

Tissue transglutaminase (TG-2) modified amniotic membrane: a novel scaffold for biomedical applications

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ABSTRACT

Background/Aims: The amniotic membrane (AM) is considered as a natural cell culture substrate and has occasionally been exploited in regenerative medicine especially for ocular surface reconstruction and dermal wound healing applications. However, its limited use infers from its relatively weak mechanical strength, difficulty during manual handling and susceptibility to proteolytic degradation *in vivo*. Therefore, in this study we aimed to enhance the mechanical and biological characteristics of the AM by enzymatic cross-linking it using tissue transglutaminase (TG): a calcium-dependent enzyme capable of forming stable $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ cross-linkages. Using a biological catalyst such as TG should not only prevent denaturation during sample preparation but also minimise the potential of residual chemical cross-linking agents compared to alternative methodologies.

Methods: Human AM, sourced from elective caesarean sectioning, were treated with TG, BSA and/or a no-treatment control. Samples were then compared in terms of their physical characteristics (SEM, transparency, mechanical strength, susceptibility to proteolytic degradation), biological characteristics (*in vitro* cell culture, activation of dendritic cells) and its *in vivo* biocompatibility/angiogenic capacity (chick chorioallantoic membrane (CAM) assay).

Results: Transglutaminase-treated AM exhibited enhanced mechanical strength and greater resistance to proteolytic/collagenase degradation compared to the control(s). SEM imaging of the TG-treated membrane summarised a significantly closer association and greater interconnectivity of individual collagen fibres yet it had no effect on the overall transparency of the AM. *In vitro* cell culture demonstrated no detrimental effect of TG-treatment on the AM in terms of cell attachment, spreading, proliferation and differentiation. Moreover, an “immune response” was not elicited based on extended *in vitro* culture with human monocyte-derived dendritic cells (mDC). Interestingly, the TG-treated AM still allowed angiogenesis to occur and in some instances, demonstrated an enhancement compared to the control (n = 5).

Conclusion: We hereby demonstrate that treating the AM with the cross-linking enzyme, transglutaminase, results in a novel biomaterial with enhanced mechanical and biological characteristics. Above all, this modified membrane demonstrates greater strength, maintains *in vitro* cell growth, retains optical transparency and allows angiogenesis to occur without inducing an immune response. Taken together, this study demonstrates the feasibility of transglutaminase as an alternate cross-linking treatment for the production of novel biomaterials and suggests that TG-treated AM may now be more commonly exploited as a therapeutic dressing for ocular or wound applications.

Keywords: amniotic membrane, biomaterials, cross-linking, tissue engineering, transglutaminase

1. INTRODUCTION

The human amniotic membrane (AM) which represents the innermost layer of the placenta is composed of three distinct strata, namely a single epithelial layer, the thick basement membrane and an avascular stroma [Sippel *et al.*, 2001]. Moreover, there is an absence of nerves, muscles and lymphatics from the AM structure [Toda *et al.*, 2007]. The clinical potential of the AM was first considered over 100 years ago by JW Davis as a surgical material in skin transplantation [Davis, 1910]. Since then, the exploitation of the AM has rapidly expanded and is now utilised in a variety of applications which include biological dressings (e.g. for wounds, ulcers, burns), as a tissue substitute for reconstructive surgery and as a novel cell culture scaffold material for tissue engineering and regenerative medicine applications [reviewed by Parolini *et al.*, 2009; Niknejad *et al.*, 2008; Lineen and Namias, 2008; Toda *et al.*, 2007; Hodde, 2002]. However, by far the most common usage of the AM has been in the field of ophthalmology as a treatment for a variety of ocular surface disorders. The AM itself possesses several key characteristics which make it desirable for medical and/or therapeutic applications and include its ease of sourcing, good biocompatibility, low immunological response, natural antimicrobial properties, anti-scarring and anti-fibrotic effects and also, being capable of inducing enhanced cell and cellular characteristics [Niknejad *et al.*, 2008; Dua *et al.*, 2004; Dua and Azuara-Blanco, 1999]. However, the limitations for further exploitation of the AM reside in its innate weak mechanical characteristics, susceptibility to proteolytic degradation and stability following long-term storage. Moreover, the membrane is frequently prone to crumbling/folding and requires trained and skilled manual handling to adequately exploit successfully.

The major components that contribute to the AM matrix structure include a variety of collagens, proteoglycans and non-collagenous glycoproteins (i.e. fibronectin and laminin) [Sippel *et al.*, 2001; Campbell *et al.*, 1989; Yurchenco and Ruben, 1987]. As such, several methods (derived from established methodologies involved in the cross-linking of collagens) have been employed to cross-link AM to improve its mechanical properties and prevent its rapid absorption by the body [Lee *et al.*, 2001; Fujisato *et al.*, 1999]. However, the majority of these methods are susceptible to leaving residual catalysts and/or by-products that may be toxic or cause an inflammatory response or, simply, not practical or cost effective at the large-scale [Matuda *et al.*, 1999; Ben-Slimane *et al.*, 1988; Dunn *et al.*, 1969]. Moreover, radiation treatment with γ -ray and electron beams demonstrated a significant decrease in both the tensile strength and elongation at break characteristics of the AM [Fujisato *et al.*, 1999] as well as collagen derived from native AM [Liu *et al.*, 1989]. As such, alternative methods are sought to enhance the physical characteristics of biological membranes in a more natural, efficient and practical manner but, most importantly, without inducing any detrimental effects on both the sample and/or patient.

