Biomaterials Science: Processing, Properties and Applications III.
Edited by Susmita Bose, Amit Bandyopadhyay and Roger Narayan.

BIOACTIVE ROSETTE NANOTUBE COMPOSITES FOR CARTILAGE APPLICATIONS

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ABSTRACT

With poor regenerative properties, articular cartilage has evoked intense studies to improve tissue repair and regeneration. However, traditional therapies such as using autografts and allografts remain problematic and challenging [1]. Nanotechnology is a promising technology to combine biocompatibility and bioactivity with mechanical properties for novel cartilage material development [2-5]. In this study, rosette nanotubes assembled by twin base linker molecules were used with poly(2-hydroxyethyl methacrylate) pHEMA for cartilage applications.

INTRODUCTION

In the United States, about 26% of orthopedic surgeons fix knee injuries which result from mechanical destruction, mechanical degeneration, or osteoarthritis [6,7]. Because articular cartilage is composed of an extracellular matrix and a small percentage of chondrocytes, once damaged, have a limited regeneration capability, poor self-healing results. Unlike other connective tissues, cartilage lacks a vascular system and progenitor cells [7]. Traditional methods have some disadvantages, such that autografts may affect donor-site morbidity or have limited cartilage availability, and allografts may trigger immune reactions. Currently, it is still a challenge to regenerate the damage tissue with a minimally invasive operation. Among new strategies, conventional materials with nanostructured features or novel nanomaterials are promising and can lead to rapid cartilage regeneration [2-6]. Rosette nanotubes (RNTs) composed of guanine^cytosine building blocks are one kind of self-assembled supermolecules that can be used a novel injectable cartilage healing material [8-11]. With the similar dimensions as collagen molecules, RNTs increase vitronectin and fibronection adsorption and subsequent cell adhesion and functions [12-14]. Moreover, RNTs can be functionalized with peptides, DNAs, siRNAs or anticancer drugs through the lysine chain on their surface or embedded in the hydrophobic tube center [15-17]. In this study, composites of twin based linker molecules (TBL) assembled into RNTs and poly(2-hydroxyethyl methacrylate) (pHEMA) were tested for cartilage applications. The addition of TBLs enhances the bioactivity of the composites thus establishing the potential of TBL/pHEMA composites as promising cartilage implant materials.

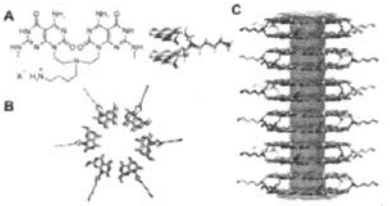

Figure 1 Schematic illustration of the self-assembling process of rosette nanotubes (RNTs) (C) from a twin G^C base (A) functionalized with an aminobutyl group (referred to as TBL or twin base linker) (B).

EXPERIMENTS

Synthesis of TBLs

TBL building blocks were synthesized according to our reported synthetic strategy [8-11], then they were dissolved in dH_2O to a final concentration of 4 mg/mL. This solution was sterilized by filtration through a 0.22 μm syringe filter.

Preparation of TBL/pHEMA/H_2O composites.

TBLs (0.01 mg/mL), initiator 2,2'-azobisisobutyronitrile (AIBN, 3 mg/mL, Sigma-Aldrich), 2-hydroxyethyl methacrylate (HEMA) monomer (5 mL, Polysciences, PA), and dH_2O were mixed to give 0, 10, 20 and 30 wt% HA/pHEMA solutions. Finally, the composites were heated in an oven at 60°C until the samples solidified completely. After polymerization, the TBL/pHEMA composites were sterilized by soaking in 70% ethanol for 20 min and exposed to ultraviolet (UV) light overnight before cell experiments.

Mechanical properties

The tensile properties of the composites were determined following the ASTM standard D638. For this, the TBL/pHEMA composite solution was placed into dog-bone shaped molds (3.18 mm in width, 4 mm in thickness and 7.62 mm in gauge length) and then into an oven (60°C, 2 h). An Instron 5882 mechanical testing system was used to determine the tensile properties of samples at a speed of 5 mm/min under dry conditions.

Cell adhesion and proliferation studies

To determine the adhesion and proliferation of chondrocytes, the cell proliferation assay (CellTiter 96, Promega) was used. Briefly, for cell adhesion, cells were seeded at 3,500 cells/cm^2 in standard cell culture media and were incubated for 4 hours. For the proliferation study, cells were seeded at 1500 cells/cm^2 for 1 day and 3 days. The dye solution was added to the cells after the end of the prescribed period for 4 h, then the stop solution was added and incubated overnight. A plate reader was used to test cell density.

