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Selecting the correct scaffold model for assessing of the dielectric response of collagen-based biomaterials

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ABSTRACT

Fish collagen (Col) was cross-linked using two methods: dehydrothermal treatment (DHT) and 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in the presence of *N*-hydroxy-succinimide (NHS). For the samples marked Col, Col-DHT and Col-EDC/NHS, dielectric properties were measured in the frequency range from 100 Hz to 100 kHz and temperatures from 25 to 145 °C. In the full temperature range, the average values of relative permittivity and dielectric losses for Col samples are lower than those recorded for Col-DHT and Col-EDC/NHS samples. The peak temperature of the dielectric parameters attributed to the denaturation temperature for Col, Col-DHT and Col-EDC/NHS, respectively, is about 75 °C, 83 °C and 89 °C. In addition, the values of these parameters are much higher in Col-EDC/NHS than in Col-DHT at the same temperature and frequency. The permittivity decrement and conductivity increment, respectively, for Col-EDC/NHS are about 62 and 32 times greater than those given by Col-DHT, which is a consequence of the EDC/NHS crosslinking action. Our electrical and dielectric studies of fish Col cross-linked by EDC/NHS or DHT provide deeper insight into the structure of collagen materials and help improve the synthesis of Col-based scaffolds for tissue engineering.

1. Introduction

Over the years, numerous studies have demonstrated that collagen-based scaffolds are known to play an important role in the biomedical application, particularly in tissue engineering of the culture of different cell types [1–8]. Collagen (Col), mostly Col type I being the principal structural protein in the extracellular matrix (ECM) of all vertebrates has been commonly used as biomaterial due to its biocompatibility, biodegradability and low immunogenicity. However, limited work on Col scaffolds for neuronal cultures has been carried out to date [9,10]. In this respect, recently we created three-dimensional (3D) materials consisting of Col alone or collagen-chondroitin sulfate (CS)-hyaluronic acid (HA) hybrid scaffolds to serve as the template and protective biomimetic niche for neurally committed stem cells [11–15]. In these works, the emphasis is placed on the studies of scaffolds consisting of Col derived from mammals. In subsequent studies, Col from alternative sources such as marine animals has gained our attention [16,17].

Fish Col compared to mammalian Col shows lower susceptibility to infections (BSE, TSE), is easily accessible at a reasonable cost and more acceptable across cultures and religions. It is known that reconstituted Col material is unstable and requires modification. To retard its biodegradation, immunotoxicity and improve the mechanical strength and thermal denaturation, physical methods (high-energy radiation, UV, and dehydrothermal treatment) or chemical crosslinking agents including water-soluble carbodiimide, glutaraldehyde, and other aldehydes, can be used [4,16,17]. Such scaffolding should mimic the properties and structure of the tissue or organ that it is intended to replace and essentially acts as an artificial ECM that aids stem cell survival and differentiation in neuronal connections. The physicochemical characteristics of the scaffold such as wettability, flexibility, mechanical stiffness, microarchitecture and electrical conductivity significantly influence the adhesion, migration, proliferation, and differentiation of seeded cells.

Literature data [18,19] suggests that the use of electroactive materials in scaffolds can evoke desirable cellular responses, especially from cells belonging to electrically excitable tissues such as skeletal muscle, nerve, cardiac tissue, and bone. Col is distinguished by the fact that each macromolecule has a coiled coil structure with three polypeptide chains, two α1 (1) and one α2 (1) chain, bound together to form a right-handed triple helical structure. The ordered triple helical structure of Col is stabilized by both intra-chain hydrogen bond and by the structural water molecules [20]. Furthermore, Col molecules have a polar orientation directed from the amino (N)-terminus towards the carboxyl (C)-terminus and exhibit dielectric and piezoelectric

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properties [21–24]. Previously, dielectric techniques have been used to study the structural characteristics of collagen-based biomaterials such as electroactive polypyrrole-collagen scaffold [25], collagen-guar gum and collagen-locust bean gum composites [26,27]. Dielectric spectroscopy was also used to study the effect of water on the molecular reorientation of collagen treated with two different classes of ionic liquids, bis-choline sulfate (CS) and 1-butyl-3-methyl imidazolium dimethyl phosphate (IDP) [28]. The optimization of the electrical stimulation process was necessary to prevent loss of human mesenchymal stem cells (hMSC) viability and influence on the differentiation into neuronal or muscular lineages. Electrical stimulation of cardiomyocytes seeded on collagen-Matrigel™ scaffolds induced their alignment and coupling, leading to synchronous contractions [29]. However, significant areas of this problem, especially the electrical conductivity of fish Col have not yet been sufficiently explained.

Under this perspective, in previous work, we focused on studies of the influence of electric field frequency and temperature on dielectric properties of fish Col crosslinked by glutaraldehyde (GA) [30]. The purpose of the present work is to compare two different methods of crosslinking: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in the presence of *N*-hydroxy-succinimide (NHS) and dehydrothermal treatment (DHT) on the dielectric response of stabilized thin films of native fish Col.

2. Materials and methods

2.1. Preparation of collagen films

Collagen type I in acetic acid solution from the fresh skin of silver carp *(Hypophthalmichtyys molitrix)* was used. The concentration of Col in the dispersion was analyzed spectrophotometrically for hydroxyproline (Pro-OH) content according to the PN-ISO 3496:2000 Standard.

