Journal of Visualized Experiments

Synthesis of Biocompatible Liquid Crystal Elastomer Foams as Cell Scaffolds for 3D Spatial Cell Cultures

--Manuscript Draft--

Manuscript Number:	JoVE55452R4
Full Title:	Synthesis of Biocompatible Liquid Crystal Elastomer Foams as Cell Scaffolds for 3D Spatial Cell Cultures
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Liquid Crystal Elastomers; 3D Porous Scaffolds; Cell Scaffolds; Cell Alignment; Cell Directionality; Biocompatible
Manuscript Classifications:	10.1.637.51.720: Polymers; 4.25: Biomedical and Dental Materials; 4.25.720: Polymers; 4.25.720.99: Biopolymers; 4.5: Macromolecular Substances; 4.5.750: Polymers; 4.5.750.78: Biopolymers; 92.23: Chemistry and Materials (General); 92.27: Nonmetallic Materials; 93.31: Engineering (General)
Corresponding Author:	Elda Hegmann Kent State University Kent, Ohio UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	ehegmann@kent.edu
Corresponding Author's Institution:	Kent State University
Corresponding Author's Secondary Institution:	
First Author:	Elda Hegmann
First Author Secondary Information:	
Other Authors:	Marianne E. Prévôt
	Senay Ustunel
	Leah E. Bergquist
	Cukelj Richard
	Gao Yunxiang
	Taizo Mori
	Lindsay Pauline
	Clements J. Robert
Order of Authors Secondary Information:	
Abstract:	We are presenting a step-by-step preparation of a 3D biodegradable foam-like cell scaffold. These scaffolds were prepared by cross-linking of star block-co-polymers featuring cholesterol units, as side-chain pendant groups, resulting in smectic-A (SmA) LCEs. Foam-like scaffolds, prepared using metal templates, feature interconnected microchannels making them suitable as 3D cell culture scaffolds. The combined properties of a regular structure of the metal foams and the elastomer properties results in a 3D cell scaffold that not only promotes higher cell proliferation compared to conventional porous templated films, but also better management of mass transport (i.e. nutrients, gases, waste). The nature of the metal template allows for easy manipulation of foam shapes (i.e. rolls or films), as well as the preparation of scaffolds of different pore sizes, for different cell studies while preserving the interconnected

	pore nature of the template. The etching process does not affect the chemistry of the elastomers, preserving its biocompatible and biodegradable nature. We are presenting here that these smectic LCEs enable the study of clinically relevant and complex tissue constructs while promoting growth, and proliferation of cells when grown on these SmA LCEs for extensive time periods.
Author Comments:	We are attaching a movie clip that we would like to incorporate during the film process, if our paper were to be accepted for publication.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

Cover Letter

KENT STATE UNIVERSITY

Benjamin Werth – Science Editor
Journal of Visualized Experiments (JoVE)

September 6, 2016

It is with pleasure that we submit the following manuscript "**Synthesis of Biocompatible Liquid Crystal Elastomer Foams as Cell Scaffolds for 3D Spatial Cell Cultures**" to be considered for publication in *Journal of Visualized Experiments*.

Liquid crystals (LCs) have been experiencing a renaissance within soft matter research, away from the established areas of liquid crystal displays, actuators and optical devices LCs are anticipated to one up an new area, LC bioscience, and to play a major role over the next decade. We believe that the unique properties of new LC materials will continue to attract tremendous interest in LC elastomers aside from actuation, lasing and sensing applications.

We are presenting here a complete a step-by step-protocol for the preparation of foam-like biocompatible, biodegradable liquid crystal elastomers (LCEs) based on our initial proof-of-concept materials, where special focus is placed on tunable porosity and its impact on cell response. This manuscript highlights the preparation method outlined on our ACS Macro Lett. 2016, 5, 4–9 manuscript following slight modifications from the synthesis and preparation published previously, also by us, in Macromolecular Bioscience (*Macromol. Biosci.* **2015**, *15*, 200-214 (featured on *Materials Views*, Link). This methology allows for better design of 3D scaffolds with a more regular fully interconnected porosity, providing a more dynamic and realistic 3D environment for cell growth that can potentially mimic native architectures and better spatially cell-cell interaction.

We believe that our methodology presented here is quite noteworthy and will resonate with the soft matter, materials science and biomaterials communities. This work is a true multi-disciplinary effort with experts from various disciplines highlighting the nature of materials chemistry at the interface to biology.

Gao, Clements and Hegmann designed the procedures described in the manuscript. Gao, Ustunel, Bergquist and Prévôt performed the synthetic experiments and analyzed the synthetic data. Cukelj and Mori conducted all cell experiments and confocal data analysis, supervised by Clements. Finally, Hegmann wrote the manuscript with contributions from all authors.

We trust that your team and the readership of JoVE will find this manuscript interesting and worthy of publication in your journal.

Sincerely yours,

Elda Hegmann
Assistant Professor – Liquid Crystal Institute, and Department of Biological Sciences, Kent State University, Kent (OH) USA

Liquid Crystal Institute
Chemical Physics Interdisciplinary Program
P.O. Box 5190 • Kent, Ohio 44242-0001
330-672-2654 • Telefax: 330-672-2796 • E-mail: mail@lci.kent.edu • www.lci.kent.edu

TITLE:
Synthesis of Biocompatible Liquid Crystal Elastomer Foams as Cell Scaffolds for 3D Spatial Cell Cultures

AUTHORS:
Prévôt, Marianne E.
Liquid Crystal Institute
Kent State University
Kent, USA
mprevot1@kent.edu

Ustunel, Senay
Liquid Crystal Institute
Kent State University
Kent, USA
ustunelsenay@gmail.com

Bergquist, Leah E.
Chemical Physics Interdisciplinary Program
Liquid Crystal Institute
Kent State University
Kent, USA
lbergqu1@kent.edu

Cukelj, Richard
Department of Biological Sciences
Kent State University
Kent, USA
rcukelj@kent.edu

Gao, Yunxiang
Liquid Crystal Institute
Kent State University
Kent, USA
yunxiang.gao@gmail.com

Mori, Taizo
Liquid Crystal Institute
Kent State University
Kent, USA
taizo0mori@gmail.com

Pauline, Lindsay
Department of Biological Sciences
Kent State University
Kent, USA

lpaulin2@kent.edu

Clements, Robert J.
Department of Biological Sciences
Kent State University
Kent, USA
rclement@kent.edu

Hegmann, Elda
Liquid Crystal Institute
Kent State University
Kent, USA
ehegmann@kent.edu

CORRESPONDING AUTHOR:
Elda Hegmann

KEYWORDS:
Liquid Crystal Elastomers, 3D Porous Scaffolds, Cell Scaffolds, Cell Alignment, Cell Directionality, Biocompatible

SHORT ABSTRACT:
This study presents a methodology to prepare 3D, biodegradable, foam-like cell scaffolds based on biocompatible side-chain liquid crystal elastomers (LCEs). Confocal microscopy experiments show that foam-like LCEs allow for cell attachment, proliferation, and the spontaneous alignment of C2C12s myoblasts.

LONG ABSTRACT:
Here, we present a step-by-step preparation of a 3D, biodegradable, foam-like cell scaffold. These scaffolds were prepared by cross-linking star block co-polymers featuring cholesterol units as side-chain pendant groups, resulting in smectic-A (SmA) liquid crystal elastomers (LCEs). Foam-like scaffolds, prepared using metal templates, feature interconnected microchannels, making them suitable as 3D cell culture scaffolds. The combined properties of the regular structure of the metal foam and of the elastomer result in a 3D cell scaffold that promotes not only higher cell proliferation compared to conventional porous templated films, but also better management of mass transport (*i.e.*, nutrients, gases, waste, etc.). The nature of the metal template allows for the easy manipulation of foam shapes (*i.e.*, rolls or films) and for the preparation of scaffolds of different pore sizes for different cell studies while preserving the interconnected porous nature of the template. The etching process does not affect the chemistry of the elastomers, preserving their biocompatible and biodegradable nature. We show that these smectic LCEs, when grown for extensive time periods, enable the study of clinically relevant and complex tissue constructs while promoting the growth and proliferation of cells.

INTRODUCTION:
There are several examples of biological and biocompatible synthetic materials designed for application in cell studies and for tissue regeneration aiming at cell attachment and proliferation[1-

[5]. There have been a few examples of biocompatible materials, known as liquid crystal elastomers (LCEs), that could respond to external stimuli with anisotropic molecular ordering[6,7]. LCEs are stimuli-responsive materials that combine the mechanical and elastic properties of elastomers with the optical functionality and molecular ordering of liquid crystals[8,9]. LCEs can experience changes in shape, mechanical deformation, elastic behavior, and optical properties in response to external stimuli (*i.e.*, heat, stress, light, etc.)[10-16]. Earlier studies have shown that liquid crystals (LCs) can sense the growth and orientation of cells[4,17]. It is possible then to assume that LCEs may be suitable for biologically and medically relevant applications, including cell scaffolding and alignment. We have previously reported the preparation of smectic biocompatible, biodegradable, cast-molded, and thin LCEs films featuring a "Swiss-cheese type" porous morphology[6,18]. We also prepared nematic biocompatible LCEs with globular morphology as scaffolds for cell growth[19,20]. Our work was aimed at tuning the mechanical properties of the materials to match those of the tissue of interest[21]. Also, these studies focus on understanding elastomer-cell interactions, as well as cellular response when the elastomers are subject to external stimuli.

The main challenges were in part to tailor the porosity of the LCEs to allow for cell attachment and permeation through the elastomer matrix and for better mass transport. The porosity of these thin films[6] allowed for cell permeation through the bulk of the matrix, but not all pores were fully interconnected or had a more regular (homogeneous) pore size. We then reported on biocompatible nematic LCE elastomers with globular morphologies. These nematic elastomers allowed for the attachment and proliferation of cells, but the pore size ranged only from 10-30 μm, which prevented or limited the use of these elastomers with a wider variety of cell lines[19,20].

Previous work by Kung *et al.* relating to the formation of graphene foams using a "sacrificial" metal template showed that the obtained graphene foam had a very regular porous morphology dictated by the chosen metal template[22]. This methodology offers full control of porosity and pore size. At the same time, the malleability and flexibility of the metal template allow for the formation of different template shapes prior to foam preparation. Other techniques, such as material leaching23, gas templating[24], or electro-spun fibers[25,26] also offer the potential for the preparation of porous materials, but they are more time consuming and, in some cases, the pore size is limited to only a few micrometers. Foam-like 3D LCEs prepared using metal templates allow for a higher cell load; an improved proliferation rate; co-culturing; and, last but not least, better mass transport management (*i.e.*, nutrients, gases, and waste) to ensure full tissue development[27]. Foam-like 3D LCEs also appear to improve cell alignment; this is most likely in relation to the LC pendants sensing cell growth and cell orientation. The presence of LC moieties within the LCE appears to enhance cell alignment with respect to cell location within the LCE scaffold. Cells align within the struts of the LCE, while no clear orientation is observed where the struts join together (junctions)[27].

