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Self-assembled Rosette Nanotubes for Tissue Engineering Applications

Linlin Sun,[1] and Thomas J. Webster[1*]

Abstract—**Self-assembled supermolecules have been studied intensively in the last decades. Interacting with nature tissues at a subcellular scale, rosette nanotubes have shown promising properties for various applications in tissue engineering. The purpose of this study was to design, fabricate and test the ability of biomimetic rosette nanotubes for the regeneration of cartilage and skin tissues.**

Keywords-Rosette nanotubes; cartilage; skin regeneration; chondrocytes; keratinocytes; fibroblasts

I. INTRODUCTION

Nanotechnology approaches could be used to facilitate the design of injectable, biocompatible, biodegradable materials for tissue engineering applications. Furthermore, optimal nano-modified surfaces or nanostructures can be functionalized with growth factors, cytokines or peptides, which further enable to accelerate the regeneration process as well as inhibit scar formation [1,2].

Self-assembled synthetic materials formed by noncovalent bonds have raised more and more interests for tissue regeneration. With the similarity of natural molecules, self-assembled supermolecules by bottom-up approaches can form nanostructures with novel functionalities. Twin-based linker (TBL) molecules self-assemble into rosette nanotubes (RNTs) with hydrophilic lysine chain displayed outside and form hydrophobic inner in the tube center. This nanotube structure is stable in aqueous environment and has 3.4 nm in diameter and microns in length [3-5]. In our previous report, they are good scaffold to mimic the geometrical interaction between hydroxyapatite crystals and collagen fibrils in bone. Also, RNTs have shown biomimetic functions and versatile delivery capabilities [6-9]. In this study, one type of rosette nanotubes (termed twin-based linker (TBL) (Figure 1)) and poly(2-hydroxyethyl methacrylate) (pHEMA) were used to generate biocompatible, bioactive, and injectable composites for cartilage and skin applications.

II. EXPERIMENTS

A. Synthesis of TBLs

TBLs were synthesized according to previously reported methods [3, 4]. For cell studies, TBLs was 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.

B. 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 60ºC 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.

C. 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). 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.

D. Cell adhesion and proliferation studies

The adhesion and proliferation of chondrocytes (ATCC, CRL-2846), keratinocytes (Invitrogen, C0015C) and skin fibroblasts (ATCC, CCL-110) were tested by the cell proliferation assay (CellTiter 96, Promega). For cell adhesion, cell seeding density was 3,500 cells/cm^2. cells were cultured in standard cell culture media and incubated for 4 hours. For proliferation studies, cells were seeded at 3500 cells/cm^2 for 1, 3 and 5 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.

E. 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).

F. GAG synthesis

For chondrocyte differentiation studies, chondrocytes were seeded at a seeding density of 10,000 cells/cm^2 onto the

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L. Sun is with the Bioengineering Program and Department of Chemical Engineering, College of Engineering, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 USA (e-mail: sun.linl@husky.neu.edu)

T. J. Webster is with the Bioengineering Program and Department of Chemical Engineering, College of Engineering, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 USA (*Corresponding author: phone: 617.373.6585; e-mail: th.webster@neu.edu)

substrates. Cells were cultured for 3 and 5 days under standard cell culture conditions with chondrogenic medium. Glycosaminoglycan (GAG) concentration was measured spectrophotometrically by a 1- 9- dimethylmethylene blue (DMMB) dye assay.

G. Statistical analysis

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

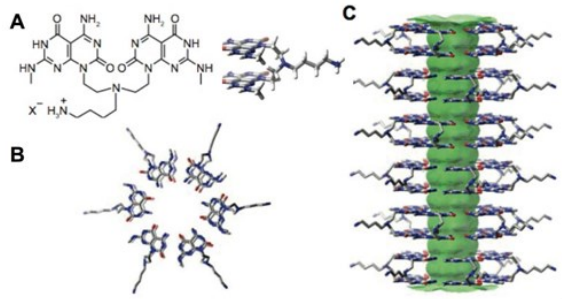

Figure 1 Schematic illustration of the self-assembling process of rosette nanotubes (RNTs) (C) from a twin G^C base (A) functionalized with an aminobutyl group (referred to as TBL or twin base linker) (B).

