak at 26.5°. This diffraction pattern evidenced the turbostratic structure of AC consisting of randomly oriented graphitic carbonaceous layers [32]. The larger d_{002} value (0.341 nm) for AC in comparison to that of graphite (0.335 nm) confirmed the turbostratic carbon structure [33]. This result was also supported by SAED results of AC (figure 1), which displayed blurred rings corresponding to the low crystallite ordering [31]. The PL showed a broad peak around 21°, which confirmed the amorphous structure of PLGA. All the composite samples showed this characteristic peak of PLGA. However, the XRD pattern of PLCN composite did not reveal any characteristic peak for CNTs. This might be due to its amorphous nature, lower concentration and the absence of CNTs at the surface of the composite. In contrast, PLGN displayed a sharp peak for GNPs, and PLAC exhibited a small peak for AC confirming the presence of reinforcements in the polymer matrix.

The chemical composition and intermolecular interactions in the PLGA composites were explored by performing FTIR analysis (figure 4(b)). The FTIR spectra of CNTs showed a peak at 3728 cm\(^{-1}\) attributing to the free O–H groups, while the peak at 2361 cm\(^{-1}\) can be assigned to the O–H groups from the strongly hydrogen-bonded carboxyl groups [34]. Two peaks for C–H stretching were observed at 2922 and 2850 cm\(^{-1}\), whereas, the peaks for C=O stretching were obtained at 1738 and 1682 cm\(^{-1}\). A broad peak for C=C stretching at 1535 cm\(^{-1}\) and a weak peak at 1026 cm\(^{-1}\) correspond to C=C stretching and C–O stretching. The GNPs IR spectra showed
characteristic peaks at 3725, 1682, and 1535 cm\(^{-1}\), which can be assigned to free O–H groups, \(\text{C}=\text{O}\) and \(\text{C}–\text{C}\) stretching. The presence of \(\text{C}=\text{O}\) and O–H groups in the FTIR spectra of both CNTs and GNPs, confirmed their carboxylic acid functionalization. Furthermore, AC showed weak intensity peaks for O–H and \(\text{C}–\text{C}\) bond stretching at around 3728 and 1527 cm\(^{-1}\), respectively. The pure PLGA (PL) sample showed sharp peaks at 1742, 1451, 1188, and 1087 cm\(^{-1}\) attributing to \(\text{C}=\text{O}\), C–H, C–O–C, and C–O stretching, respectively. The above-mentioned characteristic peaks for PLGA were also found in all PLGA composites. In addition, PLCN and PLGN showed a sharp peak for \(\text{C}=\text{O}\) stretching at around 1682 cm\(^{-1}\), confirming the presence of carboxylic acid functionalized reinforcements in the PLGA matrix. The free O–H groups (3728 cm\(^{-1}\)) were also found in all the composites.

The water contact angle measurements were performed to evaluate the effect of different reinforcements on the surface hydrophobicity of the PLGA. The PL sample showed a high water contact angle (\(\sim 95^\circ\)), indicating its highly hydrophobic nature (figure 4(c)). This hydrophobic nature of PLGA is a major concern as this could lead to its unfriendly behavior to cells and proteins [35]. A significant decrease in contact angle was obtained with the addition of carbon-based reinforcements. The decrease may be owing to the comparative hydrophilic nature of the carbon reinforcements exposed to the composite surfaces. The oxygen-containing functional groups in the CNTs, GNPs, and AC have hydrogen-bond interactions with the water leading to a decrease in contact angle and thus, an increase in the hydrophilicity of the composites. Both PLCN and PLGN showed almost similar contact angles i.e. 70° and 68°, respectively, whereas PLAC showed a slightly higher contact angle (79°). This is due to the presence of more carboxylic acid groups on functionalized CNTs and GNPs than on the AC used in this study. The difference in position-dependent contact angles for all composite samples was not significant, indicating the proper dispersion of carbon reinforcements in the PLGA matrix. Thus, the contact-angle results suggest that the presence of oxygen-containing carbon biomaterials significantly improves the hydrophilicity of the PLGA composites, making them suitable for protein and cell attachment.