Transglutaminase (EC 2.3.2.13) are a group of ubiquitously distributed enzymes capable of catalysing the linkage of peptides or proteins together to form multimers, via an ϵ (γ -glutamyl)lysine cross-link, using the side chains of lysine and glutamine residues [Griffin *et al.*, 2002]. Moreover, transglutaminases (TG) are also able to covalently bind primary amine containing compounds to peptide bound glutamine facilitating the modification of proteins [Griffin *et al.*, 2002]. As such, these enzymes have frequently been exploited in a wide range

of commercial applications including the modification of food, textiles and cosmetics [Cortez *et al.*, 2002; Kuraishi *et al.*, 1997; Bailey *et al.*, 1996; Rasmussen *et al.*, 1996; Ishii *et al.*, 1994]. However, the greatest potential by far has been the enzymes' exploitation in therapeutic and regenerative medical applications due to its ability to enhance both the mechanical and biological characteristics of key biological substrates. As such, novel biomaterials and biological substitutes derived from several biological molecules (e.g. collagen, fibrin, gelatin and chitosan) have been developed over the years and currently pursuing a pathway towards clinical and therapeutic potential [Lorentz *et al.*, 2010; Collighan and Griffin, 2009; Yung *et al.*, 2007; Chau *et al.*, 2005; Broderick *et al.*, 2005; McDermott *et al.*, 2004; Orban *et al.*, 2004; Ito *et al.*, 2003; Greenberg *et al.*, 1991].

In this study, we aim to demonstrate the efficacy and effect of treating the AM with TG and evaluating the resultant mechanical and biological properties of the modified membrane with a view of its exploitation as a novel biomaterial.

2. MATERIALS AND METHODS

All chemicals were purchased from Sigma Aldrich (Poole, UK) unless otherwise stated. Sterile preparation of stock solutions and chemicals were performed either by filtration through a 0.22µm Whatmann sterile filter and/or autoclaving at 121°C at 1bar for 45 minutes. Handling and disposal of the AM was performed in accordance with local human biological regulations and all AM manipulation performed under a sterile environment, using aseptic techniques, where possible.

Ethics approval

Human AM were collected from mothers undergoing elective caesarean sectioning and obtained with prior written consent and the approval of the University of Nottingham Medical School/Nottingham Research Ethics Committee. Membranes were obtained from patients who delivered healthy, near-term children with no gestational issues and the entire study performed under the principles of the Declaration of Helsinki.

Amniotic membrane preparation, processing and treatment

Amniotic membranes were prepared in accordance with a previously established protocol [Hopkinson *et al.*, 2006]. Briefly, the chorion was manually separated from the amnion and discarded before the remaining membrane treated with 0.5% levofloxacin in PBS to remove residual blood. Persistent blood-stained AM edges were removed by dissection. AM samples were acquired from the uniform and translucent areas adjacent to the placental disc (proximal amnion) and placental disc (distal amnion) before being placed at -80°C in PBS for long-term storage and to ensure no transmission of diseases to occur (> 6 months).

Prior to immediate use and under a sterile environment, AM samples were thawed at 37°C before being washed twice with fresh PBS and then cut into squares with dimensions approximately 1cm x 1cm. Triplicate samples were acquired from the same locality where possible and then incubated, 24h, with 1% (w/v) tissue transglutaminase (TG; guinea pig liver) in reaction buffer (2mM CaCl₂, 1mM DTT in PBS), 1% (w/v) bovine serum albumin (BSA) in PBS and PBS only (untreated control) in a humidified-atmosphere incubator at 37°C and with 5% CO₂. Samples were kept on rocking platform (Stuart Gryo-Rocker SSL3, default settings: 21) throughout the incubation step. Following treatment, AM were removed and washed once with 2mM EDTA in PBS and then once more with PBS solution only. Samples were then used immediately where possible or stored at 4°C, in PBS, and used within 5 days following initial treatment. In all experiments, the following controls were included in the set-up and used for comparative studies. Inactivated TG (TG pre-treated with 2mM EDTA and without the addition of CaCl₂), BSA-treatment as a protein control and tissue culture plastic (TCP) controls where applicable.

Transglutaminase activity assay

Transglutaminase cross-linking activity was determined using a slightly modified version of the hydroxamate assay developed by Folk and Chung [Folk and Chung, 1985]. Briefly, 25 μ l of TG sample was mixed with 75 μ l of Reagent A (0.2M sodium acetate (pH 6.0), 0.03M CBZ-Gln-Gly, 0.1M hydroxylamine, 0.01M glutathione, 5mM CaCl₂, 5mM DTT) in a well of a 96-well plate. Following incubation at 37°C for 10 minutes, 75 μ l of Reagent B (1 volume of 3N HCl, 1 volume 12% trichloroacetic acid, 1 volume 5% FeCl₃.6H₂O (dissolved in 0.1N HCl)) was added to the well in order to terminate the reaction by the co-formation of an iron complex. The resultant absorbance was read at 492nm using an Optima FLUOstar[®] plate reader (BMG LabTech, Aylesbury, UK). A unit of transglutaminase activity is defined as the amount of enzyme catalysing the formation of 1 μ mol of hydroxamic acid per minute under the described reaction conditions.

Scanning electron microscopy (SEM)

Macro- and micro-scope assessment of the AM samples was performed using SEM. Briefly, untreated and treated AM samples were fixed 24h with 3% (v/v) glutaraldehyde, washed with PBS before being treated with 1% (v/v) osmium tetroxide solution for 2 hours. Following this fixation step, samples were washed with distilled water and then processed through a series of 10 minute dehydration steps involving ethanol (25%, 50%, 70%, 90%, 95% and 100%) before being left to air-dry at room temperature (19°C). Samples were then mounted on aluminium SEM stubs using double-sided carbon tape and then gold coated, for 3 min, under an argon atmosphere. Coated samples were examined using a variable pressure JOEL 6060LV unit operating at an accelerating voltage of 10 kV. Image analysis was carried out using the in-built SEM Control User Interface software (version 6.57) and digital imaging system.

