A novel pressed porous silicon-polycaprolactone composite as a dual-purpose implant for the delivery of cells and drugs to the eye

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Abbreviations: BSS ophthalmic balanced salt solution; DMEM Dulbecco's Modified Eagle's Medium; EGF epidermal growth factor; FBS fetal bovine serum; FDA fluorescein diacetate; PBS phosphate buffered saline; PCL polycaprolactone; pSi nanostructured porous silicon; SEM scanning electron microscopy; TEM transmission electron microscopy.

response. These novel pressed pSi-PCL materials have potential for delivery of both small and large drugs that can be released in active form, and can support the growth of mammalian cells.

Keywords: polycaprolactone; nanostructured porous silicon; composite biomaterial; drug release; cell proliferation; inflammation

Highlights

- We fabricated novel pressed porous silicon-polycapralactone composite materials
- The materials supported the attachment and growth of mammalian cells
- Small molecule drugs and biologics were loaded into the composites
- The materials elicited a weak foreign body response under the rat conjunctiva
- Composite materials may be of use for the transfer of cells and drugs to the ocular surface

ABSTRACT

Dysfunction of corneal epithelial stem cells can result in painful and blinding disease of the ocular surface. In such cases, treatment may involve transfer of growth factor and normal adult stem cells to the ocular surface. Our purpose was to develop an implantable scaffold for the delivery of drugs and cells to the ocular surface. We examined the potential of novel composite biomaterials fabricated from electrospun polycaprolactone (PCL) fibres into which nanostructured porous silicon (pSi) microparticles of varying sizes (150-250 μm or <40 μm) had been pressed. The PCL fabric provided a flexible support for mammalian cells, whereas the embedded pSi provided a substantial surface area for efficient delivery of adsorbed drugs and growth factors. Measurements of tensile strength of these composites revealed that the pSi did not strongly influence the mechanical properties of the polymer microfiber component for the Si loadings evaluated. Human lens epithelial cells (SRA01/04) attached to the composite materials, and exhibited enhanced attachment and growth when the materials were coated with fetal bovine serum. To examine the ability of the materials to deliver a small-drug payload, pSi microparticles were loaded with fluorescein diacetate prior to cell attachment. After 6 hours (h), cells exhibited intracellular fluorescence, indicative of transfer of the fluorescein diacetate into viable cells and its subsequent enzymatic conversion to fluorescein. To investigate loading of large-molecule biologics, murine BALB/c 3T3 cells, responsive to epidermal growth factor, insulin and transferrin, were seeded on composite materials. The cells showed significantly more proliferation at 48 h when seeded on composites loaded with these biologics, than on unloaded composites. No cell proliferation was observed on PCL alone, indicating the biologics had loaded into the pSi microparticles. Drug release, measured by ELISA for insulin, indicated a burst followed by a slower, continuous release over six days. When implanted under the rat conjunctiva, the most promising composite material did not cause significant neovascularization but did elicit a macrophage and mild foreign body

1. Introduction

Porous silicon (pSi) shows potential as a biocompatible scaffold for applications in orthopaedics (Li et al., 1998; Coffer et al., 2005; Whitehead et al., 2008; Anderson et al., 2010), oncology (Zhang et al., 2005; Mann et al., 2011; Park et al., 2011) and ophthalmology (Low et al., 2006; Cheng et al., 2008; Low et al., 2009; Kashanian et al., 2010). For use in ophthalmic implants designed to deliver drugs or cells to the eye, the advantages of pSi include its large surface area for drug-loading and cell attachment, and good ocular biocompatibility (Low et al., 2009; Kashanian et al., 2010). However, pSi is somewhat inflexible, is opaque, and shards of the membrane form of the material exhibit sharp edges. We have previously demonstrated that a composite material comprising a soft polymer such as polycaprolactone (PCL) encasing pSi particles mitigated some of these issues (Kashanian et al., 2010). Nevertheless, challenges with drug loading and release remained. Here, we describe a novel dual-function composite of pSi and PCL, pressed pSi-PCL. The composite consists of an electrospun (non-woven) PCL fabric with nanostructured pSi microparticles pressed into the outside of the fibres. An advantage of the newer composites is that drugs, including biologics such as proteins and peptides, can be loaded into the pSi particles (McInnes et al., 2015) *after* fabrication of the PCL material, thereby avoiding exposure to heat and solvents.

Our focus is on disorders that result in loss or dysfunction of the adult corneal epithelial stem cells normally residing at the limbus, at least in humans (Notara et al., 2010; Ordonez & Girolamo, 2012; Joe & Yeung, 2014). A number of biomaterials have been assessed as scaffolds (Feng et al., 2014) for transfer of corneal epithelial stem cells and transient-amplifying cells to the ocular surface (Joe & Yeung, 2014), and silk fibroin shows promise for corneo-limbal reconstruction (Harkin et al., 2011; Bray et al., 2012). We aimed to engineer a biomaterial suitable for ocular surface repair. Herein, we assessed the loading and

release of both small molecule and macromolecular drugs into pressed pSi-PCL composite materials, and their ability to support mammalian cell attachment and growth. Implantation of the material beneath the conjunctiva of rats was performed to assess biocompatibility.

2. Materials and methods

2.1 Reagents

Ophthalmic balanced salt solution (BSS) was obtained from Alcon Laboratories Inc (Fort Worth, TX, USA). Dulbecco's Modified Eagle's Medium (DMEM) was sourced from JRH Bioscience (Lanexa, KA, USA). L-glutamine, penicillin, streptomycin sulphate, insulin, transferrin and sodium selenite were all obtained from Invitrogen (Mount Waverley, VIC, Australia). Epidermal growth factor (EGF) was obtained from ProSpec-Tany TechnoGene Ltd. (Ness-Ziona, Israel). Chloroform, fluorescein diacetate (FDA) and ethanol were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Bovogen Biologicals (Essendon, VIC, Australia). Hoechst 33342 dye was purchased from Molecular Probes (Eugene, OR, USA).

2.2 Fabrication of pressed pSi-PCL composites

PCL was obtained from Aldrich (Milwaukee, WI, USA). Nonwoven fabrics of PCL were prepared as described previously (Kashanian et al., 2010; Fan et al., 2011). Briefly, a 25% by weight solution of PCL (MW 65 kDa) in chloroform was placed in a 5 ml glass syringe fitted with a 21 gauge needle, with an applied potential of 20 kV and tip to grounded Al collector distance of approximately 20 cm. Samples of pSi were generously provided by Professor L Canham and Dr A Loni, pSiMedica Ltd. Films of pSi were first produced by electrochemical

anodization of p-type (0.01-0.02 Ohm cm) single-crystal silicon wafers in hydrofluoric acid, followed by separation of a pSi membrane from the bulk Si and subsequent milling of the membrane. The particles were sieved to two size ranges: 150-250 μm, and <40 μm. For fabrication of composite materials, two different methods were evaluated. *Method A.* To embed pSi microparticles in the outer surface of microfibres of PCL, the particles were warmed to a temperature above the polymer melting point, followed by brief exposure to the PCL fabric, forcing a modest blending between the contacting interfaces. Specifically, pSi particles were heated in an oven at 110°C, then immediately transferred to a glass plate. A 1x1 cm^2 piece of PCL fabric was physically pressed on to the hot particles. The hot particles caused localized melting of the PCL fibres and thus resulted in the pSi being partially embedded in the polymer. Loadings of the order of 5-6% pSi by mass were obtained, with retention of PCL fibre morphology. *Method B.* Improved embedding of pSi microparticles in the PCL was achieved by the brief addition of chloroform to pSi microparticles after removal from the oven, resulting in a surface etch/dissolution of the PCL surface and better adhesion of the pSi particles to the PCL matrix. This processing step was followed by an additional electrospinning event, to physically entrap more pSi into the composite. Specifically, pSi particles were heated in an oven at 220°C for 10 minutes (min), then transferred to a cold glass plate. One drop of chloroform was added to the pSi and quickly mixed. After ~10 sec, a 1x1 cm^2 piece of PCL fabric was physically pressed on to the moist pSi particles. An additional thin layer of PCL fibres was then generated on to the pSi-PCL, resulting in the formation of a "net". Loadings of the order of 32-34% pSi by mass were obtained, with retention of PCL fibre morphology.

