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Amino and carboxyl plasma functionalization of collagen films for tissue engineering applications

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ABSTRACT

Type I collagen films have been functionalized on their surfaces by plasma treatment with carboxyl and amino groups to improve their potential for grafting bioactive molecules. The physico-chemical properties of the plasma-treated films were evaluated and compared to the untreated materials by water contact angle, SEM and AFM. The presence of new functional groups on the film surfaces has been assessed by ATR-FTIR spectra after chemical derivatization. Moreover, the biocompatibility of the plasma-treated films was studied with MG-63 human osteoblast-like cells, evaluating cell proliferation, viability and morphology at 1, 3 and 7 days.

1. Introduction

The term collagen usually refers to a group of well-known proteins widespread in vertebrates, representing the chief structural protein accounting for approximately 30% of all vertebrate body protein. More than 90% of all extracellular proteins in the tendon, cartilage and bone, and more than 50% in the skin, consist of collagen [1,2]. Collagen and its architectural arrangement [3,4] in connective tissue drives prominent biological roles, such as mechanical strength [5] and activation of the blood clotting cascade [6].

Collagen is nowadays widely used as a biomaterial for several tissue engineering applications [4,7] due to its biocompatibility [8–10], to the expanding medical applications of biomaterials and connective tissue research, together with the effective production of medical-grade collagen, that allows collagen products to be successfully placed on the market. In addition, collagen is particularly suitable as biomaterial for tissue engineering since it can be prepared in a number of different forms including strips, sheets, sponges, hydrogels and beads depending on the final applications [11,12]. Nevertheless, the lack of mechanical resistance of highly porous, non-mineralized collagen matrices limits their application as stress-bearing scaffolds for hard-tissues repair [13,14]. Many efforts are devoted to the improvement of the physical, chemical and biological features of collagen devices. Several procedures have been proposed for the preparation of collagen matrices with improved mechanical properties for bone and cartilage repair applications, such as chemical glycation [15,16], cross-linking by chemical [10] or enzymatic [17] reactions, or even by intrafibrillar biosilicification [18].

Another key issue in the development of collagen scaffolds is the improvement of its biomimetic properties [19–21]; the development of collagen based functional materials has recently gained increasing interest, since such materials could be useful as scaffolds for promoting cell growth, for accelerating the wound healing process or, for example, for the development of artificial skin or for cartilage repair [22,23]. Toward this aim collagen has been engineered for example by adding other proteins, such as elastin, fibronectin or glycosaminoglycans or combining it with other molecules [20,21,24–26], such as liposomes [27] to enhance the mechanical strength of the medical devices.

Thus, the synthesis of collagen scaffolds that can be easily functionalized with a wide variety of functional groups for further bioconjugation reactions [28–32], allowing at the same time the formation of stable collagen triple helices is a relevant research area. Moreover, surface modification of the scaffolds can enhance the surface material performance in a cell environment, modifying surface properties such as hydrophylicity, cell adhesion or cell selectivity.

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In this respect, plasma treatment is a promising technique for the surface modification of polymeric materials and improvement of biomaterial biocompatibility [33,34]. In the last years, plasma treatment has been gaining popularity as a versatile and biocompatible technique for surface modification of materials [35–39]. Plasma treatments can be used for the introduction of different functionalities, including COOH and OH functionalities through air [40–43], O_2 [44] or inert gases (Ar, He) plasmas [40,45] (in the latter case, it is the post-exposure to air of the treated materials that leads to the introduction of oxygen-containing functionalities) and amines (through, for example, NH_3 plasmas). The use of plasma modification techniques offers several major advantages over the conventional chemical methods; first of all, plasma modifies the surface of the materials without affecting their morphology or their physical and chemical bulk properties. Secondly, plasma does not alter the mechanical properties of the outer layer of an implant, as observed with other techniques: for example, wet chemical treatment of a surface will cause a partial degradation of the polymer chains at the surface, leading to a decrease of the mechanical strength and to a faster degradation. Third, plasma treatments enable to uniformly modify the materials surfaces, regardless of the geometry, and can be used on complex objects. Finally, the use of hazardous solvents and/or chemicals can be avoided, rendering the material compatible with biological systems. An extremely wide range of surface modifications can be realized with different low-pressure plasmas. Hence, plasma surface modification has been employed in many technological fields, such as lubrication surfaces, bio-absorbable polymer, biocompatibility enhancement, bone internal fixation devices, diagnostic biosensors [46–51].

Despite the great number of examples on plasma modification techniques, to the best of our knowledge, only few reports have appeared to date on plasma modification of surfaces of collagen-based biopolymers [52,53], while several examples concern the surface plasma treatment of materials of different chemical nature, followed by collagen immobilization [54–56].

