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# Multifunctional and stable bone mimic proteinaceous matrix for bone tissue engineering

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ABSTRACT

Biomaterial surface design with biomimetic proteins holds great promise for successful regeneration of tissues including bone. Here we report a novel proteinaceous hybrid matrix mimicking bone extracellular matrix that has multifunctional capacity to promote stem cell adhesion and osteogenesis with excellent stability. Osteocalcin-fibronectin fusion protein holding collagen binding domain was networked with fibrillar collagen, featuring bone extracellular matrix mimic, to provide multifunctional and structurally-stable biomatrices. The hybrid protein, integrated homogeneously with collagen fibrillar networks, preserved structural stability over a month. Biological efficacy of the hybrid matrix was proven onto tethered surface of biopolymer porous scaffolds. Mesenchymal stem cells quickly anchored to the hybrid matrix, forming focal adhesions, and substantially conformed to cytoskeletal extensions, benefited from the fibronectin adhesive domains. Cells achieved high proliferative capacity to reach confluence rapidly and switched to a mature and osteogenic phenotype more effectively, resulting in greater osteogenic matrix syntheses and mineralization, driven by the engineered osteocalcin. The hybrid biomimetic matrix significantly improved *in vivo* bone formation in calvarial defects over 6 weeks. Based on the series of stimulated biological responses *in vitro* and *in vivo* the novel hybrid proteinaceous composition will be potentially useful as stem cell interfacing matrices for osteogenesis and bone regeneration.


## 1. Introduction

Scaffolds with a capacity to repair and regenerate tissues including bone have undergone significant developments over the last few decades. In bone tissue engineering, scaffolds must satisfy a number of criteria including bioactivity, compatible surfaces for the control of cell fate, degradability within an appropriate time frame for substitution by neo-tissue, and three dimensional (3D) structures for inducing osteogenesis and angiogenesis [1].

The surface of scaffolds is considered of particular importance to optimize the biological processes including initial cellular recognition, tissue specific commitment and subsequent repair and regeneration. Mimicking the interface to the tissue of concern is by far the most promising strategy to appropriately regulate cellular and tissue reactions to ensure the success of scaffolds clinically. In fact, the extracellular matrix (ECM) of the tissue provides the best micro-environmental conditions for the cells that are hosted and reside within it, and contain the necessary components required for tissue maintenance and recovery in the event of damage and disease [2].

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Bone is a highly organized tissue that comprises the major part of human hard tissues, and performs both mechanical and biological functions. Bone ECM consists of collagen (Col) and also non-collagenous proteins such as fibronectin (FN), osteocalcin (OC), osteopontin (OPN), and bone sialoprotein (BSP) [3,4]. As the most abundant ECM component, Col is involved primarily in providing mechanical strength and aiding in the formation of mineralized bone matrix. Therefore, Col has been widely used in various clinical and tissue engineering applications. The use of Col as an interface of biomaterials has been researched, with results demonstrating that Col-surfaces enhance the attachment, proliferation, and differentiation of osteoblastic/progenitor cells *in vitro*, and subsequent bone formation *in vivo* [5–8].

Aside from the structural proteins, adhesive proteins such as fibronectin (FN), vitronectin, and laminin mediate cellular recognition of the underlying matrix through integrin signaling. FN is known to be a potent and dominant adhesive protein. FN contains three types of modules: I, II, and III [9], and with respect to cell adhesion, the ninth and tenth domain of FN module III ($FNIII_{9\text{-}10}$) contains Arg-Gly-Asp (RGD) and the synergic sequence Pro-His-Ser-Arg-Asn (PHSRN) is recognized to enhance cell adhesion, proliferation and differentiation of osteoblasts and progenitor cells [10,11]. However, the efficiency of the adhesive protein alone is not sufficient to stimulate differentiation and maturation (mineralization) of progenitor/stem cells in bone regenerative processes. In other words, some ECM proteins are critically involved in the differentiation and late stage mineralization of bone. Among else, OC is highlighted in this study. OC, the most abundant non-collageneous protein in bone [4], accelerates mineral formation and growth due to its high affinity to the mineral crystals. In fact, OC has been recognized to be the key marker for late stage osteogenesis of progenitor/stem cells [12,13].

