Para-amino benzoic acid doped micro-grooved carbon fibers to improve strength and biocompatibility of PLA-PEG

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ABSTRACT Para-amino benzoic acid (PABA), a folic acid related metabolite, was first introduced to fabricate micro-grooves and improve hydrophilicity over surfaces of carbon fibers (CFs). Then, engineered CFs/poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) biocomposites were fabricated by a solvent casting/particulate leaching method. We found that introducing small hydrophobic PABA molecules and fabricating patterned structures would lead to benign integrated interfaces between CFs and the PLA-PEG matrix. Specifically, the compressive strength of CFs/PLA-PEG was improved from 3.98 to 5.48 MPa. In addition, the CFs/PLA-PEG biocomposites significantly accelerated the adhesion and proliferation of pre-osteoblasts with minimized cytotoxicity. By comparing the cytocompatibility of L929 and MC₃T₃ cells cultured on different modified PLA-PEG composites, it could be concluded that PABA-CFs not only overcame the limitation of poor strength of PLA-PEG, but also improved the cell growth. These results indicate that the PABA-CFs reinforced PLA-PEG biocomposites could be a potential alternative for tissue engineering scaffolds.

Keywords: carbon fibers, PABA modification, biocomposites, mechanical strength, biocompatibility

INTRODUCTION

Carbon in multi-scale forms including carbon nanotubes and carbon nanofibers, have been developed as potential candidates for tissue engineering due to their enhanced mechanical stiffness and prominent biocompatibility [1–5]. CFs, as reinforcement phase, also offer alternative potential as scaffolds in orthopedics. Non-immunogenic CFs

are well tolerated by the recipient and will evoke strong body immune reaction [6,7]. Specifically for instance, carbon-based scaffolds have been fabricated with similar morphologies and functions of tendon and ligament for implantation [6]. The long-term stability of CFs implanted into osteo-chondral defects within *in vivo* histological investigation also verifies CFs' feasibility and potential development in orthopedic surgery [8]. Besides, fiber-like materials will make a guidance for cell growth direction, which is an inviting role to mimic bone constitute such as trabecular [9]. However, the advantages of CFs are still limited at their maximum performance. Critical issues are the lack of investigation into the interaction between CFs and polymer matrix and the understanding of underneath interaction mechanism. It has been well-known that performance of CFs for medical applications is considered to be related with the physical and chemical properties of their surfaces [10,11]. Recently, many efforts have been endeavored to graft hydrophilic groups onto CFs' surfaces [12–15]. For example, ceramic powders were imbedded into precursor poly-acrylonitrile fibers during spinning process so as to establish direct chemical bonds with bone tissue after implantation [6]. Hydroxyapatite, as a primary constituent in bone tissue, is often considered as an additive into scaffold materials to obtain osteo-like integration between host and guest under high mechanical strength. In order to address the above mentioned concerns in obtaining maximum performance of CFs in the polymer matrix, we first introduced para-amino benzoic acid (PABA) as a surface modification agent to improve CFs' surface status

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as well as micro-grooved patterns. Our rationality is based on the following considerations: (1) PABA is a kind of folic acid related small molecule, which can be used as a moderate acidic modification agent [16]; (2) CFs of excellent mechanical performance are potential reinforcement for scaffold materials [15,17].

Co-polymer poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) has been widely used as scaffold materials for tissue engineering due to its excellent biocompatibility and highlighted feasibility of structural modification [18–28]. However, the poor mechanical property and hydrophobicity are main limiting factors for further applications [29,30]. As reported, introducing hydrophilic additives into hydrophobic substrate can improve cell adhesion and three-dimensional in-growth [31,32]. To achieve both good mechanical properties and hydrophilicity, one effective strategy is to introduce an enforcing phase which meantime can improve the hydrophilicity. Modified CFs are our first choice.

We first used PABA to modify CFs. PABA is one of critical components for folic acid synthesis in our metabolic system, which is considered to be beneficial to cell growth and division [33]. CFs modified with PABA (PABA-CFs) might not only obtain preferable hydrophilic groups over surfaces but also maintain the prominent mechanical strength. Compressive performance and biocompatibility of PABA-CFs were characterized and compared with untreated CFs (Control CFs) and CFs modified with nitric acid (HNO_3 -CFs). We hope that such modified CFs/PLA-PEG biocomposites have potential to be developed as bone tissue engineering scaffold materials.

