
Proceedings Paper:
Thanarak, J., Mohammed, H., Pashneh-Tala, S. et al. (2 more authors) (2019) Enhanced collagen production from human dermal fibroblasts on poly(glycerol sebacate)-methacrylate scaffolds. In: 2018 11th Biomedical Engineering International Conference (BMEiCON). 2018 11th Biomedical Engineering International Conference (BMEiCON), 21-24 Nov 2018, Chiang Mai, Thailand. IEEE . ISBN 978-1-5386-5724-9

https://doi.org/10.1109/BMEiCON.2018.8609928

Enhanced Collagen Production from Human Dermal Fibroblasts on Poly(glycerol sebacate)-methacrylate Scaffolds

Jeerawan Thanarak[a], Hauwa Mohammed[a], Samand Pashneh-Tala[a], Frederick Claeyssens[a,b], Nicola Green[a,b]
[a]Department of Materials Science and Engineering,
University of Sheffield, Sheffield, UK, S3 7HQ
[b]INSIGNEO Institute for in silico Medicine,
Sheffield, UK, S1 3JD

Abstract Poly(glycerol sebacate)-methacrylate (PGS-M) is a photocurable form of polyglycerol sebacate (PGS) that has recently been shown to be suitable for use as a scaffold for tissue engineering. It has the benefits of PGS, including biocompatibility and biodegradability, while also being much simpler to process into a variety of 3D structures. Cell compatibility has already been demonstrated on the 30% methacrylated PGS-M scaffolds. However no studies have yet assessed the collagen produced by cells growing on the PGS-M scaffold. Here we demonstrate that 50% methacrylated PGS-M 3D scaffolds are able to support the culture of human dermal fibroblasts for 1 week. We also show that collagen production is enhanced compared with the same cells growing on tissue culture plastic, with the cells producing approximately 50% more total collagen after 1 week in culture. These results go further to demonstrate the suitability of the PGS-M scaffolds for generating ECM based constructs for soft tissue engineering.

Index Terms tissue engineering, collagen, scaffold, fibroblasts, 3D cell culture, soft tissue

I. Introduction

The extracellular matrix (ECM) is the non-cellular, 3D scaffold found in all tissues. In addition to providing the structure within which the cells grow, it plays important roles in cell differentiation, cell-cell communication, homeostasis and morphogenesis [1]. The main fibrous proteins components of the ECM are collagen and elastin, with collagen making up ~30% of the total protein mass of a human [2]. Collagen provides the tensile strength for the tissue, while also controlling cell-matrix adhesion, chemotaxis and tissue development [3].

ECM scaffolds derived from decellularized mammalian tissues have been used to generate tissue engineered constructs for tissue repair, and have already shown success in both preclinical and clinical studies. These scaffolds appear to have benefits over synthetic scaffolds, including improved biological compatibility, reduced encapsulation and the potential for in vivo remodeling and an improvement in tissue regeneration [4–7]. Furthermore, if the ECM is derived from the same type of tissue that it is being used to repair, it will possess the correct tissue architecture. However, this process still requires a source of tissue; there are limited donor supplies of the appropriate cadaveric human tissue, while xenogeneic ECM carries potential disease risks and ethical concern. The ECM used in this way is often derived from porcine small intestine submucosa (SIS). Although widely used in tissue engineering research [8] and the clinic for both wound management [8,9] and the repair of multiple tissue types [10–12], SIS has poor mechanical properties that may limit its usefulness.

