Helical Rosette Nanotubes as a Biomimetic Tissue Engineering Scaffold Material

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Introduction:

With the rapid development of orthopedic implant technology, various bone implant surgeries and procedures (such as bone fractures, repairing defects, and hip and knee replacements) are routinely performed. Although these surgeries are usually successful operations for up to 15 years, implant failure due to loosening of the prostheses does occur for active or heavy patients. Other patients receiving implants sometimes suffer from inflammation, infection, and foreign body responses which cause intense pain and increase operating costs at the hospital. For this reason, scientists are pursuing a new generation of orthopedic tissue engineering scaffold materials which possess not only mechanical properties similar to those of physiological bone but also cytocompatible surface properties that can more successfully promote the formation and bonding of juxtaposed bone [1].

Helical Rosette Nanotubes (HRN) are such a type of novel soft organic nanomaterial obtained through the self-assembly of low-molecular-weight synthetic molecules in water. The building blocks of HRN possess key elements for their sequential self-assembly towards the formation of stable nanotubular architectures. In Figure 1a is the guanine-cytosine (G^C) motif, which possesses the Watson-Crick H-bond donor-donor-acceptor array of guanine and acceptor-acceptor-donor array of cytosine. It is possible to further functionalize the G^C motif for different applications as well. For example, the amino acid side chain (K) can be replaced by RGD (arginine-glycine–aspartic acid), a peptide sequence known to enhance cell adhesion and proliferation [2,3]. Furthermore, six G^C motifs undergo spontaneous self-assembly under physiological conditions first into a supermacrocycle (rosette, figure 1b). Then, through non-covalent interactions such as H-bonds, base stacking interactions and hydrophobic effect, the rosettes form a stable tubular stack with a hollow core 11 Å across (figure 1c) [4].

Equally as promising, previous research [1,5] has shown that cell adhesion and proliferation can be greatly enhanced on materials with grain size less than 100 nm, including nanophase metals, ceramics, and polymers. When HRN-K1 are deposited on implant surfaces, they will confer a nanometric network that resembles collagen, a nanoscale constituent in bone. Since reproducing such features and dimensions on implant surfaces are critical in bridging materials with living systems in order to achieve a stronger and more robust integration/bond between the implant and tissue, it is thus anticipated that HRN would be better-suited for orthopedic applications compared to conventional implant materials such as titanium (Ti) which does not mimic the nanometric features of bone. Previous studies have also shown that osteoblasts adhered more on specialized versions of

HRN-K1 when coated on Ti surfaces compared to uncoated Ti [6]. This phenomenon may be attributed to the presence of amino acids side chains (such as lysine) as well as to the biologically-inspired nanometric features that HRN-K1 form when cast on Ti. Moreover, because HRN-K1 can undergo a phase transition from a liquid to a viscous gel when heated to 60°C or when added directly to serum-free media at body temperatures, HRN-K1 may provide an exciting therapy to heal bone fractures and defects. However, for this realization, the strength of HRN must be increased by combining it with durable and biocompatible matrices such as hydrogels.

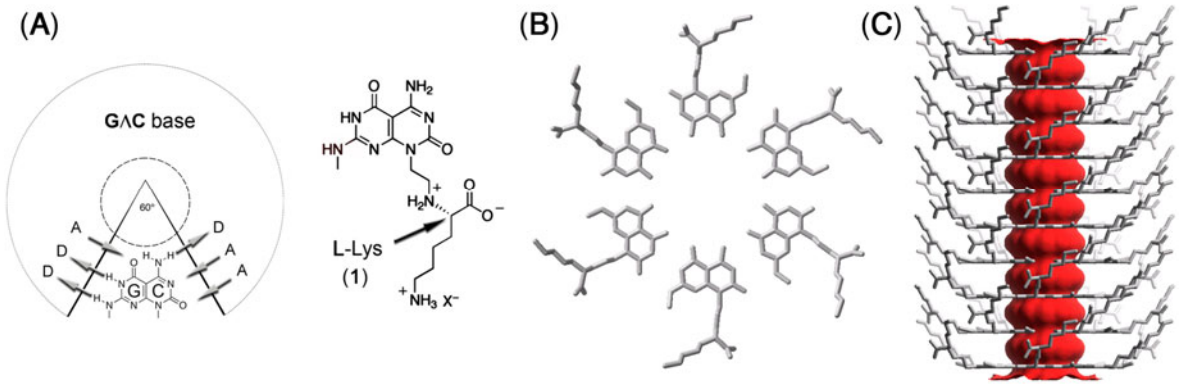


Figure 1: Self-assembly of helical rosette nanotubes with lysine side chain (HRN-K1). (a) Guanine-cytosine (G^C) motif and K1 module. (b) Rosette supermacrocycle formed by six G^C motifs maintained by 18 H-bonds. (c) Helical rosette nanotube with a 3.5 nm diameter and several μm in length.

