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Nitric oxide-releasing poly(vinyl alcohol) film for increasing dermal vasodilation

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ABSTRACT

Pathological conditions associated with the impairment of nitric oxide (NO) production in the vasculature, such as Raynaud's syndrome and diabetic angiopathy, have stimulated the development of new biomaterials capable of delivering NO topically. With this purpose, we modified poly(vinyl-alcohol) (PVA) by chemically crosslinking it via esterification with mercaptosuccinic acid. This reaction allowed the casting of sulfhydrylated PVA (PVA-SH) films. Differential scanning calorimetry and X-ray diffractometry showed that the crosslinking reaction completely suppressed the crystallization of PVA, leading to a non-porous film with a homogeneous distribution of -SH groups. The remaining free hydroxyl groups in the PVA-SH network conferred partial hydrophylicity to the material, which was responsible for a swelling degree of ca. 110%. The PVA-SH films were subjected to an S-nitrosation reaction of the –SH groups, yielding a PVA containing S-nitrosothiol groups (PVA-SNO). Amperometric and chemiluminescence measurements showed that the PVA-SNO films were capable of releasing NO spontaneously after immersion in physiological medium. Laser Doppler-flowmetry, used to assess the blood flow in the dermal microcirculation, showed that the topical application of hydrated PVA-SNO films on the health skin led to a dose- and time-dependent increase of more than 5-fold in the dermal baseline blood flow in less than 10 min, with a prolonged action of more than 4 h during continuous application. These results show that PVA-SNO films might emerge as a new material with potential for the topical treatment of microvascular skin disorders.

1. Introduction

Cutaneous endothelial dysfunction is usually associated with cardiovascular disease and can be a symptom of a more general micro-vessel endothelial dysfunction, which can have an impact upon diabetic wound healing and cutaneous infections [1,2]. Cutaneous vasoconstriction can be elicited through local or whole body cooling or can be a pathological condition, such as that present in patients with Raynaud's syndrome, for whom a prolonged digital vasospasm of the hands, feet, nose, ears, and nipples has been associated with impaired synthesis of endogenous nitric oxide (NO) or impaired sensitivity to NO and in whom benefits have been obtained with supplementation with L-arginine, a precursor for NO synthesis [3], and with topical application of an NO-generating system capable of stimulating an increase in both microcirculatory volume and flux [4]. In addition to reflecting the generalized microvascular function, NO also mediates skin vasodilation and has several other physiological and pathophysiological roles, including the regulation of platelet adhesion, smooth muscle cell proliferation, and immune system function [5]. More recently, it has been shown that the topical delivery of exogenous NO may improve wound healing in both the inflammatory and proliferative phases [6].

The potential beneficial effects that can be obtained from the topical delivery of NO to healthy skin or in wounds have stimulated the development of new NO-releasing biomaterials. Hydrogels have been used for this purpose due to their specific properties, such as lubricity and the ability to hydrate wounds [7]. For example, the topical application of Pluronic F127 hydrogels charged with S-nitroso-*N*-acetylcysteine (SNAC) and GSNO has been shown to produce local vasodilation in healthy volunteers and in healthy and streptozotocin-induced diabetic rats [2,8]. Other therapeutic actions obtained in such applications include the acceleration of wound healing [6,9,10] and an antinociceptive effect [11] in animal models. Although flowing hydrogels offer some advantages in topical applications, the possibility of using flexible hydrophilic polymeric films capable of delivering NO is also attractive because these films can be applied as bandages, which may be more appropriate in cases where the absorption of exudate is also desirable [12–14]. Poly(vinyl alcohol) (PVA) is a highly hydrophilic polymer with low toxicity and good biocompatibility, largely used

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in the alimentary, pharmaceutical and cosmetic industries [15]. However, the advantages associated with the PVA hydrophilicity cause difficulties associated with its low mechanical properties and dissolution after contact with an aqueous environment. These limitations have led to several strategies for obtaining insoluble crosslinked PVA membranes. These involve physical crosslinking through the application of freezing thawing cycles [14] and the use of different chemical crosslinking processes, such as γ irradiation [16], self-crosslinking [17] and chemical reactions with glutaraldehyde [18], propionic acid [19] and sulfonic acid [20]. The drawbacks of most chemical crosslinkers in medical applications are their intrinsic toxicity and the necessity of removing toxic reaction residues. There is therefore an effort for finding friendlier crosslinking reagents for preparing PVA hydrogels for biomedical uses. In this work, we devised a strategy for avoiding toxic reagents in the chemical crosslinking of PVA and, at the same time, for functionalizing the crosslinked PVA with an NO-releasing moiety for allowing local NO delivery for topical applications. This strategy was based on the esterification of the hydroxyl groups of PVA with mercaptosuccinic acid (MSA), a sulfhydryl (–SH)-containing dicarboxylic acid, capable of establishing intra- and intermolecular ester linkages with the PVA chains. MSA has low toxicity and is already used in medical applications for treating intoxication by metals [21–23]. The byproduct of its reaction with PVA is water, and hydrochloric acid can be used as a catalyst. The –SH groups present in the crosslinked PVA (PVA-SH) can be subsequently S-nitrosated with nitrous acid (HONO), generating a crosslinked PVA functionalized with S-nitrosothiol groups (PVA-SNO) where, in stoichiometric conditions, the only reaction byproduct is also water. In addition, due to steric factors, the MSA crosslinks must leave large unreacted PVA chain segments in the vicinity of their bonding sites, thus preserving part of the hydrophilicity of the starting PVA. As a property of the primary S-nitrosothiols, PVA-SNO films are capable of releasing NO spontaneously by a thermal reaction, thus opening a new perspective for using PVA-SNO films as a NO releasing biomaterials in topical applications for increasing skin vasodilation or for accelerating wound healing.