We have recently synthesized a unique fusion protein, comprising OC and $FNIII_{9\text{-}10}$ (OC-FN), which was aimed at regulating sequential functions of mesenchymal stem cells both in the initial adhesion and late osteogenesis processes [13,14]. Based on this, here we design bone ECM mimic matrix where Col was introduced as the structural support and network of the OC-FN. In particular, we engineered OC-FN with collagen binding domain (CBD) to form a structurally hybridized ECM network with better physicochemical stability. Some engineered proteins containing CBD have already shown good performance with Col materials [15,16]. Moreover, we induced sufficient fibrillogenesis of Col, which is known to be a key process to preserve Col stability and its native functions. The monomeric Col in solution form does not resemble the naturally occurring functional fibrillar form of collagen (fCol). The Col coating with poor fibrillar formation led to a lack of appropriate cellular responses, such as cell attachment and proliferation of osteoblasts [6,17].

Collectively, the proteinaceous matrix constituted of Col-OC-FN is considered to resemble the native bone ECM and to favor stem cell functions including initial adhesion and late osteogenic differentiation while preserving the structural stability. In particular, here we coupled the protein matrix to a 3D porous scaffold made of an easily manufacturable and clinically approved synthetic biopolymer, polycaprolactone (PCL). The scaffold is widely used due to appropriate biodegradation rate and mechanical strength, but the lack of cell recognition site and tissue specific activity has been indicated as a major limitation for bone regenerative scaffolds [18,19]. Therefore, this approach of tailoring the biopolymer scaffold surface constructs with a bone supportive ECM matrix is a facile promising approach for development of next generation bone scaffolds. The processes to generate the protein hybridized scaffolds are described and their biological impacts are demonstrated in terms of mesenchymal stem cell responses *in vitro* and bone formation *in vivo*.

## 2. Materials and methods

### 2.1. Construction and purification of recombinant fusion protein, CBD-OC-FN

To construct the CBD-OC-FN, OC-FN DNA was initially amplified by the CBD-OC-Sac forward primer, 5′-GGCGAGCTCACCAAAAAAACCCTGCGTACCCTCGAGTACC TGTATCAATGGCTG-3, the pBAD-His reverse primer, 5′-ACGGCGTTTCACTTCTGAG T -3′. PCR was conducted for 30 cycles of 1 min at 55 °C (annealing), 2 min at 72 °C (extension), and 1 min at 94 °C (denaturation). Amplified PCR products were digested with *SacI* and *HindIII*. After ligation into pBAD-HisA-OC-$FN_{III9\text{-}10}$ vector, pBAD-HisA-CBD-OC-$FN_{III9\text{-}10}$ constructs were produced. TOP10 *Escherichia coli*, selected clones were grown in LB medium containing ampicillin overnight at 37 °C after transformation. When the absorbance of cultures reached 0.6 ($A_{600}$), induction was initiated with L-arabinose (0.02% w/v) and incubated at 20 °C for 6 h. The bacterial pellets were harvested by centrifugation at 6000 × *g* for 10 min, lysed, and sonicated. A soluble extract obtained were centrifuged for 30 min at 14,000 × *g* in a refrigerated centrifuge and the supernatant was purified by affinity to a nickel-nitrilotriaceticacid resin (Invitrogen). To determine the protein, western blots were performed using a peroxidase conjugate of a monoclonal anti-polyhistidine antibody (SC-8036 HRP, SantaCruz). The degree of purification of the fusion protein was measured by carrying out sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mw = 47 kDa) using SDS-PAGE at 10% (v/v).