EXPERIMENTAL SECTION

Materials

The poly-acrylonitrile based T800 un-sized CFs with certain tensile strength were lab-made. PLA-PEG (PLA:PEG=40:1, $M_n=798,50 \text{ g mol}^{-1}$, $\rho=1.3126 \text{ g cm}^{-3}$) was supplied by Jinan Daigang Biomaterials Co., Ltd, China. PABA, nitric acid (HNO_3), acetic acid (HAc) and sodium hydroxide (NaOH) were bought from Sinopharm Chemical Reagent Co., Ltd., China. Dulbecco's modified Eagle's medium (DMEM) and α -DMEM were bought from Hangzhou Gino Biomedical Technology Co., Ltd., China. The 3-[dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide (MTT) were bought from GrandSky Biotechnology Institute, China. All chemicals were used without further purification and supplied by Sinopharm Chemical Reagent Co., Ltd., China.

Surface modification of CFs and scaffold preparation

PABA and HNO_3 were used to remove surface sizing agent of CFs as well as enhance the integration between CFs and PLA-PEG matrix. A schematic diagram of the whole modification process is depicted in Fig. 1. The CFs and PABA were mixed with the mass ratio of 1:50 at 70°C for 2 h, and a small amount of NaOH was then added as catalyst agent. The CFs were then transferred and immersed into diluted acetic acid solution (2 wt.%) for another 24 h. Distilled water, absolute ethyl alcohol and tetrahydrofuran were used in sequence to remove the residue. The CFs were finally dried at 70°C in an oven. The modification with HNO_3 route followed the similar way as mentioned above.

Figure 1 Diagram of CFs modification process applied in the work.

The scaffold samples were prepared based on solvent casting/particle leaching method according to our previous study [17]. Pure PLA-PEG scaffolds and their composite scaffolds modified with 0.5 and 2.0 wt.% CFs or PABA treated CFs were prepared, respectively.

Characterization methods

Mechanical test

The mechanical properties of CFs and CFs/PLA-PEG composite scaffolds were tested by a monofilament tensile tester (XQ-1, Donghua University, China) and a universal testing machine (WDW-2.0, Changchun Kexin Test Instrument Co., Ltd., China), respectively. For testing the tensile property of CFs, the samples were made by attaching a filament to a gauge paper frame with 20 mm distance as shown in Fig. 2. The specimens were set up to test by cutting the paper case and using a crosshead speed of 2 mm min⁻¹ to break, as reported elsewhere [34]. No less than 30 valid data were tested. The diameters of the CF monofilaments were measured via a multifunction optical microscope (CYG-055C, Shanghai Institute of Optics Instruments, China). For testing the compressive property of the scaffolds, the samples were cut into cubes with side length of 10 mm. The compressive ratio was set as 50% using a falling speed of 0.5 mm min⁻¹. At least three samples were measured for each group.

Morphology observation

The surface textures of the CFs and CFs/PLA-PEG biocomposites were characterized by scanning electron microscopy (SEM, JSM-5600LV, JEOL Co., Ltd, Japan). Due to good conductivity of CFs, only the scaffold samples needed to be fixed on a cupreous stub and sparked with a gold layer

with the thickness of 15 nm in advance to produce conductive surfaces, and then morphologies were observed at an accelerating voltage of 10 kV.

Fourier transform infrared spectrum analysis

The Fourier transform infrared spectroscopy (FT-IR, NEXUS-670, Nicolet Co., Ltd, USA) was used to identify the infrared absorption peaks of such hydrophilic groups as -NH₃ and -COOH to verify the modification results by treating with PABA. FT-IR spectra were collected during the range of 4000–500 cm⁻¹ with the resolution of 5 cm⁻¹.

Hydrophilicity evaluations

Hydrophilicity of the biocomposites was determined by testing the swelling ratio $R(\%)$ in deionized water for 24 h at room temperature and then calculated according to the Equation (1), where M_0 is the initial weight and M is the wet weight of samples after soaking [35]. Each swelling ratio measurement was repeated three times.

$$R(\%) = \frac{M - M_0}{M_0} \times 100\% \quad (1)$$

The contact angles of biocomposites were measured by video optical contact angle measurement (OCA40Micro, Dataphysics Co., Ltd., Germany). Each sample was measured at five points to get the mean contact angles.