3.2. In vitro swelling and degradation studies

The in vitro swelling degrees of the PLGA composite samples after 24 h of incubation in PBS are shown in figure 5(a). As expected, pure PLGA showed the least
swelling percentage, indicating its hydrophobic nature. The reinforcement of carbon biomaterials in the PLGA matrix leads to an increase in the swelling percentage. This significantly improved swelling of all the composite samples is due to the oxygen-containing functional groups on the surface of carbon reinforcements, which tend to absorb more PBS, increasing its swelling percentage. The PLGN composite showed the highest swelling percentage (~40%) due to the presence of more hydrophilic groups exposed on its larger surface area (due to the sheet-like structure of GNPs) when compared to the PLCN and PLAC samples.

The in vitro degradation of the PLGA composites was evaluated in terms of weight loss after soaking in PBS for 6 weeks and the results are shown in figure 5(b). Initially, all samples showed a slight reduction of weight for the first few days. Similar to the swelling studies, the pure PLGA sample showed the lowest degradation percentage. However, with the incorporation of carbon reinforcements in the PLGA matrix, the degradation rate was accelerated. Until three weeks, all the composites showed almost the same weight loss. After three weeks, a dramatic increase in weight loss was observed in the PLGN sample. The fast degradation process of the composite samples may be due to a higher interaction of carbon biomaterials with the PLGA matrix and the PBS. Also, the functionalized CNTs and GNPs create hydrophilic pockets in the composites, which could hasten the hydrolytic degradation of PLGA. Furthermore, the better interaction of the sheet-like structure of the GNPs with PLGA matrix accelerated the degradation of the PLGN composites. The degradation results are also supported by the results of the swelling studies and contact angle. The increase in hydrophilicity of the composites speeds up the degradation process leading to increased weight loss of the composite samples. The pH of the PBS was also measured periodically, as the degradation of PLGA leads to acidic byproducts. The change in pH of the degradation medium was synchronized with the weight loss of all the samples (figure 5(c)). With degradation time, the pH was found to decrease for all the samples. In comparison with pure PLGA (PL), the composite (PLCN, PLGN, PLAC) samples showed lower pH (acidic). Hence, from the studies it can be concluded that the carbon reinforcements improved the degradation rate of the PLGA matrix.

3.3. In vitro bioactivity studies
To mimic the natural bone mineralization process, in vitro bioactivity studies were performed by immersing the samples in SBF for 21 days. As confirmed by FESEM results (figure 6), the PL sample showed a much lower amount of apatite formed, which is in agreement with the literature [6]. In contrast, a thick layer of apatite was observed on all the composite samples.
samples after 21 days. The presence of oxygen functionalities on the surface of the different carbon reinforcements improved the bioactivity of the composites. The carboxyl groups present on the functionalized CNTs and GNPs trigger the apatite formation, leading to more bioactivity of PLCN and PLGN. Therefore, both PLCN and PLGN showed dense apatite formation, whereas in PLAC slightly less apatite was observed.

The negatively charged functional groups present on the carbon reinforcements (CNTs, GNPs, and AC) acted as nucleation points for ionic clusters by attracting positively charged $\text{Ca}^{2+}$ ions followed by the deposition of negatively charged $\text{PO}_4^{-}$ ions, forming an apatite layer. Similarly, more $\text{Ca}^{2+}$ and $\text{PO}_4^{-}$ ions get deposited from SBF, forming a thick apatite layer. The homogeneous layer of apatite on all the composites confirmed the proper dispersion of the reinforcements in the PLGA matrix. Along with the surface charge, the surface morphology has also been reported to influence the apatite formation [36]. In the present study, the increase in surface roughness due to the carbon reinforcements might have provided more nucleation sites on the composite surfaces [37]. Furthermore, the elemental analysis of the apatite confirmed the presence of Ca and P. The Ca/P molar ratio was observed to be 1.8 (PL), 1.65 (PLCN), 1.66 (PLGN), and 1.59 (PLAC). The Ca/P ratio of all the composite samples was close to that of stoichiometric hydroxyapatite (~1.67). Thus, the results demonstrate that the rough surface and presence of carbon-based biomaterials with oxygen functionalities triggers the self-assembly of apatite.

3.4. In vitro hemocompatibility studies

The applicability of a biomaterial to be used in contact with blood can be evaluated from its hemolytic and blood-clotting behavior. The results of hemolysis assay represented in the form of percentage hemolysis are shown in figure 7(a). The values for positive and negative control were 100% and 0%, respectively. The PL sample showed the least hemolysis, and was found to increase for the carbon-reinforced PLGA composites. This increase in hemolysis values might be due to the roughness induced by the carbon reinforcements. The PLCN sample showed the highest hemolysis values, which is due to the toxic impurities retained during CNT synthesis. However, the increase was not very significant and the results for all the samples were within the permissible limit (<5%) set by ISO 10993-4 standards [25].