### 2.2. Affinity assay of the fusion protein to collagen

For the affinity assay of the CBD-containing OC-FN fusion protein to collagen matrix, the collagen-coated 96-well culture plates were treated with either CBD-OC-FN or OC-FN without CBD at different concentrations (0.5, 1 and 2 μg/ml) and then incubated for 5 h at room temperature. After incubation, each sample was exposed to a horseradish peroxidase (HRP)-conjugated His antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 37 °C. A colorimetric substrate for the HRP was then added (Pierce, Rockford, IL), and the absorbance at 450 nm was read on a spectrophotometer. The non-treated collagen-coated dish was used as a control. Results are presented as optical density (OD) values averaged from three replicate samples (n = 3).

### 2.3. Fibrillar formation of proteins and surface tailoring of scaffolds

The collagen type I solution (60-30-810, First link Ltd.) was diluted in PBS and allowed for fibrillar formation upon immersion in incubator at elevated temperatures. To prepare Col/CBD-OC-FN solutions, the CBD-OC-FN fusion protein was added to the Col solution in PBS at different concentrations (fusion protrein:Col = 1:100, 1:200, and 1:1000) before fibrillar formation. The degree of fibrillar formation of the protein solutions was characterized via turbidity test. The turbidity was monitored at 313 nm with varying incubation time, temperature and protein concentration.

As the framework of scaffolds, macrochanneled PCL were fabricated using a RD (Ez-ROBO3, Iwashita) as described previously [20]. Scaffold was designed and controlled by CAD program. Prior to fabrication of the 3D scaffolds, PCL (MW 80,000, Sigma–Aldrich) were dissolved in acetone and then ball milled at 50 °C. The temperature of the slurry was maintained via a thermo-jacket during the fabrication. The struts were formed in an ethanol bath for the solidification of the slurry via the needle (0.33 mm). The scaffolds were designed as size of 9 × 9 mm and with 6 layers.

For surface of PCL scaffolds was then tailored with the protein solutions. For this, the PCL scaffolds were first hydrolyzed and activated with a cross-linker (EDC-NHS). The pretreated scaffold was then immersed into the different protein solutions for predetermined time period at room temperature. The protein adherence to the surface was assessed by the detection of Col using Sirius Red staining method. Direct red 80 (365548, Sigma) dissolved in saturated picric acid was added to the scaffolds and then left for 1 h at room temperature. The stained samples were washed completely with 0.01 N HCl. The surface-adhered Col was quantified by elution with 200 μl of 0.5 M NaOH. The eluted solution was transferred to 96-well plates, and the optical density was measured at 540 nm. The values were converted to a Col concentration when referenced to a standard curve of Col solution [6]. The optical density (OD) by Sirius red assay showed a linear relationship with Col concentration; $OD = 1.643 \times Col\ concentration + 0.0269$ ($R^2 = 0.99$).

The CBD-OC-FN distribution within Col matrix was examined by TEM after dropping the protein solution on a Cu-grit, followed by staining with Au nanoparticles (ab154873, abcam) conjugated with OC-antibody. The CBD-OC-FN within the Col-bound scaffolds was also directly visualized by immunostaining against OC. The scaffolds tailored with the proteins were put into OC antibody (1:200, SC-365797, Santacruz). After treatment with a blocking buffer to block nonspecific binding (5% skim milk), anti-mouse-Alexa Fluor 555 (1:500, A21422, Invitrogen) was used for conjugation with primary antibody. The fluorescence image was obtained by confocal laser scanning microscopy (CLSM, Carl Zeiss 510L, Germany). The intensity from the fluorescence image, corresponding to the quantity of fusion protein in the hybrid protein network, was measured using image J program (NIH).

An indirect ELISA protocol was also used for the quantification of the fusion protein. After the adhesion, the non-bound protein solution was transferred into the 96 well of ELISA plates and incubated overnight at 4 °C. After blocking, the His-probe antibody (1:500, SC-8036, Santacruz) and secondary antibody (1:500, SC-2005,