Mechanical testing and characterisation (Love wave)

The mechanical strength of the untreated and treated AM was assessed using a Love wave surface acoustic device (donated by CSIRO Materials Science and Engineering, Australia). Love wave devices are layered structures consisting of a piezoelectric substrate and a guiding layer which couples the acoustic wave to the near surface. Typically two inter-digital transducers, separated by a propagation path, are embedded at the interface between the substrate and guiding layer. These transducers are used to transmit and receive the acoustic waves in a delay line configuration (see figure: caption for figure: Schematic of Love wave device consisting of a piezoelectric substrate, guiding layer and interdigital transducers. A test sample is shown along the propagation path). The loading of a sample along the propagation path of the device induces damping in the wave ($\Delta\alpha$) and a change in the wavenumber ($\Delta\beta$) [G Kovacs, MJ Vellekoop, R Hauéis, GW Lubking, A Venema, "A Love wave sensor for (bio)chemical sensing in liquids, Sensors and Actuators A, 43 1994 p38-43"]. These changes can be studied using perturbation theory and expressed in terms of the complex shear modulus (G^*) as follows:

$$G^* = \frac{1}{S^2 \rho} \left[\left(\frac{\Delta \alpha}{\beta} \right)^2 - \left(\frac{\Delta \beta}{\beta} \right)^2 \right] + 2i \frac{1}{S^2 \rho} \frac{\Delta \alpha}{\beta} \frac{\Delta \beta}{\beta} \quad (1)$$

where S is the device sensitivity and ρ is the sample density and β is the unperturbed wavenumber [V Raimbault, D Rebiere, C Dejous, M Guirardel, J Pistre, JL Lachaud, High frequency microrheological measurements of PDMS fluids using SAW microfluidic system, Sensors and Actuators B, 144, 2010, p467-471]. The real part of the complex shear modulus can be used to assess the mechanical strength of the loaded sample.

In the current work the Love wave device consisted of two inter-digital piezoelectric transducers (6mm separation path length) on a ST-cut quartz substrate which were coated with a guiding layer of silicon dioxide and operated at a frequency of 118MHz. A control unit provided power to the device and an oscilloscope was used to detect the received signals; a background signal reading was initially obtained using an unloaded device before a sample of the membrane (0.3cm x 0.3cm) was loaded and the resultant signal determined. Signals were digitised and analysed using Matlab (The Mathworks, Cambridge, UK) before the mechanical strength of the membranes assessed using Equation 1. For the corresponding calculations, the density of the hydrated AM was taken to be the same as water (997.6Kg/m³ at 23°C).

Transparency assay

The optical characteristics of the AM were assessed using a simple modification of the transparency assay as previously described by Ma and colleagues [Ma *et al.*, 2010]. Briefly, membranes were placed over a sample of waterproof text card and the resulting image captured using a Fujitsu F80EXR 12MP digital camera.

Proteolytic degradation of AM (using collagenase)

The susceptibility to proteolytic degradation of the untreated and treated AM substrates was assessed using a controlled digestive assay involving microbial collagenase (*C. histolyticum*, Type IA, 0.5-5.0 FALGPA units/mg solid). In short, AM samples were washed twice with PBS, a single wash with distilled water and then incubated with a 1mg/ml, 0.2mg/ml or 0.002mg/ml collagenase solution. Samples were kept in a humidified-atmosphere incubator at 37°C and with 5% CO₂ for selected time-points before being washed twice with PBS. Samples were then viewed using a Vevo VMS-004 Discovery microscope and images captured using the in-built camera and Q-Capture software. In addition, supernatants were also collected from each of the individual membrane samples and the protein concentration quantified using the Bio-Rad DC protein assay kit (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions before the absorbance was read at 750nm using a Tecan Infinite® M200 PRO plate reader (Tecan, Reading, UK).

Cell culture and AM-cell preparation

The human epithelial cell line, U5637, derived from a grade II urinary bladder carcinoma was used during its low (3-10) passage numbers (LGC Standards, Middlesex, UK). These cells

were cultured and maintained as an adherent monolayer in T-flasks in complete media (RPMI 1640 supplemented with 10% (v/v) FBS, 2mM L-glutamine and 1% (v/v) penicillin-streptomycin). Flasks were kept in a humidified-atmosphere incubator at 37°C and with 5% CO₂. Cells were routinely passaged to ensure that they were between 50% and 90% confluency at all times. For detachment, a standard trypsinisation protocol was performed using 0.25% (w/v) trypsin/2mM EDTA in PBS.

Dendritic cells (DC) were generated from peripheral blood monocytes of healthy volunteers as previously described [Horlock *et al.*, 2007]. Briefly, PBMC were separated from heparinised whole blood by a standard density gradient centrifugation protocol using Histopaque® 1077 (Sigma, Poole, UK). Monocytes were purified using the MACS™ anti-CD14 microbeads separation kit (Miltenyi Biotec, Woking, UK) and the recovered cells cultured in RPMI medium supplemented with 10% (v/v) FCS, 2mM L-glutamine and 1% (v/v) penicillin-streptomycin solution, 250U/ml recombinant human (rh) IL-4 and 50ng/ml rhGM-CSF (R&D Systems, Oxford, UK) to promote differentiation to DC. As such, cells obtained using these methodologies are termed monocyte-derived dendritic cells (mDC) hereinafter. mDC phenotype was assessed using flow cytometry for a selection of cell surface markers (see below) and demonstrated typical phenotype of immature DC (Horlock *et al.*, 2007). PBS pre-wetted 0.5cm² AM squares were placed flat into the wells of a 24-well plate before being seeded with 100µl of the appropriate cells at a concentration of 8.35x10⁶ cells/ml (U5637) and 6.03x10⁵ cells/ml (DC). Plates were then incubated for 20 minutes in a humidified-environment, at 37°C and with 5% CO₂, before an additional 200µl of complete media was added to each sample.

