Self-assembled, Nanometer-rough Cartilage Sealants for Orthopaedic Applications

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Introduction

Articular cartilage defects are widespread, because of intensive sports activities, genetic disorders (like rheumatoid arthritis) and osteoarthritis. Due to cartilage loss, patients usually have significant pain and are limited in joint motion. Today, more than 20 million Americans suffer from arthritis [1]. It is the leading cause of disability in people over the age of 55 in the United States [2]. Except for artificial joint replacements (usually including metals) with their associated short functional life-span, there is no appropriate long-lasting treatment for cartilage damage. Currently, many researchers have focused on promoting cartilage tissue growth to repair the joints of patients instead of relying on complete joint replacement surgeries [3] [4].

Autologous chondrocyte implantation/transplantation (ACI/ACT) is a cellular based therapy already in clinical use [5]. For ACI/ACT, chondrocytes are seeded onto synthetic [6~8] or naturally derived [8~9] matrix scaffolds (implant materials) that would be sutured into the joint. However, current histological and clinical data of ACI/ACT are conflicting and highly controversial. One of the complications associated with these methods is the lack of cellular and consequently tissue binding between engineered cartilage and natural tissue [10].

Because nanophase materials (or materials with one dimension less than 100nm in at least one direction) are able to mimic the dimensions of constituent components of natural cartilage, bone and the transitional zone of cartilage (for example, due to the presence of collagens, glycosaminoglycans (GAGs) and hydroxyapatite), they have great potential to be successful alternative implant materials to what is being proposed today.

One type of nanomaterials, helical rosette nanotubes (HRNs, Figure 1), are novel biomimetic self-assembled supramolecular structures, whose basic building blocks are guanine (G) and cytosine (C) DNA base-pairs which can solidify into a viscous gel [11~13]. The G^C heteroaromatic bicyclic base possesses the Waston-Crick donor-donor-acceptor of guanine and the acceptor-acceptor-donor of cytosine. G^C undergoes a hierarchical self-assembly process under physiological conditions to form a six-membered

supermacrocycle by the formation of 18 hydrogen bonds. Because of electrostatic forces, base stacking interactions and hydrophobic effects, the rosettes form a stable stack with an inner channel 11Å in diameter. An amino acid side chain (lysine) was chosen to impose chirality and surface chemistry on the HRNs. Importantly, the lysine side chain (with an amine group and a carboxyl group) provided the possibility to functionalize a variety of drugs onto HRNs (for example, to functionalize peptide growth factors through peptide bonds). In addition, some small bioactive molecules could be trapped in the inner channels of HRNs by hydrogen bonds; this is another choice for drug delivery by HRNs. Hopefully, two drug carrier sites for HRNs could show different release rates to fit specific requirements of drugs. Thus, because HRNs can: 1) increase viscosity when heated to low temperatures (40~60°C); 2) be easily functionalized with a high-density of peptides and drugs; 3) mimic the nanostructure of natural cartilage, bone and the transitional zone between them; 4) create a surface environment which improves protein (like collagen and fibronectin) absorption as well as enhance cell adhesion and subsequent functions; 5) be injected into cartilage defect sites and in situ repair damaged cartilage; and 6) biodegrade with the recovery of natural tissue, HRNs are expected to serve as a novel, multifunctional implant material to regenerate cartilage/bone.