2.3 Characterization by electron microscopy

Nanoporous features in individual pSi microparticles were imaged by transmission electron microscopy (TEM) using a JEOL JEM 2100 and by scanning electron microscopy (SEM) using a JEOL JSM 6400. Fibre diameter and scaffold morphology in the pSi-PCL composites were also characterized by SEM.

2.4 Characterization of biomaterial tensile strength

Evaluation of tensile strength was carried out on selected pSi-PCL composites (as well as PCL fiber only controls) with an approximate cross-sectional area of 1×10^{-6} m^2 using a Hampden H-6310 tension-testing machine (Hampden Engineering Corp., East Longmeadow, MA, USA).

2.5 Small molecule loading into pSi microparticles and quantification of drug release

For loading of FDA, 10 mg of pSi microparticles were immersed in a solution of 250 g/L FDA in chloroform for 1h at 70°C. Percent loading of FDA by mass was assessed by thermogravimetric analysis (TGA) using a Seiko Instruments TG/DTA 220 unit. To quantify FDA release (Mogal et al., 2014), FDA-loaded pSi microparticles were immersed in 100 μL of PBS pH 7.4. Samples were assayed in triplicate and stored in an incubator at 37°C between sampling events. For each sampling event, the microparticles were centrifuged, and the entire supernatant was withdrawn for analysis and replaced with 100 μL of fresh PBS. The sample to be tested was diluted into 900 μL of 0.1 M NaOH and the fluorescein peak at 490 nm was read on a NanoDrop UV-Vis (Thermo Scientific, Wilmington, DE, USA) under ambient conditions. The total FDA concentration was determined by summation of the 490 nm readings at all time-points. The earlier time points were then presented as a percentage of total

accumulated release.

2.6 Cell attachment and culture on pSi-PCL composite material

Pieces of composite material were sterilised by immersion in 70% ethanol for 5 min, then washed three times with sterile Dulbecco's A phosphate buffered saline (PBS), pH 7.2. The pieces were then transferred into sterile 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and held in place by polytetrafluoroethylene O-rings. Prior to seeding cells, the material was immersed in DMEM containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin sulphate and 0.29 mg/ml glutamine (complete medium) for 5 min. Human lens epithelial cells designated SRA01/04, the kind gift of Professor V. Reddy, Kellogg Eye Centre, University of Michigan, Ann Arbor, MI, USA, were seeded at $1x10^5$ cells per well in 200 μl complete medium. Cells were maintained at 37°C with 5% CO_2 in air for 6 or 24 h.

2.7 Enhanced attachment and growth of cells on composite materials

The ability of growth factors loaded in the pSi to enhance attachment and growth of cells was examined. Initial experiments focussed on the use of FBS. Composite materials were sterilised and washed as above, then incubated with FBS for 2 h at room temperature. Material incubated in serum-free medium was used as a control. FBS was removed and SRA01/04 cells were seeded as above. After 6 or 24 h of culture, the medium was replaced with complete medium containing 10 nM Hoechst 33342 dye and incubated for 30 min. Unbound dye was removed by washing three times with complete medium. The cells were imaged on an Olympus IX71 inverted microscope or mounted on a slide in complete medium and imaged on an Olympus BX50 fluorescence microscope. 2D blind deconvolution was applied using

AutoDeblur 7.5.4 image deconvolution software (MediaCybernetics, San Diego, CA, USA) to improve image sharpness.

2.8 Loading and release of insulin, transferrin, selenite and EGF into and from pSi-PCL composite materials

Pieces of composite material were transferred to a 48 well plate, air-dried and exposed to UV radiation in a laminar air-flow hood for 5 min. Test composites were incubated in 500 µl of 1 mg/ml insulin, 0.55 mg/ml transferrin, 0.67 µg/ml sodium selenite and 10 µg/ml EGF for 16 h at 4°C in a humidified box, to maintain activity of the biologics. Insulin, transferrin, and sodium selenite improve cell growth in serum-free or serum-low medium (Bottenstein et al., 1979). Composites incubated in PBS were used as controls. The materials were washed 5 times in sterile PBS and transferred to a 96 well plate. EGF is a potent mitogen for many mammalian cells, but not for SRA01/04 cells. Thus, BALB/c 3T3 cells (the kind gift of Dr. E. Lousberg, Adelaide University, Adelaide, Australia), which are EGF-responsive (Rubin et al., 1991), were seeded on the materials at $2x10^4$ cells per well and incubated at 37°C and 5% CO_2 in air for 48 h. The number of cells in each well was enumerated using the CellTiter 96® AQueous One Solution (Promega, Madison, WI, USA), according to the manufacturer's instructions.

The release of biologics from the pSi-PCL composite materials was first measured functionally, by their ability to induce proliferation in BALB/c 3T3 cells. pSi-PCL materials were loaded as described above. Conditioned medium was prepared by incubating the pSi-PCL composite material in 400 µl DMEM at 37°C for 24 h, transferring to fresh medium for 24 h and finally into fresh medium for 96 h. Unloaded pSi-PCL composite material was used as a control. A proliferation assay with BALB/c 3T3 cells was performed using the CellTiter

96® AQueous One Solution assay as described above, with 50 µl conditioned medium used in place of the pSi-PCL composite materials. Next, the release of insulin from the composite materials was measured. Materials were loaded with insulin, transferrin, and selenite as above and incubated in 200 µl serum free DMEM in a 97 well plate at 37 °C. Samples were taken at the end of day 1, day 2 and day 6. The medium was replaced with fresh serum-free DMEM at each time point. The amount of insulin in the conditioned medium was assayed using the Insulin Human ELISA kit (Abcam, Cambridge, MA, USA), according to the manufacturer's instructions.

2.9 Biocompatibility of composite materials in the rat eye

All animal studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approval for experimentation was obtained from the institutional Animal Welfare Committee of Flinders University. Inbred Sprague-Dawley rats were bred within the institution, maintained in 50% humidity with a 12 h light-dark cycle, and were allowed access to food and water *ad libitum*.

