Spatiotemporal release of VEGF from biodegradable polylactic-co-glycolic acid microspheres induces angiogenesis in chick chorionic allantoic membrane assay

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Abstract

While vascular endothelial growth factor (VEGF) is an acknowledged potent pro-angiogenic agent there is a need to deliver it at an appropriate concentration for several days to achieve angiogenesis. The aim of this study was to produce microspheres of biodegradable polylactic-co-glycolic acid (PLGA) tailored to achieve sustained release of VEGF at an appropriate concentration over seven days, avoiding excessive unregulated release of VEGF that has been associated with the formation of leaky blood vessels. Several formulations were examined to produce microspheres loaded with both human serum albumin (HSA) and VEGF to achieve release of VEGF between 3 and 10 ng per ml for seven days to match the therapeutic window desired for angiogenesis. *In vitro* experiments showed an increase in endothelial cell proliferation in response to microspheres bearing VEGF. Similarly, when microspheres containing VEGF were added to the chorionic membrane of fertilised chicken eggs, there was an increase in the development of blood vessels over seven days in response, which was significant for microspheres bearing VEGF and HSA, but not VEGF alone. There was an increase in both blood vessel density and branching – both signs of proangiogenic activity. Further, there was clearly migration of cells to the VEGF loaded microspheres. In summary, we describe the development of an injectable delivery vehicle to achieve spatiotemporal release of physiologically relevant levels of VEGF for several days and demonstrate the angiogenic response to this. We propose that such a treatment vehicle would be suitable for the treatment of ischemic tissue or wounds.

Keywords: Chorioallantoic membrane; Vascular endothelial growth factor; Angiogenesis; Polylactic-co-glycolic acid; Sustained release

1 Introduction

Ischemic wounds can be linked to many debilitating diseases such as myocardial infarction, diabetes mellitus and chronic limb ischemia. The damage can affect a variety of tissues including myocardium, nerve, skin and skeletal muscle. The ability to enhance tissue perfusion in an ischemic wound through re-vascularisation is still the main challenge despite the major advances in surgical techniques (Tenna et al., 2014). Therapeutic angiogenesis (Takeshita et al., 1994) is a promising strategy to treat ischemic wound diseases which can be enhanced by employing VEGF to promote neovascularisation (Yoon et al., 2004). This concept has attracted many researchers since the mid-90s and despite the promising findings from numerous *in vitro* and *in vivo* animal studies (Cartland et al., 2016; Takeshita et al., 1994; Yu et al., 2015); there are inconsistencies from clinical trials (Eppler et al., 2002; Henry et al., 2001; Kusumanto et al., 2006). This was attributed to the short half-life of VEGF and susceptibility to degradation when injected directly in its solution form (Eppler et al., 2002; Kleinheinz et al., 2010). Overcoming this limitation by increasing the VEGF dose can be toxic at high systemic levels (Karvinen et al., 2011; Tayalia and Mooney, 2009). Therefore, targeted VEGF delivery using controlled release formulations appears to be a better approach to promote neovascularisation at the target tissue. This type of localised delivery requires a careful understanding of the microenvironment required for physiologic angiogenesis, which is dependent on spatiotemporal presentation of the delivered VEGF (Blau and Banfi, 2001).

Polymeric materials present a promising approach in controlling tissue VEGF presentation at the target tissue. They can provide physical protection of the payload until it is released. This also includes tailored release kinetics in term of timing in addition to achieving localised delivery with a minimally invasive injectable formulation (Silva and Mooney, 2007). Such polymeric formulations employ a lower VEGF dose for achieving a better outcome in term of

angiogenesis compared to direct injection of the VEGF solution and with less adverse effects (Eppler et al., 2002). The following represents examples of different polymeric materials that have been tested for VEGF targeted delivery include; fibrin gel (Sacchi et al., 2014), hyaluronic gel (Chen et al., 2012) in addition to PLGA microspheres, nanoparticles or scaffold (des Rieux et al., 2011; Jiang et al., 2015; Yu et al., 2015).

PLGA is a biodegradable/biocompatible, FDA approved material which has been used for localised growth factors delivery. Depending on the MW and lactide to glycolide ration, PLGA release kinetics can followes biphasic (Porporato et al., 2012) or Triphasic profile (Bhardwaj et al., 2008). This starts with initial burst release of the materials on the surface of the microspheres ~~follwoed~~followed by a significant decline with a lag phase which may be followed by a second burst release phase when massive polymer backbone degradation is reached over time (Bhardwaj et al., 2008; Faranesh et al., 2004). Differen PLGA based formulations were used to induce angiogenesis and enhance wound healing (Chereddy et al., 2015; Lee and Lee, 2009; Yu et al., 2015). However, this is still wide diversity in terms controlling the dosing, release kinetics and treatment duration to avoid limitation related to dose fluctuation or continuous release for unnecessary extended time.

