Microporous collagen spheres produced via thermally induced phase separation for tissue regeneration

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ABSTRACT

Collagen is an abundant protein found in the extracellular matrix of many tissues. Due to its biocompatibility, it is a potentially ideal biomaterial for many tissue engineering applications. However, harvested collagen often requires restructuring into a three-dimensional matrix to facilitate applications such as implantation into poorly accessible tissue cavities. The aim of the current study was to produce a conformable collagen-based scaffold material capable of supporting tissue regeneration for use in wound repair applications. Microporous collagen spheres were prepared using a thermally induced phase separation (TIPS) technique and their biocompatibility was assessed. The collagen spheres were successfully cross-linked with glutaraldehyde vapour, rendering them mechanically more stable. When cultured with myofibroblasts the collagen spheres stimulated a prolonged significant increase in secretion of the angiogenic growth factor, vascular endothelial growth factor (VEGF), compared with cells alone. Control polycaprolactone (PCL) spheres failed to stimulate a similar prolonged increase in VEGF secretion. An enhanced angiogenic effect was also seen in vivo using the chick embryo chorioallantoic membrane assay, where a significant increase in the number of blood vessels converging towards collagen spheres was observed compared with control PCL spheres. The results from this study indicate that microporous collagen spheres produced using TIPS are biologically active and could offer a novel conformable scaffold for tissue regeneration in poorly accessible wounds.

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1. Introduction

Collagen is the most abundant naturally occurring protein found in connective tissue and thus constitutes a major component of the extracellular matrix. Due to high sequence homology between species, collagen exhibits low cytotoxicity and immunogenicity and high biocompatibility, making it one of the most promising biomaterials for tissue engineering. Collagen-based scaffolds have been used in a variety of tissue engineering applications, including cartilage [1], ligament [2], heart valve [3], peripheral nerve regeneration [4] and skin substitutes [5]. This has been explored using gels [6], films [7], sheets [8], sponges [9,10], tubes [11,12], plugs [13–15] and spheres [16,17].

One type of complication for which collagen products are attracting increasing interest are fistulae. Zenoderm® (lyophilized porcine dermis) and AlloDerm® (acellular human cadaveric dermis) have been shown to be successful in closing oroantral fistulae and rectovaginal fistulae, respectively [18,19]. Injectable Permacol® (acellular porcine dermal collagen) has also been evaluated in an experimental porcine model of perianal fistulae [20]. Small intestinal submucosa (SIS), a naturally occurring biomaterial derived from porcine small intestine that is rendered acellular during processing and promotes remodeling when used as a xenograft, has been used in a variety of tissue engineering applications, including vascular [21–23], bladder [24] and abdominal wall repair [25], and is currently the most extensively studied collagen-based product for fistula repair.

The success of SIS in fistula healing [26,27] has led to the development of the first medical device for perianal fistula repair, the Surgisis® AFP™ Fistula Plug, a conically shaped plug composed of lyophilized porcine submucosa.

However, despite initial success rates of up to 80% [28], subsequent studies have reported success rates as low as 12.5% [29]. One of the main reasons for failure of the AFP™ Fistula Plug is extrusion of the device from the fistula tract. This is most likely due to a lack

of rapid integration of the scaffold into surrounding host tissue resulting from poor conformability of the device in irregular-shaped tracts. Spheres are likely to be advantageous in the application to inaccessible wounds, such as fistulae, due to their conformity to irregular shapes associated with wounds and efficient packing of the wound cavity.

Previous methods used to prepare collagen spheres include the water-in-oil emulsion technique [30] and dispersion of a collagen solution in water-immiscible organic solvents at 0–25 °C, followed by the solidification of the spheres by raising the temperature to 30–40 °C [31]. However, one difficulty associated with these methods is the complete removal of any residual oil or solvent. An alternative technique to prepare spheres, which avoids the use of oils and solvents, is thermally induced phase separation (TIPS) [32], which involves the delivery of droplets of a solution into liquid nitrogen followed by freeze-drying.

The aim of the current study was to fabricate and characterize highly porous collagen spheres using TIPS and assess their biocompatibility and angiogenic properties.

2. Materials and methods

2.1. Fabrication of collagen and poly(e-caprolactone) spheres

One percent w/v collagen dispersion was prepared by adding 0.4 g of Type I collagen derived from calf skin (Sigma–Aldrich, Poole, UK) to 40 ml of 0.5 M acetic acid and stirring at 200 rpm at room temperature overnight.

Poly(e-caprolactone) (PCL) (Sigma–Aldrich, UK) was added to dimethyl carbonate (DMC; Sigma–Aldrich) at a ratio of 1:6 (w/v) in a 50 ml Falcon™ tube. To facilitate dissolution of PCL, the Falcon™ tube was placed in a beaker of water heated to 60 °C and stirred at 200 rpm until the PCL had completely dissolved.

The collagen and PCL solutions were extruded into 50 ml Falcon tubes containing liquid nitrogen at a rate of 12 droplets min^{-1} at a distance of 5 cm from the surface of liquid nitrogen using a stainless steel nozzle (inner diameter of 0.17 mm; outer diameter of 0.35 mm; Nisco Engineering AG, Switzerland) attached to a 1 ml polypropylene syringe. The droplets solidified upon contact with liquid nitrogen forming spheres and were placed in a −70 °C freezer for 1 h to allow the liquid nitrogen to evaporate. The tubes were transferred to an upright freeze-drier (Model EF03, Edwards Modulyo, West Sussex, UK) and the frozen collagen and PCL spheres were lyophilized for 24 h to sublimate the acetic acid and DMC, respectively, and yield microporous spheres. The PCL spheres served as the negative control throughout the study.

