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Original Research Article

Effects of graphene/PLGA composite scaffolds on proliferation and differentiation of bone marrow mesenchymal stem cells

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ABSTRACT: Objective: This study aimed at fabricating three-dimensional porous graphene (G)/poly(lactic-co-glycolic acid (PLGA) composite scaffolds and establishing the potential for further application of G/PLGA porous scaffolds in bone tissue engineering. **Methods:** Different concentrations of graphene was mixed with PLGA (G/PLGA, wt. ‰: 0, 0.5‰, 5‰). **Results:** Scanning electron microscopy confirmed the inner connected porous structure of the three-dimensional G/PLGA scaffold as well as the uniform distribution of graphene in the scaf-folds. CCK-8 test indicated that G/PLGA porous scaffolds had no obvious cytotoxicity. Compared with BMSCs seeded on PLGA scaffold, the ALP activity of BMSCs seeded on the G/PLGA scaffolds increased and the expression of bone related genes was significantly up-regulated with increase of G concentration. G/PLGA porous scaffold containing 5‰ graphene showed more obvious effects on osteogenic differentiation. **Conclusions:** The G/PLGA three-dimensional porous scaffold prepared in this research possessed good biocom-patibility and could promote osteogenic differentiation of BMSCs in vitro. Thus, it has been expected to be used as a scaffold for bone tissue engineering.

KEYWORDS: Graphene; Poly(lactic-co-glycolic acid); Porous scaffold; Bone tissue engineering

The treatment of bone tissue defects is a frequent clinical problem. Currently, the traditional autologous bone graft remains the "gold standard" for clinical treatment of bone defects. However, autologous bone grafting adds additional trauma to the patient, and the future prospects are not ideal due to insufficient bone sources, donor limitations, increased number of surgeries, and prolonged healing period[1]. Compared with traditional bone grafting, the use of bone tissue engineering technology to repair bone defects has the advantages of less damage, a wide range of sources, accurate reconstruction of the morphology of the bone in the defect area, and no obvious antigenicity. Therefore, bone tissue engineering technology is expected to provide a new strategy for clinical treatment of bone tissue defects. As one of the three elements of bone tissue engineering, the stent material can regulate the functions of seed cell survival,

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colonization, migration, proliferation and differentiation, which in turn affect the regeneration of tissue morphology and function[2–4].

In recent years, poly(lactic-co-glycolic acid) [poly (lacticco-glycolic acid), PLGA] has been widely used in the construction of tissue engineering scaffold materials due to its good biodegradability and biocompatibility. However, PLGA hydrophobicity leads to poor cell adhesion[5] and PLGA does not have good osteoinductive properties[6], and these drawbacks limit the further application of PLGA materials in the field of bone tissue engineering. Graphene (G) is a two-dimensional carbon nanofiller with good electrical and optical properties. It has been reported that graphene mixed with polycaprolactone (PCL) can improve the hydrophilic and mechanical properties of PCL materials[7]. In addition, graphene coatings were reported to have strong bone-enabling properties[8]. Therefore, this study was proposed to compound graphene with PLGA to form G/PLGA composite scaffolds in order to improve the physicochemical properties of PLGA scaffolds and enhance their osteogenic properties, and to investigate the potential use of G/PLGA composite scaffolds as bone tissue engineering.

1 Materials and Methods

1.1 Experimental animals

Eight 4-week-old male SD rats, body mass (200±20) g, were provided by the animal house of the Ninth People's Hospital of Shanghai Jiaotong University School of Medicine, Animal License No.: SCXK(Shanghai) 2012–0007.

1.2 Main reagents and instruments

DMEM culture medium (Hyclone, USA), fetal bovine serum (FBS) (Zhejiang Tianhang Biotechnology Co., Ltd.), trypsin (Gibco, USA), CCK-8 kit (Dojido, Japan), BCIP/NBT alkaline phosphatase colorimetric kit, alkaline phosphatase phosphatase (ALP) assay kit (Shanghai Biyuntian Biotechnology Co., Ltd.), Dexamethasone, β-glycerophosphate sodium, vitamin C (Sigma, USA), FITC-ghostile peptide, DAPI, SYBR Green RT-PCR kit (Shanghai Yuan Sheng Biotechnology Co., Ltd.), BCA protein quantification kit (Thermo Sci-entific, USA), RNA reverse transcription kit (Takara, Japan), Graphene (Suzhou Hengqiu Graphene Technology Co., Ltd.), PLGA (Jinan Daigang Biotechnology Co., Ltd.). Scanning Electron Microscope (SEM) (S3400, Hitachi, Japan), Laser Confocal Microscope (LSM800, Zeiss, Germany).