Even with the highest levels of spatiotemporal control of VEGF release using polymeric carrier materials, there is still a need for a better control to achieve physiological angiogenesis rather than angioma or aberrant vascular structures (Banfi et al., 2005; Karvinen et al., 2011). During angiogenesis, the niche concentration of VEGF rather than the dose will determine normal or aberrant angiogenesis. Therefore, despite the importance of the control over timing and positioning of VEGF release, the local VEGF concentration should be maintained within an effective concentration range but bellow supra-physiological concentrations to induce physiological angiogenesis (Ozawa et al., 2004).

According to the literature, the angiogenic effect of VEGF can be achieved at a range of 5–20 ng/mL with a plateau effect at 50 ng/mL (Yue and Tomanek, 2001). No further improvement can be seen above this saturation level with the possibility of having aberrant angiogenesis at higher concentrations (Banfi et al., 2005). Another important aspect is the duration of the VEGF treatment, although exposure time can be extended for weeks to achieve angiogenic effect (Amsden et al., 2010; Bhardwaj et al., 2008; Cleland et al., 2001), a few days of localised VEGF treatment can be enough to elicit angiogenic effect and wound healing (Chereddy et al., 2015).

The aim of this study is to provide an engineered formulation from PLGA microspheres to deliver a therapeutic dose of VEGF in the right place and for an appropriate length of time to induce angiogenesis. PLGA has a long history of safety as a biocompatible/biodegradable material. The formulation will be engineered to achieve localised delivery for up to seven days of effective VEGF concentrations to promote angiogenesis over an appropriate therapeutic window. The delivery should not persist beyond the period in which VEGF is required. Such a formulation can be delivered either by injection for localised delivery to say cardiac tissue or by topical delivery to for example chronic non-healing ulcers.

2 Materials and methods

2.1 Materials

Poly(vinyl alcohol) (PVA, molecular weight: 13,000–23,000 Da, 87–89% hydrolysed), human serum albumin (HSA), polyethylene glycol (PEG, molecular weight: 1500 Da), stannous octoate/Tin(II) 2-ethylhexanoate (Sn(Oct)2), dimethyl sulphoxide (DMSO), sodium hydroxide (NaOH), sodium dodecyl sulphate (SDS) were obtained from Sigma–Aldrich, UK. Poly (D,L-lactide-co-glycolide) (PLGA) polymers with acid end groups and lactide:glycolide ratios of 50:50 (product name DLG 4A, lot number LP782, 59 kDa) and 85:15 (product name DLG 5A, lot number LP831, 62 kDa) were purchased from Evonik, USA. D, L-Lactide and glycolide monomers were obtained from Lancaster Synthesis, Ward Hill, MA, USA and PURAC, Gorinchem, Netherlands, respectively. The Pierce Micro bicinchoninic acid (BCA) protein assay kit and HPLC grade solvents (dichloromethane (DCM) and acetone) were purchased from Fisher Scientific UK Ltd, Loughborough, UK. Recombinant human VEGF 165 was purchased from Peprotech, UK.

2.2 Fabrication of microspheres

Synthesis of the PLGA–PEG–PLGA triblock copolymer occurred by ring opening polymerisation of the D,L-lactide and glycolide monomers in the presence of PEG and the (Sn(Oct)2), catalyst under a dry nitrogen atmosphere. Details regarding triblock polymer synthesis and characterisation can be found in White et al. (White et al., 2013). In brief, PEG was dried first under vacuum and stirring at 120 °C for 3 h followed by temperature increase to 150 °C. The monomers were then added to the reaction vessel under dry nitrogen atmosphere and left to equilibrate for 30 min. Then after, the catalyst was added and the reaction was allowed to proceed for 8 h. The resultant copolymer was dissolved and precipitated in water to remove un-reacted monomers. The triblock copolymer was then dried under vacuum to remove residual water and stored at −20 °C until required. The molecular weight (5886) and polydispersity index of the copolymer (1.14) were determined using Gel Permeation Chromatography (PL-GPC 120, Polymer Labs) with differential refractometer detection. Chloroform was employed as an eluent, with two columns (30 cm, PolarGel-M) in series calibrated against polystyrene standards.

