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Sterilization of collagen scaffolds designed for peripheral nerve regeneration: Effect on microstructure, degradation and cellular colonization

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ABSTRACT

In this study we investigated the impact of three different sterilization methods, dry heat (DHS), ethylene oxide (EtO) and electron beam radiation (β), on the properties of cylindrical collagen scaffolds with longitudinally oriented pore channels, specifically designed for peripheral nerve regeneration. Scanning electron microscopy, mechanical testing, quantification of primary amines, differential scanning calorimetry and enzymatic degradation were performed to analyze possible structural and chemical changes induced by the sterilization. Moreover, *in vitro* proliferation and infiltration of the rat Schwann cell line RSC96 within the scaffolds was evaluated, up to 10 days of culture. No major differences in morphology and compressive stiffness were observed among scaffolds sterilized by the different methods, as all samples showed approximately the same structure and stiffness as the unsterilized control. Proliferation, infiltration, distribution and morphology of RSC96 cells within the scaffolds were also comparable throughout the duration of the cell culture study, regardless of the sterilization treatment. However, we found a slight increase of chemical crosslinking upon sterilization (EtO < DHS < β), together with an enhanced resistance to denaturation of the EtO treated scaffolds and a significantly accelerated enzymatic degradation of the β sterilized scaffolds. The results demonstrated that β irradiation impaired the scaffold properties to a greater extent, whereas EtO exposure appeared as the most suitable method for the sterilization of the proposed scaffolds.

1. Introduction

Various anisotropic structures, with longitudinally oriented pores or fibers, show the potential to improve peripheral nerve regeneration, by providing prompt contact guidance to Schwann cells and axons [1–5]. This is particularly important in cases of large nerve gaps (>3 cm), where cell migration within the defect, even in the presence of bridging tubular conduits, may be significantly impaired, thus delaying axon regrowth and compromising a time-effective reconnection of the nerve stumps. Chronic denervation of the distal end, resulting in the degeneration of the distal endoneurial tubes, usually occurs within 18–24 months after injury and must be avoided to restore functional nerve connections.

Collagen-based scaffolds, either in the form of tubular conduits or aligned templates, have been shown to be promising substrates for nerve regeneration in several preclinical models, due to the excellent biocompatibility and cell-adhesive nature of collagen [6,7]. While aseptic laboratory processing and/or common disinfection methods (*e.g.* 70% ethyl or isopropyl alcohol) are considered acceptable for preclinical investigations, translation of collagen scaffolds into clinical use requires a careful selection of an appropriate sterilization method [8]. International standards on medical devices (EN 556 – "Sterilization of medical devices") require a sterility assurance level (SAL) of 10^{-6}, meaning that the probability of device contamination must be less than one in a million. In order to comply with such a high degree of sterility, terminal sterilization is the preferred method over aseptic processing, due to enhanced repeatability and intrinsically higher assurance of microbiological safety [9]. Common sterilization processes adopted for medical devices, such as moist or dry heat, irradiation and ethylene oxide infiltration, inactivate microbial and viral contaminants by disrupting or denaturing their proteins, enzymes and nucleic acids [10]. Terminal sterilization of collagen-based devices can thus lead to simultaneous partial collagen denaturation, crosslinking and/or chain scission, thus potentially compromising the biological functionality and ultimately the clinical performance of the device. This is why failure to carefully address the issue of sterilization early enough in product development can hinder an effective and fast translation into clinical use. In general, the selection of a suitable sterilization method should be based on its safety and impact on the material properties, in relation to the intended

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application [10], while the specific parameters of sterilization (*e.g.* radiation dose) should be set for each type of product depending on its bioburden [11]. Other aspects like scalability, cost and duration may be taken into account in the choice of the optimal sterilization method.

With the ultimate aim of identifying a suitable sterilization method for linearly oriented collagen scaffolds specifically designed for peripheral nerve regeneration, in this work we analyzed the effect of three different sterilization processes, among those acceptable for the sterilization of medical devices, on the morphological, mechanical, chemical and biological properties of the scaffolds. Specifically, dry heat (DHS), ethylene oxide (EtO) and β irradiation (β) treatments were selected for the comparative analysis due to their potential suitability for protein-based materials. Dry heat sterilization, performed at a temperature of 160 °C for 2 h to kill resistant spores, represents a feasible and straightforward option for collagen-based devices, unlike moist heat which would completely denature the protein [6]. However, both partial denaturation and dehydrothermal crosslinking take place during the treatment, thus requiring the examination of the impact of such changes [12,13]. In this study, DHS was considered a "laboratory only" reference sterilization treatment because it requires minimal device packaging for proper heat transfer and is less cost effective, making it less attractive for large-scale, industrial applications. Conversely, ethylene oxide (EtO) and β irradiation are common methods of choice for medical devices [10,14–16]. EtO is an exceptional sterilizing agent with bactericidal, sporicidal and virucidal activity even at low temperatures, which ensures high flexibility of the sterilization process [15,17–20]. However, EtO is known to react with the amine groups of lysine and hydroxylysine in the collagen molecules, thus affecting the triple helix stability, altering the degradation rate and potentially compromising the collagen bioactivity [10,21]. Moreover, extensive degassing and aeration are needed to reduce the content of toxic EtO residue in the final product [10,15]. Similar to γ irradiation, electron beam or β irradiation can modify the collagen structure by chain scission and/or crosslinking, generally leading to increased degradation rate [14,19]. Nonetheless, structural damage induced by β irradiation is generally inferior to the one caused by γ irradiation due to its higher dose rates (typically kGy/s *vs.* kGy/h), which lead to short exposure times and thus rapid dissipation of radiation by-products (*e.g.* free radicals) [14,19].