2.2. Cross-linking of collagen spheres

To increase the mechanical strength of the collagen spheres and render them more resistant to degradation in aqueous solution, the lyophilized collagen spheres were cross-linked with glutaraldehyde. Previous studies using glutaraldehyde as a cross-linking agent for collagen have involved immersing the material in the cross-linking agent. However, collagen spheres prepared in the current study immediately lost their structure upon immersion in the glutaraldehyde solution (data not shown). Therefore, the spheres were cross-linked with vapour from glutaraldehyde for 24 h. Vapour from two concentrations of glutaraldehyde (2.5 and 25 vol.% in phosphate-buffered saline (PBS)) was assessed to determine which would be the most effective. Briefly, collagen spheres were transferred to 10 cm Petri dishes lined with foil to prevent static attachment of the spheres to the dish. In a fume hood, the dishes were placed on an orbital shaker (200 rpm) in a container measuring 0.04 m^3. The bottom of a 10 cm Petri dish filled with

10 ml of glutaraldehyde solution was also placed in the container. The container was sealed with foil and the spheres were allowed to cross-link for 24 h.

2.3. Measurement of collagen and PCL sphere size

The diameter of collagen and PCL spheres was measured from photomicrographs of the spheres taken at 20× magnification using a light microscope (Olympus BX50) attached to a video camera (CoolSNAP-Pro$_{cf}$ Colour (Media Cybernetics, Inc). The mean diameter of the spheres was measured using image-processing software (Image-Pro® Plus, Version 4.5.0.19 (Media Cybernetics, Inc)) calibrated to the video microscopy system. The average sphere size was expressed as the mean diameter ± standard error of the mean of 10 spheres.

2.4. Scanning electron microscopy

Surface and cross-sectional morphology of the collagen and PCL spheres was examined by scanning electron microscopy (SEM). The spheres were mounted onto aluminium stubs via adhesive carbon tabs and sputter-coated with gold/palladium for 3 min in an argon atmosphere (Polaron E5000; Polaron, UK) and viewed under a scanning electron microscope (Jeol JSM-5410LV) operated at 20 kV.

2.5. Measurement of glutaraldehyde cross-linking

Glutaraldehyde reacts with the ϵ-amino groups of lysine, cross-linking them together [33]. The extent of glutaraldehyde cross-linking of collagen spheres was determined by measuring the concentration of free primary amine groups remaining using 2,4,6-trinitrobenzenesulfonic acid (TNBS), based on a previously described method [34]. Briefly, 5 mg of spheres was added to 1 ml of 4% w/v NaHCO$_3$ and incubated for 30 min at room temperature. Following the addition of 1 ml of 0.5% w/v TNBS in 4% w/v NaHCO$_3$, the tubes were incubated at 40 °C for 2 h. The samples were hydrolysed at 60 °C for 90 min after the addition of 3 ml of 6 M HCl. The reaction mixture was diluted with 5 ml of MilliQ water and cooled to room temperature. One hundred microlitres of the solution was transferred to wells of a 96-well plate and the absorbance measured at 420 nm. A blank was prepared using the same procedure, except that HCl was added prior to the TNBS solution. The absorbance values were correlated to the concentration of free amino groups using a calibration curve obtained with twofold serial dilutions of a stock solution of 10 mg of glycine in 100 ml of 4% w/v NaHCO$_3$.

2.6. Cell and culture conditions

Human colonic myofibroblasts (CCD-18Co) were obtained from the American Type Culture Collection (Rockville, MD) (CRL-1459). This cell line was used as myofibroblasts have recently been found to be present in perianal fistulae [35]. Cells were cultured in complete medium (Eagle's minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 1% non-essential amino acids, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin (Gibco)) at 37 °C in 5% CO$_2$/95% humidity. Cells were passaged every 3–4 days.

2.7. Measurement of VEGF secretion from cells cultured with spheres

CCD-18Co cells (passage 14–20) were seeded in a 96-well cell culture plate (3×10^3 cells well^{-1}) in 170 μl of complete medium (as described above) and cultured for 4 days at 37 °C in 5% CO$_2$/95% humidity. The medium was removed and cells were washed once with PBS. The collagen and PCL spheres were

UV-sterilized in a cell culture cabinet for 30 min, with orbital shaking at 200 rpm. The spheres were placed in PBS and the air within the pores of the spheres was displaced under vacuum. When the vacuum was removed, the spheres became impregnated with PBS and sank. Twelve spheres were transferred to the 96-well plate in replicates of five. One hundred and seventy microlitres of complete medium was added per well and the spheres were cultured with the cells at 37 °C in 5% CO_2/95% humidity for 10 days. Cells alone served as a negative control. Conditioned medium was collected at 1 day intervals and replaced with 170 µl of fresh medium. Conditioned medium was stored at −70 °C until further analysis. The amount of VEGF secreted from cells following 1–10 days of culture with collagen or PCL spheres was measured using a quantitative sandwich enzyme immunoassay (DuoSet DY293B Quantikine® human VEGF; R&D Systems, UK).