More recently there has also been interest in generating ECM scaffolds in the laboratory through the culture of human smooth muscle cells or fibroblasts on biodegradable synthetic scaffolds in vitro. The cells produce the ECM as the synthetic scaffold degrades. After an extended culture period the sample is decellularized, leaving behind an ECM-based scaffold with the same morphology as the original synthetic scaffold. This process has been used to generate off-the-shelf vascular grafts with mechanical properties similar to native human blood vessels [6,13]. The collagen structure and organization within these scaffolds has also been further enhanced by applying mechanical loading to the cells during culture [14]. This requires a synthetic scaffold with a degradation rate in the order of 8 to 10 weeks, which is also sufficiently elastic to effectively transduce the mechanical strain to the cells growing on the scaffold. Previous studies have used a polyglycolic acid scaffold, however there are currently no reported studies which aim to optimize the synthetic scaffold for collagen production.

We have recently reported a photocurable form of poly(glycerol sebacate) (PGS) with improved processing capabilities for use in soft tissue engineering applications: PGS-methacrylate (PGS-M)[10]. In this form, PGS is methacrylated resulting in a prepolymer which is photocurable in combination with a photoinitiator and light at the appropriate wavelength [15]. The properties of the PGS-M scaffold are tunable by varying the extent of methacrylation, and thus there is the potential to optimize collagen production by changing the mechanical characteristics of the scaffold.

Here we describe the growth of dermal fibroblasts on a 50% methacrylated PGS-M scaffold and investigate the impact of the scaffold on production of collagen.

II. Materials and Methods

All chemical reagents were obtained from Sigma Aldrich, UK, unless otherwise stated.

A. Synthesis of PGS-M Scaffold

The PGS-M prepolymer was synthesized following the protocol described by Pashneh-Tala et al. [15]. Briefly, PGS prepolymer was formed using the melt-polycondensation reaction (120°C, under nitrogen gas, 24h) using equimolar amounts of sebacic acid and glycerol (Fisher Scientific, UK). Water was removed under vacuum and the reaction allowed to continue for 36 h. The prepolymer was dissolved in a 1:4 (w/v) mixture of dichloromethane (Fisher Scientific, UK) and methacrylic anhydride (0.5 mol/mol PGS hydroxyl groups). An equimolar amount of triethylamine was then slowly added, followed by 4-methoxyphenol (1 mg/g of PGS prepolymer). The reaction was performed at 0°C and allowed to rise to room temperature over 24 h. The resulting solution was then washed with 30 mM hydrochloric acid (Fisher scientific, UK) at 1:1 (v/v), dried with calcium chloride (Fisher scientific, UK) and the dichloromethane removed via rotary evaporation, under vacuum.

The PGS-M prepolymer was mixed with toluene and Hypermer™ B246 at 10:10:1 (w/w) ratio, before being mixed with photoinitiator diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide/2-hydroxy2-methylpropiophenone, blend, 0.4 g/g PGS prepolymer). After 5 minutes mixing, water was added (8 ml/g PGS prepolymer), to increase scaffold permeability and porosity [16]. The solution was poured into a PDMS (poly(dimethylsiloxane)) mold (3.5 cm (l) x 1.5 cm (w) x 0.2 cm (d)) (Fig. 1A) and exposed to UV light (100W, OmniCure Series 1000 curing lamp) for 5 minutes on each side to photocure.

The scaffold produced (Fig. 1B) was washed in methanol then dH_2O, each for 4 days, with solvents refreshed daily to remove uncured prepolymer and photoinitiator. The scaffold was then autoclaved (121°C, 15 minutes) in dH_2O to sterilize, and stored at room temperature prior to use.

B. Culture of cells on scaffolds

Human dermal fibroblasts from primary dermal tissue were obtained with informed consent (REC 15/YH/0177) and processed and stored in accordance with the Human Tissue Act 2004. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 x 10^{-3} M glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 0.625 µg ml^{-1} amphotericin B. Cultured fibroblasts were harvested using trypsin (0.025%)/EDTA (0.01%) solution, and 1 x 10^5 cells seeded in each well of a 6 well plate, or on sterile PGS-M scaffolds. Scaffolds were seeded using very small volumes of cells (<20 µl) which were placed onto the scaffold and allowed to adhere at 37°C for one hour, before the scaffolds were fully submerged in culture media. Cells were then allowed to grow for 1 week at 37°C, 5% CO_2, in a humidified atmosphere. After 24 hours cells were supplemented with 50 µg/ml ascorbic acid 2 phosphate, except where indicated. Scaffolds were cultured for 1 week, with the culture medium replaced every 2-3 days. Control experiments were also set up with cells growing in 6 well tissue culture plastic (TCP) plates.