PLGA microspheres were produced by double emulsion method from PLGA 50:50 or 85:15+/−30% triblock. Briefly, 1 g of PLGA or 0.7 g PLGA plus 0.3 g triblock was dissolved in 5 ml DCM. The aqueous solution was prepared by dissolving 9.88 mg HSA plus 0.12 mg VEGF in 100 µl DW. The aqueous solution was added to the polymer solution, followed by homogenisation for two minutes at 9000 rpm using a Silverson L5M homogeniser (Silverson Machines,

UK) to form the primary water-in-oil emulsion. The latter was then homogenised in 350 ml of 0.3% polyvinyl alcohol at 2100 rpm, the resultant water-in-oil-in-water double emulsion was left stirring for four hours to allow DCM evaporation. The hardened microspheres were harvested via series of washing with distilled water and centrifugation followed by lyophilisation (Edwards Modulyo, IMA Edwards, UK) until dry then stored at −20 °C. Control microspheres were manufactured with 10 mg HSA protein in 100 µl distilled water in the primary emulsion. Another control was prepared without HSA, of 120 µg VEGF 165 in 100 µl DW in the primary emulsion.

2.3 Scanning electron microscopy (SEM)

The surface morphology of the fabricated particles was examined using SEM. Microspheres were mounted on aluminium stubs (Agar Scientific, UK) and gold coated using a Balzers SCD030 gold sputter coater (Balzers Union Ltd., Lichtenstein) with an argon rate of 26 mA for 5 min. The structural morphology of the microspheres was examined using a JEOL 6060L scanning electron microscope imaging system (JEOL Ltd., Hertfordshire, UK) at 10 kV ionising radiation.

2.4 Microsphere size analysis

The mean microsphere diameter and size distribution were investigated using a Coulter LS230 particle size analyser (Beckman, UK). Briefly, 50 mg/ml in double deionised water were sized with agitation to prevent particle settling. Microsphere size distribution was then determined as a function of the microsphere diffraction and plotted as a function of volume percentage.

2.5 Entrapment efficiency for total protein

The measurement of the entrapment efficiency of protein within the microspheres was a modification of techniques proposed by Sah (1997) and Morita et al. (2001). Briefly, 10 mg of PLGA microspheres were added to 750 µl DMSO and left until the sample dissolved then 2150 µl of 0.02% SDS in 0.2 M NaOH was added and left for 1 h. The Micro BCA protein assay kit was used to quantify the total protein content and compared against a standard curve of HSA. Sample (150 µl) and BCA working reagent (150 µl) were mixed and incubated for 2 h at 37 °C and the absorbance at 562 nm measured using a plate reader (Infinite M200, Tecan UK Ltd., Reading, UK).

The method for measurement of VEGF165 entrapment efficiency within the microspheres was modified from a protocol previously described by Jiang et al. (2002). PLGA microspheres (10 mg) were digested in 1 ml of 1 mM sodium hydroxide (NaOH) for 2 h and then neutralized with 1 ml of 1 mM hydrochloric acid (HCl). The solution was then centrifuged (16000*g*, Sigma 1–16 K) for 15 min at room temperature. VEGF quantification was achieved using an ELISA Quantikine Kit (R&D Systems, UK). Following the manufacturer's protocol, 50 µl of the assay diluent (RD1W) and 200 µl of the sample solution were added into a pre-coated 96 well plate with a mouse monoclonal antibody against VEGF then incubated for 2 h at room temperature. After three washes with buffer, 200 µl/well of a horseradish peroxidase polyclonal antibody against VEGF was added for 2 h at room temperature followed by another washing step before a final incubation for 20 min with 200 µl/well of a substrate solution (1:1 hydrogen peroxide and TMB chromogen). The reaction was stopped using 50 ml of 2 N sulphuric acid, the optical density was measured at 450 nm using a plate reader (Infinite M200, Tecan UK Ltd., Reading, UK). VEGF concentrations in the samples were determined using a standard curve of known concentrations of VEGF supplied with the kit.

2.6 Protein release

Total protein release from earlier publications proved to be a reliable method to characterise release kinetics for different growth factors (Kirby et al., 2016; White et al., 2013). *In vitro* studies on the controlled release of VEGF165 encapsulated within microspheres were performed in aqueous buffers over 3 weeks. Aliquots (25 mg) of the microspheres (triplicate samples from each batch) were suspended in 1 ml phosphate buffered saline (PBS, pH 7.4) and samples were left on a 3-dimensional shaker (Gyrotwister, Fisher Scientific UK Ltd) at 10 rpm in a humidified incubator at 37 °C. Samples were taken every day in the first week then continued every two days where 1 ml PBS supernatant was removed and replaced with fresh PBS. The removed supernatants were filtered using 0.2 µm filters then stored frozen until required and were then assayed for total protein content using a Micro BCA assay kit. Absorbance values were correlated to a standard curve of HSA in order to determine the total protein content.