Biological assessments

Cytotoxicity evaluations

Mouse fibroblasts (L929) and osteoblasts (MC₃T₃) were provided by Cell Bank of Shanghai Institute for Biological Science, Chinese Academy of Science. The samples were pretreated by exposure to gamma radiation (25 kGy) for

Figure 2 Schematic drawing for testing (a) the tensile strength of CF monofilament and (b) compressive strength of the CFs/PLA-PEG biocomposites. The inset is a specimen photograph.

sterilization. All the samples were rinsed by phosphate buffer solution (PBS, 0.01 mol L⁻¹, pH 7.2, Sigma Co., Ltd., USA) three times and pre-wet in the culture medium overnight before cell seeding. The cells were seeded at a density of 2×10⁴ cells per well (24-well plate) for cell proliferation assay. The cell viability was evaluated using 3-[dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide (MTT, The Grandsky Biotechnology Institute, China) assay after 1, 3, 5 and 7 days according to the literature [36]. The optical density (OD) value at 492 nm was measured using a fluorescence micro-plate reader (MULTISIKAN MK3, Thermo, USA).

The morphologies of cells spreading on materials were observed by SEM at a density of 5×10⁴ cells per well (24-well plate). Cell-material constructs of 3-day culture were rinsed with PBS three times to remove unattached cells and then fixed with 5 wt.% glutaraldehyde at 4°C for 2 h, followed by rinsing with PBS. A series of gradient ethanol solutions of 30, 50, 70, 80, 90, 95 and 100 wt.% were utilized for dehydrating and air proof overnight.

Live-dead assay

The nuclei and cytoplasm of live cells were visualized using immune fluorescence staining for DAPI (Best Bio Biotech Co., Ltd., Shanghai, China) and phalloidin (3-Bio Co., Ltd., China) according to the manufacturers' protocols, respectively. Firstly, cell-material constructs of 3-day culture were rinsed three times with PBS to remove unattached cells, and then fixed with 8 wt.% glutaraldehyde at 4°C for 12 h before staining. Afterwards the cell-material constructs were washed with PBS twice before observation under the confocal laser scanning microscope (CLSM, LSM-700, Zeiss, Germany).

Statistical analysis

One way ANOVA statistical analysis was performed to compare the cyto-compatibility of L929 and MC₃T₃ cultured on different materials. *P* value of 0.05 was selected as the significance level and the data were indicated with (*) for *P* < 0.05.

RESULTS AND DISCUSSION

Evaluations of CFs properties

CFs present characteristics of brittle materials according to their stress-strain curves [10,36]. The effects of different modifications on CFs' mechanical properties are discussed in this paper, where dispersion is one important indicator affecting the quality reliability. Weibull model compiled in Visual Basic 6.0 was carried out to analyze the strength

dispersion and explain the failure statistics of CFs subjected to tensile loads. The probability of survival of fiber $F(\sigma_i)$ and probability density function (PDF) $f(\sigma_i)$, based on the two-parameter Weibull distribution, are given in Equations (2) and (3) respectively, where σ_i is the tensile strength, m is the Weibull shape parameter and σ_0 is the Weibull scale parameter.

$$F(\sigma_i) = 1 - \exp\left[1 - \left(\frac{\sigma_i}{\sigma_0}\right)^m\right] \quad (2)$$

$$f(\sigma_i) = \frac{m}{\sigma_0^m} \sigma_i^{m-1} \exp\left[-\left(\frac{\sigma_i}{\sigma_0}\right)^m\right] \quad (3)$$

We used Weibull mode to compare the strengths of CFs (Shown in Fig. 3). According to the PDF, modified CFs with PABA and HNO₃ are considered to bear lower tensile strength (4.07 and 3.61 GPa) than the control groups (4.15 GPa) (Fig. 3a). The slopes of linear lines in Fig. 3b are estimates of the Weibull shape parameters (the volume) and represent the variability within each set of data. Tensile strength of PABA-CFs exhibits a little larger data variation than those of HNO₃-CFs, which is consistent with the conclusion from the PDF curves. Chemical oxidation with PABA and HNO₃ creates more surface defects, leading to the decrease of mechanical strength and survival probability, as depicted in their survival and hazard functions (Figs 3c and d). Although CFs treated by HNO₃ result in more defects and increasing hydrophilicity, a big loss in tensile strength happens meantime (Table 1), which is considered to be disadvantageous for CFs as reinforcement materials. Herein PABA-CFs are developed as a modification agent discussed in our following study.

