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► **To cite this version:**

Joseph Hardwicke, Bing Song, Ryan Moseley, David W Thomas. INVESTIGATION OF THE POTENTIAL OF POLYMER THERAPEUTICS IN CORNEAL RE-EPITHELIALISATION. *British Journal of Ophthalmology*, 2010, 94 (12), pp.1566. 10.1136/bjo.2009.177295 . hal-00574836

**HAL Id: hal-00574836**

**<https://hal.science/hal-00574836>**

Submitted on 9 Mar 2011

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# INVESTIGATION OF THE POTENTIAL OF POLYMER THERAPEUTICS IN CORNEAL RE-EPITHELIALISATION

Joseph Hardwicke\*, Bing Song, Ryan Moseley, David W Thomas

Wound Biology Group, Cardiff Institute of Tissue Engineering and Repair (CITER),  
Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University,  
Heath Park, Cardiff CF14 4XY, UK.

\*Corresponding Author

**ABSTRACT:** We describe the first use of a bioresponsive polymer therapeutic agent in the promotion of corneal re-epithelialisation after injury in an *ex vivo* whole-eye organ culture model. A polymer-protein conjugate consisting of dextrin and recombinant human epidermal growth factor (rhEGF) was synthesized and applied as a single dose to a 2mm *ex vivo* corneal ulcer, in culture. Enhanced wound healing was observed in response to dextrin-rhEGF, when exposed to  $\alpha$ -amylase, compared to controls. This highlights the potential for polymer therapeutics to provide a platform for bioresponsive drug/protein delivery in the field of ophthalmology.

**KEY WORDS:** cornea, wound healing, animal study, pharmacology

**INNOVATION:** The descriptor “polymer therapeutic” is an umbrella term used to describe polymeric drugs, polymer–drug conjugates, polyplexes, polymer–protein conjugates and polymeric micelles, to which a drug can be bound<sup>1</sup>. In the context of polymer-protein conjugates, the stability and solubility of proteins can be enhanced, and their biological half-life and immunogenicity reduced, via polymer conjugation<sup>2-3</sup>. To this date, although polymer therapeutics have been licensed for use in humans (Table 1), they have not been investigated widely in the field of corneal wound healing.

We have been developing a novel controlled release formulation of recombinant human EGF (rhEGF), conjugated to the *degradable* polysaccharide, dextrin<sup>4</sup>. This provides a controlled delivery platform for the release of rhEGF in response to endogenous  $\alpha$ -amylase, which is present in the tear film<sup>5</sup>. When conjugated to the dextrin polymer, the bioactivity of the growth factor is decreased, but an increase in protein stability is afforded. Bioactivity can be fully restored after polymer degradation by  $\alpha$ -amylase, and the dextrin breakdown products, maltose and isomaltose are further metabolised and/or excreted<sup>6</sup>. This Polymer-masking UnMasking Protein Therapy hypothesis PUMPT hypothesis<sup>7</sup>, has been shown to stimulate dermal keratinocyte proliferation *in vitro*, and enhances both the half-life of rhEGF and the stability in response to wound proteolysis<sup>4</sup>.

In animal studies, EGF stimulated proliferation and DNA synthesis of corneal epithelium and increased the epithelial healing rate<sup>8-13</sup>. The initial hope of EGF to progress to a licensed product for clinical use in the early 1990's<sup>14-16</sup>, did not come to fruition. The lack of significant effects from EGF, in humans, applied post penetrating keratoplasty<sup>17</sup>, did not match the previous findings from animal studies. The

aforementioned clinical studies have involved the repeated administration of EGF, due to the short half-life and the action of the tear film<sup>18</sup>.