Total protein synthesis

Chondrocytes were seeded at a seeding density of 10,000 cells/cm^2 onto the substrates. Cells were cultured for 3 and 5 days under standard cell culture conditions with chondrogenic medium. Total protein content in the cell lysates was measured using a commercial BCA™ Protein Assay Reagent Kit (Pierce Biotechnology) and following the manufacturer's instructions. Aliquots from the supernatants

of the protein-containing cell lysates (150 μl) were mixed with the reagent solutions and incubated at 37°C for 2 h. Optical absorbance was measured at 562 nm on a spectrophotometer (SpectraMax 340PC, Molecular Devices).

GAG synthesis

For chondrocyte differentiation studies, chondrocytes were seeded at a seeding density of 10,000 cells/cm^2 onto the substrates. Cells were cultured for 3 and 5 days under standard cell culture conditions with chondrogenic medium. Glycosaminoglycan (GAG) concentration was measured spectrophotometrically with a 1- 9- dimethylmethylene blue (DMMB) dye assay.

Statistical analysis

Numerical data were analyzed with a Student's t-test to make pair-wise comparisons. Statistical significance was considered at $p<0.05$.

RESULTS

TBLs and pHEMA composites were prepared and their mechanical and cytocompatibility properties were investigated. Tensile tests confirmed that when increasing H_2O concentration, the tensile strength of the composites decreased (Figure 2). There was no statistically significant difference between the tensile strengths of the 20% H_2O and 30% H_2O TBL/pHEMA composites. The tensile strength of the composites with 20% or 30% H_2O content was similar to cartilage tissue (5-20 MPa). In the cell adhesion study, chondrocytes adhered more to the TBL conjugated pHEMA composites than that on samples without TBLs (Figure 3). The cell number on the 0.01 mg/mL TBL/pHEMA with 20% H_2O composites was significantly greater than that of the non-TBL composites. The difference in chondrocyte adhesion between 20% H_2O and 30% H_2O was not statistically significant. In the proliferation study, TBLs effectively increased chondrocyte density after 3-days of culturing compared to formulations without TBL (Figure 4). Moreover, TBL/pHEMA composites stimulated chondrocytes to synthesize more protein and GAGs (Figure 5 and 6).

DISCUSSION

PHEMA-based scaffolds have several key attributes that make them promising for tissue regeneration, including biocompatibility and tunable mechanical properties. Most importantly, pHEMA-based materials have been approved for clinical use in a number of applications, including contact lenses, blood-contacting implants and drug delivery devices [17-19]. RNTs have a similar dimension as natural collagen in the cartilage extracellular matrix (about 1.5 nm in diameter and 300 nm in length) [8-11]. As well as their low toxicity, RNTs can be tailored for specific tissue engineering applications by functionalizing them with peptides or pharmaceutical agents. Such tailored chemistries can be placed covalently on the exterior of the RNTs or down the middle of the RNTs, allowing for prolonged drug release [15, 16]. The bioactive RNTs alone enhanced the initial adsorption of proteins (vitronectin and fibronectin) from blood and subsequent cell functions [12-16]. In this study, RNTs as an additive were mixed into pHEMA substrates to promote cartilage regeneration. As expected, the nanotube gel enhanced the total protein and GAG synthesis from chondrocytes (Figure 5 and 6). The cell function results observed here are consistent with previous studies from our group using this composite in cooperation with hydroxyapatite nanoparticles to enhance bone cell regeneration [20]. Since cartilage has limited blood flow in comparison to bone tissues, it is possible to treat the RNTs/pHEMA composites with the addition of growth factors to increase regeneration efficacy in the future.

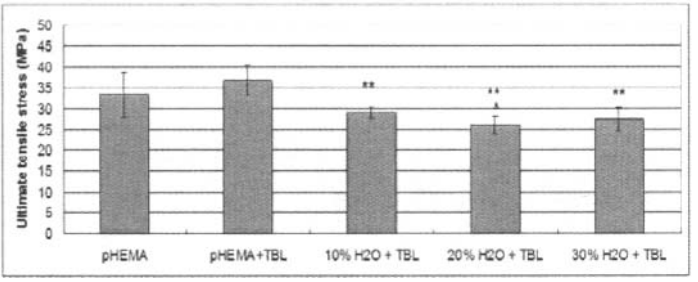

Figure 2. Ultimate tensile strength of TBL/HA/pHEMA composites. The Instron 5882 mechanical testing system was used to determine the tensile properties of samples at a speed of 5 mm/min under dry conditions. Data = Mean ± SEM, (N=3). (*) $p<0.05$ compared to pHEMA composites. (**) $p<0.05$ compared to TBL /pHEMA composites.