Overall, our LCE cell scaffold platform as a cell support medium offers opportunities to tune the elastomer morphology and elastic properties and to specifically direct the alignment of (individual) cell types to create an ordered, spatial arrangements of cells similar to living systems. Apart from providing a scaffold capable of sustaining and directing long-term cellular growth and proliferation, LCEs also allow for dynamic experiments, where cell orientation and interactions may be modified on the fly.

PROTOCOL:

Note: The following steps for the 3D LCE foam-like preparation using the 3-arm star block copolymer are shown in Figure 1. For nuclear magnetic resonance (NMR) characterization, the spectra are recorded in deuterated chloroform ($CDCl_3$) at room temperature on a Bruker DMX 400-MHz instrument and internally referenced residual peaks at 7.26. Fourier transform infrared (FT-IR) spectra are recorded using a Bruker Vector 33 FT-IR spectrometer using attenuated total reflectance mode. For each step of the following protocol, it is important to wear appropriate personal protective clothing (PPE).

1. Synthesis of α-chloro-ε-caprolactone (monomer) (according to the procedure in Jérôme *et al.*[28]); Step P1.

1.1. Before beginning the synthesis, purify 27 g of 3-chloroperbenzoic acid as follows:

1.1.1. In 800 mL of distilled water, add 1.28 g of sodium phosphate monobasic monohydrate and 8.24 g of sodium phosphate dibasic heptahydrate. Adjust the pH to 7.4 (using sodium hydroxide or hydrochloric acid) and reserve 30 mL of this solution; this is the buffer solution.

1.1.2. Using a separatory funnel, dissolve 3-chloroperbenzoic acid in 35 mL of diethyl ether. Wash the organic solution with 10 mL of buffer solution (prepared in step 1.1.1.). Repeat the wash three times.

1.1.3. Add 3 g of sodium sulfate directly to the organic solution; this drying agent absorbs water from the organic solution.

1.1.4. Filter the solution to remove the drying agent. Concentrate the filtrate under reduced pressure by using a rotary evaporator at 850 mbar and 40 °C.

1.2. Solubilize 18.5 g of purified 3-chloroperbenzoic acid in 150 mL of dry dichloromethane by stirring in an ultrasonic bath; this process typically takes 20 min. Place the solution inside a separatory funnel.

1.3. Inside a two-neck, round-bottom flask, dissolve 13.1 g of 2-chlorocyclohexanone in 15 mL of dry dichloromethane using a magnetic stirrer under nitrogen gas. Keep stirring.

1.4. Fit the separatory funnel containing 3-chloroperbenzoic acid solution (from step 1.2) to the two-neck flask in step 1.3. Flush the system with nitrogen gas. Adjust the opening of the separatory funnel so that the chloroperbenzoic acid solution falls dropwise into the 2-chlorocyclohexanone solution (1 drop every other second) and continue stirring the mixture under nitrogen gas for 96 h.

1.5. Cool the reaction mixture to -20 °C for 1 h to precipitate the *m*-chlorobenzoic acid (*m*-CBA) byproduct.

1.6. Filter the *m*-chlorobenzoic acid (*m*-CBA) and wash the remaining solution with saturated solutions of sodium thiosulfate, sodium bicarbonate, and sodium chloride.

1.7. Remove the solvent under reduced pressure using a rotary evaporator at 850 mbar and 40 °C. Purify the pale yellow, viscous liquid by distillation under reduced pressure at 2.3 Torr and 96 °C.

1.8. Monitor the success of the synthesis using the following ^{1}H NMR peaks. ^{1}H NMR (400 MHz, $CDCl_3$, δ[ppm]): 4.75-4.68 (m, 1H, C*H*ClCO), 4.37-4.26 (m, 1H, CH_2O), 4.18-4.05 (m, 1H, CH_2O), and 2.06-1.58 (m, 6H,−CH_2−)[6,27].

2. Synthesis of α-three-arm star block copolymer (SBC-αCl) by ring opening copolymerization (Sharma *et al.*[6] and Amsden *et al.*[29]); Step P2.

2.1. Before synthesis, silanize a 20-mL ampoule by filling it with a 2% (v/v) solution of 1H,1H, 2H,2H-perfluorooctyltriethoxysilane in toluene and stirring for about 24 h. Rinse with isopropyl alcohol and dry it by placing it in an oven at 140 °C for 30 min.

2.2. Add 3.64 g of ε-caprolactone, 0.5 g of α-chloro-ε-caprolactone, and 0.25 mL of glycerol to the ampoule. Mix using a vortex for 1 min.

2.3. Add 4.90 g of *D*,*L*-Lactide to the ampoule and purge it with nitrogen. Place the ampoule in an oven at 120 °C to melt the *D*,*L*-Lactide; this process typically takes about 2 h. Mix again using a vortex to make sure that all contents are well mixed and add 66 µL of tin(II)2-ethylhexanoate ($Sn(Oct)_2$) to the ampoule.

Note: *D*,*L*-Lactide will cool down during this process and will need to be re-heated in the oven to melt.

2.4. Vigorously mix one last time using the vortex and flush with nitrogen.

2.5. Close the ampoule with a rubber stopper. Place a needle connected to a vacuum tube (the house vacuum is usually enough) through the rubber stopper. Turn on the vacuum and, using a flame, melt the long neck of the glass, twisting slowly until the glass collapses on itself. Be careful to not melt the rubber stopper. Once the ampoule is flame-sealed, place it in a sand bath or an oven at 140 °C for 48 h.

2.6. Take out the ampoule and let it cool at room temperature.

2.7. Break the ampoule at the sealed mark and dissolve the highly viscous liquid by adding 10 mL of dichloromethane. Transfer the solution to a separatory funnel.

2.8. Prepare a flask containing 100 mL of cold methanol (chilled using a dry ice/acetone bath at a temperature around -78 °C). Fix the separatory funnel (step 2.7) on top of the flask. Adjust the opening of the separatory funnel so that a drop falls every other second (dropwise).

2.9. Collect the white precipitate by filtration (using a paper filter) and dry it in a vacuum oven at 50 °C.

2.10. Monitor the success of the synthesis using the following ^{1}H NMR and FT-IR peaks. ^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 5.29-5.03 (m, COC*H*CH_3), 4.43-4.25 (m, C*H*Cl), 4.24-4.12

(m, C*H*$_2$O), 4.11-4.03 (t, *J* = 4.6 Hz, C*H*$_2$O), 3.80-3.68 (m, C*H*C*H*$_2$), 3.09-2.64 (broad, s, O*H*), 2.39 (t, *J* = 4.5 Hz, α– *H*), 2.33 (t, *J* = 5.1 Hz, α-*H*), and 1.77-1.25 (m, C*H*$_2$, C*H*$_3$); FT-IR (1/λ [cm^{-1}]): 2 932 (s), 2 869 (m), 1 741 (s), 961 (s), 866, and 735 (m)[6,27].

2.11. To prepare a more hydrophilic 3D LCE, prepare a linear block copolymer (LBC) instead of an SBC, following the ring opening copolymerization (ROP) procedure described above.

2.11.1 Add 0.3 g of polyethylene glycol (PEG), 3.15 g ε-caprolactone, 1.0 g of α-chloro-ε-caprolactone, and 5.0 g of *D*,*L*-Lactide to the silanized vial mix.

3. Synthetic modification of α-Cl-Three arm SBC to α-N$_3$-Three Arm SBC (SBC-αN$_3$) (according to Sharma *et al.*[6]); Step P3.

3.1. In a round-bottom flask and under nitrogen, dissolve 5 g of SBC-αCl in 30 mL of dry N,N'-dimethylformamide.

3.2. Add 0.22 g of sodium azide and allow to react overnight at room temperature.

Caution: Sodium azide is toxic; wear appropriate personal protective clothing (PPE).

3.3. Remove the N,N'-dimethylformamide under reduced pressure using a rotary evaporator at 11 mbar and 40 °C. Dissolve the mixture in 30 mL of toluene. Centrifuge the solution thrice at 2,800 x *g* for 15 min to remove the salt formed. Evaporate the toluene under reduced pressure using a rotary evaporator at 77 mbar and 40 °C.

3.4. Monitor the success of the azide substitution using ^{1}H NMR and FT-IR peaks.

Note: ^{1}H NMR spectrum was similar to the parent SBC-αCl, with the exception of the appearance of a peak at 3.90 ppm related to the azide group; FT-IR (1/λ [cm^{-1}]): 2,928, 2,108 (s, azide), 1,754 (s), 1,450 (s), 960 (m), 865 (s), 733 (s), and 696 (m).

4. Synthesis of Cholesteryl 5-hexynoate (LC moiety) (according to Sharma *et al.*[6] and Donaldson *et al.*[30]); Step P4.

4.1. In a round-bottom flask, mix 3 g of 5-hexynoic acid and 130 mL of dichloromethane. Cool to 0 °C using an ice bath.

4.2. In another round-bottom flask, mix 8.28 g of N,N'-dicyclohexylcarbodiimide, 10.3 g of cholesterol, and 0.2 g of 4-dimethylaminopyridine.

4.3. Transfer the 5-hexynoic acid solution dropwise to the flask containing the cholesterol mixture and maintain the final mixture at 0 °C for 1 h.

4.4. Allow the mixture to warm up to room temperature overnight.

4.5. Remove the resulting dicyclohexylurea precipitate by filtration using a grade 415 paper filter and discard it.

4.6. Concentrate the filtrate under reduced pressure using a rotary evaporator at 850 mbar and

40 °C. Dissolve the collected residue in 150 mL of hexane.

4.7. Evaporate the solvent under reduced pressure using a rotary evaporator at 335 mbar and 40 °C. Add 350 mL of ethanol to the oily residue to collect the final product. Wash the off-white solid formed with ethanol and dry the solid product under vacuum at 50 °C.

4.8. Monitor the synthesis using the following ^{1}H NMR and FT-IR peaks. ^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 5.39 (d, *J* = 4.7 Hz, 1H, C*H*=C), 4.70-4.58 (m, 1H, C*H*OCO), 2.44 (t, *J* = 2.5 Hz, 2H, CH_2CO), 2.34 (m, 2H, CH_2-C*H*=), 2.31 (m, 2H, CH_2CH_2-COO), 2.28 (s, 1H, *H*C≡C), 2.27 (d, *J* = 2.3 Hz, 2H, ≡C-CH_2), 2.07-1.06 (m, 2H, CH_2, C*H*), 0.94 (s, 3H, CH_3), 0.89 (d, *J* = 1.8 Hz, 3H, CH_3C*H*), and 0.88 (d, *J* = 1.8 Hz, 6H, CH_3-C*H*), 0.67 (s, 3H, CH_3). FT-IR (1/λ [cm^{-1}]): 2830-2990 (broad and strong peak), 2,104 (m), 1695 (s), 1,428 (m), 1,135 (m), 999(s), 798 (s), and 667 (s).