1.3 Methods

1.3.1 G/PLGA composite scaffold preparation.

Dissolve 0.05 g PLGA in 0.5 mL chloroform to form a homogeneous polymer solution. Add different amounts of graphene (G/PLGA, wt. ‰: 0, 0.5‰, 5‰), stir well, add 1 g of sodium bicarbonate powder to the 12-well plate, freeze-dry after casting and molding, and obtain the G/PLGA composite scaffold after ultra-pure water immersion. the scaffold with 0‰ of G content is the PLGA-only scaffold.

1.3.2 Experimental grouping.

According to the presence or absence of graphene and the amount of graphene content, there were four groups in this study, the control group was the no-material blank group (CON group), experimental group A was the simple PLGA scaffold group with G content 0 (PLGA group), experimental group B was the G/PLGA composite scaffold with G content 0.5‰ (G/PLGA-0.5 group), and experimental group C was the G/PLGA composite scaffold with G content 5‰ (G /PLGA-5 group).

1.3.3 SEM inspection.

In this experiment, SEM was used to observe the surface microstructure and cross-sectional morphology of the prepared G/PLGA composite scaffolds. Sample preparation: the samples of different groups of scaffold materials were sprayed with gold and then observed by SEM with an accelerating voltage of 10 kV.

1.3.4 Isolation and culture of rat BMSCs.

SD rats were disarticulated and executed, the

long bones of the lower limbs were removed under aseptic conditions, the ends were cut, and the bone marrow cavity was rinsed with DMEM high sugar complete medium (containing 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin) by syringe aspiration and the bone marrow was collected, centrifuged at 1800 r/min for 10 min, the supernatant was discarded, and the cells were resuspended. The cells were observed under light microscope, and when 80% or more of the cells were fused, cell passages were performed, and the third generation BMSCs were selected for in vitro experiments.

1.3.5 G/PLGA composite scaffold adhesion assay.

The materials were first sterilized and put into 12-well plates. Logarithmic growth phase third generation BM-SCs were taken, trypsin digestion was performed to obtain cell suspensions, and cell density was adjusted to 1×105 cells/mL after counting, and 1 mL of cell suspension was added to each well. After 24 h of incubation, the original medium was discarded, the cells were fixed after washing with phosphate buffer (PBS), and the cytoskeleton was labeled with FITC-ghost cyclic peptide and the nucleus was labeled with DAPI, and the cells were fluorescently stained. After staining, the cells were placed inoculum side down on a slide and observed under a laser confocal microscope.

1.3.6 G/PLGA composite scaffold proliferation assay.

According to ISO10993–5-2009, material extracts were prepared and collected from different groups of materials for the assay of cytotoxicity. The logarithmic growth phase third generation BMSCs were taken, trypsin digested and made into cell suspensions, counted, and then 5000 cells were inoculated into each well and cultured for 4 h. The medium was replaced with material extracts. After 24 and 72 h of incubation, the original medium was discarded, and 100 μL of fresh culture medium was replaced in each well. 10 μL of CCK-8 reagent was added to each well according to the instructions of the CCK-8 kit, and the absorbance (OD) values were measured at 490 nm with an enzyme marker after 1.5 h of incubation at 37℃ .

1.3.7 G/PLGA composite scaffold ALP staining and activity assay.

According to reference[9], dexamethasone, sodium β-glycerophosphate, and vitamin C were used to prepare osteoinduction solution according to the steps and ratios, and stored away from light at 4°C. The materials were first sterilized and put into 12-well plates. Take logarithmic growth phase third generation BMSCs, make cell suspension after trypsin digestion, adjust the cell density to 1 × 105 cells/mL after counting, add 1 mL cell suspension to each well, and after 7 d of culture in osteogenic induction solution, ALP staining and activity assay were performed. ALP staining: after digesting the cells after 7 d of culture with trypsin, collect them by blowing and access them into blank culture plate, and after the cells are completely attached to the wall, according to ALP semi-quantitative assay: Cellular proteins were extracted using cell lysate, protein concentration was measured by BCA method, and ALP semi-quantitative assay was performed according to the instructions of ALP assay kit, and the results were expressed as OD/mg total protein concentration.

1.3.8G/PLGA composite scaffold RT-PCR assay.