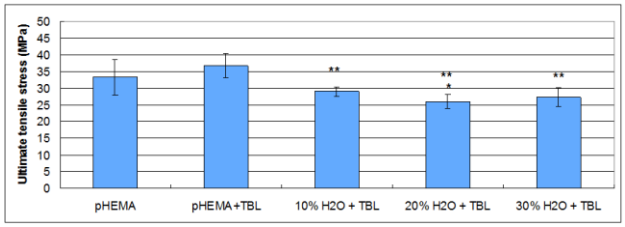

Figure 2. Ultimate tensile strength of TBL/HA/pHEMA composites. The Instron 5882 mechanical testing system was used to determine the tensile properties of samples at a speed of 5 mm/min under dry conditions. Data = Mean ± SEM, (N=3). (*) $p<0.05$ compared to pHEMA composites. (**) $p<0.05$ compared to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

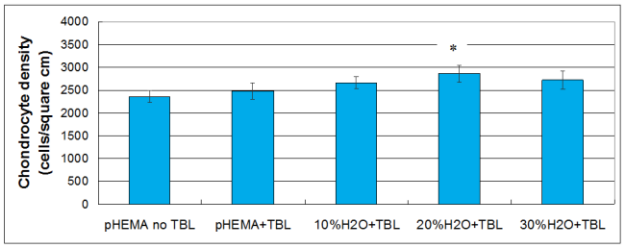

Figure 3. Chondrocyte cell adhesion on TBL/pHEMA composites for 4 h. Data = Mean ± SEM, (N=3). P< 0.05, compared to pHEMA no TBL samples. (*) $p<0.05$ compared to pHEMA without TBL composites.

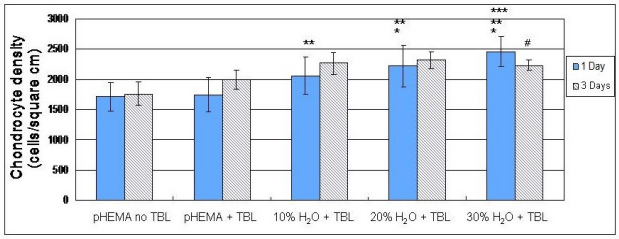

Figure 4. Chondrocyte density on pHEMA composites containing no TBL, TBL with 10%, 20%, or 30% H_2O after 1 and 3 day of culturing. All the composites contained TBLs (0.01 mg/ml). Values are mean ± SEM; n=3. (*) $p<0.05$ compared to pHEMA without TBL composites after 1 day of culturing. (**) $p<0.05$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

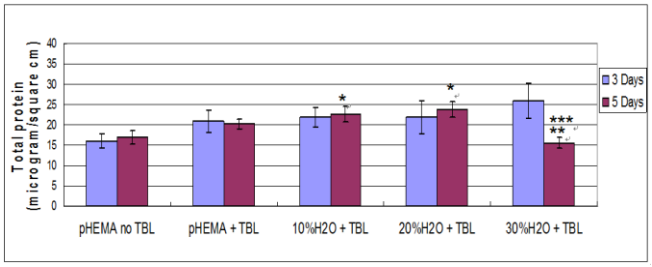

Figure 5. Chondrocyte functions of total protein synthesis after 3 and 5 day culturing. Values are mean ± SEM; n=3. (*) $p<0.05$ compared to pHEMA with TBL composite after 5 days. (**) $p<0.05$ compared to pHEMA with TBL composite after 5 days. (***) $p<0.05$ compared to pHEMA with TBL and 20% H_2O composite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