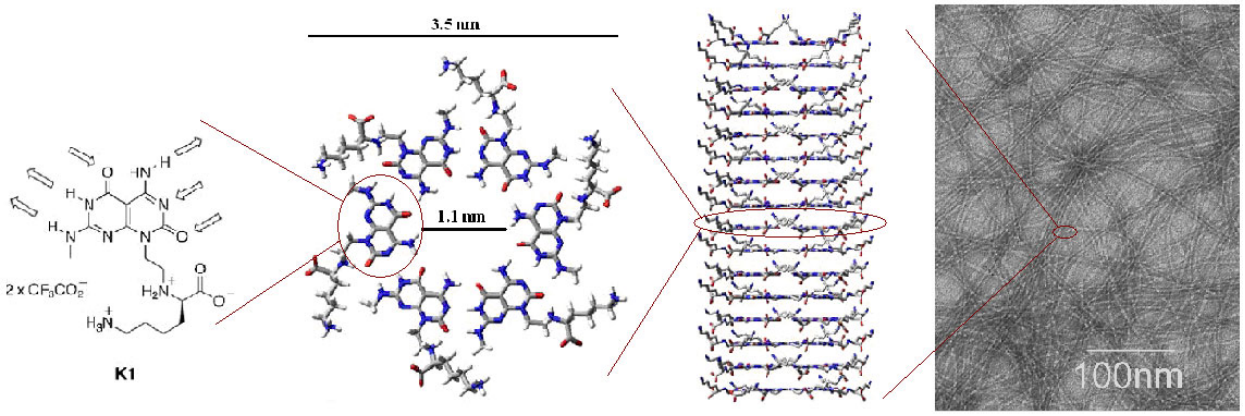

Figure 1. HRNs (with a lysine side chain) undergo spontaneous self-assembly under physiological conditions.

To further mimic the natural cartilage/bone interface and to improve the mechanical properties of engineered cartilage/bone, select biomaterials can be added to HRNs, like alginate ($(C_6H_8O_6)_n$). Alginate, just like GAGs (glycosaminoglycans, one of the major components in cartilage), belongs to the polysaccharide family. It is considered a biocompatible material for cells as it generally does not interfere with cellular functions. Many studies have involved alginate as the material to encapsulate mesenchymal stem cells and promote their osteogenic/chondrogenic differentiation [14, 15]. In this study, the mechanical properties of select hydrogel/HRN composites were tested. In addition, electrospinning was used to generate three-dimensional, implantable, composite HRN fibers containing fibroblast-like type-B synoviocytes (SFB cells). Importantly, results showed that HRNs enhanced hydrogel adhesive strength to fractured collagen and created a scaffold with nanometer-rough surface structures that promoted SFB cells adhesion and viability. In this manner, this study provided an alternative cartilage regenerative material produced by

nanotechnology techniques that can be injected as a liquid, solidify at body temperatures under short periods of time, have suitable mechanical properties to cartilage, and promote SFB cell viability and adhesion.

Materials

SFB cells were isolated from synovial membranes, which were digested with 0.1% trypsin and 0.4% collagenase Ⅱ from knee joints of 4-months old female pigs. DMEM/F-12 Ham media supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (P/S; Hyclone) was used to culture the cells under standard cell culture conditions, that is, a sterile, 37 ^{o}C, humidified, 5% CO_2 / 95% air environment.

Collagen casings #320 purchased from Nippi (Japan) were cut into 10.0 ± 0.5 cm * 2.0 ± 0.05 cm * 0.050 ±0.002 mm pieces. 70% ethanol was used to clean the collagen casing surface. PEG (molecular weight 20,000) was purchased from Sigma.

Methods

Mechanical Testing

PEG was dissolved in double distilled water to a concentration of 0.2mg/mL. HRNs were added to the PEG solution to reach different concentrations. 50μL of a PEG solution was added to collagen casing pieces in a 2cm*2cm adhesive area. Two pieces of collagen casings were adhered together by the adhesive area, then heated at 60^{o}C for 10mins. After 24 hours, until two pieces of collagen casings adhered together tightly, a tensile force was applied on the collagen casing. The tensile strength increased gradually until the adhesive area separated. The maximum tensile load was recorded by the computer.

Contact Angle Measurements

PEG with/without HRNs were placed on the collagen casings and contact angles measured using a static contact angle meter. An auto pipette was employed with the meter to ensure that the volume of the solution droplet was the same (20 μL) for each collagen casing.

Electrospinning Encapsulations

For electrospinning experiments, three different hydrogel solutions were prepared separately in DMEM medium: 10% polyvinyl acetate (PVA), 5% PVA and 3% alginate, and 5% PVA, 3% alginate and 0.01 mg/mL of HRNs solution. Each solution was placed into a 10 ml syringe. The positive lead from a high voltage supply was connected via an alligator clip to the external surface of the needle. A rectangular aluminum foil was used as a static collector and connected to the ground. The tip-to-collector distance was 15 cm. The flow rate of the solution was 2 ml/h and was dispensed automatically. The voltage was kept at 20 kV. TEM and SEM were used to analyze the nano/sub-micro fibers of the composite.

