Materials Science and Engineering C 63 (2016) 439–449

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Pullulan microcarriers for bone tissue regeneration

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ARTICLE INFO

Article history:
Received 3 July 2015
Received in revised form 2 February 2016
Accepted 1 March 2016
Available online 4 March 2016

Keywords:
Pullulan
Bone tissue engineering
Microspheres
Biomimetic mineralization
SF coating

ABSTRACT

Microcarrier systems offer a convenient way to repair bone defects as injectable cell carriers that can be applied with small incisions owing to their small size and spherical shape. In this study, pullulan (PULL) microspheres were fabricated and characterized as cell carriers for bone tissue engineering applications. PULL was cross-linked by trisodium trimetaphosphate (STMP) to enhance the stability of the microspheres. Improved cytocompatibility was achieved by silk fibroin (SF) coating and biomimetic mineralization on the surface by incubating in simulated body fluid (SBF). X-ray diffraction (XRD), scanning electron microscopy (SEM) and fluorescent microscopy analysis confirmed biomimetic mineralization and SF coating on microspheres. The degradation analysis revealed that PULL microspheres had a slow degradation rate with 8% degradation in two weeks period indicating that the microspheres would support the formation of new bone tissue. Furthermore, the mechanical tests showed that the microspheres had a high mechanical stability that was significantly enhanced with the biomimetic mineralization. *In vitro* cell culture studies with SaOs-2 cells showed that cell viability was higher on SF and SBF coated microspheres on 7th day compared to PULL ones under dynamic conditions. Alkaline phosphatase activity was higher for SF coated microspheres in comparison to uncoated microspheres when dynamic culture condition was applied. The results suggest that both organic and inorganic surface modifications can be applied on PULL microspheres to prepare a biocompatible microcarrier system with suitable properties for bone tissue engineering.

1. Introduction

Regenerative medicine offers safer approaches through tissue engineering for treatment of defective or diseased tissues compared to autograft or allograft applications. Microspheres stand as novel carriers for cells, macromolecules, and drugs in regenerative medicine [1]. Cell microcarrier systems have several advantages like providing high surface area to volume ratio for maximizing cell number during cell expansion, thereby space saving for culturing and being economical when culture media and additives are considered for cell culturing [2]. Among all materials used in microsphere systems, polymers are the most commonly used and commercialized group due to their unique properties such as the biodegradability, versatility and the ease of processability as reported with dextran [3], chitosan [4] and pullulan [5], respectively. Apart from their use as carrier systems for expanding cells *in vitro*, the use of microcarrier systems, especially those made up of biodegradable materials receive increasing interest for repairing or replacing damaged and diseased tissues through the delivery of cells or microtissues developed *in vitro* [6].

In the present study, pullulan, a natural origin biodegradable polymer was used as the main component of microcarriers. PULL is a polysaccharide composed of α-(1, 6) linked maltotriose units via α-(1, 4) glycosidic bond which add unique properties to the polymer [7]. Non-toxicity, lack of immunogenicity, ease of derivatization with functional groups [8], biocompatibility and biodegradability [9] are among the properties of PULL that make it an ideal material for tissue engineering. Various studies showed that PULL, alone or together with other polymers, is used for different purposes; i) as nanoparticles of oxidized PULL with Jeffamines® crosslinking for drug delivery [10], ii) as hydrogel composite with dextran fibers [11], iii) for scaffold formation in bone tissue engineering [12], iv) for vascular wall reconstruction in the form of composites with other polymers [13].

As stated by Park et al. the composition of microcarriers, the surface properties such as chemistry and topography significantly influence the attachment and proliferation of cultured cells [14]. Besides this, the microstructure and mechanical properties are also important criteria for cell microcarriers [15]. Therefore, in the current study surface and mechanical properties of PULL microspheres were improved in two different ways; by SF coating and by biomimetic mineralization with incubation in simulated body fluid (SBF). SF is a fibrous polymer isolated

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http://dx.doi.org/10.1016/j.msec.2016.03.002
0928-4931/

from silk and it is widely used in medicine, food [16], cosmetics [17], and chemical industry [18]. As a coating material, SF was shown to support the structure of theophylline tablets used for drug delivery [19].

For bone tissue regeneration, the ideal matrix must mimic natural bone tissue where the matrix is composed of mainly collagen type I and hydroxyapatite. Apatite deposition occurs on collagen type I fibrils with the coordination of multiple proteins that act as nucleation centers for mineralization [20]. Previously, PULL/dextran/nanocrystalline hydroxyapatite particles (nHA) composite was used in the form of a scaffold, in which nHA activates early calcification and osteoid tissue formation on the defect site by mimicking the chemistry and architecture of natural bone tissue [12]. To mimic the mineral composition of bone, the biomimetic surface coating was proposed by incubating biomaterials in simulated body fluid (SBF). Biomimetic mineralization is known to enhance the osteogenic differentiation, hence, it is used as a strategy to increase the cytocompatibility of biomaterials intended for bone tissue engineering [21].

The present study compares both organic and inorganic surface modifications on PULL microspheres for increasing bone regeneration potency. Calcium carbonate was used in microsphere preparation as porogen expecting that pores and rough surface would increase cell attachment and proliferation. The SF coated and surface mineralized PULL microspheres were prepared and characterized for the first time in literature. The microspheres were characterized and optimized with respect to degradation, particle size, and structural-morphological properties. The *in vitro* osteogenic potency of the microspheres was investigated using SaoS-2 cell line. Herein, we report that these microspheres would be particularly useful as an injectable microcarrier system for bone tissue regeneration.

