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Mineralization potential of cellulose-nanofibrils reinforced gelatine scaffolds for promoted calcium deposition by mesenchymal stem cells

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ABSTRACT

Cellulose-nanofibrils (CNFs) enriched gelatine (GEL) scaffolds were fabricated *in-situ* by the combined freeze-thawing process and carbodiimide crosslinking chemistry. The original- and variously surface anionised CNFs (carboxylated/CNF-COOH/, and phosphonated with 3-AminoPropylphosphoric Acid/CNF-COOH-ApA/) were used in order to tune the scaffolds' biomimetic structure towards a more intensive mineralization process. The pore size reduction (from 208 ± 35 μm to 91 ± 35 μm) after 50% *v/v* of CNFs addition to GEL was identified, while separated pore-walls' alignment *vs.* shorter, dense and elongated pores are observed when using 80% *v/v* of original-CNFs *vs.* anionised-CNFs, all of them possessed osteoid-like compressive strength (0.025–0.40 MPa) and elasticity (0.04–0.15 MPa). While randomly distributed Ca^{2+}-deficient hydroxyapatite/HAp/(*Ca*/P ≈ 1.4) aggregates were identified in the case of original-CNF prevalent scaffolds after four weeks of incubation in SBF, the more uniform and intensified deposition with HAp-like (*Ca*/P ≈ 1.69) structures were established using CNF-COOH-Apa. The growth of Mesenchymal Stem Cells (MSCs) was observed on all CNF-containing scaffolds, resulting in more extensive Ca^{2+} deposition compared to the positive control or pure GEL scaffold. Among them, the scaffold prepared with the 50% *v/v* CNF-COOH-ApA showed significantly increased mineralization kinetic as well as the capacity for bone-like patterning in bone tissue regeneration.

1. Introduction

The artificial materials developed for bone regeneration as an immunologically safer replacement of autographs and allographs is a challenging, yet highly demanding area. The osteo-inductive materials shall present an instructive, dynamic and spatially heterogeneous constellation of micro-to-nano-structural, compositional and mechanical cues for stem or progenitor cells' initiation towards osteoblastic lineage [1]. At the same time, they need to be biodegradable, enabling the host tissue to regenerate itself in a predicted and predefined way by ensuring a stable material-tissue interface through the implant resorption process [2], thus creating mechanical properties similar to the bone it is replacing [3]. The human bone is a complex composite tissue, comprised of 20–40% of organic phase (type I collagen and non-collagenous proteins), 10% of water, and 50–70% of mineral phase (of which the inorganic component is mainly calcium phosphate/CaP and hydroxyapatite/HAp/being very similar to the mineral known as $Ca_5(PO_4)_3(OH)$), and with small amounts of carbonate, magnesium, and acid phosphate [4]. The presence of non-collagenous proteins is believed to play a major role in the mineral phase formation during *in vivo* osteogenesis, where the bone sialoproteins (consisting of glutamic acid - related carboxylate and phosphoserine residues - related phosphate regions) act as an enhancer, while osteocalcin, osteopontin and chondroitin-4-sulfate act as mineralization inhibitors [5]. The understanding of their functions may help in optimization of the surface and bulk characteristics of the implant material, thus to provide a desirable response within the biological niche.

The materials used most frequently are biopolymers (collagen, gelatin, fibrin, chitosan, hyaluronic acid, alginate, *etc.*) or bioactive and osteo-conductive inorganics (HAp [6], bioactive glasses), the latter being brittle, poorly compatible to the defect site and even inflammatory [7]. A recent alternative are organic-inorganic hybrids [8], such as chitosan/demineralized bone matrix [9], collagen/$CaCO_3$ [10], poly(lactic-*co*-glycolic acid)-loaded collagen/hyaluronic acid [11], ormoglass (*Si*-containing CaP)-enriched electrospinned polycaprolactone [12], chitosan/tricalcium phosphate/alginate [13], platelet derived growth factor-enriched chitosan/tricalcium phosphate [14], beta-tricalcium phosphate granules/methylcellulose/hyaluronic acid [15], RGD (Arg-Gly-Asp)-enriched PEGDA/HAp [16], rhBMP-2-loaded RGD-modified alginate hydrogel within polycaprolactone electrospun mesh [17], *etc.* Their varieties are nanocomposites of nanoapatite/collagen/magnetite [18], bacterial-

