Video Article

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

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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.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55452/>

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^{1,2,3,4,5}. 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.*)^{10,11,12,13,14,15,16}. 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 interest²¹. 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 films⁶ 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 template²². 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 templating²⁴, 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 development²⁷. 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)

- Before beginning the synthesis, purify 27 g of 3-chloroperbenzoic acid as follows:
 - 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.
 - 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.
 - Add 3 g of sodium sulfate directly to the organic solution; this drying agent absorbs water from the organic solution.
 - 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.
- 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.
- 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.
- 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.
- Cool the reaction mixture to -20 °C for 1 h to precipitate the *m*-chlorobenzoic acid (*m*-CBA) byproduct.
- Filter the *m*-chlorobenzoic acid (*m*-CBA) and wash the remaining solution with saturated solutions of sodium thiosulfate, sodium bicarbonate, and sodium chloride.
- 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.
- Monitor the success of the synthesis using the following ¹H NMR peaks. ¹H NMR (400 MHz, CDCl_3 , δ [ppm]): 4.75-4.68 (m, 1H, CHClCO), 4.37-4.26 (m, 1H, CH_2O), 4.18-4.05 (m, 1H, CH_2O), and 2.06-1.58 (m, 6H, $-\text{CH}_2-$)^{6,27}.

2. Synthesis of α -Three-arm Star Block Copolymer (SBC- α Cl) by Ring Opening Copolymerization (Sharma *et al.*⁶ and Amsden *et al.*²⁹)

- 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.
- Add 3.64 g of distilled 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.
- 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 ($\text{Sn}(\text{Oct})_2$) 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.
- Vigorously mix one last time using the vortex and flush with nitrogen.
- 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 not to melt the rubber stopper. Once the ampoule is flame-sealed, place it in a sand bath, an oven, or appropriate heating element at 140 °C for 48 h.
- Take out the ampoule and let it cool at room temperature.
- 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.

- 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 about two drops fall every other second (dropwise).
- Collect the white precipitate by filtration (using a paper filter) and dry it in a vacuum oven between 50 and 60°C .
- Monitor the success of the synthesis using the following ^1H NMR and FT-IR peaks. ^1H NMR (400 MHz, CDCl_3 , δ [ppm]): 5.29-5.03 (m, COCHCH_3), 4.43-4.25 (m, CHCl), 4.24-4.12 (m, CH_2O), 4.11-4.03 (t, $J = 4.6$ Hz, CH_2O), 3.80-3.68 (m, CHCH_2), 3.09-2.64 (broad, s, OH), 2.39 (t, $J = 4.5$ Hz, $\alpha\text{-H}$), 2.33 (t, $J = 5.1$ Hz, $\alpha\text{-H}$), and 1.77-1.25 (m, CH_2 , CH_3); FT-IR ($1/\lambda$ [cm^{-1}]): 2,932 (s), 2,869 (m), 1,741 (s), 961 (s), 866, and 735 (m)^{6,27}.

Note: 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.

- 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 -N₃-Three Arm SBC (SBC- α N₃) (According to Sharma *et al.*⁶)

- In a round-bottom flask and under nitrogen, dissolve 5 g of SBC- α Cl in 30 mL of dry N,N' -dimethylformamide.
- 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).
- 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 $2,800 \times g$ for 15 min to remove the salt formed. Evaporate the toluene under reduced pressure using a rotary evaporator at 77 mbar and 40°C .
- Monitor the success of the azide substitution using ^1H NMR and FT-IR peaks.
NOTE: ^1H NMR spectrum was similar to the parent SBC- α Cl, with the exception of the appearance of a peak at 3.90 ppm related to the azide group; FT-IR ($1/\lambda$ [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)