2.7 Proliferation assay

Human dermal microvascular endothelial cells (HDMEC, passage 3 to 4) cells were seeded into 12 well plates pre-coated with gelatine (2 ml of 0.1% porcine gelatine (Sigma, UK) in PBS at 4 °C overnight) at a density of 25,000 cells per well in 2 ml endothelial cell media (Promocell, UK) supplemented with 5% foetal calf serum. Cells were then treated with VEGF 165 (0, 3, 10, or 30 ng/ml) or loaded PLGA microspheres were disinfected by incubation in a solution of penicillin and streptomycin (1000 IU/mL and 1000 µg/mL respectively (Sigma Aldrich, UK)) in PBS at 4 °C overnight prior to use. Microspheres were centrifuged at 180*g* and washed 3x in PBS. They were then re-suspended in culture media at 1 mg/ml or 3 mg/ml final concentration in Transwell inserts and placed in the culture well. After 24 h, cells were stained with MTT for 40 min at 37 °C (0.5 mg/ml in PBS). After three gentle washes with PBS, MTT was eluted in 200 µl of Cellusolve (Sigma, UK).

2.8 Assessment of proangiogenic properties of PLGA microspheres loaded with VEGF using the CAM assay

Fertilized chicken (Gallus domesticus) eggs were purchased from Medeggs (UK) and incubated from day 2 post-fertilization until day 8 at 37 °C/40% humidity in an egg incubator (RCOM Suro20, R-Com UK). A square window (1 cm^2) was then cut out of the shell using a Dremel multi-tool (Dremel, USA) exposing the CAM. 20 µl of PBS containing 1 mg of PLGA microspheres was pipetted onto the CAM. In addition to untreated eggs, 15 eggs (each) were implanted with HSA microspheres, VEGF microspheres, or VEGF and HSA loaded microspheres. The shell window was resealed with Parafilm (Bemis Flexible Packaging, USA) and adhesive tape. Eggs were then placed at 37 °C/40% humidity in an egg incubator until day 14 post fertilisation. CAM were then exposed and photographed, after which CAM were then retrieved, and the eggs sacrificed. The CAM tissue was fixed in 3.7% paraformaldehyde (Sigma Aldrich, USA) in dH2O for 1 h at room temperature before being embedded in OCT medium (Leica, Germany) for 20 min before snap freezing in liquid nitrogen. Sections (20 µm) were cut by cryostat (Leica CM1860UV (Leica Germany)) and stained with Haematoxylin & Eosin (H&E) according to the standard protocol for frozen slides. Slides were then mounted with DPX (Sigma-Aldrich, USA) and imaged at 100× (Motic microscope, Motic, China).

2.9 Statistics

All values presented are an average of at least 3 experiments, ANOVA, were used to determine statistical significance, a P value of <0.05 was deemed to be significant.

3 Results

3.1 PLGA microsphere characterisation for size, morphology, encapsulation efficiency, and release kinetics

In this study, HSA loaded microspheres were fabricated by a water in oil in water emulsion method. The different formulations were made from PLGA 50:50 or 85:15 in the presence or absence of PLGA–PEG–PLGA triblock copolymer. Formulation A (PLGA50:50), Formulation B (PLGA 50:50 plus 30% PLGA-PEG-PLGA triblock), formulation C (PLGA 85:15) and formulation D (PLGA 85:15 plus 30% PLGA-PEG-PLGA triblock). The generated microspheres gave an average diameter range of 70–80 um as seen in Fig. 1A-D. The mean particle size diameter of the formulations were as follow, A (79.6 µm ± 24.4), B (81.4 µm ± 23.9), C (70.4 µm ± 23.7), D (75 µm ± 24.5).

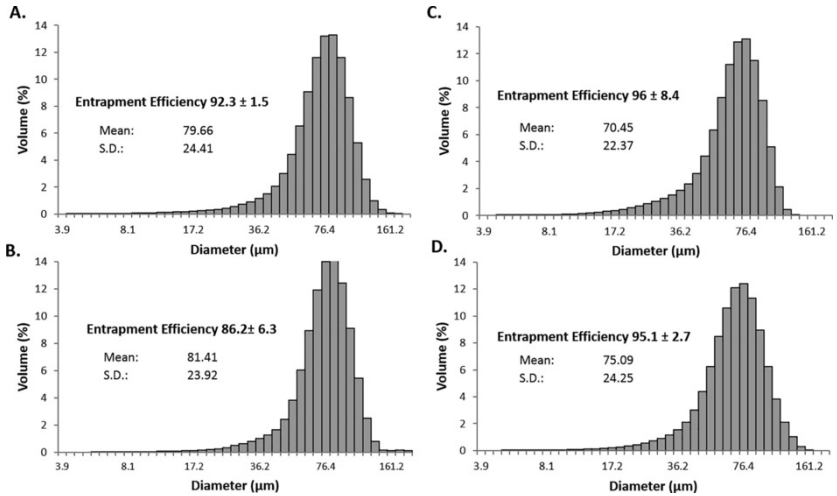

Fig. 1 Representative size distribution results of PLGA microspheres and entrapment efficiency for HSA protein with average size diameter for formulations, A (PLGA50:50), B (PLGA 50:50 plus 30% PLGA-PEG-PLGA triblock), C (PLGA 85:15) and D (PLGA 85:15 plus 30% PLGA-PEG-PLGA triblock).

