pISSN 1738-2696 · eISSN 2212-5469
https://doi.org/10.1007/s13770-016-9130-1
ORIGINAL ARTICLE

TERM

Chitosan/Graphene and Poly(D, L-Lactic-co-Glycolic Acid)/Graphene Nano-Composites for Nerve Tissue Engineering

S. Soltani[1], M. Ebrahimian-Hosseinabadi[1]*, A. Zargar Kharazi[2]

[1]Department of Biomedical Engineering, Faculty of Engineering, University of Isfahan, Isfahan, Iran
[2]Biomaterials and Tissue Engineering Group, Advanced Medical Technology Department, Isfahan University of Medical Science, Isfahan, Iran

This study aimed at examining and comparing the fabrication process, electrical conductivity, and biological properties of Chitosan/Graphene membranes and poly(D, L-lactic-co-glycolic acid) (PLGA)/Graphene membranes. Nano-composite membranes were made using chitosan or PLGA matrix, and 0.5–1.5 wt.% graphene nano-sheets as the reinforcement material; all the membranes were fabricated through solution casting method. Fourier transform infrared spectroscopy and X-ray diffraction results indicated that the graphene had been uniformly dispersed in polymeric matrix. The membranes with 1.5 wt.% graphene appeared to have the highest value of electrical conductivity among all the examined the membranes and this growth was about 10^6 in comparison with neat polymers. Since the Chitosan 1.5% graphene membrane was found to have the highest proliferation after 72 hours by MTT [3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay of PC12 cell line ($p<0.05$), it is promising to consider nano-composite membrane for nerve tissue engineering applications.
Tissue Eng Regen Med 2016;13(6):684-690

Key Words: Nerve tissue engineering; Graphene; Chitosan; Poly(D, L-lactic-co-glycolic acid); PC12 cell line

INTRODUCTION

Nerve regeneration is a complex biological action; repairing peripheral nerve gap involves the usage of autologous nerve grafts. However, there are inherent disadvantages of autologous nerve grafting, including the limited supply of donor nerves, the loss of function at the donor nerve graft site, the need for a second surgery, and a mismatch between the donor nerve and the recipient site. Over recent years, tissue engineering approaches have provided some alternative methods to be used instead of autologous nerve graft. These methods involve fabrication of scaffolds with lots of biomaterial, and then seeding stem cells on them to produce an artificial tissue that is suitable for implantation [1-4].

Polymer-based nano-composite materials have attracted the attention of researchers in a wide range of fields, especially as scaffolds, because of their controllable features. In recent years, there has been a great interest in using natural polymers like Chitosan and synthetic polymer like poly(D, L-lactic-co-glycolic acid) (PLGA) as composite matrixes. Chitosan was chosen for scrutiny in the present study because it is one of the second-abundant natural biopolymers on the earth, and is structurally a copolymer of β [1,4]-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose, that is generally obtained by deacetylation of chitin. Because of its biocompatibility, biodegradability, multiple functional groups, antibacterial, its solubility in aqueous medium, and its low cost, chitosan has been investigated for several decades for a wide spectrum of applications [5-7]. PLGA is mostly used as the synthetic biodegradable and biocompatible polymer and is certified by the US Food and Drug Administration; PLGA could be applied for such medical uses as drug delivery, absorbable suture, and tissue engineering. However, whereas chitosan and PLGA have numerous advantages, their mechanical and electrical properties are not good enough to satisfy a wide range of applications [8-10].

Graphene is a single layer of carbon atom that consists of rings with a hexagonal lattice structure. Although graphene is categorized as carbon allotropes, it possesses distinct properties that no other carbon molecules, such as graphite and other allo-

Received: December 19, 2015
Revised: March 4, 2016
Accepted: March 14, 2016
***Corresponding author:** M. Ebrahimian-Hosseinabadi, Department of Biomedical Engineering, Faculty of Engineering, University of Isfahan, Azadi Sq., Isfahan 81746-73441, Iran.
Tel: 98-31-3793-5617, Fax: 98-31-3793-2771
E-mail: m.ebrahimian@eng.ui.ac.ir

tropes, have. Its electrical conductivity, for instance, is up to 2×10^3 S/cm. Other properties of graphene, such as superior elasticity and adsorption of protein, and low molecular weight substances, may change the direction of stem cell differentiation and neural cell proliferation. Its elasticity value is reported to be 0.1−1 terapascal [11-17]. Thus, graphene nano-sheets are ideal reinforcements for the nano-composite membranes that could be used in nerve tissue engineering applications such as nerve guidance channel. Indeed, a series of experiments has already been conducted on using the nano-sheets of graphene oxide, or reduced graphene oxides, as nano-fillers to improve the conductivity or the strengths of polymers [18-24], but in this research the membranes were prepared with pure (99%) graphene nano-sheets to affirm the ability of pure graphene without any functionalized reaction and through a cheaper and easier method.