5. Synthetic modification of α-N_3-Three Arm SBC to α-Cholesteryl-Three Arm SBC (SBC-αCLC) via an Azide-Alkyne Huisgen Cyclo-Addition Reaction ("click" reaction) to obtain SBC-Chol (according to Sharma *et al.*[6]); Step P5.

5.1. In a round-bottom flask, dissolve 1 molar equivalent of SBC-αN_3 (1.5 g) in 100 mL of freshly distilled tetrahydrofuran (THF). Add 1.2 molar equivalent of cholesteryl 5-hexynoate (1.94 g), 0.1 molar equivalent of copper(I)iodide (0.06 g), and 0.1 molar equivalent of triethylamine (0.03 g). Stir the mixture overnight at 35 °C under nitrogen.

5.2. Evaporate the solvent under reduced pressure using a rotary evaporator at 357 mbar and 40 °C.

5.3. Dissolve the residual mixture in 80 mL of dichloromethane and centrifuge for 5 min at 2,800 x *g* at room temperature to remove unreacted materials and side products.

5.4. Monitor the synthesis using the following ^{1}H NMR and FT-IR peaks. ^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 7.54 (s, CH=C-triazole), 5.43-5.34 (m, C=CH cholesterol), 5.10-5.06 (m, $COCHCH_3$), 4.68-4.55 (m, O-CH cholesterol), 4.24-4.19 (m, CH_2O), 4.18-4.13 (t, *J*=5.0 Hz, CH_2O), 4.11-4.05 (t, *J*=4.4 Hz, CH_2O), 2.47-2.41 (t, *J*=4.9 Hz, $COCH_2$), 2.31-2.25 (m, $COCH_2$), 2.07-1.02 (m, CH_2, CH_3), 1.05–1.03 (s, CH_2, CH_3), 0.96-0.92 (d, *J*=3.3 Hz, CH_2, CH_3), 0.91-0.87 (dd, *J*=1.9 Hz, J=1.8 Hz, CH_3), and 0.71-0.68 (s, CH_3). FT-IR (1/λ [cm^{-1}]): 3 260 (s), 2 920 (s), 1 710 (s), 1 460 (s), 1 370 (s), 1 240 (m), 1 190 (s), 733 (s), and 668 (s).

6. Synthesis of 2,2-bis(1-caprolactone-4-yl)propane (crosslinker, BCP) (according to Gao *et al.*[27] and Albertsson *et al.*[31]); Step P6.

6.1. In a round-bottom flask, prepare a solution containing 10.8 g of 2-bis(4-hydroxy-cyclohexyl)propane and 52 mL of acetic acid.

6.2. Prepare a solution containing 11 g of chromium trioxide in 50 mL of acetic acid and 80 mL of distilled water. Add this solution dropwise to the solution prepared in step 6.1 while maintaining the mixture temperature between 17 and 20 °C (*e.g.*, in a water bath); this dropwise process takes 2 h. Once the process is complete, allow the solution to stir for about 30 min.

6.3. Add 50 mL of 2-propanol. Stir the solution overnight at room temperature.

6.4. Concentrate the dark purple solution under reduced pressure using a rotary evaporator at 137 mbar and 40 °C. Add 300 mL of distilled water to precipitate; the precipitate should be light purple.

6.5. Filtrate the crude product using a grade 415 paper filter. Wash the solid material with ~ 250 mL of distilled water or until the solid becomes white.

6.6. Dissolve the solid material in 15 mL of benzene at 40 °C and let it recrystallize at 25 °C.

6.7. Add 8.34 g of dry diketone dissolved in dry dichloromethane and a solution containing 6.0 g of 3-chloroperbenzoic acid in 75 mL of dichloromethane to the flask.

6.8. Reflux the solution at 40 °C for 24 h.

6.9. Cool the reaction mixture to -20 °C for 10 min to precipitate the *m*-chlorobenzoic acid byproduct.

6.10. Remove *m*-chlorobenzoic acid by filtration (using a paper filter) and concentrate the solution under reduced pressure.

6.11. Wash the viscose crude product with 200 mL of 2-heptanone and dry the precipitate under vacuum at 50 °C overnight.

6.12. Monitor the synthesis using the following ^{1}H NMR and FT-IR peaks. ^{1}H NMR (400 MHz, $CDCl_3$, δ [ppm]): 4.42-4.37 (dd, *J* = 14.2, 7.4 Hz, 2H, C*H*$_2$OC=O), 4.21-4.15 (t, 2H, *J* = 11.3 Hz, C*H*$_2$OC=O), 2.80-2.75 (ddt, *J* = 14.3, 6.5, 1.6 Hz, 2H, C*H*$_2$COO), 2.63-2.57 (tt, *J* = 13.3, 2.1 Hz, 2H, C*H*$_2$COO), 2.04-1.93 (M, 4H, -C*H*$_2$CH$_2$OC=O), 1.70-1.56 (m, 4H, -C*H*$_2$ C*H*$_2$COO), 1.48-1.38 (m, 2H, -C*H*C(CH$_3$)$_2$), and 0.84 (s, 6H, C*H*$_3$C-).

7. Creation of porous 3D elastomer scaffold using either hexamethylene diisocyante (HDI) or 2,2-bis(1-caprolactone-4-yl)propane (BCP)[27] as crosslinkers (according to Gao *et al.*[27]); Step P7.

7.1. Prepare three-arm elastomer mixture using 0.75 g of SBC-αCLC by adding 0.25 mL of HDI (or 0.45 mL of BCP) and 0.24 mL of ε-caprolactone monomer. Add 60 µL of Sn(Oct)$_2$. If using BCP instead of HDI, mix SBC-αCLC and BCP using a vortex and place them in an oven at 140 °C until the BCP fully melts and dissolves (this step can take up to 2 h). Once the BCP has been dissolved, take it out of the oven and, the Sn(Oct)$_2$, and vortex.

7.2. Prepare a "sacrificial" nickel foam template by cutting a 1 x 2 cm metal piece. Roll it from one of the short ends so that the final roll is approximately a 1 x 1 cm metal piece (see Figure 4).

7.3. Put the nickel foam in a glass vial or aluminum foil pack and pour the mixture prepared in step 7.1 to fully cover the foam for 2 min. Remove the excess mixture with a Pasteur pipet. Leave it in an oven overnight at 80 °C.

7.4. Peel off the aluminum foil or break the glass. Using a razor blade, shave the elastomer around the metal foam to expose the nickel metal.

7.5. Prepare 1 M iron(III)chloride ($FeCl_3$) solution in 100 mL of water. Place the foam in a flask and add 70 mL of $FeCl_3$ solution. Stir for three days at room temperature and change the $FeCl_3$ solution every day. Before each change, stir the foam with ionized water for 0.5 h.

Note: The etching process is typically completed after three days. To ensure that the etching process is completed, perform tactile compression tests until the foams feel soft. Foam resistance to tactile compression tests indicates the presence of a residual metal template.

7.6. Rinse the elastomer foam with ethanol and place in a vacuum oven overnight at 40 °C.

7.7. Characterize the materials using scanning electron microscopy (SEM), differential scanning calorimetry (DSC), mechanical compression tests, and thermal gravimetric analysis (TGA)[27].

Note: For preparing a more hydrophilic 3D LCE, replace the SBC with LBC (making sure that the LBC also contains an LC moiety) following the steps described in step 7.5.

8. Seeding of elastomer scaffold with SH-SY5Y neuroblastoma cells and culture using sterile techniques; Step P8.

8.1. Sterilize the elastomer by washing it twice with 50 mL of 70% of ethanol. Perform UV irradiation for 10 min and wash with 50 mL of 70% of ethanol. Rinse it twice with 50 mL of sterile water and 50 mL of phosphate-buffered saline (PBS). Place the elastomers in 24-well culture plates.

8.2. Prepare cell growth medium for SH-SY5Y containing 90% Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep).

8.3. After counting the cells using a hematocytometer, prepare 1.5×10^5 SH-SY5Y cells suspended in 100 μL of growth medium (seed solution). Add the solution on top of the elastomers, making sure to form a drop.

8.4. Incubate the seeded elastomers at 37 °C and 5% CO_2 for 2 h to promote cell adhesion. Add 0.5 mL of growth medium. Incubate again at 37 °C with 5% CO_2.

8.5. Change the medium every other day after washing with 0.5 mL of PBS.

9. Microscopic imaging of elastomer construct; Step P9.

9.1. Fix the cells grown on elastomers with 4% paraformaldehyde (PFA) in PBS for 15 min. Rinse with 3 mL of PBS three times for 5 min each and place the elastomer with the fixed cells in a culture dish with an attached coverslip.

Caution: PFA is toxic. Wear appropriate personal protective clothing (PPE).

9.2. Stain the fixed samples with 0.1% of 4',6-diamidino-2-phenylindole (DAPI) in 500 µL of PBS for 10 min and rinse twice with 1 mL of PBS for 5 min.

9.3. Immediately image the elastomers using confocal microscopy with DAPI fluorescence, acquiring image stacks that span the sample.

Note: Here, image stacks were acquired using a 20x objective and a 60x objective.

9.4. Analyze the optical confocal image stacks using ImageJ[32].

REPRESENTATIVE RESULTS:

This report shows the preparation method of a porous 3D LCE as a scaffold for cell culture using a nickel metal template. The obtained 3D LCE demonstrates a complex interconnected channel network that allows for easy cell infiltration, as well as more suitable mass transport[27]. It was found that cells are able to fully penetrate the interconnected channel network and are also able to align within the LCE. Here, a metal nickel foam (99% Ni, density of 860 g/cm^2) was selected following a similar approach to the graphene foam preparation previously reported by Kung *et al*[22]. The metal foam was polymer casted, with all metal struts fully covered. The selection of the metal foam size and density is important, as it imparts the final morphology of the LCE and can help in mimicking the tissue environment of interest.