2.8. Cell viability

Following the collection of conditioned medium from the collagen and PCL spheres at the final time-point of 10 days, the viability of the cells was determined by performing the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [36]. Briefly, 100 µl of fresh medium and 10 µl of 5 mg ml^{-1} MTT in PBS were added per well. After 4 h incubation at 37 °C, 100 µl of 20 vol.% sodium dodecyl sulphate:50 vol.% dimethylformamide was added per well and incubated at room temperature overnight in the dark to solubilize the formazan crystals. One hundred microlitres of the solubilized formazan solution was transferred to wells of a fresh 96-well cell culture plate and the absorbance measured at 540 nm.

2.9. Degradation of collagen spheres

The degradation rate of the collagen spheres was assessed over 10 weeks. Collagen spheres were UV-sterilized and the trapped air within the spheres was displaced as described above. Five collagen spheres were placed in 1.5 ml microfuge tubes containing 500 µl of PBS in triplicate for each time-point (1 and 3 days, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 weeks) and incubated at 37 °C. At each time point, photographs of the spheres were taken to visually assess the extent of degradation. The supernatants were collected and stored at −70 °C. At each time point, the microfuge tubes containing spheres for subsequent time points were inverted three times and then left to settle for 2 min. To account for the movement and replacement of tissue fluid that is likely to occur in vivo, half of the solution, i.e. 250 µl of PBS, was removed and replaced with fresh PBS. The microfuge tubes were inverted a further three times to mix and then returned to incubation at 37 °C until the next time point.

2.10. In vivo analysis of collagen spheres

The chick embryo chorioallantoic membrane (CAM) assay, an in vivo model for evaluating the angiogenic properties of materials, was used to assess the collagen spheres compared with control PCL spheres. Fertilized brown Goldline chicken eggs were incubated horizontally at 37.5 °C in a humidified incubator (Lyon, Technologies Inc, USA) for 3 days. At day 3 of incubation, a false air sac was created directly over the CAM by aspirating 2 ml of albumin from the acute end of the egg using a 21-G gauge needle (BD Microlance™ Hypodermic Needle, Becton–Dickinson, UK) attached to a 5 ml polypropylene syringe (BD Plastipak™, Becton–Dickinson, UK). A circular window, approximately 3 cm in diameter, was cut into the shell with small dissecting scissors to reveal the embryo and CAM vessels. The window was sealed with Sellotape® and the eggs were returned to the incubator for a further 4 days. At day 7 of incubation, the spheres to be implanted were sunk in

PBS, as described above, and placed between the secondary and tertiary blood vessels branching off the main blood vessel attached directly to the embryo, approximately 2 mm from secondary blood vessel and 5 mm from the junction of the two vessels. Filter paper discs (6 mm in diameter) saturated with 5 µl of bFGF (200 ng) served as a positive control indicating angiogenic activity in the CAM assay. Because the filter paper discs were larger and could not be implanted in the same way, they were placed in sparsely vascularized areas of the CAM. Each type of sample was implanted in duplicate on separate CAMs, allowing as much distance between the two samples as possible to prevent interference. Following implantation, the samples placed on the CAMs were imaged using a digital stereo microscope (Leica MZFL111 and Leica software (IM (Image Manager) 1000) at 20× magnification. The windows were resealed with Sellotape® and eggs incubated for a further 3 or 7 days in a humidified incubator at 37.5 °C. At day 3 or 7 post-implantation (day 10 or 14 of incubation), images of the samples on the CAM were captured as described above to assess changes in vascularization. These images were used for the quantification of blood vessels surrounding the biomaterials. To assess angiogenesis, the number of blood vessels converging towards the spheres was counted manually. Only microvessels (blood vessels less than 10 µm in diameter), as determined using a scale placed on images, were counted as previously described [37]. All studies using the CAM assay were conducted in compliance with institutional ethical use protocols.

2.11. Statistical analysis

Data are expressed as means ± standard error of the mean of the indicated number of replicates. The data were subjected to a Kolmogorov–Smirnov (KS) test for normality and found to follow a normal distribution. Comparison between groups was performed using unpaired two-tailed t-tests. Statistical significance was accepted at the $p < 0.05$ level. Calculations were performed using GraphPad Prism Version 4.00 statistical software (GraphPad Software, San Diego, USA).

3. Results

3.1. Collagen and PCL spheres

SEM analysis revealed the surface of the uncross-linked collagen spheres was rough and highly porous (Fig. 1). The internal structure was also highly porous and interconnected. Cross-linked collagen spheres revealed a similar spherical structure to the uncross-linked collagen spheres but the surface pores were reduced in size compared with uncross-linked collagen spheres. The internal porous structure of the cross-linked collagen spheres appeared to be similar to that of the uncross-linked spheres. SEM images of the collagen spheres indicate the pore sizes range from approximately 5–10 µm in diameter.

SEM analysis of the PCL spheres revealed well-formed spheres with a relatively smooth surface containing pores of a cylindrical shape arranged in a chevron-like pattern (Fig. 1). The internal porous structure of the PCL spheres consisted of interconnected cylindrical pores, a large number of which were lined with ladder-like structures.