This study provides a report of the preparation of nano-composite membranes with solution casting method because this method is the green fabrication process [22]. The membranes include Chitosan and PLGA, as matrixes, and 0.5−1.5 wt.% graphene nano-sheets, as the reinforcement materials. Moreover, the electrical conductivity and biological properties were investigated and compared for all membranes in order to select the most suitable membrane for nerve tissue engineering applications.

MATERIALS AND METHODS

Materials

Chitosan, MTT [3-(4,5-di-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide], and Trypsin-ethylene diamine tetra-acetic acid (EDTA) were supplied by Sigma-Aldrich (Germany). Chitosan has medium molecular weight and viscosity between 200−800 cP. PLGA 75:25 was purchased from Purac biochem bv (the Netherland). Graphene powder was bought from Neutrino (Iran). Acetic acid (glacial) and chloroform were purchased from Merc (Germany). Finally, phosphate buffered saline (PBS), fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were supplied by Bioidea (Iran). All of these materials were used as received.

Fabrication of nano-composite membranes

The chitosan/graphene membranes were produced by the solution-casting method. Graphene powder was first dispersed in an aqueous solution of 1 wt.% acetic acid by ultra-sonication for 12 h. Then chitosan was dissolved in this graphene suspension by stirring (ARE VELP, Italy) for 3 hours with 300 rpm. This way, composite membranes with different graphene contents (0, 0.5, 1, and 1.5 wt.%) were produced. The disc-like membranes were first dried at room temperature and then in oven at 50°C overnight to completely remove acetic acid. The procedures for the preparation of PLGA/graphene nano-composite films are as follows. Dispersion of graphene nano-sheets was obtained by mixing them in chloroform solvent, followed by ultra-sonication for 12 h. Then, the dispersion was mixed with PLGA polymers at certain concentration and then was mixed by magnetic stirring for 2 h. Composite membranes with different graphene contents (0, 0.5, 1, and 1.5 wt.%) were produced. The disc-like membranes were first dried at room temperature and then in oven at 50°C overnight to completely remove chloroform.

Instruments

The fourier transform infrared (FT-IR) spectra for chitosan/graphene nano-compsoite membranes were recorded [FT-IR-6300 (400−4000 cm^{-1}) JASCO, Japan] with attenuated total reflectance accessory. The wide-angle X-ray diffraction (XRD) patterns of chitosan/graphene membranes were recorded (XMD-300 UNISANTIS, Switzerland). The morphology of the surface and cross section of the membranes were measured by scanning electron microscopy (SEM), (435 VP LEO, UK) with an acceleration voltage of 16 kV. The electrical conductivity of the films was measured using a standard four-probe method (KETHLY instrument, USA).

Cell culture study

Proliferation rate of PC12, with informed consent considering ethical issues, were studied on neat and composite membranes and were compared with a control sample by MTT assay. PC12 (Pasteur Institute of Iran, NCBI code: C153) is a rat pheochromocytoma cell line; this neuronal fibroblasts-like cell is one of the usual cell lines for MTT assay of nervous implants like nerve guidance condiutes [4,12]. PC12 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution (penicillin, streptomycin, amphotericin, and gentamycin) under standard cell culture conditions (i.e., 37°C, 5% CO_2/95% humidity). After reaching 80−90% confluency, the cells were detached by a 0.25% solution of trypsin-EDTA and viable cells were counted by trypan blue assay.

Prior to cell seeding, the chitosan/1.5 wt.% graphene membranes and PLGA/1.5 wt.% graphene membranes were cleaned in 75% ethanol solution [13], sterilized for 30 min. under ultraviolet light, washed 2 times with PBS and incubated with DMEM for 12 h at 95% humidity. After the samples were placed in a 24 well polystyrene (as a negative control) culture plate, cells were further seeded onto the top of the samples at a density of 20×10^3 cells/well and cultured with medium at 37°C, 5% CO_2 and 95% humidity. During the cell culture, the medium was replaced every other day. For investigation of proliferation after 1 and 3 days of cell seeding in a 24-well plate, cells were washed with

PBS to eliminate nonviable cells, and were incubated with fresh medium containing 10% MTT dye solution (5 mg/mL). After 4 h of incubation at 37°C in 5% CO_2, the cells were pipetted into a new 24-well plate. The absorbance of the content of each well was measured at 545 nm using a multiwell microplate reader (100-TS NIKON, USA). Three repeated measures were conducted for each group.

