3D Bio-Printing Fabrication and Properties of Graphene Dispersion-based Hybrid Scaffolds

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Abstract. Peripheral nerve is an important tissue of human body, which is distributed in various parts of human body, and has the function of connecting central nervous system and other organs. The self-repair ability of the nervous system is poor. However, 3D bio-printed neural tissue-engineered scaffolds offer a promising solution. In this study, graphene (Gr) was dispersed by a certain method, and then blended with gelatin (Gel) and sodium alginate (SA) to prepare a mixed bio-ink. Performance of different ratios of bio-ink was evaluated to determine the best printing ratio of the material. Four groups of Gr/Gel/SA scaffolds (The concentrations of graphene were 0%, 0.02%, 0.08% and 0.2% (w/v%), respectively) were prepared by using 3D printing technology. The mechanical strength, contact angle, degradation rate and water absorption of bio-scaffolds were compared to select the most suitable scaffold to support cell proliferation and differentiation, PC12 cells were used to study the biocompatibility of the scaffolds. This article aims to get the most optimized scaffolds.

1. Introduction

With the development of industrial mechanization and transportation, people have more and more opportunities for high-risk work, the incidence of spinal cord and peripheral nerve injuries has increased significantly. Peripheral nerve injury has become one of the most critical health problems in the world [1]. The nervous system has limited ability to repair its nerve damage which usually requires external adjuvant therapy [2]. At present, the main method for clinical repair of peripheral nerve defects is severance reconnection, but excessive tension at the severance may cause secondary neuronal necrosis. Allogeneic or xenogeneic nerves are the ideal nerve source, but treatment failure often results in severe immune rejection [3]. Therefore, repairing nerves is one of the most challenging areas. Tissue engineering is an interdisciplinary field of life science and materials science, and provides a potential alternative platform for implantation of nerve cells.

3D bio-printing can accurately build cell-loaded biomaterials into functional structures with complex structures [4]. Compared with traditional bionic scaffold construction methods in tissue engineering, it supports a wider range of applications with a richer and variable scaffold morphology [5]. Due to its rapid prototyping and control ability in material preparation, 3D bio-printing opened up a new perspective for the development of different types of nerve scaffolds which can be used for the treatment of specific patients. 3D bio-printing technology has great potential in medical tissue engineering applications.
Biological 3D printed materials must have the ability to provide a good growth environment for cell proliferation in order to cultivate tissues with complex structures and physiological functions. Biological materials that are commonly used are gelatin, alginate and extracellular matrix. Among those, gelatin has been widely used in biological direct printing technology [6]. Gelatin can be physically cross-linked in an aqueous solution above 40°C and form an α-helical structure reversibly below 30°C. The structure of physical gel comes from the intermolecular triple helices assembly is similar to collagen [7]. Sodium alginate is a natural linear polysaccharide anionic copolymer [8], which can be mixed with polyvalent cations during the printing process for immediate polymerization. Carbon-based nanomaterials have been widely studied for biomedical applications [9]. Graphene (Gr) is a carbon-derived nanomaterial [10]. Due to its excellent electrical conductivity, high tensile strength, and simple functional group bonding, it has versatile applications in the biomedical field.

The preparation of scaffolds is a major focus of tissue engineering, and there are many methods for preparing scaffolds. There are many shortcomings in the traditional preparation method of neural tissue engineering scaffolds, such as limited shape of the scaffolds and difficulty in controlling the aperture. Compared with electrostatic spinning technology, 3D printing is more convenient, faster and easier to control, 3D printing technology can overcome the limitations of traditional scaffold preparation methods in shape and process consistency and can also achieve the goal of high-precision scaffolds preparation. In this study, we solved the graphene dispersion problem and compounded graphene, gelatin and sodium alginate to produce a conductive biodegradable hydrogel and 3D printing scaffolds are produced using biodegradable hydrogel. Gelatin and sodium alginate have poor mechanical properties, hence the addition of Gr to these materials was expected to enhance the mechanical properties and biocompatibility of these scaffolds. The study also aimed to lay the foundation for the role of electrical stimulation in PC12 cell survival and neurite growth, which may lead to new applications.

2. Materials and Methods

2.1. Preparation of Biological Ink
A small amount of BYK190 (Water-based dispersant), 1-Butyl-3-methylimidazolium Bromide (BmimBr) and Poly urethane (PU) resin were added to a certain amount of graphene slurry, and it was sufficiently dispersed by shearing at 1500 rpm for three hours using a high-speed shear disperser to obtain a relatively stable graphene dispersion system. Next, gelatin and sodium alginate were separately added to the graphene dispersion system to obtain a gelatin blend system and a sodium alginate blend system at a certain concentration. The two blends of a certain concentration were then mixed in equal volumes and stirred in a 37°C water bath for 2 h. The mixture was degassed for 30 min to obtain a Gr/gelatin (Gel)/sodium alginate (SA) blended bio-ink.