The preparation of the polymer casting is based on the crosslinking of a glycerol-based three-arm star block copolymer (SBC) using ring-opening polymerization (ROP). The monomers are ε-caprolactone, modified ε-caprolactone (synthesized for the purpose of adding a halogen atom that will later be substituted by an LC moiety), and *D*,-*L*-lactide (Figure 2). The final product is a hydrophobic 3-arm SBC. SBCs with 4 and 6 arms, made by replacing the glycerol node with pentaerythritol and dipentaerythritol, respectively, have been previously reported[18]. For a more hydrophilic SBC, the central node was replaced with oligoethylene oxide (see the protocol notes). However, replacing glycerol with oligoethylene oxide results in a linear block copolymer[27]. We used a modified synthesis from Younes *et al*[29]. The modified version allows for the use of a modified ε-caprolactone with a halogen group in two different positions, alpha and gamma to the carbonyl[6]. Once the SBC is formed, the modified ε-caprolactone block suffers a displacement of the halogen atom with an azide group. The displacement of the halogen group by the azide group is confirmed by the appearance of the 2,100-cm^{-1} band using attenuated total reflectance (ATR) FT-IR (Figure 3). The azide group is later used to covalently attach the LC moiety (cholesteryl hexynoate) to the star block co-polymer using alkyne-azide Huisgen's cycloadditon reaction (also known as a "click reaction"), obtaining the final star block copolymer with a side-chain cholesterol pendant unit (SBC-Chol). The formation of the triazole ring is confirmed by the disappearance of the ,2100-cm^{-1} band and the appearance of a singlet observed at 7.30 ppm in the ^{1}H NMR spectra (see Figure 3). We have recently reported on the effect of the placement of a halogen group either alpha (*α*-Br) or gamma (γ-Cl) to the carbonyl on the functionalized *ε*-CL[6]. It should be noted that replacing the central node in the SBC co-polymers with 4-arm and 6-arm central cores has an effect on the mechanical properties of the obtained LCEs because the central nodes serve as both initiators and intrinsic cross-linkers[18].

Once the SBC-Chol has been prepared and fully characterized, the crosslinking process using a metal template is the last step for foam preparation. The SBC-Chol was mixed with

hexamethylene diisocyanate (HDI, crosslinker), ε-caprolactone, and $Sn(oct)_2$ (ROP catalyst, see step 7.1). The bis-caprolactone (BCP) crosslinker was replaced with HDI, as previously reported. The metal foam is cut and shaped to the desired form (Figure 4). Two paths for foam preparation, namely the primary porosity ($LCEF_{PP}$) and primary/secondary porosity ($LCEF_{P+SP}$), have been previously reported. Here, the $LCEF_{P+SP}$, or "dipping," method, is presented, where the nickel template is quickly dipped in the polymer mixture. The metal foam is placed inside a container made of aluminum foil or a scintillation vial containing the polymer mixture, with all metal struts fully immersed in the polymer mixture. The excess polymer mixture is carefully removed. This polymer-covered nickel template is placed overnight, still inside the container, in an oven at 80 °C. Crosslinking is carried out in the presence of the catalyst for about 2 h. After the crosslinking process, the polymer-covered nickel template is removed from its container by peeling off the aluminum foil or breaking the glass container. The excess polymer is carefully shaved from the polymer-covered nickel template to expose the edges of the nickel template and placed inside a container with a saturated $FeCl_3$ solution (Figure 5). After a few hours, the nickel is almost completely removed, and only the LCE foam is rinsed with deionized (DI) water to eliminate the nickel/$FeCl_3$ solution. The LCE foam is again placed inside a container with a saturated $FeCl_3$ solution and rinsed with DI water two more times. After the etching process is complete and the entire nickel template is fully eliminated, the LCE foam shows an interconnected channel network with hollowed struts (Figure 6). Figure 6 shows the internal LCE foam morphology observed using scanning electron microscopy (SEM). The LCE foam struts are hollow, and the overall regular morphology is contingent upon the nickel foam template.

Previously, the use of BCP required several steps to keep it in solution (*i.e.*, melted), because BCP starts to recrystallize as soon as the solution cools down by a few degrees (from 140 °C to room temperature to add the $Sn(Oct)_2$; see step 7.1). This makes the manipulation of the metal foam and polymer mixture challenging prior to crosslinking. The use of HDI as a crosslinker allowed for a faster crosslinking time than when using BCP. BCP is a solid at room temperature, with a melting point of 140 °C, whereas HDI is a liquid at room temperature. The choice of crosslinkers directly affects the preparation time and, as in our case, reduced the crosslinking temperature from 140 to 80 °C, also reducing elastomer preparation.

The LCE foams were tested using compression-deformation (Movie 1). A 70% reduction in LCE size is observed, with no effect on overall dimensions. Upon the release of compression from the LCE, it fully recovers its original shape and size. It is believed that the presence of the liquid crystal moieties in the LCE material is critical, as elastomers prepared under the same condition without the cholesterol-*ε*-caprolactone block did not recover their original shape and collapsed into themselves.

Once LCE foams were obtained, they were ready to be seeded with neuroblastomas (SH-Sy5Y) using standard cell culture techniques. LCE foams were washed twice in 70 % ethanol and rinsed thrice with PBS prior to cell seeding. Within 2-3 days of cell seeding, the cells were observed to attach to the walls of the 3D LCE network. To fully study cell attachment and expansion within the LCE network, cells were fixed after 30 days (Figure 7). Cells were fixed, stained with DAPI, and imaged using confocal microscopy. The cells were found to have proliferated, attached, and expanded to extensively cover the 3D LCE scaffold network. Furthermore, detailed analysis revealed cell nuclei elongation, which in most cases was not affected by the curved sections of

the 3D LCE scaffold network. Cell elongation can also be correlated to cell alignment and is most likely the result of the smectic-A phase characteristic of the LCE presented. This was previously observed in C2C12 cells grown after 14 days[27]. In this study, we show that the cells continue to proliferate for more than 14 days; this is not restricted to C2C12 cells alone. The use of the LCE foams can be expanded to almost any cell line. Cells growing on 3D LCE foam-like scaffolds benefit from higher mass transport efficiency compared to 2D scaffolds. In 2D scaffolds, cells usually grow in layers, on top of one another. Cells growing on the top layers are the only ones that have full access to all nutrients, gas, and waste removal (mass transport). Cells within the lower layers are unable to access nutrients, and there is a higher degree of cell death in this case. Within 3D scaffolds, cells have a more efficient (compared to 2D scaffolds) access to nutrients, growth factors, and gases, permitting long-term cell and tissue regeneration studies. Cell waste management (removal of waste) using the 3D LCE foam-like scaffolds is also more effective than in the 2D scaffolds. The porosity (and/or other structural properties) of the LCEs allows for rapid mass transport and increased cell loading compared to less porous (or other) matrices, allowing media and cellular access to central regions of the matrix. This LCE platform is tunable to support the growth of many types of primary and immortalized cell lines, including muscle, nerve, and skin, among others, as well as multi-cellular culture systems. Essentially, this is one of the benefits of the platform, since it can be used to grow many different cell types and systems for extended periods. In addition, the ability to grow multiple layers of cells in the construct more closely emulates natural environments.

FIGURE LEGENDS:

Figure 1: General procedure describing the step-by-step preparation and characterization of the LCE foams. See the protocol section for details.

Figure 2: Crosslinking scheme of 3-arm SBC. The crosslinking scheme of the 3-arm SBC using biscaprolactone or HDI as cross-linkers in the presence of a nickel metal template for the preparation of foam-like LCEs is shown.

Figure 3: Scheme of 1,2,3-triazole formation (successful "click" reaction) followed by ^{1}H NMR and FT-IR. The displacement of the halogen group by the azide group is confirmed by the appearance of the 2,100-cm^{-1} band using attenuated total reflectance (ATR) FT-IR. The formation of the triazole ring is confirmed by the disappearance of the 2,100-cm^{-1} band and the appearance of a singlet, observed at 7.30 ppm in the ^{1}H NMR spectra.

Figure 4: Optical images of various foam shapes prior to crosslinking. The metal foam is cut and shaped to the desirable form, as shown.

Figure 5: Nickel foam before (A) and after (B) crosslinking.

Figure 6: LCE foam morphology observed using scanning electron microscopy (SEM). Representative SEM images of LCE foams on SBC-based (a and b) and LBC-based (c and d) elastomers using Ni-860 as the metal template are shown. Arrows indicate hollowed struts.

Figure 7: Confocal micrographs displaying DAPI-stained nuclei of SH-SY5Y cells attached to the elastomer foam 30 days after seeding. 2D images were stacked in the *z*-direction,

and cross-section images in the *xz*- and *yz*-planes were created. The images show that SH-SY5Y cells (bright spots) attached and expanded within the walls of the hollow channels in the elastomer foam. The cells spatially expanded into multiple layers and extended over 100 μm through the construct (as shown in the *xz*- and *yz*-plane image cross sections).

Movie 1: Video images of the deformation and recovery of an LCE roll.

DISCUSSION:

Liquid crystalline elastomers have recently been studied as biocompatible cell scaffolds due to their stimuli responsiveness. They have been proven to be ideal platforms as cell scaffolds. However, an important factor to keep in mind when preparing and designing a new LCE scaffold is porosity. The incorporation of leachable solids[23] or gases does not always result in homogeneous porosity or fully interconnected pores. The use of a metal template that can be etched out not only offers the opportunity to have a more organized and regular internal structure, but also allows for the selection of pore size and density. This is directly related to the metal template previously used for the preparation of graphene foams[22]. Metal templates also provide the opportunity to form shapes prior to LCE crosslinking, permitting a vast array of possibilities to create sophisticated and complex architectures with regular porosity designed to closely resemble endogenous environments. Foam LCEs prepared using this methodology allow for the improved study of cell material and, more importantly, spatial cell-cell interactions, a feat not possible within two-dimensional (2D) environments. 2D cell scaffolds do not allow the cells to freely interact with neighboring cells. In addition, the cells typically grow in monolayers (or directly on top of one another), without full access to space for growth and, more importantly, for interaction. Specific spatially interacting cell types, such as neurons and glial cells, are of paramount importance when designing constructs as potential implants or long-term experimental platforms designed to mirror living systems.

Our modular, solvent-free LCE synthesis using a metal template, presented here, helps adjust cell adhesion by tuning the hydrophobic/hydrophilic balance by choosing the proper central node and ratio of monomers[7,27]. This is relevant for cell seeding in order to avoid an extra step that includes the addition of a Matrigel layer, usually done to promote cell attachment. The amount and size of the central node, as well as the crosslinker, play critical roles in the final LCE, as they affect the thermal and mechanical properties of LC elastomers. In addition, this also permits the tuning of the biodegradability rates, as presented before, to fit the full regeneration of tissue as the LCE degrades[6]. Currently, we have ongoing experiments focusing on investigating how the type of cross-linker, central core, and replacement of *D,L*-lactide for *L*-lactide affects the elastic properties, cell adhesion, proliferation, and cell alignment.