During blood clot formation, thrombin transforms into fibrin fibers, which entrap the hemoglobin. The quantity of the entrapped hemoglobin increases with time and hence, decreases the free hemoglobin. The free hemoglobin can be used as an indicator to assess the anticoagulation property of a biomaterial. The absorbance values of samples at 540 nm are shown in figure 7(b). According to this assay, a higher absorbance value indicates thromboresistance (i.e. less chance of clot formation) of the material.

From the results, it is evident that the absorbance values of all the samples are significantly higher than...
the control (cover slips). In addition, the blood incubated with the PL samples had lower absorbance values when compared to composite samples, suggesting rapid clotting in the case of the PL samples. These results imply that all the composite samples (PLCN, PLGN, PLAC) have good anticoagulation property. This is due to the improved hydrophilicity of the PLGA composites reinforced with oxygen-containing carbon biomaterials. The enhanced hydrophilicity reduces the platelet adhesion on the composite surfaces, leading to better anticoagulation property. The lowest contact angle of PLGN leads to the best hemocompatibility among all the samples. Therefore, from both the hemolysis and anticoagulation assays, it can be concluded that the PLGA composites reinforced with carbon possess good hemocompatibility.

### 3.5. Protein adsorption studies

The adsorption of proteins on the surface of an implant is among the leading events that occur immediately after implantation. This adsorbed protein further mediates the cell attachment and other biological responses. Thus, to elucidate cellular response towards a developed composite, it becomes important to evaluate the protein adsorption on its surface. In this study, BSA was used as a model protein, because of its structural and functional similarity to that of human serum albumin. The results for BSA protein adsorption on the surfaces of different PLGA composites are shown in table 1.

The PL sample showed the least protein adsorption. However, an increase was observed on the PLGA reinforced with carbon materials. The oxygen functionalities present on the surface promoted the protein adsorption of the composites \[38, 39\]. This might be due to the interaction of the surface hydrophilic groups with the functional groups of BSA via van der Waals and electrostatic forces. The high ionic strength buffer (i.e. PBS) used to perform this study further encouraged these interactions. Another reason for increased protein adsorption might be the roughness caused by carbon reinforcement, which provided larger surface areas for proteins to adsorb. Carbon nanomaterials like CNTs and GNPs induced more protein adsorption on the PLCN and PLGN composites, as the π electron cloud present on these graphite structures interact with the hydrophobic part of the BSA proteins. Also, the presence of more functional groups on the sheet-like (2D) structure of the GNPs resulted in the highest protein adsorption on the PLGN samples. The protein adsorption on PLAC was almost similar to that on PLCN. The high adsorption properties of AC played an important role in such results. Thus, the existence of carbon biomaterials in the PLGA matrix improved the protein adsorption on the composites making them suitable for cell attachment.

### Table 1. Protein adsorption on the PLGA composites.

| Samples | Protein adsorption (μg/μl) |
|---------|----------------------------|
| PL      | 256.15 ± 17.02             |
| PLCN    | 326.23 ± 10.56             |
| PLGN    | 357.40 ± 10.23             |
| PLAC    | 322.01 ± 18.6              |

### 3.6. Tensile properties

The mechanical properties of pure PLGA and PLGA reinforced with CNTs, GNPs, and AC were assessed by tensile strength, Young’s modulus, and energy at break. The typical stress–strain curves for PLGA and PLGA composites are shown in figure 8. All the composites demonstrated a significantly higher tensile strength when compared to the pure PLGA sample. The addition of 1 wt% of CNTs, GNPs, and AC in the PLGA matrix resulted in a 1.6, 2.0, and 1.2-fold increase in tensile strength of the composites, respectively (table 2). Similarly, the Young’s modulus and energy at break were also found to increase, making the composites stiffer and tougher in comparison to pure PLGA. The PLAC sample showed weaker tensile properties in comparison to the other two composites. This might be due to the better dispersion of functionalized nano-sized CNTs and GNPs in the PLGA matrix. Also, the functional groups present on the surface of the CNTs and GNPs form a hydrogen bond...
improve the mechanical strength of the composites, although all the carbon reinforcements were found to break, respectively, when compared to the PL sample. The sheet-like structure of GNPs provides a larger surface topography and accordingly regulate their shape and cytoskeletal organization. The morphology of the osteoblast cells cultured on the PLGA composites was evaluated by FESEM, as shown in figure 9(b). It can be seen from the images that the cells were round in shape and less spread out on the PL sample. However, on the PLGA–carbon composite sample the cells were more spread out and elongated. The propagation of filopodia was evident on the composite samples. Cells were found to adhere to each other with cellular microextensions. The osteoblast cells cultured on the PLGN sample showed spindle-like morphology, whereas on the PLGN sample the cells were fully spread out and formed a group.

