Research Article

Biocompatibility of Poly(Lactic-Co-Glycolic Acid) - Graphene Oxide Nanoplatelets Composite Using Cryopreserved Human Stem Cells

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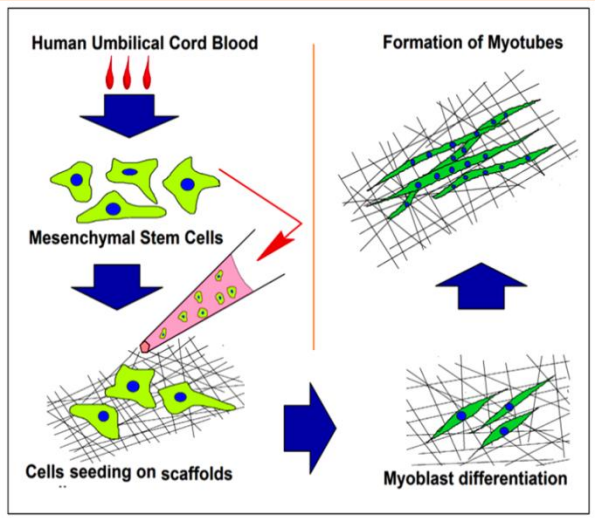
Abstract

The development of a suitable scaffold material for muscle tissue regeneration *in vivo* is still a major challenge. We reported excellent biocompatibility of electrospun meshes of poly(lactic-co-glycolic acid, PLGA)-graphene oxide nanoplatelets (GO) composite with graphene oxide concentration within the percolation threshold (f_c~0.78wt% GO) and non-toxicity limit (~20µg GO/mL solution). Cryopreserved human mesenchymal stem cells (hMSCs) were used showing myobast differentiation and myotube formation with increased cell viability. These results confirmed high potential of GO-PLGA scaffold meshes for skeletal muscle tissue regeneration or other biomedical applications like wound healing and drug delivery.



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Introduction

Over the last decade, various nanomaterials (nano diamond, graphene and carbon nanotubes) have been used with suitable polymer to meet the requirements of the desired scaffolds for tissue engineering (TE) [1]. Biopolymers, bioceramics and their polymer composites had already been extensively used for different biomedical applications [2, 3] because these materials possessed desired biocompatibility, biodegradability and good solubility in organic solvents. Interestingly, addition of very small amount (~0.3-0.5wt%) of graphene oxide nanoplatelets (GO) in different polymers like poly(methyl methacrylate) (PMMA) [4] and polyvinyl alcohol PVA [5] enhanced both conductivity (σ) and dielectric constant of the resulting composites along with mechanical stability [6, 7]. Enhancement of conductivity of the scaffold by adding suitable bioactive fillers also enhances biocompatibility along with conductivity [3]. Recently several reports on the tissue engineering applications of GO-polymers (PLGA, PCL etc.) composites have been made [8-10]. However, very little or no work has been done to show the biocompatibility of such composite meshes suitable for different biomedical applications using human stem cells or human cord blood drive stem cells. Most of the earlier studies were confined to the use of animal cell lines [3, 10]. So the study of biocompatibility of scaffolds using human stem cells is important. Moreover, in those studies with graphene oxide - polymer composites [3, 8], biocompatibility had been investigated using arbitrarily higher concentration of GO. Graphene oxide concentration in polymer above 50µg/ mL was reported to be toxic to the human cells [11]. Biocompatibility of the scaffold increases with increasing GO concentration as the conductivity of the composite increases with GO concentrations and also showed percolation threshold behaviour [12] in conductivity. But for biomedical applications, an optimum GO concentration must be used and that concentration should be kept within the non toxicity limit as well as below the said percolation threshold for better mechanical stability and biodegradability.

For the GO-PCL and GO-PLGA composite the conductivity threshold appeared around 0.75-0.78wt% [12]. Above the said percolation threshold, conductivity and hence biocompatibility increase was observed at the cost of mechanical stability and biodegradability [13].