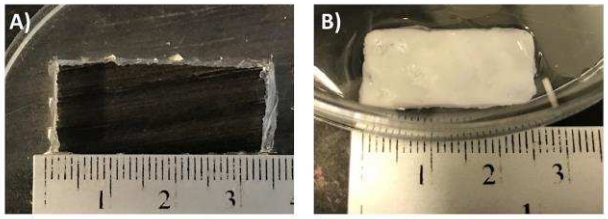

Fig. 1. The PDMS mold (A) used to form the PGS-M prepolymer during the photocuring step, and the PGS-M scaffold (B) generated from this process

At the end of the experiment, scaffolds were transferred to fresh 6 well plates. Scaffolds or cells growing on TCP were either analyzed for cell metabolic activity via the MTT assay, or collagen content via the Sirius red assay.

C. MTT Assay

Scaffolds or cells growing on TCP were washed with PBS and incubated with MTT-ESTA (0.5 mg mL^{-1} in PBS) for 70 minutes at 37°C. Acidified isopropanol (200 µL) was used to solubilize the formazan and absorbance was measured at 540 nm with a protein reference of 630 nm subtracted. Activity was calculated as a percentage of the control.

D. Sirius Red Assay

Scaffolds or cells growing on TCP were washed with PBS and fixed with 4% formaldehyde for 20 min, before being washed 3x in PBS. Total cellular collagen production was quantified by staining with a 0.1% Picrosirius red solution (0.1% Direct Red 80 in saturated picric acid) for 18 hours at room temperature. The remaining solution was washed away with deionized water and the resulting stain was removed by rocking at room temperature with methanol:0.2 M sodium hydroxide (1:1) for 30 minutes. The absorbance of the resulting solution was then measured at 490 nm

III. Results

The synthetic process used in this study was able to generate PGS-M scaffolds of the desired dimensions (Figure 1B). These scaffolds could then be sterilized and used to support fibroblast growth. Cell metabolic activity assays showed that viable human dermal fibroblasts could be observed on the 50% methacrylated PGS-M scaffolds after 1 week in culture (Fig. 2A), demonstrating the biocompatibility of the PGS-M scaffold. However the metabolic activity measured at the end of the week was significantly lower (P=0.0005) than that observed for the same cells growing on the TCP control. However, interestingly, Figure 2B shows that the cells growing on the PGS-M scaffolds generated approximately 50 % more total collagen over the course of the week in culture compared to the same cells on the TCP control (P<0.0001).

Taking into account the reduced cell numbers on the PGS-M scaffolds compared to the control it can be seen that there is an approximately 10 fold increase in the amount of collagen produced by the cells (Fig. 2C) when grown on the PGS-M scaffolds compared to the TCP control (P<0.0001).

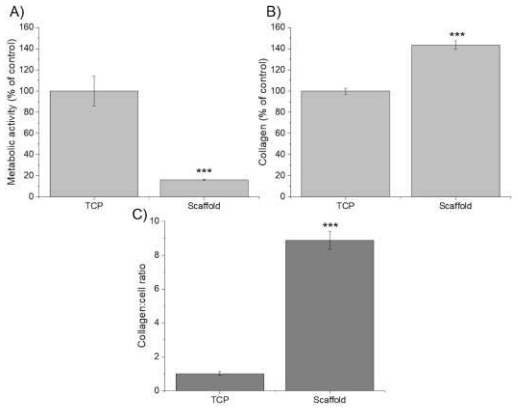

Fig. 2. The cellular metabolic activity (A) and total collagen (B) detected after 1 week of human dermal fibroblast culture on TCP or 50% PGS-M scaffold. The ratio of total collagen to metabolic activity is also shown (C). Results shown are mean from n=3 samples, error bars are standard deviation.

