Studies Toward the Development of Orthopaedic Tissue Engineering Material Based on Self-Assembled Rosette Nanotubes

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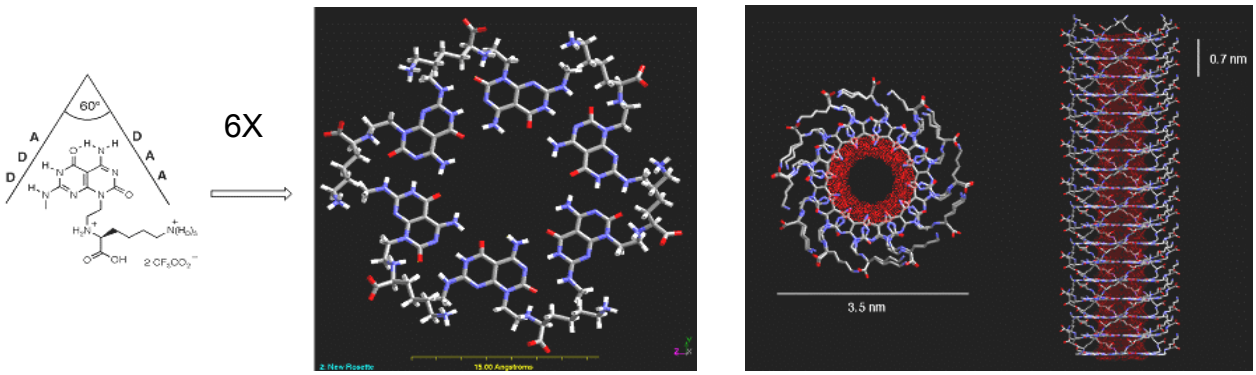
Introduction

Orthopaedic biomaterials research has moved into making "smart" tissue engineered materials that can be replaced to restore normal function and integrity of bone. Conventional approaches have not been able to design and fabricate bone implants that last longer than 15 years once implanted. New materials designed to possess biologically-inspired chemistries and nanoscale architectures, which mimic native occurrences are anticipated to extend implant lifetime and reduce early failures.

Due to the unique nanoscale properties of some components in bone such as collagen (~300 nm in length) and hydroxyapatite crystals (~20-80 nm in length, ~4-6 nm in thickness), nanomaterials have been proposed as the next generation of improved implant materials [1, 2]. There is increasing evidence that osseointegration is promoted on nanostructured surfaces such as ceramics, metals, polymers and composites thereof [1-6]. Yet, current orthopaedic materials such as titanium (Ti) do not possess desirable nanometer surface features, which is believed to be a reason why Ti sometimes fails clinically. The knowledge that cells *in vivo* interact with nanometer-sized structures led us to study the impact of helical rosette nanotubes (HRN), a nanotubular assemblage with biologically-inspired chemistries, on bone cell attachment.

Helical rosette nanotubes (HRN) are a new class of organic materials assembled from a single bicyclic block featuring the complementary hydrogen bonding arrays of Guanine and Cytosine, the G/C motif (Fig 1a) [7, 8]. This building block self-assembles spontaneously in water to form a six-membered cycle (called rosette) maintained by 18 H-bonds, which stack up to form a nanotube with a hollow core ~1 nm across and up to several millimeters long (Fig 1b) [7]. Because of their mechanism of formation, these constructs can serve as very stable scaffolds for the synthesis of functional nanotubular assemblies with predefined chemical and physical properties. For instance, a variety of functional groups suited for different applications can be attached to the G/C motif (in place of lysine in Fig 1a).



(a) (b)

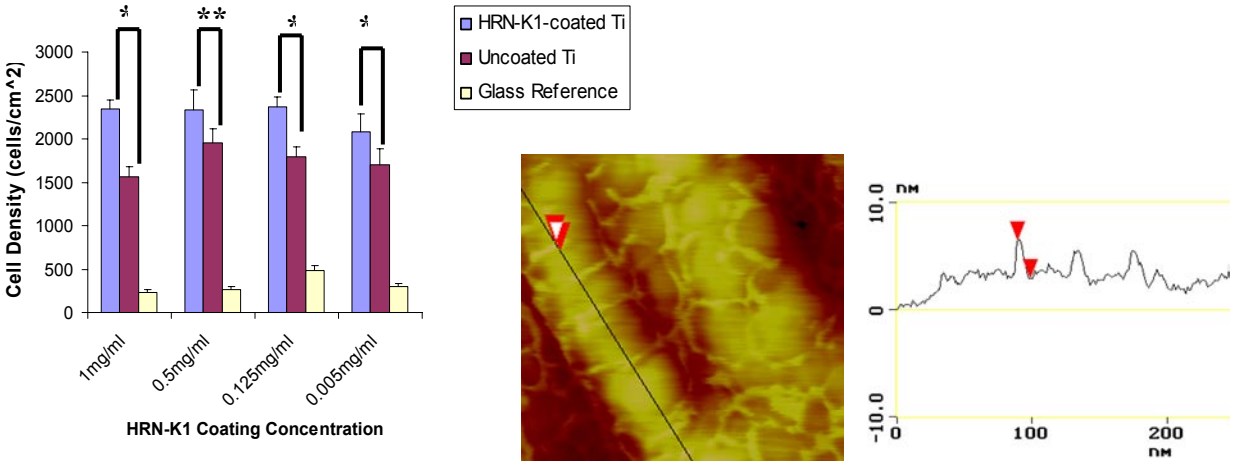
Figure 1: Building blocks of helical rosette nanotubes (HRN). (a) The G/C motif possesses the Watson-Crick H-bond donor-donor-acceptor (DDA) array of guanine and acceptor-acceptor-donor (AAD) array of cytosine (*left*). Six GC motifs self-assemble via 18 H-bonds to form the rosette (*right*). (b) Second level of organization produces a stack with a hollow core ~1 nm across and up to several millimeters long. Top view (*left*). Side view (*right*) [7]