2. Materials and methods

2.1. Materials

Poly(vinyl alcohol) (99% hydrolyzed, nominal MW 85,000–146,000), mercaptosuccinic acid (2-sulfanylbutanedioic acid) 97%, hydrochloridric acid (HCl) 37%, phosphate buffer saline (PBS), pH 7.4, glutathione (GSH), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), (5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Aldrich Chemical Company, Co. Milwaukee, WI, USA), sodium nitrite ($NaNO_2$) (Fisher Chemicals, Fair Lawn, NJ, USA) and ascorbic acid 99% and copper(I) chloride (CuCl) (Synth LTDA, São Paulo, Brazil) were all of analytical grade and were used as received. S-nitrosoglutathione (GSNO) was synthesized according to the procedure described in Ref. [24].

2.2. Synthesis of poly(vinyl alcohol) crosslinked with mercaptosuccinic acid

The esterification of PVA with MSA was performed by mixing a previously dissolved 5 wt% PVA solution with mercaptosuccinic acid (final concentration 10 wt %) in the presence of 0.15 mol L^{-1} HCl (pH 0.8). The reaction was conducted under reflux and N_2 atmosphere at 100 °C over 3 h in a two-neck, round-bottomed flask on a heating plate with controlled temperature (IKA, model C-MAG HS 7). After this time, the temperature was reduced to 70 °C, and a vacuum system was used to remove ca. 2/3 of the water volume from the reaction flask to shift the equilibrium toward the product.

2.2.1. Casting of sulfhydrylated PVA films

After cooling the product to room temperature, the viscous solution was poured onto square acrylic molds (15 × 15 cm) and was allowed to dry in an oven at 70 °C for 72 h. The dry films were removed from the molds by immersion in deionized water up to their complete swelling. The wet free films had a thickness of ca. 500 μm and were further immersed in deionized water (ca. 10 cm^2 of films in l L of water) for 72 h with total water replacement every 24 h, for the removal of reaction residue. After the washing procedure, the PVA-SH films were dried again in an oven at 70 °C for 72 h and stored in a desiccator under activated silica gel for further use.

2.2.2. Quantification of free sulfhydryl groups in sulfhydrylated PVA films

Free sulfhydryl groups in the sulfhydrylated PVA films were quantified using the Ellman assay. A 2.0 mM solution of DTNB in 50 mM of sodium bicarbonate and a 1.0 M solution of TRIS with the pH adjusted to 8.0 with HCl, were used in the assay. A calibration curve was obtained using a stock GSH solution (1.0 M) as standard. Aliquots of five variable volumes of GSH solution were placed in 2 mL eppendorf tubes. Each tube received 100 μL of TRIS solution and 50 μL of DTNB solution and had the final volume adjusted to 2 mL with deionized water. The UV–VIS spectrum of each solution was measured in a quartz cuvette (1 cm optical path) and analyzed in a diode array spectrophotometer (Hewlett-Packard, model 8453). Three individual samples of PVA-SH films of ca. 10 mg were incubated with 1850 μL of deionized water, 100 μL of TRIS and 50 μL of DTNB under magnetic stirring in eppendorf tubes for 5 min under protection from room light. The solution from each tube was transferred to a quartz cuvette and had its UV–VIS spectra recorded as above. The absorption measurements for the calibration curve and for the sulfhydryl quantification in the samples were taken at 412 nm. Measurements were performed in triplicate and are expressed as the means ± SD.

2.3. S-nitrosation of sulfhydrylated PVA films

The S-nitrosation of the PVA-SH films was conducted by immersing the films in a nitrous acid solution (40 mmol L^{-1} $NaNO_2$ in 1.2 mol L^{-1} HCl) for periods of 5, 7 and 10 min, yielding films with increasing S-nitrosation degrees designated as PVA-SNO. After S-nitrosation, the PVA-SNO films were washed with ca. 20 mL of deionized water per cm^2 of film and were immersed in liquid nitrogen. To assure complete removal of the excess nitrosating solution from the films in the washing process, the washing water was analyzed by recording its UV–VIS spectra until the disappearance of the absorption band of the nitrous acid (see Fig. S-1).

The frozen films were dried by lyophilization at −80 °C and 70 mTorr for 24 h and were stored in a desiccator with activated silica gel under N_2 atmosphere. The kinetics of the S- nitrosation reaction was monitored spectrophotometrically to characterize the time necessary for complete S-nitrosation of the free sulfhydryl groups of the PVA-SH films. This monitoring was achieved by fixing films of 1 × 2 cm in a Teflon® holder with a window of 0.7 × 1.5 cm. This holder was immersed in the nitrosating solution, prepared as above. At time intervals of 0.5 min in the first 6 min and 3 min in the next 6 min, the Teflon® holder containing the films was removed from the nitrosating solution, washed with a flow of ca. 20 mL of deionized water in each face of the film and briefly dried with a nitrogen flow. The holder was then taken to the spectrophotometer, and the UV–VIS spectrum of the film was recorded.