Firstly, the materials were sterilized and put into 12-well plates. Logarithmic growth phase third generation BMSCs were taken, trypsin digested and made into cell suspension, and the cell density was adjusted to 1×105 cells/mL after counting, and 1 mL of cell suspension was added to each well. After 7 d of osteogenic induction medium culture, RNA was extracted and reverse transcribed to obtain cD-NA, and RT-PCR was performed to detect the expression of osteogenic genes, including ALP, runt-related transcription factor-2 (RUNX2), osteocalcin (OCN) and bone sialoprotein (BSP). The relative expression of the target genes in each group of samples was set as a

reference value (set to 1) in the CON group, and the fold change of the standard was used as the basis and recorded as the mean ± standard deviation.

Table 1 Sequences of primers

Gene Name	Primer sequences (5'–3')
BSP	F : AGAAAGAGCAGCACGGTTGAGT
	R : GACCCTCGTAGCCTTCATAGCC
ALP	F : TATGTCTGGAACCGCACTGAAC
	R : CACTAGCAAGAAGAAGAAGCCTTTGG
OCN	F : GCCCTGACTGCATTCTGCCTCT
	R : TCACCACCTTACTGCCCTCCTG
RUNX2	F : ATCCAGCCACCTTCACTTACACC
	R : GGGACCATTGGGAACTGATAGG

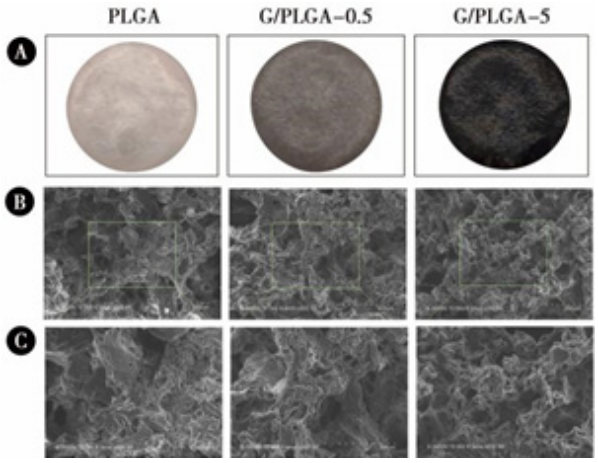
1.4 Statistical analysis

All experiments were repeated more than three times, and experimental data were expressed as mean ± standard deviation and compared between groups using one-way analysis of variance (One Way ANO-VA) and the Tukey-Kramer method. $p < 0.05$ was considered a statistically significant difference. All data were statistically analyzed using SPSS 19.0 software.

2 Results

2.1 Surface morphology characterization of G/PLGA composite scaffolds

The surface morphological characteristics and SEM results of the G/PLGA composite scaffold are shown in **Figure 1**. the PLGA scaffold is white, and the color of the scaffold material gradually becomes darker with the increase of graphene content (**Figure 1A**). the SEM results (**Figure 1B**) show that the prepared PLGA scaffold, G/PLGA-0.5 scaffold, G/ The pore walls of the PLGA scaffold were smooth, and the uniformly dense distribution of graphene particles was visible on the surface of the pore walls of the G/PLGA-5 scaffold, while the graphene content on the surface of the G/PLGA-0.5 scaffold was relatively small.



A: Big picture; B, C: SEM picture and partial enlargement picture

Figure 1 The gross view images and SEM images of structure of different scaffolds

2.2 Adhesion of BMSCs and material cytotoxicity

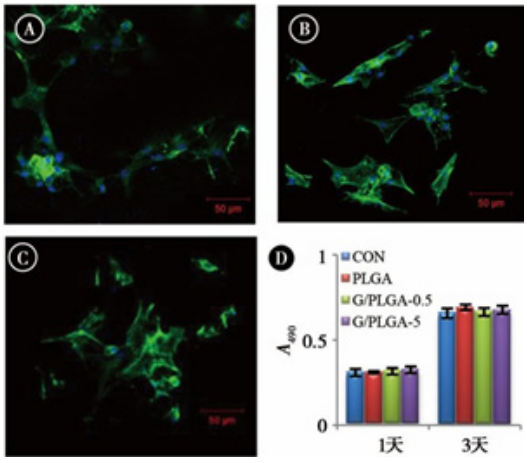
The results of CCK-8 assay are shown in **Figure 2**. The OD values of cells cultured with the extracts of the three experimental groups after 24 h and 72 h were not statistically significant ($P > 0.05$) compared with those of the cells in the CON group, and the OD values at 72 h were significantly higher than those at 24 h. This indicates that all three materials were not significantly cytotoxic and none of the three materials affected the proliferation of BMSCs in a short period of time.

