Self-assembled Rosette Nanotubes for Bone Regeneration

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Statement of Purpose: Rosette nanotubes (RNTs) are novel biomimetic self-assembled drug delivery devices. With the self-assembly process of RNTs, different drug combinations can be chemically functionalized on the nanotubes (such as short peptides or growth factors). In addition, RNTs can be injected into the physiological environment as a liquid and then solidify into a viscous gel at body temperatures. RNTs are similar in size to collagen in bone and cartilage. Previous studies have shown that RNTs are biocompatible and increase the adhesion and function of osteoblasts (bone-forming cells) compared to other commonly used orthopedic implant materials (like hydrogels and Ti). Thus, it is hoped that RNTs can serve as an in situ, curable, multiple drug delivery vehicle to improve bone cell adhesion and long term functions.

In this study, three short peptides from bone morphogenetic protein-7 (BMP-7) were selected and tested to enhance osteoblast cell proliferation and long-term functions. Moreover, RNTs were functionalized with three short peptides and KRSR (a known bioactive peptide for osteoblasts). The functionalized nanotubes were characterized by nuclear magnetic resonance (NMR), mass spectrometry (MS), elemental analysis and scanning electron microscopy (SEM). Importantly, results showed that BMP-7 short peptides were bioactive for osteoblast functions. Moreover, RNTs were functionalized with different peptides and self-assembled or co-assembled with such peptides or peptide combinations. In addition, functionalized RNTs embedded in hydrogel (pHEMA) were demonstrated to enhance osteoblast adhesion. In this manner, this *in vitro* study provided a self-assembled implant material which relies on nanotechnology to deliver multiple drugs for bone regeneration.

Methods: For proliferation studies, osteoblasts (ATCC, CRL-11372, cultured in DMEM, 10% FBS, 1% P/S under standard cell culture conditions) were separately seeded at 1000 cells/cm^2 into a 24 well plate under standard cell culture conditions with 100μg/mL of synthetic peptide-a (SNVILKKYRN), b (KPSSAPTQLN) and c (KSNVILKKYRN) and their various combinations or 200ng/mL BMP-7. At the end of 1, 3 and 5 days, osteoblasts were fixed, stained and counted under fluorescence microscope.

For differentiation studies, osteoblasts (cultured in DMEM with 10% FBS, 1% P/S, 50 μg/mL l-ascorbic acid, 10 mM β-glycerophosphate) were seeded in 24-well plates (50,000 cells/cm^2). At the end of 7, 14, and 21 days, total protein content and alkaline phosphatase activity were determined via commercially available kits. Calcium deposited by osteoblasts were dissolved in HCl and tested for calcium concentration using a commercially available kit.

For functionalizing studies, peptides were synthesized under standard solid phase synthesis protocol on Wang resins, and mixed with RNT units, Dichloroethene, sodium triacetoxy borohydride and N,N-Diisopropylethylamine for 36 hours. After the functionalized peptides were cleaved and decapped with Trifluoroacetic acid, SEM, UV, MS and elemental analysis were used for characterization and to determine the self/co-assembly process of functionalized RNTs.

For bioactivity tests, KRSR functionalized RNTs were embedded in 0.5mL pHEMA gel per well in 24 well cell culture plates. 3500 cells/cm^2 osteoblasts were seeded in each well under standard cell culture conditions. After 4 hours, osteoblasts density counted under a fluorescence microscope.

Statistics were performed using a one-tail *t*-Test.

Results: For proliferation studies, after 3 and 5 days, osteoblast number increased when cultured with peptide-b, c, BMP-7 and any peptide combinations with peptide-b compared to controls. Moreover, osteoblasts in the presence of peptide-b had a more spread morphology than controls. For osteoblast long term function studies, peptide-a, c and all combinations including peptide-c increased calcium deposition and alkaline phosphatase activity from osteoblasts after 2 and 3 weeks (Figure 1).

For functionalizing studies, MS and elemental analysis confirmed the chemical structure and components of the RNT units functionalized with peptides. In addition, SEM analysis (Figure 2) and UV proved the self/co-assembly of the RNT units with different peptides. Compared with RNTs with a lysine side chain, a larger diameter of RNTs functionalized with BMP-7 peptides confirmed a high density of peptide outside the nanotubes. For bioactivity tests, KRSR peptide was still bioactive after functionalized onto RNTs.

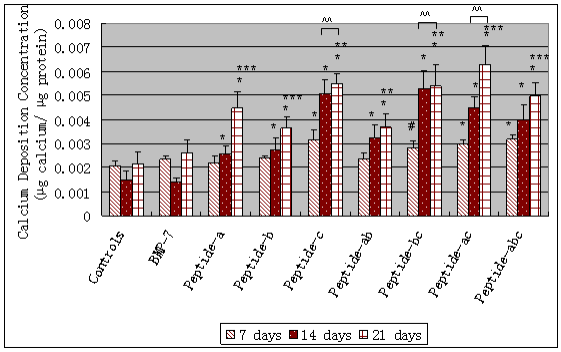

Figure 1. Osteoblast calcium deposition of osteoblasts (per unit protein). Data are mean ± SEM (N=3). #p<0.1 compared to controls. *p<0.05 compared to controls. ^^p<0.05. **p<0.05 compared to the same samples at 7 days and ***p<0.05 compared to the same samples at 7 days and 14 days.

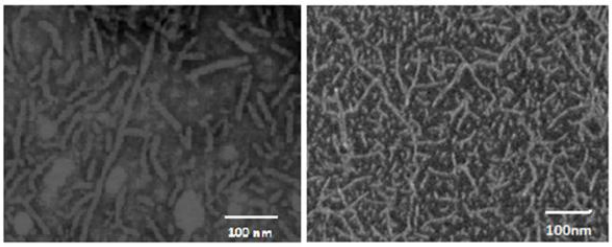

Figure 2. RNTs functionalized with peptide-c and RNTs with lysine side chains.

Conclusions: Results of this study demonstrated that peptide-b from BMP-7 enhanced osteoblast proliferation, while peptide-c from BMP-7 promoted osteoblast long term functions. Most importantly, after functionalizing with different peptides, RNT units were still able to self/co-assemble into biologically-inspired nanotubes. Especially, KRSR was still bioactive after functionalized onto RNTs. In this manner, RNTs can be modified to have different combinations of peptides. In summary, RNTs are nanostructured self-assembled materials promising for multi-drug delivery for orthopaedic applications.

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