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Cardiomyocyte behavior on biodegradable polyurethane/gold nanocomposite scaffolds under electrical stimulation

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ABSTRACT

Following a myocardial infarction (MI), cardiomyocytes are replaced by scar tissue, which decreases ventricular contractile function. Tissue engineering is a promising approach to regenerate such damaged cardiomyocyte tissue. Engineered cardiac patches can be fabricated by seeding a high density of cardiac cells onto a synthetic or natural porous polymer. In this study, nanocomposite scaffolds made of gold nanotubes/nanowires incorporated into biodegradable castor oil-based polyurethane were employed to make micro-porous scaffolds. H9C2 cardiomyocyte cells were cultured on the scaffolds for one day, and electrical stimulation was applied to improve cell communication and interaction in neighboring pores. Cells on scaffolds were examined by fluorescence microscopy and scanning electron microscopy, revealing that the combination of scaffold design and electrical stimulation significantly increased cell confluency of H9C2 cells on the scaffolds. Furthermore, we showed that the gene expression levels of Nkx2.5, atrial natriuretic peptide (ANF) and natriuretic peptide precursor B (NPPB), which are functional genes of the myocardium, were up-regulated by the incorporation of gold nanotubes/nanowires into the polyurethane scaffolds, in particular after electrical stimulation.

1. Introduction

Cardiovascular diseases pose the highest risk of death in the world, according to the American Heart Association Statistics. Every 34 s one American dies by heart attack, stroke or other cardiovascular problems [1]. Currently, treatment options following a myocardial infarction (MI) and subsequent congestive heart failure are still limited. Pharmacological agents increase the blood flow but limit ventricular remodeling events and increase cardiac output [2]. Mechanical devices, such as the left ventricular assist device (LVAD), can only be applied to a limited group of patients [3]. The only successful treatment option for a severe MI to date is heart transplantation [4]; however, the lack of suitable donors significantly restricts this option.

As cardiovascular diseases remain a major cause of morbidity and mortality, new strategies in cardiovascular treatments attract much attention. Among all cardiovascular diseases, MI is one of the key reasons for heart failure, resulting in heart dysfunction and progressive death of cardiomyocytes when normal heart function cannot be restored afterwards [5]. Cell therapy has so far shown only little improvement of cell retention and long-term survival [6]. Instead, biocompatible 3D scaffold materials might provide a feasible solution, as some structures may improve cell retention, survival and even cell differentiation [7,8]. These kinds of scaffolds or patches can, in principle, be directly implanted on the infarcted tissue with or without cells after MI [9].

Typically, tissue engineering for cardiovascular regeneration is based on producing biomimetic and biodegradable materials for scaffold fabrication [4] that ideally integrate signaling molecules and induce cell migration into the scaffolds [10,11].

A material suitable for a tissue engineering-based approach to treat myocardial infarction should provide an environment that is predisposed to improve electromechanical coupling of the cardiomyocytes with the host tissue [10,12], as well as cardiomyocyte adhesion [9]. This adhesion is essential for the proliferation of cardiomyocytes and for ventricular function. Materials suitable for application in cardiac tissue engineering include natural polymers, such as decellularized myocardium [13], collagen [14], alginate [15], fibrin [16], as well as synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA), their copolymers [17,18], and polyurethane (PU) [19,20].

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Among the above-mentioned materials, PUs are considered a major class of applicable elastomers because of their good biocompatibility and biodegradability, their high flexibility, and their excellent mechanical properties [21,22]. The stiffness of heart muscle varies from 10 kPa in the beginning of the diastole to 500 kPa at the end of the diastole, therefore an elastic material having a stiffness in this range would be required for cardiac engineering [23]. Such Young's moduli are obtained with biodegradable PUs [19,24], which can be synthesized by using vegetable oils as polyol and aliphatic diisocyanate, resulting in typical degradation times of several months. Among different grades of PUs, castor oil-based PU shows no toxicity, is low in cost, and is available as a renewable agricultural resource [25–27]. This grade of biodegradable PUs has already been widely applied in biomedical engineering, including materials for peripheral nerve regeneration, cardiovascular implants, cartilage and meniscus regeneration substrates, cancellous bone substitutes, drug delivery carriers and skin regeneration sheets [28–30].

Furthermore, tissue engineering applications require that cells are embedded into the material. Much progress has recently been made in order to fabricate porous polymer scaffolds, in particular by using salt leaching techniques [31–33]. The success of this method has been shown for a variety of soft and hard polymers [34–37], and we have recently established this procedure for PU [38].

Although many PU-based materials have been developed for providing vascular grafts, only few PU scaffolds have so far been studied in the context of myocardial tissue engineering [39,40], even though PU is easy to implant into muscle tissue, because it is stiffer than typical hydrogels. An important goal for myocardial tissue engineering must be the fabrication of materials that allow for the synchronization of electrical signals, and thus enhance the contraction of cardiomyocytes in the scaffold material so that a homogeneous total contraction of the engineered patch is guaranteed. In the study presented here, we fabricated a biodegradable nanocomposite material by incorporating gold nanotubes/nanowires into PU scaffolds so that the wired material structure can mimic the electromechanical properties of the myocardium.