Cell viability and proliferation

Cell viability was assessed using the CellTiter AQ One Solution Cell Proliferation™ (MTS) assay kit (Promega, Southampton, UK) according to the Manufacturer's instructions. Briefly, assays were performed under reduced lighting by the addition of 20µl of CellTiter AQ reagent into the relevant samples containing 100µl of culture medium (or variations thereof using a 1:5 ratio of the AQ reagent:culture media). Samples were then incubated in a humidified-atmosphere incubator at 37°C and with 5% CO₂ for 60 minutes before the absorbance was read at 492nm using an Optima FLUOstar® plate reader (BMG LabTech, Aylesbury, UK).

Cell attachment and spreading

At specified time-points following initial cell seeding, samples were washed once with PBS before being fixed in 4% (w/v) paraformaldehyde for 20 minutes. Samples were then washed twice with PBS, permeabilised by the addition of 0.1% (v/v) Triton X-100 in PBS (15 minutes), washed twice again with PBS and then stained using a dual staining protocol consisting of May-Grunwald stain (0.25% (w/v) in methanol) (15 minutes), wash in PBS, and Giemsa stain (0.4% (w/v) in methanol, diluted 1:50 with water) (20 minutes). A final two washes with distilled water were performed before samples were left to air-dry (room temperature) and

imaged through a Nikon SM21500 microscope and images captured using the in-built Nikon Digital DS-L1 image capture unit. Pictures were analysed using Scion Image™ software (Scion Corporation, Maryland, USA). Spread cells were distinguished and characterised based upon the presence of a clear halo of cytoplasm surrounding their nucleus as previously described by Jones and colleagues [Jones *et al.*, 1997].

Flow cytometry (expression of cell surface markers)

Expression of cell surface markers on mDC was assessed by the addition of monoclonal antibodies, conjugated to the fluorophores, FITC, PE or PCy5, with specificity for individual cell surface markers. All antibodies and isotype controls were purchased from Beckman Coulter (High Wycombe, UK) unless otherwise stated. The panel of antibodies used in this investigation include: PE-CD11c (clone BU15, IgG1), FITC-CD54 (clone 84H10, IgG1), PCy5-CD83 (clone HB15a, IgG2b), PE-CD86 (clone HA5.2B7, IgG2bκ), PE-CD206 (clone 3.29B1.10, IgG1), PE-CD209 (clone AZND1, IgG1) and PCy5-HLA-DR (Immu-357, IgG1). Additionally, an unstained sample and the appropriate isotype controls were included in each batch analysis to address autofluorescence and non-specific binding, respectively. Surface staining was performed as previously described [Royer *et al.*, 2010]. Cells were harvested and washed twice with PBA (0.5% (w/v) BSA and 0.1% (w/v) sodium azide in PBS). In the final wash, the supernatant was removed and 5µl of each of the antibodies of interest were added to the resultant cell pellet before being vortexed and incubated, in the dark, for 20 minutes at 4°C. Samples were then washed with PBA before the addition of 600µl of fixing solution (0.5% (v/v) formaldehyde, in PBS) and then stored in the dark, at 4°C, until acquisition using a flow cytometer. A maximum of 7 days was allowed for the storage of the fixed samples prior analysis. Data for each cell surface marker was acquired using a Beckman Coulter EPICS Altra™ flow cytometer (Beckman Coulter, High Wycombe, UK). Each sample population was gated to only include cells of interest based on their forward scatter (cell size) and side scatter (cell granularity) profiles and on the basis of the expression of certain cell surface markers (i.e. CD11c and/or HLA-DR for DC lineage). A total of 8000 events were collected for each sample. Raw data were analysed using WinMDI 2.9 software (build #2, 6-19-2000; Scripps Research Institute. <http://facs.scripps.edu/software.html>) and the absolute mean fluorescence intensity (MFI) value for each marker was determined. Absolute MFI value is defined as: [absolute MFI] = [MFI value of sample] – [MFI value of isotype/unstained sample]

Endocytosis of DC

Assessment of endocytosis was performed by measuring the uptake of FITC-dextran as previously described [Garcia-Nieto *et al.*, 2010]. mDC were collected and washed with cold PBS (4°C) before being resuspended in cold serum-free RPMI medium (4°C) and split into four eppendorfs (i.e. $\sim 5 \times 10^5$ cells/ml in 500µl per eppendorf) per sample. FITC-dextran (Sigma, Poole, UK) was then added, at a final concentration of 1mg/ml, to two of the eppendorfs while the remaining two were left unstained. One each of the stained and unstained samples were then placed into a humidified incubator (37°C and 5% CO₂) for 90 minutes. The remaining stained and unstained samples were placed at 4°C for the same time

period. Following incubation, cells were washed once with cold serum-free RPMI medium (4°C) followed by a wash with cold PBS (4°C). Samples were then fixed by the addition of 600µl of cold fixing solution (0.5% (v/v) formaldehyde in PBS, at 4°C) and immediately processed using flow cytometry.

Chick chorioallantoic membrane (CAM) assay

The angiogenic capacity of the AM was assessed using the chick chorioallantoic membrane (CAM) assay. Briefly, the assay was performed on fertilised 4-day-old eggs (Henry Stuart, Fakenham, UK) by the direct addition of a 0.5cm² AM sample onto the chorion. Following a further 6-day incubation at 37°C, the eggs were chilled at 11°C for 1 hour and then the localised area surrounding the AM/chorion viewed using a Leica MZ16F light microscope and images captured using the in-built camera and Q-Capture software. Quantification of vessels was achieved by visual manual counting of the number secondary vessel formation per mm² area as previously described [Saif *et al.*, 2010].