The initial experiments were supplemented with ascorbic acid 2 phosphate in the culture medium. However, once it had been established that the fibroblasts produced significantly more collagen on the scaffolds than in 2D culture, further studies were performed to ascertain if the addition of ascorbic acid 2 phosphate was in any way beneficial. Figure 3 shows the cell metabolic activity and total collagen from dermal fibroblasts after 1 week in culture on TCP or PGS-M scaffolds in the absence or presence of 50 $\mu g\ ml^{-1}$ ascorbate acid-2-phosphate. The results show a similar response to that observed in the initial experiments, with a significant reduction in metabolic activity and a significant increase in total collagen on the scaffolds compared to tissue culture plastic. However, removal of the ascorbic acid had no significant impact on collagen production or cell proliferation in either 2D or 3D culture.

IV. Discussion

Glass coverslips coated with a low Mw 30 % PGS-M film and 3D scaffolds with the same 30% methacrylation have previously been shown to support both fibroblast and smooth muscle cell growth at comparable levels to uncoated glass surfaces [15]. However, this is the first time that fibroblast growth on 50 % methacrylated PGS-M scaffolds has been reported. Although the metabolic activity of the cells growing on these scaffolds, as measured by the MTT assay, shows a significant decrease in activity compared to the TCP control. This suggests fewer cells growing on the scaffold than on TCP after 1 week.

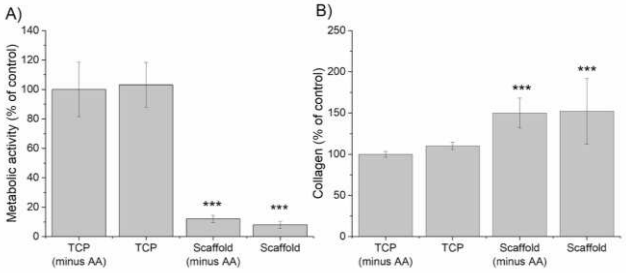

Fig. 3. The impact of removing the ascorbic acid-2-phosphate supplement (AA) from the culture medium upon cell metabolic activity (A) and collagen production (B) when cells are culture for 1 week on TCP or 50% PGS-M scaffolds. Results shown are mean from n=3 samples, error bars are standard deviation.

It has been noted that the responsiveness of the MTT assay appears to be greatly reduced when measuring cell metabolic activity in PGS-M scaffolds. Other methods to evaluate cell metabolic activity may be more suitable, such as the resazurin cell viability assay [17]; alternatively improved cell proliferation data may be more readily obtained through the quantification of total DNA present [18]. However, it was also noted that many cells did not attach to the scaffold, with a large number of cells observed growing instead on the TCP around the scaffold during the experiments. This reduced cell attachment is, in part at least, likely to have led to a reduction in cell numbers after 1 week in culture. Further studies are therefore required to determine whether the observed reduction in cell attachment on the scaffolds was a result of the change in the extent of methacrylation, or another factor, such as the cell seeding process, which may have resulted in cells being washed onto the TCP before cell attachment to the scaffold could take place.

Despite the indication of reduced cell numbers growing in the PGS-M scaffolds, the total amount of collagen produced was significantly higher than for the cells growing on TCP. Other studies report similar findings, with an increase in collagen production when cells are grown on other 3D scaffolds compared to 2D [19,20]. In particular, the non-methacrylated version of this scaffold, PGS, has already been shown to enhance collagen production [21,22], compared to cells grown in 2D. This is perhaps unsurprising, since 3D scaffolds would be expected to facilitate growth of fibroblasts in a more biologically relevant environment, where the cells are better able to perform their normal functions of matrix formation and remodeling [23]. The PGS-M scaffold used in this study has similar properties to the PGS scaffold, being both biocompatible and biodegradable, but has the added advantage that it is simple to synthesize and can be readily photocured to generate a variety of 3D structures [15]. The additional evidence, generated in this study, that collagen production is also enhanced indicates that PGS-M shows considerable potential for use in soft tissue engineering.