3.2. Glutaraldehyde cross-linking of collagen spheres

Following 24 h cross-linking with 2.5 vol.% glutaraldehyde vapour, the mean diameter of collagen spheres was significantly reduced to 1.74 ± 0.03 mm, compared with uncross-linked collagen spheres at 2.30 ± 0.02 mm in diameter ($p < 0.0001$) (Fig. 2).
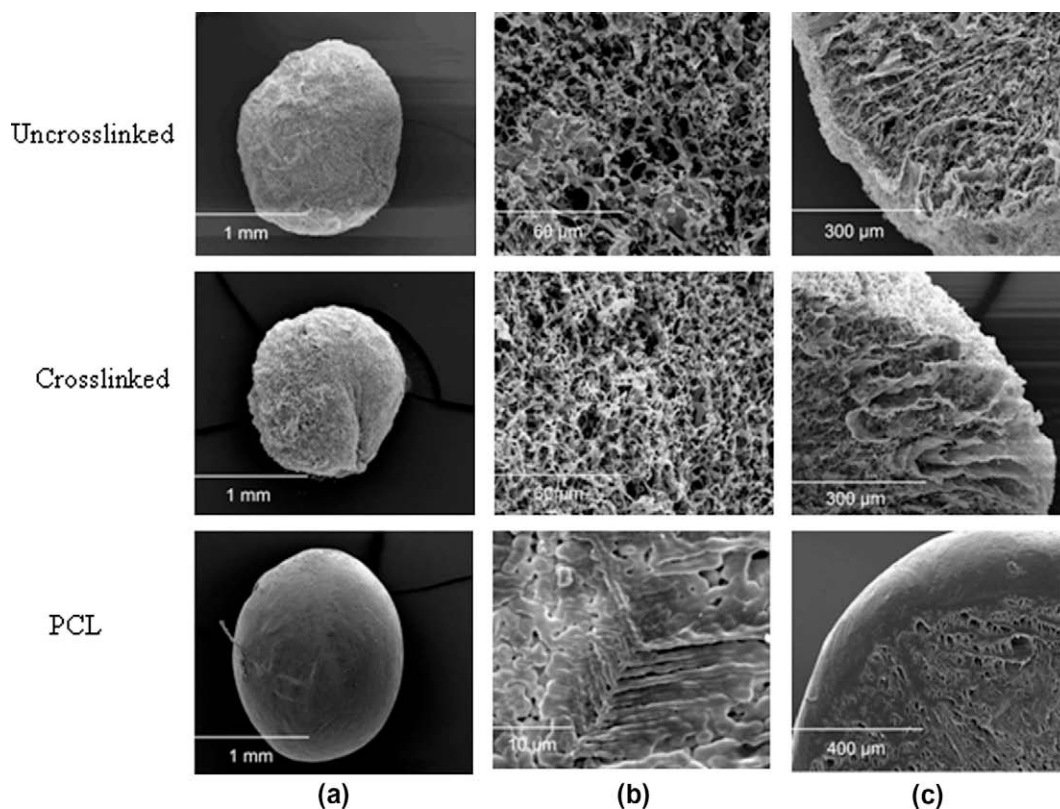
Fig. 1. SEM of uncross-linked and glutaraldehyde-cross-linked collagen spheres and PCL spheres. (a) Whole sphere; (b) surface of sphere; (c) internal structure of sphere.
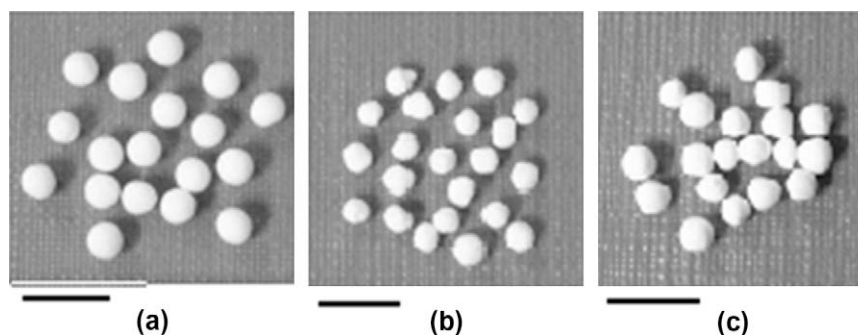

Fig. 2. Collagen spheres: (a) uncross-linked; (b) after 24 h of cross-linking with 2.5 vol.% glutaraldehyde vapour. (c) PCL spheres. Scale bar = 5 mm.

The concentration of free lysine residues measured in uncross-linked collagen spheres was 22.33 µg ml^{-1}. Cross-linking of the collagen spheres with 2.5 ($p < 0.05$) and 25 vol.% ($p < 0.01$) glutaraldehyde vapour for 24 h resulted in a significant reduction in free lysine residues compared with uncross-linked collagen spheres (Fig. 3). There was no significant difference in the amount of free lysine residues between the two concentrations of glutaraldehyde. It was therefore decided to use the lower concentration of glutaraldehyde (2.5 vol.%) to cross-link the collagen spheres.

3.3. Cell viability

At the end of the 10 day study period, the viability of cells was determined using the MTT assay (Fig. 4). The viability of cells cultured with the collagen spheres was significantly reduced compared with cells cultured alone ($p < 0.001$). Likewise, the viability of cells following 10 days culture with PCL spheres was also significantly reduced compared with cells cultured alone ($p < 0.001$).

