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# Functional biopolymer-based matrices for modulation of chronic wound enzyme activities

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ABSTRACT

Collagen, collagen/hyaluronic acid (HA) and collagen/HA/chitosan (CS) sponges loaded with epigallocatechin gallate (EGCG), catechin (CAT) and gallic acid (GA) were developed and evaluated as active chronic wound dressings. Their physico-mechanical properties, biostability, biocompatibility and ability to inhibit in vitro myeloperoxidase (MPO) and collagenase—major enzymes related with the persistent inflammation in chronic wounds—were investigated as a function of the biopolymer composition and the polyphenolic compound used. The results demonstrated that the molecular weight of HA influences significantly the bulk properties of the obtained materials: higher elastic modulus, swelling ability and biostability against collagenase were measured when HA with higher molecular weights (830 and 2000 kDa) were added to the collagen matrices. The addition of CS and the polyphenols increased further the biostability of the sponges. Preliminary in vitro tests with fibroblasts revealed that the cells were able to adhere to all sponges. Cell viability was not affected significantly by the addition of the polyphenols; however, the presence of CS or high molecular weight HA in the sponge composition was associated with lower cellular viability. Finally, all specimens containing polyphenols efficiently inhibited the MPO activity. The highest inhibition capacity was observed for EGCG ($IC_{50}$ = 15 ± 1 μM) and it was coupled to the highest extent of binding to the biopolymers (>80%) and optimal release profile from the sponges that allowed for prolonged (up to 3–5 days) effects.



## 1. Introduction

Chronic (non-healing) wounds are characterized by a persistent inflammation during which numerous mutually causative cellular events lead to overexpression of proteolytic and neutrophil-derived oxidative enzymes. For example, total matrix metalloproteinases (MMPs) activity, being predominantly collagenases, is up to 30-fold greater in chronic than in acute wound fluids [1], resulting in a protease/antiprotease imbalance and excessive breakdown of the extracellular matrix (ECM). The proteolytic damage of the tissue is further promoted by oxidation of the natural protease inhibitors with hypochlorous acid (HOCl) generated by the main neutrophil enzyme, myeloperoxidase (MPO) [2]. It is believed that bringing the counts of the deleterious chronic wound enzymes down to the levels found in acute wounds would allow healing to progress [3]. Several approaches have been proposed and different biopolymer-based dressings have been developed in order to achieve this effect. Among them, collagen—the most abundant structural component of the ECM—is largely employed in dressings to create optimal moist conditions for wound healing and absorb proteases from wound exudates [4]. In addition, collagen ensures fibroblasts adhesion and growth, leading to the formation of new granulation tissue and epithelium at the wound site [5]. Hyaluronic acid (HA), a non-sulfated glycosaminoglycan (GAG), is another important moisturizing constituent of the ECM. HA macromolecules inhibit the expression of some MMPs to a different extent, depending on their molecular weight [6]. Chitosan (CS), sharing structural similarities with naturally occurring GAGs, possesses intrinsic antimicrobial properties and accelerates early phase wound healing [7]. The assembling of collagen, HA and/or CS into composite materials has displayed advantages in tissue regeneration applications over the use of any of these biopolymers alone [8,9].

Wound dressings, on the other hand, require high stability during the extended treatment duration combined with low frequency of dressing changes as recommendable for chronic wounds treatment. This stability-at-use might be compromised by the intrinsic susceptibility of the biopolymer components to enzymatic degradation in contact with biological fluids. The application of suitable

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chemical cross-linkers, e.g. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), usually applied in combination with N-hydroxysuccinimide (NHS) to form "zero length" bonds between polymers [10], overcomes their enzymatic degradability. Moreover, the multifactorial nature of the chronic wounds does also require upgrading of the biopolymer platforms with active agents to more efficiently interfere at molecular level with the biochemical events governing the ECM breakdown. The latter can be achieved by topical application of bioactive molecules, e.g. enzyme inhibitors.

Natural polyphenolic compounds recently received a great deal of attention in medicine owing to their antioxidant, antimicrobial, anti-inflammatory and consequently wound healing promoting properties [11]. Their ability to complex with proteins and polysaccharides has been exploited for improving the biopolymers stability to enzymatic degradation [12]. Moreover, the propensity of these compounds to bind proteins presumably accounts for the fact that polyphenols were able to inhibit virtually every enzyme tested in vitro [11]. Our previous studies indicated that controlled inhibition of major chronic wound enzymes could be achieved by the application of plant polyphenolic extracts [13,14].

The current study focuses on the development of a sponge-like material that combines the intrinsic properties of the individual biopolymers—collagen, hyaluronic acid and chitosan—in a composite dressing and the optimization of the biopolymer composition of such dressing for chronic wound treatment. The obtained sponges were additionally loaded with bioactive polyphenols from *Camellia sinensis* to modulate the activity of chronic wound enzymes and further enhance the biostability of the materials. Tuning of the physico-mechanical, biological and functional properties of the composite sponges is discussed as a function of the biopolymer composition and the used bioactive agent.

