Materials Science and Engineering C 80 (2017) 165–173

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Glycerol-plasticised silk membranes made using formic acid are ductile, transparent and degradation-resistant

Benjamin J. Allardyce [a], Rangam Rajkhowa [a], Rodney J. Dilley [b], Sharon L. Redmond [b], Marcus D. Atlas [b], Xungai Wang [a,*]

[a] Deakin University, Institute for Frontier Materials, Geelong, Australia
[b] Ear Science Institute Australia and Ear Sciences Centre, School of Surgery, University of Western Australia, Australia

ARTICLE INFO

Article history:
Received 27 January 2017
Received in revised form 11 May 2017
Accepted 16 May 2017
Available online 17 May 2017

ABSTRACT

Regenerated silk fibroin membranes tend to be brittle when dry. The use of plasticisers such as glycerol improve membrane ductility, but, when combined with aqueous processing, can lead to a higher degradation rate than solvent-annealed membranes. This study investigated the use of formic acid as the solvent with glycerol to make deformable yet degradation-resistant silk membranes. Here we show that membranes cast using formic acid had low light scattering, with a diffuse transmittance of less than 5% over the visible wavelengths, significantly lower than the 20% transmittance of aqueous derived silk/glycerol membranes. They had 64% β-sheet content and lost just 30% of the initial silk weight over 6h when tested with an accelerated enzymatic degradation assay, in comparison the aqueous membranes completely degraded within this timeframe. The addition of glycerol also improved the maximum elongation of formic acid derived membranes from under 3% to over 100%. They also showed good cytocompatibility and supported the adhesion and migration of human tympanic membrane keratinocytes. Formic acid based, silk/glycerol membranes may be of great use in medical applications such as repair of tympanic membrane perforation or ocular applications where transparency and resistance to enzymatic degradation are important.

1. Introduction

Silk fibroin based membranes have shown promise as a biomaterial with applications ranging from cutaneous wound repair [1,2], to corneal regeneration [3,4] and for tympanic membrane repair [5–9]. Although regenerated silk membranes are simple to produce, they are typically brittle when dry [10], exhibiting high tensile strength but low breaking elongation [11]. One way to improve the maximum elongation of silk membranes is to add the polyol plasticiser glycerol [12–15], that at concentrations of >20% w/w also induces conformational change, increasing the crystalline content and rendering the blended membranes water insoluble [12]. This removes the need to induce crystalline silk II structure through soaking in an alcohol such as ethanol. Aqueous silk/glycerol membranes are water insoluble, but show a high proportion of hydrophilic, non-crystalline domains, particularly α-helices [12], which have been associated with a higher rate of enzymatic degradation [11,16,17]. It is therefore predicted that aqueous glycerol-based membranes would degrade significantly faster than membranes with a higher crystalline content, such as ethanol-annealed non-plasticised silk membranes.

In contrast to aqueous membranes, silk membranes cast from lyophilised silk foam regenerated in a polar solvent such as formic acid have a significantly higher crystallinity than as-cast aqueous membranes, making them water insoluble without any solvent annealing [6,18–21]. These membranes also demonstrate higher maximum elongation when dry compared with aqueous silk membranes [6]. By combining formic acid based silk solution with glycerol, it may be possible to produce flexible, plasticised membranes that offer better resistance to proteolytic degradation (because of a higher crystalline content). The use of membranes with particular biomechanical properties has specific relevance in surgery. This study therefore compared the physical and mechanical properties of aqueous silk/glycerol membranes with formic acid silk/glycerol membranes. Membrane surface metrology was compared in order to determine if the addition of glycerol significantly impacted surface roughness. Cell migration and cell viability were also compared between the materials. The glycerol content was set at 40% w/w for both membranes, above the critical 20% concentration required to make the aqueous membranes insoluble [22]. This concentration was chosen to maximise membrane stability since membranes containing 20% glycerol were still partially soluble [22]. The 40% concentration was chosen to further enhance ductility and ensure maximum water stability. Where appropriate, the properties of non-plasticised (i.e. no glycerol) aqueous and formic acid based silk membranes were included

* Corresponding author.
E-mail address: xungai.wang@deakin.edu.au (X. Wang).

http://dx.doi.org/10.1016/j.msec.2017.05.112
0928-4931/

in order to provide experimental context to the changes in properties conferred by the glycerol to each membrane type.

2. Materials and methods

2.1. Preparation of silk membranes

Reeled, undegummed fibres from bivoltine *Bombyx mori* silkworms were purchased from a silk reeling facility (Automatic Silk Reeling Unit, Ramanagaram 562159 Factory, Ramanagaram, India). Fibres were degummed for 30 min at 98 °C using 2 g/L sodium carbonate (Sigma-Aldrich, St. Louis, MO, USA), and 1 g/L unscented olive oil soap (Vasse Virgin, Wilyabrup, Western Australia, Australia). Degumming was carried out using a rotary textile dyeing machine (Ahiba IR Pro, Datacolor, Lawrenceville, USA). Degummed fibres were dried overnight at 40 °C then dissolved with 9.3 M lithium bromide for 5 h at 60 °C. Dissolved silk solution was dialysed at 4 °C for 3 days against deionised water (dH_2O) to obtain aqueous silk solution with a concentration of between 4 and 5% w/v as calculated by gravimetric analysis. The silk solution from each batch was diluted to 4%; to make aqueous membranes, this solution was mixed with the required amount of glycerol, then cast into Petri dishes on a level surface and allowed to dry for 24 h. To make formic acid based membranes, 4% silk solution was divided up into 50 mL tubes and frozen at −80 °C for 24 h. Frozen silk was then transferred to a pre-chilled FreeZone freeze-drier and dried for 3 days (Labconco, Kansas City, MO, USA). Freeze-dried silk foam was sliced into small pieces with a scalpel and dissolved in 99% formic acid for 1 h on an orbital mixer at room temperature. Dissolved samples were centrifuged at 7000 × *g* for 2 min to remove bubbles, mixed with the appropriate amount of glycerol, cast into 55 mm diameter Petri dishes and allowed to dry for 24 h in a fume hood. The final thickness of all membranes was 50 μm.