Experimental

This investigation involved seeding osteoblasts (bone-forming cells) on a titanium (Ti) substrates coated with HRN. Two HRNs functionalized with different amino acids were investigated: 1) lysine (HRN-K1) and 2) arginine (HRN-R1). Detailed experimental methods are outlined in [9]. Briefly, Ti were coated with HRN by simple adsorption for 30 min. Osteoblasts were allowed to attach for 1 hr after which they were fixed and stained with Hoeschst (for protein studies, either serum or serum-free media were used). Five random fields of adherent cells were counted *in situ* per substrate and statistical analysis was performed. All experiments were run in triplicate and repeated 3 times. Uncoated Ti served as a positive control while glass served as a reference. HRN adsorption on Ti was confirmed by imaging substrates with atomic force microscopy (AFM). Heated samples of HRN-K1 were examined by transmission electron microscopy (TEM).

Results and Discussion

Compared to uncoated Ti and glass, there was a statistical difference in the number of osteoblasts that adhered on Ti coated with HRN-K1 (Fig 2a) and HRN-R1 (not shown) after 1 hr. Preferential cell adherence was comparable in both HRN-K1 and HRN-R1. There was no statistical difference between the various HRN-K1 concentrations studied. Fig 2b and 2c show the adsorption of HRN-K1 on Ti surface.

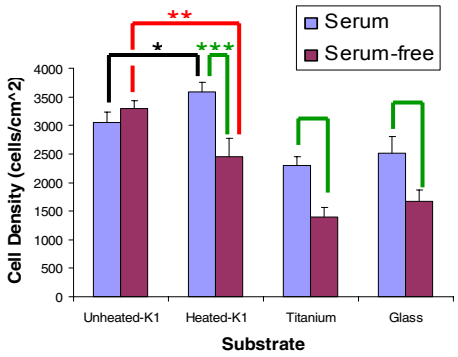


(a) (b) (c)

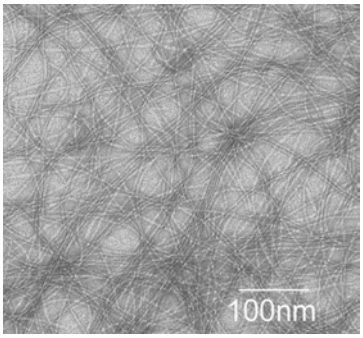
Figure 2: (a) Enhanced OB adhesion on HRN coated Ti. Data are mean ± SEM; n=3; $^{*}p < 0.01$, $^{**}p < 0.10$ (T-test) when compared to uncoated Ti. (b) AFM image shows HRN-K1 formed networks on the Ti surfaces (scan size: 638nm; scan rate: 2 Hz). (c) Arrow heads on one HRN. Section analysis indicates a height of 3.5 nm, which is consistent with the computed diameter of one HRN [7].

Ti was chosen as a substrate because it is one of the currently used orthopaedic implants. The HRN utilized in this study were chosen because 1) they are both positively charged and 2) both lysine and arginine set the stage for later incorporation of KRSR and RGD peptide sequences on HRN, respectively. These sequences are known to enhance bone cell adhesion [9]. In addition, it has been shown by *in vitro* and rat models that lysine and arginine play therapeutic roles in osteoporosis and fracture healing [10]. The protein studies show that while proteins are necessary in the heated-HRN-K1 samples for increased OB adhesion, in the absence of proteins, heated-HRN-K1 still performed better than uncoated Ti. Proteins were not necessary for enhanced OB adhesion on unheated-HRN-K1 coated Ti (showed no difference in the number of adherent OB under both serum and serum-free

conditions) (Fig 3a). TEM of a heated sample of HRN-K1 shows the formation of networks, bundles and sheets of HRN on the TEM grid (Fig 3b).



(a)



(b)

Figure 3: (a) Protein study. OB adhesion on HRN-K1 coated Ti under serum and serum-free conditions. Data are mean ± SEM; n = 3; $^{*}p < 0.1$; $^{**}p < 0.05$; $^{***}p < 0.01$ (T-test) when compared as indicated by bars. (b) TEM of a heated sample of 1mg/ml HRN-K1 showing the formation of networks, bundles and sheets of HRN.

The protein studies led us to speculate that HRN may resemble certain properties of proteins that when coated on Ti could provide signals for OB adhesion [11]. This data contradicts conventional wisdom that proteins are necessary for OB adhesion [12-15] and thus, warrants further studies of HRN in bone tissue engineering. Growth factors [16] and/or select bone recognition peptide sequences [17] that preferentially attract bone cell adhesion can be tethered to HRN to improve OB adhesion beyond those observed here. In addition, temperature, concentration and pH factors, which affect self-assembly of these nanotubes, can be manipulated to obtain a viscous and highly moldable hydrogel. This property is very well-suited for fabricating three dimensional constructs, which can potentially repair bone fractures or act as cell delivery vehicles in cartilage transplant. These avenues are currently being investigated.

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