Fig. 2 A-D show representative micrographs for the microspheres shape and morphology from the different formulations that show spherical, non-aggregated regular shaped microspheres with a smooth continuous surface.

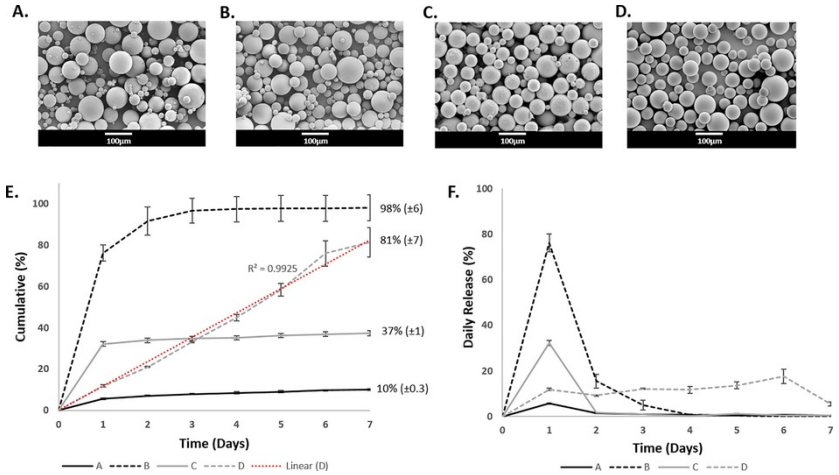

Fig. 2 Morphology of microspheres and release kinetics. Representative micrographs of PLGA microspheres formulations (A, B, C, D). Cumulative HSA release for the formulations over seven days, data suggest linear release kinetics for formulation D with $R^2 = 0.99$ (E). Daily HSA release for formulations over seven day. Results expressed as mean ± SEM.

Efficient HSA protein loading within microspheres from the different formulations was achieved using the double emulsion method; A 92% (±2), B- 86% (±6), C- 96% (±8) and D- 95% (±3). The *in vitro* release kinetics from formulations A-C shows a traditional biphasic release pattern, which started with the initial burst on day one and was followed by a decline on day two then a lag phase until the study ended on day seven. Different release profiles were evident with formulation D with zero order release as seen in Fig. 2 E. The data from daily HSA release during the seven-day period can be seen in Fig. 2 F, there was a lower initial burst in A compared to other formulations with an average daily HSA release of 1.4% (±0.7). The incorporation of triblock copolymer in B increased the initial burst to the highest level among other groups; the average release of HSA was 14% (±10.5). There was an intermediate burst release with C in relation to other formulations of interest with an average daily release of 5.3% (±4.4). A sustained release profile was obtained with D with no burst and an average daily release of 11.6% (±1).

3.2 VEGF release and bioactivity

Formulation D was selected to achieve a sustained seven days VEGF release to induce an angiogenic effect in the *in ovo* CAM model. Therefore, with approximately 10% daily release, the dose of VEGF loaded microspheres was calculated to give the desired release pattern with an effective VEGF concentration of 10–20 ng/mg microspheres/day (Fig. 3).

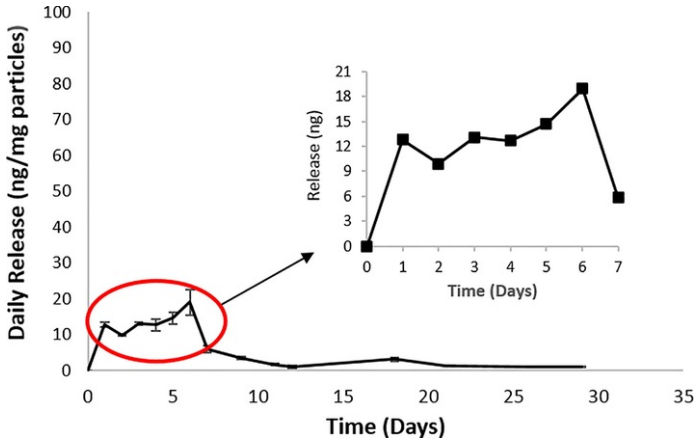

Fig. 3 Daily VEGF release from formulation D (PLGA 85:15 plus 30% PLGA-PEG-PLGA triblock) based on total protein release over 30 days, with magnified area for the first seven days. Results expressed as mean ± SEM.