Composite materials were cut into 2x2 mm^2 pieces, immersed in 70% ethanol for 5 min, washed in sterile ophthalmic BSS and exposed to UV radiation overnight. Animals were anaesthetised using inhaled isoflurane in 2 L/min oxygen, delivered via a nose-cone. A subconjunctival pocket was created by blunt dissection of the superior, temporal and inferior conjunctiva of the right eye of each of 6 rats. A piece of the composite material was placed in each pocket and secured with interrupted 10-0 monofilament nylon sutures (Alcon Laboratories, Fort Worth, TX, USA). Chloromycetin ointment 1% (Aspen Pharmaceuticals, St Leonards, Australia) was applied and the eyelid was sutured shut for 24 h. In a further 3 rats, mock surgery was performed, without implantation of the biomaterial (suture-only controls).

Eyes were monitored weekly at the operating microscope for 8 weeks, at which time the rats were euthanized. Enucleated eyes were fixed in buffered formalin, embedded in paraffin wax, sectioned at 5 μm, and stained with haematoxylin and eosin (H&E) for end-point histology. Sections were assessed at the light microscope by a qualified pathologist (SK).

3. Results

3.1 Composite structure

High porosity pSi (81%) was used in all composite materials. The pore diameter of this pSi was in the range of 10-20 nm as determined by high resolution TEM (Fig. 1A, B). In composites produced by method A (pSi-PCL material A), brown-black pSi particles were apparent by visual inspection but pSi loading was relatively low, at 5-6% by mass (Fig. 1C). In composites produced by method B (pSi-PCL material B), a nearly continuous pSi film was evident to the unaided eye (Fig. 1D), with significant pSi loading of 32-34% by mass. Two different pSi particle size ranges were utilized in scaffold preparation. Scanning electron micrographs of composites produced by method A are illustrated in Fig. 1E and G (pSi particles 150-250 μm and <40 μm, respectively) and those produced by method B are shown in Fig. 1F and H (pSi particles 150-250 μm and <40 μm, respectively).

The effect of pSi particle loading on the tensile strength of these composites was also evaluated. A PCL-only microfiber control sample exhibited a value of 1.8 ± 0.4 MPa; Method A pSi-PCL composite (5-6% Si), 1.2 ± 0.2 MPa; Method B pSi-PCL composite (32-34% Si), 1.6 ± 0.2 MPa. Thus the presence of the pSi particles embedded on the outer surface of the PCL does not significantly impact the mechanical strength of these relatively small microfibers.

3.2 Small drug loading into and release from pSi-PCL composite materials

The model small molecule drug FDA was loaded into pSi-PCL material A. The percent loading of FDA by mass, as assessed by thermogravimetric analysis, was 7.8% of pSi particle mass for larger pSi particles (150-250 μm) and 10.9% for the smaller particles (<40 μm). The rate of drug release was then measured (Fig. 2). The majority of the FDA release occurred within the first 4 h: 63% and 50% for the larger (150-250 μm) and smaller (<40 μm) pSi particles, respectively. Release then slowed over the remainder of the two-day period, reaching 92% for the composite pressed with the larger pSi microparticles and 75% for the composite pressed with the smaller pSi microparticles at 48 h.

3.3 Transfer of FDA to cells grown on pSi-PCL composite materials

Using pSi-PCL material A, the feasibility of transferring drugs from the loaded pSi component of the material into cells was assessed. FDA is itself non-fluorescent, but intracellular enzymatic cleavage converts FDA to the fluorescent product fluorescein, which accumulates within the cells (Rotman et al., 1966). This property allows assessment of cell viability, as the esterase required for cleavage is intracellular and is only active in live cells. A human lens epithelial cell line, SRA01/04 was used as a model mammalian ocular cell type. Attachment of SRA01/04 cells to the composites was observed at 6 h after seeding. The cells appeared evenly distributed and were present on the PCL fibres as well as pSi particles. Transfer of FDA to the cells was confirmed by the detection of fluorescein in all FDA-loaded materials, but not in the unloaded control material (Fig. 3A). Detection of fluorescein

indicated that the cells were viable. Compared with the material with smaller (<40 μm) microparticles (Fig. 3B), the fluorescent signal was stronger in the material with the larger (150-250 μm) microparticles (Fig. 3C).

3.4 Enhanced attachment and growth of cells on FBS loaded composite material

Loading of peptide or protein drugs into the pSi composite materials was performed with pSi-PCL prepared by method B, to maximise macromolecular drug loading. Although cell attachment to uncoated pSi-PCL material B was observed at 6 h, there was little evidence of cell growth at 24 h (Fig. 4A, 4C). FBS, a model mixture of growth factors, has previously been shown to improve mammalian cell attachment to pSi membranes (Low et al., 2006). Enhancement of cell attachment to, and growth on, pSi-PCL material B composites that had been loaded with FBS was next assessed. Coating with FBS improved cell attachment and growth of SRA01/04 cells (Fig. 4B, 4D). The cells colonised the available surface area on the coated materials by 24 h and as before, grew on both the PCL fibres and the pSi particles.

3.5 Loading and release of biologics in active form from composite materials post-fabrication

All subsequent experiments were performed with composites prepared by method B into which the larger size range of pSi microparticles (150-250 μm) had been pressed, to maximise drug loading. The functional efficacy of specific biologics loaded into pSi-PCL material B on attached cells was next assessed. A mixture of epidermal growth factor, insulin, transferrin and sodium selenite was tested, as this combination of biologics induces proliferation in serum-deprived murine BALB/c 3T3 fibroblasts. Cells seeded on biologic-loaded and washed pSi-PCL material B showed significantly more proliferation than cells grown on unloaded composite material (Fig 5A). Cells seeded on polymer PCL fibres without pSi but pre-

incubated with biologics did not show increased proliferation, indicating the biologics had loaded into the pSi microparticle component of the composite material.

The release of loaded epidermal growth factor, insulin, transferrin and sodium selenite into cell culture medium was demonstrated by the effect of this culture medium on the proliferation of serum-deprived BALB/c 3T3 cells. The PCL-only material was not used in this experiment, as the previous experiment had demonstrated the biologics had loaded only into the pSi component of the composite material. Release was monitored over 6 days with samples taken on day 1, day 2 and a third sample consisting of release from days 3-6. DMEM conditioned by incubation of epidermal growth factor, insulin, transferrin and sodium selenite loaded pSi-PCL composite materials induced proliferation in BALB/c 3T3 cells (Fig 5B), in comparison with medium conditioned with unloaded pSi-PCL. Samples taken on day 1 of the release elicited the largest proliferative response, indicating a large amount of the loaded biologics had been released on day 1. However samples collected between days 3-6 also induced cell proliferation, indicating the continuing release of growth factors over this time-frame.

Finally, the release of insulin from the material was quantified by ELISA over 6 days, with samples taken on day 1, day 2 and a third sample consisting of release from days 3-6, as before. More insulin was released from the composite materials on day 1 than thereafter (Table 1). The rate of release decreased over time, in keeping with the observations from the functional release assay above. Taken together, these data suggested an initial burst release of the epidermal growth factor, insulin, transferrin and sodium selenite, followed by a slow release as the pSi microparticles dissolved.