- 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.
- 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.
- 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.
- Allow the mixture to warm up to room temperature overnight.
- Remove the resulting dicyclohexylurea precipitate by filtration using a grade 415 paper filter and discard it.
- 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.
- 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 .
- Monitor the synthesis using the following ^1H NMR and FT-IR peaks. ^1H NMR (400 MHz, CDCl_3 , δ [ppm]): 5.39 (d, $J = 4.7$ Hz, 1H, $\text{CH}=\text{C}$), 4.70-4.58 (m, 1H, CHOCO), 2.44 (t, $J = 2.5$ Hz, 2H, CH_2CO), 2.34 (m, 2H, $\text{CH}_2\text{-CH}=\text{C}$), 2.31 (m, 2H, $\text{CH}_2\text{CH}_2\text{-COO}$), 2.28 (s, 1H, $\text{HC}=\text{C}$), 2.27 (d, $J = 2.3$ Hz, 2H, $\equiv\text{C-CH}_2$), 2.07-1.06 (m, 2H, CH_2 , CH), 0.94 (s, 3H, CH_3), 0.89 (d, $J = 1.8$ Hz, 3H, CH_3CH), and 0.88 (d, $J = 1.8$ Hz, 6H, $\text{CH}_3\text{-CH}$), 0.67 (s, 3H, CH_3). FT-IR ($1/\lambda$ [cm^{-1}]): 2,830-2,990 (broad and strong peak), 2,104 (m), 1,695 (s), 1,428 (m), 1,135 (m), 999 (s), 798 (s), and 667 (s).

5. Synthetic Modification of α -N₃-Three Arm SBC to α -Cholesteryl-Three Arm SBC (SBC- α CLC) via an Azide-Alkyne Huisgen Cyclo-addition Reaction ("Click" Reaction) to Obtain SBC-Chol (According to Sharma *et al.*⁶)

- In a round-bottom flask, dissolve 1 molar equivalent of SBC- α N₃ (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.
- Evaporate the solvent under reduced pressure using a rotary evaporator at 357 mbar and 40°C .
- Dissolve the residual mixture in 80 mL of dichloromethane and centrifuge for 5 min at $2,800 \times g$ at room temperature to remove unreacted materials and side products.
- Monitor the synthesis using the following ^1H NMR and FT-IR peaks. ^1H NMR (400 MHz, CDCl_3 , δ [ppm]): 7.54 (s, $\text{CH}=\text{C}$ -triazole), 5.43-5.34 (m, $\text{C}=\text{CH}$ 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 ($1/\lambda$ [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)

- 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.

2. Prepare a solution containing 11 g of chromium trioxide in 50 mL of acetic acid and 8 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.
3. Add 50 mL of 2-propanol. Stir the solution overnight at room temperature.
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.
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. Dissolve the solid material in 15 mL of benzene at 40 °C and let it recrystallize at 25 °C.
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.
8. Reflux the solution at 40 °C for 24 h.
9. Cool the reaction mixture to -20 °C for 10 min to precipitate the *m*-chlorobenzoic acid byproduct.
10. Remove *m*-chlorobenzoic acid by filtration (using a paper filter) and concentrate the solution under reduced pressure.
11. Wash the viscous crude product with 200 mL of 2-heptanone and dry the precipitate under vacuum at 50 °C overnight.
12. Monitor the synthesis using the following ¹H NMR and FT-IR peaks. ¹H NMR (400 MHz, CDCl₃, δ [ppm]): 4.42-4.37 (dd, *J* = 14.2, 7.4 Hz, 2H, CH₂OC=O), 4.21-4.15 (t, 2H, *J* = 11.3 Hz, CH₂OC=O), 2.80-2.75 (ddt, *J* = 14.3, 6.5, 1.6 Hz, 2H, CH₂COO), 2.63-2.57 (tt, *J* = 13.3, 2.1 Hz, 2H, CH₂COO), 2.04-1.93 (m, 4H, -CH₂CH₂OC=O), 1.70-1.56 (m, 4H, -CH₂CH₂COO), 1.48-1.38 (m, 2H, -CHC(CH₃)₂), and 0.84 (s, 6H, CH₃C-).