VEGF loaded microspheres were fabricated with or without HSA carrier protein. Confirmation that this VEGF was released in the therapeutic range was obtained in subsequent experiments evaluating the biological response

to the microspheres (Fig. 4A). VEGF treatment of HDMEC cells *in vitro* caused a dose dependant increase in the rate of proliferation between 3 and 30 ng over 24 h, peaking at an increase of approximately 35% over control (Fig. 4D). Fig. 4D also shows that VEGF associated with the PLGA microspheres was released and had a bioactive effect on the HDMEC cells. VEGF released from PLGA microspheres caused an increase in proliferation compared to HSA only microspheres. This increase was significant where the microspheres had a combination of HSA and VEGF at a dose of 1 mg/ml but not 3 mg/ml. The released VEGF stimulated endothelial cell proliferation to an extent comparable to the addition of VEGF at a concentration of approximately 5 ng per ml. This was achieved by delivering microspheres in the concentration range of 1–3 mg per ml.

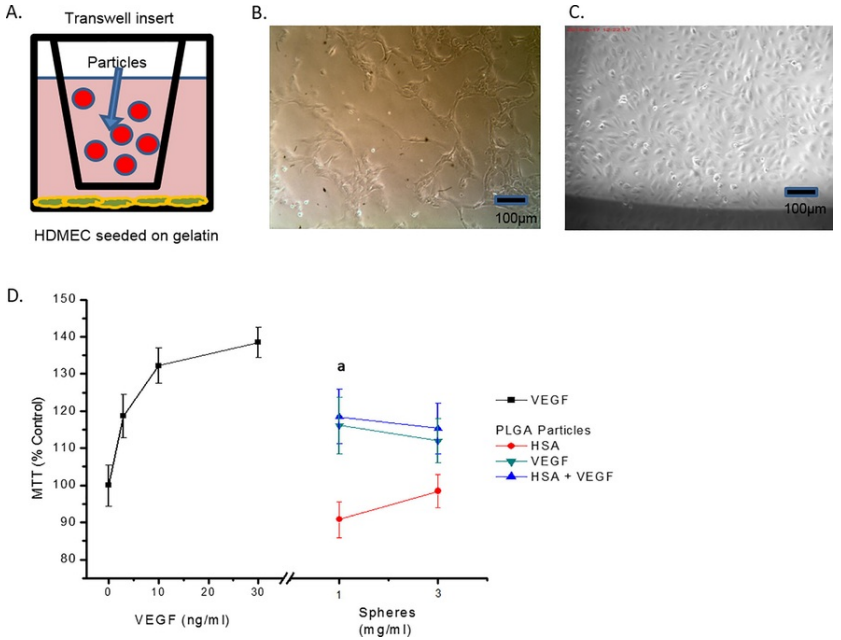

Fig. 4 The experimental design in which PLGA microspheres releasing VEGF were placed in a Transwell insert over a monolayer of HDMECs seeded on gelatine (A). The appearance of the cells on gelatine at low (B) and high density (C). The effect of adding VEGF to HDMEC cultures measuring the increase in proliferation indirectly via an MTT metabolic essay and the effect of microspheres containing HSA, VEGF alone and HSA plus VEGF (D). Results expressed as mean ± SEM, significance determined by ANOVA ([a]indicates $P < 0.05$).

3.3 Effect of the VEGF releasing microspheres on new blood vessel formation *in ovo* using the CAM assay

The value of the CAM assay is that it shows the development of new blood vessels over seven days allowing quantitation of the proangiogenic effect of a treatment. Addition of microspheres induced an increase in new blood vessel formation, and this was significant when VEGF was delivered with HSA.

VEGF release from PLGA microspheres stimulated an increase in vessel formation, which can be seen in photographs of the CAM in Fig. 5 A to C. There is a visible increase in both small and large vessels, and the number of junctions between vessels (an indication of increased branching) of the chorioallantoic membrane of fertilised eggs. Quantitation of the numbers of small and large vessels and junctions between the HSA, VEGF and VEGF and HSA samples is shown in Fig. 5 D to F. There is a significant increase in the number of small vessels, large vessels, and branches, when the CAM is treated with PLGA microspheres loaded with HSA and VEGF compared to HSA alone. PLGA microspheres loaded with only VEGF did show an increase in small vessels, large vessels, and junctions, relative to HSA loaded PLGA microspheres; but this was not found to be statistically significant. For reference, Fig. 5A shows an example of the typical large and small vessels and junctions.