2.2. Preparation of Bio-Scaffolds
Gr/Gel/SA bio-ink was prepared by the above method, and finally the ratio of Gel/SA was selected to be 6: 2 according to the comprehensive performance of the material, and the addition ratio of graphene was 0%, 0.02%, 0.08% and 0.2%. Adjusted the printer parameters and prepared the scaffolds into 8 mm by 8 mm by 2 mm pieces. Then they were crosslinked with 2w/v% Ca2+ solution immediately after bio-printing, and put them in deionized water for 1 h after the crosslinking reaction was completed to wash away excess Ca2+. Put the cross-linked scaffolds into the refrigerator at 4°C for pre-freezing overnight. After the pre-freezing was completed, they were pre-frozen at -80°C for 2h in the freeze dryer, and finally freeze dried in the vacuum dryer. The freeze-dried scaffolds could be used for subsequent testing.

2.3. CCK Test Detection Biocompatibility of Bio-Scaffolds
The proliferation of PC12 cells on the scaffolds was quantitatively detected by the CCK experiment, and the proliferation of PC12 cells inoculated on the scaffold for 1, 3, 5, and 7 days was tested. CCK solution was configured before the experiment, and its formula was 1:10 ratio between CCK stock solution and base medium, which was enough to be used in the experiment. After the experiment, the
remaining CCK solution could be refrigerated away from light and could be used later. Avoid the light during the whole process, took out the scaffold inoculated with PC12 cells, used a pipette to suck the remaining medium in the well plate, added the same amount of CCK solution to the well containing the cell-scaffold complex. After the operation was completed, put the well plate in the incubator. Took out after incubating for 2 hours in the incubator, used the pipette to suck the liquid in the well and moved it to the 96-well plate, then used a microplate reader to detect its OD value.

2.4. Fluorescence Staining to Observeest the Proliferation of PC12 Cells on the Bio-Scaffolds
First, the staining agent was prepared in the base medium, and 10 ml hoechst 33258 reagent, 2 ml calcein-AM reagent, and 2 ml PI reagent were added to 1 ml base media. Removed the scaffolds that had cultured PC12 cells for 1, 5, 7 days from the incubator, used a pipette to aspirate the remaining medium, then added 0.5 ml of staining agent to each well with the scaffolds, and placed them in the incubator for half an hour, and finally took out the well plate and observed them under a fluorescence microscope.

3. Results and Discussions
3.1. Characterization of Biological Ink
It can be seen from Figure 1 that the slopes of all the curves were negative, and the shear viscosity of the polymer material decreased with the increase of shear rate, indicating that the materials were all pseudo-shaped fluids. The higher absolute value of the slope of the material after cross-linking indicated that the material was easier to shear thinning after cross-linking. In addition, comparing the two groups of materials before and after cross-linking, it was found that the shear viscosity of the material decreased with the increase of graphene content before or after cross-linking, indicating that graphene will affect the shear viscosity of the material.

![Figure 1](image1.png)

**Figure 1.** sodium alginate (SA), 0%-graphene (Gr)/gelatin (Gel)/SA, 0.02%-Gr/Gel/SA, 0.08%-Gr/Gel/SA, 0.2%-Gr/Gel/SA, 0%-Gr/Gel/SA/Ca2+, 0.02%-Gr/Gel/SA/Ca2+, 0.08%-Gr/Gel/SA/Ca2+ and 0.2%-Gr/Gel/SA/Ca2+ ten groups of materials’ shear viscosity.

