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Ultra thin hydro-films based on lactose-crosslinked fish gelatin for wound healing applications

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ABSTRACT

This study focuses on the development and characterization of an ultra thin hydro-film based on lactose-mediated crosslinking of fish gelatin by Maillard reaction. Lactose results in the only efficient crosslinker able to produce resistant to handling hydro-films when compared to conventional crosslinkers such as glutaraldehyde or genipin (tested at 25 and 37 °C in phosphate buffer saline solution (PBS)).The disappearance of the peak related to the N-containing groups (XPS) and the images obtained by SEM and AFM demonstrate the highly ordered nano-scaled structure of lactose-crosslinked gelatin, confirming the crosslinking efficiency. This dressing presents high hydrophilicity and mild occlusivity, as shown by the swelling curve (max swelling at 5 min) and by the occlusion factor of 25.17 ± 0.99%, respectively. It demonstrates high stability to hydrolysis or cell-mediated degradation. Moreover, ISO 10993-5:2009 biocompatibility assay results in undetectable cytotoxicity effects. Spreading, adhesion and proliferation assays confirm the excellent adaptability of the cells onto the hydro-film surface without invading the dressing. Finally, the hydro-film enables the controlled delivery of therapeutic factors, such as the epidermal growth factor (EGF). This study demonstrates that lactose-mediated crosslinking is able to produce ultra thin gelatin hydro-films with suitable properties for biomedical applications, such as wound healing.

1. Introduction

In the last ten years, hydrogels composed on natural-origin proteins (e.g. fibrin and collagen) have been widely studied with the aim of developing new biomaterials imitating as much as possible the extracellular matrix (ECM) (Holzapfel et al., 2013). On the one hand, hydrogels are considered excellent biomedical systems for their three-dimensional imitating ECM framework, for their swelling behavior (Feng et al., 2016) and for their response to different environmental stimuli, such as temperature or pH, which allow the releasing of loaded drug in a controlled manner (Raafat, 2010). On the other hand, the use of natural proteins is essential since synthetic hydrogels do not possess the intrinsic ability to support tissue organization (Lim et al., 2013).

Among other natural proteins, gelatin can be considered an ideal candidate for hydrogel formation and a highly valuable material for biomedical applications owing to its multiple advantages (Malafaya et al., 2007; Santoro et al., 2014). Firstly, it is relatively inexpensive, with an excellent film forming capacity. Secondly, it has been recognized as a "generally recognized as safe" (GRAS) material by the FDA, with excellent biocompatibility and biodegradability. Thirdly, as a denatured protein, gelatin is much less antigenic than others such as collagen. Moreover, the gelatin chains contain arginine-glycine-aspartic (RGD) motifs, known to be important in promoting cell adhesion, thereby improving the biological behavior over polymers that lack these adhesion

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sequences. Lastly, it possesses diverse functional groups for chemical modifications, such as coupling with crosslinkers and targeting-ligands, properties that can be exploited in drug delivery. Especially, gelatin has the capacity to electrostatically interact with drugs to form a polyion complex which paves the way to the creation of new drug controlled release systems, regulated by multiple ionic interactions.

Nevertheless, films based on gelatin show some drawbacks due to their hydrophilic nature, especially moisture sensitivity. In fact, films may swell, partially dissolve or disintegrate when immersed in aqueous solutions and, thus, the use of crosslinkers becomes fundamental to improve their mechanical properties. The selection of a chemical crosslinking agent is critical since the residual crosslinker could cause toxic side effects. Among these reagents, glutaraldehyde (GTA) has been widely used, although its cytotoxicity has been largely demonstrated (Reddy et al., 2015). More recently, genipin (GP) is receiving increasing attention due to the fact that it is less toxic than aldehydes (De Clercq et al., 2016), but further studies are being developed in search of a crosslinker which does not cause any toxic effect. In this regard, some sugars (e.g. ribose, fructose) have been used as crosslinkers for their ability to interact with gelatin through a harmless process called Maillard reaction (Etxabide et al., 2015a).

Thus, the focus of this work was to develop and characterize a novel ultra thin hydro-film (≈50 μm) based on fish gelatin and lactose (Lac) as an alternative to the commonly used GTA- and GP-mediated crosslinking. First, the crosslinking degree, surface properties, occlusivity, swelling capacity and structure of the dressing were assessed. With the aim of evaluating safety issues, cytotoxicity assays were performed following the ISO 10993-5:2009. The biological behavior of the hydro-film was also studied in cell cultures in terms of cell adhesion, spreading, proliferation and infiltration. Additionally, epidermal growth factor (EGF), widely known for stimulating cell growth, proliferation, and differentiation, was embedded in the hydro-film in order to test the suitability of this approach to deliver therapeutic agents. To our knowledge, this is the first attempt to develop an ultra thin fish gelatin hydro-film by Lac-mediated crosslinking, which may have a considerable potential for several biomedical applications such as wound healing.