The limitations of this protocol are: (1) the limited variety of commercially available metal (nickel) foams and their limited range in strut dimensions, (2) the requirement that the LCE or any other polymer/elastomer material must chemically resist the conditions of the metal etch (here, an $FeCl_3$ etch), and (3) the fact that cell alignment appears to be limited to the liquid crystal modified elastomers reported here (the parent non-LCE elastomers did not show any appreciable cell alignment).

In conclusion, it has been previously shown that SmA LCEs can be prepared using our modular

synthesis procedure. Additional biocompatible LC moieties can be incorporated to explore their mechanical properties and new liquid crystalline effects on cell proliferation and, particularly, cell alignment. It is known that mechanical properties, particularly Young's moduli values, are critical for cell adhesion, proliferation, and cell alignment. The results here show that the porosity of SmA LCEs can be tuned through the use of a metal template to provide an effective way to control the pore size and to tailor the LCE shape (*i.e.,* overall morphology). Dipping the Ni template into the SCB polymer mixture, followed by etching the Ni template provides an easy approach for new LCE morphologies. The foam-like LCEs described here provide cells (here, SH-Sy5Y, a standard cell model to study neuronal function) with a more realistic, 3D environment for growth and interaction. Allowing multiple cell types to grow in multiple layers dispersed throughout the elastomer closely mimics endogenous neural environments and offers the intriguing possibility of more sophisticated 3D tissue culture experiments. The use of tunable and dynamic LCEs to mimic native architectures paves the way for next-generation cell models for longitudinal and clinically relevant cell studies.

ACKNOWLEDGMENTS:
The authors would like to thank Kent State University (collaborative research grant and support for the Regenerative Medicine Initiative at Kent State − ReMedIKS) for the financial support of this project.

DISCLOSURES:
The authors have nothing to disclose.

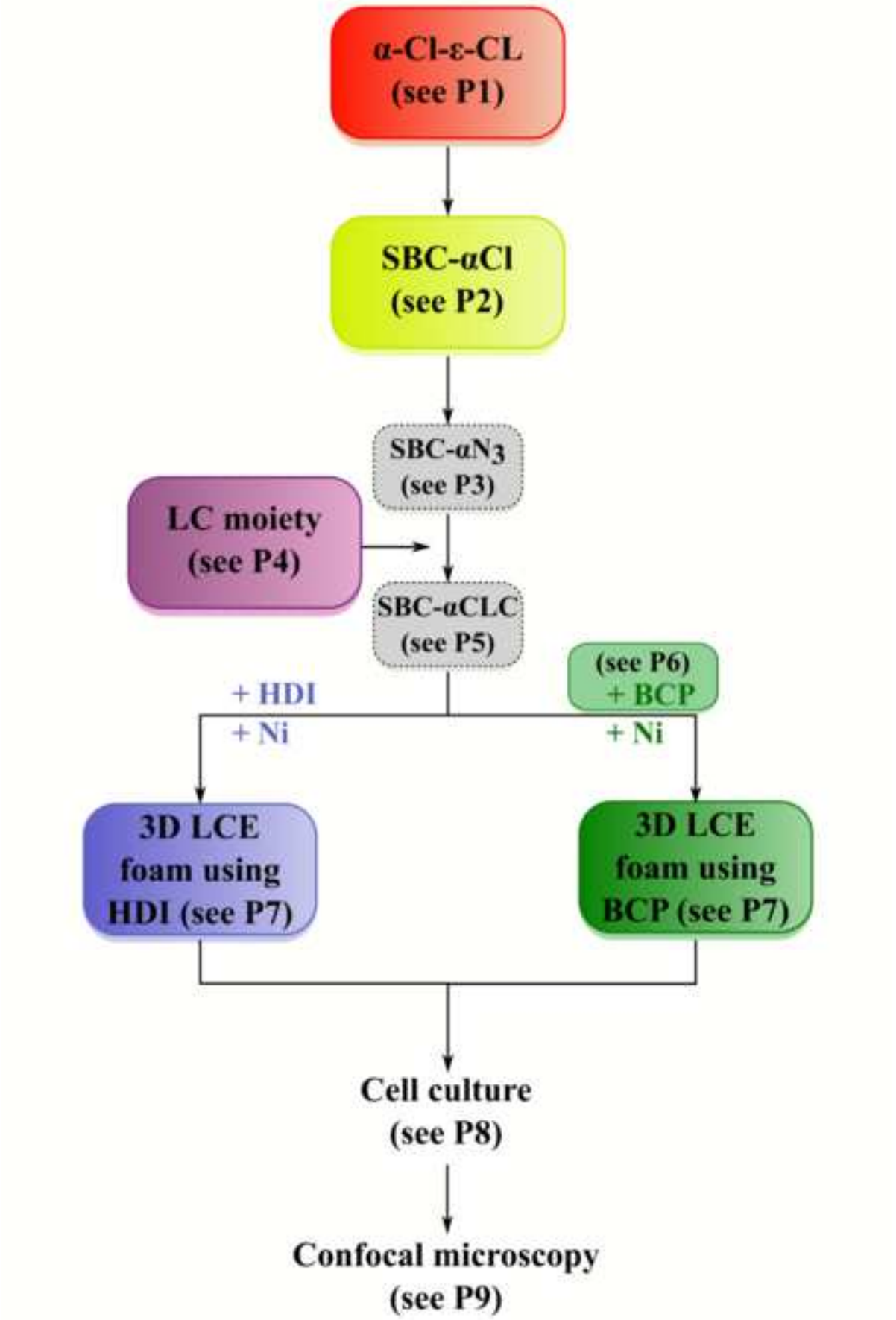
REFERENCES:
1 Khor, E. & Lim, L. Y. Implantable applications of chitin and chitosan. *Biomaterials.* **24** (13), 2339-2349, doi:10.1016/s0142-9612(03)00026-7 (2003).
2 Chung, H. J. & Park, T. G. Surface engineered and drug releasing pre-fabricated scaffolds for tissue engineering. *Adv. Drug Deliv. Rev.* **59** (4-5), 249-262, doi:10.1016/j.addr.2007.03.015 (2007).
3 Yakacki, C. M. & Gall, K. Shape-Memory Polymers for Biomedical Applications. *Shape-Memory Polymers.* **226** 147-175, doi:10.1007/12_2009_23 (2010).
4 Agrawal, A. *et al.* Stimuli-responsive liquid crystal elastomers for dynamic cell culture. *J. of Mat. Res.* **30** (4), 453-462, doi:10.1557/jmr.2014.392, (2015).
5 Agrawal, A., Yun, T. H., Pesek, S. L., Chapman, W. G. & Verduzco, R. Shape-responsive liquid crystal elastomer bilayers. *Soft Matter.* **10** (9), 1411-1415, doi:10.1039/c3sm51654g (2014).
6 Sharma, A. *et al.* Biocompatible, Biodegradable and Porous Liquid Crystal Elastomer Scaffolds for Spatial Cell Cultures. *Macromol. Biosci.* **15** (2), 200-214, doi:10.1002/mabi.201400325, (2015).
7 Yakacki, C. M. *et al.* Tailorable and programmable liquid-crystalline elastomers using a two-stage thiol-acrylate reaction. *RSC Adv.* **5** (25), 18997-19001, doi:10.1039/c5ra01039j, (2015).
8 deGennes, P. G., Hebert, M. & Kant, R. Artificial muscles based on nematic gels. *Macromolecular Symposia.* **113** 39-49, doi:10.1002/masy.19971130107, (1997).
9 Fleischmann, E.-K. & Zentel, R. Liquid-Crystalline Ordering as a Concept in Materials Science: From Semiconductors to Stimuli-Responsive Devices. *Angew. Chem. Int. Ed.* **52** (34),

8810-8827, doi:10.1002/anie.201300371, (2013).
10 Finkelmann, H., Kim, S. T., Munoz, A., Palffy-Muhoray, P. & Taheri, B. Tunable mirrorless lasing in cholesteric liquid crystalline elastomers. *Adv. Mater.* **13** (14), 1069-1072, doi:10.1002/1521-4095(200107)13:14<1069::aid-adma1069>3.0.co;2-6, (2001).
11 Artal, C. *et al.* SHG characterization of different polar materials obtained by in situ photopolymerization. *Macromolecules.* **34** (12), 4244-4255, doi:10.1021/ma001928e, (2001).
12 Camacho-Lopez, M., Finkelmann, H., Palffy-Muhoray, P. & Shelley, M. Fast liquid-crystal elastomer swims into the dark. *Nat. Mater.* **3** (5), 307-310, doi:10.1038/nmat1118, (2004).
13 Yamada, M. *et al.* Photomobile polymer materials: Towards light-driven plastic motors. *Angew. Chem. Int. Ed.* **47** (27), 4986-4988, doi:10.1002/anie.200800760, (2008).
14 Ohm, C., Brehmer, M. & Zentel, R. Liquid Crystalline Elastomers as Actuators and Sensors. *Adv. Mater.* **22** (31), 3366-3387, doi:10.1002/adma.200904059, (2010).
15 Fleischmann, E.-K. *et al.* One-piece micropumps from liquid crystalline core-shell particles. *Nat. Commun.* **3**, doi:10.1038/ncomms2193, (2012).
16 Herzer, N. *et al.* Printable Optical Sensors Based on H-Bonded Supramolecular Cholesteric Liquid Crystal Networks. *J. Am. Chem. Soc.* **134** (18), 7608-7611, doi:10.1021/ja301845n, (2012).
17 Lockwood, N. A. *et al.* Thermotropic liquid crystals as substrates for imaging the reorganization of matrigel by human embryonic stem cells. *Adv. Funct. Mater.* **16** (5), 618-624, doi:10.1002/adfm.200500768, (2006).
18 Sharma, A. *et al.* Effects of structural variations on the cellular response and mechanical properties of biocompatible, biodegradable, and porous smectic liquid crystal elastomers, *Macromol. Biosci.*, DOI:10.1002/mabi.201600278; (2016) In press.
19 Bera, T. *et al.* Liquid Crystal Elastomer Microspheres as Three-Dimensional Cell Scaffolds Supporting the Attachment and Proliferation of Myoblasts. *ACS Appl. Mater. Interfaces.* **7** (26), 14528-14535, doi:10.1021/acsami.5b04208, (2015).
20 Bera, T., Malcuit, C., Clements, R. J. & Hegmann, E. Role of Surfactant during Microemulsion Photopolymerization for the Creation of Three-Dimensional Liquid Crystal Elastomer Microsphere Spatial Cell Scaffolds. *Front. Mater.* **3** (31), doi:10.3389/fmats.2016.00031, (2016).
21 McKee, C. T., Last, J. A., Russell, P. & Murphy, C. J. Indentation Versus Tensile Measurements of Young's Modulus for Soft Biological Tissues. *Tissue Eng. Part B Rev.* **17** (3), 155-164, doi:10.1089/ten.teb.2010.0520, (2011).
22 Kung, C.-C. *et al.* Preparation and characterization of three dimensional graphene foam supported platinum-ruthenium bimetallic nanocatalysts for hydrogen peroxide based electrochemical biosensors. *Biosens. Bioelectron.* **52** 1-7, doi:10.1016/j.bios.2013.08.025, (2014).
23 Amsden, B. Curable, biodegradable elastomers: emerging biomaterials for drug delivery and tissue engineering. *Soft Matter.* **3** (11), 1335-1348, doi:10.1039/b707472g, (2007).
24 Sinturel, C., Vayer, M., Morris, M. & Hillmyer, M. A. Solvent Vapor Annealing of Block Polymer Thin Films. *Macromolecules.* **46** (14), 5399-5415, doi:10.1021/ma400735a, (2013).
25 Riboldi, S. A. *et al.* Skeletal myogenesis on highly orientated microfibrous polyesterurethane scaffolds. *J. Biomed. Mater. Res. A.* **84A** (4), 1094-1101, doi:10.1002/jbm.a.31534, (2008).
26 Chung, S., Moghe, A. K., Montero, G. A., Kim, S. H. & King, M. W. Nanofibrous