In the present study, GO-impregnated biomimetic PLGA matrices with GO concentration within nontoxicity limit (20μg/ mL solution for human stem cells [11]) were fabricated via an electrospinning process and preliminary cell-scaffold interaction was study using cord blood derived stem cells similarly to our previous work [8] with GO-PCL scaffolds using higher GO concentrations. The widely used PLGA was chosen because of its low percolation threshold limit (f_c ~0.78wt% GO [13]) and this composite appeared to be a novel one with immense prospect for *in-vivo* tissue regeneration. Observed myoblast differentiation of stem cells and oriented myotubes formation on such PLGA-GO electrospun scaffold meshes with low GO concentration (within non toxicity limit) confirmed excellent biocompatibility and potential of the PLGA-GO composites for next generation TE and biomedical applications like wound healing and drug delivery.

Experimental

Chemicals and methods

GO was prepared as before [8] from pristine graphite powder. For making GO-PLGA composite, GO was added at a concentration of 20μg/ml in PLGA-DMF (dimethyl fluoride) solution (which was within the non-toxicity limit [11]) and stirred for two hr producing homogeneous solution. The obtained colloidal mixture was loaded into a 12mL plastic syringe with a stainless-steel needle (diameter ~0.60mm) and used for making fibrous meshes by using electrospinning machine (PICO ESPIN, India). The needle for electrospinning was connected to the high voltage supply (~20kV selected by adjusting the voltage by several trials) and the flow rate of the solution was adjusted to 1.5mL/h. The fibrous meshes were collected on aluminum foil placed at a distance of 12 cm from the needle tip. Electrospun collagen (0.10g/ml acetic acid) meshes were also prepared for control using similar technique. The mesh films were air dried and sterilized for one hr by UV (wavelength ~ 254nm and power 15W). Previously isolated cord blood derive mesenchymal stem cells [8, 13] were cryo-preserved (in liquid nitrogen with temperature controlling set up), which were used for testing biocompatibility of the scaffolds and myoblast differentiation potential.

The GO-polymer composite samples were characterized by X-ray diffraction (XRD) (Philips Shiffert 3710 diffractometer using Cu-K_α radiation source) analysis, scanning electron microscopy (SEM: JEOL JSM 6400) and field emission scanning electron microscopic (FESEM: FESEM Model JEM-2010, JEOL) studies. Raman spectroscopy (HORIBA JOBIN Yuon: Exciting wavelength 514nm with Argon ion laser), and Fourier transform infrared (FTIR: Perkin–Elmer spectrum 100 FTIR with a 4 cm^{-1} resolution) spectroscopic studies were carried out to characterize the samples. Frequency dependent dielectric permittivity (ε) and electrical conductivity (σ) values of PLGA-GO films (1cm diameter and ~0.1mm thickness) and meshes (20 mm x20 mm, n=5) were measured using HP impedance analyzer (Model 4941) [14, 15] and constant current and voltage sources. These studies indicated the importance of GO surface change for the enhancement of conductivity and dielectric constant of the composite and hence biocompatibility of the composite meshes.

Stem cells culture on PLGA-GO and biocompatibility

Cord blood derived mesenchymal stem cells, hMSCs, used for cell culture were isolated from human umbilical cord blood (UCB) similarly to our earlier work with GO-PCL and GO-PLGA composite scaffolds [3, 9, 12, 13]. All normal procedures of isolation of stem cells and formalities maintained were already reported [16].

The UCB derived mesenchymal stem cells, hMSCs (5×10^3 cells/mL), were directly seeded onto the PLGA- GO mesh (areas ~45 mm^2) and control substrates separately in a 12 well cell culture plate and cultured with skeletal muscle differentiation media (90 v/v%), supplemented with FBS (10 v/v%) and 100X antibiotic-antimycotic solution (1 v/v% approximately) and kept under incubation at 37^{o}C and 5% CO_2 condition. In addition, insulin like growth factor 1 (IGF-1) was added (5ng/mL) to enhance the differentiation process.