**METHODS:** We have evaluated the efficacy of single dose dextrin-rhEGF in an *ex vivo* organ culture model of corneal wound healing<sup>19-20</sup>. The synthesis and characterization of the conjugate was reproducible (Figure 1)<sup>4</sup>. Two-month old male Wistar Han rats (250-300g; Charles River Laboratories, Margate, UK), were sacrificed by CO<sub>2</sub> asphyxiation, confirmed by cervical dislocation. Wounding was performed within 1 hour of sacrifice. The globe was irrigated with Balanced Salt Solution (BSS Plus<sup>®</sup>; Alcon Laboratories, Hemel Hemstead, UK) containing 0.1% EDTA. A full-thickness 2 mm corneal abrasion was produced by excision. The globe was then enucleated. Tissue culture medium (DMEM supplemented with penicillin G, 100U/mL; streptomycin sulphate, 100µg/mL; amphotericin B, 0.25µg/mL; L-glutamine, 2mM (Invitrogen; Paisley, UK): serum-free media; SFM) was supplemented with study compounds, as shown in Table 2, and added to the globes (n = 4). Specimens were maintained at 37 °C / 5% CO<sub>2</sub>, for 64 h. Wound healing was assessed by digital wound photography and fluorescein staining. After 64h, globes were fixed in 10% formal saline, embedded in wax, sectioned and stained with H&E. Statistical analyses were undertaken using GraphPad Prism<sup>®</sup>, version 4.00 (GraphPad Software, San Diego, USA). Data were compared using a one-way ANOVA, with a Bonferroni post-test, for group analysis. Results were expressed as a mean and standard deviation (S.D.). Statistical significance was considered at a probability of  $P < 0.05$ .

**RESULTS:** This *ex vivo* model of corneal wound healing was sensitive to stimulation by foetal calf serum (FCS) and by dextrin-rhEGF. In the conjugated form, the rhEGF promoted a significant increase in the rate of corneal wound healing, with wound closure by 48 hours, in the presence of human physiological levels of  $\alpha$ -amylase<sup>23-24</sup> compared to free rhEGF. With the addition of exogenous  $\alpha$ -amylase, at physiological concentrations, rhEGF was released, or “unmasked” from the polymer, thereby restoring bioactivity (Figures 2 and 3).

There was a significant improvement in wound healing ( $P < 0.006$ ), in response to serum (SCM; Group 2), compared to the serum-free (SFM; Group 1) and  $\alpha$ -amylase (Group 3) controls, at 24 h (Figure 4a). All wounds in the SCM group (Group 2) had fully re-epithelialised by 48 h, whilst the average wound areas in the serum-free controls (Groups 1 and 3), were  $24.5 \pm 8.1\%$  and  $31.4 \pm 5\%$ , respectively, of that at time 0 h ( $P < 0.001$ ). In the unconjugated rhEGF Groups (Groups 4 and 5), there were no significant effects on wound re-epithelialisation ( $P > 0.05$ ), at either concentration, and neither resulted in total wound re-epithelialisation. Wound areas, at 64 h, were  $5.6 \pm 11.3\%$  and  $23.4 \pm 21.4\%$ , of the total wound area at time 0 h, for the 10  $\mu\text{g/mL}$  rhEGF, and 1  $\mu\text{g/mL}$  rhEGF Groups, respectively (mean  $\pm$  S.D.) (Figure 4b).

In the dextrin-rhEGF conjugate Study Groups (Groups 6 to 8), there was failed re-epithelialisation, in the absence of physiological concentrations of  $\alpha$ -amylase (Group 6; “masked”), with an abrasion of  $23.4 \pm 5.5\%$  of that at time 0, persisting at 64 h. This was not significantly different to the SFM control (Group 1) ( $P > 0.05$ ). Upon the addition of  $\alpha$ -amylase (93 i.u./L; “unmasking”), there was a significant decrease in the wound area, at 24 h, in both the 1  $\mu\text{g/mL}$  rhEGF equivalent (Group 7) and 10  $\mu\text{g/mL}$  rhEGF equivalent (Group 8) Groups ( $P < 0.006$ ). The dextrin-rhEGF

conjugate was significantly more effective, at decreasing the wound area, at 24 h, at the lower dose (1  $\mu\text{g/mL}$  rhEGF equivalent), than at the higher dose (10  $\mu\text{g/mL}$  rhEGF equivalent) ( $P = 0.027$ ) (Figure 4c).