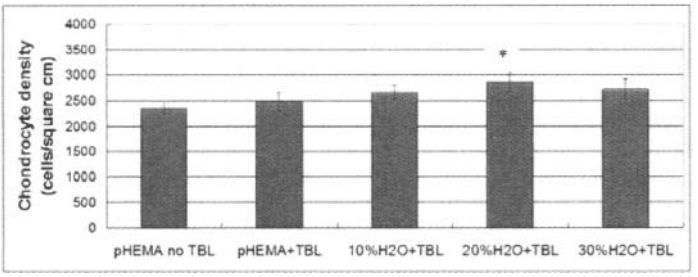

Figure 3. Chondrocyte cell adhesion on TBL/pHEMA composites for 4 h. Data = Mean ± SEM, (N=3). $p< 0.05$, compared to pHEMA no TBL samples. (*) $p<0.05$ compared to pHEMA without TBL composites.

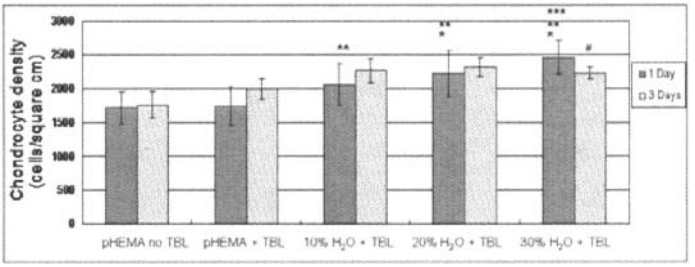

Figure 4. Chondrocyte density on pHEMA composites containing no TBL, TBL with 10%, 20%, or 30% H_2O after 1 and 3 day of culturing. All the composites contained TBLs (0.01 mg/ml). Values are mean ± SEM; n=3. (*) $p<0.05$ compared to pHEMA without TBL composites after 1 day of culturing. (**) $p<0.05$ compared to pHEMA with TBL composite after 1 day. (***) $p<0.05$ compared to pHEMA with TBL and 10% H_2O composite after 1 day. (#) $p<0.05$ compared to pHEMA without TBL composites after 3 days.

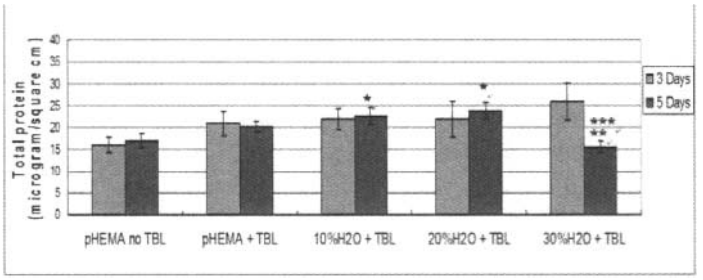

Figure 5. Chondrocyte functions of total protein synthesis after 3 and 5 day culturing. Values are mean ± SEM; n=3. (*) $p<0.05$ compared to pHEMA with TBL composite after 5 days. (**) $p<0.05$ compared to pHEMA with TBL composite after 5 days. (***) $p<0.05$ compared to pHEMA with TBL and 20% H_2O composite after 5 days.

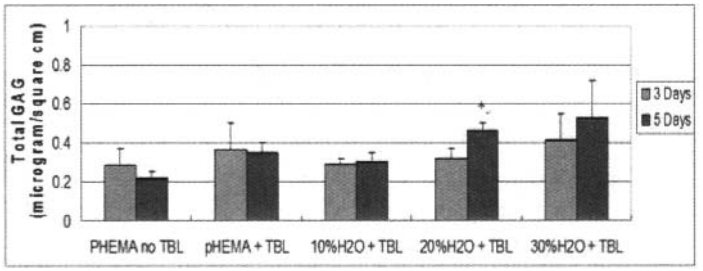

Figure 6. Chondrocyte functions of GAG synthesis after 3 and 5 day culturing. Values are mean ± SEM; n=2. (*) $p<0.05$ compared to pHEMA no TBL composite after 5 days.

CONCLUSIONS

The present study indicated that the tensile strengths of the composites were closer to those of cartilage tissue with 20% or 30% water. All composites containing TBLs had higher chondrocyte density than composites without TBLs. The composite with 20% H_2O had the highest cell density among these five groups. In particular, TBL/pHEMA/20%H_2O composites had the highest chondrocyte adhesion density after 3 days of culturing. The addition of TBLs increased chondrocyte differentiation including total protein and GAG synthesis. Therefore, TBL were effective at increasing the bioactivity of the composites, and TBL/pHEMA composites are promising as cartilage implant materials.

ACKNOWLEDGEMENTS

The authors acknowledge Audax Medical, Inc. for financial assistance, Canada's Natural Science and Engineering Research Council, Canada's National Research Council, and the University of Alberta.

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