Results and Ddiscussion

Figure 1 showed the X-Ray diffraction patterns of the PLGA and PLGA-GO composite. XRD of GO indicated the characteristic graphene oxide peak around $2\theta = 11.1^0$ corresponding to a d-spacing of 0.78 nm [10]. XRD patterns of PLGA (Figure1a) showed amorphous character while PLGA-GO composite exhibited XRD peak (Figure 1b) at 21.65^0 indicating crystalline phase of the polymer due to the presence of GO. The well-dispersed low concentration GO acted as nucleating agents and thus the crystallinity of the composites was improved. Inset of Figure 1b showed

the micrograph of the electrospun meshes with ~85-90% porosity. Raman spectra of thin GO (Figure 1c) indicated the characteristic graphene oxide peaks at frequencies around 1338 and 1600cm^{-1}, respectively, for the G and D band usually assigned to the E_{2g} phonon of Csp^2 atoms and a phonon breathing mode of symmetry A_{1g}. The presence of GO was also observed from the PLGA-GO Raman spectra (Figure 1d).

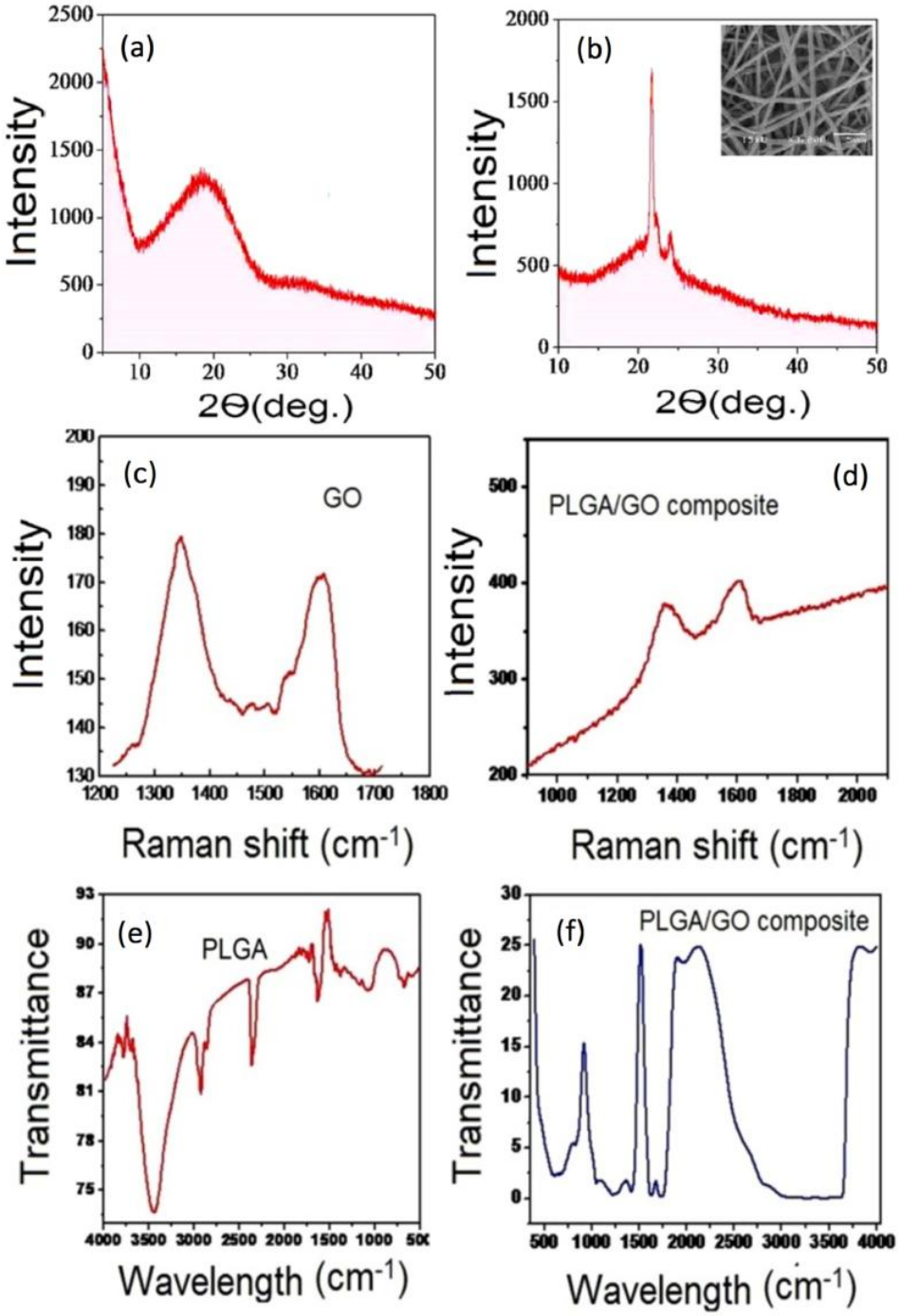

Figure 1 (a) XRD patterns of pure PLGA and (b) PLGA-GO Composite indicating sharp crystalline peaks. Inset of (b) shows the SEM micrograph of the electrospun PLGA-GO composite fibrous meshes. (c) Raman spectra of GO and (d) PLGA-GO composite meshes. FTIR spectra of (e) PLGA and (f) PLGA-GO composite meshes.

The Fourier transform infrared (FTIR) spectra of PLGA and PLGA-GO composite were shown in Figure 1e and 1f for comparison. The absorption bands observed in the PLGA-GO at 1727 cm^{-1} indicated carbonyl stretching. The bands at 1295 cm^{-1} and 1240 