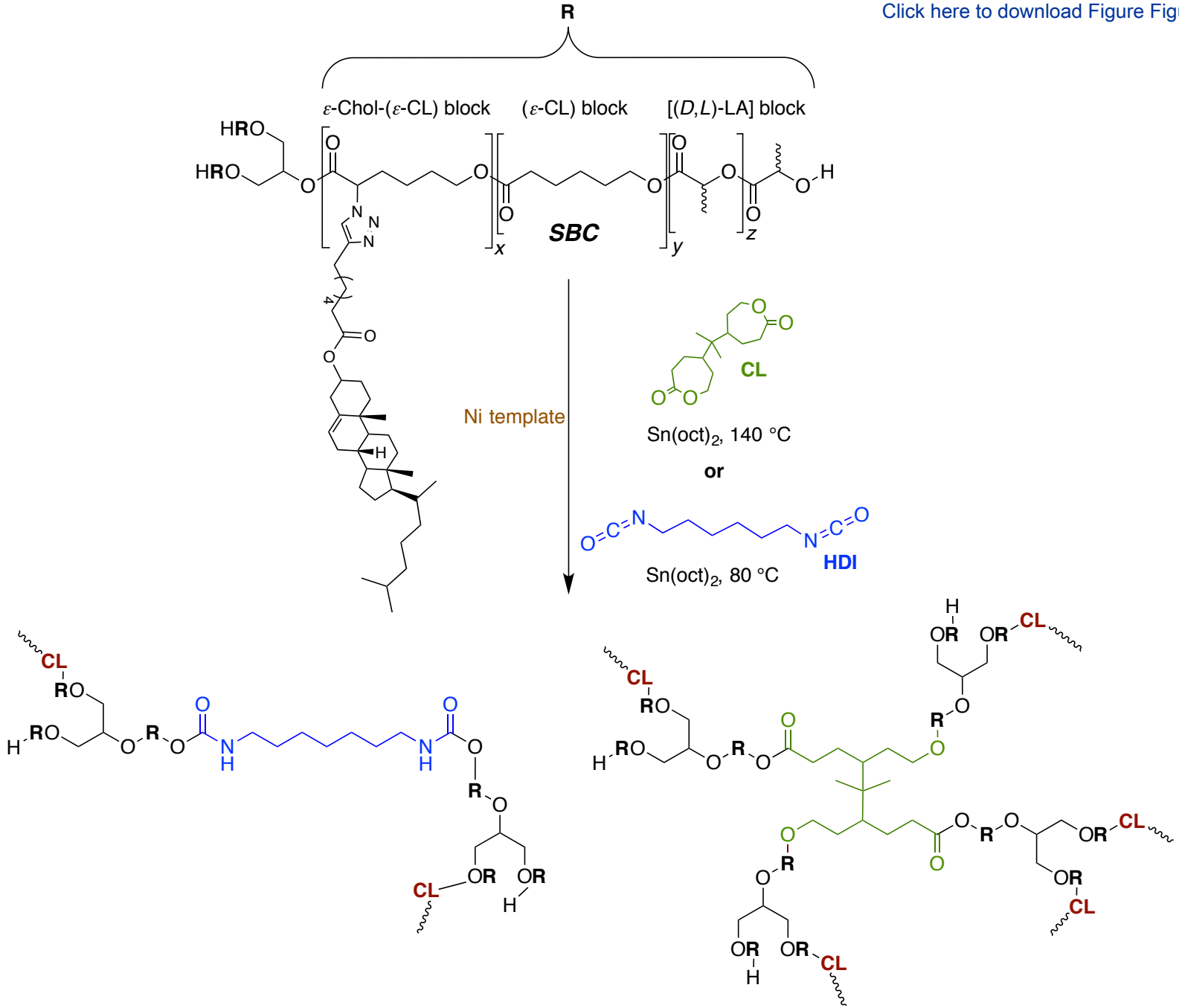
scaffolds electrospun from elastomeric biodegradable poly(L-lactide-co-epsilon-caprolactone) copolymer. *Biomed. Mater.* **4** (1), 9, doi:10.1088/1748-6041/4/1/015019, (2009).
27 Gao, Y. X. *et al.* Biocompatible 3D Liquid Crystal Elastomer Cell Scaffolds and Foams with Primary and Secondary Porous Architecture. *ACS Macro Lett.* **5** (1), 14-19, doi:10.1021/acsmacrolett.5b00729, (2016).
28 Lenoir, S. *et al.* Ring-opening polymerization of alpha-chloro-is an element of-caprolactone and chemical modification of poly(alpha-chloro-is an element of-caprolactone) by atom transfer radical processes. *Macromolecules.* **37** (11), 4055-4061, doi:10.1021/ma035003l, (2004).
29 Younes, H. M., Bravo-Grimaldo, E. & Amsden, B. G. Synthesis, characterization and in vitro degradation of a biodegradable elastomer. *Biomaterials.* **25** (22), 5261-5269, doi:10.1016/j.biomaterials.2003.12.024, (2004).
30 Donaldson, T., Henderson, P. A., Achard, M. F. & Imrie, C. T. Chiral liquid crystal tetramers. *J. Mater. Chem.* **21** (29), 10935-10941, doi:10.1039/c1jm10992h, (2011).
31 Palmgren, R., Karlsson, S. & Albertsson, A. C. Synthesis of degradable crosslinked polymers based on 1,5-dioxepan-2-one and crosslinker of bis-epsilon-caprolactone type. *J. Pol. Sci. A Polym. Chem.* **35** (9), 1635-1649, doi:10.1002/(sici)1099-0518(19970715)35:9<1635::aid-pola5>3.0.co;2-q, (1997).
32 Rasband, W. S., *ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA,* http://imagej.nih.gov/ij/ 1997-2015.

