

## Supplementary Materials

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**Supplementary Figure 1** *In vivo* confocal images of pig corneas grafted with CLP-PEG implants and unoperated control corneas at 12 months post-operation.

## Supplementary Methods

### CLP-PEG production and characterization

The 38 amino acid long CLP peptide, Cys-Gly-(Pro-Lys-Gly)<sub>4</sub>(Pro-Hyp-Gly)<sub>4</sub>(Asp-Hyp-Gly)<sub>4</sub>, was synthesized on a Symphony automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, U.S.A.). 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) (ChemPep Inc., Wellington, FL, USA) was used as the activating reagent. The 0.1mmol scale synthesis used Fmoc-Gly-PEG-PS resin (Applied Biosystems, Life Technologies Europe BV, Sweden) and amino acids in each coupling. The resulting peptides were cleaved from the resin by treatment with a mixture of trifluoroacetic acid (TFA), water and triisopropylsilane (TIS) (95:2.5:2.5 v/v; 10 mL per gram of polymer). They were then filtered and the TFA was evaporated. CLP peptide then was precipitated by the addition of cold diethyl ether, centrifuged and lyophilized. Prior to use, CLP was resuspended in milli-Q water.

Aqueous solutions of 8-arm PEG-maleimide and purified CLP were mixed at a molar ratio of PEG-maleimide:CLP (1:5) at room temperature under Good Laboratory Practice (GLP) conditions. After four days of continuous stirring, a homogenous solution of the bio-conjugate was dialyzed using a 12-14 kD MW cut off tubing (Spectrum Laboratories, Inc., CA, US) for 2-3 days to remove the unreacted reactants. After dialysis, the solution was lyophilized and stored at 4°C until further use.

The bio-conjugation of CLP with 8-arm PEG Maleimide was characterized using <sup>1</sup>H NMR, on an Oxford 300 MHz spectrometer (Varian, CA, US) at room temperature. Briefly 1% solutions of PEG-maleimide and CLP-PEG were made in

C<sub>2</sub>D<sub>6</sub>OS (Dimethyl-d<sub>6</sub> sulfoxide, Armar Chemicals, Döttingen, Switzerland). The resonance of deuterated solvent (C<sub>2</sub>D<sub>6</sub>OS,  $\delta = 2.5$ ) was used as an internal standard.

The triple helical structure of CLP and CLP-PEG were evaluated using Chirascan™ CD Spectrometer (Applied Photophysics Ltd., Surrey, UK). Briefly, 1% of sample solutions were prepared and a quartz cell of 0.1 cm path length was used to record the CD spectra at 180-260 nm wavelengths, at a scan rate of 1nm/s at room temperature.

### **CLP-PEG and recombinant human collagen-phosphorylcholine hydrogels**

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (MO, USA). A T-piece syringe mixing system we previously described<sup>1</sup> was used to make CLP-PEG hydrogels. 500mg of 12% (w/w) CLP-PEG was taken into a 2ml glass syringe and calculated volumes of N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) were added to the syringe mixing system. The molar equivalents of CLP-PEG-NH<sub>2</sub>: EDC was 1:2 and the molar ratio of EDC: NHS was 1:1. All reagents were thoroughly mixed prior to moulding the hydrogel into flat sheets or cornea shape implants.

Benchmark hydrogels comprising Recombinant Human Collagen type III (RHC; Fibrogen Inc., San Francisco, CA, USA) with 2-methacryloyloxyethyl phosphorylcholine (MPC; Paramount Fine Chemicals Co. Ltd, Dalian, China) (RHC-MPC) were fabricated using our previously published protocol<sup>2</sup>.

### **Characterisation of hydrogels**

All samples were tested in triplicate. After demoulding, the hydrogels were kept in PBS for 24 hours. The wet weight of hydrogels ( $W_0$ ) was obtained after gentle

blotting on filter paper to remove surface water. The samples were then dried under vacuum until a constant weight ( $W$ ) was obtained. The total equilibrated water content of the hydrogels ( $W_t$ ) was calculated according to the following equation:  $W_t = \{(W - W_0)/W\} \times 100\%$

Optical properties such as transmission of white light and back-scattering were measured at room temperature using a custom-built instrument.<sup>2</sup> The percent transmission of the samples was compared to the open beam intensity. The relative percent of light back scattered from the collimated beam by the sample was measured with a circular array of 8 photodiodes each at 30 degrees off axis.

The mechanical properties of CLP-PEG hydrogels were measured by using an Instron universal test machine (Biopuls 3343, High Wycombe, UK) equipped with Bluehell2 software. The measurements were carried out under water-immersion at 37°C. Dumb-bell shaped hydrogels of 500µm thick with a gauge length of 14 x 6 mm and grips at each end of 10 x 6 mm, were cast in moulds. The mechanical testing was carried out using a 50N load cell with a crosshead speed of 10 mm/min.