cm^{-1} represented the C-O and C-C stretching bonds. The bands at 1239 cm^{-1} and 1175 cm^{-1} were the asymmetric C-O-C stretching bonds indicating characteristic absorption of PLGA. The FTIR spectrum of PLGA-GO (Figure 1f) indicated an intense band at 3438cm^{-1} which was attributed to stretching of the O-H band of

CO-H. The band at 1639cm^{-1} was associated with stretching of the C=O bond of the carbonyl groups. Deformation of the C-O band was observed at 1017cm^{-1}.

From FTIR spectroscopy, evidences of different types of oxygen functionalities on GO were exhibited. The ultra violet (UV) spectrum of GO exhibited a maximum at 371nm, characteristic feature of the π-π* transition of aromatic C-C bonds. Similar peaks were also observed in GO-PCL [8, 13] as well as in PLGA-GO at 460 nm. The ionic bonds, the π-π stacking forces created by the sp^2 bonding and hydrophobic interaction between molecules allowed graphene to adsorb proteins and low molecular weight chemicals which is important for the biocompatibility of graphene oxide.

Conductivity and dielectric permittivity of the composite

The GO concentration dependent conductivity and dielectric permittivity were shown in **Figure 2**. Increase of both conductivity and dielectric permittivity were observed with increase of GO concentration indicating percolation threshold around 0.78wt%. These studies indicated the importance of GO surface change for the enhancement of conductivity of the GO-polymer composite. The increase of conductivity of the scaffold embedded with GO caused the enhancement of biocompatibility of the scaffold [3].

