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Multiple tissue response modifiers to promote angiogenesis and prevent the foreign body reaction around subcutaneous implants

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ABSTRACT

Dexamethasone-releasing PLGA poly(lactic-co-glycolic acid) microsphere/PVA (polyvinyl alcohol) hydrogel composite coatings have been shown to prevent the foreign body reaction (FBR) to subcutaneous implants in small and large animal models. Such coatings were developed to extend the lifetime of implantable biosensors. However, long-term exposure of tissue to low levels of dexamethasone results in a reduction in blood vessel density due to the anti-angiogenic effect of dexamethasone. This mild effect, while not threatening to the subject's health, may interfere with analyte detection and the sensor response time over the long-term. The present work is focused on the development of coatings that deliver combinations of three tissue response modifiers (TRMs): dexamethasone, VEGF (vascular endothelial growth factor) and PDGF (platelet derived growth factor). Dexamethasone, VEGF and PDGF prevent the FBR, increase angiogenesis and promote blood vessel maturation (which increases blood flow), respectively. To minimize any potential interference among these three TRMs (for example, PDGF increases fibrosis), the relative doses of dexamethasone, VEGF and PDGF were adjusted. It was determined that: *a*) all three TRMs are required for maximum promotion of angiogenesis, blood vessel maturation and prevention of the FBR; *b*) VEGF has to be administered at higher doses than PDGF; *c*) an increase in dexamethasone dosing must be accompanied by a proportional increase in growth factor dosing; and *d*) modification of the TRM ratio can achieve a constant capillary density throughout the implantation period which is important for applications such as biosensors to maintain sensitivity and a stable sensor baseline. Moreover, an osmosis-driven process for encapsulation of proteins in PLGA microspheres that showed low burst release was developed.

1. Introduction

Biosensors, defined as analytical devices that detect biological analytes, have progressed in their development and miniaturization such that fully-implantable sensors (usually in the subcutaneous tissue) will become a reality in the near future [1–12]. These sensors will provide real-time, continuous monitoring of analytes, such as glucose, which are currently being monitored intermittently. Diabetic patients are a major group that will benefit from the realization of implantable biosensors, since tight blood glucose control is paramount to diabetic health.

Once implanted, biosensors are attacked by the body's defense mechanism, a cascade of events collectively known as the foreign body reaction (FBR) which results in the encapsulation of biosensors in a fibrous membrane that isolates them from the surrounding tissue and thus renders them ineffective [13,14]. The FBR and its prevention have been a research focus during the past decades; the most common method to prevent the FBR is by the continuous administration of an anti-inflammatory agent, most commonly dexamethasone [6,13–17]. Dexamethasone is popular due to its high efficacy and potency which result in only small amounts of the drug being required for long-term action. However, long-term exposure of a tissue to low levels of dexamethasone results in reduction in blood vessel density in the local area due to dexamethasone's anti-angiogenic effect [18–20]. This mild effect, while not detrimental to the subject's health, may interfere with analyte detection and sensor response time.

Glucose diffuses passively from the blood to the subcutaneous tissue where it can be detected by an implanted biosensor. The time it takes for glucose to travel from the capillary wall to the sensor surface is dependent on the distance it has to cover, and typically ranges from 6 to 15 min [21]. Long-term exposure of dexamethasone decreases the number of capillaries available for glucose diffusion to the sensor, as well as increases the average distance between the capillaries and the sensor surface. Both effects may significantly increase sensor response lag time as well as sensitivity. Accordingly, growth factors that promote angiogenesis can be administered alongside dexamethasone in order to

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prevent dexamethasone-induced ischemia and reduce glucose sensor lag times. VEGF (vascular endothelial growth factor) has successfully been administered with dexamethasone through poly(lactic-co-glycolic acid) (PLGA) microspheres embedded in a polyvinyl alcohol (PVA) hydrogel over a one-month period and an increase in capillary density was observed [22]. To further increase the capillary density around the implants, the current work focuses on administration of dexamethasone, VEGF and PDGF (platelet derived growth factor). VEGF promotes the migration of endothelial cells to the site, which form new branches on existing blood vessels [23–26]. PDGF promotes the maturation of the new branches by attracting pericytes that form an outer cellular layer on the new branches and connect venous and arterial blood flow [27–30].

It is known that angiogenesis and the FBR have some mechanistic overlap; cytokines that promote branching of existing blood vessels and recruitment of endothelial cells (primarily VEGF) exacerbate acute inflammation and inhibit chronic inflammation (which starts with the activation of fibroblasts) [31–33]. On the other hand, cytokines that promote the maturation of new blood vessel branches (primarily PDGF), *via* the recruitment of pericytes, promote chronic inflammation [34–37]. In addition, dexamethasone is well known to inhibit angiogenesis. It is therefore evident that the simultaneous release of these three molecules in a tissue will result in significant interference in their respective roles. In the current work, composite implants (referred to as composites here) containing combinations of dexamethasone, VEGF and PDGF were investigated in the subcutaneous tissue using a rat model. The tissue surrounding the implants was examined *via* histological evaluation for prevention of the FBR and concurrent promotion of angiogenesis. Adjustment of the relative ratios of the three TRMs eliminated interference among the TRMs that was observed for some of the composite combinations.