Figure

Click here to download Figure Figure 1.PNG

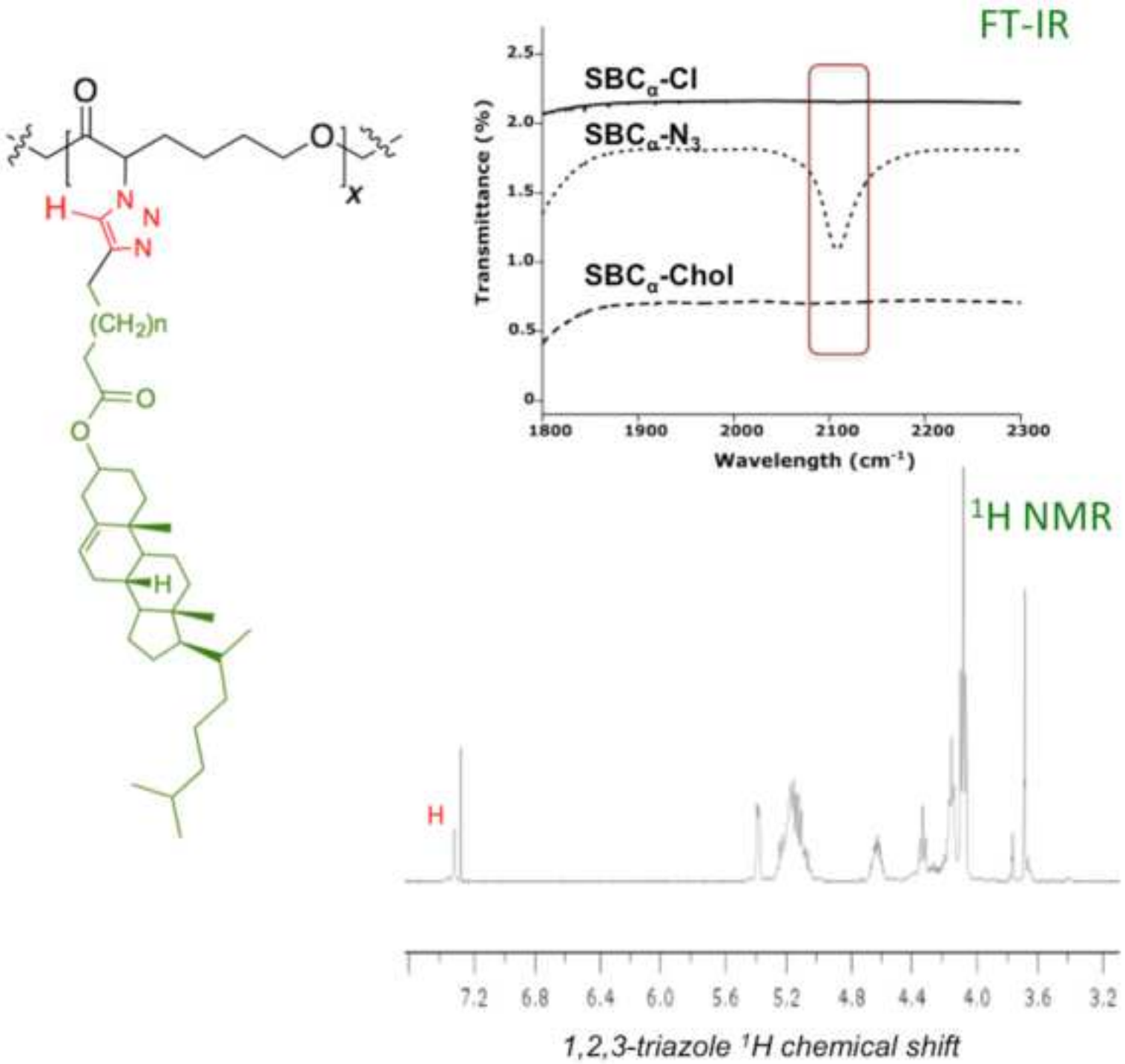


Figure



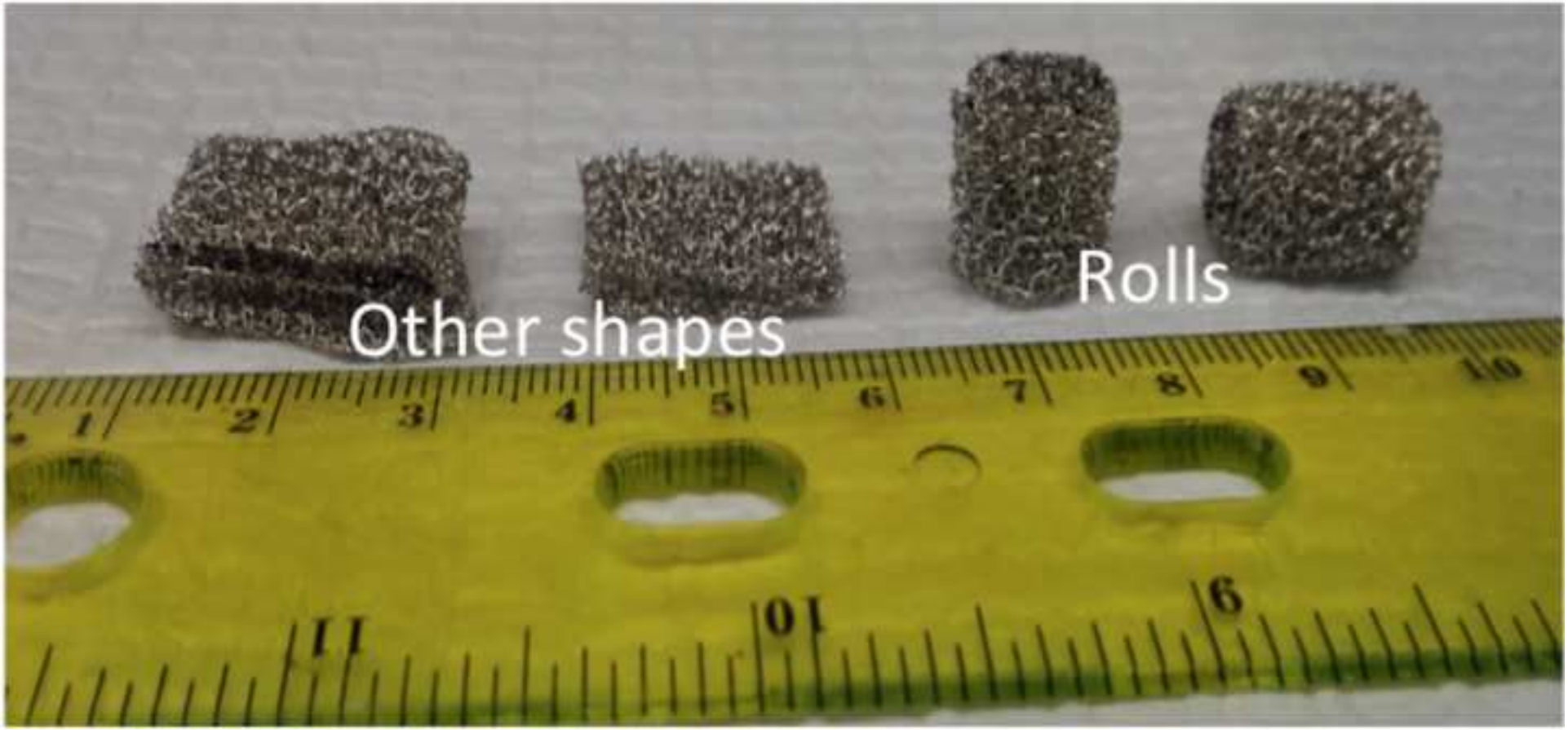
Figure

Click here to download Figure Figure 3 HD.png



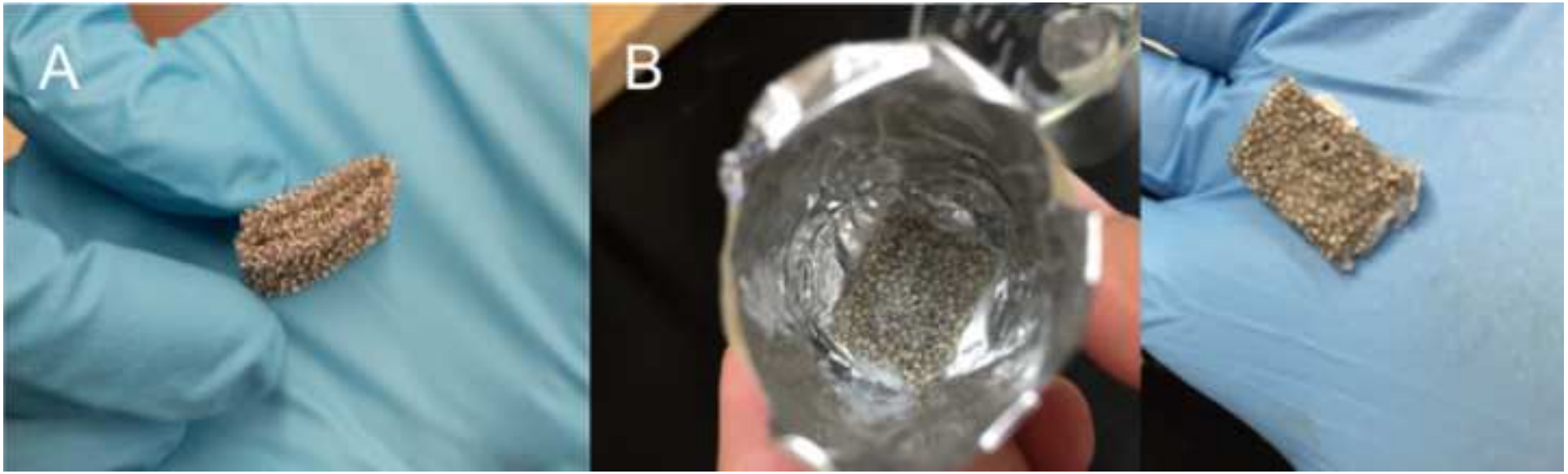
Figure

Click here to download Figure Figure 4 HD.png



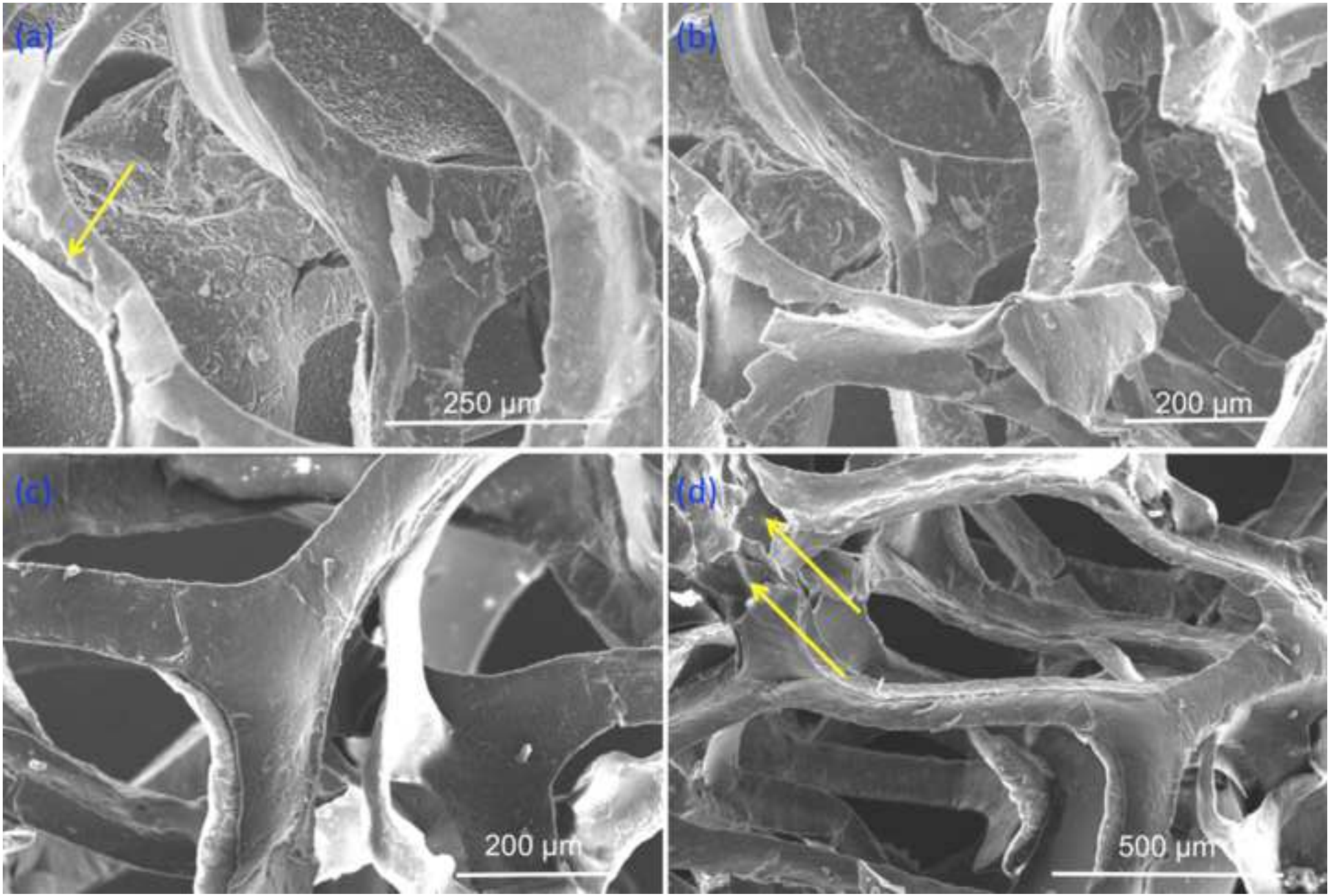
Figure

Click here to download Figure Figure 5 HD.png



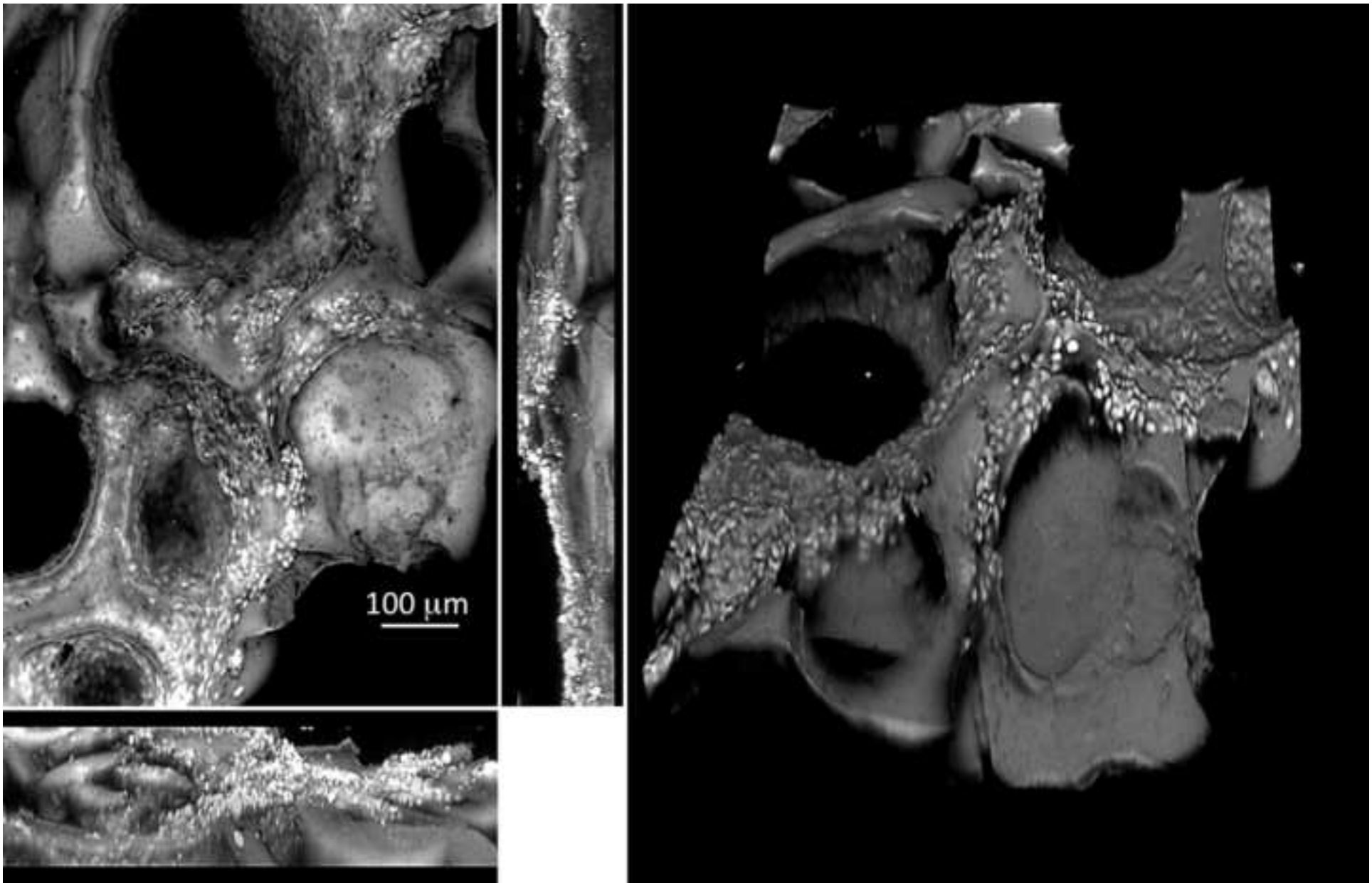
Figure

Click here to download Figure Figure 6.jpeg



Figure

Click here to download Figure Figure 7.jpeg



Name of material	Company
1H, 1H, 2H, 2H-perfluorooctyltriethoxysilane	Alfa Aesar
2-bis(4-hydroxy-cyclohexyl)propane	TCI
2-chlorohexanone	Alfa Aesar
2-heptanone	Sigma Aldrich
2-propanol	Sigma Aldrich
3-chloroperbenzoic acid, *m*- CPBA	Sigma Aldrich
4-dimethylaminopyridine	Alfa Aesar
4',6-diamidino-2-phenylindole, DAPI	Invitrogen
5-hexynoic acid	Alfa Aesar
Acetic acid	VWR
Acetone	Sigma Aldrich
Alcohol 200 proof ACS Grade	VWR
Benzene	Alfa Aesar
ε-caprolactone	Alfa Aesar
Chloroform	VWR
Cholesterol	Sigma Aldrich
Chromium(VI) oxide	Sigma Aldrich
Copper (I) iodide	Strem Chemicals
D ,*L* -Lactide	Alfa Aesar
Dichloromethane	Sigma Aldrich
Diethyl ether	Emd Millipore
N,N-Dimethylformamide	Sigma Aldrich
Dulbecco's modified Eagle medium, DEME	CORNING Cellgo
Ethanol	Alfa Aesar
Formaldehyde	SIGMA Life Science
Fetal bovine serum, FBS	HyClone
Filter paper, Grade 415, qualitative, crepe	VWR
Glycerol	Sigma Aldrich
Hexamethylene diisocyanate, HDI	Sigma Aldrich
Iron(III) chloride	Alfa Aesar
Isopropyl alcohol	VWR
Methanol	Alfa Aesar
N,N'-dicyclohexylcarbodiimide	Aldrich
N,N-Dimethylformamide	Sigma Aldrich
Nickel metal template	American Elements
Neuroblastomas cells (SH-SY5Y)	ATCC
Penicillin streptomycin	Thermo SCIENTIFIC
Polyethylene glycol 2000, PEG	Alfa Aesar
Sodium azide	VWR
Sodium bicarbonate	AMRESCO
Sodium chloride	BDH
Sodium phosphate dibasic heptahydrate	Fisher Scientific
Sodium phosphate monobasic monohydrate	Sigma Aldrich
Sodium sulfate	Sigma Aldrich
Tetrahydrofuran	Alfa Aesar
Thiosulfate de sodium	AMRESCO

Tin(II) 2-ethylhexanoate	Aldrich
Toluene	Alfa Aesar
Triethylamine	Sigma Aldrich
Trypsin	HyClone
Olympus FV1000	

Catalog number	Comment/Description
L16606	Silanizing agent
B0928	Reagent
A18613	Reagent
W254401	Solvent
278475	Solvent
273031	Reagent
A13016	Reagent
D1306	Nuclear Stain
B25132-06	Reagent
36289	Solvent
34850	Solvent
71001-866	Reagent
AA33290	Solvent
A10299-0E	Reagent
BDH1109	Solvent
C8503	Reagent
232653	Reagent
100211-060	Reagent
L09026	Reagent
320269	Solvent
EX0190	Solvent
270547	Solvent
10-013	Cell Media
33361	Solvent
F8775	Fixative
SH30071.01	Media Component
28320	Filtration
G5516	Central node (3-arm)
52649	Crosslinker
12357	Etching agent
BDH1133	Solvent
L13255	Solvent
D80002	Solvent
270547	Solvent
Ni-860	Foam template
CRL-2266	Cell line
15140122	Antibiotics
B22181	Reagent
97064-646	Reagent
865	Drying salt
BDH9286	Drying salt
S-374	Drying salt
S9638	Drying salt
239313	Drying salt
41819	Solvent
393	Drying salt

S3252	Reagent
22903	Solvent
471283	Reagent
SH30042.01	Cell Detachment

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Synthesis of Biocompatible Liquid Crystal Elastomer Foams as Cell Scaffolds for 3D Spatial Cell Cultures

Author(s): Elda Hegmann, et.al.

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Elda Hegmann
Department:	Liquid Crystal Institute
Institution:	Kent State University
Article Title:	Synthesis of Biocompatible Liquid Crystal Elastomer Foams as Cell Scaffolds for 3D Spatial Cell Cultures
Signature:	
Date:	September 6, 2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

KENT STATE UNIVERSITY