The objective of this *in vitro* study was to create an easy-to-use injectable and nanostructured 3D scaffold based on this promising novel self-assembled nanomaterial (HRN-K1) but with suitable mechanical properties (through the incorporation of hydrogels) to fill bone fractures and repair bone defects.

Materials and methods:

1. Preparation of HRN-K1 solutions:

A 0.1mg/ml HRN-K1 stock solution was formulated according to standard procedures [6]. Two HRN-K1 solutions were then prepared by diluting the stock solution with deionized water: one with the concentration of 0.01 mg/ml, the other with 0.001 mg/ml. These solutions can be stored at room temperature for at least one year.

2. Preparation of HRN-K1 hydrogel scaffolds:

2 ml 0.01 mg/ml HRN-K1 solution and 4 mg of 2,2'-azobisisobutyronitrile (Sigma cat#441090) free radical initiator were added to 2 ml 2-hydroxyethyl methacrylate (HEMA) monomer in a 10 ml vial (Sigma cat#128635) [7-9]. The mixture was blended mechanically in order to get a homogenous solution, which was then poured into a Teflon mold and heated in an oven at 45°C for several hours. After the mold was removed from the oven,

the transparent hydrogel (pHEMA) scaffold obtained was sterilized in a 70% ethanol solution at room temperature for 15 minutes and then immersed in sterile water at room temperature for 45 minutes prior to cell experiments [10]. The same procedure was applied to the preparation of hydrogel scaffolds with different HRN-K1 concentrations.

In addition, hydrogel scaffolds without HRN-K1 were separately coated with 0.01 and 0.001 mg/ml HRN-K1 solutions by simple adsorption at room temperature for 30 minutes. Uncoated hydrogels served as references.

3. Osteoblast adhesion on HRN-K1 hydrogel scaffolds:

Osteoblast (bone-forming cell) adhesion was tested on five types of substrates: #1--0.01 mg/ml HRN-K1 coated hydrogel scaffold, #2--0.01 mg/ml HRN-K1 embedded hydrogel scaffold, #3--0.001 mg/ml HRN-K1 embedded hydrogel scaffold, #4--0.001 mg/ml HRN-K1 coated hydrogel scaffold, and #5--uncoated hydrogel .

Human fetal osteoblasts (American type culture collection: CRL-11372) were seeded at 3500 cells/cm^2 onto the above five substrates. Osteoblasts were allowed to adhere in 2 ml of DMEM/F-12 Ham supplemented with 10% fetal bovine serum (FBS, hyclone), 1% Penicillin-Streptomycin (P/S, hyclone), then were cultured under standard cell culture conditions (37°C, humidified, 5% CO_2/95% air) for 4 h.

Non-adherent cells were removed through PBS sequential washings. Adherent cells were then fixed with 10% normal buffered formalin, stained with DAPI stain and counted under a fluorescence microscope.

4. Statistics:

Experiments were run in triplicate and repeated three times for each substrate. Student's t-test was used to make pair-wise comparisons. Statistical significance was considered at $p<0.1$.

Results:

Figure 2 shows the results of osteoblast adhesion on the five types of substrates of interest to this study. Osteoblasts more preferably adhered to HRN-K1 hydrogel coated scaffolds (#1, #3) and HRN-K1 embedded hydrogel scaffolds (#2, #4) than to uncoated hydrogels (#5). In the case of 0.01 mg/ml HRN-K1 coated hydrogels (#1), the result (*$p<0.001$*) was significantly greater than that of uncoated hydrogels (#5). With increasing HRN-K1 concentrations, cell density also increased (i.e, both #1 and #2 were greater than #3 and #4). The difference in adhesion between 0.01 mg/ml HRN-K1 embedded hydrogels (#2) and coated hydrogels (#1) was not statistically significant. There was no statistical difference between 0.001 mg/ml HRN-K1 embedded hydrogels (#3) and 0.001 mg/ml HRN-K1 coated hydrogels (#4) as well. The fluorescently stained cells on the various substrates are shown in figure 3 and correlate with the quantitative data presented in figure 2