It was, however, unexpected that removal of ascorbic acid-2-phosphate had no apparent effect on the culture. Ascorbic acid-2-phosphate is the stable version of Vitamin C, and is generally used in cell cultures to enhance collagen production, with around 2 to 3 fold increases in collagen production reported from fibroblasts grown in similar concentrations of ascorbic acid [20,24,25]. Furthermore, other studies demonstrate that the addition of ascorbic acid-2-phosphate also increased fibroblast proliferation alongside enhanced collagen production [26,27]. The reports however do suggest that the optimal concentration of ascorbic acid-2-phosphate is cell type dependent; furthermore since the cells used in this study were primary cells, it may be that there is some patient to patient variability in the response to ascorbic acid-2-phosphate and consequently further optimization of concentrations may be required.

It has also been shown that the application of mechanical strain during culture can enhance collagen production and or-

ganization in engineered tissues [28,29]. Therefore, the effect of mechanical stimulation of the PGS-M scaffolds on both cell proliferation and collagen production will be assessed in future experiments to determine if the increase in collagen production reported here could be further enhanced.

Further studies are required to determine the impact of varying the degree of PGS methacrylation on collagen production. Nevertheless, the rapid degradation rates described previously for PGS-M scaffolds [15], in conjunction with the improvement in collagen production demonstrated in this study mean PGS-M has the potential to be used for the in vitro generation of ECM-based constructs for soft tissue engineering.

REFERENCES

[1] Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci. 2010;123: 4195–4200.

[2] Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, et al. Molecular Biology of the Cell, Sixth Edition. Garland Science; 2014.

[3] Rozario T, DeSimone DW. The extracellular matrix in development and morphogenesis: a dynamic view. Dev Biol. 2010;341: 126–140.

[4] Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: Structure and function. Acta Biomater. 2009;5: 1–13.

[5] Greco KV, Francis L, Somasundaram M, Greco G, English NR, Roether JA, et al. Characterisation of porcine dermis scaffolds decellularised using a novel non-enzymatic method for biomedical applications. J Biomater Appl. 2015;30: 239–253.

[6] Dahl SLM, Koh J, Prabhakar V, Niklason LE. Decellularized Native and Engineered Arterial Scaffolds for Transplantation. Cell Transplant. 2003;12: 659–666.

[7] Taylor DA, Sampaio LC, Ferdous Z, Gobin AS, Taite LJ. Decellularized matrices in regenerative medicine. Acta Biomater. 2018; doi:10.1016/j.actbio.2018.04.044

[8] Yi S, Ding F, Gong L, Gu X. Extracellular Matrix Scaffolds for Tissue Engineering and Regenerative Medicine. Curr Stem Cell Res Ther. 2017;12: 233–246.

[9] Chang J, DeLillo N Jr, Khan M, Nacinovich MR. Review of small intestine submucosa extracellular matrix technology in multiple difficult-to-treat wound types. Wounds. 2013;25: 113–120.

[10] Barendse-Hofmann MG, van Doorn LP, Oskam J, Steenvoorde P. Extracellular matrix prevents split-skin grafting in selected cases. J Wound Care. 2007;16: 455–458.

[11] Boyd WD, Johnson WE 3rd, Sultan PK, Deering TF, Matheny RG. Pericardial reconstruction using an extracellular matrix implant correlates with reduced risk of postoperative atrial fibrillation in coronary artery bypass surgery patients. Heart Surg Forum. 2010;13: E311–6.