In the cytoskeleton fluorescence staining photos after 24 h of incubation, the green fluorescence is the FITC-ghost cyclic peptide-labeled cytoskeleton and the blue fluorescence is the DAPI-labeled nucleus. As shown in **Figure 2**, BMSCs spread better on the surface of the scaffolds in the G/PLGA-0.5 and G/PLGA-5 groups compared with the cells on the PLGA scaffolds, with the best spreading of the cells in the G/PLGA-5 group with visible cell pseudopods, suggesting that the scaffolds in the G/PLGA-0.5 and G/PLGA-5 groups may have better adhesion effects. The results of cytotoxicity assay and adhesion assay indicated that the G/PLGA composite scaffold had good biocompatibility.

2.3 ALP staining and activity detection

Figure 3 shows the results of ALP staining

and semi-quantitative activity assay, which are basically consistent. After BMSCs were cultured on the material for 7 d, the cells in the G/PLGA-5 group had significantly darker ALP staining than the PLGA and CON groups, and the ALP activity results also showed that the cells in the G/PLGA-5 group had the highest ALP activity ($P< 0.05$). It indicates that the G/PLGA-5 group had a stronger promotion effect on ALP expression in BMSCs under mineralization-inducing conditions. In contrast, the ALP activity of the G/PLGA-0.5 group was higher than that of the PLGA group although it was lower than that of the G/PLGA-5 group ($P < 0.01$). the ALP expression of the PLGA group was not significantly different from that of the CON group ($P > 0.05$). the results of ALP staining and activity assay also illustrated from the side that all three scaffold materials were biocompatible and the inoculated stem cells could sustain proliferation The results of ALP staining and activity assay also showed that all three scaffold materials were biocompatible and the inoculated stem cells could continue to proliferate and differentiate.



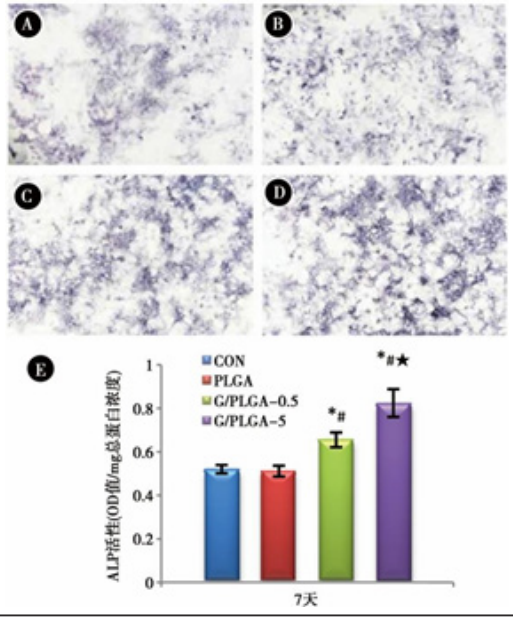
A~C: Fluorescence microscopy images of BMSCs inoculated on PLGA scaffold, G/PLGA-0.5 scaffold and G/PLGA-5 scaffold for 24 h. D: Cell proliferation assay results.

1 day
3 day

Figure 2 Adhesion of BMSCs and cytotoxicity of materials

2.4 Osteogenesis-related gene expression

RT-PCR detected the expression of osteogenic-related genes in different groups of cells, and the results showed that: the PLGA scaffold had no significant osteogenic effect on the cells, so there was no significant change in osteogenic genes, and there was no significant difference with the CON group ($P> 0.05$); the G/PLGA-0.5 scaffold and G/PLGA-5 scaffold were able to significantly upregulate the expression of osteogenic genes in BMSCs compared with the PLGA group ($P< 0.01$). The expression of osteogenic genes of BMSCs was significantly upregulated in the G/PLGA-5 group compared with the PLGA group ($P< 0.01$), where ALP and BSP genes were consistently higher at 7 d. The expression of RUNX2 was elevated early and not significantly elevated by 7 d. The trend of OCN expression was slowly increasing.). It indicates that the incorporation of a certain amount of graphene can significantly improve the osteogenic activity of PLGA scaffolds, and the increase of graphene content in a certain range can enhance the osteogenic performance.