3.2. Characterization of Bio-Scaffolds
Figure 2A showed the compression modulus of four sets of scaffolds with different graphene contents. As the pressure force increased uniformly and slowly, the samples were gradually compressed and deformed, and the stress and deformation at each position of the scaffolds were substantially consistent, indicating that the material was uniform. It can be seen from the figure that the addition of graphene increased the mechanical strength of the scaffolds, but the mechanical strength did not
increase indefinitely. The excessively high graphene content would reduce the mechanical strength of the scaffolds, which may result in the agglomeration phenomenon due to high graphene content. The agglomeration phenomenon will cause the volume fraction of graphene to decrease which will also lead to the weakening of scaffold mechanical properties. Figure 2C showed the water absorption of four groups of scaffolds with different graphene contents. The water absorption of the scaffolds in aqueous solutions at 3, 6, 9, 24, 48 and 96 hours was measured. In the first few hours, the water absorption of the scaffolds increased rapidly and then increased slowly over the next few days. As can be seen from the figure, the water absorption of the scaffolds decreased slightly with the increase of graphene, because graphene was a hydrophobic substance, so that the water absorption capacity of the scaffolds containing the graphene was lowered. After 96 hours, the water absorption rates of the Gels/SA, 0.02% Gr/Gel/SA, 0.08%Gr/Gel/SA and 0.2%Gr/Gel/SA scaffolds were 1100.5 ± 18.96%, 1041.2 ± 11.21%, 1019.5 ± 29.65% and 1031.8 ± 15.54%, respectively. The higher water absorption rate of scaffolds indicated that the scaffolds had good hydrophilicity and could meet the requirements of cell proliferation and adhesion on the scaffolds. The contact angles of the prepared scaffolds were also determined (Figure 2B). As the graphene content increased, the contact angles of the scaffolds became larger and larger, indicating that the hydrophilicity of the material was getting worse. The contact angles of the Gels/SA, 0.02%Gr/Gel/SA, 0.08%Gr/Gel/SA and 0.2%Gr/Gel/SA scaffolds were 56.21 ± 1.24°, 64.38 ± 2.45°, 96.20 ± 0.25° and 116.37 ± 3.54°, respectively. Figure 2D showed a graph of the scaffolds degradation rate. During the first three days of degradation, the scaffold degradation rate was high, and then the degradation of the scaffold began to be gentle. It may be that the content of Na ions in the degradation solution was reduced, and the degradation of the scaffolds tended to be slow. As the graphene content increased, the degradation rate of the scaffolds gradually fell down. Because the addition of graphene increased the inorganic content of the material, and made the hydrophilicity of the material worse, the porosity was smaller, resulting in slower degradation, which ultimately led to a decrease in the scaffold degradation rate.

Figure 2. Physical and chemical properties of bio-scaffolds. (A) Mechanical strength of bio-scaffolds. (B) Contact angle of 0%Gr/Gel/SA, 0.02%Gr/Gel/SA, 0.08%Gr/Gel/SA and 0.2%Gr/Gel/SA. (C) Water absorption rate of 0%Gr/Gel/SA, 0.02%Gr/Gel/SA, 0.08%Gr/Gel/SA and 0.2%Gr/Gel/SA. (D) Degradation rates of 0%Gr/Gel/SA, 0.02%Gr/Gel/SA, 0.08%Gr/Gel/SA and 0.2%Gr/Gel/SA. ***p<0.001; **p<0.01.
3.3. Compound Fluorescence Staining to Observe Proliferation of PC12 Cells

Investigation was also conducted to exam the proliferation of PC12 cells. As shown in Figures 3, cells were seeded on four groups of scaffolds with different graphene contents and cultured for five days to qualitatively evaluate the distribution and viability of the cells. It can be seen from the figure that PC12 cells grew well in all scaffolds, with a large number of living cells and a very small number of dead cells. With the increase of culture time, the cells proliferated a lot. Compared with Gel/SA scaffolds, graphene-added scaffolds had more living cells, and they were more capable of stretching and growing cells adherent. Figure 3 showed that these scaffolds improved PC12 cell proliferation and distribution, and the effect of scaffolds would be better after adding graphene. In order to further investigate the biological activities of cells cultured on the scaffolds, cell proliferation was also quantitatively measured.

![Figure 3. Viability and distribution of PC12 on 0.08%Gr/Gel/SA scaffold 1, 5, 7 day after inoculation (Scale: 500 μm).](image)

3.4. Biological Performance of Bio-scaffolds

As shown in Figure 4, during the first three days of cell culture, the viability of cells became higher and higher with the content of graphene increased, indicating that graphene could promote cell proliferation in the early stage of in vitro culture. The cell viability of Gels/SA, 0.02%Gr/Gel/SA, 0.08%Gr/Gel/SA and 0.2%Gr/Gel/SA scaffolds on the third day were 54.70±2.61%, 58.45±1.23%, 59.54±6.14, and 70.91±9.14%, respectively. After the cells were cultured in the all scaffolds for more than five days, the cell viability did not increase with the increase content of graphene. This showed that after a long period of culture, the excessively high graphene content would have a greater and greater negative impact on the cells, and the cyto-toxicity of the scaffold would become more and more obvious. However, compared to the Gel/SA scaffold, the scaffolds containing graphene were still more advantageous for cell proliferation in vitro.
Figure 4. The cell viability rate of PC12 inoculated on the bio-scaffolds. **p< 0.01; *p< 0.05.

4. Conclusion
In this paper, graphene/gelatin/sodium alginate biological ink was successfully prepared. The bio-ink Gel: SA=6:2 was the most suitable for 3D printing, and the bionic scaffold was successfully prepared. The physical and chemical properties of the scaffolds were investigated and PC12 cells were inoculated into the scaffold to examine the biocompatibility of the scaffold. It was found that the right amount of graphene will improve the mechanical properties of the scaffolds, and the graphene will be evenly dispersed in the scaffolds. After further optimization, the scaffold with Gr/Gel/SA ratio of 0.08:6:2 was found to have better water absorption, porosity, compression modulus, hydrophilicity and cellular compatibility.

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