Statistical analysis

All the experimental data were expressed as mean±standard deviation (SD). Statistical analysis was carried out using single-factor analysis of variance. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The structure of the composite membrane was studied by XRD. The XRD pattern of nano-composites with different percentages of graphene is shown in Figure 1. Accordingly, the characteristic peak of aggregated graphene sheets was so weak that it was possibly overlapped by the peak of polymer at $2\theta=21.3°$, indicating that most graphene sheets were exfoliated and uniformly dispersed in the polymer matrix. Furthermore, the incorporation of graphene did not affect the crystalline

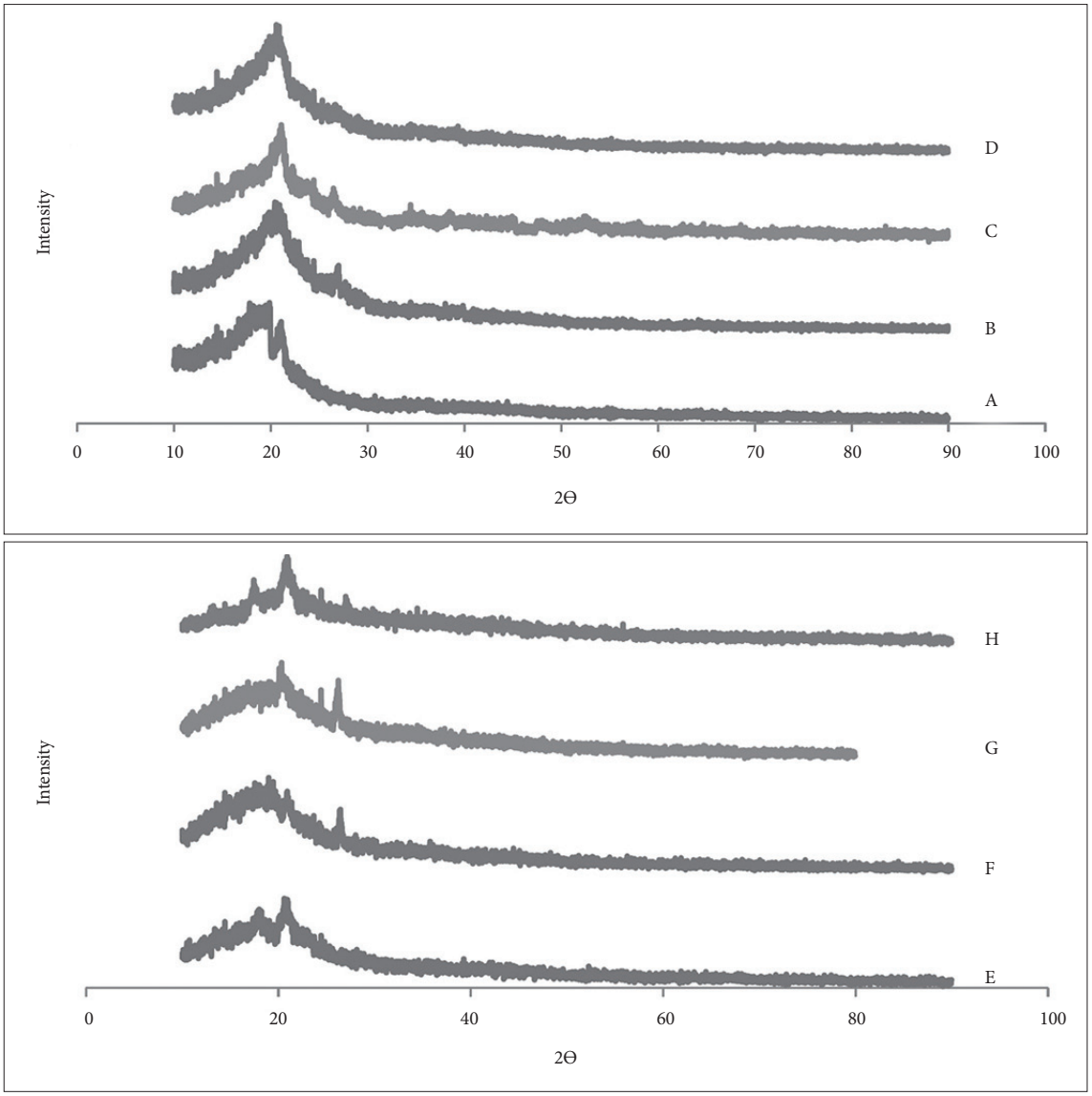

Figure 1. XRD patterns of (A) neat chitosan, (B) chitosan/0.5 wt.% graphene, (C) chitosan/1 wt.% graphene, and (D) chitosan/1.5 wt.% graphene composites. (E) Neat PLGA, (F) PLGA /0.5 wt.% graphene, (G) PLGA /1 wt.% graphene, and (H) PLGA /1.5 wt.% graphene composites. XRD: X-ray diffraction, PLGA: poly(D, L-lactic-co-glycolic acid).

structure of polymer. The nano-composite membranes with graphene contents in the range 0−1.5 wt.% had almost the same XRD patterns, implying that they had similar crystalline structures [6,8].

FT-IR experiments were carried out to investigate the interaction between graphene and polymers. As shown in Figure 2, in the spectrum of chitosan, there were two characteristic absorbance bands centered at 1653 and 1563 cm^{-1}, which corresponded to the C=O stretching vibration of -NHCO- and the N-H bending of -NH2, respectively. Compared with pure chitosan, these two peaks also appeared in the spectra of chitosan/graphene nano-composites. Additionally, for PLGA, there were 5 charac-