3.4. VEGF secretion from cells cultured with collagen or PCL spheres

The amount of VEGF secreted from cells cultured with collagen or PCL spheres was measured at days 1–10 of culture (Fig. 5). Collagen spheres stimulated a progressive increase in VEGF secretion from cells between days 4–10 of culture. A significant increase in VEGF secretion was seen at days 2, 3, 7, 9 and 10 of culture compared with cells cultured alone.

PCL spheres initially stimulated a significant increase in VEGF secretion at days 1 and 2 compared with cells cultured alone. However, from day 3 onwards, cells cultured with PCL spheres secreted significantly reduced levels of VEGF compared with cells cultured alone (Fig. 5).

3.5. Collagen sphere degradation

Glutaraldehyde cross-linked collagen spheres were immersed in PBS and incubated at 37 °C for a period of up to 10 weeks
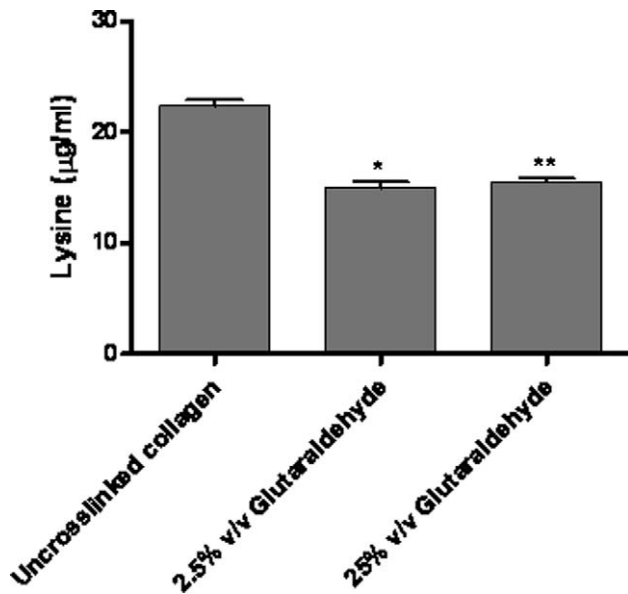
Fig. 3. Available free lysine residues following 24 h glutaraldehyde cross-linking. The concentration of free lysine residues available in collagen spheres was significantly reduced following 24 h of cross-linking with 2.5% (*$p < 0.05$) and 25% (**$p < 0.01$) glutaraldehyde vapour compared with uncross-linked collagen spheres.
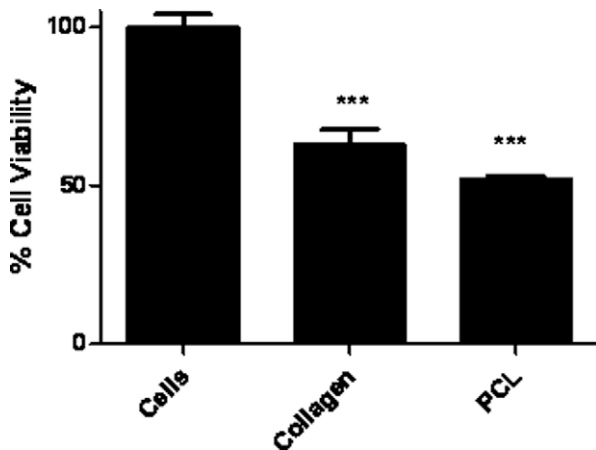

Fig. 4. Cell viability after 10 days of culture with collagen or PCL spheres. Cell viability was significantly reduced compared with cells cultured alone (***$p < 0.001$).

Fig. 5. Secretion of VEGF from cells after 1–10 days of culture with (a) collagen and (b) PCL spheres. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(Fig. 6). Visual inspection revealed that the spheres remained intact for up to 4 weeks, after which they started to lose their spherical structure and clump together. After 5 weeks the spheres had completely lost their spherical structure, and by week 10 the collagen fibres had dispersed into solution.