## 2. Materials and methods

### 2.1. Materials

Collagen type I from bovine skin was kindly supplied by Lohmann & Rauscher International GmbH & Co. (Germany). Hyaluronic acid sodium salts of different molecular weights (Mw; 6830 and 2000 kDa) from Lifecore Biomedical (USA) and ultrapure chitosan (Mw ~50 kDa) from KitoZyme (Belgium) were used for the experiments. Collagenase from *Clostridium histolyticum* (277.50 U $mg^{-1}$ solid, one unit liberates peptides from collagen equivalent in ninhydrin colour (at 570 nm) to 1.0 μmol of leucine in 5 h at pH 7.4 and 37 °C in the presence of calcium ions), N-(3-[2-Furyl]Acryloyl)-Leu-Gly-Pro-Ala (FALGPA), green tea polyphenols (epigallocatechin gallate, catechin and gallic acid), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) and N-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich. Highly purified MPO from human leukocytes (1550 U $mg^{-1}$ solid, one unit will produce an increase in absorbance at 470 nm of 1.0 $min^{-1}$ at pH 7.0 and 25 °C, calculated from the initial rate of reaction using guaiacol substrate) was obtained from Planta Natural Products (Austria). EnzChek® kit was purchased from Life Technologies (Spain). All other reagents were of analytical grade purchased from Sigma–Aldrich and used as received.

### 2.2. Preparation of biopolymer sponges

All individual biopolymer solutions were prepared at a concentration of 1% (w/v). Collagen and CS were dissolved in 1% acetic acid, while HAs were dissolved in distilled water. The prepared solutions were mixed as follows: collagen/hyaluronic acid (CHA) in 9:1 weight ratio, and collagen/hyaluronic acid/chitosan (CHACS) in the ratio 4.5:1:4.5. The mixtures were homogenized for 20 min, poured in Petri dishes with 3 mm thickness and freeze-dried. The obtained sponges were cross-linked using 96% ethanol containing EDAC (50 mM)/NHS (10 mM) for 24 h, thoroughly washed with distilled water and freeze-dried again. CHA specimens were named after the Mw of the used HA (CHA6, CHA830, CHA2000). The CHACS specimen was prepared using HA with Mw of 830 kDa.

### 2.3. Loading of the biopolymer sponges with polyphenols

The cross-linked sponges (10 mg) were loaded with polyphenols (1 ml of 1 mM aqueous solutions of epigallocatechin gallate (EGCG), catechin (CAT) or gallic acid (GA)) by simple immersion at 4 °C for 24 h. The impregnation time was chosen according to the literature to ensure equilibrium loading of the polyphenols [15]. Thereafter the samples were freeze-dried.

### 2.4. Characterization of the cross-linked biopolymer sponges

#### 2.4.1. Fourier transform infrared spectroscopy (FTIR)

Infrared spectra of the sponges were recorded using a Perkin Elmer Spectrum 100 FT-IR spectrometer utilizing the attenuated total reflectance technique. Spectra were averaged over 75 scans.

#### 2.4.2. Tensile testing

Strip-shaped sponges (30 × 10 × 3 mm) were subjected to uniaxial tensile testing in a wet state at room temperature using a universal mechanical testing machine INSTRON 5540. After removing the excess of phosphate buffer saline (PBS, pH 7.4) by gentle contact with filter paper, the specimens were stretched at a cross-head speed of 5 mm $min^{-1}$ until failure. Five samples were tested per each experimental group.

### 2.5. Extent of collagenase adsorption and biostability of the biopolymer matrices

Collagenase binding onto the cross-linked sponges was studied in 50 mM PBS (pH 7.4, 40 mM $CaCl_2$) containing 2 μg $ml^{-1}$ collagenase incubated for 24 h at 37 °C. Thereafter, the supernatants were collected and analysed for the remaining collagenase activity using EnzChek®. Briefly, the remaining in the supernatants collagenase was left to cleave a gelatin–fluorescein conjugate in darkness at room temperature for 6 h, after which the fluorescence was measured (495/515 nm) using a microplate reader Infinite M200, Tecan (Austria). The results are expressed as the percentage of collagenase binding onto the materials ($n = 5$), inversely proportional to the enzymatic activity in the supernatant.

Biodegradation tests were carried out in vitro by incubation of the sponges in 50 mM Tris–HCl (pH 7.4, with 40 mM $CaCl_2$) containing 10 or 60 μg $ml^{-1}$ collagenase at 37 °C for 24 h. After complete hydrolysis of the supernatants in 6 N HCl for 24 h at 90 °C and neutralization with 6 N NaOH, the hydroxyproline (Hyp) content was determined using the method previously described by Reddy and Enwemeka [16]. The calculation was based on the assumption that collagen contains ~14% Hyp [17]. Five samples were tested per each experimental group.

### 2.6. Polyphenols binding onto the biopolymer sponges and release studies

Polyphenols uptake by the sponges was estimated by comparison of the total phenol content in solution before and after incubation with sponges ($n = 3$) using the Folin–Ciocalteu spectrophotometric method [18]. The calibration curves were prepared using different concentrations of polyphenols. The experiments for the cumulative release of polyphenols were started by