[12] Fallon A, Goodchild T, Wang R, Matheny RG. Remodeling of extracellular matrix patch used for carotid artery repair. J Surg Res. 2012;175: e25–34.

[13] Dahl SLM, Kypson AP, Lawson JH, Blum JL, Strader JT, Li Y, et al. Readily available tissue-engineered vascular grafts. Sci Transl Med. 2011;3: 68ra9.

[14] Huang AH, Lee Y-U, Calle EA, Boyle M, Starcher BC, Humphrey JD, et al. Design and Use of a Novel Bioreactor for Regeneration of Biaxially Stretched Tissue-Engineered Vessels. Tissue Eng Part C Methods. 2015;21: 841–851.

[15] Pashneh-Tala S, Owen R, Bahmaee H, Rekštytė S, Malinauskas M, Claeyssens F. Synthesis, Characterization and 3D Micro-Structuring via 2-Photon Polymerization of Poly(glycerol sebacate)-Methacrylate–An Elastomeric Degradable Polymer [Internet]. 2018. doi:10.3389/fphy.2018.00041

[16] Wong LLC, Ikem VO, Menner A, Bismarck A. Macroporous Polymers with Hierarchical Pore Structure from Emulsion Templates Stabilised by Both Particles and Surfactants. Macromol Rapid Commun. 2011;32: 1563–1568.

[17] O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem. 2000;267: 5421–5426.

[18] Quent VMC, Loessner D, Friis T, Reichert JC, Hutmacher DW. Discrepancies between metabolic activity and DNA content as tool to assess cell proliferation in cancer research. J Cell Mol Med. 2010;14: 1003–1013.

[19] Rothan HA, Djordjevic I, Bahrani H, Paydar M, Ibrahim F, Abd Rahmanh N, et al. Three-dimensional culture environment increases the efficacy of platelet rich plasma releasate in prompting skin fibroblast differentiation and extracellular matrix formation. Int J Med Sci. 2014;11: 1029–1038.

[20] Osman NI, Roman S, Bullock AJ, Chapple CR, MacNeil S. The effect of ascorbic acid and fluid flow stimulation on the mechanical properties of a tissue engineered pelvic floor repair material. Proc Inst Mech Eng H. 2014;228: 867–875.

[21] Hagandora CK, Gao J, Wang Y, Almarza AJ. Poly (glycerol sebacate): a novel scaffold material for temporomandibular joint disc engineering. Tissue Eng Part A. 2013;19: 729–737.

[22] Gao J, Ensley AE, Nerem RM, Wang Y. Poly(glycerol sebacate) supports the proliferation and phenotypic protein expression of primary baboon vascular cells. J Biomed Mater Res A. 2007;83: 1070–1075.

[23] Rhee S. Fibroblasts in three dimensional matrices: cell migration and matrix remodeling. Exp Mol Med. 2009;41: 858.

[24] Tajima S, Pinnell SR. Regulation of collagen synthesis by ascorbic acid. Ascorbic acid increases type I procollagen mRNA. Biochem Biophys Res Commun. 1982;106: 632–637.

[25] Russell SB, Russell JD, Trupin KM. Collagen synthesis in human fibroblasts: Effects of ascorbic acid and regulation by hydrocortisone. J Cell Physiol. 1981;109: 121–131.

[26] Hata R, Senoo H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. J Cell Physiol. 1989;138: 8–16.

[27] Phillips CL, Combs SB, Pinnell SR. Effects of ascorbic acid on proliferation and collagen synthesis in relation to the donor age of human dermal fibroblasts. J Invest Dermatol. 1994;103: 228–232.

[28] Kim BS, Nikolovski J, Bonadio J, Mooney DJ. Cyclic mechanical strain regulates the development of engineered smooth muscle tissue. Nat Biotechnol. 1999;17: 979–983.

[29] Seliktar D, Black RA, Vito RP, Nerem RM. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. Ann Biomed Eng. 2000;28: 351–362