3.6 Biocompatibility of materials in the rat eye

Unloaded pSi-PCL B material was implanted under the conjunctiva of adult inbred Sprague-Dawley rats to investigate *in vivo* biocompatibility. Immediately after implantation, pSi particles were visible at the operating microscope (Fig. 6A) but had dissolved by 8 weeks, at which stage the PCL fabric was still visible (Fig 6B). Vessels grew towards and encircled the sutures (Fig 6B). At 8 weeks post implantation, the animals were euthanized and their eyes were collected for histological analysis. At end-point histology, the position of the implant was marked by a thin fibrous capsule, with some neovascularization observed around the site (Fig. 6C). A foreign body response mediated by macrophages and foreign body-type giant cells was also visible (Fig 6D). The neovascular response to the sutures in the suture-only control rats was similar to the response against the implants. Overall, the implants were well tolerated by the animals and did not cause significant acute inflammation or a lymphocyte-predominant immune response.

4. Discussion

In this study, we developed a dual-purpose composite material of pSi and PCL suitable for the transfer of drugs and cells to the eye. We designed a straightforward method of pressing pSi microparticles on to a sheet of non-woven PCL fabric. Examination by SEM demonstrated that the pSi particles were partially embedded in the PCL but remained exposed on the surface, to permit drug loading *after* preparation of the composite material. This conferred a significant advantage over similar materials in which pSi particles were completely embedded within the PCL fibres (Kashanian et al., 2010), as exposure of many drugs to solvents or high temperatures during fabrication of the biomaterial would inevitably lead to drug denaturation and loss of activity. We further demonstrated that soaking the pSi particles in chloroform prior to pressing caused localised etching of the PCL fibres, improving attachment of pSi

microparticles, and that particle retention could further be improved by the deposition of a thin layer of PCL strands on top of the particles. Together, these improvements led to increased pSi particle loading compared with previous methods (Kashanian et al., 2010), providing a high drug loading capacity. The PCL fabric base conferred a degree of flexibility to the materials, while the addition of nanostructured pSi microparticles allowed the loading of both small drugs, as exemplified by fluorescein diacetate, and peptide or protein biologics, as exemplified by insulin. The presence of the pSi particles did not significantly alter the measured mechanical strength of the PCL. Observed tensile strength values were in the 1.2-1.8 MPa range, consistent with literature values for high-porosity PCL scaffolds possessing fiber diameters in the micrometer range (Eshraghi & Das, 2010; Sant et al., 2011).

The small molecule FDA is non-fluorescent and readily taken up by cells. Intracellular esterases cleave FDA to the fluorescent product fluorescein, which is retained within living cells (Rotman et al., 1966). Human lens epithelial cells, chosen as a convenient source of ocular epithelial cells, when seeded on to composite PCL-pSi materials demonstrated green fluorescence, indicating that FDA released from the composites had been taken up and converted to fluorescein by viable cells. The cells grew on composite materials irrespective of pSi microparticle size. However, more fluorescent cells were observed on materials with larger (150-250 μm) rather than smaller (<40 μm) pSi particles, possibly because such particles were able to be loaded with more of the model drug. Cells growing on or near a pSi microparticle appeared brighter than more distant cells. The former cells would therefore have been exposed to higher local concentrations of FDA than the latter.

Human lens epithelial cells attached to, and grew upon, the pSi-PCL composite materials. A coating of FBS, which contains a cocktail of growth factors and proteins, improved cell attachment and growth, and cells colonised the available surface area within 24 h. We also demonstrated loading and release of a mixture of biologic agents containing

epidermal growth factor, insulin and transferrin into the composite materials, using the proliferation of BALB/c 3T3 cells grown in serum-deprived medium (Bottenstein et al., 1979) as a readout. This functional assay confirmed that the biologics were released from the composites in an active form. PCL fabric without embedded pSi microparticles did not elicit cell proliferation, indicating that the proteins loaded into the pSi. The release profile of biologics from the composite materials was also investigated. Loaded pSi-PCL was incubated in cell culture medium, which was then used to induce proliferation of the BALB/c 3T3 cells. The release profile showed an initial burst release followed by a gradual release over 6 days, as the pSi particles dissolved. Such a time-frame is likely sufficient for transfer of corneal epithelial progenitor cells to the eye and their subsequent migration on to the ocular surface.

Use of the composite as an ocular implant requires it to be biocompatible in the eye. The biocompatibility of both pSi and PCL have previously been demonstrated individually (Low et al., 2009; Ang et al., 2006) and together as a composite material (Kashanian et al., 2010) for use at the front of the eye. Different PCL composites have recently been tested as potential scaffolds for transplantation of retinal progenitor cell to the back of the eye (Baranov et al., 2014). Furthermore, PCL is an FDA-approved material. We assessed the biocompatibility of our novel pressed pSi-PCL composites by implantation under the rat conjunctiva, away from the visual axis. The flexibility of the material allowed for easy implantation into pockets created in the subconjunctival space by blunt dissection. Implantation of the materials did not cause significant inflammation. Moderate neovascularization was observed, however, the degree of neovascularization was similar in suture-only controls. A foreign body type response mediated by histiocytes and foreign body type macrophages was also observed, again similar to that observed in the suture-only controls. Overall, the materials demonstrated acceptable biocompatibility following implantation into the subconjunctival space in rats.

Ex vivo expanded cells must be transferred to the eye on a scaffold (Pellegrini et al., 1997), of which human amniotic membrane is the most commonly used (Tseng et al., 1998). Human amniotic membrane contains a number of growth factors (Meller et al. 2011). However, the use of human amnion is associated with regulatory issues, including the risk for transfer of infectious agents. We envision that our novel composite materials might serve as useful scaffolds for the transfer of cells and growth factors or drugs to the eye in ocular surface disease.

5. Conclusions

Pressed pSi-PCL is an easily fabricated composite material. The composite is versatile, being suitable for delivery of either drugs or cells, or both, to the eye. The pSi microparticles can be pre-loaded with drugs such as FDA before fabrication of the composite, and transfer of FDA to cells grown on the composites *in vitro* was demonstrated. Coating of the composite material at room temperature with FBS after fabrication was also possible, and resulted in enhanced ocular cell attachment and growth. Loading of biologic agents was demonstrated, and the biologics were released in an active form. This novel composite material provides several advantages over pSi or PCL alone, and is superior to previously-described composite materials for drug loading and release. The ability to load the material with growth factors and to support the attachment and growth of ocular epithelial cells suggest a potential application in the treatment of ocular surface disease. Other potential avenues of investigation with this composite material, such as its utility to act as an implantable niche for limbal stem cells, remain to be explored.

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References

Anderson MC, Olsen R. Bone ingrowth into porous silicon nitride. J Biomed Mater Res 2010;92:1598-605.

Ang LP, Cheng ZY, Beuerman RW, Teoh SH, Zhu X, Tan DT. The development of a serum-free derived bioengineered conjunctival epithelial equivalent using an ultrathin poly(epsilon-caprolactone) membrane substrate. Invest Ophthalmol Vis Sci 2006;47:105-12.

Baranov P, Michaelson A, Kundu J, Carrier RL, Young M. Interphotoreceptor matrix-poly(ϵ-caprolactone) composite scaffolds for human photoreceptor differentiation. J Tissue Eng 2014;5:2041731414554139. doi: 10.1177/2041731414554139.

Bottenstein J, Hayashi I, Hutchings S, Masui H, Mather J, McClure DB, et al. The growth of cells in serum-free hormone-supplemented media. Methods Enzymol 1979;58:94-109.

Bray LJ, George KA, Hutmacher DW, Chirila TV, Harkin DG. A dual-layer silk fibroin scaffold for reconstructing the human corneal limbus. Biomaterials 2012;33:3529-38.

