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Characterization and evaluation of whey protein-based biofilms as substrates for in vitro cell cultures

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Abstract

Whey proteins-based biofilms were prepared using different plasticizers in order to obtain a biomaterial for the human keratinocytes and fibroblasts in vitro culture. The film properties were evaluated by Fourier Transform Infrared Spectroscopy (FTIR) technique and mechanical tests. A relationship was found between the decrease of intermolecular hydrogen bond strength and film mechanical behavior changes, expressed by a breaking stress and Young modulus values diminishing. These results allow stating that the film molecular configuration could induce dissimilarities in its mechanical properties. The films toxicity was assessed by evaluating the cutaneous cells adherence, growth, proliferation and structural stratification. Microscopic observation demonstrated that both keratinocytes and fibroblasts adhered to the biofilms. The trypan blue exclusion test showed that keratinocytes grew at a significantly high rate on all the biofilms. Structural analysis demonstrated that keratinocytes stratified when cultured on the whey protein-based biofilms and gave rise to multi-layered epidermal structures. The most organized epidermis was obtained with whey protein isolate/DEG biofilm. This structure had a well-organized basal layer under supra-basal and corneous layers. This study demonstrated that whey proteins, an inexpensive renewable resource which can be obtained readily, were non-toxic to cutaneous cells and thus they could be useful substrates for a variety of biomedical applications, including tissue engineering.

Keywords: Whey proteins; Biofilms; Cell culture

1. Introduction

A lot of natural and synthetic polymers have been explored as biomaterials, particularly as scaffolds for tissue engineering and the controlled release of drugs. While the properties of synthetic polymers are limited, natural polymers provide many important benefits, including degradability, biocompatibility and biological stability [1–5]. Thus, there is continuous ongoing research on natural materials with particularly desirable tissue-specific properties that can be used for many specific biomedical applications.

Whey proteins, also known as milk serum proteins, are widely used in food products because of their high nutritional value and ability to form gels [6,7] and emulsions [8]. It has also been recently shown that they can be used for bioencapsulation and delivery systems [9]. Whey protein beads have been successfully produced using an emulsification/cold-gelation process to protect fat-soluble bioactive molecules sensitive to gastric pH [9]. Another important functional property of whey proteins is their ability to form biofilms in the presence

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of plasticizers [10,11]. These components, generally polyols, can modify the chemical–physical and mechanical properties of films [10,11], their adhesion strength [12], and their permeation behavior [10,11], which in turn can affect their release properties [13]. The macroscopic properties of protein films are strongly dependent on the relationships between structural proteins within the network and their interactions. Some chemical products, like plasticizers, play an important role in protein conformation and induce specific molecular changes as the film formation progresses. To better understand the protein film formation it is essential to comprehend the determining relationships between the molecular mechanisms of the process and the film macroscopic properties.

Widely used in food applications, the whey proteins provide great potential for biomedical applications too, including controlled release systems [9,14]. Previous studies have shown that whey proteins can also act as bioactive molecules. The hydrolysis of whey proteins by digestive enzymes generates bioactive peptides that can exert many physiological effects in vivo, including on the gastrointestinal, cardiovascular, endocrine, immune, and nervous systems [15,16]. Whey protein hydrolysates can also improve the human keratinocytes growth in culture [17]. All these properties suggest that whey protein materials appear to be good candidates for biomedical applications. However, it is essential to demonstrate that they are non-toxic and biocompatible. The first aim of this study is to evaluate the impact of different plasticizers on the whey protein-based biofilms physical–chemical properties using Fourier Transform Infrared Spectroscopy (FTIR) and mechanical tests. The second one is to evaluate the whey protein-based biofilms toxicity by assessing their impact on the adherence, growth, proliferation, and structural stratification of model skin cells. Two biofilm types were produced: the first one using whey protein isolate (WPI) plasticized with diethylene glycol (DEG), ethylene glycol (EG) and glycerol (GLY) and the second one using the major whey protein, β-lactoglobulin (BLG), plasticized with DEG.

2. Materials and methods

2.1. Materials

WPI and BLG were obtained from Davisco (Food International, Inc., Le Sueur, MN, USA). WPI contains 92.5% (dry matter) total protein with 78% BLG, 16.6% α-lactalbumin (ALA), and 5.4% bovine serum albumin (BSA). BLG is a purified fraction of WPI ($>92\%$) obtained from fresh, sweet dairy whey using unique ion-exchange technology. The GLY, EG, and DEG plasticizers were of analytical grade. GLY and EG were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA) while DEG was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Whey protein biofilm preparation

Film-forming solutions were prepared by dissolving WPI or BLG in distilled water (10% w/v) according to the McHugh and Krochta method [10]. The solutions were stirred for 90 min at room temperature. The pH was adjusted to 7 and the film forming solution were incubated in a water bath at 80 °C for 30 min to denature the proteins and then cooled to 23 °C. After an additional 60 min of stirring, plasticizers were added in equal volumes. These mixtures were stirred for 30 min to form homogeneous solutions and then centrifuged to eliminate air bubbles (2300 rpm, 10 min). Then the casting solution has been spread out on 140 mm-diameter Petri dishes and the film was obtained by the solvent evaporation in an oven at 23 °C for 20 h. The relative humidity was maintained at 43% using a saturated K_2CO_3 solution. The films were peeled from the Petri dishes using a spatula and stored (T: 23 °C and RH: 43%) until used.

2.3. Physico-chemical characterization

2.3.1. Infrared measurements

The infrared spectra (2 cm^{-1} resolution) were recorded using a Magna 560 Nicolet spectrometer (Madison, WI, USA) equipped with a mercury–cadmium–telluride (MCT) detector. The spectrometer was continuously purged with dry, CO_2-free air. For transmission measurements, the protein solutions were placed in a thermostated cell (6 μm Mylar space). Buffer contribution was substracted as described previously [24]. Film spectra were measured on a horizontal attenuated total reflectance (ATR) crystal (ZnSe). Each spectrum is the average of 128 scans apodized with the Happ-Genzel function. Subtractions and Fourier self-deconvolutions were performed using the software provided with the spectrometer (Omnic software) to study the amide I region of the proteins. Band narrowing was done with a full-width at half-height of 18 cm^{-1} and a resolution enhancement factor of 2.

2.3.2. Mechanical properties

An Instron Universal testing instrument (model 5565, Instron Engineering Corporation, Canton, MA, USA) equipped with a 50 N strength captor was used to measure the film tensile properties according to ASTM D882-91. Initial grip separation and crosshead speed was set at 50 and 24 mm/min, respectively. The film strips were 80 mm long and 6.35 mm wide. The film thickness (± 0.001 mm) was measured by a digital micrometer (Mitituyo, Aurora, IL, USA) in five random positions: the average thickness of each film was used to determine the mechanical properties. Load–displacement curve were recorded during the test: based on this data, breaking stress (σ_b), breaking strain (ε_b), and Young modulus (E) values were calculated for all the measurements. Tensile values were reported as averages of seven replicates for each type of film. The multiple comparison analyses was performed using Statgraphics Plus Professional V4.1 software (Manugistics Inc., Rockville, MD, USA).