Cell Adhesion and Viability

For cell viability and adhesion experiments, SFB cells were isolated from the knees of 4-month old female pigs. SFB cells were mixed with the hydrogel solution at a concentration of 500,000 cells/mL. The hydrogel solution containing cells was electrospun onto glass slides (2 cm diameter). The glass slides with cells were then cultured in DMEM medium with 10% FBS in a sterile, 37 ^{o}C, humidified, 5% CO_2 / 95% air environment for 1 day. Then, cells were stained and counted under a fluorescence microscope to exam cell viability and adhesion.

Microscopy Studies

For TEM analysis, a drop of HRN-K1 with PEG was placed on the TEM grids. Some samples were dried at room temperature directly while others were adhered on collagen casing surfaces and dried in 60 ^{o}C for 10 mins. Then, by applying the tensile force, TEM grids were pulled apart from the collagen casing. After negatively staining, all samples were imaged by TEM (Philips, EM410 PW6008) at 10,000×~235,000× operating at 100 kV acceleration voltages.

Statistical Analysis

Statistics were performed using a one-tail T-Test for n=3~9 where $p<0.05$ was considered statistically significant.

Results

Mechanical Testing and TEM Analysis

Results from this study showed that as the HRN concentration increased, the adhesive strength of PEG on collagen casings also increased, as shown in Figure 2. Transmission electron microscopy (TEM) pictures showed that in the PEG solution, 0.1mg/mL HRNs aggregated into longer ribbons (Figure 3A), which may have increased the mechanical strength of the HRNs. In Figure 3B, after heating at 60^{o}C for 10 mins, HRNs assembled into long ribbons and some separation points were found after fracture during the tensile tests. These results demonstrated that HRNs strongly adhered onto collagen casings, thus, increased the adhesive strength of PEG between two pieces of collagen casings.

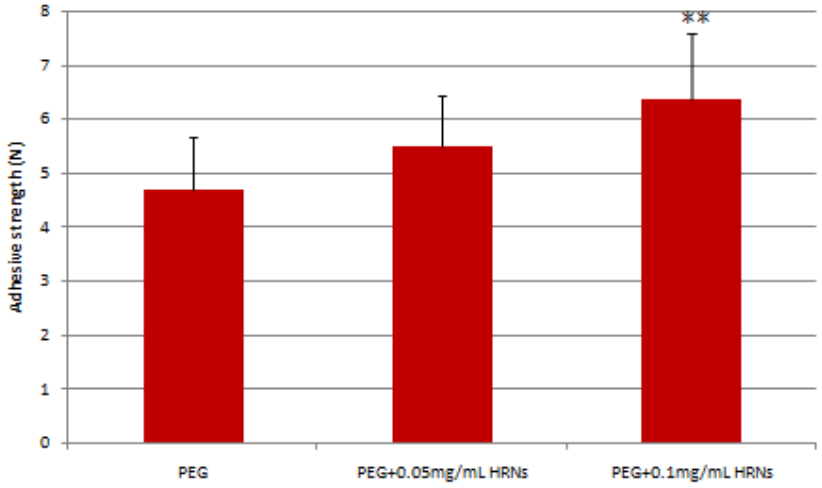

Figure 2. Increased adhesive strength of PEG in the presence of HRNs. Data are mean ± SEM (N=1, n=5). **p<0.1 compared to PEG (no HRNs).