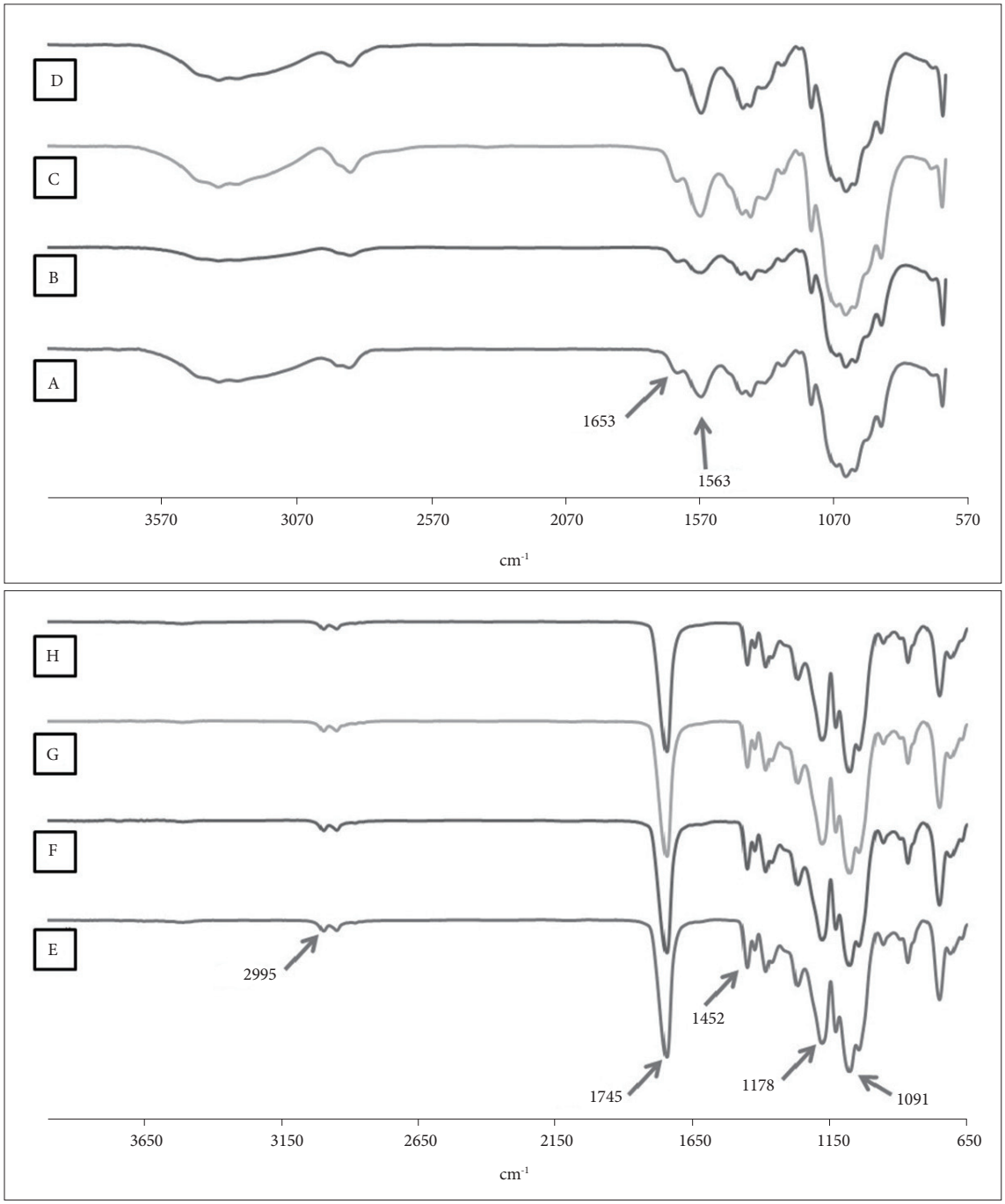

Figure 2. FT-IR spectra of (A) neat chitosan, (B) chitosan/0.5 wt.% graphene, (C) chitosan/1 wt.% graphene, and (D) chitosan/1.5 wt.% graphene composites. (E) Neat PLGA, (F) PLGA /0.5 wt.% graphene, (G) PLGA /1 wt.% graphene, and (H) PLGA /1.5 wt.% graphene composites. FT-IR: fourier transform infrared, PLGA: poly(D, L-lactic-co-glycolic acid).

teristic absorbance bands centered at 2995 (C−H), 1745 (C=O), 1452 (O−H), 1178 (C−O epoxy), and 1091 (C−O alkoxy) cm^{-1}. As shown in the spectrum of PLGA, these peaks could be seen in all weight percent of graphene from 0 to 1.5. Hence, there was no change in the structure of polymers, and the reactions between these two, polymers and graphene, were physical [8,13].

Figure 3 shows the typical SEM cross-section of neat chitosan and PLGA, chitosan/1.5 wt.% graphene composite, and PLGA /1.5 wt.% graphene composite membranes. These images indicated that the graphene nano-sheets were uniformly dispersed in the polymer matrix, and so was the case with polymer coated graphene nano-sheets [6,8]. The uniform distribution of graphene nano-sheets in the matrix amplified the electrical conductivity through the membranes, which was essential for nerve tissue engineering.

According Table 1, the conductivity of the composite membrane was the function of its graphene content. Although chitosan and PLGA are insulator polymers, the conductivity of their composite with 0.5 wt.% graphene is low. Notwithstanding, the conductivity of the composite film increased for 10^6 orders as its graphene content increased from 0.5 wt.% to 1.5 wt.%. So this composite can be a suitable candidate for nerve conduits because of electrical conductivity [6,17,20].

According to the results mentioned above, the MTT assay was done for neat chitosan and PLGA, chitosan/1.5 wt.% graphene composite and PLGA/1.5 wt.% graphene composite