Cheng L, Anglin E, Cunin F, Kim D, Sailor MJ, Falkenstein I, et al. Intravitreal properties of porous silicon photonic crystals: a potential self-reporting intraocular drug-delivery vehicle. Br J Ophthalmol 2008;92:705-11.

Coffer JL, Whitehead MA, Nagesha DK, Mukherjee P, Akkaraju G, Totolici M, et al. Porous silicon-based scaffolds for tissue engineering and other biomedical applications. Phys Stat Sol (A) 2005;202:1451-5.

Eshraghi S, Das S. Mechanical and microstructural properties of polycaprolactone scaffolds with one-dimensional, two-dimensional, and three-dimensional orthogonally oriented porous architectures produced by selective laser sintering. Acta Biomater 2010;6:2467-76.

Fan D, Akkaraju GR, Couch EF, Canham LT, Coffer JL. The role of nanostructured mesoporous silicon in discriminating in vitro calcification for electrospun composite tissue engineering scaffolds. Nanoscale 2011;3:354-61.

Feng Y, Borrelli M, Reichl S, Schrader S, Geerling G. Review of alternative carrier materials for ocular surface reconstruction. Curr Eye Res 2014;39:541-52.

Harkin DG, George KA, Madden PW, Schwab IR, Hutmacher DW, Chirila TV. Silk fibroin in ocular tissue reconstruction. Biomaterials 2011;32:2445-58.

Joe AW, Yeung SN. Concise review: identifying limbal stem cells: classical concepts and new challenges. Stem Cells Transl Med 2014;3:318-22.

Kashanian S, Harding F, Irani Y, Klebe S, Marshall K, Loni A, et al. Evaluation of mesoporous silicon/polycaprolactone composites as ophthalmic implants. Acta Biomater 2010;6:3566-72.

Li X, Coffer JL, Chen Y, Pinizzotto RF, Newey J, Canham LT. Transition metal complex-doped hydroxyapatite layers on porous silicon. J Am Chem Soc 1998 1998;120:11706-9.

Low SP, Williams KA, Canham LT, Voelcker NH. Evaluation of mammalian cell adhesion on surface-modified porous silicon. Biomaterials 2006;27:4538-46.

Low SP, Voelcker NH, Canham LT, Williams KA. The biocompatibility of porous silicon in tissues of the eye. Biomaterials 2009;30:2873-80.

Mann AP, Tanaka T, Somasunderam A, Liu X, Gorenstein DG, Ferrari M. E-Selectin-targeted porous silicon particle for microparticle delivery to the bone marrow. Adv Mater 2011;23:278-82.

McInnes SJP, Turner CT, Al-Bataineh SA, Airaghi Leccardi MJI, Irani Y, Williams KA, et al. Surface engineering of porous silicon to optimise therapeutic antibody loading and release. J Mater Chem B 2015;3:4123-33.

Meller D, Pauklin M, Thomasen H, Westekemper H, Steuhl KP. Amniotic membrane transplantation in the human eye. Dtsch Arztebl Int 2011;108:243-8.

Mogal VT, Yin CS, O'Rorke R, et al. Tuning model drug release and soft-tissue bioadhesion of polyester films by plasma post-treatment. ACS Appl Mater Interfaces 2014;6:5749–5758.

Notara M, Alatza A, Gilfillan J, Harris AR, Levis HJ, Schrader S, et al. In sickness and in health: corneal epithelial stem cell biology, pathology and therapy. Exp Eye Res 2010;90:188-95.

Ordonez P, Di Girolamo N. Limbal epithelial stem cells: role of the niche microenvironment. Stem Cells 2012;30:100-7.

Park JS, Kinsella JM, Jandial DD, Howell SB, Sailor MJ. Cisplatin-loaded porous Si microparticles capped by electroless deposition of platinum. Small 2011;7:2061-9.

Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. Lancet. 1997;349:990-3.

Rotman B, Papermaster BW. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc Natl Acad Sci USA 1966;55(1):134-41.

Rubin JS, Chan AM, Bottaro DP, Burgess WH, Taylor WG, Cech AC, et al. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci USA 1991;88:415-9.

Sant SP, Hwang CM, Lee S-H, Khademhosseini A. Hybrid PGS-PCL microfibrous scaffolds with improved mechanical and biological properties. J Tissue Eng Regen Med 2011;5:283-91.

Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. Arch Ophthalmol 1998;116:431-41.

Whitehead MA, Fan D, Mukherjee P, Akkaraju GR, Canham LT, Coffer JL. High-porosity poly(epsilon-caprolactone)/mesoporous silicon scaffolds: calcium phosphate deposition and biological response to bone precursor cells. Tissue Eng Part A 2008;14:195-206A.

Zhang K, Loong SL, Connor S, Yu SW, Tan SY, Ng RT, et al. Complete tumor response following intratumoral ^{32}P biosilicon on human hepatocellular and pancreatic carcinoma

xenografts in nude mice. Clin Cancer Res 2005;11:7532-7.

Figure legends

Figure 1. Visualisation of pSi microparticles and pSi-PCL composite materials. (A and B) High resolution TEM image of pSi microparticles (81% porosity) utilized in the composites. Individual pores can be visualized between the dense, dark regions of the image. Scale bars = 20 nm. (C) and (D) Optical images of pSi/PCL fibre composites (2x1 cm^2 pieces) prepared by method A (C) and method B (D). (E-H) SEM images of pSi /PCL fibre composites prepared by method A, with large (150-250 μm) pSi particles (E); method A, with small (<40 μm) pSi particles (F); method B, with large (150-250 μm) pSi particles (G); method B, with small (<40 μm) pSi particles (H). Scale bar = 200 μm for (E) and (G); scale bar = 50 μm for (F) and (H).

Figure 2. Fluorescein diacetate (FDA) release from loaded pSi microparticles. Filled squares: 150-250 μm microparticles; unfilled circles: small <40 μm pSi microparticles (n=3).