To investigate the functionality of these materials as cardiac patches, H9C2 rat cardiomyocyte cells were seeded on different polyurethane-gold nanotube/nanowire (PU-GNT/NW) composites. Eventually, electrical stimulation was applied to the cell-scaffold constructs in order to enhance the functional performance of cardiac scaffolds and to improve cell morphology and alignment. We used fluorescence and scanning electron microscopy as well as gene expression analysis to investigate the behavior of cardiomyocyte cells on the scaffolds. We demonstrate that the adhesion and proliferation of cells significantly depends on the amount of incorporated GNT/NW, and that an optimum concentration of 50 ppm of GNT/NW can provide the best environment for cells to achieve native cardiomyocyte function.

2. Materials and methods

2.1. Synthesis of polyurethane-GNT/NW composite scaffolds

Polyurethane-GNT/NW composites were synthesized according to our previous work [38]. In brief, gold nanotubes/nanowires (GNT/NW) were made by using template-assisted electrodeposition and mixed with castor oil/polyethylene glycol-based polyurethane (PU). Concentrations of 50 and 100 ppm of GNT/NW were used to synthesize two different composites types. For fabrication of porous scaffolds, 355–600 μm sieved table salt was added to the PU-GNT/NW solution, then the mixture of PU-GNT/NW and salt was cast in a Teflon mold of 10 mm diameter and 4 mm thickness. Afterwards, all samples were dried at room temperature for 48 h; then the porous scaffolds were placed in distilled deionized water (DDW) for 2 more days to remove the salt. In the following, we refer to the scaffolds as PU-0 for pure PU scaffolds, PU-50 for scaffolds containing 50 ppm GNT/NW, and PU-100 for those containing 100 ppm GNT/NW.

2.2. Permeability

As it is experimentally difficult to obtain 3D information about pore interconnectivity based on 2D images, Li et al. [41] suggested a simple method of soaking the samples in an ink solution and then imaging the colored sample. Accordingly, our scaffolds were soaked in a solution of common blue writing ink for 24 h and dried at room temperature. Then, a cross section of samples with a thickness of 1 mm was prepared by cutting with a surgical blade and then imaging the samples with a Nikon (TS100) inverted microscope (10× objective). This treatment provides information on the interconnectivity of pores as well as on their accessibility from neighboring pores. Porosity was calculated by ImageJ [42] using a manually set intensity threshold.

2.3. Cell culture and electrical stimulation

H9C2 rat cardiomyocytes were purchased from the European Collection of Cell Cultures (ECACC, Germany) and maintained in Dulbecco's Modified Eagle's medium (DMEM, Biochrom, Germany), supplemented with 10% fetal bovine serum (FBS, Biochrom, Germany) and 1% penicillin and streptomycin (100 U/ml, Biochrom, Germany) at 37°C and 90% humidity. H9C2 is a subclone of the original clonal cell line derived from embryonic rat heart tissue. Cells were sub-cultured regularly and used up to passage 6. Prior to the experiments, PU scaffolds were sterilized using ethylene oxide gas and placed in 10 ml of sterilized phosphate buffered saline (PBS) for 2 h. Cells (10^6) were seeded per cylindrical scaffold (diameter: 10 mm, thickness: 4 mm) and incubated overnight to allow cell attachment. On the following day, cells were stimulated using a function generator (Toellner, Germany) with a square pulse of 1 V/mm amplitude, pulse duration of 2 ms, at a frequency of 1 Hz for 15 min. This procedure was repeated on three consecutive days, once per day [43,44]. Stainless steel 304 was used as the electrode material for electrical stimulation. Compared to titanium electrodes and titanium electrodes coated with titanium nitrate, the electrical field was stable in stainless steel 304 electrodes over the whole time of stimulation [43]. The cell-scaffold constructs were left in the incubator for one more day.

2.4. Staining with Calcein and Hoechst

Calcein was used for staining viable cells and Hoechst for staining cell nuclei. Five repeats of each scaffold group were stained with both Calcein AM and Hoechst after 1 day of cell culture before stimulation and another 5 repeats of each group were stained on the fourth day after cell seeding and electrical stimulation. For Calcein staining, the samples were rinsed once with DMEM and incubated with a 1 μg/ml solution of Calcein (BD Bioscience, Germany) in DMEM for 10 min at 37 °C. Afterwards, the samples were washed with DMEM twice, stained with 10 μg/ml Hoechst 32258 (Invitrogen, Germany) in PBS and incubated for 20 min at 37 °C. Then, the samples were washed extensively with PBS and imaged using an Olympus BX43 fluorescence microscope (Olympus, Japan) with a 10× objective. Cell confluency was measured as the ratio of the area stained with Calcein to the whole surface of a scaffold in 2D images using ImageJ [42]. This test was performed in two independent experiments and at least 5 images were taken in each experiment.

2.5. Gene expression

Cells were lysed in TriSure (Bioline, Luckenwalde, Germany) and RNA extraction was performed according to the manufacturer's protocol. In order to obtain enough RNA, cells grown on 3 scaffolds were pooled. After RNA extraction, aliquots of 200 ng total RNA from each group were reverse transcribed into cDNA, using a cDNA synthesis kit (AmpTec, Hamburg, Germany) and the provided oligo dT-V primer. Subsequently the cDNAs were purified utilizing the spin columns and