In the present study, three types of Col films have been prepared: (i) Col thin film without any modification, (ii) Col thin film crosslinked by EDC with NHS, and (iii) Col film treated with DHT. Col films were prepared by casting of the dispersion at concentration 0.9% (w/v) onto glass plates and dehydrated by drying under a laminar flow at room temperature. Afterward, air dried Col films were dehydrothermally modified by heating them at 80 °C for 48 h under a vacuum of 0.05 bar. Other Col films were crosslinked using the mixture of water soluble EDC (Sigma-Aldrich, USA) with NHS (Sigma-Aldrich, USA) according to the method [16,31]. Briefly, Col films were immersed in an 80% ethanol containing 33 mM EDC and 6 mM NHS at pH = 5.5 for 4 h at room temperature. Thereafter, the matrices were washed in 0.1 M Na_2HPO_4 and then with distilled water. Finally, the Col crosslinked films were dehydrated again by slow drying under a laminar flow hood. Thus prepared thin films were submitted to dielectric studies.

2.2. Insight in some physicochemical properties of collagen films

Comprehensive biochemical, spectroscopic, morphological and structural properties of Col-EDC/NHS and Col-DHT samples were examined and evaluated and can be found in our earlier publication [16]. Col films without any further treatment were taken as reference materials. Some results of the physicochemical characterization of Col films are summarized in Table 1. Nonetheless, in the present work, compared to [16], we slightly changed the conditions for modifying Col film (Col and ethyl alcohol concentration, dehydration parameters). It needs to be highlighted that only subtle changes in the parameters of physicochemical properties of these samples were recorded compared to those given in Table 1. This may be due to several reasons. Briefly, T_d strongly depends primarily on the degree of Col crosslinking and water content and increases with the decrease in water content. For example, it has been reported that for Col type I in water solution T_d = 38 °C, for Col films after drying under a laminar flow at room temperature T_d = 77 °C but for Col sponge in the freeze-dried state T_d = 120 °C [16,32]. Moreover, it was found that Col films dehydrated at 105 °C for 24 h under a vacuum of 0.05 bar showed the similar degree of crosslinking as the samples being treated at 80 °C for 48 h under a vacuum of 0.05 bar [7,16]. Importantly, ethyl alcohol did not denature the helical structure of the Col and prevented the hydrolysis of EDC. But, Nam et al. [33] reports and discusses that there are a number of research papers on the reaction of EDC/NHS with collagen in ethanol but it is not completely clear as to how the EDC and NHS coupling reaction would be affected when the alcohol percentage in aqueous conditions changes; hence, different ethanol concentrations from 40% to 80% are being used without characterization of the coupling rate. Taking the above factors into consideration, the values of the relevant parameters given in Table 1 are taken into account in this work.

Table 1
Characteristics of some physicochemical properties of collagen film [16].

Parameter	Col non-crosslinked	Col crosslinked by EDC/NHS	Col crosslinked by DHT
Temperature denaturation [°C]	77.0	88.0	80.0
Degree of crosslinking [%]	no crosslinking	53 ± 2	26 ± 3
Porosity [%]	75.0	65.2	68.3
Water contact angle [degree]	60.5 ± 1.0	67.2 ± 1.0	62.1 ± 1.0

2.3. Analysis of Col-DHT and Col-EDC/NHS films

The dielectric properties of the Col, Col-DHT and Col-EDC/NHS films as a function of the frequency of an applied voltage across the electrodes and temperature were obtained by measuring the electrical resistance (R) and capacitance (C) of the total system [34], which is related to the electrode and the bulk materials. Measurements of the R and C were carried out using an impedance analyzer HIOKI 3522-50 LCR in the temperature, T, from 25 to 145 °C. The AC voltage of 1 V (RMS) in frequency, f, the range of 100 Hz–100 kHz, was applied to a sample placed between two silver paste electrodes with an area of $S = 20\ mm^2$ and spaced apart by $d = 0.02$ mm. These experiments were performed in the wet and dry states of the sample in an air atmosphere with the use of the measuring chamber, described in the previous paper [35]. The wet and dry states concerned respectively the same samples, air-dried at room temperature with a relative humidity of 65–70% RH and devoid of loosely bound water absorbed from the environment. Therefore, dielectric measurements include a procedure for heating a wet sample in the range of 25–145 °C (~2 h) and at a constant temperature of 145 °C (~1 h), and then after rapidly cooling it to room temperature, the heating procedure of the already dry sample as a function of temperature (~2 h) to 145 °C. These measurements carried out on the same number of samples (n = 10), taken from the Col, Col-DHT and Col-EDC/NHS group thus, on thirty samples in total. In this study, as in our previous work of the fish Col crosslinked by glutaraldehyde (GA) [30], we measured the water content in wet samples of Col, DH-DHT and Col-EDC/NHS without electrodes. After the water removal procedure, the average water content in the same number of samples (n = 3) from the Col, Col-DHT and Col-EDC/NHS groups was 14, 16 and 19%, respectively. The values of the relative permittivity, dielectric loss and conductivity of these samples were calculated from $\varepsilon' = Cd/\varepsilon_o S$, $\varepsilon'' = d/\omega\varepsilon_o RS$ and $\sigma = \omega\varepsilon_o\varepsilon''$, respectively, where ε_o is the permittivity of a vacuum ($\varepsilon_o = 8.854$ pF/m), and ω is the angular frequency ($\omega = 2\pi f$).

3. Results

Fig. 1(a and b) show the temperature dependence of the logarithm