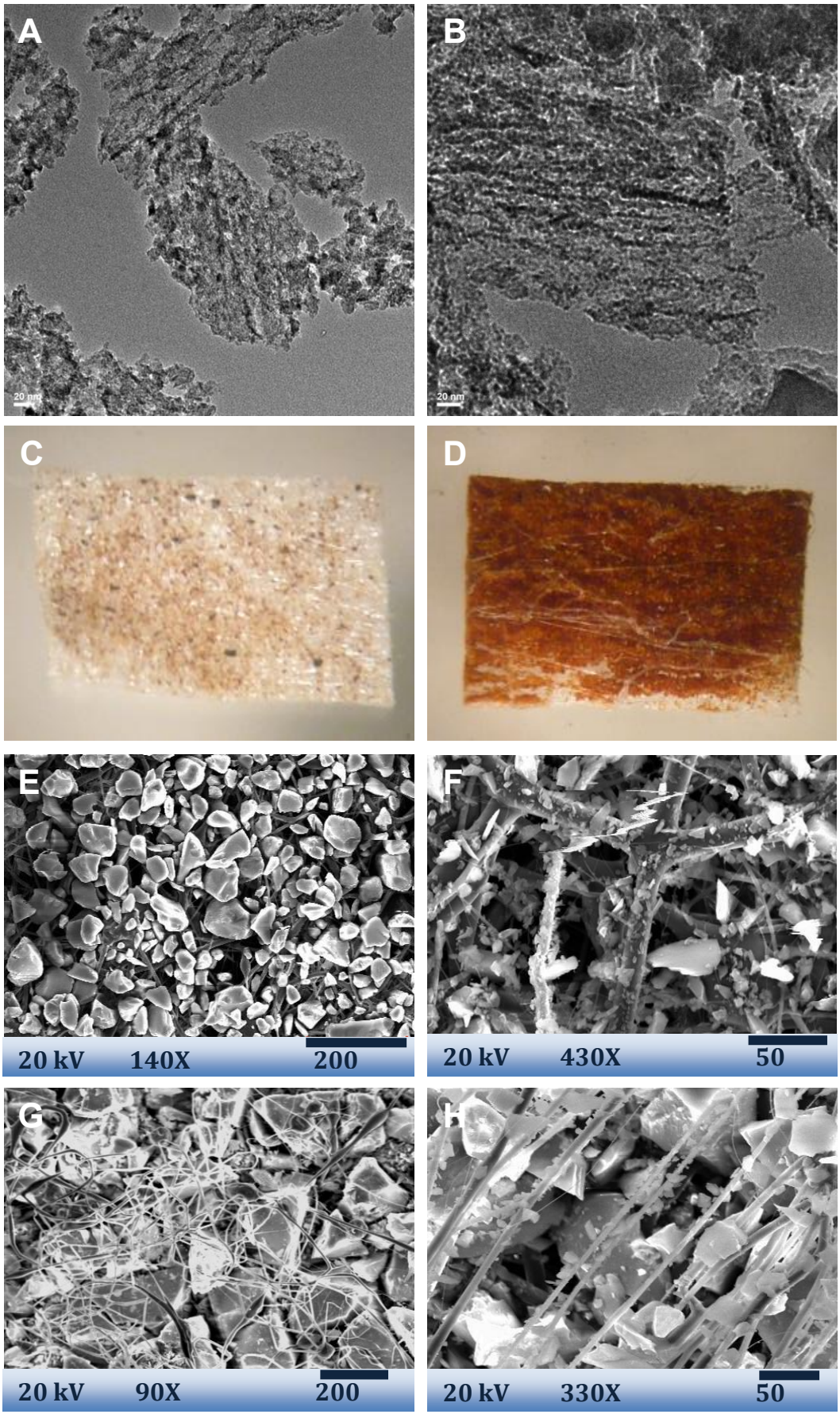
Figure 3. Fluorescein diacetate (FDA) uptake by SRA01/04 cells attached to pressed pSi-PCL composite materials 6 h after seeding on FDA loaded pSi-PCL composite materials. Cell nuclei stained with Hoechst 33342 (blue), cell cytoplasm stained with fluorescein (green), pSi particles (asterisk), scale bars = 50 μm; inserts show a high magnification image of the cells, scale bars = 10 μm. (A) No green fluorescence was observed in the unloaded materials; (B) FDA-loaded composite with <40 μm pSi particles showed faint green fluorescence; (C) FDA-loaded composite with 150-250 μm pSi particles showed bright green fluorescence. Images were sharpened using AutoDeblur 7.5.4 image deconvolution software (MediaCybernetics, San Diego, CA, USA).

Figure 4. Growth of SRA01/04 cells on FBS-coated and uncoated pSi-PCL composite materials. Images of SRA01/04 cells 24 h after seeding on pSi-PCL composite materials. (A) Uncoated pSi-PCL with <40 μm pSi; (B) FBS-coated pSi-PCL with <40 μm pSi; (C) uncoated pSi-PCL with 150-250 μm pSi; (D) FBS-coated pSi-PCL with 150-250 μm pSi.

Cells were observed on the pSi particles (asterisks) as well as on the polymer fibers (arrow). Cells showed poor attachment to uncoated materials but adhered to and colonized the FBS-coated materials. Cell nuclei stained with Hoechst 33342 (blue), scale bar = 50 µm.

Figure 5. Proliferation of BALB/c 3T3 cells in response to EGF-insulin, selenite and transferrin (EGF-ITS) released from pSi-PCL composite materials. (A) Proliferation of BALB/c 3T3 cells 48 h after seeding on pSi-PCL composite materials or PCL fibres loaded with EGF-ITS. Cells seeded on EGF-ITS loaded pSi-PCL showed significantly more proliferation than on unloaded composite material and loaded PCL *p=0.008. Cells seeded on EGF-ITS loaded PCL did not proliferate more than cells loaded on unloaded PCL, #p=0.095. (B) Proliferation of BALB/c 3T3 cells in response to medium incubated with EGF-ITS loaded pSi-PCL composite material. EGF-ITS loaded in pSi-PCL composite materials was released into cell culture medium. Medium conditioned with EGF-ITS loaded pSi-PCL composite material induced proliferation of cells compared to medium conditioned with unloaded pSi-PCL.

Figure 6. Subconjunctival implantation of pressed pSi-PCL composites in the rat. (A) and (B) Pressed pSi-PCL composite material implanted under the rat conjunctiva, immediately after implant and 8 weeks post implant respectively. Arrows mark the implants. A gauze swab is visible in the top left corner of image B (asterisk). The porous silicon particles were visible immediately after implantation but had completely dissolved at 8 weeks; (C) Haematoxylin & eosin stained section of a representative eye 8 weeks after implantation. A foreign body response mediated by macrophages and foreign body-type giant cells was observed; (D) Magnified view of the area delineated by the red box in (C), showing remnants of the composite material (arrow). (E) Response to the suture (arrow) was similar to the foreign body response against the implant. Scale bars (C) = 200 µm, (D) and (E) = 100 µm.


A
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B
20 nm
C
D
E
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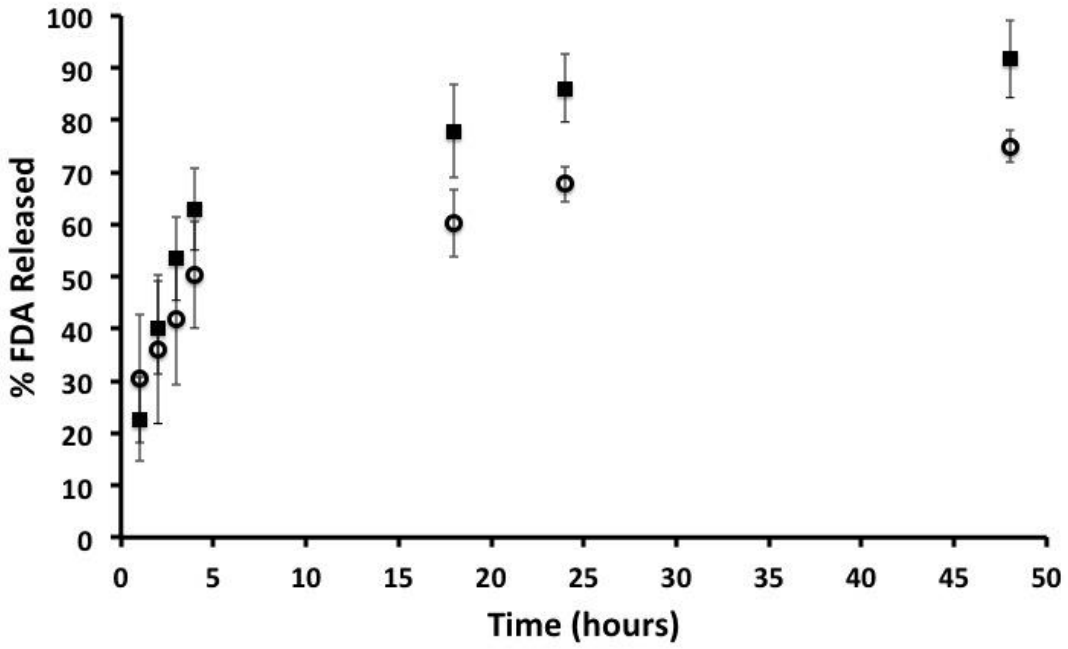
F
20 kV 430X 50
G
20 kV 90X 200
H
20 kV 330X 50