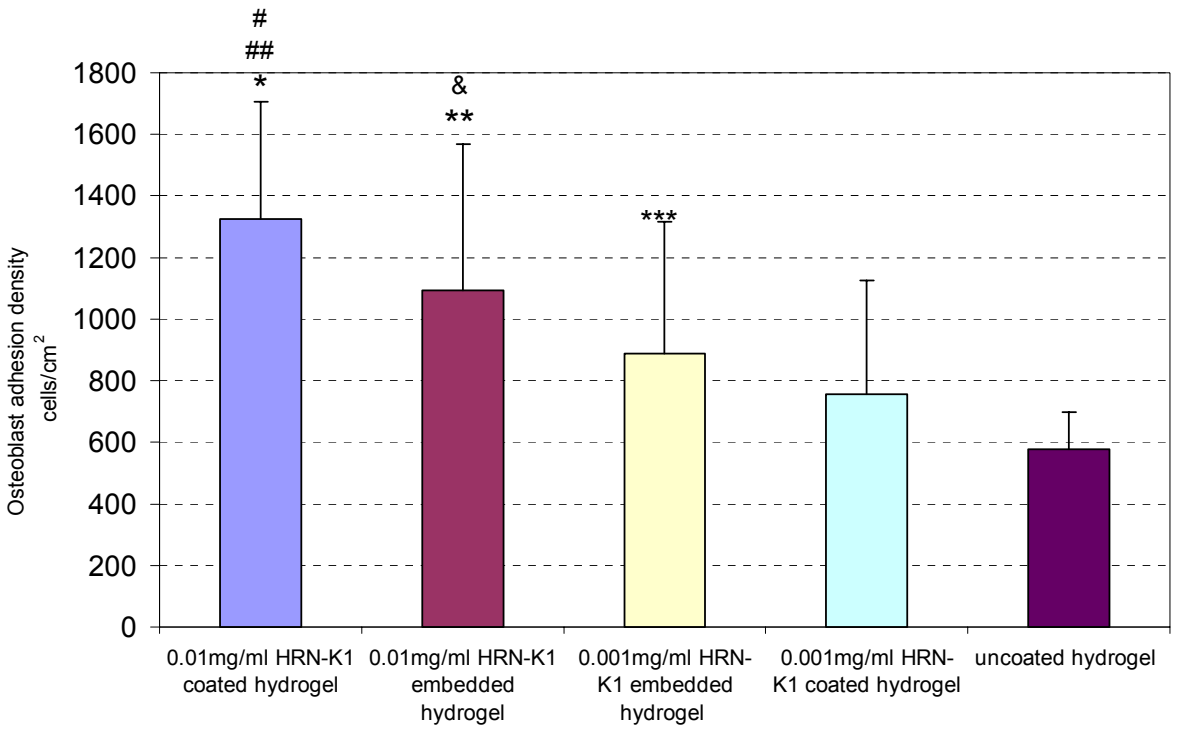
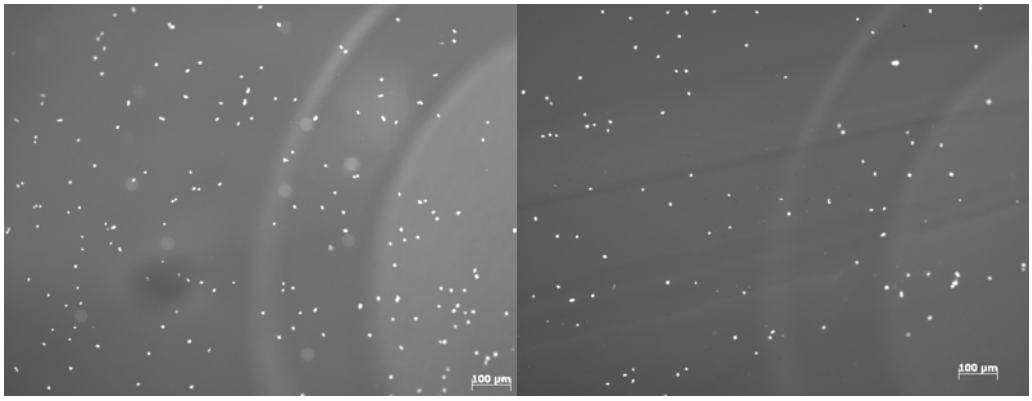
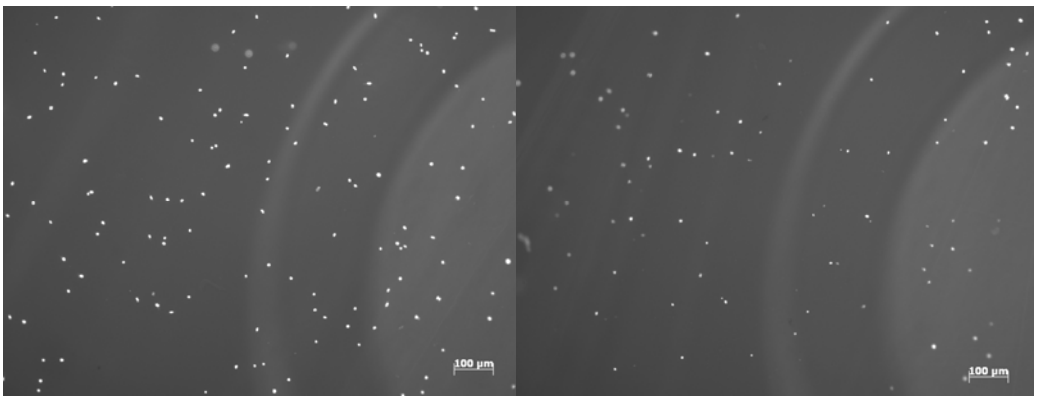