The actin filaments are cytoskeletal proteins, which mediate the cellular movement and provide structural support to the cells. The cytoskeletal organization of cells cultured on the PLGA composite samples was evaluated by confocal microscopy (figure 9(c)) after dual staining of the nuclei and actin filaments. A larger number of cells was observed on the composite samples compared to the PL sample. The reinforcement of carbon materials increases the surface roughness (especially with carbon nanomaterials such as CNTs and GNPs), which is believed to improve cell adhesion and growth. This is due to the increase in protein adsorption on rough surfaces.

It has also been reported that the presence of oxygen-containing groups shows a positive response towards cell attachment and growth [8]. Amongst the carbon-containing composites, cells cultured on PLGN displayed better cytoskeleton organization and actin expression. In the PLGN sample, the cells were distributed on the entire surface and showed numerous filamentous extensions. Many layers of well–spread osteoblast cells with filopodia were observed and actin filaments were also clearly visible. Therefore, the 2D structure of GNPs resulted in better adhesion of cells on the composite samples.

3.7.1. Cell viability

The viability and proliferation results of MG-63 cells cultured on PLGA composites are normalized and shown in figure 9(a). The results indicated that the carbon reinforcements used in this study did not cause any cytotoxicity. It is evident from the results that all the samples, including PL and PLGA composites, facilitated cell proliferation when compared to the control plate (24-well cell culture plate). Furthermore, the cell viability of all the carbon-reinforced PLGA composites was higher than that of the pure PLGA samples. This shows that the reinforcement of carbon biomaterials encourages the cell attachment and growth on the composite surfaces. The hydrophilic functional groups present on the carbon reinforcements improve the protein adsorption (section 3.5), which further increases the cell adhesion and growth. The cells proliferated faster on the PLGN composites compared with PLCN and PLAC. This is due to more functional groups and high protein adsorption of the PLGN sample on its surface. The MTT results suggested that the carbon reinforcements in the PLGA matrix potentially increase the cell proliferation on the composite samples.

3.7.2. Cell morphology

The adhesion of osteoblast cells plays an important role in the expression of cell functions such as cell proliferation and differentiation [40]. It depends on different factors like surface charge, hydrophilicity, and roughness of the material. The cells can sense surface topography and accordingly regulate their shape and cytoskeletal organization. The morphology of the osteoblast cells cultured on the PLGA composites was evaluated by FESEM, as shown in figure 9(b). It can be seen from the images that the cells were round in shape and less spread out on the PL sample. However, on the PLGA–carbon composite sample the cells were more spread out and elongated. The propagation of filopodia was evident on the composite samples. Cells were found to adhere to each other with cellular microextensions. The osteoblast cells cultured on the PLGN sample showed spindle-like morphology, whereas on the PLGN sample the cells were fully spread out and formed a group.

Alkaline phosphatase, an early and quantitative osteoblastic differentiation marker, is an important factor in the formation of hard tissue. The osteoblast cells mineralize bone matrix during their differentiation. The ALP helps in decreasing the concentration of...
extracellular pyrophosphate, which is an inhibitor of mineralization and it increases the inorganic phosphate concentration to promote mineral formation. Osteogenic differentiation of cells cultured on PLGA composites was estimated by quantifying ALP activity and the results with respect to the control (24-well plate) are shown in figure 10(a). All the samples revealed up-regulation of ALP activity in comparison to the control. Furthermore, the cells grown on composites displayed significantly higher ALP activity than those on the pure PLGA sample. Thus, carbon-reinforced PLGA composites supported higher osteoblast differentiation. Among these composites, the PLCN and PLGN samples showed better results than the PLAC sample. The nanostructured CNTs and GNPs provide better surface topography and electric properties, which play an important role in the promotion of cell differentiation. Consistent with the cell viability results (section 3.7.1), the PLGN composite has shown the best ALP expression. High protein adsorption on sheet-like structured GNPs (thus, larger area and more functional groups to interact) leads to more cell adhesion on the PLGN samples, which further expresses high ALP activity.