We report thereby the use of plasma techniques for the functionalization of CollageneType I films with CO_2 and N_2/H_2 plasmas, which are known to introduce respectively carboxylic and amino groups on the surface of polymeric materials. We investigated the physicochemical features of the modified surfaces as well as their in vitro biocompatibility in the presence of human osteoblast-like cells.

2. Materials and methods

2.1. General methods

Collagen type I from equine tendon was purchased from OPOCRIN S.p.a.; all other chemicals were purchased from Sigma–Aldrich and used without further purification. All solvents were dried over molecular sieves, for at least 24 h prior to use.

2.2. Collagen membrane preparation

Type I collagen films from equine tendon (1% collagen gel in acetic acid) were produced by a solvent-casting method as previously described [57]. Briefly, the 1% gel was diluted 1:6 w/v in ultrapure water. The suspension was homogenized at 4 °C with a mixer for 2 min at maximum speed. After removal of the aggregates, 80 mL of collagen solution was poured into a 35-mm diameter culture dish and the solvent evaporated in the fume hood for two days.

2.3. Plasma treatment

Plasma treatments of the collagen films with CO_2 and N_2/H_2 plasmas were performed in a capacitively coupled radiofrequency (RF) reactor, constituted by a cylindrical glass chamber (inner diameter 10 cm, length 30 cm), closed at each end with stainless steel flanges [58,59].

An RF power supplier was connected through a matching network to a copper ring placed around the cylindrical chamber. Two other copper rings, placed apart from the RF antenna, were grounded. Prior to depositions, the reactor was evacuated up to 10^{-1} Pa by a rotary pump. After this step, carbon dioxide (for the insertion of carboxylic groups) or nitrogen/hydrogen mixture (for the insertion of amino groups) were introduced in the chamber.

The nitrogen flow was measured directly through a flow meter (EL-Flow series F-201C by Bronkhorst) while hydrogen was introduced through a needle valve. In order to maximize the surface density of inserted amino groups, these plasma treatments were performed with a N_2:H_2 partial pressure ratio of 1:2 (P_{N2} = 13 Pa, P_{tot} = 40 Pa, power input 20 W, exposure time 4 min) [60–62]. Instead, CO_2 plasma treatments were performed at a gas pressure of 20 Pa (power input 20 W, exposure time 4 min).

After the plasma treatments, the collagen samples were kept for 10 min in the CO_2 or N_2/H_2 atmosphere and then recovered for further studies.

2.4. Water angle contact

Hydrophilic characteristics of the treated and untreated collagen films were evaluated by static contact angle (WCA) measurements, using a Data Physics OCA 20 instrument. Acid–base properties of the modified surfaces were investigated by means of contact angle measurements with aqueous solutions at different pH [63].

For this purpose, acidic and basic aqueous solutions (pH ranging from 1 to 13) were prepared by addition of appropriate amounts of HCl and NaOH to ultrapure water. Contact angle measurements were performed immediately after the plasma treatments. For every plasma treatment and aqueous solution, at least four contact angle measurements were performed.

2.5. AFM analysis

AFM measurements were performed on plasma treated collagen films cut in square size (0.5 × 0.5 cm). AFM images were collected in tapping™ mode by a MultiModeNanoscope V (Bruker) using single-beam silicon cantilever probes (Veeco RTESP: resonance frequency 300 kHz, nominal tip radius of curvature 10 nm, force constant 40 N/m). Data sets were subjected to a first-order flattening. Roughness (Ra) of the surfaces was calculated by Gwyddion 2.28 software.

2.6. Attenuated total reflectance-infrared (ATR-IR) analysis

ATR-IR absorption spectra were recorded at RT in the range 800–2000 cm^{-1} with a micro-FTIR Nicolet iN10, equipped with a micro-ATR germanium tip, under nitrogen flux, with a spectral resolution of 2 cm^{-1} and 256 accumulations. After baseline subtraction, spectra were normalized to unity with respect to the Amide I band at 1630 cm^{-1}.

2.7. Wet chemical derivatization

Chemical derivatization was carried out in glass Petri dishes. All the samples were first washed in ultrapure water and dried at room temperature.

For the ATR-FTIR analysis: the N_2/H_2 plasma-treated films were reacted twice with 2,2,2-trifluoroacetic anhydride (TFAA), using a pre-mixed solution of TFAA (0.5 M) and N,N-diisopropylethylammine (DIPEA, 0.5 M) in dry tetrahydrofuran (THF) for 30 min each