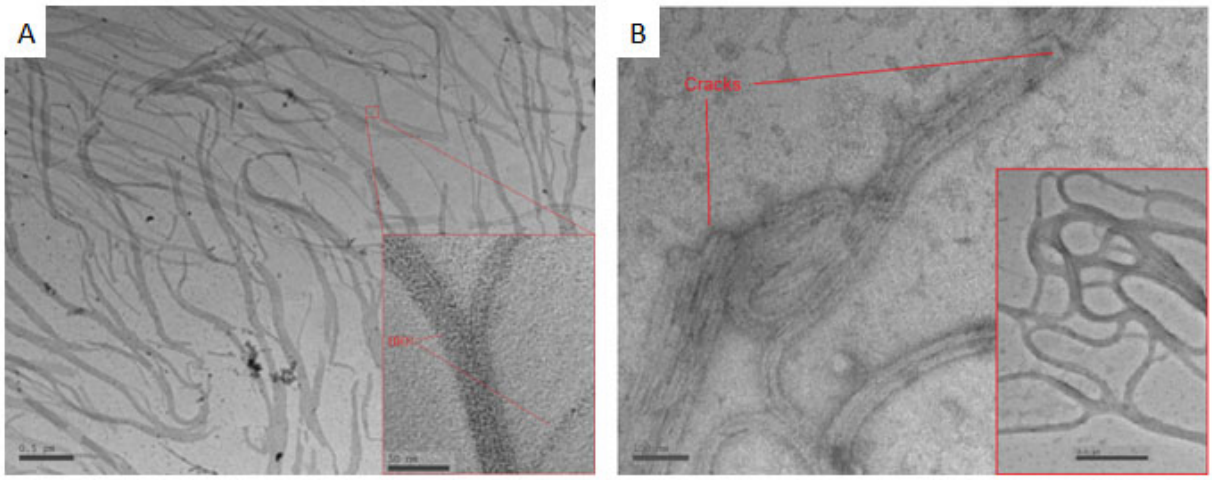

Figure 3. TEM pictures of the unheated HRN/PEG solution on a TEM grid without tension (A) and the heated HRN/PEG solution with tension (B).

Contact Angle Studies

For contact angle studies, as shown in Figure 4, with increasing HRN concentrations, the contact angle of the hydrogel solution on collagen casings decreased, which provided evidence that HRNs promoted the surface contact of PEG to increase adhesive strength.

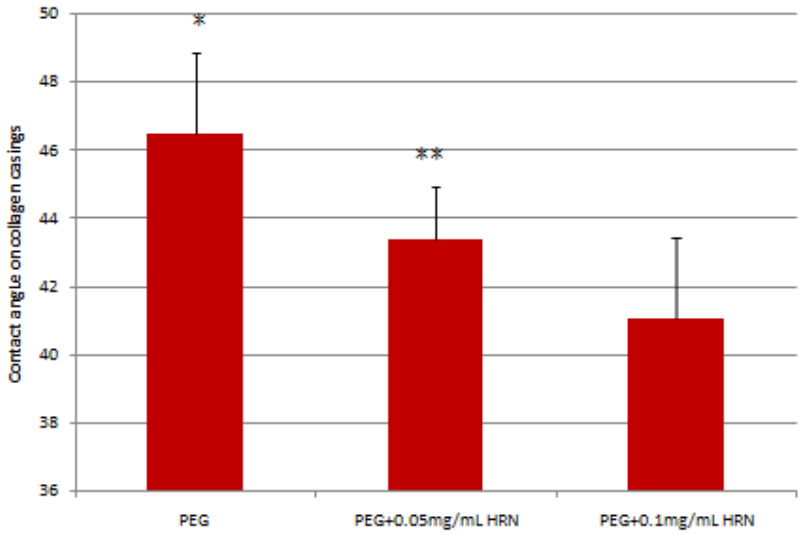

Figure 4. Decreased contact angles of PEG solution in the presence of HRNs on collagen casings. Data are mean ± SEM (N=1, n=6). *p<0.05 compared to controls (PEG only), **p<0.05 compared to controls (PEG only).

Electrospinning Encapsulation

TEM analysis showed the PVA/alginate hydrogel was electrospun into sub-micro fibers with diameters from 200~700 nm. Moreover, on these fibers, HRNs were clearly

observed when add to created nanorough scaffolds as marked by yellow arrows, as shown in Figure 5A. In Figure 5B, no HRNs are present in the hydrogels, thus, the nano-structures on the hydrogel fibers can not be observed.

