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Promoting angiogenesis with mesoporous microcarriers through a synergistic action of delivered silicon ion and VEGF

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ABSTRACT

Angiogenic capacity of biomaterials is a key asset to drive vascular ingrowth during tissue repair and regeneration. Here we design a unique angiogenic microcarrier based on sol-gel derived mesoporous silica. The microspheres offer a potential angiogenic stimulator, Si ion, 'intrinsically' within the chemical structure. Furthermore, the highly mesoporous nature allows the loading and release of angiogenic growth factor 'extrinsically'. The Si ion is released from the microcarriers at therapeutic ranges (over a few ppm per day), which indeed up-regulates the expression of hypoxia inducing factor 1α (HIF1α) and stabilizes it by blocking HIF-prolyl hydroxylase 2 (PHD2) in HUVECs. This in turn activates the expression of a series of proangiogenic molecules, including bFGF, VEGF, and eNOS. VEGF is incorporated effectively within the mesopores of microcarriers and is then released continuously over a couple of weeks. The Si ion and VEGF released from the microcarriers synergistically stimulate endothelial cell functions, such as cell migration, chemotactic homing, and tubular networking. Furthermore, *in vivo* neo-blood vessel sprouting in chicken chorioallantoic membrane model is significantly promoted by the Si/VEGF releasing microcarriers. The current study demonstrates the synergized effects of Si ion and angiogenic growth factor through a biocompatible mesoporous microsphere delivery platform, and the concept provided here may open the door to a new co-delivery system of utilizing ions with growth factors for tissue repair and regeneration.

1. Introduction

Angiogenesis is a key event in the repair and regeneration of most damaged tissues [1,2]. It is a new formation of blood vessels from existing vasculature tissue [1,3,4]. A series of angiogenic events, involving recruitment and migration of progenitor cells, their endothelial cell differentiation and subsequent maturation into a functional tubular structure, are engaged during the tissue repair process. To engineer tissue constructs *in vivo*, the angiogenesis has been implicated as the most efficient and rapid way to replenish nutrients and oxygen to cells in the tissue reconstruction [5–8].

In bone repair and regeneration, angiogenesis signs a turnover from inflammatory to tissue recover and growth phase [7,9,10]. Therefore, early angiogenesis is meant to be a more active commitment of tissue forming cells and a rapid healing of damaged tissues. In fact, the neo-formed blood vessels not only transport growth factors and cytokines necessary for the osteogenic differentiation of cells but are also a delivery route of progenitor and stem cells [11].

Many strategies have been explored to enhance the angiogenesis during bone formation, through the use of bioactive scaffolds, cells (endothelial or stem cells) and/or signaling molecules (growth factors) [12,13]. When scaffolds are designed properly to deliver cells and therapeutic molecules the angiogenic events can be

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synergistically accelerated which is ultimately helpful for bone formation. For example, nanofibrous scaffolds developed to incorporate and deliver angiogenic growth factors were shown to enhance endothelial cell maturation and tubular formation [14,15]. Furthermore, when the scaffolds were engineered to deliver co-cultured cells (endothelial and mesenchymal stem cells), the *in vivo* angiogenesis could be significantly improved [16,17].

Here we communicate a unique scaffold design to promote the angiogenesis in bone repair; spherical microcarriers holding a capacity to deliver dual therapeutic molecules ('ion' plus 'growth factor'). Produced through a sol-gel process, the microcarriers are networked with a silica glass structure and possess a high mesoporosity (with a few nanometers in pores). Due to a spherical form and proven biocompatibility, the microcarriers support excellent cellular growth enabling injectable cell delivery into defective bone tissues [18].

In particular, here we focus on the release of Si ions from the sol-gel silica microcarriers. Si ions present in the glass chemistry are to release at therapeutically-relevant level to stimulate the angiogenesis. As to the effects of Si ions on angiogenesis, several studies have previously reported primarily using bioactive glasses, where the ionic extracts (Si ions together with Ca and phosphate ions) from glasses were shown to significantly enhance the angiogenic events of endothelial cells [19–21]. The current silica sol-gel microcarriers, on the other hand, are to release only Si ions, and thus are considered a proper model to investigate the angiogenic behaviors played solely by the Si ions (without the involvement of other ions).