2. Materials and methods

2.1. Materials

Dexamethasone was purchased from Cayman Chemical Company (Ann Arbor, MI). VEGF, PDGF and their respective ELISA quantification kits were purchased from Peprotech (Rocky Hill, NJ). MicroBCA kit was purchased from Life Technologies. High-molecular weight polyvinyl alcohol (HMW-PVA, MW 133 kDa), was purchased from Polysciences, Inc. (Warrington, PA). Low-molecular weight PVA (LMW-PVA, 99% hydrolyzed, MW 30–700 kDa), bovine serum albumin (BSA), and BSA–FITC were purchased from Sigma-Aldrich (St. Louis, MO). PLGA Resomer® RG503H (inherent viscosity 0.32–0.44 dl/g) was a gift from Boehringer–Ingelheim. Methylene chloride was purchased from Fisher Scientific (Pittsburghm, PA). Sprague Dawley rats were purchased from Charles River Laboratories (Willimantic, CT).

2.2. Formulation of protein microspheres

In a 50-ml Teflon vial, 500 mg of PLGA was added with 2 ml methylene chloride (DCM). After the polymer was dissolved, 200 μl of the protein phase (100 mg/ml BSA with either 5 μg VEGF (V), 5 μg PDGF (P) or 5 μg VEGF and 2.5 μg PDGF (VP)) were added to the polymer solution. The vial was vortexed at 3000 rpm for 10 s and homogenized at 10,000 rpm for 30 s to achieve a water-in-oil primary emulsion. A secondary water-in-oil-in-water emulsion was made by adding 10 ml water phase (1% w/v of LMW-PVA, with or without 2% w/v NaCl) and vortexing at 3000 rpm for 10 s. The secondary emulsion was diluted with 10 ml of Milli-Q water to speed up polymer precipitation and microsphere formation. The diluted emulsion was kept under vacuum on a horizontal shaker at 300 rpm for 3 h. The hardened microspheres were purified to remove LMW-PVA and non-encapsulated drug *via* three centrifugation cycles (2 min, 3500 rpm), freeze dried overnight and stored at −20 °C until further use. Formulations containing 2% w/w FITC–BSA (of the total BSA amount) were prepared to visualize the protein distribution inside the microspheres. All microsphere preparation steps took place in a single vial (from polymer dissolution in the organic solvent to microsphere storage), as demonstrated in Fig. 1.

2.3. Formulation of dexamethasone microspheres

Dexamethasone-containing microspheres were prepared as above with the 200 μl protein solution replaced by 100 mg of crystalline dexamethasone. A homogenous drug suspension in the polymer solution was achieved following 2.5 min of homogenization at 10,000 rpm.

2.4. Microsphere characterization

2.4.1. Drug loading (dexamethasone)

5 mg of dried microspheres was dissolved in 1 ml DMSO and then diluted 10 times in phosphate buffered saline (PBS) pH 7.4. Dexamethasone concentration was determined *via* RP-HPLC (PerkinElmer, Inc.). Mobile phase 35% acetonitrile in water, 0.1% phosphoric acid; column C18 500 × 03 mm; and detection wavelength 240 nm.

2.4.2. Drug loading (proteins)

2 mg of dried microspheres was dissolved in 1 ml acetone, and the undissolved pellets were washed and collected *via* three centrifugation cycles (12,000 rpm, 5 min) and dried under vacuum for 30 min to remove acetone. The pellets, which contained the proteins, were reconstituted in 1 ml of PBS containing 0.1% Tween 20. VEGF and PDGF ELISA kits were used to quantify the growth factor concentration in the reconstituted solutions, as per the manufacturer's instructions. A MicroBCA kit was used to quantify BSA as per the manufacturer's instructions. The encapsulation efficiency was expressed as the percentage of protein loaded compared to the theoretical total loading.

2.4.3. Burst release

5 mg of dried microspheres was suspended in 10 ml PBS containing 0.1% Tween 20. The suspensions were incubated at 37 °C under stirring (100 rpm). After 24 h, 1 ml of suspension was centrifuged at 14,000 rpm for 5 min and the supernatant was analyzed for protein content.

2.4.4. Particle size

An Accusizer 780 (Particle Sizing Systems) was used to measure the particle size of the PLGA microspheres. 3–5 mg of dried microspheres was suspended in 1 ml of 0.1% w/v LMW PVA solution, bath-sonicated for 10 s to break any aggregates and analyzed for volume-based average size.

2.4.5. Confocal microscopy

A Nikon A1R Spectral confocal microscope was used to visualize protein distribution inside the microspheres. Microspheres were suspended in distilled water prior to imaging, and FITC–BSA was detected at excitation and emission wavelengths of 494 and 518 nm, respectively.

2.5. Fabrication of composites

A predetermined amount of PLGA microspheres were added to 1 ml of HMW-PVA solution in water (5% w/v) and the mixture was bath-sonicated for 10 s to achieve a homogenous suspension. The suspension was fed into stainless steel tubes with inner diameter 1.5 mm and subjected to three freeze–thaw cycles (2 h at −20 °C, 1 h at ambient temperature) to physically cross-link the HMW-PVA solution to form a hydrogel. The gels were removed from the tubes and cut at 5 mm length pieces, air-dried for 3 h and stored at −20 °C until further use. The different groups of composites prepared and their compositions are shown in Table 1.