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Characterization and osteogenic activity of a silicatein/biosilica-coated chitosan-*graft*-polycaprolactone

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ABSTRACT

Several attempts have been made in the past to fabricate hybrid materials that display the complementary properties of the polyester polycaprolactone (PCL) and the polysaccharide chitosan (CHS) for application in the field of bone regeneration and tissue engineering. However, such composites generally have no osteogenic activity per se. Here we report the synthesis of a chitosan-*graft*-polycaprolactone (CHS-*g*-PCL) and its subsequent characterization, including crystallinity, chemical structure and thermal stability. Upon surface-functionalization of CHS-*g*-PCL with osteogenic biosilica via the surface-immobilized enzyme silicatein, protein adsorption, surface morphology and wettability were assessed. Finally, the cultivation of osteoblastic SaOS-2 cells on the surface-functionalized CHS-*g*-PCL was followed by analyses of cell viability, mineral deposition and alkaline phosphatase activity. These characterizations revealed a composite that combines the versatile properties of CHS-*g*-PCL with the osteogenic activity of the silicatein/biosilica coating and, hence, represents an innovative alternative to conventionally used CHS/PCL composites for biomedical applications, where stable bone–material interfaces are required.

1. Introduction

Over the past decade, polymer-based composites have become the focus of many biomedical approaches that aim to develop suitable synthetic matrices and scaffolds for bone regeneration and replacement [1–3]. The demands on these materials are high; ideally they need to have bioactive, osteoinductive and osteoconductive potential. Bioactivity describes the property of a material to directly bond with host bone tissue via a hydroxyapatite-like layer. On the other hand, osteoinductive and osteoconductive materials respectively induce bone formation de novo in the absence of osteogenic factors and guide the synthesis of newly forming bone along their surfaces [4]. Composites for biomedical approaches often comprise chitosan (CHS) [5], polycaprolactone (PCL) [6], but also several other polymers [3]. CHS is a (partially) deacetylated derivative of the naturally occurring polysaccharide chitin and consists of β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. The polycationic character of CHS, in combination with the presence of reactive functional groups, enhances both adherence to negatively charged molecules (e.g. growth factors or glycosaminoglycan and proteoglycans of the extracellular matrix) and cell adhesion [7]. CHS is biocompatible, bioresorbable and moldable. However, due to its hydrophilicity CHS is brittle in the wet state and lacks the tensile strength required to match that of natural hard tissues [8,9]. PCL, on the other hand, is a linear polyester prepared by ring-opening polymerization of ε-caprolactone. PCL is biocompatible, biodegradable and has an excellent tensile strength. However, its application, particularly in tissue engineering, is hampered by its hydrophobicity, neutral charge distribution, slow degradation rate and reduced cell attachment/spreading [8–10]. Consequently, several attempts have been made in the past to combine the mutually complementary properties of PCL and CHS, e.g. by blending in miscible solvents [8] or in one common solvent [11], grafting [12,13] or crosslinking [14]. Indeed, the hydrophilic nature of CHS improves the wettability and permeability of PCL-containing composites and, thus, accelerates hydrolytic degradation of PCL. Conversely, PCL enhances the wet state mechanical characteristics of CHS. Moreover, distribution of hydrophobic regions (PCL) dispersed in a hydrophilic phase (CHS) promoted protein adsorption and cell attachment [9]. Nevertheless, since such composites have no osteogenic potential per se, recent attempts have concentrated on combining blends of CHS and PCL with nanoparticulate HA [10] and bioactive glass [15].

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Further efforts to synthesize advanced materials for bone repair and replacement have explored the osteogenic potential of nanoparticles that consist of biogeneously formed amorphous and hydrated silica (biosilica). Biosilica is the product of the enzymatically catalyzed polymerization of orthosilicate. The underlying catalytically active protein—silicatein—is the only known natural biosilicifying enzyme. Silicatein mediates via its catalytic triad the synthesis of reactive, cyclic silicic acid species from very low concentrations of the monomeric precursor at near-physiological conditions [16]. Biosilica has been shown to stimulate both mineralization activity and proliferation of human osteoblastic cells (SaOS-2) [17]. The resulting increased formation of hydroxyapatite (HA) has been correlated with an enhanced expression of both the osteoclastogenesis inhibitory factor osteoprotegerin and bone morphogenetic protein-2 [18,19]. Since then, silicatein and its silicate substrate have been applied in vivo in a poly(D,L-lactide)/poly(vinyl pyrrolidone)-based matrix as a prototypic implant material [20]. Concurrently, genetic modification of silicatein has extended the spectrum of possible biomedical application of biosilica: silicatein was fused to a biomimetic protein tag (Glutag) that conferred HA binding affinity and, hence, facilitated the coating of HA substrates with biosilica [21]. Finally, a new polyurethane dimethacrylate/methacryloxypropyltrimethoxysilane (PUDMA-*co*-MPS) copolymer was synthesized and functionalized with biosilica through the polycondensation activity of immobilized silicatein to obtain an osteogenic surface coating, using both soluble orthosilicate as well as silanol groups on the copolymer surface as enzyme substrate [22].