3.7.4. Mineralization assay
The calcium deposition on the samples cultured with cells indicates the matrix mineralization by osteogenic cell differentiation. The ARS-based assay was
performed to evaluate the matrix mineralization by quantifying the calcium content on the developed PLGA composite samples. Figure 10(b) shows the relative mineralization results normalized to the control. As expected from other biological results, the calcium deposition was lowest on the control when compared with the PL and PLGA composites. The carbon-reinforced samples demonstrated significantly higher calcium deposition than the pure PLGA sample after 7 days of cell culturing. Amongst the PLGA composites, PLGN showed the highest amount of bone nodule formation.

The functionalized reinforcements impart a negative charge to the surface of the composite. These negative surfaces encourage the heterogeneous nucleation and growth of hydroxyapatite. It has been reported that the presence of carboxyl groups induce calcium deposition even after a short period of cell culturing [38]. Upon implantation, the surface chemistry of the material plays an imperative role in regulating the cellular responses towards the implant. The cellular responses such as cell attachment, proliferation, differentiation, and matrix formation, depend on the protein adsorption on the implant. The oxygen-containing functional groups present on the three carbon reinforcements used in this study act as bioactive molecules by providing sites for protein adsorption. Also, the interaction of II electrons of graphite structure and the hydrophobic part of protein encourage protein adsorption on carbon nanomaterials (CNTs and GNP). These interactions specifically adsorb proteins, which enhance cell proliferation and differentiation, and hence, up-regulate the ALP activity and matrix mineralization.

Although the same amounts of the three carbon biomaterials were reinforced, the differences in their morphology lead to different results. The exceptional electric properties of CNTs and GNP played an important role in improving cellular responses compared to AC. Furthermore, in spite of having the same electric properties and graphitic structure, GNP showed better responses. This is mainly due to the sheet-like structure of GNP, which is different from tubular CNTs. A larger surface area of GNP, with a larger number of functional groups, was available for proteins and cells to attach. Therefore, GNP displayed stronger enhancement in cell proliferation and differentiation. From the results, it can be seen that carbon-reinforced PLGA composites not only supported cell attachment and proliferation but improved cell differentiation.

4. Conclusion

In this study, PLGA-based carbonaceous composites were successfully developed using a solvent-casting method. Three carbon materials (1 wt%: 1D CNTs, 2D GNP, 3D AC) with different structures were reinforced in PLGA matrix. The primary aim of the study was to analyze the effects of different structures of reinforcements on mechanical and in vitro biological properties of the composites. The presence of hydrophilic groups on reinforcements enabled their homogeneous dispersion in polymer matrix. All the carbonaceous composites showed improved hydrophilicity, swelling, degradation, protein adsorption, and bioactivity in comparison to the pure PLGA sample. The tensile strength of the samples was drastically improved with reinforcement of 1 wt% of each carbon material. The carbon reinforcement in the PLGA matrix stimulated the enhancement in cell biological behavior. Improvement in osteoblast cell proliferation and differentiation was observed from the ALP activity and mineralization assay. The different structures of reinforcements played an important role in tailoring the mechanical and biological properties of the composites. Both CNTs and GNP showed better results compared to AC, owing to their nano-structures, high aspect dimensional ratio, and exceptional mechanical and electric properties. Although both CNTs and GNP have similar graphitic structure and electric properties, GNP present greater advantages for reinforcing PLGA matrix over CNTs. The sheet-like 2D structure of GNP was more exposed for protein adsorption and cell attachment in comparison with the tubular CNTs. Also, their larger interface resulted in the effective load transfer between the PLGA matrix and GNP. These results suggested that the structure of reinforcement plays an important role. Overall, from the above study, it can be concluded that the PLGA-based carbonaceous composites provide a suitable microenvironment for cell proliferation and differentiation along with improved mechanical properties.

Notes

The authors declare no competing financial interest.

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