3.6. In vivo analysis of collagen spheres

The effect of collagen spheres on directed blood vessel growth was quantified by manually counting the total number of blood vessels converging towards the spheres. Filter paper discs saturated with 200 ng of bFGF, which served as positive controls, induced a positive angiogenic response characterized by a spoked wheel pattern of vascularization at both 3 and 7 days after implantation (Fig. 7). There was no directed blood vessel growth towards the PCL spheres after implantation. At 3 days following implantation, there appeared to be no blood vessels converging towards the collagen spheres; however, a spoked wheel pattern of vascular-
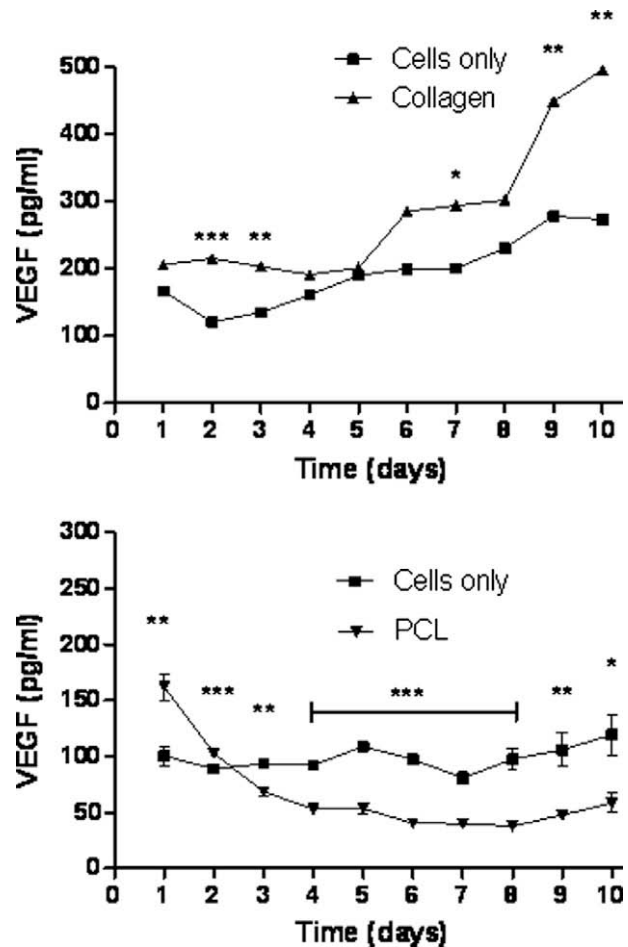
ization was evident at 7 days following implantation. At 3 days after implantation, collagen spheres stimulated a non-significant increase in the number of converging blood vessels compared with PCL spheres. At 7 days after implantation, the number of blood vessels converging towards the collagen spheres was significantly increased compared with PCL spheres ($p < 0.05$).

4. Discussion

Collagen-based medical devices are gaining interest and many implants and prosthetic devices currently exist, particularly for vascular, orthopaedic, and dental applications. Recently, the first medical device for perianal fistula repair, the Surgisis® AFP™ Fistula Plug composed of collagen has also been developed.

One reason why tissue engineering scaffolds fail is the lack of cell infiltration and subsequent integration of the device into host tissue. Insufficient porosity preventing rapid cell and tissue infiltration may explain why the Surgisis® AFP™ Fistula Plug is extruded from fistula tracts. Furthermore, the shape and rigidity of the plug is not suitable for all fistulae, especially complex fistulae that may have branched tracts and multiple openings. An alternative experimental approach for treating fistulae has involved the instillation of collagen paste into the tracts [20]. Whilst this approach theoretically allows delivery of the collagen material into complex fistulae, the collagen slurry does not provide a structured environment capable of guiding tissue infiltration. Rapid inter- and intra-tissue infiltration of the microporous structure of TIPS
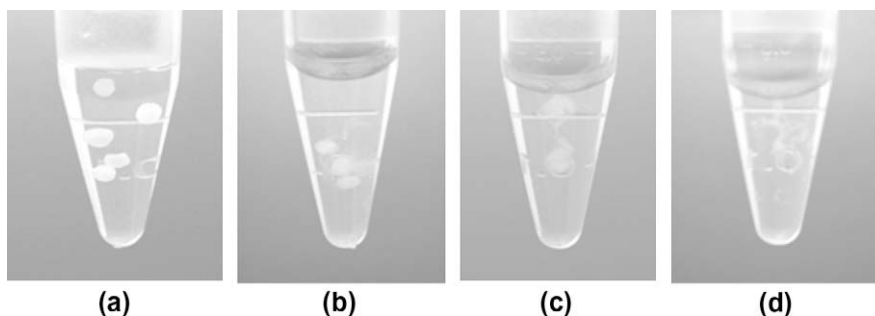
Fig. 6. Degradation of collagen spheres following immersion in PBS at 37 °C for: (a) 0 weeks; (b) 4 weeks; (c) 5 weeks; (d) 10 weeks.
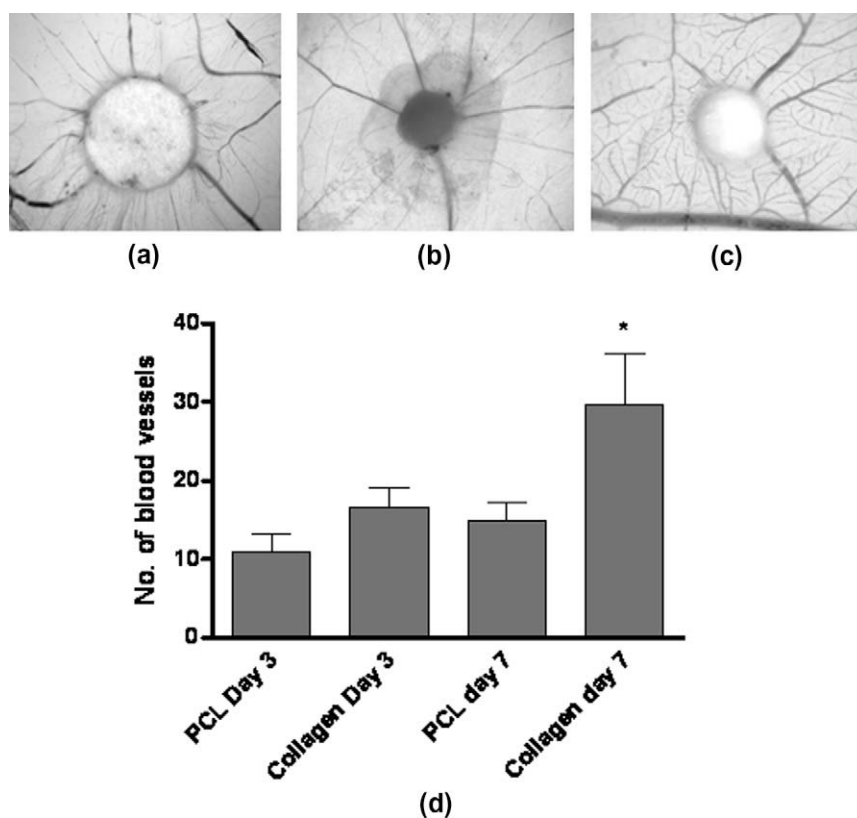