2. Materials and methods

2.1. Materials

Pullulan (PULL) was obtained from Hayashibara Japan; Mw: 200 000 g/mol. Sodium carbonate, sodium periodate and sodium borohydrate were from Riedel-de Haen (Germany). Dulbecco's Modified Eagle's Medium (DMEM), trypsin (0.25%), fetal bovine serum (FBS), streptomycin, trypsin-EDTA and bovine serum albumin (pH 7.0) were purchased from Biochrom (Germany). Dimethyl sulfoxide (DMSO) was supplied by AppliChem GmbH (Germany). Trisodium trimetaphosphate (STMP), sodium hydroxide and calcium carbonate were purchased from Sigma Aldrich, (USA). O-cresolphthalein complexone and 2-amino, 2-methyl, 1-propanol (AMP) were supplied from Sigma, (USA). Fluorescein isothiocyanite (FITC) and rhodamine B isothiocyanite (RBITC) Hoechst 33,258, calf thymus DNA, Na_2EDTA were obtained from Aldrich, (USA). Glass beads were purchased from NextAdvance, (USA).

2.2. PULL microsphere preparation and characterization

Preparation of microspheres was performed by water-in-oil emulsion method (Fig. 1). Briefly, PULL (15%, w/v) and STMP (7.1%, w/v) were dissolved in 2 mL distilled water at room temperature to obtain a homogenous solution. Calcium carbonate microparticles (90 mg, mean size of 20 μm) were then suspended in the mixture (the weight ratio = 0.42, $CaCO_3$/PULL) and NaOH powder (8% final concentration) was added to start crosslinking reaction. When the solution became viscous, it was added dropwise into mineral oil (50 mL) in a round-bottom flask under continuous stirring at 500 rpm with an overhead stirrer (Heidolph RZR 2051, Germany). Prior to the addition of water phase, Span 80 and Tween 80 (each 50 mg) were added as surfactants. The resultant emulsion was stirred for 4 h at RT and then microparticles were collected on a 70 μm cell strainer. They were washed on the strainer with acetone several times until oil was removed. The microspheres were then dried at RT and stored in desiccators for further use. For generating pores within the microspheres, they were treated with an excess amount of hydrochloric acid (10 mM), which dissolves and removes calcium carbonate thereby leaving porous and rough structures behind.

3 batch microsphere samples were used for particle size determination for which the mean particle size of microspheres in each sample was determined by measuring the diameter of 100 microspheres in the wet state. The diameter of microspheres was determined from light transmission microscopy images by using ImageJ software (ImageJ 1.48; National Institutes of Health, Bethesda, MD). The particle size of the microspheres was given as average microsphere size ± standard deviations (SD).

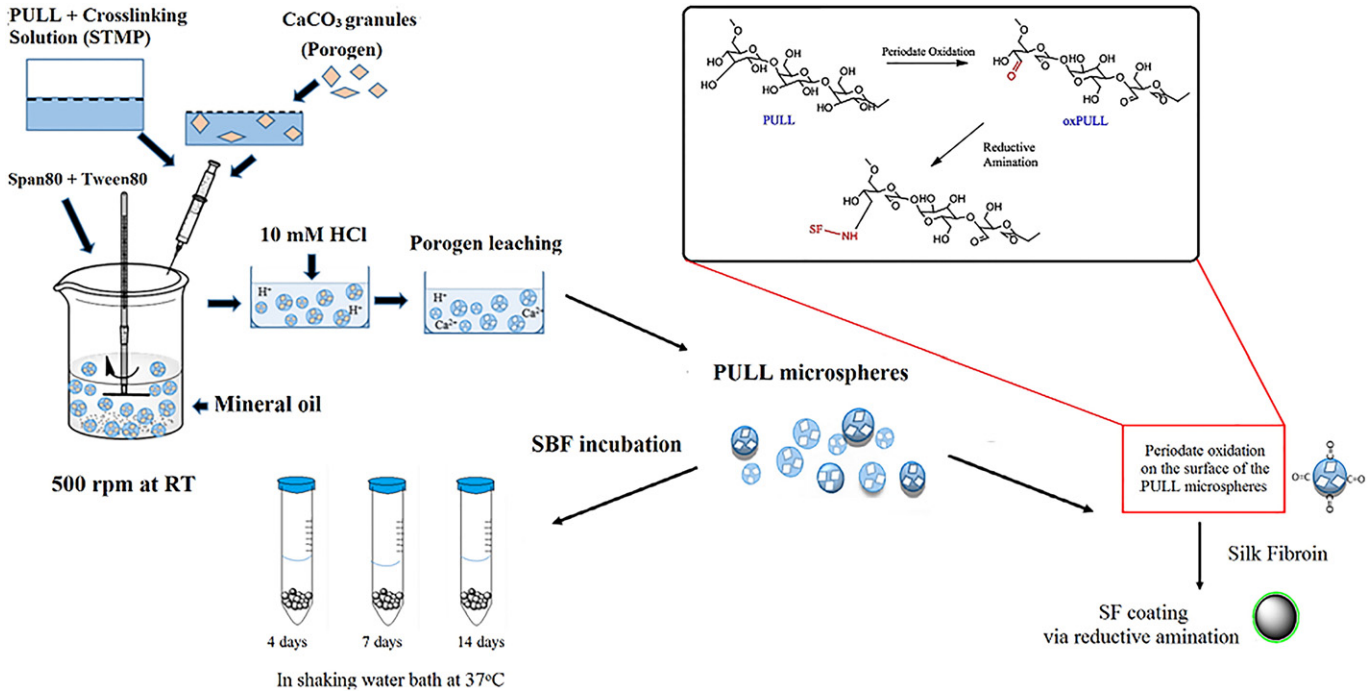

Fig. 1. Scheme showing PULL microsphere production, surface coating and biomimetic mineralization procedures.