Not only the Si ion present in the silica glass network, but a potential angiogenic growth factor, vascular endothelial growth factor (VEGF) is also incorporated through the nanopore space and delivered in a controlled manner. Among the growth factors, VEGF has been a potential angiogenic promoter, stimulating endothelial cell recruitment, proliferation, and differentiation, and the increase of vascular permeability through VEGF signaling pathway [4,22–24]. Therefore, many studies have developed scaffolds that could load and deliver VEGF to stimulate angiogenesis [23,25,26]. Not a single delivery of VEGF or other growth factors, but a co-delivery of more than two growth factors has recently shown to be more potent in stimulating angiogenesis and the subsequent tissue repair process [27–29]. For example, Co-stimulation with VEGF-A and FGF-2 exhibited significant improvement of the cell migration and neovascularization *in vivo* than single delivery [30]. Moreover, the delivery of bFGF and VEGF as dual factors in PLGA nanoparticle-modified bladder acellular matrix immediately restored vascular network and inhibited contracture of augmented bladder [31].

Here, we strategize the dual delivery of ion (Si) and growth factor (VEGF) to synergize the pro-angiogenic events. The effects of the delivered therapeutic molecules on the angiogenic behaviors are investigated in term of migration, recruitment, and tubular networking of endothelial cells *in vitro* as well as the neo-blood vessel formation *in vivo*. The design of the current study is schematically illustrated in Fig. 1a. This approach, as the first report of co-delivering ion and growth factor, is envisaged to open the door to a new class of therapeutic scaffolds in angiogenesis and tissue regeneration.

2. Materials and methods

2.1. Preparation of mesoporous silica microspheres

Mesoporous silica microspheres were prepared by a sol-gel process, as described previously [18]. Briefly, 0.1 M HCl (2.4 ml) was added to tetraethyl orthosilicate (10 ml) (TEOS, $C_8H_{20}O_4Si$, Sigma–Aldrich) with the addition of deionized water to form an acid catalyzed sol. Then, 0.08 M ammonium hydroxide (NH_4OH, 28.0% NH_3 in water, Sigma–Aldrich) was added dropwise to the sol. One hundred ml of olive oil was used as an emulsifier and the pH was adjusted at 5.0–5.5. After this, 5 ml of the sol was added dropwise to olive oil, while stirring at 95 rpm to allow gelation. Microspheres were collected, vacuum filtered, rinsed with water and ethanol, and left overnight to dry. The microspheres were then sieved between 200 and 300 μm for further experiments.

2.2. Characterizations

The morphology of the microspheres was observed by scanning electron microscopy (SEM; JEOL JSM 6510). The morphological change of the microspheres after immersion in α-minimal essential medium for 28 days was also examined. The mesopore properties were characterized using Quadrasorb SI automated surface area and pore size analyzer (Quantachrom Instruments). The porosity, specific surface area, and pore volume were determined by N_2 gas adsorption/desorption, using the Brunauer–Emmett–Teller (BET) method. Pore size distribution was obtained by the Barret–Joner–Halenda (BJH) method. Total pore volume was calculated from the amount adsorbed at a maximum relative pressure (P/P0).

2.3. Ion release test

The cumulative release of Si ion from the microspheres was measured at 37 °C up to 14 days. Microspheres were soaked in α-MEM for each time point, and then the samples were centrifuged at 3000 rpm for 3 min and the supernatant was taken for the measurement with inductively coupled plasma atomic emission spectroscopy (ICP-AES Perlin-Elmer, OPTIMA 4300V). Three replicate samples were evaluated.

2.4. Protein loading test

As a model protein to observe the loading capacity of microspheres, cytochrome C (cyt C) was used as it has molecular size and charge property similar to many growth factors [32]. For the loading test, the microspheres were added to different concentrations of cyt C in PBS. At each time point, the loaded amount was recorded using UV spectrometer at 408 nm. Furthermore, a loading isotherm of cyt C was obtained from the cyt C loaded amounts according to the following mass balance equation: $q_e = (C_0 - C_e) \times (V/W)$, where q_e is the amount of cyt C (in mg) adsorbed per mg silica microspheres, C_0 and C_e are the initial and equilibrium concentrations of cyt C (mg/ml), respectively, V is the volume of solution (ml), and W is the weight of silica microspheres used (mg).

2.5. Preparation of recombinant VEGF (with GFP)

The preparation of VEGF with green fluorescent protein (GFP) was described previously [33]. In brief, human VEGF cDNA was amplified using forward and reverse primer; 5′-GACGG TACC GCACCCAT GGCAGAAGG- 3′, and 5′-AGAAT TCTC ACCGCCTC GGCTTGTC- 3′, respectively. VEGF with GFP was amplified by using PCR with forward and reverse primer; 5′-GGAAT TCGT GAGC AAGGGCGA GGAG-3′, and 5′-TGAA TTCTA CTTGTA CAGCTC-3′, respectively.

2.6. VEGF loading and release study

The prepared VEGF was made into a solution of 100 μg/ml in