Figure 1

Figure 2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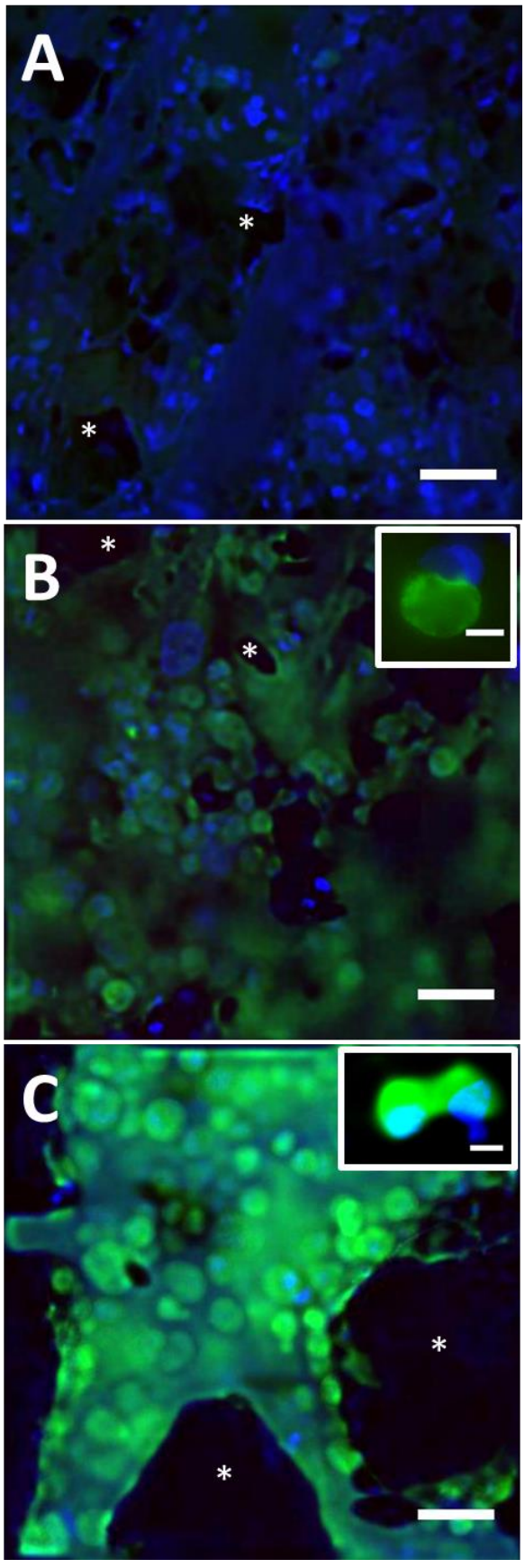


