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Effect of chemical cross-linking on gelatin membrane solubility with a non-toxic and non-volatile agent: Terephthalaldehyde

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ABSTRACT

In this paper, terephthalaldehyde (TPA) is proposed as non-toxic and non-volatile gelatin cross-linker. Optimal cross-linking parameters (TPA/gelatin ratio, temperature) were first determined from *in situ* rheological measurements on gelatin solutions and from chemical tests with 2,4,6-trinitrobenzenesulfonic acid (TNBS assays) on gelatin gel. The highest cross-linking ratio was achieved for a concentration of 0.005 g TPA/g gelatin at 60 °C. The impact of TPA cross-linking on gelatin membrane functional properties (water swelling ratio, water vapor sorption and mechanical properties) was measured. TPA cross-linking increased 17 times the liquid water resistance duration of gelatin films, and delayed the entry of vapor water in the polymer matrix for 7 days, indicating that TPA increased the hydrophobic character of the gelatin matrix.

1. Introduction

Bio-sourced polymers present advantages over synthetic polymers in term of biocompatibility, biodegradability and sustainability. Among the natural polymers, gelatin displays physico-chemical characteristics which confer enormous potential in the field of tissue engineering upon this protein [1]. Indeed gelatin is composed of a large variety of side chains and chemical functions and is water soluble. It is composed of a unique sequence of amino acids, with a high content of glycine, proline and hydroxyproline. Proline and hydroxyproline content is particularly interesting in terms of gelling effect [2]. Cross-linking of gelatin for biomaterial applications is essential because of its sol–gel temperature around 30 °C, lower than body temperature. Physical [3], enzymatic [4] and chemical cross-linking methods have already been reported for gelatin. In most cases bifunctional reagents including glutaraldehyde [5], diisocyanates [6], carbodiimides [7], genipin [8–10], polyepoxycompounds [11] and acyl azides [12] have been used. Due to the complex nature of gelatin as a polymer, especially the diversity of present functional groups, controlling the cross-linking step is challenging. In the case of dialdehyde, gelatin derivatization occurs mostly *via* the amine groups of lysine and hydroxylysine [13]. Glutaraldehyde is a widely used cross-linker for proteins and especially for gelatin [14–17] but it presents a drawback: its cell and systemic toxicity [18]. Martucci et al. [19] and Bigi et al. [15] showed that at low concentration the glutaraldehyde toxicity does not represent a hazard in the final product. But, new security regulations impose to evaluate the impact of the compound toxicity on the environment but also on the manufacturers' health [20]. GTA volatility assigns manufacturers to install specific equipment like extractor fans. There is thus a need in finding non-volatile cross-linker to simplify the operating procedures.

Gelatin based films have been successfully prepared by solution drying at temperatures lower than sol–gel temperature [21–25]. However, a cross-linking step is needed to maintain mechanical integrity of films/membranes. It has been reported that the use of highly reactive glutaraldehyde as cross-linker for membrane preparation results in heterogeneous material since in this case relatively high concentration of gelatin are used (20 wt%) [26]. This anisotropy is due to the lack of control of the cross-linking kinetics. Thus, the use of a less reactive cross-linker is necessary to get a better control of the film structuring mechanisms. The novelty of this work concerns the development of a method to cross-link gelatin with a less reactive cross-linker and above all non-toxic, to comply with biomaterial applications. Terephthalaldehyde (TPA) is a dialdehyde as GTA and can react with amine functions to form imines [27] *via* Schiff base formation [28]. TPA has already been used as cross-linker for polyvinyl alcohol [29,30] and chitosan [31] with which it forms imines *via* Schiff base formation. TPA innocuousness lies on the absence of alpha hydrogen on the aldehyde functions contrary to GTA. Indeed, it has been reported [32,33] that

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the specific position alpha of hydrogen in the molecule permits to GTA to undergo aldol-reactions giving rise to aldol-form intermediates. TPA safe attribute is explained by its inability to form these intermediates combined to its non-volatility. From our knowledge, no thorough study of TPA as gelatin cross-linker is available. This work focuses on the integration of the chemical cross-linking reaction along gel or membrane preparation by adding TPA directly in gelatin solution in order to favor homogeneous cross-linking. In a first part, the cross-linking reaction was studied through rheological measurements on gelatin solutions to depict cross-linking kinetics according to the temperature and the TPA concentration. Cross-linked gelatin percentages under gel state were then determined through the determination of residual amino groups by using TNBS assays. In a second part, cross-linking achievement on gelatin was measured by studying the behavior of cross-linked films directly immersed in water or in acetic acid, or exposed to water vapor. Finally, the mechanical properties of uncross-linked and cross-linked films were compared.