2. Experimental section

2.1. Preparation of gelatin hydro-films

5 g of type A gelatin (200 bloom, Weishardt International, LiptovskyMikulas, Slovakia) were dissolved in 100 ml of distilled water for 30 min at 80 °C under continuous stirring to obtain a homogeneous blend. After that, 10 wt% glycerol (Panreac Química, Barcelona, Spain), based on dry gelatin, was added to the solution and the pH was adjusted to 10 with NaOH (1 M). Then, the crosslinker (Lac, GP or GTA) was added to the solution, which was kept stirring for 5 min at 37 °C. Based on some previous studies, 20 wt% Lac (pure, Panreac Química, Barcelona, Spain) (Etxabide et al., 2015a), 0.75 wt% GTA (25% w/v in water, Panreac Química, Barcelona, Spain) (Chiou et al., 2008), or 0.25 wt% GP (≥ 98% HPLC purity, Sigma-Aldrich, Madrid, Spain) (Ma et al., 2013), all of them based on dry gelatin, were added to the solution as optimum crosslinker concentrations. Finally, 17 ml of hydro-film forming solution were poured into each Petri dish and left drying at room temperature for 48 h. The thickness of the hydro-films was around 50 μm. Additionally, when using Lac as crosslinker, the hydro-films peeled from the Petri dishes (designated as non-crosslinked hydro-films) were heated at 105 °C for 24 h to promote crosslinking (designated as crosslinked hydro-films). All hydro-films were conditioned in an ACS Sunrise 700 V bio-chamber (Alava Ingenieros, Madrid, Spain) at 25 °C and 50% relative humidity before testing. Moreover, according to the type of experiment to be performed, the hydro-films were cut into discs of different size using a punch (3–20 mm, JLB320CM, Boehm, France) (Fig. S**1**).

2.2. Characterization of gelatin hydro-films

The hydrolytical stability of hydro-films crosslinked with Lac, GP and GTA was assessed. The rest of the experiments were carried out using only the hydro-films crosslinked with Lac.

2.2.1. Hydrolytical stability

In vitro hydrolytical behavior of the hydro-films crosslinked with Lac, GP and GTA was studied under simulated physiological conditions using phosphate-buffered saline (PBS) solution at pH 7 for 3 days at 25 °C and 37 °C. Three samples of each film were immersed in PBS solution and the flasks were stored in an environmental chamber at 25 °C or 37 °C in order to analyze the dependence of temperature on the film hydrolytical stability. The hydro-film stability was evaluated removing the films from the medium and analyzing their handling and physical integrity.

2.2.2. Physicochemical analysis by ATR-FTIR

Fourier transform infrared (FTIR) spectroscopy was carried out on a Nicolet Nexus FTIR spectrometer equipped with horizontal attenuated total reflectance (ATR) crystal (ZnSe) (Thermo Scientific, Madrid, Spain). A total of 32 scans were performed at 4 cm^{-1} resolution. Measurements were recorded between 4000 and 800 cm^{-1}.

2.2.3. Surface analysis by XPS

The surface analysis of non-crosslinked hydro-films (prior to heating) and crosslinked hydro-films (after heating to promote Maillard reaction) was carried out. X-ray photoelectron spectroscopy (XPS) was performed in a SPECS spectrometer using a monochromatic radiation equipped with Al Kα (1486.6 eV). The binding energy was calibrated by Ag 3d5/2 peak at 368.28 eV. All spectra were recorded at 90° take-off angle. Survey spectra were recorded with 1.0 eV step and 40 eV analyzer pass energy and the high resolution regions with 0.1 eV step and 20 eV pass energy. All core level spectra were referenced to the C 1 s neutral carbon peak at 284.6 eV. Spectra were analyzed using the CasaXPS 2.3.16 software, and peak areas were quantified with a Gaussian-Lorentzian fitting procedure.

2.2.4. Hydro-films morphology by SEM and AFM

The morphology of the hydro-films surfaces and cross sections was analyzed by using a field emission scanning electron microscope Hitachi S-4800 (Hitachi High-Technologies Corporation, Tokyo, Japan) at an acceleration voltage of 15 kV. Samples were mounted on a metal stub with double-side adhesive tape and coated under vacuum with gold (JFC-1100) in an argon atmosphere prior to observation. The surface topography of the samples was studied by atomic force microscopy (AFM) under ambient conditions. AFM images were obtained using a scanning probe microscope (Nanoscope IIIa Multimode™, Bruker). Tapping mode was employed in air using an integrated tip/cantilever at 200–400 kHz resonance frequency, 0.6–1.0 Hz scan rate and 20–80 N/m force constant. Regarding sample preparation, 10 μl of solution at 80 °C was pipetted onto a piece of mica sheet (1.0 cm diameter) attached to a substrate (1.5 cm diameter) with double-adhesive tabs and conditioned at 80 °C to avoid possible conglomeration of gelatin. After that, a high speed spin (2000 rpm) was employed during 120 s to get well-expanded thin hydro-films. After that, samples were kept at room temperature in Petri dishes for 48 h to