Statistical analysis

Mean values and standard deviations were calculated from each sample and the differences between the means were compared using the Student *t*-test (two-tailed) or one-way ANOVA where appropriate with values of $p < 0.05$ considered significant.

3. RESULTS

Mechanical characteristics of TG-treated AM

Native amniotic membrane samples treated with enzymatically-active transglutaminase (quantified using the hydroxamate activity assay) in order to catalyse the formation of $\epsilon(\gamma$ -glutamyl)lysine cross-links were analysed by SEM. Figures 1 and 2 summarise the post-treatment effects on the AM following a 24h treatment with 1% (w/v) TG and 1% (w/v) BSA (as a protein control) in a humidified incubator at 37°C and 5% CO₂. At the x200 and x750 magnification levels, TG-treated AM demonstrated a closer and denser association of individual ECM fibrous matter compared to the untreated and BSA-treated AM samples which did not appear to differ themselves. Moreover, at the higher magnifications, the TG-treated AM exhibited a much smoother and uniform surface compared to the untreated membrane sample: comparable for both the apical and epithelial sides of the AM. Interestingly, the BSA-treated membrane also displayed a relatively smooth surface but was populated with a significant amount of aggregated globular (protein) material.

Samples of each membrane were subjected to mechanical analysis using a Love wave acoustic device. The device was loaded separately with each of the AM samples and the resulting frequency shift and change in signal amplitude, relative to the unloaded device, were recorded. The measured frequency shift and signal amplitude were expressed as the change in the wavenumber and damping relative to the unperturbed wavenumber, and substituted into Equation 1. The real part of the shear modulus was then determined

allowing a direct comparison of the membrane mechanical *strength* to be made. This is summarised in Figure 3. It can be seen that both the relative damping and change in wavenumber of the TG-treated AM are significantly lower ($p < 0.05$ and $p < 0.01$, respectively) than that of the untreated AM control sample. Moreover, TG-treated AM also demonstrated a significantly lower relative damping than that of the BSA-treated AM sample ($p < 0.05$). Calculation of the individual shear modulus indicate that the TG-treated AM appears to be mechanically stronger/stiffer than the BSA-treated membrane; with both protein-treated membranes being stronger/stiffer than the untreated control.

Transparency of the TG-treated AM

The transparency characteristics of the AM samples were assessed using a simple visual test: the ability to image/read specific words when the membranes were placed over sample text as documented in Figure 4. It can be seen that neither of the TG or BSA pre-treatments impaired the transparency of the AM samples.

Susceptibility to proteolytic degradation

The resistance to proteolytic degradation of the AM samples was assessed using a concentration range of microbial collagenase (*C. histolyticum*). The collage of images depicted by Figure 5 summarise the physical characteristics of the untreated, 1% (w/v) BSA-treated and 1% TG-treated AM samples when exposed to 0.2mg/ml of collagenase for different time-points. In addition, parallel supernatant samples were collected and their protein concentration determined using the colorimetric based Bio-Rad DC protein assay kit and summarised in Figure 6. Visual signs of degradation began to appear at the 24 hour time-point for the untreated AM sample but at the 72 hour time-point for the BSA-treated sample. In contrast, the TG-treated AM samples remained relatively intact up until the 144 hour time-point. This observation was further corroborated with the protein concentration which demonstrated a consistent and significant lower protein reading ($p < 0.05$) for the TG-treated AM samples compared to the BSA-treated and untreated controls at the appropriate time-points. Moreover, this degradation profile was replicated at the 1mg/ml and 0.002mg/ml collagenase concentrations [data not shown].

Cell culture characteristics of the TG-treated AM

Figure 7 shows the short-term (72 hours) cell attachment and spreading characteristics of both the U5637 cell line and mDC on TCP, untreated AM, BSA-treated and TG-treated AM samples when used as culture surfaces. Increased numbers of attached U5637 cells were found when cultured on the TG-treated AM compared to the untreated AM sample and also appeared to demonstrate an enhancement in its degree of spreading based on the extent of the cytoplasmic halo surrounding the nucleus [spreading data not shown]. In contrast, the BSA-treatment appeared to decrease the ability of the U5637 cells to attach to the membrane. No major differences were noted for mDC when cultured on the different AM-treated membranes.

The metabolic activity of the cells was quantified using the MTS proliferation assay and summarised in Figure 8. It can be seen that cellular activity for both cell types was significantly greater on the TG-treated AM compared to the other membrane samples (at the 48 hour time-point). However, the difference in this effect became negligible at the longer culture period (72 hour time-point) for both the U5637 and mDC.

Angiogenic potential of AM (CAM assay)

A representative selection of images for the untreated, BSA-treated and TG-treated AM used in the CAM assay is summarised in Figure 9. Quantification, based on the formation of secondary vessels per mm², allows the comparison of the angiogenic potential of each membrane to be made. Interestingly, none of samples appeared to inhibit angiogenesis due to the presence of clear and defined blood vessels in all the samples. In some cases, it was observed that the TG-treated AM samples allowed the development of several smaller (tertiary and quaternary) blood vessels compared to the other control conditions.

mDC phenotype and endocytic ability

Phenotypical analyses of the mDC following culture on the different substrates were achieved using flow cytometry. Absolute MFI values of the surface markers of the mDC samples are documented in Figure 10. In summary, the results suggest that no phenotype (or differentiation) changes occur following extended culture on the different AM samples compared to the untreated AM control. Interestingly, small increases in the expression of CD86, CD209 and HLA-DR were found to be induced following culture (48h) on the TG-treated AM sample compared to culture on TCP. Moreover, a decrease in CD206 expression also appeared to occur following culture on the AM samples compared to TCP at the 48 hour time-point. Analysis of the endocytic ability of the mDC was achieved using the FITC-dextran uptake assay and summarised in Figure 11. Overall, absolute MFI values increased with time but no differences were observed between the cells when cultured on the different AM substrates.