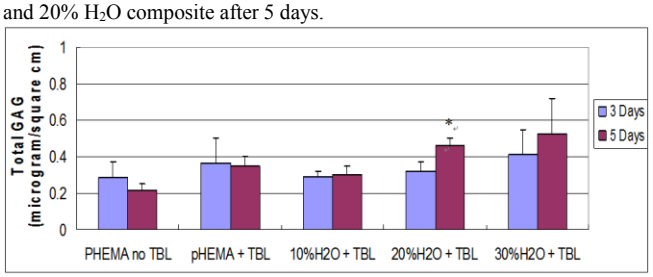

Figure 6. Chondrocyte functions of GAG synthesis after 3 and 5 day culturing. Values are mean ± SEM; n=2. (*) $p<0.05$ compared to pHEMA no TBL composite after 5 days.

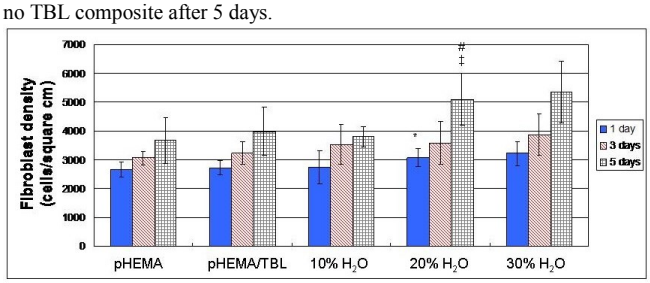

Figure 7. Fibroblast cell density on pHEMA composites after 1, 3, and 5 days of culturing. Cell seeding density was 3500 cells/cm^2. Cell density was indicated as the average number of cells/cm^2 on sample surface. Data = Mean ± STDEV, (N=1). (*) $p<0.05$ compared to pHEMA + TBL composites after 1 day. (‡) $p<0.05$ compared to pHEMA no TBL composites after 5 days. (#) $p<0.05$ compared to 10 % H_2O + TBL pHEMA composites after 5 days.

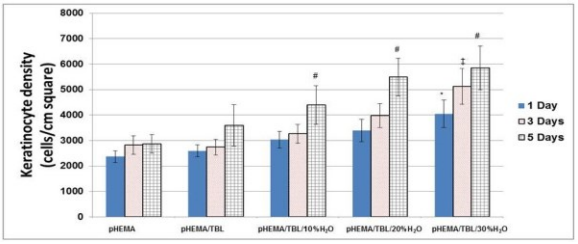

Figure 8. Keratinocyte cell density on pHEMA composites after 1, 3, and 5 days of culturing. Cell seeding density was 3500 cells/cm^2. Cell density was indicated as the average number of cells/cm^2 on the sample surface. Data = Mean ± STDEV, (N=3). (*) $p<0.05$ compared to pHEMA for 1 day. (‡) $p<0.05$ compared to pHEMA for 3 days. (#) $p<0.05$ compared to pHEMA for 5 days.

III. RESULTS AND DISCUSSION

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 composites decreased. There is no statistically significant difference between the tensile strengths of 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 skin tissue (5-30 MPa) [10]. In the cell adhesion study, chondrocytes, keratinocytes and fibroblasts adhered more to the TBL conjugated pHEMA composites than that on samples without TBLs (Fig. 3). The difference in both keratinocyte and fibroblast adhesion between 20% H_2O and 30% H_2O was not statistically significant. In proliferation study, TBLs effectively increased chondrocytes, keratinocyte and fibroblast density after 5-day culturing compared to formulations without TBLs (Fig. 4, 7, 8). Moreover, TBL/pHEMA composites stimulated chondrocytes to synthesize more protein and GAGs (Fig. 5 and 6).

IV. CONCLUSION

The aim of the present study was to develop biocompatible biomaterials based on TBL and pHEMA composites for tissue regeneration applications. The characterization of the TBL/pHEMA hydrogels revealed that due to their tunable mechanical properties and biocompatibility, they show promise for the utilization in the biomedical field.

ACKNOWLEDGEMENTS

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