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cellulose/carboxymethyl cellulose/HAp [19], mineralized collagen/collagen I/hyaluronic acid, *Ca*/alginate/gelatin/HAp [20], *etc*., which are highly promising candidates due to their excellent bioactivity and mechanical strength, as well as their promotion capacity for angiogenesis and vascularisation. However, insufficient inter-components` integration, non-homogenous degradation in body fluid, and even masking of the inorganic by the organic component [21], as well as its poor dispersibility into the biopolymeric matrix, are the main limitations that imply a need for further compositional and structural advancement to enrich them with more complex and *in-vivo* adapted functionalities.

The nanocellulose, above all the semi-crystalline and rod-like cellulose nanofibrils (CNFs) of up to 100 nm in diameter and few μm in length, have already gained significant attention in pharmaceutical and biomedical applications as wound healing [22], tissue engineering [23,24], cell therapy [25], drug delivery [26,27], and diagnostics [28], being related to their nanometer - sized features similar to natural collagen fibrils [24], large surface area, specific biomechanical characteristics, surface chemistry, good biocompatibility [29], and low cytotoxicity with tolerogenic potential to the immune system [30]. However, very limited studies exist on the usage of CNFs as applied biomaterial, such as a hydrogel-like cell culturing scaffold for human hepatic cell lines/HepaRG and HepG2 [25], porous nanocomposites for growth of L-929 fibroblasts, as well as scaffolds for cartilage tissue engineering/TE [31], which indeed makes their application highly promising [32]. Particularly the usage of CNFs in bone TE is still not presented, even though their fibrillar nature has been recognised as biomimetic architecture for the initiation of osteogenic differentiation [33]. The fabrication of three-dimensional porous materials having a pore size above the critical range for bone TE (*i.e*. >100 μm) and interconnecting pore network but, at the same time, good mechanical properties and slow degradation rate, is an additional challenge.

The aim of this study is to define the potential of CNFs-reinforced gelatine (GEL) based scaffolds to be used for bone TE. The developed scaffolds were evaluated related to their morphological, mechanical, and physico-chemical properties, as well as their bioactivity and biological response by means of osteogenic environment of Mesenchymal Stem Cells (MSCs). Special emphasis was given to the usage of surface pre-modified CNFs (carboxylated^{-} and phosphonated with biomimetic bearing moieties) in order to tune their surface chemistry towards a more intensive mineralization process [5], being essential scaffolds for bone tissue regeneration.

2. Experimental

2.1. Materials

Two types of cellulose nanofibrils (origin/CNF as obtained from bleached birch pulp of 20–50 nm in diameter and length of several μm, and carboxylated (*i.e*. TEMPO-oxidised)/CNF-COOH from parenchymal cellulose of 3–5 nm in diameter and length of 500–2000 nm, bearing 1.3 ± 0.1 mmol/g of carboxylic groups) were produced and supplied by Betulium Ltd., Finland. Type B bovine-skin gelatine (GEL) (pI of pH ~ 5 and Mw of 47 ± 32 kDa), fluorescein 5-isothiocyanate (FITC, isomer I), 1-ethyl-3(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid (MES), trinitrobenzenesulfonic acid (TNBS), 3-aminopropylphosphoric acid (ApA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay, were purchased from Sigma Aldrich, Germany, without further purification. Milli-Q water was used throughout all the experiments. All the chemicals were of analytical grades and used as received.