4. DISCUSSION

The major component of the amniotic membrane (AM) is collagen which is further interdispersed with a variety of other ECM proteins [Sippel *et al.*, 2001]. Although this matrix composition renders the AM insoluble in physiological (body water) pH, native AM is still subject to quick *in vivo* degradation due to its susceptibility to proteolytic attack and thermal instability [Fujisato *et al.*, 1999]. Moreover, further exploitation of the AM in regenerative medicine is limited by its weak mechanical characteristics, stability post-storage and difficulty during manual handling. Although several methodologies, mainly targeting the cross-linking of collagens, have been employed to improve the mechanical properties of AM, they are susceptible to leaving residual catalysts or by-products that may induce an inflammatory response [Lee *et al.*, 2001; Matuda *et al.*, 1999]. To circumvent such problems, in this study, we aimed to improve the mechanical properties of the AM by treating it with the biological enzyme, transglutminase (TG). Previous work including those of others have demonstrated that TG is capable of cross-linking collagen and its derivatives resulting in novel biomaterials with improved mechanical, biological and biocompatibility characteristics [Zeugolis *et al.*, 2010; O'Halloran *et al.*, 2006; Chau *et al.*, 2005; McHale *et al.*, 2005; Orban *et al.*, 2004; Chen *et al.*, 2003]. In addition, a significant portion of the other ECM proteins that contribute to the AM structure are also substrates that can be involved in the TG cross-linking reaction [Griffin *et al.*, 2002; Greenberg *et al.*, 1991]. Initial work using 0.01-10% (w/v) TG, considerable physical and manual handling differences of the AM were observed (data not shown) and samples were consequently treated with an optimal concentration of 1% (w/v) TG based on a combination of costs, sample preparation, physical and biological characterisation. As such, following treatment with TG, our results suggest that a physical modification/cross-linking of the native AM has occurred. SEM imaging of the untreated AM show the characteristic network of fibrous matter, most likely collagen, as previously described [Wiese, 1975]. On treating the AM with TG, a tighter and denser association between the individual fibres becomes more apparent and in addition, the lower stratum appears more uniform, smoother and with a more organised arrangement. This observation itself may be exploited as a pre- or post-treatment procedure for the standardisation of the membrane before or after long-term storage. Interestingly, a similar characteristic "bundling" of collagen type I fibrils has been reported by Chau and colleagues following treatment with microbial TG and analysed accordingly using AFM [Chau *et al.* 2007]. In contrast, the BSA-treated TG samples (which act as a protein-only control) displayed the loose fibrous network typical of native AM but also a significant amount of aggregated globular material which was absent in the untreated membrane sample and likely to be protein/component derived from the BSA. Although as described earlier, the AM is composed of distinct tissue layers which contain range of proteins, the identification of the exact proteins involved during the TG treatment is not required and is indeed beyond the scope of this study; the ultimate aim of this study is to investigate the effects of the TG-treatment on native AM prior its medical application.

Indication of change in the physical property of the modified AM was also suggested by analysis using the Love wave surface acoustic device: a large shear storage modulus represents a mechanical enhanced/stronger material. Moreover, a lower amplitude displacement value is characteristic of a more robust/stiffer material. Accordingly, our

results indicate that the TG-treated AM exhibited both a greater shear storage modulus and a lower amplitude displacement value compared to the untreated sample. Comparative studies were previously reported with collagen, fibrin and gelatin-based substrates following treatment with TG in which their corresponding mechanical properties were significantly enhanced by the cross-linking reaction [Akpalo and Larreta-Garde, 2010; O'Halloran *et al.*, 2008 and 2006; Hu and Messersmith, 2005; Broderick *et al.*, 2005]. Although not as significant as the TG-treated sample, enhancement of the mechanical properties of the BSA-treated AM also occurred. However, as BSA does not impart any cross-linking effect, the increase in stability may be attributed to an 'artefact' of BSA coating on the AM directly or by indirectly modulating the acoustic sampling/device. In addition, this enhanced physical characteristic inferred a greater resistance to proteolytic attack as previously documented for a collagen-based biomaterial [Chau *et al.*, 2005] and our results demonstrate a similar behaviour when the AM is exposed to different concentrations of collagenase. Enhanced resistance to degradation (0.02mg/ml collagenase) was maintained and observed for up to 144 hours in the TG-treated AM sample; a suitable timeframe and bioactivity for wound healing and repair to occur. In contrast, native and BSA-treated AM samples demonstrated signs of enzymatic breakdown even at the lower concentration of collagenase (0.002mg/ml) compared to a relatively intact TG-treated membrane sample at the corresponding time-points. At the highest concentration of collagenase (1mg/ml) used in this study, a level analogous to significant tissue remodelling and repair in the eye [Girard *et al.*, 1993], the majority of the membranes suffered extensive degradation within 24h although several larger membrane fragments were still present in the 48h time-point for the TG-treated AM samples. In contrast, membranes were completely solubilised at the 48h time-point for both the untreated and BSA-treated AM samples. Interestingly, MMP-1 and MMP-13 are found in the amniotic fluid at all stages of pregnancy and suggests that a close interaction between the AM and the collagenases exists [Fortunato *et al.*, 2004]. As such, although our TG-treatment may initially alter the AM's susceptibility to collagenase degradation, it still ultimately allows the process to occur which may be important for developing a localised wound healing patch (i.e. following amniocentesis).

