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Fabrication of nanofibrous macroporous scaffolds of poly(lactic acid) incorporating bioactive glass nanoparticles by camphene-assisted phase separation

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HIGHLIGHTS

- Exploring macroporous scaffolds with nanofibrous structuring.
- Incorporating bioactive glass nanoparticles to produce nanocomposite scaffolds.
- Showing excellent *in vitro* bioactivity useful for bone tissue engineering.

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ABSTRACT

Here we produced macroporous and nanofibrous scaffolds with bioactive nanocomposite composition, poly(lactic acid) (PLA) incorporating bioactive glass nanoparticles (BGnp) up to 30 wt%, targeting bone regeneration. In particular, the nanofibrous structure in the scaffolds was generated by using a bicyclic monoterpene, camphene ($C_{10}H_{16}$), through a phase-separation process with PLA-BGnp phase in chloroform/1,4-dioxane co-solvent. Furthermore, macropores were produced by the impregnation of salt particles and their subsequent leaching out, followed by freezing and lyophilization processes. The produced PLA-BGnp scaffolds presented highly porous and nanofibrous structure with porosities of 90–95% and pore sizes of over hundreds of micrometers. BGnp with sizes of ~90 nm were also evenly impregnated within the PLA matrix, featuring a nanocomposite structure. The nanofibrous scaffolds exhibited enhanced hydrophilicity and more rapid hydrolytic degradation as the incorporated BGnp content increased. The bone-bioactivity of the scaffolds was substantially improved with the incorporation of BGnp, exhibiting rapid formation of apatite throughout the scaffolds in a simulated body fluid. The developed macroporous and nanofibrous scaffolds with PLA-BGnp bioactive composition are considered as a novel 3D matrix potentially useful for bone tissue engineering.

1. Introduction

Nanocomposite three-dimensional (3D) scaffolds made of degradable polymers with the incorporation of bioactive inorganic materials have shown great potential for application to the field of hard tissue regeneration [1–3]. Scaffolds have facilitated functional recovery of damaged and diseased tissues by recruiting a population of surrounding stem or progenitor cells and guiding their regenerative processes [3–6]. Furthermore, *ex vivo* culture of such candidate cells to engineer tissue-mimicking structures also requires the use of scaffolds with appropriate mechanical and physico-chemical properties [7].

The native bone extracellular matrix (ECM) is a sort of nanocomposite comprising of a collagen fibrous network with embedded hydroxyapatite inorganic nanocrystals. This nanocomposite structure of ECM provides proper mechanical functionality to bone, as well as high strength and toughness, and consequent resistance to catastrophic failure. From the viewpoint of biomaterials, the use of bioactive inorganics significantly improves cell responses required for bone regeneration, such as osteoblast maturation and bone ECM syntheses [8–11]. The use of biopolymer is also greatly helpful for the processing of

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inorganic materials, making it possible to apply polymer technology involving low-temperatures and molding and shaping, which is a particular merit of the porous scaffold processing [12,13].

Along with the compositional aspect, the surface physical properties also dictate the biological reactions, affecting protein adsorption, adhesion and growth of cells, and their tissue-specific differentiation. As the surface is the first point of contact for cells, the surface micro/nanostructure is critical for regulating cellular processes and subsequent tissue formation [14–17]. Among the physical parameters, morphological traits, mainly those relating to nanoscale morphology, have recently been demonstrated to dominate many cellular responses. One of the most attractive morphological traits is 'nanofibrous', and the nanofibrous structure has mainly been implicated in native tissue ECM proteins such as collagen and elastin, and thus, has been considered as an ECM-mimicking nanostructure [17–19]. This is why cells recognize well the nanofibrous structured surface, even better than the flat surface, and behave more profoundly and specifically to secret ECM molecules [16–19]. In general, the nanofibrous-tailored substrates possess large surface areas that allow the adsorption of biological proteins, such as cell adhesive proteins, improving the initial cellular recognition of the surface. Moreover, the nanofibrous surfaces have demonstrated better cell proliferation and osteogenic differentiation with respect to flat substrates [16,17]. Therefore, the nanofibrous materials are considered as a promising platform for the repair and regenerative matrices of tissues, including bone.

One interesting and potential area of utilizing this nanofibrous structured platform is in electrospinning. In other words, through electrospinning processes, nanofibrous matrices can easily be implemented [18,19]. A range of biomaterials, including degradable polymers, have been electrospun into a nanofibrous form, finding extensive utility for the repair of damaged tissues, such as skin, blood vessel, cartilage, nerve and bone [18–20]. While the electrospun materials provide excellent culture substrate conditions for many different sorts of cells, one major drawback is the difficulty in providing 3D macropores within the nanofibrous structure, and shaping it into complex forms [19]. Although some efforts have been exerted by modifying the collecting part of the electrospinning apparatus, or incorporating porogen during electrospinning process, these have limitations in terms of processibility and tunability over shape and porosity [18–20]. This issue confronted in electrospinning is particularly in great need when we compare the process with other 3D scaffolding techniques, such as gas foaming, template replication, and rapid prototyping. Therefore, it is highly recommended to develop a methodology to create nanofibrous and macroporous scaffolds by utilizing possible alternative methods.