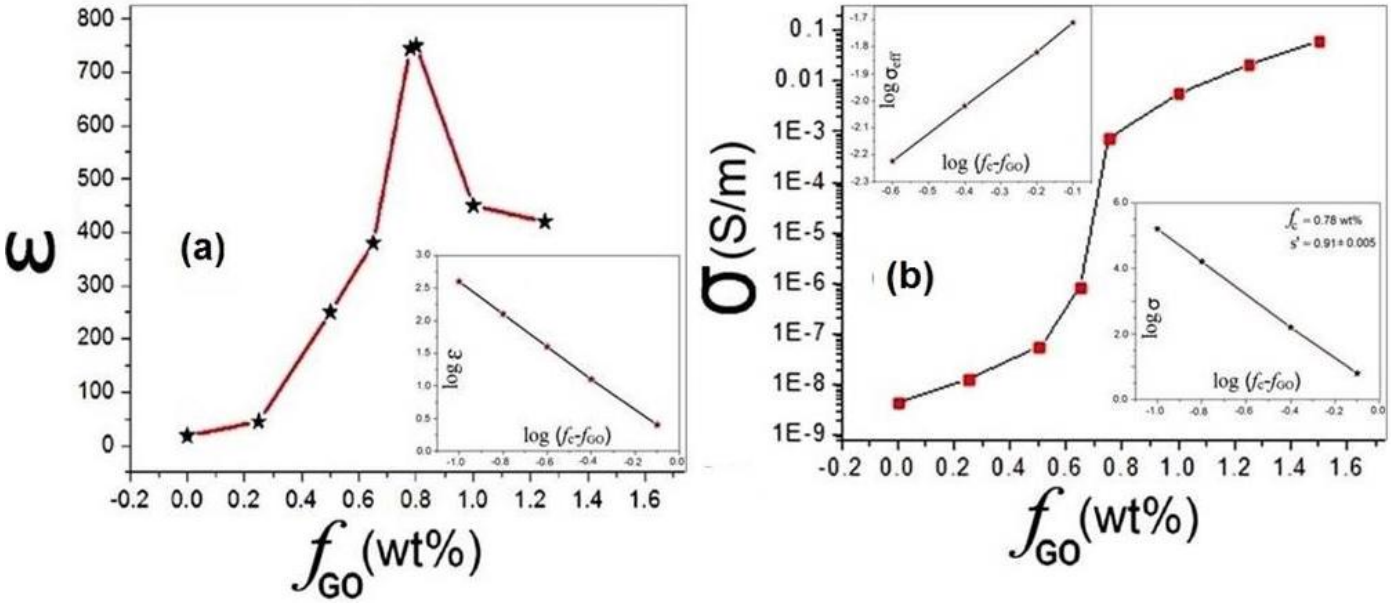

Figure 2 Dependence of effective dielectric constant ε (a) and conductivity σ (b) of the PLGA-GO composite on GO concentration f_{GO}. Inset of (a) shows the best fit of the ε data with Eq.1 (with f_c *=0.78).* The inset of (a) and (b) showed the theoretical [12] best fit data of σ data (with f_c *= 0.78*).

Myoblast differentiation on PLGA-GO fibrous meshes and cell viability

The FACS (Fluorescence-Activated Cell Sorter) analysis of the cells adhered onto the PLGA-GO mesh substrates was performed to confirm the positive expression of myogenic markers CD56 and desmin indicating the differentiation of hMSCs into skeletal muscle cells and cells phenotype (**Figure 3a-d**). Figure 3e showed the viability of myoblasts cultured on PLGA-GO scaffold meshes and on controls (collagen meshes) after 5 days of culture. Cell viability was found to increase significantly for the PLGA-GO meshes of our present investigation compared to the control surfaces. This result implied that PLGA-GO meshes were cytocompatible and supported cell proliferation.

Immunofluorescence staining

Immunostaining results confirmed differentiation of hMSCs into myoblasts via early expression of myogenin on collagen control shown in **Figures 4(a-b)** and PLGA-GO meshes (Figures 4 d-i)**.** Immunostaining of Desmin, MyoD, Myosin heavy chain (MHC) and Dystrophin for PLGA-GO meshes indicated better expression of these muscle specific antigens for PLGA-GO mesh compare to collagen control. Formation of myotubes were more aligned like natural orientation, on PLGA-GO mesh (Figure 4f) compare to that on control surface (Figure 4c).It is to be noted that both PLGA-GO and GO-PCL composite showed enhanced conductivity [13]. It was noticed that in comparison to GO-PLGA, GO-PCL [8] exhibited better biocompatibility and cell scaffold interaction. This might be due to higher

conductivity of the GO-PCL meshes compared to those of GO-PLGA. Different chemical structure s of the two interacting polymers (PCL and PLGA) might also be partially responsible for the enhance conductivity of the GO-PCL composite and biocompatibility. Therefore, the excellent biocompatibility and myoblast differentiation potentiality of PLGA-GO meshes compared to that of pure GO is considered to be due to increased conductivity (Figure 2) of the GO-PLGA composite meshes.