Fig. 7. Representative images of the excised chick CAMs implanted with various materials. Directed blood vessel growth was observed towards (a) filter paper disc + 200 ng bFGF (7 days; 10× mag) and (b) collagen spheres (7 days; 25× mag), but (c) no directed blood vessel growth was observed towards PCL spheres (7 days; 25× mag). (d) Effect of collagen and PCL spheres on the number of converging blood vessels at 3 and 7 days post-implantation. On day 7 the number of blood vessels converging towards collagen spheres was significantly increased compared with PCL spheres ($^*p < 0.05$). Day 3: collagen $n = 8$, PCL $n = 6$; day 7: collagen $n = 3$, PCL $n = 8$.

spheres composed of poly(lactide-co-glycolide) (PLGA) has been observed [38]. Therefore the properties of collagen spheres fabricated using a similar technique were explored in the current study.

SEM analysis of the collagen spheres revealed a highly interconnected porous structure both on the surface of the spheres and internally. Unlike TIPS microporous spheres fabricated from PLGA, the surface did not contain chevron-like patterns and the internal structure did not contain channel-like radial tubular macropores directed towards the centre of the sphere [32]. These differences are likely to have resulted from the different solvents used in the fabrication processes for each type of sphere.

The size of pores in the collagen spheres ranged from approximately 5 to10 μm in diameter, which would be large enough for blood vessel infiltration, as evidenced by the infiltration of blood vessels into the pores of PLGA TIPS spheres, which ranged from approximately 1 to 5 μm in diameter [38].

Whilst a porous structure is likely to benefit tissue infiltration into the spheres, it also reduces the mechanical strength of the spheres. Chemical cross-linking is frequently used to improve the mechanical properties of collagen, also rendering it resistant to dissolution, heat denaturation and enzymatic degradation. As scaffolds for soft tissue engineering should preferably remain biodegradable, cross-linking of collagen usually involves the introduction of labile bonds, which can be either enzymatically or chemically degraded in vivo [39]. One of the most commonly used collagen cross-linking agents is glutaraldehyde. Glutaraldehyde cross-linking results in the formation of covalent bonds between adjacent polymer chains. Previous studies have described immersing the material in a solution of glutaraldehyde, usually at concentrations ranging from 0.1 to 3 vol.%, but concentrations as low as 0.0075 vol.% have also been reported to be effective at reducing degradation [40–45]. The collagen spheres prepared in the current

study were found to lose their spherical structure upon immersion in glutaraldehyde, therefore the spheres were cross-linked using glutaraldehyde vapour. The significant reduction in the concentration of free lysine residues of collagen spheres cross-linked with 2.5 and 25 vol.% glutaraldehyde vapour compared with uncross-linked collagen spheres indicated effective cross-linking. There was no significant difference in the concentration of available lysine residues between collagen spheres cross-linked with 2.5 and 25 vol.% glutaraldehyde, suggesting that maximal cross-linking of collagen spheres was achieved with vapour from a solution of glutaraldehyde at a concentration near 2.5 vol.%.

The mean diameter of the collagen spheres following 24 h cross-linking with 2.5 vol.% glutaraldehyde vapour was significantly reduced compared with the diameter of uncross-linked collagen spheres. SEM analysis of the cross-linked spheres revealed reduced surface pore size, but there was no noticeable difference in internal porous structure compared with uncross-linked collagen spheres. This suggested that the vapour of 2.5 vol.% glutaraldehyde may have effectively cross-linked the external surface of the collagen spheres without completely penetrating inside the sphere.

A supply of oxygen and nutrients is essential for cell survival, hence angiogenesis, the formation of new blood vessels from pre-existing vasculature, plays an important role in tissue engineering. Previous attempts to render collagen scaffolds angiogenic have involved the immobilization of recombinant angiogenic growth factors, such as VEGF, basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β), via heparin [46–49] or the homobifunctional cross-linker SS-PEG-SS [50], or simply admixing the recombinant growth factors to the scaffolds [49]. However, the effectiveness of these scaffolds is limited due to the short half-lives of the growth factors. An alternative method recently assessed was the attachment of adult bone marrow mesenchymal stem cells and dermal fibroblasts [51]. Although this resulted in enhanced endothelial cell proliferation, sourcing the bone marrow stem cells may pose a problem.