Benjamin Werth – Science Editor
Journal of Visualized Experiments (JoVE)

December 7, 2016

It is with pleasure that we submit the newly revised version of our manuscript entitled "**Synthesis of Biocompatible Liquid Crystal Elastomer Foams as Cell Scaffolds for 3D Spatial Cell Cultures**" to be considered for publication in *Journal of Visualized Experiments.*

Please find all our responses and adjustments in this letter and the revised manuscript in red font.

Thank you for the careful Peer Review revisions that were made to the manuscript. The following editorial/peer review comments need to be addressed in order to finalize your manuscript.

• Your manuscript has been modified by your editor, please maintain the current formatting throughout the manuscript. **Please use the updated manuscript located in your Editorial Manager account (under "File Inventory") for all subsequent revisions**. The updated manuscript is also attached.

• 2.4 Note: Please add details on how to reheat to melt Lactide. Is SnOct2 added while mixing and re-heating? We have adjusted 2.3 for clarification

• 6.10: How is the filtration performed? We clarified on the text.

• 7.2: It is not clear how the roll the piece along its side. The figure does not describe how this is done. Please note that the figures are incorrectly labelled. We have clarified the preparation of rolls and correctly referred to the right figure.

• Results:

-It is not necessary to repeat description of the protocol in the results section (ex. Paragraph 3- Lines 394-408). While you revise, please ensure that all figures are cited in the results section and a description of the figure is provided (e.g. Figure 5 (lines 401-403). We are not repeating the protocol, we are discussing the results and it is important that we describe how we characterize the materials (at every step) and make sure that synthetic steps have been properly achieved.

• Figures:

-Please label the figure files to match the figure number. Currently, the figure file- Figure 4. jpeg is labelled Figure 5.

- Two file of the same Figure 5 (nickel foam before and after crosslinking) have been uploaded.

-Figure 2 (crosslinking scheme of 3-arm SBC) is missing.

All figures have been adjusted.

• The manuscript will benefit from copy editing, as there are a number of grammatical errors throughout. We have given the manuscript to native-english spoken members of our lab for corrections. If there are any more errors, we will be happy to correct them if they can be pointed out to us.

Liquid Crystal Institute
Chemical Physics Interdisciplinary Program
P.O. Box 5190 • Kent, Ohio 44242-0001
330-672-2654 • Telefax: 330-672-2796 • E-mail: mail@lci.kent.edu • www.lci.kent.edu

-Line (L) 317: Please complete the sentence within the brackets. Now line 337, it has been adjusted and detail have been given

-L 439: should be ‘cells grow in layers’ Adjusted

- Reviewer #1

- Line 386 and 388 - Be a little more specific, or cite an example, of what is mean by "cools down a few degrees" and "allowed for a faster crosslinking time." As written, it is a little vague for what the actual values might be. We have adjusted the paragraph and added a few lines for clarification.

Please discuss the details mentioned in the rebuttal letter in the Results section of the manuscript.

We trust that we have properly addressed all suggestions and your team will find this manuscript interesting and worthy of publication in your journal.

Sincerely yours,

Elda Hegmann
Assistant Professor – Liquid Crystal Institute, and Department of Biological Sciences, Kent State University, Kent (OH) USA