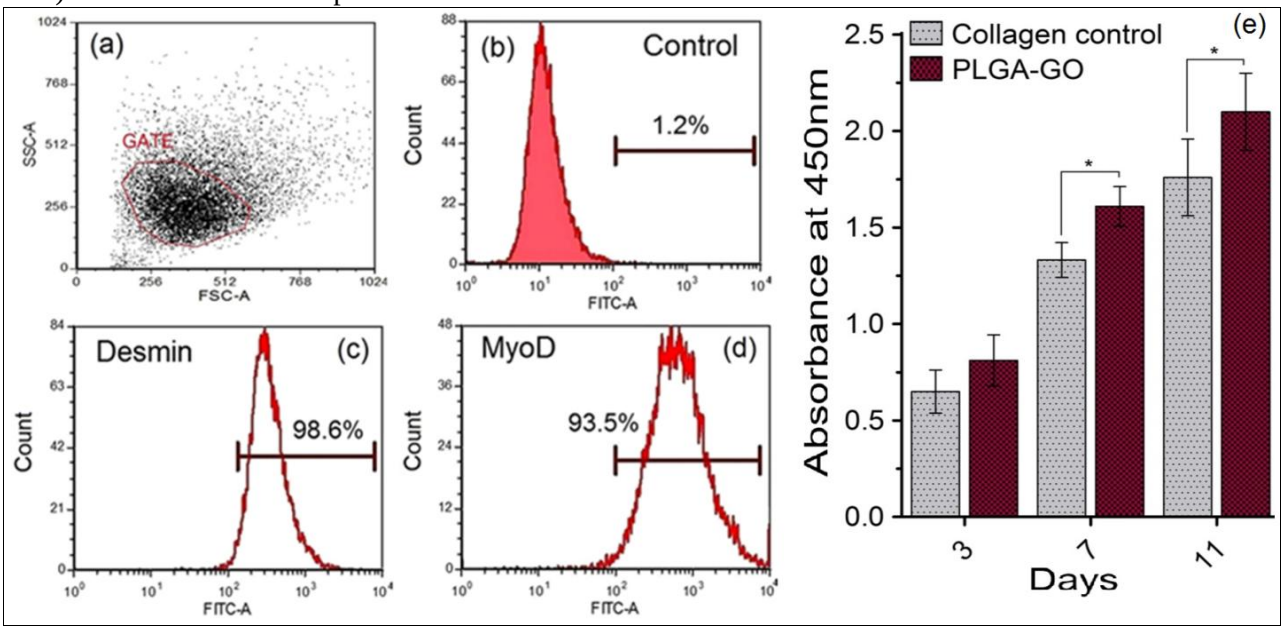

Figure 3 At passage 5, gating was done on main population discriminating the cell debris and doublet population (a). Isotype control (b). hSkMCs highly expressed for positive skeletal muscle markers desmin (98.6%) (c) and MyoD (93.5%)(d) representing skeletal muscle cells phenotype. (e) Cells viability and proliferation observed by tetrazolium salt (WST-8) assay. Results presented as the means ± standard deviation (SD). * indicates significant difference (*n=5; p<0.05*).

