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Highly porous bioresorbable scaffolds with controlled release of bioactive agents for tissue-regeneration applications

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ABSTRACT

Highly porous poly(DL-lactic-co-glycolic acid) films with controlled release of horseradish peroxidase (HRP) as a model protein have been successfully developed and studied. These films, which are prepared by freeze-drying inverted emulsions, are designed for use in tissue-regeneration applications. The effects of the emulsion's formulation and host polymer's characteristics on the film's microstructure and HRP release profile over 4 weeks were investigated. A dual pore size population is characteristic for most films, with large 12–18 μm pores and small 1.5–7 μm pores, and porosity in the range of 76–92%. An increase in the polymer content and its initial molecular weight, organic/aqueous (O:A) phase ratio and lactic acid content, or a decrease in the HRP content, all resulted in a decreased burst effect and a more moderate release profile. A simultaneous change in two or three of these formulation parameters (compared to a reference formulation) resulted in a synergistic effect on the HRP release profile. A constant HRP release rate was achieved when a composite film was used. Human gingival fibroblast adhesion to the films indicated good biocompatibility. Appropriate selection of the emulsion's parameters can therefore yield highly porous films with the desired protein-release behavior which can serve as scaffolds for bioactive agents in tissue-regeneration applications.

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1. Introduction

Tissue engineering has been described as "an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" [1]. A major area of research in tissue engineering is the development of scaffolds that elute bioactive agents. Upon implantation of such scaffolds, cells from the body are recruited to the site, thus enabling tissue formation [2]. Growth factors are essential for promoting cell proliferation and differentiation. However, direct administration of growth factors is problematic, due to their poor in vivo stability [3,4]. It is therefore necessary to develop scaffolds with controlled delivery of bioactive agents that can achieve prolonged availability as well as protection of these bioactive agents, which may otherwise undergo rapid proteolysis [5,6]. The main obstacle to successful incorporation and delivery of small molecules, as well as proteins, from scaffolds is their inactivation during the process of scaffold manufacture due to exposure to high temperatures or harsh chemical environments. Methods that minimize protein inactivation must therefore be developed. Three approaches to protein (growth factor) incorporation into bioresorbable scaffolds have recently been presented: (i) adsorption onto the surface of the scaffold [7]; (ii) composite scaffold/microsphere structures [6,8]; and (iii) freeze-drying of inverted emulsions. The third method is briefly described below.

Emulsions are metastable colloids formed by two immiscible fluids, where one is dispersed in the other in the presence of surface-active agents (surfactants) [9]. Inverted emulsions are composed of water droplets dispersed in a continuous oil (organic) phase. Emulsions are obtained by shearing two immiscible fluids, leading to the fragmentation of one phase into the other. They are metastable and their lifetime may vary considerably depending on the temperature and their composition. The instability is due to the large interfacial area, which results in a large surface energy that is associated with finely dispersed systems [10]. The technique of freeze-drying inverted emulsions is unique in being able to preserve the liquid structure in solids. Also, it is important to note that incorporation of bioactive molecules is carried out during the scaffold-production process. This fabrication process enables the incorporation of both water-soluble and water-insoluble drugs into the film in order to obtain an "active implant" that releases drugs to the surrounding in a controlled manner. Water-soluble bioactive agents are incorporated in the aqueous phase of the inverted emulsion, whereas water-insoluble drugs are incorporated in the organic (polymer) phase. Sensitive bioactive agents, such as proteins, can also be incorporated in the aqueous phase. This prevents their

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exposure to harsh organic solvents and enables the preservation of their activity. Whang et al. [11,12] used this method to prepare poly(DL-lactic-co-glycolic acid) (PDLGA) scaffolds loaded with a recombinant human bone morphogenetic protein (rhBMP-2) and investigated the effect of the rhBMP-2 release in vivo using a rat model. The rhBMP-2-incorporated scaffold induced bone formation, which confirmed the preserved bioactivity of the rhBMP-2 released from the scaffold. Contact radiography, radiomorphometry, histology and histomorphometry revealed significantly more bone in the rhBMP-2 implants than in the controls.

In the current study we investigated highly porous film scaffolds produced using by freeze-drying inverted emulsions. Horseradish peroxidase (HRP) is a relatively inexpensive enzyme and was chosen as a model protein since it is very sensitive to solvents and elevated temperatures. If proteins such as HRP can be incorporated in the films without losing their activity, these films can be loaded with growth factors and can be used for building scaffolds for tissue-regeneration applications. As mentioned above, the technique of freeze-drying inverted emulsions is currently the only method for preparation of scaffolds loaded with sensitive bioactive agents such as proteins, where loading is performed during the scaffold preparation without losing the protein's activity. The present study examined the effects of each one of the emulsion's formulation parameters and host polymer's characteristics on the film microstructure and on the resulting HRP release profile (for 28 days). In addition, the combined effects of two and three parameters were studied and a unique composite structure was developed. These have not been studied before. Human gingival fibroblast (HGF) adhesion to these novel scaffolds was also studied.