2.2. Functionalization of CNF-COOH with ApA molecules (CNF-COOH-ApA)

The ApA molecules were coupled to carboxylated-CNF under heterogeneous conditions, using carbodiimide chemistry [34]. 17.5 mg of ApA were suspended in 4 mL of Milly-Q water, and then added to 25 mL of 0.5% *w/v* of CNF-COOH dispersion in 0.05 M MES buffer, which corresponds to a 1:1 M ratio between the carboxylic groups from CNF-COOH and amino groups from ApA. The carboxylic groups of CNF-COOH were activated by addition of coupling reagents (EDC and NHS) in a 10:1 M ratio to the carboxylic groups. Two references, the first without the addition of coupling reagents and a second without the addition of ApA, were prepared in parallel. All dispersions were incubated at room temperature for 24 h under mild shaking conditions, followed by 2 days` dialyses with cellulose membrane of 1 kDa cut-off to remove all non-reacted reagents, being checked with measuring of media conductivity.

2.3. Scaffolds' preparation

Different compositions of GEL/CNF-based scaffolds were prepared by mixing 10% *w/v* of GEL solution and 2% *w/v* of CNF dispersions (CNF, CNF-COOH or CNF-COOH-ApA) in different (6/0, 5/1, 3/3, 1/5) volume ratios, being denoted as GEL6, GEL5/CNF1, GEL3/CNF3 and GEL1/CNF5, respectively; in case of using modified CNF, the respective denotation was suitably changed. 10% *w/v* of GEL solution was dissolved primarily in MES buffer (pH 5.5) under gentle stirring and temperature up to 50 °C, being followed by the addition of original or modified CNF dispersions. The EDC was added to 6 mL of respective GEL/CNF mixtures at a molar ratio of 2.5/1 with respect to the number of GEL free amino groups, and mixed with NHS in the fixed EDC/NHS molar ratio of 4/1, both dissolved in 1 mL of Milly-Q water. Immediately after mixing, dispersions were poured into Teflon Petri dishes of 50 mm and placed on a temperature-controlled (by the software programme Supercool®) Cu-plate of a self-constructed cryo-unit with a temperature set to −16 °C for 2 h to allow the cryogelation process and formation of the microporous structure, followed by an additional 24 h treatment at +4 °C in a refrigerator to achieve the crosslinking, based on our previous study [35]. For cross-linking quenching, the formed scaffolds were dialyzed against water and stored at 4–8 °C. The TNBS spectrophotometric assay using TNBS reagent was used to estimate the cross-linking degree (%), following the procedure described in our previous work [35].

2.4. Characterization

Fourier Transform Infrared Spectroscopy (FTIR) analyses were performed on freeze-dried CNF samples in order to evaluate the modification products. A Perkin–Elmer IR spectrophotometer with a golden gate Attenuated Total Reflectance (ATR) attached to a diamond crystal was used. The spectra were accumulated within 16 scans at a resolution of 4 cm^{-1} within a 4000–650 cm^{-1} range. The background air spectrum was subtracted. The spectrum 5.0.2 software programme was applied for the data acquisition analysis.

Dynamic Light Scattering (DLS) analyses were performed on both original and differently modified CNF dispersions diluted with Milly-Q water down to 0.005% *w/v*, to identify the particle sizes and ζ-potential change by using Malvern Zeta Sizer (Malvern, NanoZS, UK). The average values, together with Standard Deviations (SD) of the mean and counts were calculated from six individual measurements.

The potentiometric titration of the scaffolds containing original and differently modified CNFs was carried out to quantify the processing-dependant surface charge, as well as the CNFs' charge contribution. The titration was carried out using the dual-burette instrument Mettler Toledo T70, within an inert (N_2) atmosphere, being filled with 0.1 M HCl and 0.1 M KOH. All the solutions were prepared in deionized water with low carbonate content ($<10^{-5}$ M) achieved by boiling and cooling within an N_2 atmosphere. The titration was performed in a back and forth manner, between pH 2.5 and pH 11 (0.1 M). Different titrant volumes (0.001 to 0.25 mL) were added dynamically within 30 to 180 s periods. Blank HCl-KOH titration was carried out under the same