2.2. UV–Visible spectrophotometry

Membrane transparency over the visible wavelengths was measured using a Cary 5000 UV–Visible spectrophotometer (Agilent, Santa Clara, CA, USA) with diffuse reflectance accessory. The percentage transmittance of samples was determined by scanning from 700 to 380 nm. Samples were scanned with the certified PTFE reference standard attached to determine total transmittance and again with a light trap attached to determine the diffuse transmittance. The resulting total and diffuse transmittance scans were plotted together for each membrane type. The haziness of each sample was also quantified at 380, 550 and 700 nm using the following formula:

$$\%\ \text{Haze} = \frac{T_{\text{diffuse}}}{T_{\text{total}}} \times 100$$

2.3. Surface metrology and roughness

The surface roughness of each sample was calculated using optical profilometry. Briefly, the top and bottom of 3 membranes were imaged on a Veeco Dektak 150 Contour GT (Bruker, Billerica, MA, USA). Scans were taken at a magnification of 50× using a 2× multiplier. The output file for each scan was then imported into the open source software Gwyddion (version 2.45); the scans were corrected by plane levelling, then the root mean square (RMS) roughness (R_q) was calculated. Any missing data identified by Gwyddion was masked and excluded from roughness calculations. Roughness data was presented as the mean ± standard deviation of 3 measurements of the 3 membranes of each type. A total of 9 measurements were conducted for each membrane type.

2.4. Scanning electron microscopy

Samples in tissue culture plates were rinsed in phosphate buffered saline (PBS), fixed in 2.5% glutaraldehyde in PBS, pH 7.4 for 30 min at room temperature (RT) then dehydrated with increasing grades of ethyl alcohol for 1 h each at RT (50%, 70%, 95%, 100% super dry 2 changes). Samples were critical point dried from ethanol through CO_2 (Emitech, Laughton, UK) and sputter coated at 0.07 Torr in argon gas for 2 min at 25 kV (E5100 sputter coater, Polaron Equipment Inc., Laughton, UK). Samples were mounted on aluminium stubs and viewed in a scanning electron microscope (Philips, Eindhoven, Netherlands). Representative images were taken at 18× 200× and 500× magnification.

2.5. Membrane secondary structure

The proportion of crystalline (β-sheet and turn) and amorphous (α-helix and random coil) motifs was measured in each membrane type using a Vertex 70 Fourier transform infrared (FTIR) spectrophotometer (Bruker, Billerica, MA, USA). Scans were taken in absorbance mode over the range of 4000 to 600 cm^{-1}. A total of 3 membranes of each type were measured, with 6 scans taken per membrane (edge of the membrane, top surface, edge of the membrane bottom surface, centre of the membrane top surface, centre of the membrane bottom surface) for a total of 18 measurements per membrane type. The top and bottom surface scans were averaged, and the amide I region (1705 to 1595 cm^{-1}) was subjected to deconvolution and curve fitting using 7 known conformational positions as described previously [23]. The relative peak area of each of these 7 deconvoluted peaks was used to determine the % content of side chain, β-sheet, random coil, α-helix and β-turn. The % peak area values were expressed as the mean ± standard deviation of 6 measurements (centre and edge region of 3 separate membranes). The averaged scan of all samples per membrane type was also plotted after deconvolution.

2.6. Tensile mechanical properties

Membranes for tensile testing were sliced into 5 mm wide strips, then conditioned at 20 °C ± 2 °C and 65% ± 2% relative humidity for at least 48 h prior to tensile testing. Tensile testing was conducted using a model 5967 tester (Instron, Norwood, MA, USA) with a 100 N load cell. Samples were tested until break using a gauge length of 15 mm, an extension rate of 15 mm/min and a pre-load of 0.1 N. The thickness of each membrane was measured before cutting into strips; membranes were measured in 6 places using a three-decimal-place digital micrometre (Kinchrome, Melbourne, Australia). The average thickness of these measurements was used to calculate the cross-sectional area and subsequently, the stress and strain of each membrane. A minimum of 20 strips were tested across at least 3 membranes; tensile properties were expressed as mean ± standard deviation of these measurements.

2.7. Resistance to degradation

Membranes were tested using an accelerated in vitro enzymatic degradation study using a modified method based on previous work [6]. Membranes were conditioned to 20 °C ± 2 °C and 65% ± 2% relative humidity for at least 48 h, then cut into 5 strips per membrane. The weight of each sample was recorded using a 4 decimal place balance before the membrane strips were sterilised using UV light for 30 min. Each strip was then aseptically transferred to a 15 mL plastic tube. Control samples were incubated with 0.1 M phosphate buffered saline (PBS) pH 7.4 while experimental samples were incubated with 0.1 M PBS containing 1 mg/mL Protease XIV (Sigma-Aldrich, St. Louis, MO, USA). This was equivalent to 3.5 units per mL. Samples were incubated over 3 days; the protease solution and buffer was changed each day to maintain