**DISCUSSION:** The increased efficacy of the dextrin-rhEGF conjugate, in enhancing corneal wound healing, may be due to the sustained release phenomena<sup>4</sup>. In the *ex vivo* model described herein, the rhEGF was applied as a single dose at the outset of the experiment, and the free rhEGF may have been degraded, due to the short half-life of EGF<sup>18</sup>. Although initial wound closure rates are similar in the first 24 h between rhEGF and dextrin-rhEGF (+ $\alpha$ -amylase), this apparent difference tailed off as time progressed. In the *ex vivo* organ culture model, the pharmacokinetics of EGF will differ, but the sustained release of rhEGF, from the  $\alpha$ -amylase-mediated, degradation of the dextrin-rhEGF conjugate, may account for the enhanced wound healing in the dextrin-rhEGF study groups. The *ex vivo* model of corneal wound healing, has been shown to be reproducible, and that it is a reliable model for further analysis of polymer-protein conjugates. This is the first application of polymer therapeutics specifically designed for tissue regeneration, in such a model, and these preliminary investigations highlight the potential for the application of these therapeutics to enhance corneal wound healing. This application of a specific endogenous trigger to promote the controlled release of a bioactive peptide, may be applied to the healing and/or regeneration of other tissues in the future.

**ACKNOWLEDGEMENTS:** J.H. was funded by a Fellowship from The Healing Foundation (Registered Charity number 1078666) and the Welsh Office for Research and Development (WORD) and the authors would like to acknowledge support from EPSRC platform grant No. EP/C 013220/1.

**COMPETING INTEREST:** None declared.

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**Table 1**

<b>Name</b>	<b>Manufacturer</b>	<b>Polymer</b>	<b>Protein</b>	<b>Indication</b>
Neulasta™ (Pegfilgrastim)	Amgen	PEG	GCSF	Chemotherapy- induced neutropaenia
Pegasys™ (Peginterferon alfa)	Roche	PEG	α-IFN-2a	Hepatitis C
ViraferonPeg™ (Peginterferon alfa)	Schering-Plough	PEG	α-IFN-2b	Hepatitis C
Somavert™ (Pegvisomant)	Pfizer	PEG	Human growth hormone antagonist	Acromegaly

**Table 2**

<b>Group</b>	<b>Supplement</b>
1	-----
2	10% foetal calf serum (FCS; Invitrogen)
3	$\alpha$ -amylase (93 i.u./L, Sigma-Aldrich, Poole, UK)
4	rhEGF (1 $\mu$ g/mL)
5	rhEGF (10 $\mu$ g/mL)
6	dextrin-rhEGF (1 $\mu$ g/mL rhEGF equivalent, in PBS)
7	dextrin-rhEGF (1 $\mu$ g/mL rhEGF equivalent, in PBS) and $\alpha$ -amylase (93 i.u./L) (pre-incubated for 24 h, at 37 °C)
8	dextrin-rhEGF (10 $\mu$ g/mL rhEGF equivalent, in PBS) and $\alpha$ -amylase (93 i.u./L) (pre-incubated for 24 h, at 37 °C)

**TABLE LEGENDS****Table 1**

Polymer therapeutics that are licensed in the UK. PEG = polyethylene glycol, GCSF = granulocyte colony stimulating factor, IFN = interferon.

**Table 2**

Culture medium supplementation. PBS = phosphate buffered saline, rhEGF = recombinant human epidermal growth factor.

## **FIGURE LEGENDS**

### **Figure 1**

The PUMPT hypothesis. Succinoylated dextrin is conjugated to rhEGF using EDC and sulfo-NHS cross-linkers, to produce a dextrin-rhEGF polymer-protein conjugate. On exposure to  $\alpha$ -amylase (present in human tear fluid), bioactive rhEGF is released from the polymer in a controlled release fashion. rhEGF would induce corneal epithelial cell proliferation and migration, whilst the polymer would be metabolised to the maltose and isomaltose disaccharide units. RT = room temperature.

### **Figure 2**

Typical *ex vivo* corneal wound closure images. Panel (a) shows the healing of the corneal abrasion in serum-free conditions, over 64h. Panel (b) shows the healing of the corneal abrasion in 10% FCS-supplemented media.). Panels (c) to (h) show typical fluorescein staining of the corneal wound in the remaining Study Groups, at 48 h.

### **Figure 3**

Panel (a) shows haematoxylin / eosin staining of a section through a corneal abrasion, in the absence of FCS. Panel (b) shows haematoxylin / eosin staining of a section through a region of corneal abrasion, in presence of FCS. Both specimens were harvested at 64h. The corneal wound is still evident in the absence of FCS-supplementation (Wm = wound margin) (20 x magnification).