PABA modification produces many micro-grooves serving as defect sites over CFs surfaces because of the acidic solution etching, and such defect sites containing hydrophilic groups can share the mechanical load based on defect theory, especially when banding with other matrixes [37]. Robust combination between the modified CFs and matrix is the essential precondition for optimizing the mechanical properties of scaffolds [17], which is largely subjected to each component's surface status. Compared with the untreated CFs (depicted as Control CFs), PABA-CFs provide rougher surface for hydrophilic groups such as -COOH to graft on, which is proved by FT-IR spectra. As shown in Fig. 4d, clear visible bands near 1673 cm⁻¹ dedicated to C=O are observed, and the most intensive peak is attributed to the stretching vibration. Bands located in the range of 2852–2921 cm⁻¹ are attributed to stretching vibration from saturated hydrocarbon C-H or CH₂. These functional

Figure 3 The strength Weibull distribution and simulated functions of CFs under various modifications. (a) Probability density functions curves; (b) Weibull distribution simulation; (c) survival function curves; (d) hazard function fitting.

Table 1 Monofilament tensile data

Sample	Tensile strength (GPa)	Diameter (μm)	Breaking elongation (%)
Control CFs	4.15 ± 0.39	5.16 ± 0.21	1.50 ± 0.019
HNO ₃ -CFs	3.61 ± 0.37	4.80 ± 0.25	1.38 ± 0.013
PABA-CFs	4.07 ± 0.46	4.94 ± 0.30	1.21 ± 0.075

groups are considered to be beneficial for obtaining a robust combination with matrix and adsorbing biomolecules effectively [38,39]. Besides, CFs themselves offer a relatively benign platform for cell proliferation, especially when treated with PABA (Fig. 4c), which may facilitate nutrient substances and tune the cell response on the micro-grooved CFs' surface [40]. Based on the above results, PABA-CFs are chosen as additives in the preparation of biocomposites.

Evaluations of CFs/PLA-PEG biocomposites

An ideal scaffold material should allow sufficient space onto its surface, providing good biocompatible environment for cell proliferation and migration [41,42]. Given that the brittleness of CFs is the major limitation for their applications as suitable cell substrates, introducing another porous polymer material would provide some degree of mechanical flexibility [37]. Due to good hydrophilicity of both PLA-PEG and PABA-CFs, satisfied combinations are obtained and observed in Fig. 5. It is logical to expect that the overall performances will be a function of the respec-

tive contribution from each phase. There is an increase in porosity with increasing CFs content, which may be ascribed to the addition of PABA-CFs disorganizing the original PLA-PEG arrangement. The increasing porosity is beneficial to the exchange of nutrients among cells and transportation of metabolic wastes. At the same time, the addition of PABA-CFs reinforcement plays an important role in compensating for poor polymer mechanical property and the mechanical loss induced by increased porosity.

As shown in Fig. 5, only a small amount of PABA-CFs has a positive influence on improving the absorption capacity of scaffold materials, which meantime supports the key function of PABA-CFs in promoting hydrophilicity. The 2° change of contact angle between Figs 5b and c is not significant, the reason for this might be that more CFs are coated by the matrix. High porosity always results in poor compressive performances inevitably, while PABA-CFs reinforcement here can dominate the loss by their robust integration with polymer matrix. Generally, compressive strength for non-stress bone sites is required to be higher than 4 MPa [43]. The biocomposites containing 0.5 and

Figure 4 (a and b) SEM images of CFs at various modifications. (c) Cell viability tested by MTT (mean $\pm$ SD, $P < 0.05$) and (d) FT-IR spectra of PABA-CFs and the control group.