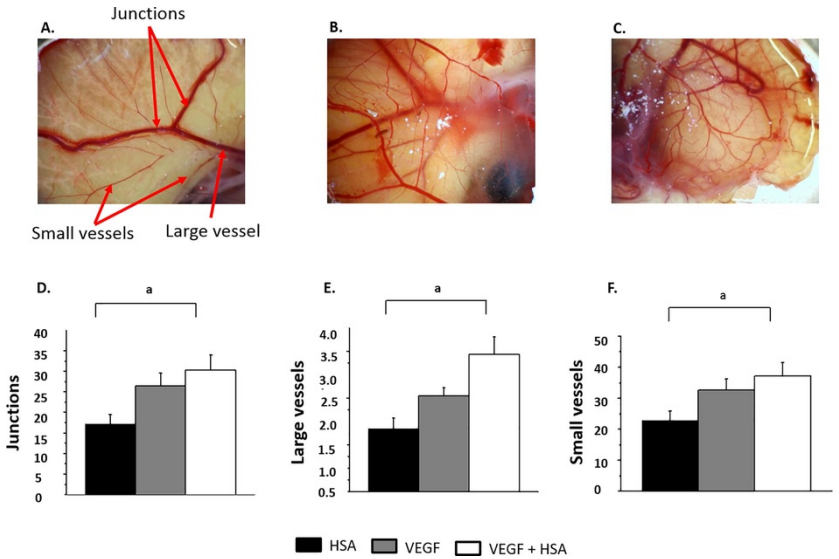

Fig. 5 *In ovo* CAM assay model for angiogenesis, bright-field microscopy images for control microspheres (A) and microspheres releasing VEGF (B&C). Quantitation of the effects of the microspheres releasing HSA, VEGF or VEGF plus HSA on the number of branches/junctions between vessels (D), the number of large vessels (E.) and the small vessels (F). Examples of each type of structure are indicated in (A). Results expressed as mean ± SEM, significance determined by ANOVA ([a]indicates $P < 0.05$).

Fig. 6 shows H&E stained histology cryosections of the CAM treated with PLGA microspheres. From the images of the chorionic membrane, HSA bearing PLGA microspheres can be seen as clear round material embedded in the chorionic membrane (Fig. 6A). Cells can be seen growing around but do not appear to be tightly coating the microspheres, there is a noticeable gap between the cells of the chorionic membrane and the microspheres. In samples where VEGF or VEGF and HSA were present, the microspheres appear to have a coating of cells, which appear to have the morphology of epithelial cells (Fig. 6B and 6C). The VEGF or VEGF and HSA bearing microspheres appear to be more tightly surrounded by cells of chorionic membrane than the HSA microspheres; there was no gap in the tissue surrounding the microspheres.

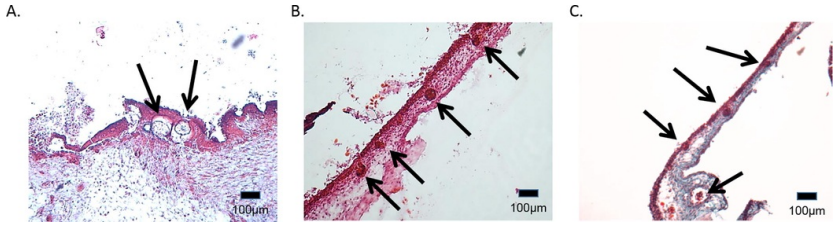

Fig. 6 H&E stained sections of CAM membranes showing the growth of tissue around PLGA microspheres containing HSA, VEGF, or VEGF plus HSA. 6A shows the presence of microspheres with HSA alone in the CAM membrane. Microspheres are evident (arrows) but there is no cellular ingrowth into them. 6B shows the ingrowth of cells into microspheres containing VEGF and 6C shows the ingrowth of tissue into microspheres containing VEGF and HSA.

4 Discussion

VEGF therapy is a promising strategy for treating ischemic wound diseases; however, the main hurdle lies in the efficient delivery of a short living molecule with direct injection into the body. Silva and Mooney (2007) suggested the low *in vivo* VEGF concentration due to the short half-life could probably explain the limited clinical success with this treatment (Silva and Mooney, 2007). To circumvent this limitation, VEGF delivery using a controlled release formulation can achieve a better presentation of short half-life molecules with enhanced local concentration and eventually avoiding systemic side effects. Nevertheless, this still requires a tight control of the localised growth factor delivery (VEGF) to maintain effective therapeutic levels during the specific period of treatment. In this study, our use of a delivery system based on injectable PLGA microspheres attempts to address some of the issues around VEGF use. We demonstrate the ability of PLGA microspheres to achieve a therapeutic dosage both *in vitro and in ovo* combined with release data to prove that this therapeutic effect would last throughout the healing phase of tissue repair. After VEGF release, the breakdown of the peptide and tissue dilution would reduce concentrations to sub-therapeutic levels as VEGF release tapers off and is overtaken by tissue clearance/metabolism. This objective was achieved by using an engineered formulation of PLGA microspheres with a release modifier (PLGA-PEG-PLGA triblock copolymer) loaded with VEGF on a carrier protein HSA. The VEGF loading was calculated based on release kinetic

from the carrier protein to give a sustained VEGF release for seven days which then drops to sub-therapeutic level.