As mentioned above, a number of novel approaches have been developed for the fabrication of composites that comprise CHS and PCL, mostly by blending. However, little research has been conducted on the grafting of both polymers and the combination of the resulting composite with silica to confer osteogenic potential for enhanced cell mineralization. Accordingly, the objective of the present work was (i) to apply an uncomplicated NHS/DCC (*N*-hydroxysuccinimide/*N*,*N*′-dicyclohexylcarbodiimide)-based method for fabrication of a chitosan-*graft*-polycaprolactone (CHS-*g*-PCL) as material for potential application in the field of bone regeneration; (ii) to determine the relevant physicochemical parameters of the grafted material; (iii) to surface-functionalize the carrier with biosilica through the polycondensation activity of the immobilized enzyme silicatein; and (iv) to evaluate both the biological parameters and the osteogenic activity of the silicatein/biosilica-coated carrier in vitro by applying the SaOS-2 osteoblastic cell model.

2. Materials and methods

2.1. Chemicals

All chemicals and kits were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless stated otherwise.

2.2. Synthesis of CHS-g-PCL

The carboxylic end groups of the aliphatic polyester PCL (70–90 kDa) were chemically modified through treatment with DCC and NHS for subsequent conjugation to CHS (140–220 kDa; degree of deacetylation, 93%) by amidation. CHS (2 wt.%) was prepared by dissolving in acetic acid (2%, v/v). Then, the pH was adjusted to 5.5 with 3 M NaOH. Upon dissolution of PCL (2 wt.%) in *N*,*N*-dimethylformamide (DMF) and dichloromethane (DCM) (50:50%; v/v), 2.5 mM of both DCC and NHS were added. After 12 h of incubation at room temperature (RT), the insoluble byproduct (dicyclohexylurea) was removed by centrifugation and subsequent filtration. Moreover, to remove residual DCC and NHS, the activated PCL solution was dialyzed against 10 volumes of DMF/DCM. Finally, 50 ml of the CHS solution were added to 12.5 ml of the activated PCL solution and left stirring for 24 h (50 °C) at pH 8. The resulting gummy-like material (CHS-*g*-PCL) was washed in ethanol (30%, v/v) and isopropanol (30%, v/v), molded according to the later application (e.g. as carrier discs with a thickness of ~250 μm), and vacuum dried. For application in cell culture, the carrier discs were sterilized in ethanol (70%, v/v) for 10 min.

2.3. Chemical and physical characterization of CHS-g-PCL

The crystallinity of the CHS-*g*-PCL samples was determined by X-ray diffraction (XRD; X'pert MPD diffractometer; PANalytical, Kassel-Waldau, Germany), using Cu K_α radiation generated at 40 kV and 40 mA. The samples were scanned from 10° to 90° with a step size of 0.02° and a count rate of 3.0° min^{-1}. Crystallinity was also determined of samples that had been incubated for 4 weeks with *Pseudomonas sp*. lipase (1 mg ml^{-1} phosphate-buffered saline (PBS), with 0.05 wt.% NaN_3; pH 7.4; 37 °C) to enzymatically hydrolyze PCL ester bonds [23]. The chemical structure of the CHS-*g*-PCL sample surfaces was characterized by Fourier transform infrared spectroscopy with attenuated total reflection (FTIR-ATR; Varian 660 spectrometer with Golden Gate ATR accessory; ThermoFisher Scientific, Schwerte, Germany). Spectra were collected by averaging 36 scans in the range of 580–4000 cm^{-1} with a resolution of 4 cm^{-1}. Spectra were also collected of those samples that had been treated with *Pseudomonas sp*. lipase. Thermogravimetric (TGA) and derivative thermogravimetric (DTG) analyses were conducted in a nitrogen environment at a heating rate of 10 °C min^{-1} from RT to 800 °C (Q500; TA-Instruments, Eschborn, Germany). For static water contact angle measurements, digital images of a water droplet (5 μl) on the investigated surfaces were taken by a UK1117 camera (EHD Imaging GmbH, Damme, Germany) and analyzed by using the EMPIX Northern Eclipse software.

2.4. Silicatein immobilization and biosilica formation on CHS-g-PCL

For surface immobilization of silicatein, the CHS-*g*-PCL samples were treated with 1,6-hexamethylenediamine (HMD) to introduce amino groups via nucleophilic attack on the carbonyl groups of PCL [24]. For this reason, the material was washed in ethanol (50%, v/v) for several hours, and then incubated in HMD (10 wt.% in isopropanol) for 3 h (37 °C). Afterwards, the samples were dried in vacuum until a constant weight was obtained. Subsequently, the samples were treated with 20 mM HCl for 30 min (RT) before they were incubated with refolded recombinant silicatein (500 μg ml^{-1} in Tris–HCl; pH 7.5). At this pH the negative charge of silicatein facilitated binding to the protonated amino groups on the CHS-*g*-PCL surface [25]. After 2 h of incubation (RT), the supernatants were collected for later analysis (see below) and silicatein immobilization was confirmed through immunodetection by using a combination of silicatein-specific primary antibodies (diluted 1:1,000 in 15% blocking solution; Roche Applied Science, Mannheim, Germany) and species-specific Cy3-labeled secondary antibodies (diluted 1:2,000; Dianova, Hamburg, Germany) [26]. Then, the samples were inspected by confocal laser scanning microscopy (cLSM; Zeiss 710, Zeiss, Göttingen, Germany; see Section 2.5). Concurrently, silicatein immobilization was quantified by Bradford protein assay (Coomassie Brilliant Blue G-250) using the protein supernatants. Moreover, immobilized silicatein was visualized by staining the bound protein with EZblue and subsequent inspection of the samples with a VHX-1000 digital microscope (Keyence, Neu-Isenburg, Germany). In addition, surface morphologies of silicatein-coated and uncoated samples were investigated by atomic force microscopy (AFM; MFP-3D BIO; Asylum Research, Mannheim, Germany) in tapping mode at RT. The instrument