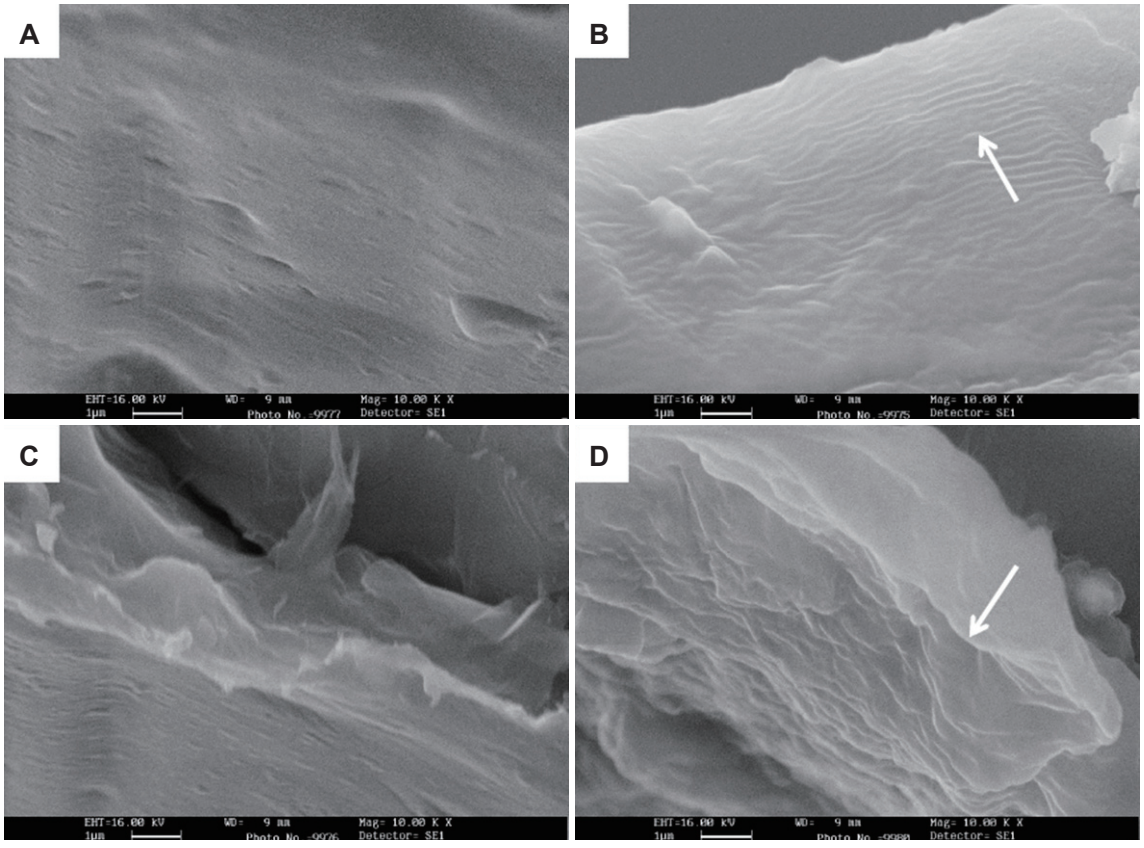

Figure 3. Typical cross-section SEM images of (A) neat chitosan, (B) chitosan/1.5wt.% graphene composite, (C) neat PLGA, and (D) PLGA /1.5 wt.% graphene composite membranes. The arrows indicate polymer coated graphene nano-sheets (magnification, ×10000). SEM: scanning electron microscopy, PLGA: poly(D, L-lactic-co-glycolic acid).

Table 1. The conductivity of nano-composite membranes

Composition of membranes	Electrical conductivity (S/m)	Composition of membrane	Electrical conductivity (S/m)
Neat chitosan	$0.201\pm0.032\times10^{-6}$	Neat PLGA	$0.234\pm0.012\times10^{-6}$
Chitosan/0.5 wt.% graphene	$0.264\pm0.020\times10^{-6}$	PLGA/0.5 wt.% graphene	$0.257\pm0.019\times10^{-6}$
Chitosan/1 wt.% graphene	0.166±0.0022	PLGA/1 wt.% graphene	0.213±0.0016
Chitosan/1.5 wt.% graphene	0.249±0.0016	PLGA/1.5 wt.% graphene	0.33±0.0024

PLGA: poly(D, L-lactic-co-glycolic acid)

membranes. *In vitro* cell culture assay was the first step for the evaluation of cell growth and interaction between membranes and cells. Figure 4 shows the results of MTT assay on membranes by PC12 cells. As it is shown, the PC12 cells could attach and grow on membranes, but Ch/1.5 G membranes improved and accelerated cell proliferation compared to cell growth on PLGA/1.5 G and neat membranes; after 24 hours of incubation, it was found that cells adhered and spread on the surface of the scaffolds and the difference between neat and composite chitosan were not considerable because of SDs. By using statistical analysis, after 1 and 3 days, cell proliferations on Ch/1.5 G membranes were significantly (p<0.05) higher in comparison to the others. In Ch/1.5 G membranes, because of existence of natural polymer as a matrix of nano-composite membranes, cell proliferation rose and as shown in Figure 5, PC12 cells created axons on this membrane. However, in PLGA/1.5 G membranes, due to a transient pH decline during hydrolysis, the cell growth and cellular proliferation did not rise as they did for chitosan/1.5 G membranes after 1 and 3 days [6,10,13].