Figure 5 (a–c) SEM images of biocomposites containing different fractions of PABA-CFs (The insets are the simulative pictures of corresponding contact angle, and red arrows denote PABA-CFs). (d and e) Swelling ratio and compressive strength (mean $\pm$ SD, $P < 0.05$).

2 wt.% PABA-CFs can stand 4.36 and 5.48 MPa compressive strength, respectively ($P < 0.05$), which satisfies the basic mechanical requirements for non-stress bone tissue.

Biocompatibility of modified CFs/PLA-PEG biocomposites

Fig. 6 displays the morphologies of L929 and MC₃T₃ cells spreading on the biocomposites containing different PABA-CFs fractions. The SEM images show the morphologies of cells on the surface, and the CLSM images show the morphologies of cells inside the biocomposites. Both pure PLA-PEG scaffolds and their biocomposites possess feasibility for cell adhesion and proliferation. The modified CFs/PLA-PEG biocomposites present some dif-

ferences with increasing PABA-CFs content after 1 day of culture. It is also demonstrated that the addition of fiber-like material can offer a reasonable guidance for cell growth [3,9]. The modified CFs/PLA-PEG biocomposites provide more space to accommodate and further wrap cells, which keeps in accordance with the following cell viability test.

Cell viability was evaluated by MTT assay of both L929 and MC₃T₃ cells. In Fig. 7, the adhesion and proliferation level of cells cultured on the modified CFs/PLA-PEG biocomposites are better than those cultured on pure PLA-PEG scaffolds, indicating the addition of PABA-CFs causes non-cytotoxicity to both types of cells. Comparing the CFs/

Figure 6 SEM and CLSM images of L929 (a–c and a₁–c₁) and MC₃T₃ (d–f and d₁–f₁) cells adhered and proliferated on the PLA-PEG scaffolds and their biocomposites with and without modified CFs.

Figure 7 MTT assay of L929 (a) and MC₃T₃ (b) cells cultured on the PLA-PEG scaffolds without and with different CFs fractions at different time points (mean $\pm$ SD, $P < 0.05$).

PLA-PEG biocomposites with different amounts of CFs, 0.5% CFs can bring better results. On the whole, the addition of PABA-CFs induces no negative effects on cell proliferation, and the CFs/PLA-PEG biocomposites show good biocompatibility.

CONCLUSIONS

PABA treated CFs with improved hydrophilicity were used to modify PLA-PEG scaffold. PABA-CFs present rich micro-grooves over the surface of PLA-PEG and maintain its basic tensile strength. The engineered CFs/PLA-PEG biocomposites possess robust combination interfaces between the CFs and polymer matrix. The PABA doped micro-grooved CFs improved both the biocompatibility and mechanical properties of the resulting scaffolds, showing their potential for applications in tissue engineering.

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Author contributions Shi Y and Wang N made the surface modifications of CFs and prepared the scaffolds; Shi Y and Li M performed the biological assessment experiments with help of Xing M; Shi Y and Li M wrote the paper with support from Wu Q. All authors contributed to the general discussion.

Conflict of interest The authors declare that they have no conflict of interest.

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对氨基苯甲酸掺杂的微槽结构碳纤维用于改善PLA-PEG的强度及生物相容性

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摘要 本文利用一种叶酸代谢产物, 对氨基苯甲酸(PABA), 来改性碳纤维(CFs), 通过在其表面形成微槽结构以达到改善其亲水性的目的. 采用溶液浇注/粒子沥滤法制备了CFs/PLA-PEG复合生物支架. 研究发现, 引入小的PABA疏水分子可以在CFs和聚乳酸-聚乙二醇(PLA-PEG)基体之间形成良性结合界面. CFs/PLA-PEG生物支架的抗压强度从3.98 MPa提高到5.48 MPa. 由于PABA的改性, 低毒性的CFs/PLA-PEG复合材料显著加速了预成骨细胞的粘附, 对细胞增殖没有明显影响. 通过比较支架上的L929细胞和MC₃T₃细胞的生物相容性, 可以认为PABA-CFs不仅克服了PLA-PEG强度差的缺陷, 而且可以促进细胞的生长, 证明了PABA-CFs增强PLA-PEG生物复合材料可作为一种潜在的组织工程支架材料.