7. Creation of Porous 3D Elastomer Scaffold Using either Hexamethylene Diisocyanate (HDI) or 2,2-Bis(1-caprolactone-4-yl)propane (BCP)²⁷ as Crosslinkers (According to Gao et al.²⁷)

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 distilled 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.
2. Prepare a "sacrificial" nickel foam template by cutting a 1 x 4 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**).
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.
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.
5. Prepare 1 M iron(III)chloride (FeCl₃) solution in 100 mL of water. Place the foam in a flask and add 70 mL of FeCl₃ solution. Stir for three days at room temperature and change the FeCl₃ 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.
6. Rinse the elastomer foam with ethanol and place in a vacuum oven overnight at 40 °C.
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

1. Sterilize the elastomer by washing it twice with 1 mL of 70% ethanol. Perform UV irradiation for 10 min and wash with 1 mL of 70% ethanol. Rinse it twice with 1 mL of sterile water and 1 mL of phosphate-buffered saline (PBS). Place the elastomers in 24-well culture plates.
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).
3. After counting the cells using a hemacytometer, prepare 1.5 x 10⁵ 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.
4. Incubate the seeded elastomers at 37 °C and 5% CO₂ for 2 h to promote cell adhesion. Add 0.5 mL of growth medium. Incubate again at 37 °C with 5% CO₂.
5. Change the medium every other day after washing with 1 mL of PBS.

9. Microscopic Imaging of Elastomer Construct

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).
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.
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.
4. Analyze the optical confocal image stacks using ImageJ³².

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 transport²⁷. 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/cm³) 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 reported¹⁸. 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 copolymer²⁷. 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 carbonyl⁶. 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 cycloaddition 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 2,100-cm⁻¹ band and the appearance of a singlet observed at 7.30 ppm in the ¹H 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 -CL⁶. 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-linkers¹⁸.

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 (LCEFP_P) and primary/secondary porosity (LCEFP_{P+SP}), have been previously reported. Here, the LCEFP_{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 FeCl₃ 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/FeCl₃ solution. The LCE foam is again placed inside a container with a saturated FeCl₃ 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 days²⁷. 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 1: General procedure describing the step-by-step preparation and characterization of the LCE foams. See the protocol section for details. [Please click here to view a larger version of this figure.](#)

Figure 2: Crosslinking scheme of 3-arm SBC. The crosslinking scheme of the 3-arm SBC using bis(4-chlorophenyl) ether or HDI as cross-linkers in the presence of a nickel metal template for the preparation of foam-like LCEs is shown. [Please click here to view a larger version of this figure.](#)

Figure 3: Scheme of 1,2,3-triazole formation (successful "click" reaction) followed by ^1H NMR and FT-IR. The displacement of the halogen group by the azide group is confirmed by the appearance of the $2,100\text{-cm}^{-1}$ band using attenuated total reflectance (ATR) FT-IR. The formation of the triazole ring is confirmed by the disappearance of the $2,100\text{-cm}^{-1}$ band and the appearance of a singlet, observed at 7.30 ppm in the ^1H NMR spectra. [Please click here to view a larger version of this figure.](#)

Figure 4: Optical images of various foam shapes prior to crosslinking. The metal foam is cut and shaped to the desirable form, as shown. [Please click here to view a larger version of this figure.](#)

Figure 5: Nickel foam before (A) and after (B) crosslinking. [Please click here to view a larger version of this figure.](#)

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. [Please click here to view a larger version of this figure.](#)

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). [Please click here to view a larger version of this figure.](#)

Movie 1: Video images of the deformation and recovery of an LCE roll. [Please click here to view this video.](#) (Right-click to download.)

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 solids²³ 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 foams²². 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 degrades⁶. 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.

Disclosures

The authors have nothing to disclose.

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