The formulation was designed to produce injectable microspheres (~80-μm diameter) through a hypodermic needle (Qutachi et al., 2014). This delivery method is a minimally invasive technique for clinical applications compared to for example the intracoronary injection for myocardial ischemia, which requires a major surgical procedure (Henry et al., 2001). The microspheres can be also applied topically using wound dressings or suitable gel formulation. Furthermore, the size of the microspheres in this study offer a unique advantage for interstitial growth factor delivery by avoiding the microspheres internalisation into cells which is expected with microspheres in the size range of 0.1–10 μm (He and Park, 2016).

For effective therapeutic angiogenesis more attention needs to be directed towards the VEGF concentration at the target tissue rather than the dose delivered (Ozawa et al., 2004). The activity of VEGF to promote proliferation and migration of vascular endothelial cells might be disrupted by the delivery of VEGF in a sub- or supra-therapeutic dosage. The duration of therapy can be another influential factor, continued delivery after the required period could lead to vessels failing to become adequately patent, or in extreme cases promote uncontrolled over-vascularisation leading to oedematous swelling or bleeding from newly formed vessels or development of an haemangioma – like tumour. Therefore for therapeutic angiogenesis, the minimum fluctuation in VEGF release associated with zero order release kinetics for the required duration may be more appropriate than the conventional bi/triphasic release pattern from other PLGA based formulations (Bhardwaj et al., 2008).

The release mechanism through PLGA polymer reflects a complex interplay between drug diffusion through the matrix, polymer degradation and swelling (Sandor et al., 2001). The transient initial burst phase of the encapsulated molecules can be attributed to the diffusion of surface molecules within the polymer matrix (Langer, 1990). This is followed by a sharp decline, which continues depending on the polymer characteristics until the polymer matrix become soft due to polymer degradation (Park, 1995). The conventional PLGA microspheres, might not be the best option for growth factors delivery due to the risk the increased core acidity rooted to the degradation by-products which could be responsible for compromising the bioactivity (Hines and Kaplan, 2013) and inducing an auto-catalytic degradation of the polymer matrix (Sandor et al., 2001). However, VEGF is an acid stable growth factor with enhanced bioactivity at acidic pH (Goerges and Nugent, 2004). Furthermore, PLGA-PEG-PLGA triblock copolymer is also an FDA approved polymer (Chen et al., 2017) which works as a release modifier by providing hydrophilic micro-channels with the hydrophobic polymer matrix to facilitate the growth factor release by diffusion and minimise the accumulation of acidic by products (Qodratnama et al., 2015). This enhances steady state release and appears to achieve a constant zero order release with PLGA 85:15.

Difficulty in preserving growth factor's biological activity is another challenge for therapeutic application. Although the PLGA matrix provides a physical protection for sensitive molecules following injection into the body, the payload still has to go through a harsh environment during microspheres fabrication. The use of a carrier protein such as HSA provides a reliable protection for the bioactive molecules to preserve biological activity. Denaturation of growth factors is expected to commence at the interphase between aqueous and organic solvent during emulsion formation. This is probably due to partial solvation and hence changing in the structural confirmation of the growth factor molecule. The presence of HSA at high concentration at the interphase will work as shielding agent at the interphase and preserve the growth factor's biological activity (van de Weert et al., 2000).

Vascularisation is under tight regulated control in the body which is essential to prevent hypoxia/nutrient starvation/waste build up after tissue damage. The use of VEGF initially showed promise as a therapy but it's use and effects can vary in the body with low or hyper-physiological dose of VEGF leading to ineffective or malformed leaky vessels as in haemangioma formation (Ozawa et al., 2004). Physiological angiogenesis can be achieved at 20 ng/mL which appears to be plateaued at 50 ng/mL (Yue and Tomanek, 2001). The PLGA formulation in this research maintained a constant release of VEGF between 10 and 20 ng/mL for seven days, which proved to be effective in inducing angiogenesis in an *in ovo* CAM model.

5 Conclusion

In summary, the work in this research provides a formulation with engineered release kinetics to achieve effective VEGF delivery to enhance angiogenesis. The present formulation has potential as a treatment for ischemic wound diseases. The main advantage here is sustained VEGF delivery at therapeutic concentration with minimum variability over the treatment period. The polymer matrix has been designed to degrade gradually into metabolites, which are readily cleared by the body.

Declaration of interests

None.

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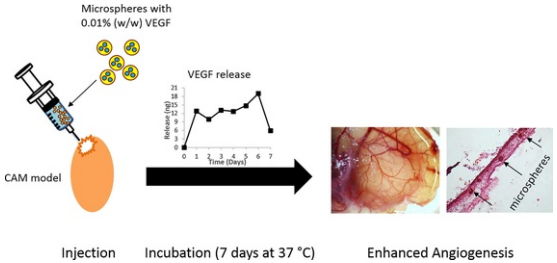

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