2. Experimental

2.1. Materials

Porcine skin gelatin (Type A, Bloom 188) and polyethylene glycol (PEG) 200 were purchased from Fluka and Sigma–Aldrich, respectively. Terephthalaldehyde (TPA, Aldrich) was used as cross-linker without further purification. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from Sigma, borax solution ($Na_2B_4O_7$) from Fluka and hydrochloric acid from Sigma–Aldrich (Fig. 1).

2.2. Preparation of gels and films

Gelatin solutions were prepared by dissolving 20 wt% gelatin powder in demineralized water at 60 °C, with mechanical stirring for about 120 min. PEG 200 was added to solutions at 6 wt%. The cross-linker, TPA (from 0.005 to 0.025 g/g of gelatin) was first dissolved in DMSO and then added to the gelatin or gelatin/PEG systems in solution at 30 °C, 40 °C or 60 °C and stirred for 1 h before casting, such as residual DMSO content in gelatin solution remained less than 0.1%. Films were prepared by dry-casting of the gelatin solution at 30 °C on flat Teflon coated sheets using a K Control Coater 101 (Erichsen Instruments). Gelatin films were allowed to dry at 20 °C under (45 ± 5)% of relative humidity (RH) for 2 days. All the membranes were stored at ambient temperature in a box (*ca.* 25 ± 5%RH) before subsequent analysis.

2.3. Thickness measurement

Film thickness was determined using a thickness gage (Mitutoyo). The given values are the average of ten measurements at different positions for each sample.

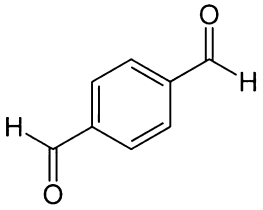

Fig. 1. Chemical structure of terephthalaldehyde (TPA).

2.4. Rheological analysis

Cross-linking kinetics at different temperatures and cross-linker concentrations were determined using a rheometer MCR 301 (Anton Paar) with parallel plate system PP50. The temperature was controlled at 25 °C with an IMC70 Peltier system. The transition from liquid to soft solid was determined using an oscillation test (1 Hz frequency, normal force set to 0 N) with strain varying from 1 to 0.01% following a logarithmic ramp.

The same equipment was used to determine the sol/gel transitions for gelatin. The ramp temperature was controlled from 40 °C to 25 °C with a 0.003 °C/s cooling rate using an IMC70 Peltier system. The transition from liquid to soft solid was determined with an oscillation test (1 Hz frequency, normal force set to 0 N) with strain varying from 1 to 0.01% following a logarithmic ramp. The sol/gel transition was defined as the temperature for which the damping factor ($\tan\delta$) became lower than 1.

The mechanical properties of the films were evaluated by extensional rheology. These tests were performed using a rheometer MCR 301 (Anton Paar) using the Universal Extensional Fixture UXF12. The temperature was controlled at 25 °C with a CTD180 Peltier system. The samples were tested in triplicate on 4 cm × 1 cm specimens.

2.5. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) assays

Determination of residual amino groups in gelatin was performed by the method described by Bigi et al. [15] with some modifications. 0.5 mg of uncross-linked and cross-linked 24 h old gels were solubilized in 6 mL of borax solution. Then 6 mL of 10% (v/v) TNBS solution was added. The solution was then stirred and heated at 30 °C for 1 h. In order to stop the reaction 12 mL of 1 M HCl was added and the solution was set aside for 30 min. The absorbance of the solutions (Abs) was determined spectrophotometrically at 347 nm after suitable dilution. The absorbance of control samples (uncross-linked gelatin – Abs_G, and TNBS/Borax/HCl only solution – Abs_{TNBS}) were also determined to provide the reference values necessary for cross-linking ratio calculation:

$$\text{Cross-linking ratio}\,(\%) = 100\left[1 - \frac{100(\text{Abs} - \text{Abs}_G)}{\text{Abs}_G - \text{Abs}_{TNBS}}\right]$$

All data shown are the average value of three replicates.

2.6. Water content

Actually, the films qualified as dried have reached a thermodynamic equilibrium after drying at 20 °C under (45 ± 5)%RH and a subsequent storage at (20 ± 2)°C under (25 ± 5)%RH. The measurement of water residual amount in dry films was performed by thermogravimetric analysis (TGA G500, TA Instruments under nitrogen flow of 100 mL/min) with a temperature ramp of 10 °C/min.

2.7. Swelling kinetics

The films were cut in 2 cm × 2 cm pieces and desiccated at 50 °C for at least 48 h. They were weighted and left at ambient temperature in 30 mL of deionized water. The swelling kinetics were determined until the samples lost their mechanical integrity, by periodically measuring the weight of the films by means of a microbalance (Sartorius CPA225D, with an accuracy of 0.00001 g), after gently blotting the surface with a tissue.