7 day
ALP activity (OD value/mg total protein concentration)

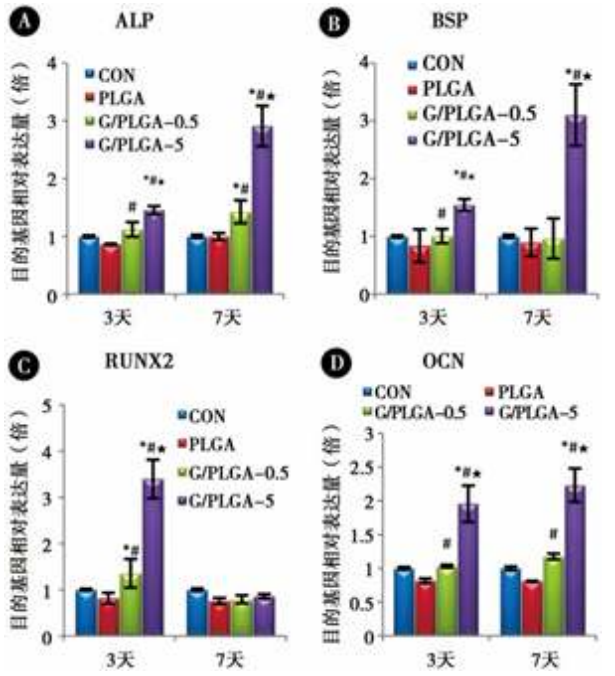
A~ D:Photographs of ALP staining in CON group, PLGA group, G/PLGA-0.5 group, G/PLGA-5 group in this order (×40) ; E:Results of ALP activity in different groups of cells; * :compared with CON group, $P< 0.01$, #:compared with PLGA group, $P<0.01$; ★ :compared with G/PLGA-0.5 group, $P<0.01$

Figure 3 Results of ALP staining and activity assay

3 Discussion

In recent years, with the increasing research

on the application of bone tissue engineering technology to the repair of oral and maxillofacial bone defects, the repair treatment of bone tissue defects has also been rapidly developed. As one of the three elements of bone tissue engineering, the scaffold material is the basic framework for cell attachment and metabolic site, as well as the main medium for cytokine loading and release, and its morphology and function directly affect the morphology and function of the constituted tissues, which is the most basic building block for constructing tissue engineering, so screening and preparing an ideal scaffold material is crucial for the development and clinical application of oral bone tissue engineering[10].



3 day
7 day
Relative expression amount of target gene (times)

A~ D: relative expression of ALP, BSP, RUNX2, OCN genes in this order; *:compared with CON group, P< 0.01, #: compared with PLGA group, P< 0.01; ★ : compared with G/PLGA-0.5 group, P<< 0.01

Figure 4 Real-Time PCR analyses for the expression of osteogenic differentiation related genes

In this experiment, G/PLGA scaffolds were prepared by compounding graphene with PLGA, and porous three-dimensional scaffolds were constructed by using sodium bicarbonate poreogenesis. SEM observed the porous structure of G/PLGA scaffold materials, with pores penetrating each other and graphene uniformly distributed, which increased the specific surface area of the scaffolds and improved the surface properties of PLGA materials. The results of cell adhesion test also illustrated that the addition of graphene could improve the surface properties of PLGA, and the cells spread better on the scaffold material with higher graphene content. Some scholars believe that graphene is cytotoxic and needs to be used with caution[11], while others have confirmed that small doses of graphene do not affect cell proliferation[12]. In this experiment, a small dose of monolayer graphene was compounded with PLGA scaffold, and the experimental results confirmed that the small dose of graphene was not significantly cytotoxic and did not affect the proliferation of BMSCs.

Nayak et al[8] and Lee et al[12] attached graphene films to different media to examine their effects on the proliferation and differentiation of human mesenchymal stem cells, and the results showed that graphene films did not affect cell proliferation and had significant osteogenic induction of cells. Another study reported that cranial bone repair with graphene hydrogels in rats was better than the control group in repairing cranial bone defects; the in vitro biological assay results also showed that graphene hydrogels promoted osteogenic differentiation of human mesenchymal stem cells[14–15]. This is consistent with the trend of ALP activity and RT-PCR assay in this experiment, and the G/PLGA scaffold incorporating graphene has superior osteogenic induction than PLGA scaffold, and the G/PLGA-5 scaffold with higher graphene content can significantly increase the expression of osteogenic-related genes and ALP compared with the blank control group, indicating that the G/PLGA-5 scaffold has good promotion of osteogenic induction. The G/PLGA-5 scaffold can be subsequently used for further in vivo and in vitro experiments to verify its osteogenic effect and investigate its osteogenic mechanism. In addition, since graphene is a nanomaterial, its metabolism and degradation in vivo need to be considered, which also needs further experimental confirmation.

In this paper, we initially demonstrated that a small dose of graphene could improve the surface properties of PLGA and significantly increase the bone-enabling activity of PLGA, and the bone-enabling activity of G/PLGA scaffold was positively correlated with the content of graphene in a certain range. The G/PLGA-5 scaffold material constructed in this experiment has good biocompatibility and bone-enabling properties and is expected to be a new bone tissue engineering scaffold material.

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