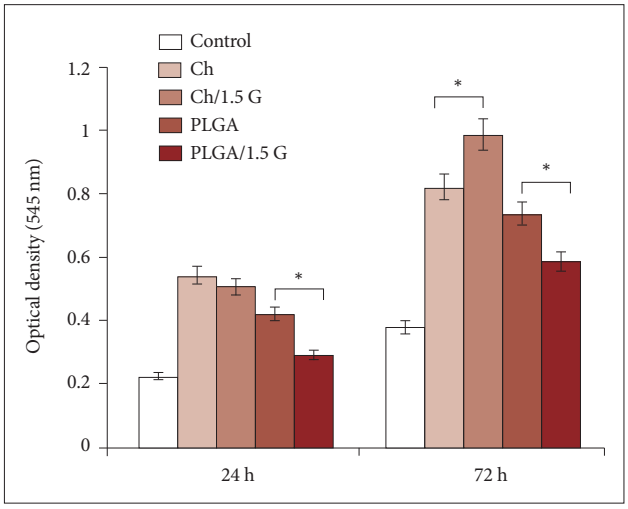

Figure 4. The comparison of PC12 cells proliferation on neat chitosan and PLGA, chitosan/1.5 wt.% graphene composite (Ch/1.5 G) and PLGA /1.5 wt.% graphene composite (PLGA/1.5 G) membranes after 24 and 72 hours.(*p<0.05). PLGA: poly(D, L-lactic-co-glycolic acid).

CONCLUSION

FT-IR spectroscopy and X-ray diffraction results indicated that graphene was uniformly dispersed in polymeric matrix, and there was a physical interaction between polymer and graphene. Conductivity measurements of the chitosan /1.5% graphene membrane and PLGA/1.5% graphene membrane were carried out using a four-point probe that showed these nanocomposites were more conductive than the other examined samples. After 3 days in cell culture, the chitosan/1.5 wt.% graphene membranes exhibited the maximum proliferation of the PC12 cells. As a consequence, it could be construed that chitosan/1.5 wt.% graphene (Ch/1.5 G) nano-composite membranes are promising biomaterials for nerve tissue engineering applications. Finally, conducting supplementary *in vitro* and *in vivo* tests are recommender for further research in this area.

Acknowledgements

The authors acknowledge the support by University of Isfahan and Isfahan University of Medical Sciences in Iran.

Conflicts of Interest

The authors have no financial conflict of interest.

Ethical Statement

There are no animal experiments carried out for this article.

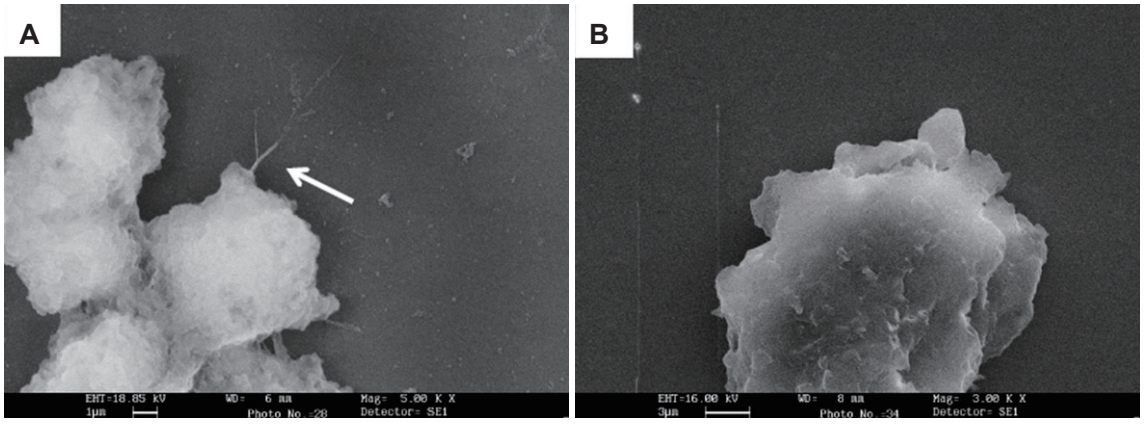

Figure 5. SEM images of PC12 cells on the composite membranes. chitosan/1.5 wt.% graphene composite (A) and PLGA /1.5 wt.% graphene composite (B). The arrow indicate axons of cell on the membrane. SEM: scanning electron microscopy, PLGA: poly(D, L-lactic-co-glycolic acid).