Figure 3

Figure 4

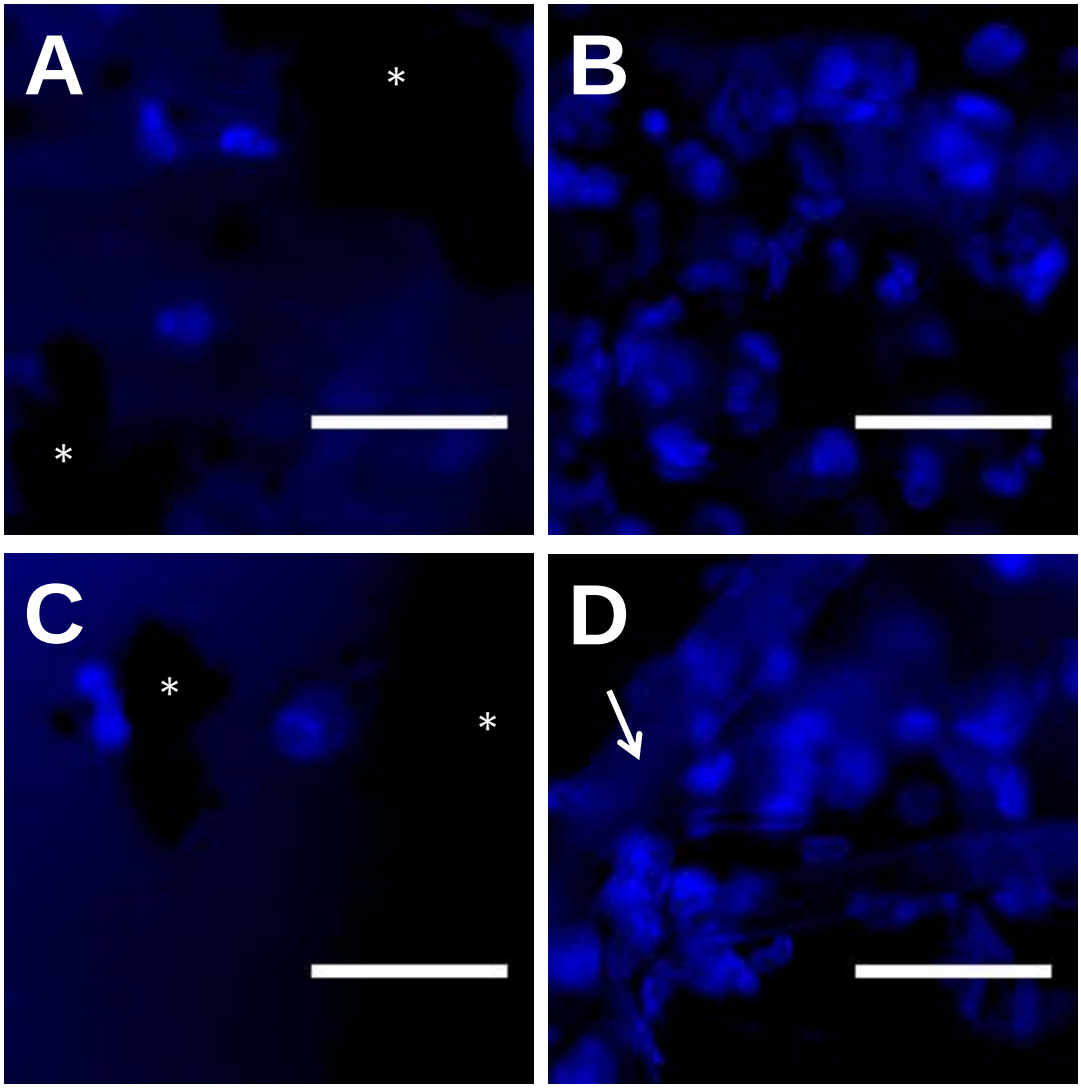
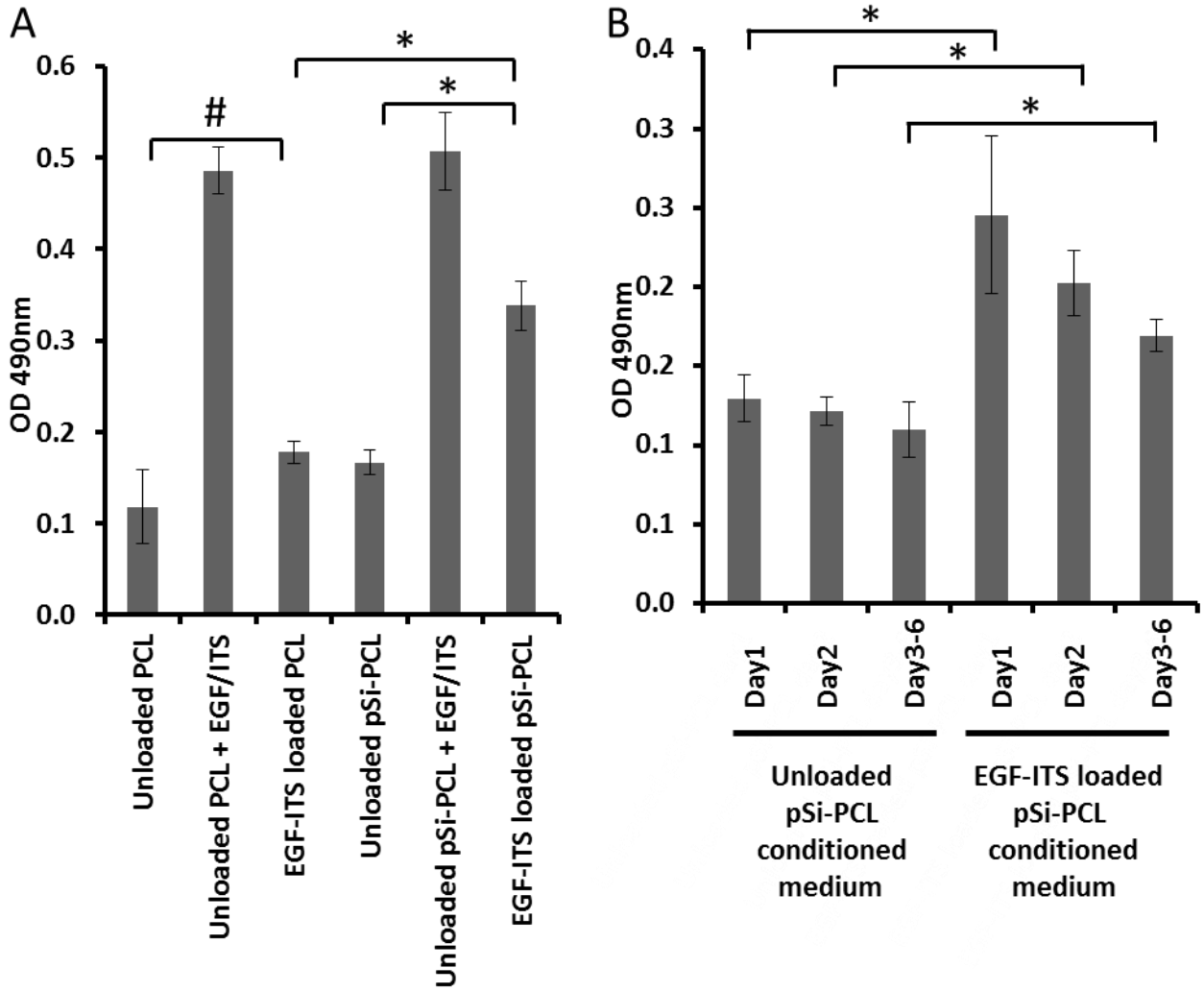


Figure 5


A
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Unloaded PCL
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EGF-ITS loaded PCL
Unloaded pSi-PCL
Unloaded pSi-PCL + EGF/ITS
EGF-ITS loaded pSi-PCL
B
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Day1
Day2
Day3-6
Day1
Day2
Day3-6
Unloaded pSi-PCL conditioned medium
EGF-ITS loaded pSi-PCL conditioned medium

Figure 6

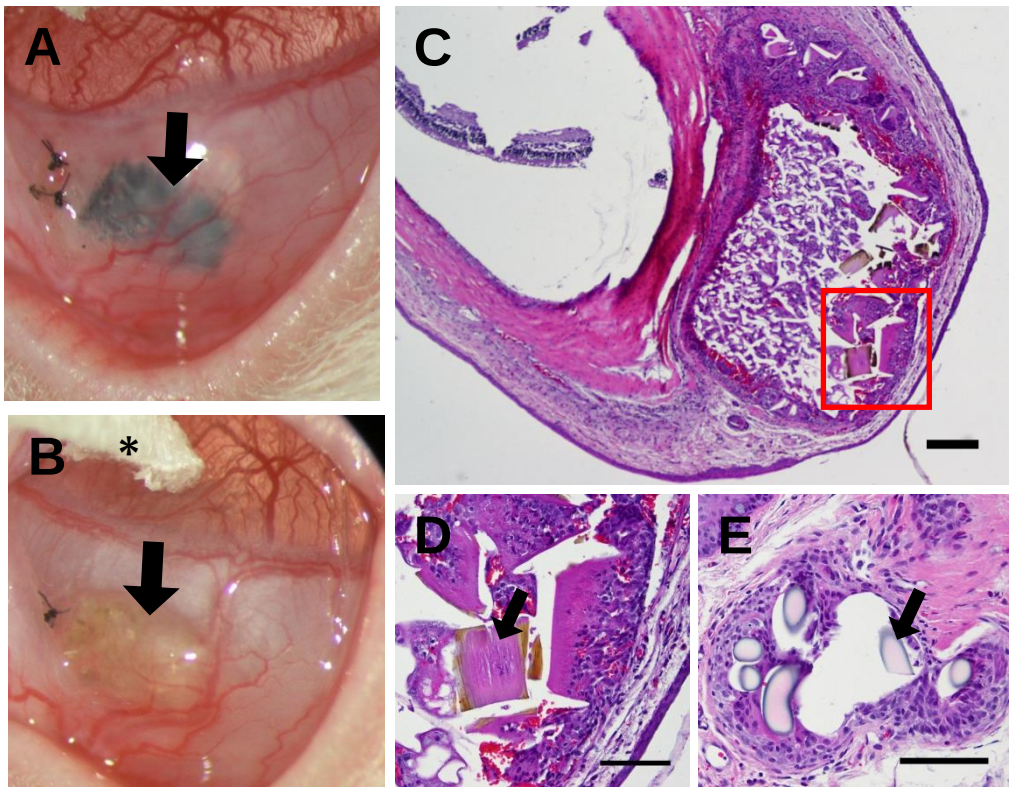

Table 1 Rate of release of insulin from loaded pSi-PCL material B

Day of release	Insulin release (ng insulin/mg pSi-PCL B) mean ± standard deviation[1]
Day 1	91 ± 1.9
Day 2	18 ± 0.7
Days 3-6	6 ± 3.0

[1] Means of 3 biological replicates (individual pieces of pSi-PCL); data are representative of two separate experiments.