2. Materials and methods

2.1. Materials

Most of the bioresorbable porous films used herein were made of 50/50 PDLGA, inherent viscosity (i.v.) = 0.56 dl g^{-1} (in $CHCl_3$ at 30 °C), MW ≈ 83 kDa. Certain films were made of the following polymers: relatively low MW 50/50 PDLGA, i.v. = 0.4 dl g^{-1} (in $CHCl_3$ at 30 °C), MW ≈ 50 kDa, relatively high MW 50/50 PDLGA, i.v. = 1.13 dl g^{-1} (in $CHCl_3$ at 30 °C), MW ≈ 185 kDa and 75/25 PDLGA, i.v. = 0.65 dl g^{-1} (in $CHCl_3$ at 30 °C), MW ≈ 103 kDa. Poly(DL-lactic acid) (PDLLA), inherent viscosity (i.v.) = 0.55 dl g (in $CHCl_3$ at 30 °C), MW ≈ 80 kDa was used for the outer layers of the composite film only. All polymers were purchased from Lakeshore Biomaterials, Inc., AL, USA.

HRP with an initial enzymatic activity of 500 U mg^{-1}, 40 kDa, Aldrich, served as a protein model.

A BCA™ Protein Assay Kit was used for measuring the protein content of solutions with a relatively high (20–2000 μg ml^{-1}) protein content, and a Micro BCA™ Protein Assay Kit was used for measuring the protein content of solutions with a relatively low (0.5–40 μg ml^{-1}) protein content. These were purchased from Pierce.

2.2. Porous film preparation

PDLGA was dissolved in $CHCl_3$ to form an organic solution. An aqueous solution containing HRP was poured into the organic solution (in a test tube) and homogenization of the emulsion was performed using a Kinematica PT-3100 Polytron homogenizer operated at 5000 rpm for 3 min. We have previously found that these processing conditions are optimal, i.e. preserve most of the enzymatic activity (94–100%) and yield homogeneous emulsions for all examined formulations [13].

An emulsion formulation containing 17.5% w/v 50/50 PDLGA (i.v. = 83 kDa) in the organic solution, 1% w/w drug in the aqueous medium (relative to the polymer load), and an organic/aqueous (O:A) phase ratio of 4:1 v/v was used as the reference formulation. Additional formulations were used in order to investigate the effect of the emulsion's parameters on the film's porous structure and on the HRP release profile from the film. These included variations in polymer content (15%, 20% and 25%), HRP loading (0.5% and 2%), O:A phase ratio (2:1, 6:1 and 8:1), copolymer composition (75/25) and polymer MW (50 and 185 kDa). In the first stage of each studied series only one parameter was changed and the others were left the same as in the reference formulation. In the second stage of the study, two or three formulation parameters were changed simultaneously.

Each emulsion was poured into an aluminum dish and then frozen immediately in a liquid nitrogen bath. The samples were then placed in a pre-cooled (−100 °C) freeze-dryer (Virtis 101 equipped with a nitrogen trap) capable of working with organic solvents (the freezing temperature of the condenser was approximately −105 °C) and freeze-dried in order to preserve the microstructure of the emulsion-based films. Drying was performed in two stages: (i) the freeze-dryer chamber pressure was reduced to 100 mTorr while the temperature remained at −100 °C; (ii) the condenser was then turned off after 5 h and its plate temperature gradually increased to room temperature while the pressure was monitored between 100 and 700 mTorr. During this step the liquid nitrogen trap condensed excess water and solvent vapors. The samples were stored in desiccators until use.

2.2.1. Composite film preparation

A composite film, which was composed of three layers, contained a porous 50/50 PDLGA film sandwiched between two PDLLA layers. The parameters of the 50/50 PDLGA inner layer are: 25% w/v polymer, MW = 83 kDa, O:A = 8:1 and 0.5% w/w HRP. The parameters of the PDLLA outer layers are: 17.5% w/v polymer, MW = 80 kDa and O:A = 2:1. The outer film layers did not contain HRP. The composite film preparation was performed as follows: the inner 50/50 PDLGA layer was first prepared as described above and then immersed in the PDLLA emulsion, frozen immediately in liquid nitrogen and freeze-dried.

2.3. In vitro HRP release studies

Small 1.5 cm × 2.0 cm films (four samples) were immersed in phosphate-buffered saline (PBS) at 37 °C for 28 days in order to determine the HRP release kinetics from these structures. The release studies were conducted in closed glass tubes containing 2.0 ml PBS medium. The medium was removed (completely) periodically, at each sampling time, and fresh medium was introduced. Sampling was carried out at 0.5, 1, 2, 3, 7, 14, 21 and 28 days.

The HRP content of each medium sample was determined using the micro BCA assay method, by measuring absorbance at 562 nm, using a SpectraMax 340 PC^{384} plate reader spectrophotometer. Cumulative HRP release profiles were determined relative to the initial amount of HRP in the composite fibers (HRP released during the incubation period + the residue remaining in the fibers). Four repetitions were performed for each sample. Results are presented as means ± SD.

2.4. Residual HRP recovery from the films

Residual HRP recovery from the films used for in vitro release experiments was conducted using a previously described method [14]. In short, the films were placed in 1 ml sodium dodecyl sulfate (SDS)/NaOH 5%/0.1 M solution for 48 h at 37 °C. Following extraction, the HRP concentration was estimated using a micro BCA assay method as described above.

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