Cells cultured with collagen spheres secreted significantly more VEGF throughout the present study than control cells. This suggests that the collagen spheres themselves have a stimulatory effect on VEGF secretion from cells. The angiogenic potential of the collagen spheres was further verified in vivo using the CAM assay, where an increased number of blood vessels converging towards the collagen spheres was observed compared with control PCL spheres. This was consistent with recent findings by Yao et al. [52], who also observed the rearrangement of blood vessels into a spoked wheel pattern in response to collagen 7 days after implantation on the CAM.

Angiogenic activity has also been observed following implantation of porcine SIS, which became rapidly infiltrated with host cells and blood vessels before gradually being replaced by host tissue [53–56]. This is thought to be due to the presence of bFGF, TGF-β and VEGF in SIS [57,58]. In addition to VEGF, other growth factors, including bFGF, TGF-β and tumour necrosis factor-α, have been found to stimulate blood vessel formation in the CAM [59–61]. Therefore, it is possible that the effects observed in the current study are due to the presence of inherent angiogenic factors in type I collagen.

Recently, collagen matrices cross-linked with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide in conjunction with N-hydroxysuccinimide were found to exhibit increased angiogenic activity compared with uncross-linked collagen when assessed using the CAM assay [52]. The current study did not compare the angiogenic activity of glutaraldehyde cross-linked collagen spheres compared with uncross-linked spheres, therefore the angiogenic response observed could be partly attributable to the cross-linking agent. In addition, it is possible that the vascular response observed in the CAM assay is due to an inflammatory response to the collagen spheres. However, the increased levels of VEGF secretion in vitro suggest that it may be at least a partly VEGF-driven response.

The rate of VEGF release from collagen scaffolds immobilizing or loaded with the growth factor is dependent on the rate of degradation of the scaffold [50]. Although VEGF was not loaded into the microporous collagen spheres, the ability of the scaffolds to stimulate VEGF secretion from surrounding cells and/or the release of inherent VEGF will also depend on the rate of degradation of the spheres. Native collagen degrades rapidly, but cross-linking serves to maintain the original mechanical and structural integrity of the scaffold over a longer period of time, thus delaying its degradation. Therefore, the extent of cross-linking of the material affects the rate of degradation. Varying degradation rates of glutaraldehyde-cross-linked collagen implants have been reported, ranging from a few weeks [62,63] to several months [64]. In the current study, a loss of the spherical structure of the collagen spheres was observed as early as 4 weeks following immersion in PBS at 37 °C. This was most likely due to the incomplete cross-linking of the collagen spheres, which may have resulted in the collapse of the internal structure at an early stage. The instability of the collagen spheres in physiological fluids may suggest a reason for concern, particularly in the metalloproteinase-rich environment of fistulae. It is possible that complete cross-linking of the spheres may have resulted in delayed degradation. An increase in the concentration of glutaraldehyde is correlated with a decrease in collagen solubilization [65]; hence the use of a higher concentration of glutaraldehyde in the current study may have delivered a reduced degradation rate of the collagen spheres. Although no significant difference in cross-linking was found in the current study with 25 vol.% glutaraldehyde, as determined by the TNBS assay, concentrations higher than 25 vol.% may be required to significantly improve the cross-linking of the spheres. Alternatively, cross-linking the collagen spheres with carbodiimide, which is less toxic than glutaraldehyde during degradation, may prove more effective. As cross-linking of collagen by UV radiation is reported to result in partial denaturation, the cross-linking of the collagen spheres using UV light was not investigated in this study; however, the spheres were sterilized by exposure to UV light for 30 min and this may have contributed to some further cross-linking of the spheres. Studies have reported cross-linking of collagen following exposure to UV light for 60 min [66] and significant increases in the modulus of collagen hydrogels after exposure to UV for 30–45 min [67]. However, even after this process, the spheres were still found to lose their structure, hence it is possible that UV cross-linking of the collagen spheres prepared in the current study may have also been ineffective.

Adequate vascularization is a key aspect of tissue regeneration and wound healing, where it is required for the delivery of oxygen and nutrients to infiltrating tissues and the removal of waste products. The results from the current study indicate that collagen microspheres may promote vascularization when implanted in vivo, most likely through their ability to stimulate VEGF secretion from cells in the local milieu.

The primary aim of the current study was to determine if collagen spheres could be produced via TIPS and whether they possessed angiogenic activity. Future studies will be required to ascertain whether the microporous collagen spheres can promote blood vessel integration. In the current study the duration the implants were left on the CAM assay was limited to 7 days. Therefore, to substantiate the current findings, further long-term studies are required that use in vivo models with implantation of the spheres into either subcutaneous pockets or fistula-like tracts to determine if blood vessels are able to enter the implant compared with PCL control spheres. Since the collagen spheres tested in vitro appeared

to be fragile, this approach will also provide information not available from the current study on how the collagen spheres respond to compressive forces encountered in vivo, along with their degradation in vivo in the presence of proteases, and the ability of different cell types to colonize the collagen spheres. The inclusion of VEGF-blocking antibodies into such in vivo model systems would also clarify whether glutaraldehyde-induced inflammation contributes to the angiogenic response observed in the current study.

In conclusion, microporous collagen spheres may provide a biologically active scaffold for wounds, such as fistulae, that are difficult to access. Their structure will allow efficient packing into the wound cavity whilst their ability to stimulate vascularization will promote tissue regeneration.

Acknowledgements

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