REFERENCES

1. Ortigüela ME, Wood MB, Cahill DR. Anatomy of the sural nerve complex. J Hand Surg Am 1987;12:1119-1123.
2. Mackinnon SE, Hudson AR. Clinical application of peripheral nerve transplantation. Plast Reconstr Surg 1992;90:695-699.
3. Gu X, Ding F, Williams DF. Neural tissue engineering options for peripheral nerve regeneration. Biomaterials 2014;35:6143-6156.
4. Ghasemi-Mobarakeh L, Prabhakaran MP, Morshed M, Nasr-Esfahani MH, Ramakrishna S. Electrical stimulation of nerve cells using conductive nanofibrous scaffolds for nerve tissue engineering. Tissue Eng Part A 2009;15:3605-3619.
5. Yang X, Tu Y, Li L, Shang S, Tao XM. Well-dispersed chitosan/graphene oxide nanocomposites. ACS Appl Mater Interfaces 2010;2:1707-1713.
6. Wang X, Bai H, Yao Z, Liua A, Shi G. Electrically conductive and mechanically strong biomimetic chitosan/reduced graphene oxide composite films. J Mater Chem 2010;20:9032-9036.
7. Rana VK, Choi MC, Kong JY, Kim GY, Kim MJ, Kim SH, et al. Synthesis and drug-delivery behavior of chitosan-functionalized graphene oxide hybrid nanosheets. Macromol Mater Eng 2011;296:131-140.
8. Yoon OJ, Jung CY, Sohn IY, Kim HJ, Hong B, Jhon MS, et al. Nanocomposite nanofibers of poly(d, l-lactic-co-glycolic acid) and graphene oxide nanosheets. Compos Part A 2011;42:1978-1984.
9. Park JJ, Yu EJ, Lee WK, Ha CS. Mechanical properties and degradation studies of poly(D,L-lactide-co-glycolide) 50:50/graphene oxide nanocomposite films. Polym Adv Technol 2014;25:48-54.
10. Houchin ML, Topp EM. Physical properties of PLGA films during polymer degradation. J Appl Polym Sci 2009;114:2848-2854.
11. Zhang Y, Nayak TR, Hong H, Cai W. Graphene: a versatile nanoplatform for biomedical applications. Nanoscale 2012;4:3833-3842.
12. Ryu S, Kim BS. Culture of neural cells and stem cells on graphene. Tissue Eng Regen Med 2013;10:39-46.
13. Fan H, Wang L, Zhao K, Li N, Shi Z, Ge Z, et al. Fabrication, mechanical properties, and biocompatibility of graphene-reinforced chitosan composites. Biomacromolecules 2010;11:2345-2351.
14. Sayyar S, Murray E, Thompson BC, Gambhir S, Officer DL, Wallace GG. Covalently linked biocompatible graphene/polycaprolactone composites for tissue engineering. Carbon 2013;52:296-304.
15. Verdejo R, Bernal MM, Romasanta LJ, Lopez-Manchadoa MA. Graphene filled polymer nanocomposites. J Mater Chem 2011;21:3301-3310.
16. Alekseev A, Chen D, Tkalya EE, Ghislandi MG, Syurik Y, Ageev O, et al. Local organization of graphene network inside graphene/polymer composites. Adv Funct Mater 2012;22:1311-1318.
17. Bunch JS. Mechanical and electrical properties of graphene sheets [Dissertation]. Ithaca: Cornell University, 2008.
18. Tkalya E, Ghislandi M, Alekseev A, Koninga C, Loos J. Latex-based concept for the preparation of graphene-based polymer nanocomposites. J Mater Chem 2010;20:3035-3039.
19. Liang J, Huang Y, Zhang L, Wang Y, Ma Y, Guo T, et al. Molecular-level dispersion of graphene into poly(vinyl alcohol) and effective reinforcement of their nanocomposites. Adv Funct Mater 2009;19:2297-2302.
20. Chung DDL. Review electrical applications of carbon materials. J Mater Sci 2004;39:2645-2661.
21. Zhu Y, Murali S, Cai W, Li X, Suk JW, Potts JR, et al. Graphene and graphene oxide: synthesis, properties, and applications. Adv Mater 2010;22:3906-3924.
22. Pan Y, Wu T, Bao H, Li L. Green fabrication of chitosan films reinforced with parallel aligned graphene oxide. Carbohydr Polym 2011;83:1908-1915.
23. Ahadian S, Estili M, Surya VJ, Ramón-Azcón J, Liang X, Shiku H, et al. Facile and green production of aqueous graphene dispersions for biomedical applications. Nanoscale 2015;7:6436-6443.
24. Akhavan O, Ghaderi E, Shirazian SA, Rahighi R. Rolled graphene oxide foams as three-dimensional scaffolds for growth of neural fibers using electrical stimulation of stem cells. Carbon 2016;97:71-77.