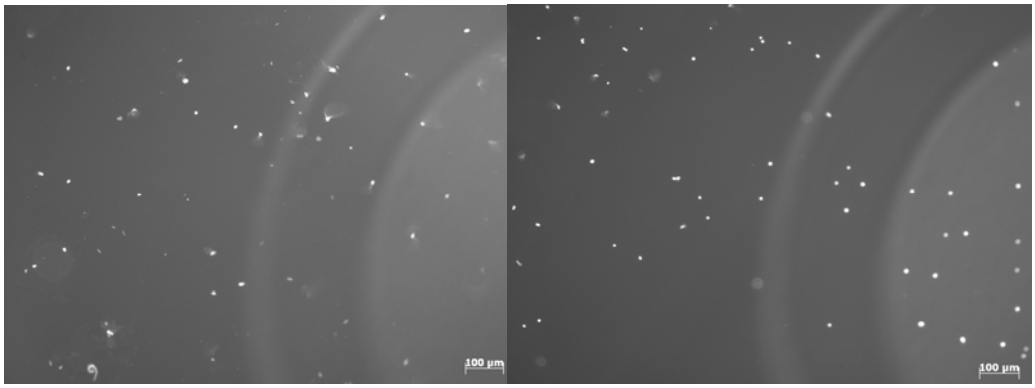
Figure 2. Enhanced osteoblast adhesion on 0.01 mg/ml HRN-K1 coated hydrogels. Data are mean ±SEM; n=3. * *p<0.001,* ** *p<0.01,* *** *p<0.05* when compared to uncoated hydrogels; # *p<0.005* when compared to 0.001 mg/ml HRN-K1 coated hydrogels; ## *p<0.05* when compared to 0.001 mg/ml HRN-K1 embedded hydrogels; & *p<0.1* when compared to 0.001 mg/ml HRN-K1 coated hydrogels.