The most frequently exploited application of the AM is in ophthalmology as a novel treatment for acute ocular surface diseases and has resulted in a variety of commercial therapeutic products coming to the market (e.g. ArBlast[®], AmbioDry[®], AmnioGraft[®] and ProKera[®]). However, an important stipulation of the biomaterial (especially for corneal regeneration and repair) is that it should allow a degree of transparency to occur; in effect, allow sight or vision [Connon *et al.*, 2010; Nakamura *et al.*, 2004 and 2003; Koizumi and Kinoshita, 2003]. As reported in our investigation, the TG-treated AM demonstrates enhanced mechanical strength and stiffness without affecting its transparency compared to the untreated membrane. However, this *transparency assay* is a subjective test and the transmission of light and/or refractive index would need to be determined using alternative established methodologies. Coincidentally, these visual characteristics would not be relevant if the AM were to be used as a wound dressing or patch for tissue regeneration or repair.

The attachment, spreading and proliferation characteristics of the U5637 cells and mDC were slightly enhanced during culture on the TG-treated AM compared to the untreated

membrane. Although no major increases were observed in these biological characteristics, the results suggest that the TG treatment itself did not damage or cause any detrimental effect to the native membrane which has already been established as a natural cell culture substrate [Connon *et al.*, 2010]. Moreover, the effect of the AM on cell differentiation and its interaction with immune cells were assessed via culture with mDC. DC are specialised antigen presenting cells which serve as sentinels of the immune system and play a key role in the initiation of immune responses and activation of adaptive immune cells such as T-cells. DC are amongst the first immune cells that come in contact with foreign antigens and constantly monitor/sample their microenvironment for the presence of such material using different mechanisms including endocytosis. DC maturation, as evidenced by up-regulation of surface markers such as CD83, CD86 and HLA-DR, is usually observed after encountering “danger signals” and could indicate immune activation [Banchereau *et al.*, 2000; Banchereau and Steinman, 1998]. Our results suggest that no significant long-term phenotype or functional changes occur for the mDC cultured on any of the AM samples. However, interestingly, small increases in the expression of CD86, CD209 and HLA-DR were found to be induced following 48h of culture on the TG-treated AM sample compared to culture on TCP. This observation may be explained by a previous finding within our group which reported that culturing mDC on either a fibronectin or laminin substrate enhanced their endocytic ability and altered their phenotype [Garcia-Nieto *et al.*, 2010]; clearly this may be a possibility as ECM components of the ECM include both fibronectin and laminin [Sippel *et al.*, 2001]. As such, these results suggest that induction of an inflammatory response may be minimal (with no detrimental changes in the basic function or activation status of DC) when the AM is exploited for *in vivo* use. Furthermore, TG has already been shown to be widely distributed in its native form in the ciliary body, zonular fibres and blood vessel walls in the eye [Raghunath *et al.*, 1999]. Based on the enhancement of the aforementioned biological characteristics, the TG-treated AM may also be considered as a novel tissue engineering scaffold for cell culture applications.

Although it is widely reported that the AM exhibits an anti-angiogenic effect, the exact mechanism by which this occurs has yet to be fully elucidated [Dua *et al.*, 2004, Hao *et al.*, 2000; Bennett *et al.*, 1980 and 1982]. As several studies have also demonstrated a panel of growth factors and/or cells within the AM that may contribute to the upregulation of angiogenesis [Wolbank *et al.*, 2009; Kim and Tseng, 1995; Magnatii *et al.*, 1989], it may be feasible to suggest that the AM *itself* may solely dictate the pro- or anti-angiogenic response when used in a clinical setting. Our results suggest that native AM, following long-term storage and extensive wash steps during preparation/manipulation, allows angiogenesis to occur when exploited in a CAM assay. Interestingly, following treatment with TG, this membrane still retains the pro-angiogenic characteristic and in some cases (where n = 5), there even appeared to be an enhancement in the number and quality of vessels formed. A negligible improvement/effect on angiogenesis was observed with the BSA-treated AM compared to the untreated AM sample. However, care must be taken when interpreting this data due to the variability in models (i.e. egg sample) and effects derived from species cross-specificity.

5. CONCLUSIONS

In summary, we demonstrate that treating native AM with TG leads to enhancement and improvement in the mechanical properties of the membrane which is likely due to a change in its physical structure and susceptibility to proteolytic degradation. Interestingly, this treatment does not change the visual transparency of the membrane nor affect its biological/biocompatibility characteristics. Taken together, these findings suggest that the TG treatment on the AM may be exploited as a method to produce novel biomaterials for cell culture applications and/or regenerative therapy or as a treatment to stabilise and standardise membrane preparation before or after long-term storage.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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AUTHOR CONTRIBUTIONS

DYSC: design of experiments, TG activity, cell culture, DC work, flow cytometry, CAM assay, drafting of manuscript, final approval of manuscript; SB: cell culture, AM manipulation, DC work, flow cytometry, transparency assay, degradation and protein assay, CAM assay, final approval of manuscript; MLM: design of experiments, mechanical testing, drafting of manuscript, final approval of manuscript; VH: SEM, degradation and protein assay, drafting of manuscript, final approval of manuscript; NLT: design of experiments, AM preparation, final approval of manuscript; HSD: procurement of AM, funding, final approval of manuscript; FRAJR: design of experiments, funding, final approval of manuscript; AMG: design of experiments, funding, final approval of manuscript.