The resistance of CLP-PEG and RHC-MPC hydrogels to collagenase was determined as we previously described.<sup>3</sup> *Clostridium histolyticum* (Sigma-Aldrich, St.Louis, USA). Hydrogels were placed in a vial containing 5U/mL collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St.Louis, USA) in 0.1M Tris-HCl (pH 7.4) and 5mM CaCl<sub>2</sub> at 37°C. The collagenase solution was changed at every 8 hours and the percent residual mass of the sample was measured at different time points.

### **Subcutaneous implantation in rats**

After approval from the local ethical committee (Linköpings Djurförsöksetiska Nämnd) and in compliance with the Swedish Animal Welfare

Ordinance and the Animal Welfare Act, CLP-PEG hydrogel samples, 1 cm diameter x 500 µm thick, were implanted subcutaneously into rats as per ISO 10993-6 to test for local reaction to CLP-PEG hydrogels. Each sample was inserted into a subcutaneous pocket created by blunt dissection in the paravertebral region of the back of 9 week old Wistar rats. A total of 4 samples were evaluated in a total of 4 rats. Implantation sites were checked for healing, skin appearance, and re-growth of hair post-surgery. After 90 days, the rats were euthanized and the implants were harvested and fixed in 4% paraformaldehyde for histopathological examination following haematoxylin and eosin staining (H&E).

#### **CLP-PEG implantation and evaluation in mini-pig corneas**

In preparation for clinical translation, in compliance with the OECD Principle of Good Laboratory Practice (GLP), ENV/MC/CHEM (98) 17, 1997, and with local ethical permission from Stockholms Norra Djurförsöksetiska Nämnd, one CLP-PEG implant was grafted into one cornea each of four Gottingen mini-pigs (Ellegaard, Denmark) by anterior lamellar keratoplasty (ALK), at Adlego Biomedical AB (Solna, Sweden). Animals were intubated and anaesthetized prior to surgery. The right cornea of each pig eye was cut with a 6.5 mm circular trephine to a depth of 500 µm, and the corneal button was then manually dissected with a diamond knife and removed. Hydrogel implants were cut with a 6.75 mm diameter trephine and placed into the surgical bed. A piece of clinical human amniotic membrane (HAM) (St:Erik's Eye Hospital, Stockholm) was placed over the implant to suppress undesired inflammation and the implants were kept in place with overlying sutures (10/0 monofilament nylon). Upon completion of the surgery, an antibacterial and anti-inflammatory ophthalmic suspension (Tobrasone with 3 mg/ml dexamethasone and

1mg/ml tobramycin, Alcon, Sweden) was administered. The maintenance dose was 1 drop, 3 times daily for 5 weeks. The unoperated contralateral corneas and RHC-MPC hydrogels served as controls.

The health status of all animals were monitored throughout the 12 month study. The corneas and implants were evaluated before surgery, at 5 weeks and then at 3, 6, 9 and 12 months after surgery. The examinations were performed by a surgeon who was blinded to which animals received CLP-PEG or RHC-MPC hydrogels. These examinations included slit lamp biomicroscopy (to evaluate haze from a 0 to +4 scale, any neovascularization and general health of the eye), Schirmer's tear test to assess tear function, pachymetry to determine any changes in corneal thickness and *in vivo* confocal microscopy (Heidelberg HRT3 with a Rostock Cornea Module, Heidelberg Engineering GmbH, Dossenheim, Germany) to assess in-growth of corneal cells, nerves and any blood vessels or inflammatory cells.

### **Evaluation of regeneration**

At 12 months post-operation, the mini-pigs were euthanized after their final clinical eye examination. The entire implanted cornea and unoperated contralateral controls were dissected out with 2-3 mm of the surrounding sclera. A central 3 mm diameter, full-thickness sample was trephined out and snap frozen for collagen analyses. The remaining cornea was divided into pieces, with half fixed in 4% buffered paraformaldehyde and processed for histopathological examination by a 3<sup>rd</sup> party certified veterinary pathologist (BioVet AB, Sollentuna, Sweden).