### **Figure 4**

Panel (a) shows the corneal wound healing (% reduction in wound area compared to 0

h), over 64 h, in response to FCS (serum-containing media) and serum-free controls. There was a significant decrease ( $*P < 0.006$ ) in wound area, in response to FCS, when compared to serum-free controls. Panel (b) shows the corneal wound area reduction, in response to free rhEGF. The initial rate of wound area reduction (over the first 24 h), was not significantly different ( $P > 0.05$ ) from FCS, but the effect was not sustained over 64 h. Panel (c) shows the corneal wound area reduction, in response to dextrin-rhEGF. In the “masked” ( $\alpha$ -amylase-free) state, the bioactivity of dextrin-rhEGF was reduced to that of serum-free controls ( $P > 0.05$ ), but upon the addition of  $\alpha$ -amylase, at physiological concentrations, bioactivity was significantly restored ( $**P < 0.006$ ). Dextrin-rhEGF was most effective at the 1  $\mu\text{g}/\text{mL}$  concentration ( $***P = 0.027$ ), compared to the conjugate at 10  $\mu\text{g}/\text{mL}$  concentrations.

Figure 1. Hardwicke *et al*

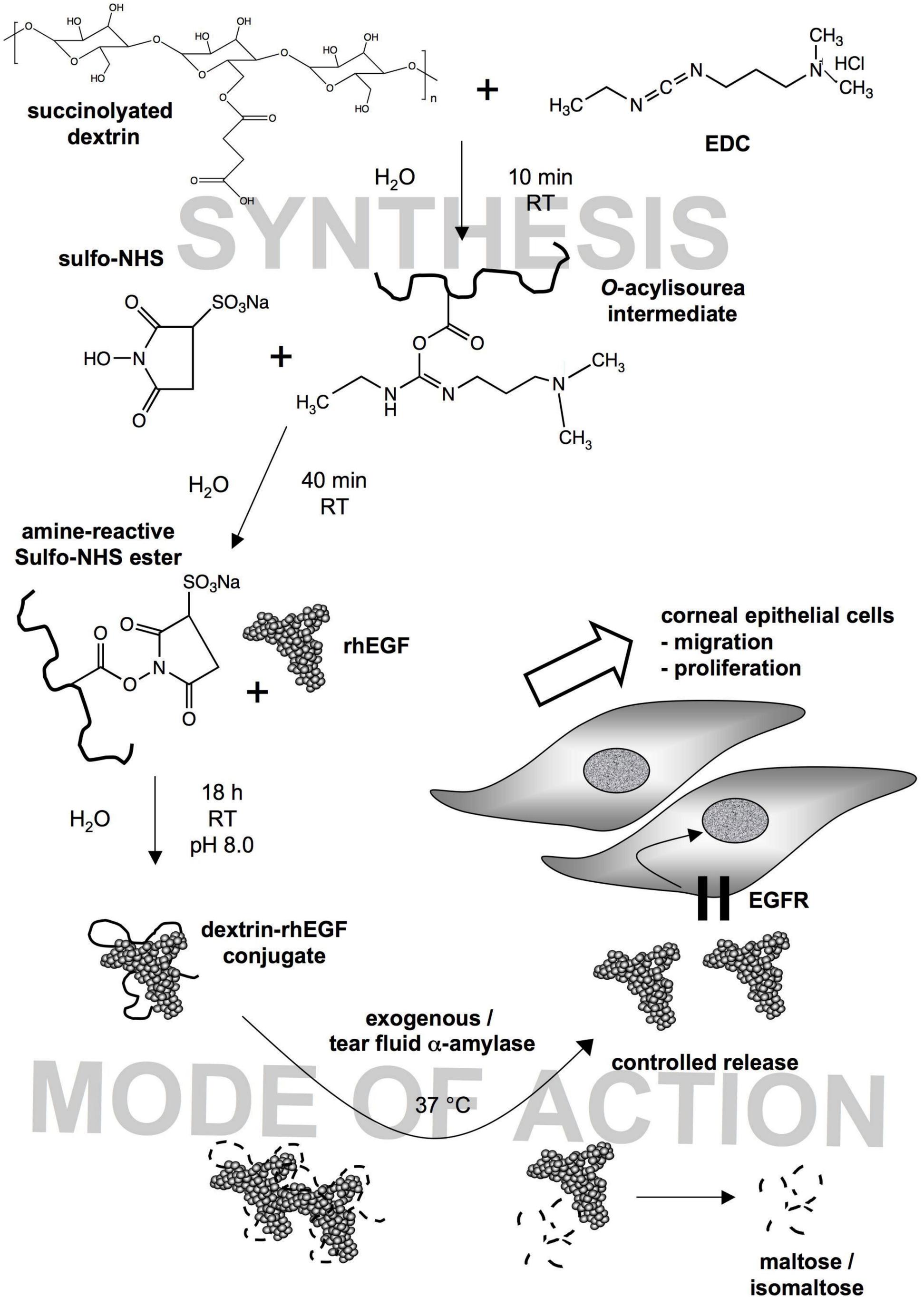


Figure 2. Hardwicke *et al*

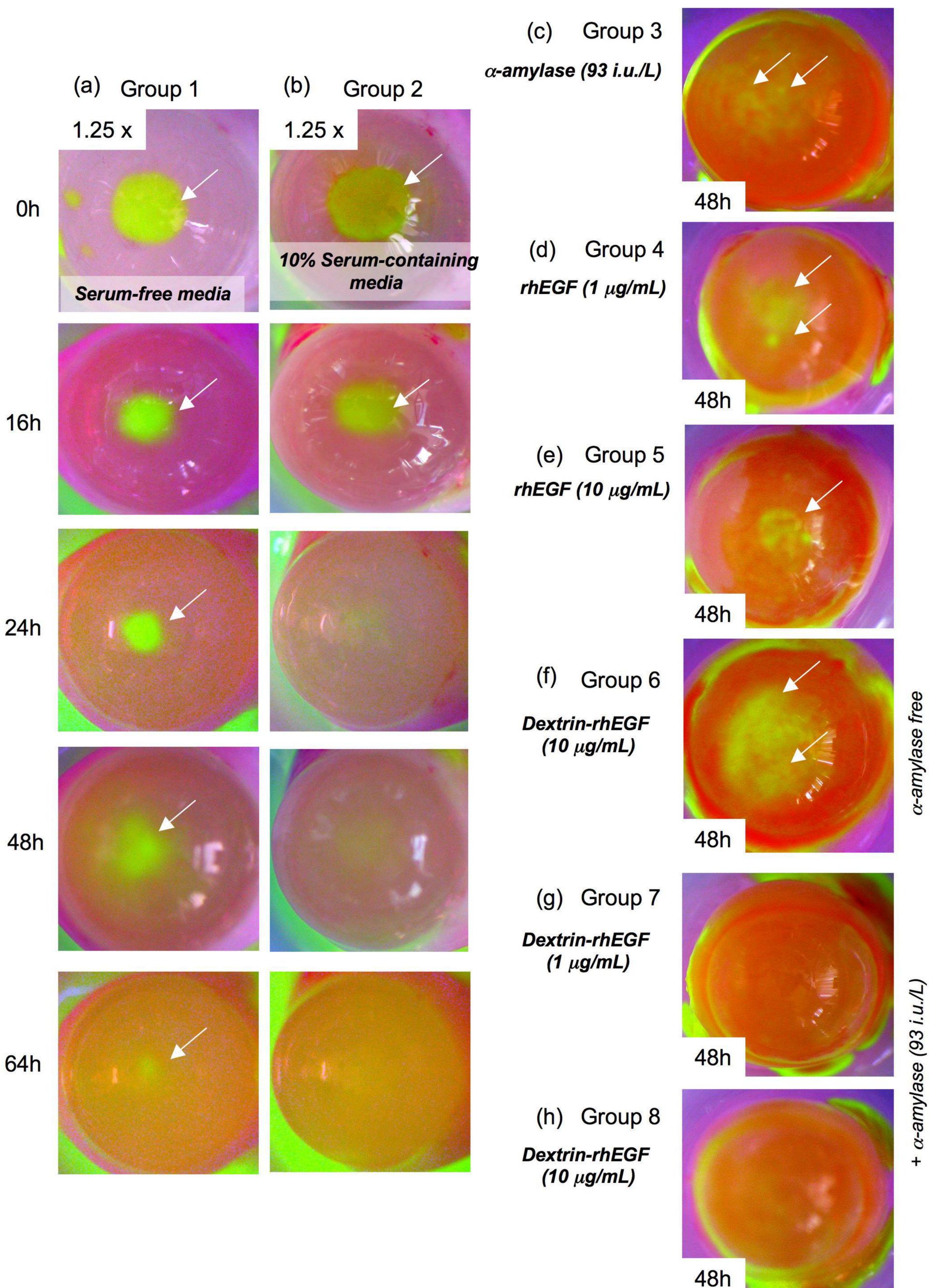


Figure 3. Hardwicke *et al*

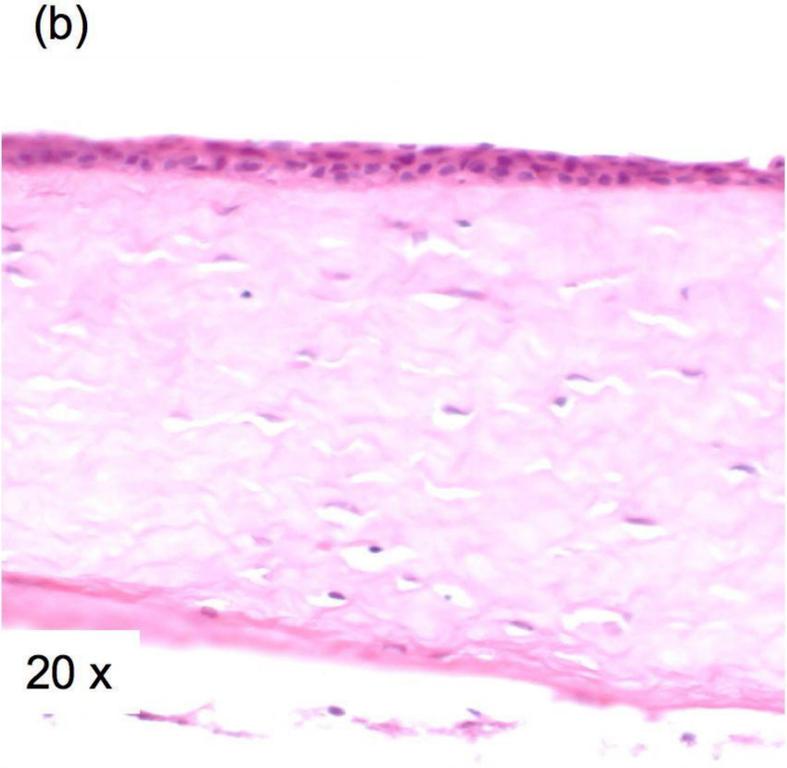
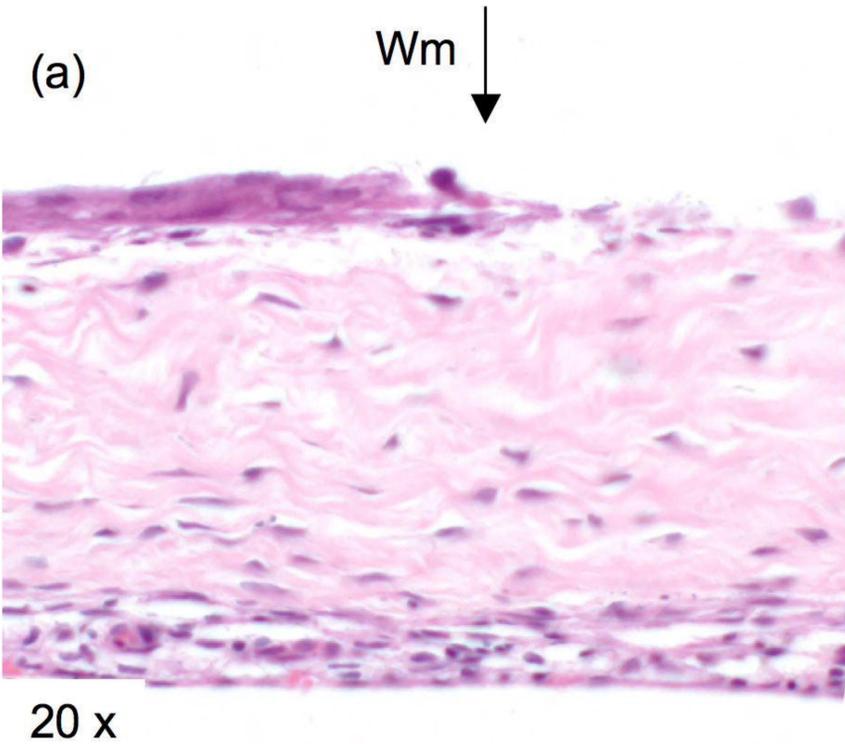


Figure 4. Hardwicke *et al*