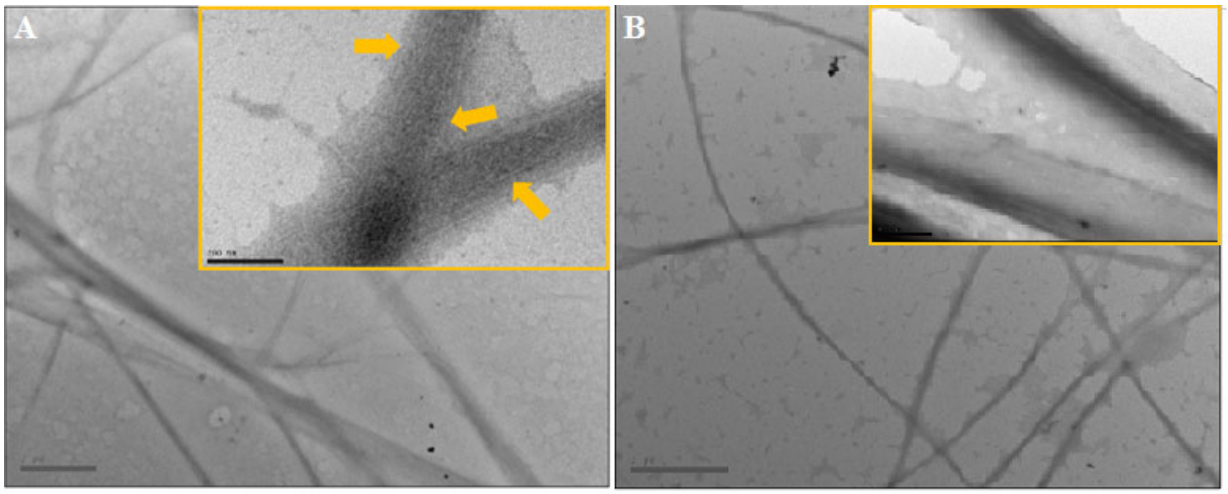

Figure 5. Electrospinning hydrogel fibers with HRNs (A) and without HRNs (B).

Cell Viability and Adhesion Studies

After 1 day of cell culture, cell numbers for the PVA/alginate/HRN samples were the highest compared to any other scaffold created in this study. In addition, cell viability (live cell numbers/total cell numbers) was similar on all of the scaffolds, as shown in Figure 6.

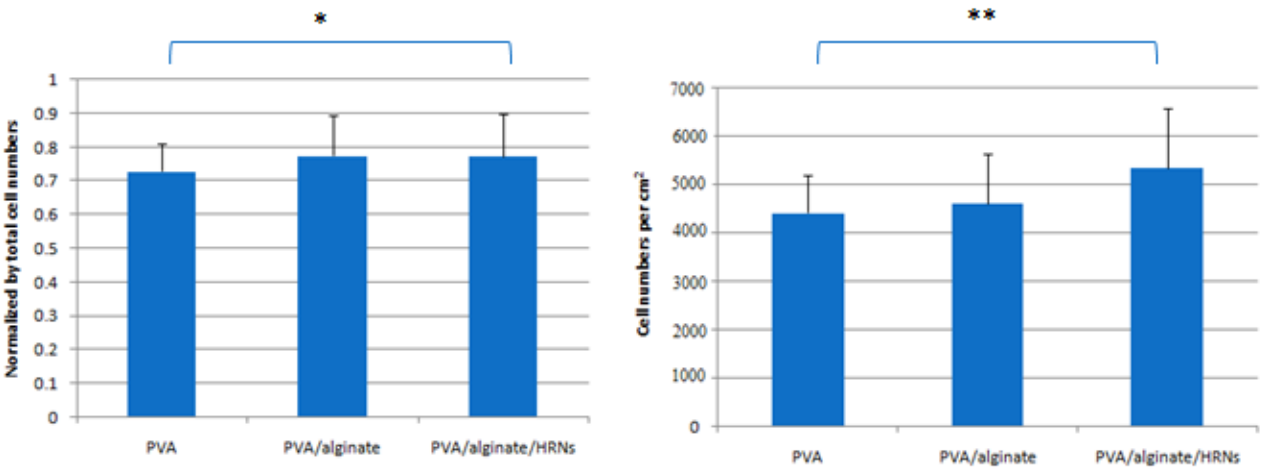

Figure 6. SFB cell viability (left) and adhesion (right) after 1 day culture. Data are mean ± SEM. *p>0.1 compared to PVA alone, *p<0.1 compared to PVA alone. HRNs increased cell numbers.

Discussion and Conclusions

Results of this study demonstrated that HRNs (novel, nanostructured self-assembled materials) increased the adhesion strength to collagen and can be directly electrospun with

synovial cells into a 3D fiber mesh at the site of surgery to increase cell adhesion. HRNs may accomplish this by increasing surface energy of hydrogel composites (as demonstrated by decreased contact angles) and provide for nanostructured surface features known to promote cell functions. HRNs appear to provide a supportive matrix for cartilage precursor cells and that they can be used to deliver drugs or growth factors useful in cartilage tissue engineering. Therefore, electrospun HRNs might be a useful scaffold for engineering a cartilage bio-composite for grafting into cartilage defects.

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