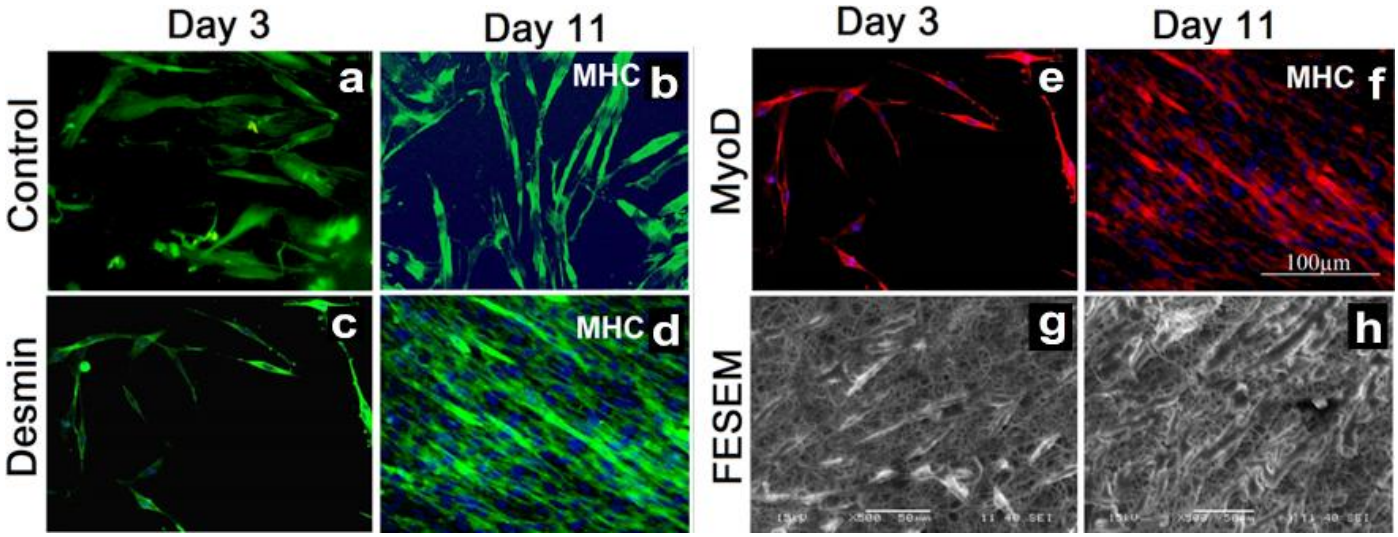

Figure 4 For PLGA-GO meshes, results of immunostaining using Desmin and Myosin heavy chain (MHC) on collagen control (a,b) after 3 and 11 days of culture (d-f). Nuclei were counterstained with DAPI (blue). FESEM analysis of myoblast cells grown on PLGA-GO meshes on (g,h).

FESEM was carried out for further confirmation of cellular attachment on PLGA-GO meshes after 3, and 11 days of culture (Figure 4g, h). Similar trend of aligned myotubes formation was found from FESEM micrographs. A better interconnectivity of the PLGA-GO fibrous mesh and enhanced conductivity and dielectric constant due to GO might play a combined role at the cell anchoring sites for oriented cell proliferation on PLGA-GO meshes. It is however, observed that for higher concentration of GO in the scaffold [8, 12], biocompatibility does not increase appreciably. This is important for in-vivo applications as in wound healing using antibacterial graphene oxide-polymer nano-

composites. Detailed analysis of the cell-scaffold interaction elucidating the origin of excellent biocompatibility of the GO embedded polymer scaffold associate with GO surface charge will be published elsewhere.

Conclusions

PLGA-GO nanocomposite fibrous meshes with low GO concentration within nontoxicity limit showed excellent biocompatibility, using cord blood stem cells, which was comparable with those obtained with higher GO concentrations. Addition of GO enhanced both conductivity and dielectric constant of the composites due to percolation behavior. The present results demonstrated enhanced biocompatibility of conducing PLGA-GO fibrous meshes, compared to GO or PLGA, showing excellent myoblast differentiation and self-aligned myotube formation with human mesenchymal stem cells. Increased biocompatibility of GO and GO-polymer composites were attributed to the surface charge and nano-flake structure of graphene oxide. The use of GO-based polymer composite meshes might be considered as the most potentials substrates for future generation tissue engineering and other biomedical applications (like wound healing and drug delivery).

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