In this study, we evaluated the structural and chemical variations induced in linearly oriented collagen-based scaffolds by DHS, EtO and β sterilizations, through multiple analyses including scanning electron microscopy, mechanical tests, differential scanning calorimetry, free amine quantification and enzymatic degradation. Furthermore, with specific focus on the intended application for peripheral nerve regeneration, the *in vitro* interaction of the rat Schwann cell line RSC96 with the sterilized scaffolds was assessed, by monitoring the cellular proliferation and infiltration up to 10 days in culture.

2. Materials and methods

2.1. Materials

Type I collagen derived from calfskin and approved for clinical use was purchased from Symatese Biomateriaux (Chaponost, France). All other chemicals and cell culture media were obtained from Sigma-Aldrich (Milan, Italy) unless otherwise stated, and used as received.

2.2. Scaffold fabrication

Collagen scaffolds with nearly axially oriented pores were prepared *via* unidirectional freezing of a collagen suspension followed by freeze-drying, according to a protocol described in the literature with slight modifications [22,23]. Briefly, dry collagen flakes were suspended in distilled water (2% w/v) and kept for 5 h under mild agitation. While mixing, the collagen suspension was refrigerated on ice to avoid heating and consequent denaturation of the collagen fibers. The slurry was then centrifuged at 6000 rpm for 10 min to remove air bubbles and injected into PVC plastic tubes (3 mm diameter, 30 mm length). The tubes were sealed at one end with Parafilm® and placed in stand-up position onto a copper plate that was put on the shelf of a freeze-dryer (Virtis Advantage), precooled to −40 °C. After completely freezing, the collagen suspension was sublimated under vacuum (<100 mTorr) for 17 h at a temperature of 0 °C, in order to obtain cylindrical porous scaffolds with longitudinally aligned pore channels [22,23].

2.3. Scaffold crosslinking

With the aim of enhancing the handling properties and the mechanical stiffness of the collagen scaffolds, as well as their resistance against enzymatic degradation, two consecutive crosslinking treatments were performed [12,24]. Firstly, right after freeze-drying the soft collagen matrices were carefully extracted from the PVC tubes, placed into aluminum foil envelopes and dehydrothermally (DHT) crosslinked at 120 °C for 24 h ($p < 100$ mTorr). This treatment is well known to slightly crosslink the collagen, without affecting the scaffold pore structure [22,24–26]. Then, additional crosslinking was performed by soaking preliminarily hydrated DHT treated scaffolds in a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS), at a molar ratio of 5:2 (EDAC:NHS) for 2 h, according to a protocol reported in the literature [27]. After extensive rinsing with distilled water, the doubly crosslinked scaffolds (DHT + EDAC) were finally re-lyophilized.

Due to the porosity gradient typically achieved by uniaxial freezing along the length of the scaffolds [22], only the central region of the specimens was considered for further investigation. More precisely, a 10-mm long cylinder was isolated from each scaffold by means of two transverse cuts performed at distances of approximately 10 mm from the extremities.

2.4. Scaffold sterilization

Crosslinked collagen scaffolds, obtained as described above, were sterilized by one of the following methods: dry heat sterilization (DHS), EtO infiltration (EtO) and β irradiation.

DHS was performed in house by placing the scaffolds in a vacuum oven at 160 °C for 2 h. Similar to DHT crosslinking, aluminum foil was used for scaffold packaging.

Conversely, EtO and β treatments were commercially outsourced (Bioster SpA, Seriate-BG, Italy), and performed in accordance with EN ISO 11135 and EN ISO 11137, respectively. For both sterilization processes, the scaffolds were placed in 35 mm Petri dishes and packaged in self-sealing sterilization pouches. Ethylene oxide sterilization was performed by exposure to an EtO atmosphere (concentration of 321 g/m^3), at a relative humidity of 65%, for 8 h at 50 °C. The scaffolds were then aerated with warm airflow (46 °C) at atmospheric pressure for 48 h to remove toxic EtO. Post-sterilization analysis performed by Bioster SpA showed that the residual amount of EtO in the scaffolds was lower than 1 $\mu g/cm^2$. With regard to β sterilization, scaffolds were electron-beam irradiated at a nominal dose of 20 kGy, using a 10 MeV energy source.

Where appropriate, non-sterilized samples were used as a reference, to evaluate the changes induced by each of the sterilization treatments under investigation.

2.5. Pore analysis

Scanning electron microscopy (SEM) was performed on the collagen scaffolds ($n = 4$ for each scaffold type), with the double aim of assessing the average pore size and orientation achieved by the scaffold processing, before and after EDAC crosslinking, and verifying possible microstructural changes ascribable to the sterilization methods. Longitudinal and transverse sections, the latter corresponding to the ends of the