Here we focus on the phase-separation method, wherein an appropriate use of solvents and freezing conditions allows for the generation of a nanofibrous network of biopolymers with a porous structure. Previously, Ma et al. has been working on methodology to produce nanofibrous polymer scaffolds by means of a phase-separation method [17,21,22]. The method is a solvent-based process requiring a freezing step to separate the two phases of material and solvent, which is ascribed to the immiscibility between the phases, and a subsequent removal-off the crystallized solvent phase. The resultant material part exerts various nano/microstructures, like nanofibrous morphology. The nanofibrous scaffolds of poly(lactic acid) (PLA) or PLA-gelatin using organic solvents have been successfully produced, and have shown to recruit several cell types and to stimulate differentiation into osteoblastic lineage [17,21,22], suggesting that the nanofibrous surface is particularly interesting as a scaffold for bone regeneration. Recently, we have also investigated the generation of a nanofibrous biopolymer, poly(hydroxyl butyrate-*co*-valerate) (PHBV), by the phase-separation method [16]. In particular, a novel solvent, camphene, was used to create nanofibrous structured PHBV. Because of the high freezing-point and ease-of-sublimation, the camphene phase interconnecting the material part could be liberated easily under relatively mild conditions, suggesting it to be a promising candidate source for creating nanofibrous structure of biopolymers [16].

We aimed to utilize the camphene-assisted method in the creation of novel nanofibrous 3D scaffolds made of PLA and bioactive glass nanoparticles (BGnp) for bone regeneration. The BGnp were prepared by a sol–gel method with sizes of less than a hundred nanometers. Thus, the prepared BGnp are considered to provide excellent bioactivity for bone cell functions and bone formation, compared to the hydrophobic and less bioactive PLA polymeric scaffold. Macropore generation was exploited by inducing freezing temperatures as high as possible with proper choice of solvents and camphene, and additionally through salt impregnation – leaching out method. Here we report first on the preparation of the macroporous nanofibrous PLA scaffolds with the incorporation of different concentrations of BGnp, and then we briefly examined the properties, particularly those of *in vitro* bone-bioactivity (apatite forming ability) in a simulated body fluid.

2. Experimental procedures

2.1. Bioactive glass nanoparticles (BGnp)

The BGnp were prepared by a sol–gel method, as described elsewhere [23]. In brief, a binary composition $85SiO_2/15CaO$ (mol %) of BGnp was synthesized by a ultra-sound assisted base-catalyzed sol–gel method using poly(ethylene glycol), (PEG; $(C_2H_4)_nH_2O$, M_n: 10,000) as a template. PEG of 5 g was completely dissolved in methanol at pH adjusted to 12.5 by adding NH_4OH. 0.179 g of Ca $(NO_3)_2 \cdot 4H_2O$ of was then dissolved in the solution, and 0.895 g tetraethyl orthosilicate (TEOS) was then diluted with methanol and added to the solution, with the application of a high-power ultra-sound using a Sonoreactor, LH700S ultra-sonic generator (Ulsso Hitech) operating at 20 kHz and 700 W. The output power was 220 W in a 10-s on/10-s off cycle for 20 min. After 24 h, the white precipitate was separated by centrifugation at 5000 rpm using a Mega17 R centrifuge (Hanil Science), and was then washed and re-dispersed with water and ethanol. The precipitate was dried at 70 °C and was then calcined at 700 °C under air for 5 h.

2.2. Preparation of nanofibrous PLA-BGnp macroporous scaffolds

For the preparation of the nanofibrous structured scaffolds, the camphene-assisted phase-separation method was introduced. First, pure PLA composition was used to prepare a nanofibrous structure by varying the concentration of camphene ($C_{10}H_{16}$, Sigma–Aldrich). For this, PLA was dissolved at 3 wt% in a co-solvent of chloroform/1,4-dioxane of 1/4, within which different concentrations of camphene (camphene/PLA ratio of 0, 2, and 4 by weight) were dissolved. The mixture solution was poured into a plastic mould, and was then allowed to freeze at −20 °C overnight, during which the camphene-PLA solution was frozen solid. The frozen samples were freeze-dried for 3 days to sublimate the solution and camphene, and to create the porous-structured scaffold. In order to provide macropores within the scaffold, salt-particles were added to the PLA-camphene solution. NaCl particles of 200–500 μm diameter were first added to a plastic mould, followed by the addition of