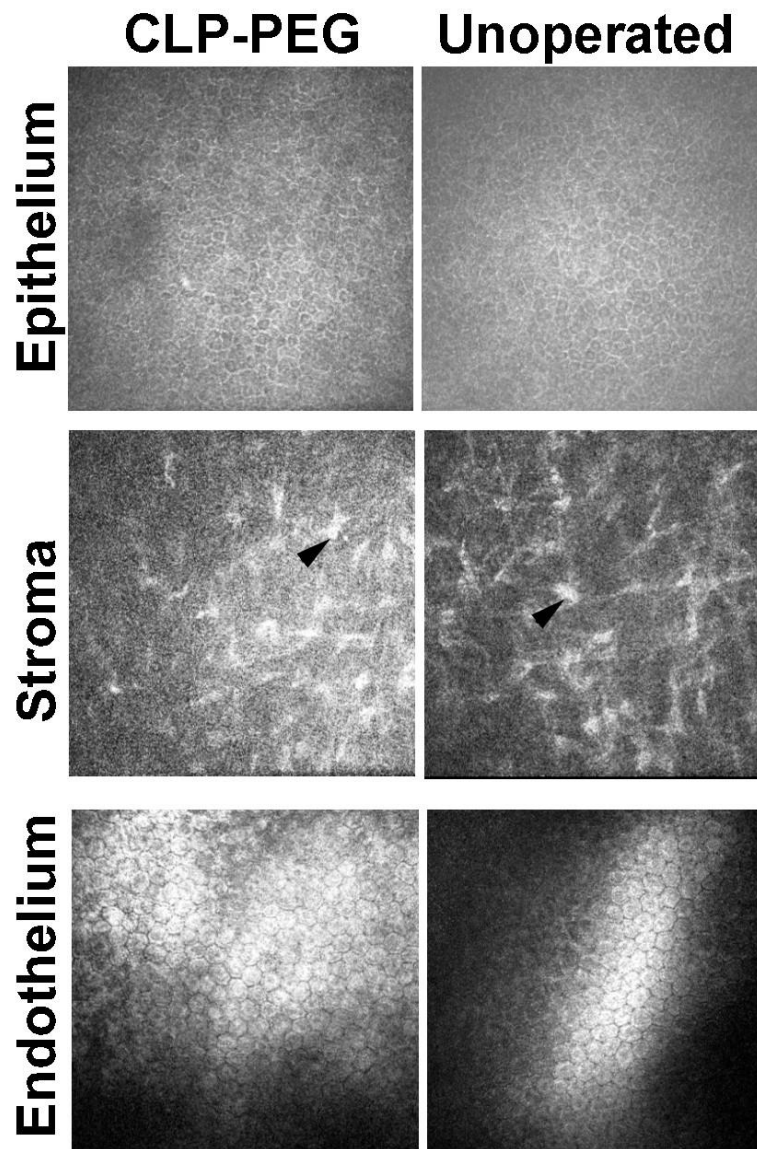
For protein analyses, each frozen 3 mm diameter sample from operated and control corneas was weighed and re-suspended in 10 mM HCl containing 1 mg/mL pepsin. The samples were digested with porcine pepsin at 2-8°C for 96h and the

soluble fraction (supernatant) was recovered by centrifugation. An aliquot of the supernatant was mixed with NuPAGE 4X LDS sample buffer (Life Technologies) denatured at 75°C for 8 minutes and analyzed on 3-8% Tris-acetate gels under non-reducing conditions. Proteins were visualized by staining with Gelcode Blue. Stained gels were scanned and band intensity was quantitated using a GE Healthcare ImageQuant350 equipped with ImageQuant TL image analysis software version 7.0.

To determine if the initial CLP-PEG implant had been completely remodeled or was still present within the regenerated neo-corneas, FTIR spectra of CLP and PEG-maleimide alone, and non-implanted CLP-PEG hydrogel was compared to cornea samples from CLP-PEG implanted eyes and control eyes. The samples were scanned between 4000 to 400  $\text{cm}^{-1}$  using VERTEX 70 FTIR spectrometer (Bruker, Billerica, MA, USA) at the resolution of 4  $\text{cm}^{-1}$  averaging 200 scans.

#### **Supplementary References**

1. M. M. Islam, M. Griffith and K. Merrett, *Methods Mol. Biol.*, 2013, **1014**, 157-164.
2. D. Priest and R. Munger, *Ophthalmol. Vis. Sci.*, 1998, **39**, S352.
3. C. Deng, F. Li, J. M. Hackett, S. H. Chaudhry, F. N. Toll, B. Toye, W. Hodge and M. Griffith, *Acta Biomater.*, 2010, **6**, 187-194.



**Supplementary Fig. 1:** *In vivo* confocal microscopic images of pig corneas grafted with CLP-PEG implants and unoperated control corneas at 12 months post-operation. Epithelial coverage and stromal cell ingrowth have resulted in neo-corneal tissues similar to the natural, unoperated cornea. Arrowheads indicate stromal cells. The endothelium was left intact during the surgery.