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FIGURE LEGENDS

Figure 1. Scanning electron microscopy (SEM) images of untreated and treated amniotic membrane (AM) samples. Untreated, 1% (w/v) BSA-treated and 1% (w/v) TG-treated AM samples (24h, at 37°C and 5% CO₂) were mounted onto aluminium stubs, gold coated under an argon atmosphere before being imaged using a JOEL 6060LV variable pressure SEM operating at 10kV accelerating voltage at x200, x750 and x1500 magnifications

Figure 2. Scanning electron microscopy (SEM) images of selective sides of untreated and treated amniotic membrane (AM) samples. Untreated and 1% (w/v) TG-treated AM samples (24h, at 37°C and 5% CO₂) were mounted onto aluminium stubs, gold coated under an argon atmosphere before being imaged using a JOEL 6060LV variable pressure SEM operating at 10kV accelerating voltage at x1500 and x1000 magnifications

Figure 3. Mechanical characteristic profiles of untreated and treated amniotic membrane (AM) samples. 0.3cm² samples of untreated, 1% (w/v) BSA-treated and 1% (w/v) TG-treated AM were assessed using a Love wave surface acoustic device operating at 118MHz and 6mm separation path length. The induced damping relative to the unperturbed wavenumber ($\Delta\alpha/\beta$), the change in wavenumber relative to the unperturbed wavenumber device a ($\Delta\beta/\beta$) and shear storage modulus were determined by deducting the residual background reading of the unloaded device from the sample readings. Results are the mean values \pm SD from three independent samples with density taken as 997.6Kg/m³. Statistical analysis was performed using a Student's *t*-test with comparisons between the untreated AM and TG-treated AM samples and values corresponding to ($p < 0.05$) represented with *.

Figure 4. Transparency characteristics of untreated and treated amniotic membrane (AM) samples. 0.5cm² samples of untreated, 1% (w/v) BSA-treated and 1% (w/v) TG-treated AM were assessed for their transparency by overlaying the membrane samples onto a piece of waterproof text card and the resulting image captured with a Fujitsu F80EXR 12MP digital camera

Figure 5. Susceptibility to collagenase degradation of untreated and treated amniotic membrane (AM) samples. 0.5cm² samples of untreated, 1% (w/v) BSA-treated and 1% (w/v) TG-treated AM were exposed to 0.2mg/ml of microbial collagenase (*C. histolyticum*) in a humidified-atmosphere incubator, at 37°C and with 5% CO₂, for several selected time points. Samples were then viewed using a Veho VMS-004 Discovery microscope and images captured using the in-built camera and Q-Capture software

Figure 6. Supernatant protein concentration of untreated and treated amniotic membrane (AM) samples following exposure to collagenase. Residual solubilised protein in the

supernatants of the AM samples were assessed using the Bio-Rad DC protein assay kit following exposure to 0.2mg/ml of microbial collagenase (*C. histolyticum*) in a humidified-atmosphere incubator, at 37°C and with 5% CO₂, for several selected time points before the absorbance was read at 750nm using an Tecan Infinite® M200 PRO plate reader. Results are the mean values ±SD from three independent experiments with each having triplicate samples. Statistical analysis was performed using a Student's *t*-test with comparisons between the untreated AM and TG-treated AM samples and values corresponding to ($p<0.05$) represented with *, ($p<0.01$) represented with ** and ($p<0.001$) represented with ***. AM = untreated AM, BAM = BSA-treated AM, TAM = TG-treated AM, TCP = tissue culture plastic

Figure 7. Attachment and spreading characteristics of U5637 cells and mDC on untreated and treated amniotic membrane (AM) samples. After 72 hours of culture on the substrates in a humidified-atmosphere incubator, at 37°C and with 5% CO₂, cells were washed before being fixed in 4% (w/v) paraformaldehyde, permeabilised using 0.1% (v/v) Triton X-100 in PBS and stained with May-Grunwald and Giemsa stains. Samples were then viewed with Nikon SM21500 microscope (x20 magnification) and images captured using the in-built Nikon Digital DS-L1 image capture unit. TCP = tissue culture plastic

Figure 8. Metabolic activity characteristics of U5637 cells and mDC on untreated and treated amniotic membrane (AM) samples. (A) U5637 cells and (B) mDC were cultured on the substrates in a humidified-atmosphere incubator, at 37°C and with 5% CO₂, cells for the appropriate time points before metabolic activity was determined using the CellTiter AQ solution according to the manufacturer's instructions. Sample absorbances were read at 492nm using an Optima FLUOstar® plate reader. Results are the mean values ±SD from three independent experiments with each having triplicate samples. Statistical analysis was performed using a Student's *t*-test with comparisons between the untreated AM and TG-treated AM samples and values corresponding to ($p<0.05$) represented with *. Comparisons between the BSA-treated AM and TG-treated AM samples with values corresponding to ($p<0.05$) are represented with ⁺. AM = untreated AM, BAM = BSA-treated AM, TAM = TG-treated AM, TCP = tissue culture plastic

Figure 9. Angiogenic potential of the untreated and treated amniotic membrane (AM) samples. The angiogenic potential of the AM samples was determined using the chick chorioallantoic membrane (CAM) assay. 0.5cm² AM samples were initially placed onto the chorion of a 4 day old egg and then, following 6 days of incubation at 37°C, the AM/chorion was viewed using a Leica MZ16F light microscope (x40 magnification) and images captured using the in-built camera and Q-Capture software. Images are a representative of 5 independent experiments.

Figure 10. Phenotype of mDC cultured on untreated and treated amniotic membrane (AM) samples. Summary of absolute MFI expression for mDC surface markers when cultured on the different substrates at the 48h time-point. [absolute MFI = (MFI of sample) – (MFI isotype/unstained)]. Samples were analysed using a Beckman Coulter EPICS Altra flow cytometer with 5000 events sampled per condition. Values represent average value from n=2 experiments

Figure 11. Endocytic characteristics of mDC cultured on untreated and treated amniotic membrane (AM) samples. Summary of absolute MFI expression for FITC-dextran uptake of mDC when cultured on the different substrates at the 48h time-point. [absolute MFI = (MFI of sample) – (MFI isotype/unstained)]. A 4°C control sample was also included in the corresponding MFI calculations. Samples were analysed using a Beckman Coulter EPICS Altra flow cytometer with 8000 events sampled per condition. Values represent average value from n=2 experiments