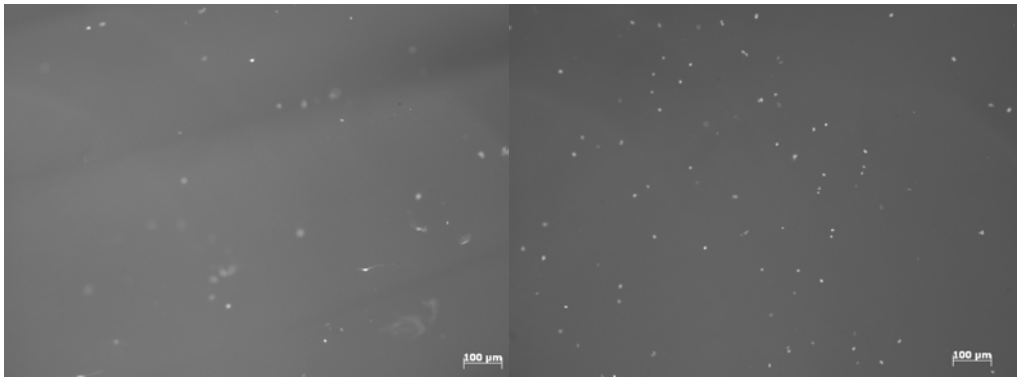
0.01 mg/ml HRN-K1 coated hydrogels



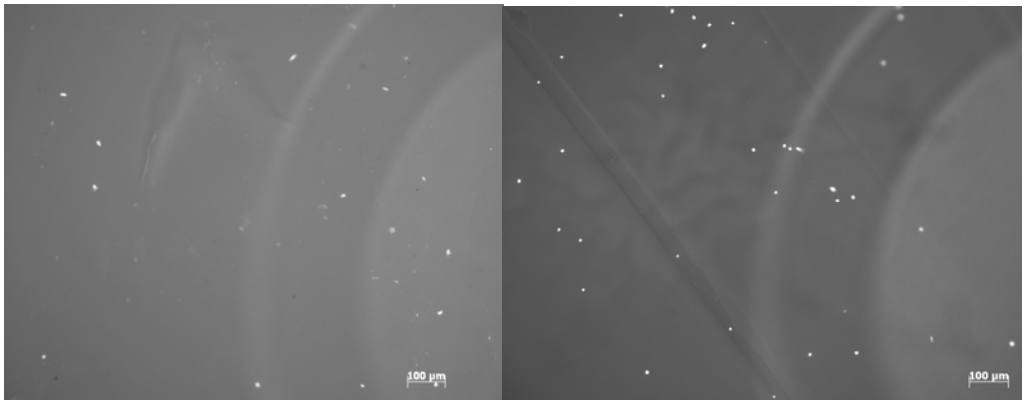
0.01 mg/ml HRN-K1 embedded hydrogels



0.001 mg/ml HRN-K1 embedded hydrogels



0.001 mg/ml HRN-K1 coated hydrogels



hydrogel controls

Figure 3. Increasing fluorescently stained osteoblasts on HRN-K1 coated or HRN-K1 embedded hydrogels compared to plain hydrogel controls.

Discussions:

There is much current interest in the use of liquid injectable materials that can stiffen into a scaffold once in the human body. The formation of injectable scaffolds onto which cells are able to develop new bone tissue at the site of fracture is a promising method to repair bone fractures and defects. Due to their excellent properties, HRN-hydrogel based injectable scaffolds may be suitable for treating irregularly shaped defects. The ultimate goal of this study is to design a novel self-assembled material composite, making it possible to treat small bone fractures simply through their addition to body fluids immediately after the patient enters the emergency room. One possible approach to form injectable scaffolds is via the combination of aqueous hydrogels and HRN-K1. Our research has focused on hydrogel scaffolds partly because they possess suitable mechanical properties and they have already been extensively used in tissue engineering and drug delivery applications [11,12]. We envision that the combination of HRN-K1 and elastic hydrogels could lead to a new generation of bonelike composites with unique mechanical properties.

The present study provided evidence of enhanced osteoblast adhesion on HRN-K1 hydrogel scaffolds even at a very low HRN-K1 concentration of 0.001 mg/mL. Hydrogel scaffolds coated with HRN-K1 at 0.01 mg/mL significantly improved osteoblast adhesion compared to the experiments with lower concentrations of HRN-K1 (0.001 mg/mL) and dramatically outperformed uncoated hydrogels. These results are consistent with previous studies that focused on investigating HRN-K1 as a potential orthopedic coating material [6,13]. Higher concentrations of HRN-K1 hydrogel scaffolds may display more lysine side chains on the biomaterial surface, thus possibly mimicking lysine-rich bovine bone proteins known to enhance osteoblast adhesion, proliferation and differentiation [14]. It is evident from these preliminary results that the HRN-K1 hydrogel scaffolds have the potential to be used as injectable scaffolds for tissue engineering applications and, thus, warrant further attention.

Conculsions:

In summary, the potential of HRN-K1 hydrogels as novel tissue engineering injectable scaffold materials for orthopedic applications was investigated. This study demonstrated that these materials are attractive for osteoblast adhesion due to their nanoscale dimensions and chemical make up. The 0.01 mg/ml HRN-K1 coated hydrogels significantly improved osteoblast adhesion compared to the other substrates. Future investigations include incorporating peptide sequences for enhanced specificity, hydroxyapatite for improved mechanical properties [15-17], and growth factors (such as BMP-2) to stimulate cell growth and bone tissue formation.

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